The Epidemiology of *Clostridium perfringens* type A on Swine Farms in Ontario and the Perceived Role in Neonatal Piglet Enteritis

by

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ABSTRACT

THE EPIDEMIOLOGY OF *CLOSTRIDIUM PERFRINGENS* TYPE A ON SWINE FARMS IN ONTARIO AND PERCEIVED ROLE IN NEONATAL PIGLET ENTERITIS

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To study the distribution of *Clostridium perfringens* and toxin genes, 48 swine farms were visited and 354 fecal samples were collected. The isolates recovered from lactating sows, gestating sows, grower-finishers, and manure pits were less likely to possess consensus gene *cpb2* compared to those from suckling pigs ($P<0.05$). The relative importance of different pathogens associated with neonatal piglet diarrhea was identified. A total of 237 neonatal diarrhea cases were submitted to the Animal Health Laboratory, University of Guelph between 2001 and 2010. The combined frequencies for cases involving enterotoxigenic *Escherichia coli*, *Clostridium perfringens* type A, rotavirus, and *Clostridium difficile* accounted for 56% of the total cases. A survey was administered to 22 practitioners and 17 pathologists for the diagnosis of *C. perfringens* type A. The majority (95%) of practitioners were moderately to very confident of their diagnosis, but almost half (41%) of the pathologists were not confident of their diagnosis.

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CHAPTER ONE

INTRODUCTION, LITERATURE REVIEW, AND OBJECTIVES

1. INTRODUCTION

Neonatal diarrhea represents a major problem in the early stages of pig production. In North America, piglets are typically weaned at 21 days of age with an ideal weight of 6.5 kg or greater. The entry weight of the weaned piglet to the nursery is an important determinant of growth rate and survival. The industry target for pre-weaned mortality is below 10%. Diarrheal disease is a common reason for weaning weights to be below target and pre-weaning mortality to become unacceptably high, resulting in significant economic loss. An outbreak of neonatal diarrhea can occur for several reasons, including when a new pathogen enters a farm due to a lapse in biosecurity, when the sow has not been exposed to the pathogen, or when conditions allow for an endemic pathogen to create disease (usually as a result of the pathogen challenge exceeding the immunity of the piglet).

In the past few decades, major classical infectious causes of piglet neonatal diarrhea in Canada have included: enterotoxigenic *Escherichia coli* (ETEC), transmissible gastroenteritis virus (TGEV), rotavirus, and *Isospora suis* (coccidia). Reports of neonatal piglet diarrhea caused by *Clostridium perfringens* type C are absent in Canada. ETEC are Gram-negative, anaerobic bacteria that can cause secretory diarrhea through bacterial adherence to the piglet intestine and production of enterotoxins. Bacterial adherence is mediated by five major fimbrial adhesins, including F4, F5, F6, F7, and F18. TGEV are enveloped, pleomorphic coronaviruses that attach and destroy mature villous cells. Porcine rotaviruses are non-enveloped icosahedral viruses (divided

into serogroups A, B, C, and E), which replicate in the villous epithelial cells and cause villous atrophy. *I. suis* causes coccidiosis when infectious oocysts are ingested and rapid replication of the parasite destroys the intestinal cells. TGEV, rotavirus, and *I. suis* are pathogens that may cause malabsorptive diarrhea in neonatal piglets. Specific prevention strategies are available for infection caused by ETEC (commercial vaccines) and *I. suis* (anti-coccidial medication), while recommendations have been developed to prevent TGEV and rotavirus from entering the herd (e.g. sanitation and reducing fomite transmission). Compared to previous decades, there is a relative decrease in neonatal diarrhea cases associated with ETEC, TGEV, rotavirus, and *I. suis*, which may be due to changes in farm management factors, advanced diagnostic techniques, emerging diseases, or other factors. Current research is being directed to identifying and controlling emerging diseases, and improving diagnostic techniques and prevention strategies for pathogens that are known to cause neonatal piglet diarrhea.

There has been an increase in the proportion of cases attributed to clostridia in the past decade, particularly *Clostridium perfringens* type A and *Clostridium difficile*. *Clostridium* species are ubiquitous strict-to-tolerant Gram-positive anaerobes that can be found in almost any environment and the gastrointestinal tract of warm-blooded species. The pathogenic mechanism of *C. perfringens* type A is currently unknown, and some studies have suggested that strains possessing the beta2 toxin gene (*cpb2*) are associated with neonatal diarrhea. The pathogenic mechanism of *C. difficile* is also unknown, but it is believed to cause colitis through the production of monomeric toxins, including toxin A (TcdA), toxin B (TcdB), and ADP-ribosyltransferase. *C. difficile* toxinotype V is associated with disease in piglets. This review will cover the major infectious causes of

neonatal diarrhea in pigs: ETEC, TGEV, rotavirus, *I. suis*, and *C. perfringens* type C. In addition, it will also include information about what is currently known about *C. perfringens* type A and *C. difficile*, the emerging infectious causes of neonatal piglet diarrhea.

2. LITERATURE REVIEW

2.1. Causes of Piglet Diarrhea and a Description of the Disease

2.1.1. Enterotoxigenic *Escherichia coli*

Escherichia coli are Gram-negative, non-spore-forming, facultative anaerobic bacteria. They are abundant in the environment and the mammalian gastrointestinal tract. While most *E. coli* strains do not cause disease, some strains are major pathogens. The ideal temperature range for *E. coli* growth is 37 to 42 °C; neutral pH is ideal for bacterial growth, but some *E. coli* strains that cause diarrhea can tolerate exposure to pH 2.0 [1]. The rod-shaped bacterium has a diameter of approximately 0.5 μm, and length of 1.0 to 3.0 μm; the periplasm consists of a single peptidoglycan layer [1]. *E. coli* usually have fimbriae (or pili), and most common *E. coli* strains have F1 (or type 1 pili) [2]. The traits characterizing different pathotypes of *E. coli* are often encoded by additional fimbriae (F antigens), and serotypes are commonly used to subdivide species based on virulence traits [2]. Complete serotyping involves determination of O and K (somatic), H (flagellar), and F antigens; antisera are available for typing isolates with proven or suspected pathogenicity [2]. Diagnostic laboratories often have a limited spectrum of antisera, which may be adequate, since isolates from a given region maintain their characteristic antigenic makeup [2].

Enterotoxigenic *E. coli* (ETEC) are a major cause of diarrhea and death, commonly observed in pigs aged 0-4 days [3]. Both the bacterial adherence (mediated by highly host-specific fimbriae) and production of enterotoxins are necessary for the disease to occur; the bacteria attach to and colonize the intestinal lining, and then secrete enterotoxins [3]. Fimbriae are classified by diverse nomenclature based on serological reactivity or receptor specificity; the first fimbrial adhesins demonstrated on swine ETEC were thought to be capsular antigens and were named K88 and K99 [3]. The current nomenclature for ETEC strains is based on serological activity using F designation, hence, K88 and K99 are called F4 and F5, respectively [3]. Susceptibility of pigs to ETEC infection is dependent on the expression and exposure of fimbrial receptors on the host intestine, which may be affected by pig lineage and age [3]. Porcine ETEC isolates produce sixmajor fimbrial adhesins, including F4 (K88), F5 (K99), F6 (987P), F7, F18, and F41; isolates may produce more than one fimbrae, and F4 is further divided into different antigenic forms (F4ab, F4ac, and F4ad) [3]. F18 is also divided into different antigenic forms, including F18ab and F18ac; strains that express F18ab are associated with edema disease [4].

The enterotoxins produced by ETEC include heat labile enterotoxin (LT), heat stable enterotoxin type A (STa), heat stable enterotoxin type B (STb), Shiga toxin type 2e (Stx2e), or enteroaggregative *E. coli* heat-stable enterotoxin 1 (EAST1) [1,3,4]. LT has a classic holotoxin structure with AB subunits which activate the G protein (increase intracellular cAMP levels), inhibiting water and NaCl absorption, and stimulating an increase in Cl^- and $HCO3^-$ by crypt cells [1,3]. The STs are small heat stable polypeptides, but STb is antigenically and genetically unrelated to STa [1, 3]. STa binds

to guanylate cyclase on the host cell membrane, stimulating an increase in cyclic GMP levels, reducing intestinal absorption of NaCl and water [1,3], while STb stimulates cyclic-nucleotide-independent fluid secretion into the gut [1]. Stx2e (also known as edema disease factor) produces lesions associated with edema disease in pigs [3, 4]. When the toxin enters circulation, it can destroy endothelial cells of small vessels, causing blood clots, hemorrhage, ischemic necrosis, and edema in vital organs including the brain [4]. EAST1 has been found in pig ETEC isolates, but its role in developing diarrhea is unknown [3, 4].

Diarrhea may be observed 2 to 3 hours after birth and affects single pigs or whole litters, with high mortality in the first few days of life [3]. Piglets can lose 30 to 40% of total body weight due to fluid loss and die from dehydration in severe cases, and some cases of infection are so rapid that death occurs before development of diarrhea [3]. On the other hand, diarrhea may be mild, with no evidence of dehydration [3]. The feces are clear and watery or vary in color from white to various shades of brown [3]. Pigs less severely affected can recover if treated appropriately. In cases of ETEC complicated by shock, apparently healthy pigs die suddenly or decline rapidly with cyanosis of the extremities [3]. Gross lesions may include stomach dilation, venous infarcts on the greater stomach curvature, and dilation and congestion of the small intestine; in ETEC infection complicated with shock, characteristic lesions include blood-tinged intestinal contents, marked congestion of the small-intestinal and stomach walls, and mild to moderate intestinal hyperemia [3]. Histologically, layers of *E. coli* can be observed adhering to the mucosal epithelium of the jejunum and ileum. Histological lesions may include vascular congestion in the lamina propria with hemorrhage into the intestinal

lumen, increased numbers of neutrophils and macrophages, and some villous atrophy; severe villous necrosis, hemorrhage, and microvascular fibrinous thrombi may be observed in the lamina propria of the stomach, small intestine, and colon in ETEC infection complicated with shock [3].

ETEC infection in neonatal piglets is effectively controlled by vaccinating sows, typically at 6 weeks and 2 weeks prior to farrowing; effective commercial vaccines are available for purified fimbria or killed whole-cell bacterins, which usually contain strains producing F4, F5, F6, and F41 [3]. In cases where vaccination is ineffective, it is important to identify the serotype of the pathogen and possible inclusion into an autogenous vaccine [3]. Antibiotic therapy can be used in treating ETEC infection. Different ETEC isolates vary in antibiotic sensitivity, and broad-spectrum antibiotics may be used until the results of antibiotic sensitivity are known [3].

2.1.2. Transmissible Gastroenteritis Virus

Transmissible Gastroenteritis (TGE) in pigs was first reported in 1946 in the United States and thereafter it was reported in other swine-producing countries [5]. In the 1980s, a mutant of TGEV became widespread and was known as porcine respiratory coronavirus (PRCV) [5]. A serological study revealed that subclinical infection of PRCV increased the seroprevalence of TGEV/PRCV antibodies in swine herds [6], and the economic impact of TGE has not been evaluated since the spread of PRCV [5].

Transmissible Gastroenteritis Virus is an enveloped, pleomorphic coronavirus with a diameter between 60 to 160 nm, and surface projections that are 12 to 25 nm long; the genome consists of positive-sense, single-stranded RNA, and a viral envelope derived from the host cell [5]. The virus is photosensitive, stable in freezing temperatures, and

labile at room temperature; virus particles can be inactivated by several chemical solutions, such as bleach, sodium hydroxide, iodines, and chloroform [5]. TGEV are resistant to trypsin, and relatively stable in pig bile and pH 3, allowing the virus to survive in the stomach and small intestine [5]. TGEV contains 4 structural proteins: a large surface glycoprotein (spike or S), small membrane protein (E or sM), integral membrane glycoprotein (M), and nucleocapsid protein (N) [5]. The S protein binds to the host intestinal receptor, and functions in virus neutralization, virus-cell attachment, membrane fusion, and hemagglutination [5].

TGE may occur in endemic or epidemic forms. In the epidemic form, the virus spreads rapidly in a susceptible herd, causing sows to develop anorexia and agalactia, and diarrheal disease with high mortality in piglets less than 3 weeks of age; the disease becomes self-limiting as sows acquire immunity, then the herd either becomes free of the virus or chronically infected [5]. Endemic TGE occurs when the virus is able to survive in a herd and infect susceptible animals, and this is more likely to occur in very large herds or herds with continual introduction of naïve gilts [5]. Vaccination against TGEV in a subset of animals may also allow the virus to survive on the farm [6]. In endemic TGE, clinical signs are less severe and cause low mortality. TGE outbreaks typically occur in winter because the virus is stable when frozen and kept away from sunlight or heat; possible reservoirs of TGEV between winter epidemics include continuous flow grower-finisher farms or neighboring TGEV-positive pig farms [5].

The virus enters the pig via the oral and nasal routes, and survives the low pH stomach environment and duodenal proteolytic enzymes. The incubation period of TGEV is 18 hours to 3 days, and infection spreads rapidly through the herd affecting most

animals in 2 to 3 days; carrier pigs can shed the virus for 2 weeks [5]. Clinical signs of epidemic TGE infection of suckling pigs are transient vomiting, watery and yellowish diarrhea, rapid weight loss, dehydration, and high morbidity and mortality; most pigs under 7 days of age will die after showing the first clinical signs [5]. Clinical signs of endemic TGE are less severe and cause low mortality.

Gross lesions may reveal distended stomach and small intestine with curdled milk, areas of hemorrhage on the stomach, and yellow foamy fluid in the small intestine; the intestinal wall is thin and transparent, and the villi are markedly shortened [5]. TGEV destroys mature villous cells (i.e. not the crypt epithelium), and regeneration of the villous may take up to 10 days in neonatal piglets [5]. Histological lesions reveal marked villous atrophy in the jejunum and ileum, and rarely in the proximal duodenum [5]. Villous atrophy is often more severe in newborn pigs compared to 3-week-old pigs, suggesting that neonates are at higher susceptibility of TGE infection; factors affecting age-dependent resistance include greater regenerative ability of villous epithelial cells in older pigs, resistance of newly replaced enterocytes to TGEV infection, absence of the apical tubulovesicular system (where the virus replicates) in pigs older than 3 weeks of age, and a greater virus dose is likely needed to infect an older pig compared to a younger pig [5].

There are no antiviral agents available specifically for the treatment of TGE, but TGE antibodies (IgA) found in sow milk provides a high degree of passive immunity to the suckling pig [5]. Some precautions to prevent TGEV from entering the herd include introducing only pigs that are known to be free of TGE, are serologically negative, and/or have been quarantined for 2 to 4 weeks before entering the herd [5]. If a TGE outbreak

occurs, pigs should only be moved to another herd after a period of at least 4 weeks from the last sign of disease in order to reduce spread of the disease [5]. Precautions should be taken to minimize disease transmission through animal vectors, such as starlings which feed around swine and can spread TGEV between herds during winter [5]. Feces from pigs infected with TGEV can be carried on fomites, such as footwear, clothing, feeds, and equipment. When TGE has occurred in a herd that has not yet been exposed, sows should be purposely exposed to the virus 2 weeks before farrowing, such as through minced intestines of infected piglets [5]. Commercial vaccines (attenuated or killed virus) are available, but they do not induce sufficient immune responses in the gut and result in irregular levels of active and passive immunity [5].

2.1.3. Rotavirus

Rotaviruses are opportunistic pathogens causing gastroenteritis in young humans and animals. They are ubiquitous, very stable to solvents, extreme temperatures, and a pH range of 3.0 to 9.0 [7]. The 70-nm icosahedral virus particle is non-enveloped, with short spikes which resemble a wheel; the viral capsid is composed of three protein layers, including the outer (VP7 and VP4), intermediate (VP6), and the inner (VP2) layers, all of which are required for a virus to be infectious [7]. The genome is composed of 11 segments of double-stranded RNA and RNA-dependent RNA polymerase (VP1), which encode 6 core viral structural proteins (VP1 to 3, VP4, VP6, and VP7) and 6 non-structural proteins [7]. VP4 forms the spikes of the viral particle, and are involved in hemagglutination [7]. Seven serogroups of rotavirus (A to G) are classified based on VP6 antigens, and porcine rotaviruses belong to serogroups A, B, C, and E; however, group E rotavirus has only been detected in a single herd outbreak that occurred in the United

Kingdom [7]. Group A rotaviruses are the most common agent causing diarrhea in humans and pigs, which are further classified into 10 G types and 7 P types based on antibody responses to VP7 and VP4, respectively [7]. Rotaviruses can remain infective for 7 to 9 months at room temperature, and feces stored at 10°C can remain infectious for 32 months; although they are resistant to many disinfectants, rotaviruses can be inactivated by solutions containing ethanol or bleach [7]. Rotavirus enters the pig via the fecal-oral route, and the virus replicates in the villous epithelial cells of the jejunum and ileum, and the cecum or colon [7]. Enterocytes are lysed, causing villous atrophy and subsequent malabsorptive diarrhea [7]. Group A rotaviruses can infect 1 to 6-week-old pigs, and is most common in 3-week-old pigs after maternal antibodies provided by colostrum decline [7, 8]. The virus is shed for 1 to 14 days in the feces [7]. Group C rotaviruses are widespread in adult swine, and may cause sporadic diarrhea in suckling and recently weaned pigs [7].

Diarrhea may occur in 1-to-41 day old suckling pigs, which is generally uncomplicated and lasts for a few hours to several days [7, 8]. Feces are yellow or white, watery to creamy, and flocculant [8]. Morbidity is usually less than 20%, and mortality is less than 15% [7]. Gross lesions may appear before or with the onset of diarrhea (most severe in pigs 1 to 14 days of age). The stomach usually contains food; the small intestine, cecum, and colon are thin-walled, flaccid, and dilated with watery, flocculant, yellow or gray fluid [7]. The intestinal lacteals do not contain chyle, and associated mesenteric lymph nodes are small and tan [7]. Histologically, degeneration of epithelial cells are observed 16-18 hours post-inoculation, and severe villous atrophy by 24-72

hours post-inoculation and the villi are eroded or covered by squamous epithelial cells [7]. Crypt hyperplasia may also occur [7].

There is no specific treatment for rotavirus infection, and it is difficult to eradicate rotaviruses from swine herds because they are ubiquitous [7]. A study showed that nurseries using continuous flow had significantly lower rates of rotavirus infection compared to an all-in/all-out flow, suggesting that the piglets exposed to rotavirus were able to develop an active immune response under partial protection of maternal antibodies [9]. Commercial vaccines for attenuated rotavirus and inactivated rotavirus are available for sows and nursing pigs, but the efficacy of current vaccines is uncertain or poor [7].

2.1.4. *Isospora suis*

Coccidiosis caused by *Isospora suis* is an important protozoal disease in pigs; the obligate intracellular parasite has development stages inside the host and in the external environment. The oocysts are spheroid to spherical, with a diameter between 19.4 to 22.5 µm, and a smooth single-layer capsule 1.5 µm thick [10]. The coccidia life cycle is divided into three phases: sporogony, excystation, and endogenous development. During sporogony, unsporulated oocysts are passed in the feces and sporulate within 12 hours to become infectious and environmentally resistant, which is dependent on temperature (ideal range is 20°C and 37°C) and moisture [10]. When the oocysts are fully sporulated, they are resistant to most disinfectants [10]. Each oocyst contains 2 sporocysts with 4 sporozoites each. When the infectious sporulated oocysts are ingested, sporozoites are released into the intestinal lumen and then enter the intestinal cells to replicate (exycstation) [10, 11]. The sporozoites divide many times to produce offspring

(endogenous development), which in turn enter other intestinal cells. There are two distinct types of asexual stages in the endogenous life cycle. Sporozoites enter enterocytes and become binucleated type-1 meronts, and each meront produces two type-1 merozoites (within 24 hours) with a characteristic side-by-side appearance [10]. Several divisional cycles occur producing many type-1 merozoites, which in turn produce type-2 meronts and type-2 merozoites (seen as early as 1 day post-inoculation) [10]. Following the asexual stages are the sexual stages, consisting of microgamonts and macrogamonts [10]. The microgametes produce bi-flagellated microgametes, which fertilize uninucleated macrogamonts to form oocysts, and can be found in the feces 5 days post-inoculation [10]. Rapid replication causes intestinal cells to be destroyed; multiplication mostly occurs in the ileum and jejunum, and in the cecum and colon for heavy infections [10, 11].

Diarrhea occurs in pigs between 7 and 11 days of age, with yellow to gray diarrhea being a major clinical sign, low to moderate mortality, and high morbidity; the feces are initially loose or pasty and become more fluid as infection progresses [10]. Piglets covered with liquid feces appear damp and give off a rancid odour; they will continue to nurse, develop a rough hair coat, dehydrate, and lose weight [10]. The severity of disease depends on the amount of oocysts ingested by the piglet. Lesions develop around 4 days post-inoculation and are associated with the presence of asexual stages; in severe cases, piglets may show signs of coccidiosis before sexual stages are produced [10]. In severely affected piglets, gross lesions are characterized by fibrinonecrotic membrane in the jejunum and ileum [10]. Microscopic lesions include villous atrophy, villous fusion, crypt hyperplasia, and necrotic enteritis; tall columnar

enterocytes may be destroyed, exposing the lamina propria or replaced by flattened immature cells [10].

The primary source of *I. suis* for suckling pigs is unknown, but the parasite can be transmitted through contaminated farrowing crates once established on the farm [10]. When infected pigs recover, they are resistant to subsequent infection [10]. Sows do not appear to be a major source of infection for nursing pigs, therefore anticoccidial drugs in the sow ration and colostrum will not protect piglets from developing coccidiosis [10]. Coccidiosis is readily controlled by administering toltrazuril orally to pigs at 3 to 5 days of age. Improving sanitation to remove oocysts from the farrowing environment is the most efficient preventative measure for reducing neonatal coccidiosis. This involves removing animals from the building, cleaning the crates to remove organic debris, disinfection for several hours or overnight with at least 50% bleach or ammonia compounds, and steam-cleaning [10]. Crate-to-crate contamination with oocysts should be avoided, as they can be carried on boots, clothing, or other animals; controlling rodent population may also reduce mechanical transmission of oocysts [10].

2.1.5. *Clostridium perfringens*

Clostridium perfringens are divided into five toxinotypes from A to E according to differential production of the major toxins alpha (CPA), beta (CPB), epsilon (ETX), and iota (ITX) [12, 16]. C. *perfringens* of any type are able to produce enterotoxin (CPE) and beta2 toxin (CPB2) [13].

2.1.5.1. Toxins

The production of potent toxins is a characteristic of clostridia. This section reviews some aspects of toxin production by *C. perfringens* and their role in different enteric diseases of animals as a background to understanding the potential role of *C. perfringens* type in neonatal enteritis in pigs. Overeating is a common prerequisite for ruminant enterotoxemia caused by type D organisms, which provides a rich environment for bacterial proliferation, toxin production and absorption [21]. In addition, anti-trypsin factors in colostrum may also contribute to enterotoxemia in neonatal animals [21]. CPA is encoded by chromosomes [14], and possesses phospholipase C activity [21]. The toxin is not thought to be important in animal disease, since experimental studies have indicated that pure inoculation of CPA does not produce significant lesions to ligated gut loops [12, 18]. The enterotoxin gene (*cpe*) can be present on the chromosome or plasmid [14], and encodes a pore-forming toxin [13, 21]. Although CPE is not thought to be important in animal disease, it has been associated with human disease: type A isolates carrying chromosomal *cpe* have been isolated in human food poisoning cases, while type A isolates carrying plasmid *cpe* have been isolated from antibiotic-associated diarrhea or sporadic diarrhea cases [14, 22]. The CPB gene (*cpb*) resides on a plasmid [14] and encodes a trypsin-susceptible, pore-forming toxin [21]. The association of CPB with serious enteric disease in neonatal farm animals is because of the trypsin-inhibiting effect of colostrum and with serious disease in humans is associated with the trypsin inhibiting effect of some foodstuffs, such as cassava. ETX is encoded by the *etx* gene found on plasmids [14]. When proteolytically activated, ETX increases cell permeability, [21] and

causes edema in the intestines and colon [14]. ITX, encoded by two genes (*iap* and *ibp*) [14], is a binary toxin which disrupts the cellular cytoskeleton [14, 21].

CPB2, the toxin which has been linked to neonatal porcine enteritis, is a 28-kDa protein encoded by the *cpb2* gene which resides on a large plasmid [18, 23]. The toxin mechanism is currently unknown, but purified toxin was lethal to mice (minimal lethal dose 3 μg) and cytotoxic for CHO cells [23]. Experimental evidence showed that antibodies raised against CPB2 reacted weakly to CPB, and antibodies raised against CPB did not react to CPB2, indicating a weak immunological relationship [23]. Like CPB, CPB2 is susceptible to trypsin; CPB2 had minor effects on the cell actin filament network (but not the cytoskeleton), and the toxin induced hemorrhagic necrosis of the intestinal wall in the ileal loop test [23]. There are two variant of CPB2, encoded by either a consensus gene or an atypical gene, which displays approximately 70% DNA identity to consensus *cpb2* [24]. The purified atypical CPB2 had approximately 10-fold less toxicity on Caco-2 cells compared to consensus CPB2 [24]. Experimental evidence revealed that most porcine (type A and type C) isolates carried consensus *cpb2* and generally expressed consensus CPB2, whereas most non-porcine isolates carried atypical *cpb2* and generally did not express atypical CPB2 [24]. The non-porcine isolates that carried consensus *cpb2* were expressed at different levels, since some isolates carried a cryptic gene due to a frame-shift mutation [24]. The condition that leads to gene expression for production of CPB2 is unknown [24], although expression is under control of the VirR/VirS regulation [21]. Jost and colleagues also determined whether the presence of *cna*, which encodes a collagen adhesion protein (CpCna), is correlated with the presence of consensus *cpb2* residing on the same plasmid [25]. The study found that

cna was present in 70% of total *cpb2* positive *C. perfringens* isolates, but in 98% of porcine isolates; both genes were regulated at the transcription level by VirR/VirS (*cpb2* was up-regulated, whereas *cna* was down-regulated by this system). Jost and colleagues proposed that temporal expression and adhesion of CpCna is required in early infection, while CPB2 is expressed later to mediate tissue damage [25].

Prevalence studies on the presence of *cpb2* in *C. perfringens* isolates were evaluated in different species. One study reported the presence of *cpb2* in 92% of neonatal swine *C. perfringens* isolates with enteritis compared to 11% of normal swine isolates, and *cpb2* was found in 47% of isolates from calves with enteritis or abomastitis; the presence of *cpb2* in *C. perfringens* isolates from other species ranged from 12% in goats, 20% in camelids, cervids, sheep and horses, and 35% in dogs and birds [17]. *C. perfringens* isolates from the environment, animal feed, and food had a low occurrence of *cpb2* [17]. Some studies reported the presence of *cpb2*-positive isolates from diarrheic piglets, but *cpb2* was not detected in the isolates of control piglets [17, 18]. These studies suggest that CPB2 is either involved in the pathogenesis of *C. perfringens* type A enteritis in piglets or alternatively may be a marker of virulence [13]. However, other surveys have indicated that *cpb2* can be detected in half the isolates of normal pigs [21], so that the specific association with enteritis may be less clear than previously suggested. Some studies have suggested an association of *cpb2*-positive *C. perfringens* isolates in horses with fatal progression of typhlocolitis, particularly if they were treated with aminoglycoside antibiotics [20, 26]. The *cpb2* gene in equine isolates was reported to be cryptic due to a single nucleotide deletion encoding a premature stop codon, but treatment with aminoglycoside antibiotics (gentamycin or streptomycin) induced *cpb2*

gene expression presumably by frame-shifting at the ribosome level [26]. A case report also indicated that CPB2-toxigenic *C. perfringens* type A were recovered from a foal with colitis that was treated with gentamicin [27]. In contrast, a study that analyzed *C. perfringens* isolates in broiler chicken populations from necrotic enteritis outbreaks revealed that *cpb2* was found exclusively in healthy bird isolates [28]. The presence of *cpb2* gene reported in *C perfringens* isolates from human diarrheal cases was below 15% in food-poisoning isolates, but it was found in 75% of isolates for antibiotic-associated or sporadic diarrhea in addition to CPE [29]. In summary, some studies suggest that *cpb2* positive *C. perfringens* isolates may be involved in enteric disease, at least for certain species, including piglets [17, 18], calves [17], horses treated with aminoglycoside antibiotics [20, 26], and humans with antibiotic-associated or sporadic diarrhea [29]. However, the role of *cpb2* is unclear, since the gene can also be isolated from *C. perfringens* in apparently healthy animals, including pigs [21] and chickens [28].

2.1.5.2. *Clostridium perfringens* type C

Clostridium perfringens type C infection is characterized by hemorrhagic (and often fatal) necrotic enteritis in 0-to-5-day old piglets [12, 13, 21]. The organism can be found in small numbers in the intestine of healthy animals, which proliferate to 10^8-10^9 colony-forming units (CFU)/g of intestinal contents and induce disease under appropriate conditions [13]. *C. perfringens* type C attach to jejunal epithelial cells and proliferate along the basement membrane, causing cell desquamation, hemorrhage, and extensive necrosis of the lamina propria (necrosis may extend to all layers of the intestine) [12, 13]. CPB is a key factor in tissue damage, but the specific role of the toxin in pathogenesis of type C enteritis is unclear [13]. Experimental evidence indicated that gut loop

inoculations with broth cultures of *C. perfringens* type C induced typical lesions, but necrosis was not due to inoculation of CPB alone [13]. Piglets younger than 4 days old are more susceptible to type C enteritis due to trypsin secretion deficiencies and colostral protease inhibitors, and the cause of death is likely due to intestinal damage and toxemia [13]. It was suggested that CPB2 may also play a role in pathogenesis [13].

In non-immune herds, epidemic *C. perfringens* type C enteritis causes high morbidity (affected litters can reach 100%) and high mortality, with several piglets that die within 12-36 hours after birth [12, 13]. The disease becomes endemic when herd immunity rises, and mild outbreaks may occur over a period of several months [13]. Bloody diarrhea may appear as early as 12 hours after birth; it is commonly observed in 3-day-old piglets, but rarely affects pigs older than 1 week of age [12, 13]. Gross lesions are typically observed in the small intestine (jejunum), and possibly the cecum and spiral colon; lesions are occasionally confined to the large intestine [13]. The lesions consist of intestinal and mesenteric hyperemia, fibrinonecrotic (pseudomembranous) enteritis with emphysema, bloody gut contents, red mesenteric lymph nodes, and adhesions may develop between intestinal loops [13]. The hallmark microscopic lesion is hemorrhagic necrosis of the intestinal wall, which usually progresses to affect all intestinal layers [13]. The luminal surface is covered by degenerated epithelial cells, inflammatory cells (pseudomembrane), and large bacilli with square ends and spores [13]. *C. perfringens* type C outbreaks are effectively controlled through vaccination [21]; commercial vaccines with type C toxoid generally eliminate disease within one farrowing cycle, and injections should be given to sows at 2-3 weeks before farrowing [12]. Affected piglets may be treated with oral antimicrobials (ampicillin or amoxicillin) or ceftiofur [12].

2.1.5.3. *Clostridium perfringens* type A

C. perfringens type A are a part of the normal flora in the swine intestine, found in large numbers in the colon and smaller numbers in the small intestine. These bacteria colonize the piglet intestine and multiply to 10^9 CFU/g of intestinal contents within 12 hours after birth, and they are found in the highest populations in non-medicated 1-to-7 day old piglets, along with *E. coli* [21]. The population of *C. perfringens* type A then decreases to 10^6 CFU/g at weaning age, as other species of bacteria establish their populations in the piglet intestinal flora [21]. It is currently not possible to differentiate between isolates that are normal flora and those of disease-causing strains, therefore the epidemiology of type A enteritis is highly speculative [13]. The pathogenesis of type A enteritis has been proposed to involve bacterial proliferation and production of CPB2 [21]. Toxins are produced in the jejunum and ileum, and bacterial attachment and invasion are uncommon [13]. Although the mechanism of CPB2 is unknown, it is thought to stimulate secretory diarrhea and mild inflammation of villous tips [21].

Diarrhea attributed to *C. perfringens* type A can occur in piglets during the first week of age, but is most commonly seen in piglets born within 48 hours [12, 13, 21]. Clinical signs are described as non-hemorrhagic and mucoid diarrhea with creamy or pasty feces lasting for up to 5 days [12, 13, 21]. The gross and microscopic lesions associated with type A enteritis are not specific [13]. Necropsy may reveal the small intestine as flaccid, thin-walled, and gas-filled with watery contents and no blood [12]. Microscopic lesions may involve superficial villus tip necrosis and fibrin accumulation, which are most severe in the jejunum and ileum with colonization by *C. perfringens* [12, 13]. There are no unequivocal descriptions of the reproduction of neonatal enteritis in

piglets using characterized strains of *C. perfringens*, type A, and it is well known that this organism colonizes the stomach and small intestine in the neonatal piglet [32]. Most if not all aspects of neonatal enteritis associated with *C. perfringens* type A in piglets require to be investigated to confirm that these bacteria truly are associated with disease.

There are currently no specific treatment or control strategies for *C. perfringens* type A enteritis. Some success has been reported for autogenous vaccination, or feedback of infected feces and intestinal contents to the sow [30]. Sanitation of the farrowing facility alone was also reported to control the problem in some cases [30]. Veterinarians consistently reported that tylosin injections were an effective treatment for piglets affected with type A enteritis [30].

2.1.6. *Clostridium difficile*

Clostridium difficile are commonly found in the colon of healthy animals, but toxin production leads to intestinal lesion development [13]. They are encapsulated, motile spore-formers with adherence factors, and sporulate readily in contrast to *C. perfringens* [13]. Reports of disease associated with *C. difficile* in swine emerged in the early 1980's, and the diagnosis for this disease has increased in the past decade [21]. Surveys suggest high prevalence of infection (e.g. 90% of herds), but the actual disease prevalence is unknown [21]. In humans, *C. difficile* is associated with approximately 25% of antibiotic-associated diarrhea cases [12, 21]. *C. difficile* is frequently compared using ribotypes or toxinotypes; toxinotype V can be found in human disease, and is also frequently associated with disease in piglets [21]. The pathogenesis of *C. difficile* is thought to be mediated by monomeric toxins of at least 3 toxin classes: toxin A (TcdA), toxin B (TcdB), and ADP-ribosyltransferase [21]. TcdA and TcdB have 45% amino acid

similarity [21]; these toxins enter cells and disrupt the activity of Rho, a triphosphate (GTP) binding protein involved in cytoskeletal system regulation, causing cell rounding and death [14, 21]. TcdA and TcdB are similar in their basic structure, but do not demonstrate significant immunological cross-reaction, and antitoxin antibodies for these toxins do not show cross-neutralization [14]. ADP-ribosyltransferase is a binary, pore-forming toxin which attaches to and enters enterocytes, disrupting the cytoskeleton leading to cell death [21].

Diarrhea typically occurs in 1-to-7 day old piglets, rarely with respiratory distress and sudden death [12, 13, 21]. Gross lesions may include mesocolonic edema, with pasty to watery yellow feces that fill the large intestine [12]. A study by Yaeger and colleagues determined that the presence of mesocolonic edema had 42% positive predictive value, but *C. difficile* toxin was not detected in any pigs without mesocolonic edema [31]. The lack of gross lesions may contribute to underdiagnosed cases of *C. difficile* enteritis [13]. The microscopic hallmark lesions of *C. difficile* infection are multifocal mucosal epithelial erosions, with suppuration and exudation of colon [21]. There is currently no commercial product available for the prevention of *C. difficile* infection, but susceptibility tests show that virginiamycin administered to sows before and after farrowing may be useful [12]. In addition, tylosin can be used to treat affected piglets [12].

2.2 Etiological Diagnosis of Piglet Diarrhea

An accurate diagnosis of neonatal diarrhea outbreak in the herd requires the veterinary practitioner to observe pigs in the environment: it is important to determine whether healthy piglets get diarrhea or whether piglets with compromised health develop

diarrhea, in addition to sow health and immune status [21]. Necropsy should be performed on piglets at various levels of disease, and fresh tissues should be selected from non-medicated, acutely affected piglets for examination [21]. In addition, samples obtained for microscopic examination from euthanized piglets should be immediately fixed in 10% formalin [21]. Diarrhea may involve more than one etiological agent. Diagnosis of the agent causing diarrhea is generally based on clinical signs, age of onset, mortality pattern, gross and microscopic lesions, and the isolation of suspected pathogen(s); for chronic disease cases, the history of infection in the herd should also be considered. A presumptive diagnosis can be made by determining the fecal pH [3]. For example, secretory diarrhea caused by ETEC infection has an alkaline pH, whereas malabsorptive diarrhea caused by transmissible gastroenteritis virus or rotavirus has an acidic pH [3].

ETEC infection is indicated by the presence of Gram-negative bacteria adhering (multi-focally or diffusely) to the villus surface of the small intestine of infected pigs; Gram staining of impression smears from the ileum reveals large numbers of Gram-negative bacilli (hemolytic or non-hemolytic *E. coli*) [3, 4]. Fimbrial adhesions may be detected by serologic assays such as slide agglutination, immunofluorescence, and enzyme-linked immunosorbent assay (ELISA) [3]. Genotypic analysis of ETEC strains are commonly used to define virotypes [3]; multiplex polymerase chain reaction (PCR) can identify genes for the virulence factors F4, F5, F6, F18, and F41, and genes for the toxins LT, STa, STb, and Stx2e [4].

Diagnosis of TGE may be accomplished through several methods by detection of viral antigen, detection of viral nucleic acids (PCR), detection of virus (electron

microscopy), isolation and identification of virus, and/or detection of a significant antibody response [5]. The most common method for diagnosing TGE is the detection of viral antigen using immunohistochemistry, but they require pigs in early stages of diarrhea [5].

Diagnosis of rotavirus infection requires detection of virus, viral antigen, or viral RNA. The virus is shed in the highest concentrations in the first 24 hours after the onset of diarrhea, and samples should be collected from pigs in the acute phase of disease [7]. A number of methods can be used to detect rotavirus, including electron microscopy, immunohistochemistry (IHC), ELISA, latex agglutination, and reverse-transcription polymerase chain reaction (RT-PCR) [7]. Commercial kits of ELISA are frequently used for detecting antigens in fecal samples and intestinal contents [7]. The most widely used methods for rotavirus detection and genotyping is RT-PCR, which provides a sensitive method for genotyping G and P types for rotavirus group A, and for detecting group B and C rotaviruses [7].

Diagnosis of coccidiosis caused by *Isospora suis* is most quickly achieved by finding oocysts in the feces of infected piglets [10]. Feces should be collected for fecal smears or flotations from several litters that have been showing clinical signs for 2-3 days, the peak time for oocyst production [10]. The oocysts of *I. suis* have characteristic structures between the oocyst wall and sporont known as hazy bodies [10].

Diagnosis of *Clostridium perfringens* type C is based on isolation of large numbers of *C. perfringens* by bacterial culture, followed by genotyping of the isolates and detection of CPB toxin [12]. The colonization of large Gram-positive rods in the intestines of affected pigs may support the diagnosis [12]. CPB can be detected using

ELISA, but the toxin breaks down readily in intestinal contents, thus failure to detect the toxin should not be used as a basis of ruling out type C enteritis [13].

The diagnostic method for *C. perfringens* type A enteritis is not well established. Diagnosis is currently based on isolation of large numbers of *C. perfringens*, genotyping the isolates as type A, and ruling out other known enteric pathogens that cause disease [13]. The presence of *cpb2* has also been used by some to confirm diagnosis; there is currently no commercial test available for detecting CPB2 toxin in isolates [13].

Diagnosis of *C. difficile* can be confirmed with toxin detection (TcdA or TcdB) via ELISA [12]. Isolation of *C. difficile* alone is not sufficient for diagnosis as there can be toxigenic and non-toxigenic strains; in addition, some strains only produce TcdB [12]. ELISA kits for detecting *C. difficile* toxin are inexpensive, fast, and sensitive; however, the toxin is labile and will denature if the specimen is not collected from acutely affected individuals and immediately frozen [30].

2.3 Control and Prevention of Piglet Diarrhea

Specific prevention and/or treatment are available for some pathogens causing neonatal enteric diseases. Effective commercial vaccines are available for preventing infection caused by ETEC [3] and *C. perfringens* type C [21], and anti-coccidial medication is available for treatment of *I. suis* [10]. Although commercial vaccines are also available for infections caused by TGEV and rotavirus, the efficacy of these products to control the diseases is unknown. Instead, certain precautions and sanitation procedures should be taken to prevent the viruses from entering the herd [5, 7]. There is a lack of specific prevention and treatment for *C. perfringens* type A and *C. difficile*, which are emerging enteric diseases of neonatal piglets. Regardless of the pathogen involved in

neonatal diarrhea, basic treatment and control of disease requires the consideration of three critical risk factors, including sow immunity, adequate colostrum for effective passive transfer (IgG) with continued milk ingestion (IgA), and consistent management of the microenvironment, hygiene, and husbandry [21]. It is important to enhance the passive immunity of piglets, for example, by exposing replacement gilts to feces of older sows in order to increase antibody titers for pathogens present in the environment. It is also important to pay attention to sow lactation diet, feed intake, sow comfort, and farrowing crate design to ensure adequate and effective transfer of colostrum and milk to the litter [7]. Supportive therapy can alleviate starvation, dehydration, and acidosis of affected piglets [5]. Although parenteral treatment with fluids, electrolytes, and nutrients are effective at controlling some of the clinical impact of diarrhea, it is not always practical under farm conditions. The environment for piglets should be warm (preferably above 32°C), draft-free, and dry; water or nutrient solutions should also be freely accessible to infected pigs [5]. These measures may reduce mortality in pigs that are infected at more than 3 days of age [5]. Cross-fostering of infected or susceptible litters onto immune sows may also be useful in controlling outbreaks [5, 7]. Ideally, suckling piglets should become subclinically infected with the pathogens in the environment while they are protected by passive antibodies, in order to develop active immunity and prevent subsequent infection. Management practices should be designed to reduce the amount of pathogen exposure to susceptible pigs and to boost the level of passive immunity. The farrowing room should be disinfected and thoroughly dried before introduction of sows.

3. OBJECTIVES

As a contribution to improving understanding of neonatal enteric disease in pigs, and in particular to understand better the role of *C. perfringens* type A, the objectives of this thesis were: (i) to compare the distribution of *Clostridium perfringens* and selected toxin genes among pigs at different stages of production, and to identify farm factors affecting this distribution, (ii) to identify the frequency and trends of different pathogens contributing to neonatal piglet diarrhea in Ontario swine farms from 2001 to 2010, and (iii) to investigate how Canadian veterinary practitioners diagnose, treat, and control neonatal piglet diarrhea associated with *C. perfringens* type A, and how this compares to the way in which veterinary pathologists in selected parts of the world diagnose neonatal piglet diarrhea associated with *C. perfringens* type A.

REFERENCES CITED

1. Welch RA: The Genus *Escherichia.* In *Prokaryotes*. Volume 6. 3rd edition. Edited by Dworkin M, Falkow S, Rosenberg E. New York: Springer Science, Business Media LLC; 2006:60-71.

2. Fairbrother JM, Carlton G: *Escherichia coli* Infections. In *Diseases of Swine*. 9th edition. Edited by Straw BE, Zimmerman JJ, D'Allaire S, Taylor DJ. Ames, Iowa: Iowa State University Press, 2006:639-641.

3. Fairbrother JM: Neonatal *Escherichia coli* Diarrhea. In *Diseases of Swine*. 9th edition. Edited by Straw BE, Zimmerman JJ, D'Allaire S, Taylor DJ. Ames, Iowa: Iowa State University Press, 2006:641-649.

4. Francis DH: Enterotoxigenic *Escherichia coli* infection in pigs and its diagnosis. *J Swine Health Prod* 2002, 10(4):171-175.

5. Saif LJ, Sestak K: Transmissible Gastroenteritis and Porcine Respiratory Coronavirus. In *Diseases of Swine*. 9th edition. Edited by Straw BE, Zimmerman JJ, D'Allaire S, Taylor DJ. Ames, Iowa: Iowa State University Press, 2006:489-516.

6. Wesley RD, Woods RD, McKean JD, Senn MK, Elazhary Y: Prevalence of coronavirus antibodies in Iowa swine. *Can J Vet Res* 1997, 61:305–308.

7. Yuan L, Stevenson GW, Saif LJ: Rotavirus and Reovirus. In *Diseases of Swine*. 9th edition. Edited by Straw BE, Zimmerman JJ, D'Allaire S, Taylor DJ. Ames, Iowa: Iowa State University Press, 2006:435-454.

8. Bohl EH: Rotaviral diarrhea in pigs: Brief review. *J Am Vet Med Assoc* 1979, 174:613–615.

9. Dewey C, Carman S, Pasma T, Josephson G, McEwen B: Relationship between group A porcine rotavirus and management practices in swine herds in Ontario. *Can Vet J* 2003, 44:649–653.

10. Lindsay DS, Dubey JP: Coccidia and Other Protozoa. Rotavirus and Reovirus. In *Diseases of Swine*. 9th edition. Edited by Straw BE, Zimmerman JJ, D'Allaire S, Taylor DJ. Ames, Iowa: Iowa State University Press, 2006:861-73.

11. Mundt HC: *Isospora suis* infection in piglets. *J Anim Protozooses* 2005, 20(1):1-12.

12. Songer JG, Taylor DJ: Clostridial Infections. In *Diseases of Swine*. 9th edition. Edited by Straw BE, Zimmerman JJ, D'Allaire S, Taylor DJ. Ames, Iowa: Iowa State University Press, 2006:613-27.

13. Songer JG, Uzal FA: Clostridial enteric infections in pigs. *J Vet Diag Invest* 2005, 17:528-536.

14. McClane BA, Uzal FA, Miyakawa MF, Lyerly D, and Wilkins T: The enterotoxic clostridia. In *Prokaryotes*. Volume 4. 3rd edition. Edited by Dworkin M, Falkow S, Rosenberg E. New York: Springer Science, Business Media LLC; 2006:698-752.

15. Wiegel J, Tanner R, Rainey FA: An Introduction to the Family Clostridiaceae. In *Prokaryotes*. Volume 4. 3rd edition. Edited by Dworkin M, Falkow S, Rosenberg E. New York: Springer Science, Business Media LLC; 2006:654-678.

16. Garmory HS, Chanter N, French NP, Bueschel D, Songer JG, Titball RW: Occurrence of *Clostridium perfringens* b2-toxin amongst animals, determined using genotyping and subtyping PCR assays. *Epidemiol Infect* 2000, 124:61–67.

17. Bueschel DM, Jost BH, Billington SJ, Trinh HT, Songer JG: Prevalence of cbp2, encoding beta2 toxin, in *Clostridium perfringens* field isolates: correlation of genotype with phenotype. *Vet Microbiol* 2002, 94:121–129.

18. Waters M, Savoie A, Garmory HS, Bueschel D, Popoff MR, Songer GJ, Titball RW, McClane BA, Sarker MR: Genotyping and phenotyping of beta2-toxigenic *Clostridium perfringens* fecal isolates associated with gastrointestinal diseases in piglets. *J Clin Microbiol* 2003, 41:3584–3591.

19. Manteca C, Daube G, Jauniaux T, Linden A, Pirson V, Detilleux J, Ginter A, Coppe P, Kaeckenbeeck A, Mainil JG: A role for the *Clostridium perfringens* beta2 toxin in bovine enterotoxaemia? *Vet Microbiol* 2002, 86:191–202.

20. Herholz C, Miserez R, Jacques N, Frey J, Popoff M, Gibert M, Gerber H, Straub R: Prevalence of β2 Toxigenic *Clostridium perfringens* in Horses with Intestinal Disorders. *J Clin Microbiol* 1999, 37(2):358-361.

21. Schwartz KJ: Clostridium-associated diseases in swine. *In Proceedings of American Association of Swine Veterinarians: 7-10 March 2009; Dallas.*

22. Lindström M, Heikinheimo A, Lahti P, Korkeala H: Novel insights into the epidemiology of *Clostridium perfringens* type A food poisoning. *Food Microbiol* 2011, 28(2):192-198.

23. Gibert M, Jolivet-Renaud C, Popoff MR: Beta2 toxin, a novel toxin produced by *Clostridium perfringens*. *Gene* 1997, 203:65-73.

24. Jost BH, Billington SJ, Trinh HT, Bueschel DM, Songer JG: Atypical *cpb2* genes, encoding beta2-toxin in *Clostridium perfringens* isolates of nonporcine origin. *Infect Immun* 2005, 73:652–656.

25. Jost BH, Billington SJ, Trinh HT, Songer JG: Association of genes encoding beta2 toxin and a collagen binding protein in *Clostridium perfringens* isolates of porcine origin. *Vet Microbiol* 2006, 116:173-182.

26. Vilei EM, Schlatter Y, Perreten V, Straub R, Popoff MR, Gibert M, Gröne A, Frey J: Antibiotic induced expression of a cryptic *cpb2* gene in equine β2-toxigenic *Clostridium perfringens*. *Mol Microbiol* 2005, 579(6):1570-1581.

27. Hazlett MJ, Kircanski J, Slavic D, Prescott JF: Beta 2 toxigenic *Clostridium perfringens* type A colitis in a three-day-old foal. *J Vet Diagn Invest* 2011, 23:373–376.

28. Chalmers G, Bruce HL, Hunter DB, Parreira VR, Kulkarni RR, Jiang YF, Prescott JF, Boerlin P: Multilocus sequence typing analysis of *Clostridium perfringens* isolates from necrotic enteritis outbreaks in broiler chicken populations. *J. Clin. Microbiol* 2008, 46:3957–3964.

29. Fisher DJ, Miyamoto K, Harrison B, Akimoto S, Sarker MR, McClane BA: Association of beta2 toxin production with *Clostridium perfringens* type A human gastrointestinal disease isolates carrying a plasmid enterotoxin gene. *Mol Microbio* 2005, 56(3):747-62.

30. Lehe K: Successful control of clostridial enteritis: A report from the back forty. *In Proceedings of American Association of Swine Veterinarians: 7-10 March 2009; Dallas.*

31. Yaeger M, Funk N, and Hoffman L: A survey of agents associated with neonatal diarrhea in Iowa swine including *Clostridium difficile* and porcine reproductive and respiratory syndrome virus. *J Vet Diagn Investig* 2002, 14:281-287.

32. Smith WH, Jones JET: Observations on the alimentary tract and its bacterial flora in healthy and diseased pigs. *J Path Bact* 1963, 84:387-412.

CHAPTER TWO

The Epidemiology of *Clostridium perfringens* on Ontario Swine Farms

1. INTRODUCTION

Clostridium perfringens type A is considered by some to be one of the most common causes of diarrhea in neonatal piglets [1, 2]. However, the pathogenic basis of *C. perfringens* type A diarrhea is unclear, and the current diagnostic methods for this disease are not specific.

Clostridium perfringens are ubiquitous, Gram-positive anaerobes that can be isolated from many environments, and their spore-forming ability allows them to persist in the swine ecosystem [2]. Currently, *C. perfringens* are divided into five toxinotypes (A to E) depending on their production of four major toxins. Isolates of any toxinotype may produce enterotoxin (CPE) and beta2 toxin (CPB2) [3].

In the past decade, diagnosis of neonatal piglet diarrhea due to *C. perfringens* type A has increased, and has been associated with increased pre-weaned mortality [1]. Disease can develop in suckling pigs in the first week of life, and typical clinical signs include non-hemorrhagic, mucoid diarrhea seen within 48 h of birth, and lasting for 5 d [2]. The current method of diagnosis is generally based on isolation of large numbers of *C. perfringens* type A possessing the consensus CPB2 gene (*cpb2*) detected by polymerase chain reaction (PCR) from the feces or intestinal contents of the piglets and the exclusion of other known causes of neonatal diarrhea [3]. There is currently no commercial test available for detecting CPB2 in isolates [1, 3]. Most porcine isolates carry and express *cpb2* [4].

There is poor understanding of most aspects of *C. perfringens* type A as a possible cause of neonatal diarrhea in piglets, and the prevalence and types of *C. perfringens* present in Ontario swine farms is unknown. The objectives of this study were to compare the distribution of *C. perfringens* and selected toxin genes among pigs at different stages of production, and to identify farm factors affecting this distribution.

2. MATERIALS AND METHODS

2.1. Farm Selection

Forty-eight farms in Ontario were conveniently selected and visited once or twice between two periods of sample collection from August to December 2010, and from January 2011 to May 2011. For the first period of sample collections, 28 farms were visited. One farm was visited twice in the first period, and visited again in the second period. In the second period, a total of 11 farms from the first period were visited again, and 20 farms were visited for the first time. Some farms that were visited from the first period were not re-visited because they did not have suckling pigs or they were no longer in business.

2.2. Sample Collection

In both periods of sample collection, for farrow-to-finish farms, pooled-fecal samples were collected from gestating sows, weanling pigs, and grower-finisher pigs, and in addition a pooled sample was taken from the manure pit. In the second period of sample collection, fecal samples were also collected from lactating sows and their litters. A fecal sample of the lactating sow was collected by randomly selecting a sow, and manually obtaining a rectal sample with a gloved hand. A pooled-fecal sample of the

lactating sow's litter was also obtained by selecting three suckling pigs in the litter and collecting the feces from the piglet's rectum. If suckling pigs did not defecate enough during the sampling process, fresh piglet feces were obtained from the farrowing area of the litter. Three fecal samples were collected from lactating sows, and three pooled fecal samples were collected from their respective litters from each farrowing operation.

A pooled-fecal sample of gestating sows was obtained by selecting 6 sows from different areas of the barn, and obtaining a rectal sample. A pooled-fecal sample of weanling pigs or grower-finisher pigs was obtained from randomly selecting 6 pens, observing the pigs defecating, and then immediately collecting the fresh feces from the floor of the pens. On grower-finisher farms, 3 pooled-fecal samples were taken from the pigs present, as well as a pooled manure pit sample. All farms on the study used a liquid manure system and a sample from the holding tank or manure pit was obtained by filling a plastic bottle from the manure pit. Liquid manure from three locations in the pit and at three different depths was combined to create the pooled sample.

2.3. Questionnaire

A survey was administered to collect information on each farm for different management factors including the following: type of operation (farrowing-to-finish, farrowing-to-feeder, or grower-finisher operation), total number of barns, herd size (number of sows, gilts, weanling pigs, and grower-finisher pigs only), sow flow (all-in/all-out or continuous), weanling flow (all-in/all-out or continuous), grower-finisher flow (all-in/all-out or continuous), number of farrowing rooms and crates, the presence of other agricultural species (other species), drug usage and vaccination, history of diarrhea outbreak in suckling pigs or post-weaning pigs in the past year, and treatment of

the diarrhea problem. A total of 46 farms were surveyed; two farms were not surveyed because they were no longer in business.

2.4. Bacterial Isolation

For the bacterial culture, samples were weighed and four to five serial 10-fold dilutions were performed in phosphate buffered saline, pH 7.2. Fecal dilutions were plated onto selective SFP (Shahadi Ferguson Perfringens) medium (Difco, Detroit, MI, USA). The SFP media contains 5% egg yolk emulsion (Oxoid, Nepean, ON) and 12 μg/ml kanamycin sulphate and 30 IU/ml polymyxin B sulphate (SFP Selective Supplement, Oxoid). The plates were transferred to an anaerobic jar, with anaerobic atmosphere provided by GasPak (BD, Sparks, MD, USA) and incubated at 37°C overnight for 1-2 d. Colonies with characteristic *C. perfringens* colonial and microscopic morphology, as well as lecithinase activity on the egg yolk agar, were counted and counts converted to colony-forming units (CFU) per gram.

2.5. Real Time Fluorogenic Multiplex Polymerase Chain Reaction

Clostridium perfringens colony lysates were examined by multiplex polymerase chain reaction (PCR) for toxin genes using the method described by Albini and colleagues [5]. Five isolates per sample were genotyped for *cpa*, consensus *cpb2* (*cpb2*) and atypical *cpb2* (*atyp-cbp2)*. The isolates recovered during the first period of the study were also genotyped for the beta toxin (*cpb*), enterotoxin (*cpe*), epsilon toxin (*etx*), and iota toxin (*iap*), NetB toxin (*netB*) and large clostridial cyototoxin (*tpeL*). Since these were all absent they were not examined in the second period. The primer sets for the major toxin genes and *cpb2* used were designed using the reported nucleotide sequences [5]. The primer sets for *netB, tpeL*, and *atyp-cpb2* were designed for this study using

standard approaches and appropriate bacterial control strains. The Roche LightCycler® 480 SW 1.5 software package was used to analyze the data. Relevant positive control strains were included in all batched PCR tests.

2.6. Data Analysis

The data were entered in spreadsheet (Microsoft Excel 2007; Microsoft Corp., Redmond, WA) and imported to Stata 10 Intercooled for Windows XP (StataCorp LP, College Station, TX) for statistical analysis.

The Spearman's rank correlation test was used to analyze the correlation between these toxin genes in lactating sows and their litters.

A mixed linear regression modeling method with farm as a random effect was used to analyze the association between *C. perfringens* type A count in fecal samples and independent variables. Two separate logistic regression models with farm as a random effect were fitted to analyze the association between the presence of *cpb2* and *atyp-cpb2*, respectively in *C. perfringens* type A isolates and independent variables. Univariable regression was performed for linear regression for *C. perfringens* count ($\log_{10}$ CFU/g) (Appendix 2.1) and logistic regression for the presence of *cpb2* in *C. perfringens* type A (Appendix 2.5). The predictor variables with $P<0.20$ were selected for inclusion in multivariable analyses. Pair-wise correlation coefficients were calculated between independent variables, and coefficients with an absolute value greater than 0.8 were considered colinear. A manual stepwise procedure was used to build the models. A variable was identified as a confounder if it changed the coefficient of the main effects by 20% or more when the potential confounder variable was removed. Interaction was evaluated between all independent variables and the stage of production. Each interaction

term was assessed for statistical significance with the main effects model, and the interaction terms with $P<0.05$ were selected for inclusion in multivariable analysis. Interaction terms that were not significant in the final model were removed if removal of the interaction term did not result in a significant likelihood ratio test (logistic regression), or if the removal of the interaction term resulted in a smaller Bayesian Information Criteria (BIC) in the linear regression model.

For linear multivariable analysis (Appendix 2.4)., a $\log_{10}$ transformation was used on the *C. perfringens* count (CFU/g) in order to meet the normal distribution and homoscedasticity assumptions for the outcome of interest. The Cook-Weisberg test was performed for the linear multivariable model with farm as a fixed effect, and the assumption of homoscedasticity was met if $P>0.05$. The Shapiro-Wilk normality test was used to test the normal distribution of $\log_{10}$ CFU/g with farm as a fixed effect, and the assumption of normality was met if $P>0.05$. The standardized residuals were graphically assessed for outliers, and the leverage values were graphically assessed.

The logistic regression multivariable model with farm as a fixed effect for the presence of *cpb2* in *C. perfringens* type A isolates (Appendix 2.9) was assessed for goodness-of-fit, which was indicated by $P>0.05$ in the Hosmer-Lemeshow test. Pearson residuals were graphically assessed for outliers, and the leverage values were graphically assessed. The delta-beta values were graphically assessed for covariate patterns that have an influence on the model.

2.3. RESULTS

A total of 354 fecal samples were collected. Of the pooled-fecal samples, 37 were collected from gestating sows, 99 from grower-finisher pigs, 44 from weanling pigs, and

60 from the manure pit. Fecal samples were also collected from 57 lactating sows and 57 pooled samples from their suckling pigs. Overall, *C. perfringens* was isolated from 225 (63.6%) of 354 fecal samples (98% of suckling pigs, 34% of weanling pigs, 18% of grower-finisher pigs, 89% of gestating sows, 96% of lactating sows, and 75% of manure pit samples). A farm was considered positive for *C. perfringens* isolate if *cpa* was detected in the isolate of at least one fecal sample, and a farm was considered positive for consensus *cpb2* if it was detected in the *C. perfringens* isolate of at least one fecal sample. A total of 42 farms (87.5%) were positive for *C. perfringens*, and 25 farms (52%) were positive for *cpb2*. *C. perfringens* was not recovered from samples collected on 6 (12.5%) of 48 farms, all of which were grower-finisher operations. Within-herd prevalence of *C. perfring*ens type A ranged between 0 and 100% with a mean of 53.5 ± 32%.

3.1. *Clostridium perfringens* Count

The mean of $\log_{10}$ CFU/g of *C. perfringens* in fecal samples is shown in Table 2.1. The total mean count was 2.7±2.4 $\log_{10}$ CFU/g. The distribution of independent variables on 46 surveyed farms is shown in Table 2.2. Independent variables with a significant level of $P<0.20$ that were initially included in the multivariable linear regression analysis were the stage of production, type of operation, total swine herd size, and sampling period. In multivariable analysis, the $\log_{10}$ *C. perfringens* count was higher in suckling pig fecal samples compared to that of weanling pigs, grower-finisher pigs, and the manure pit ($P<0.05$) (Table 2.3). The $\log_{10}$ *C. perfringens* count was higher in gestating sow samples, lactating sow samples, or the manure pits, respectively, compared to that of weanling pigs and grower-finisher pigs ($P<0.05$). The $\log_{10}$ *C. perfringens*

count tended to be higher overall in the second sampling period, but this was not significantly different ($P>0.05$); the sampling period was included in the final model because it was a confounder in that the association between stage of production and *C. perfringens* count varied in the first and second period of study. In the final model, there was an interaction between production stage and the presence of other agricultural species ($P<0.05$) (Table 2.3).

Potential outliers were identified for the weanling fecal samples of two farms (Appendix 2.2): the weanling fecal samples from these two farms had a high *C. perfringens* count of 6.0 and 6.7 $\log_{10}$ CFU/g, respectively. Potential extreme observations (leverage) were identified for the weanling fecal samples of two farms, of which *C. perfringens* was not isolated (Appendix 2.3).

3.2. *Clostridium perfringens*-associated Toxin Genes

The distribution of *cpb2* and *atyp-cbp2* genes among *C. perfringens* isolates cultured from fecal samples is presented in Table 2.1.

The independent variables initially included in the multivariable logistic analysis were the stage of production, presence of *atyp-cpb2*, type of operation, presence of other species, herd size, and sampling period. The type of operation, presence of other species, and herd size did not significantly affect the likelihood of detecting *cpb2*-positive *C. perfringens* isolates in the final model ($P>0.05$). The variable of vaccination was not included in the statistical analysis because none of the farms surveyed vaccinated sows or pigs for *C. perfringens*.

Cpb2 was more likely found in the isolates of suckling pigs compared to those of lactating sows, gestating sows, and grower-finisher pigs, and manure pits ($P<0.05$) (Table 2.4). The likelihood of finding *cpb2* in *C. perfringens* isolates recovered from weanling pigs was higher compared to that of gestating sows (OR=13, $P=0.011$) and grower-finisher pigs (OR=15, $P=0.027$).

The Intraclass Correlation Coefficient (ICC) that compared the within-farm and between-farm variance was 0.24 and 0.25, respectively for linear regression and logistic regression, indicating a low level of clustering in which the farm-level variance of *C. perfringens* count and the presence of *cpb2* accounted for almost a quarter of the total variation.

The likelihood of detecting *cpb2* was higher in *atyp-cpb2* positive isolates compared to *atyp-cpb2* negative isolates ($P<0.001$) (Table 2.4). The percentages of samples that had *atyp-cpb2* were 51% in *cpb2*-positives samples, and 6% in *cpb2*-negative samples.

C. perfringens isolates recovered from lactating sows (OR=0.20, P=0.001), weanlings (OR=0.20, P=0.039), and the manure pits (OR=0.32, P=0.022) were less likely to be positive for *atyp-cpb2* than isolates recovered from suckling pigs. The correlation of *cpb2* or *atyp-cpb2* in isolates of lactating sows and in isolates from their litters was not statistically significant.

Potential outliers were identified for fecal samples from two farms (Appendix 2.6). On one farm, *cpb2* nor *atyp-cpb2* were detected in the isolates of a suckling pig fecal sample. On the other farm, *cpb2* was not detected in the *atyp-cpb2* positive isolates of the manure pit fecal sample. Potential extreme observations were identified for the

suckling pig fecal samples of one farm: the *cpb2*-positive isolates were only recovered from two of three fecal samples (Appendix 2.7). A potential influential covariate pattern was identified for one farm; the *cpb2* gene was not detected in the isolates from a grower-finisher fecal sample, but *cpb2* was detected in the isolates from the manure pit fecal sample (Appendix 2.8).

The isolates recovered during the first period of the study were all negative for *cpb*, *cpe*, *etx*, *iap*, *netB* and *tpeL*. For this reason, they were not examined in the second period.

4. DISCUSSION

The current study of enteric *C. perfringens* in pigs identified novel findings that may increase the understanding of the epidemiology of infection and possible diagnosis of the suckling pig diarrhea thought to be caused by this organism. The study is the first to examine systematically the quantitative prevalence of *C. perfringens*, as well as an expanded range of toxin genes, in pigs of different ages on multiple farms.

The role of *C. perfringens* type A in neonatal piglet diarrhea is not well understood. From 2004 to 2009, *C. perfringens* type A has been the second most commonly diagnosed infectious agent in neonatal piglet diarrhea cases submitted to the Iowa State University Veterinary Diagnostic Lab and the trend appears to be increasing [2]. With the relative decrease in prevalence of other common pathogens known to cause swine enteric illness, including *Escherichia coli*, transmissible gastroenteritis virus, and coccidia [2,6], and studies that identified an association between *cpb2*-positive *C. perfringens* type A and isolates from diseased piglets [7,8,9], *C. perfringens* type A is speculated to be an emerging pathogen associated with neonatal piglet diarrhea. Some

prevalence studies conducted on infectious causes of piglet diarrhea did not include *C. perfringens* type A in the analysis [6, 10, 11], perhaps because of the nonspecific diagnostic criteria for this disease. However, prevalence studies conducted in United States, Germany, and Japan could not identify any etiological agents respectively in 22%, 58%, and 17% of the total diarrheic pigs examined [6,10,11], which indicated that there could be unknown pathogens involved in causing the disease. There is a need to resolve whether *C. perfringens* type A is a true pathogen associated with neonatal piglet diarrhea or commensals of the normal piglet flora. It is possible that some strains of *C. perfringens* are involved in neonatal enteric disease while others are commensals. The specific role of *CPB2* in *C. perfringens* enteritis is unknown, and the presence of *cpb2* has been suggested as a virulence marker for *C. perfringens* isolates [3]. There is a great diversity within *C. perfringens* strains as demonstrated by a study conducted in the midwestern United States, but the strains recovered from scouring piglets could not be differentiated genetically from strains recovered from non-scouring piglets [12]. Similarly, a recent study in Ontario demonstrated that CPB2 was expressed in healthy and diarrheic piglets, and that quantifying the number of *cpb2*-positive *C. perfringens* type A in the intestinal contents was not a useful approach for making a diagnosis of *C. perfringens* type A enteritis in diarrheic piglets [13]. It is possible that healthy piglets possess toxigenic strains of *C. perfringens* type A, but the manifestation of enteric disease may be caused by multiple factors, such as rapid bacterial proliferation and toxin production, unknown toxins, or environmental factors [12, 13].

Our results showed that the number of *C. perfringens* type A isolates was higher in suckling pigs compared to pigs in other stages of production. High numbers of *C.*

perfringens were also isolated from gestating and lactating sows. The pattern of decline in colonization by *C. perfringens* with age has been noted previously [14]. The decrease in *C. perfringens* count for fecal samples in weanling and grower-finisher pigs was expected, since the population of *C. perfringens* decreases in the pig intestine as other bacterial species establish their populations [2]. The numbers of *C. perfringens* declined in pigs that reach the grower-finisher stage, which may be partly due to changes in management factors including the use of antimicrobials during the nursery and grower-finisher stages. The usage of antimicrobials during the grower stage may interrupt the gut microflora and decrease the number of *C. perfringens*. Host-related factors that affect the decline in colonization may include changes in large intestinal microflora with time, age-related changes in host physiology, or the development of intestinal immunity. Many *C. perfringen*s isolated from pigs are relatively unusual among isolates from different animal sources in that they belong to a clonal population [15]. Unusually, these isolates are characterized both by being consensus *cpb2*-positive and by expressing CPB2 [4]. There thus appears to be an adaptation of this lineage to swine. On the basis of the present study, the adaptation of this lineage appears to be to the young pig, and notably to the suckling piglet. However, we did not examine the isolates made here for the phylogenetic relationships.

Most of the suckling pig isolates in this study were positive for consensus *cpb2* as noted in other studies [4, 7, 8, 9]. The gestating sow isolates were usually not positive for *cpb2* and lacked the *atyp-cpb2* gene that was found in lactating sow and suckling pig isolates, which appear to be specifically colonized by *cpb2*-positive strains [3]. It is possible that the sows in the lactating stage acquired *cpb2*-positive *C. perfringens* strains

from suckling pigs in the litter, but the strains were not always maintained when the suckling pigs are weaned and sows returned to the gestating stage. However, there was no significant correlation of the presence of *cpb2* and *atyp-cpb2* in *C. perfringens* type A isolates from sows and their litters, suggesting that the source of the strains in suckling pigs may be different from sows, or that *cpb2* and/or *atyp-cpb2* positive isolates are more likely to proliferate in suckling pigs. Interestingly, there was a dominance of *atyp-cpb2* isolates in *C. perfringens* isolated from grower-finisher pigs. It is possible that other factors contributed to the decline in consensus *cpb2*-positive *C. perfringens* isolates seen in grower-finisher pigs, such as antimicrobial usage. A negative association between *cpb2*-positive *C. perfringens* isolates and resistance to erythromycin and clindamycin was documented in an Ontario study, but they did not differentiate the isolates possessing consensus *cpb2* and atypical *cpb2* [16]. The isolates from the manure pit were unexpectedly similar to isolates from suckling pigs, suggesting a selective advantage of possessing *cpb2* gene in both this environment and in the suckling pig intestine. Speculatively, the manure pit may represent an ecologically unstable environment, which may also be typical of the suckling pig intestine. This study identified genes in the most abundant isolate of *C. perfringens*, so that the less abundant genotypes may not have been detected. *C. perfringens* isolates positive for *cpb2* were more likely to be positive for *atyp-cpb2*, suggesting a linkage between these genes. Further research on the genetic diversity of *C. perfringens* type A isolates in clinically healthy and diarrheic suckling pigs, and the conditions of which *cpb2* and *atyp-cpb2* toxin genes are expressed may help clarify the role of this agent in neonatal piglet diarrhea.

Farms were surveyed for a variety of management factors that might affect the epidemiology of enteric *C. perfringens*. The number of *cpb2*-positive farms appeared higher in farrowing operations compared to grower-finisher operations because *cpb2* was mostly present in *C. perfringens* isolates of suckling pigs. In multivariable analysis most of the management factors did not appear change the distribution of *cpb2*-positive farms, including pig flow, antibiotic usage, and history of diarrhea. This may suggest that *cpb2*-positive isolates were ubiquitous and farm management factors did not affect the distribution of *cpb2*-positive isolates. Excluding sow fecal samples, there was a decrease in *C. perfringens* count for samples that came from mixed farms (where other agricultural species were present). However, there was an increase in *C. perfringens* count in fecal samples that came from lactating or gestating sows compared to suckling pigs in mixed farms. This may suggest that the sows were harboring *C. perfringens* isolates that originated from other species.

Since the farms in our study were conveniently selected, the results may not apply to the general population of Ontario swine farms. The difference in bacterial count of *C. perfringens* in fecal samples from pigs at different stages of production might have been affected by the sampling procedure, since samples for weanling pigs and grower-finisher pigs were collected from feces in the pen flooring area while the samples for sows and suckling pigs were collected from the rectum. The fecal samples of gestating sows, grower-finishers, and weanlings were pooled. It was possible that some individual pigs were negative for *C. perfringens* or isolates with selected toxin genes, but the pig fecal samples were deemed positive if *C. perfringens* or isolates with selected toxin genes were found in any isolate of the pooled fecal samples.

This is the first time that *C. perfringens* type A from swine has been examined for *netB* and *tpeL,* genes that are common in isolates from chickens with necrotic enteritis [17]. Their absence supports the idea of host adaptation of strains of *C. perfringens*, as apparent here in the association of *cpb2*-positive strains with piglets. The absence of *cpb* is consistent with the absence of *C. perfringens* type C in enteric disease in swine and other animals in Ontario. The lack of *cpe* positive *C. perfringens* isolates confirms earlier studies [18, 19].

5. CONCLUSION

To our best knowledge, this is the first study on the epidemiology of *C. perfringens* in the swine population. This study provides baseline data from healthy pigs at different stages of production as well as in the manure pits for future studies to investigate the role of *C. perfringens* in swine enteric diseases. The study suggests that if C. *perfringens* type A are involved in neonatal enteritis, there may be strains with specific characteristics that cannot be identified by the existing genotyping system. Further work is required to determine whether this is the case.

REFERENCES CITED

1. Lehe K: Successful control of clostridial enteritis: A report from the back forty. *In Proceedings of American Association of Swine Veterinarians: 7-10 March 2009; Dallas.*

2. Schwartz KJ: *Clostridium*-associated diseases in swine. *In Proceedings of American Association of Swine Veterinarians: 7-10 March 2009; Dallas.*

3. Songer JG, Uzal FA: Clostridial enteric infections in pigs. *J Vet Diag Invest* 2005, 17:528-536.

4. Jost BH, Billington SJ, Trinh HT, Bueschel DM, Songer JG: Atypical *cpb2* genes, encoding beta2-toxin in *Clostridium perfringens* isolates of nonporcine origin. *Infect Immun* 2005, 73:652–656.

5. Albini S, Brodard I, Jaussi A, Wollschlaeger N, Frey J, Miserez R, Abril C: Real-time multiplex PCR assays for reliable detection of *Clostridium perfringens* toxin genes in animal isolates. *Vet Microbiol* 2008, 127:179–185.

6. Yaeger M, Funk N, and Hoffman L: A survey of agents associated with neonatal diarrhea in Iowa swine including *Clostridium difficile* and porcine reproductive and respiratory syndrome virus. *J Vet Diagn Invest* 2002, 14:281-287.

7. Garmory HS, Chanter N, French NP, Bueschel D, Songer JG, Titball RW: Occurrence of *Clostridium perfringens* b 2-toxin amongst animals, determined using genotyping and subtyping PCR assays. *Epidemiol Infect* 2000, 124:61–67.

8. Bueschel, DM, Jost BH, Billington SJ, Trinh HT, Songer JG: Prevalence of cbp2, encoding beta2 toxin, in *Clostridium perfringens* field isolates: correlation of genotype with phenotype. *Vet Microbiol* 2002, 94:121–129.

9. Waters M, Savoie A, Garmory HS, Bueschel D, Popoff MR, Songer GJ, Titball RW, McClane BA, Sarker MR: Genotyping and phenotyping of beta2-toxigenic *Clostridium perfringens* fecal isolates associated with gastrointestinal diseases in piglets. *J Clin Microbiol* 2003, 41:3584–3591.

10. Wieler LH, Ilieff A, Herbst W, Bauer C, Vieler E, Bauerfeind R, Failing K, Klos H, Wengert D, Baljer G, and Zahner H: Prevalence of enteropathogens in suckling and weanling suckling pigs with diarrhea in southern Germany. *J Vet Med B* 2001, 48:151-159.

11. Katsuda K, Kohmoto M, Kawashima K, and Tsunemitsu H: Frequency of enteropathogen detection in suckling and weanling pigs with diarrhea in Japan. *J Vet Diagn Invest* 2006, 18:350-354.

12. Baker AA, Davis E, Rehberger T, and Rosener D: Prevalence and diversity of toxigenic *Clostridium perfringens* and *Clostridium difficile* among swine herds in the midwest. *Appl Environ Microbiol* 2010, 76:2961-2967.

13. Farzan A, Kircanki J, DeLay J, Soltes G, Songer GJ, Friendship R, Prescott JF: An investigation into the association between *cpb2*-encoding C. perfringens type A and diarrhea in neonatal piglets. *Can J Vet Res, in press.*

14. Smith WH, Jones JET: Observations on the alimentary tract and its bacterial flora in healthy and diseased pigs. *J Path Bact* 1963, 84:387-412.

15. Jost HB, Trinh TH, Songer GJ: Clonal relationships among *Clostridium perfringens* of porcine

origin as determined by multilocus sequence typing. *Vet Microbiol* 2006, 116:158-65.

16. Slavic D, Boerlin P, Fabri M, Klotins KC, Zoethout JK, Weir PE, Bateman D: Antimicrobial susceptibility of *Clostridium perfringens* isolates of bovine, chicken, porcine, and turkey origin from Ontario. *Can J Vet Res* 2011, 75:89–97.

17. Chalmers G, Bruce HL, Hunter DB, Parreira VR, Kulkarni RR, Jiang YF, Prescott JF, Boerlin P: Multilocus sequence typing analysis of *Clostridium perfringens* isolates from necrotic enteritis outbreaks in broiler chicken populations. *J. Clin. Microbiol* 2008, 46:3957–3964.

18. Kanakaraj R, Harris DL, Songer GJ, Bosworth B: Multiplex PCR assay for detection of *Clostridium perfringens* in feces and intestinal contents of pigs and in swine feed. *Vet Microbiol* 1998, 63:29-38.

19. Klaasen HL, Molkenboer MJ, Bakker J, Miserez R, Häni H, Frey J, Popoff MR, van den Bosch JF: Detection of the beta2 toxin gene of *Clostridium perfringens* in diarrhoeic piglets in The Netherlands and Switzerland. *FEMS Immunol Med Microbiol* 1999, 24(3):325-32.

TABLES

Table 2.1: Log_{10} CFU/g of *C. perfringens* in fecal samples and the positive proportion of toxin genes.

	Log_{10} (CFU/g)	Percentage of positive samples		
	Mean ± SD	*cpb2*	*atyp-cpb2*	Total No. of Samples
Suckling pigs	5.0 ± 1.70	77	44	57
Lactating Sows	4.0 ± 1.86	26	16	57
Gestating Sows	4.3 ± 1.85	5	0	37
Weanling pigs	1.3 ± 1.87	18	7	44
Grower-finisher pigs	0.6 ± 1.24	2	5	99
Manure Pit	2.7 ± 1.82	27	17	60
Total	2.7 ± 2.38	25	15	354

Table 2.2: The distribution of independent variables among 46 surveyed farms.

Independent Variable		Number of *cpb2*-positive farms (%)	Number of *cpb2*-negative farms (%)	Univariable analysis *P*-value
Type of operation	Farrowing (n=30)	22 (73)	8 (27)	0.012
	Grower-finisher (n=16)	4 (25)	12 (75)	
Other species	Not present (n=23)	13 (57)	10 (43)	0.004
	Present (n=23)	13 (57)	10 (43)	
Herd Size	Small (<1000) (n=9)	3 (33)	6 (67)	Referent
	Medium (≥1000-3000) (n=28)	17 (61)	11 (39)	0.272
	Large (>3000) (n=9)	6 (67)	4 (33)	0.018
Sow Flow	All-in/all-out (n=16)	12 (75)	4 (25)	0.417
	Continuous (n=14)	10 (71)	4 (29)	
Weanling Flow	All-in/all-out (n=23)	16 (70)	7 (30)	0.326
	Continuous (n=8)	7 (87.5)	1 (12.5)	
Grower-Finisher Flow	All-in/all-out (n=27)	13 (48)	14 (52)	0.327
	Continuous (n=13)	7 (54)	6 (46)	
Antibiotic usage	Yes (n=7)	7 (100)	0	0.194
	No (n=23)	15 (65)	8 (35)	
Diarrhea	Yes (n=11)	9 (82)	2 (18)	0.562
	No (n=19)	13 (68)	6 (32)	

Table 2.3: Linear regression comparing association of *C. perfringens* (log_{10} CFU/g) and independent variables.

Parameters		Coefficient	Standard Error	95% Confidence Interval	*P*-value
Stage	Piglet	Referent			
	Lactating sow	-1.9	0.367	(-2.7)-(-1.2)	<0.001
	Gestating sow	-1.4	0.438	(-2.2)-(-0.50)	0.002
	Weanling	-3.8	0.398	(-4.6)-(-3.1)	<0.001
	Grower-finisher	-4.4	0.413	(-5.2)-(-3.6)	<0.001
	Manure pit	-1.7	0.403	(-2.5)-(-0.93)	<0.001
Other species	Absent	Referent			
	Present	-0.93	0.484	(-1.9)-(-0.019)	0.055
	Present x Gestating sow	1.5	0.626	0.30-2.75	0.015
	Present x Lactating sow	1.8	0.533	0.79-2.9	0.001
Sampling Period	First	Referent			
	Second	0.34	0.234	(-0.12)-(-0.80)	0.144

Table 2.4: Logistic regression comparing association of *cpb2* positive *C. perfringens* isolates and independent variables.

Parameters		Odds Ratio	Standard Error	95% Confidence Interval	*P*-value
Stage	Piglet	Referent			
	Lactating sow	0.069	0.0437	0.020-0.24	<0.001
	Gestating sow	0.020	0.0189	$3.2x10^{-3}$-0.13	<0.001
	Weanling	0.26	0.216	0.052-1.3	0.103
	Grower-finisher	0.017	0.0185	$2.0x10^{-3}$-0.14	<0.001
	Manure pit	0.11	0.0677	$3.2x10^{-2}$-0.37	<0.001
Atyp-cpb2	Negative	Referent			
	Positive	19	12.0	5.7-65	<0.001

CHAPTER THREE

A Retrospective Study of the Etiological Diagnosis of Diarrhea in Suckling Pigs between 2001 and 2010

1. INTRODUCTION

Neonatal piglet diarrhea is a major cause of pre-weaning mortality in the swine farrowing operation, resulting in significant economic lost. The relative importance of different diseases contributing to neonatal piglet diarrhea is changing, possibly because of changes in husbandry and management practices, or advances in diagnostic techniques or emergence of new diseases. Reports in the United States have indicated an increase in neonatal piglet diarrhea cases attributed to *Clostridium difficile* in a survey conducted in 2000 [1], and in *Clostridium perfringens* type A in diagnostic laboratory submission data from 2004 to 2009 [2]. The reports also indicate a relative decrease in diagnosed cases for *E. coli*, TGEV, and *C. perfringens* type C compared to retrospective data from previous decades [1,2].

In recent years, clostridial infections as causes of neonatal diarrhea in piglets have gained increased prominence. *Clostridium spp.* are ubiquitous, Gram-positive anaerobic bacteria with spore-forming ability. *C. perfringens* are divided into 5 toxinotypes [2,3]. Some studies have found an association between *C. perfringens* type A strains containing the beta2 toxin gene (*cpb2*) and neonatal piglet diarrhea [4,5]. The current diagnostic method for *C. perfringens* type A enteritis is not specific and is generally based on isolation of large numbers of *C. perfringens* type A from the intestinal contents, absence of other pathogens known to cause diarrhea, and the detection of *cpb2* through polymerase chain reaction (PCR) [3]. Diarrhea associated with *C. difficile* can also occur

in piglets between 1 to 7 days of age, with some reports also including sudden death and scrotal edema [2,3]. Diagnosis of *C. difficile*-associated neonatal piglet diarrhea is generally confirmed with demonstration of colonic lesions and toxin detection via commercially available enzyme-linked immunosorbent assay (ELISA) kits [1,3].

The purpose of the study was to use passive laboratory surveillance data to identify the frequency and trends of different pathogens contributing to neonatal piglet diarrhea in Ontario swine farms from 2001 to 2010.

2. MATERIALS AND METHODS

2.1. Data Collection

The data were provided by the Animal Health Laboratory (AHL) at the University of Guelph, and included laboratory submissions from Ontario swine farms from 2001-2010. The data included gastrointestinal cases (GIT case) where live or dead pigs between 1 to 7 days of age were submitted. A GIT case was defined as a case submitted for one or more of the following reasons observed in piglets: enteritis, diarrhea, scours, unthriftiness, weight loss, sudden death, or increased mortality. The data set for GIT cases submitted from January 2001 to April 2007 was provided in text file, and the data set for GIT cases submitted from May 2007 to December 2010 was provided in a spreadsheet (Microsoft Excel 2007; Microsoft Corp., Redmond, WA).

2.2. Diagnostic Method

The diagnosis of the pathogen(s) involved in a GIT case was based on necropsy examination of submitted pigs, identifying suspected pathogen(s) based on gross and histological lesions, and performing tests to identify the suspected pathogen(s). Tests for

identifying bacteria or viruses were performed on the feces or contents of the small intestine, colon, or cecum of the pigs. The diagnosis of colibacillosis required evidence of colonization by short rod-shaped bacilli at the intestinal mucosa, isolation of *E. coli,* and identification of enterotoxigenic *E. coli* (ETEC) strains through agglutination serotyping or genotyping through gel-based polymerase chain reaction (PCR). Diagnosis of transmissible gastroenteritis (TGE) required evidence of atrophic enteritis, and identification of TGE virus by a positive fluorescent antibody test (FAT) or positive immunohistochemistry (IHC). Diagnosis of rotavirus required evidence of atrophic enteritis, and identification of rotavirus through a positive FAT or positive latex agglutination test (LAT), which detected rotavirus A. When other types of rotavirus infection were suspected, multiplex real-time reverse-transcriptase polymerase chain reaction (real time RT-PCR) was performed which detected rotavirus A and C, or gel-based RT-PCR which detected rotavirus B. The PCR tests for detecting rotavirus were available from September 2010 onward [6]. Diagnosis of coccidiosis caused by *I. suis* required evidence of villus atrophy and/or *Isospora* merozoites, schizonts or oocysts in the cytoplasm of the mucosal epithelial cells, and identification of oocysts in the feces through positive sucrose wet mount or fecal flotation tests. Sucrose wet mount of intestinal contents also identified *Cryptosporidium parvum.* Diagnosis of *C. perfringens* type A required evidence of long rod-shaped bacilli colonization in the small intestine, isolation of *C. perfringens,* and genotyping of *C. perfringens* strains through gel-based PCR, which detected the toxinotype (A, B, C, D, E) and required evidence that the isolates were positive for *cpb2*. Diagnosis of *C. difficile* required evidence of colitis, detection of *C. difficile* toxins (A and B) via enzyme-linked immunosorbent assay

(ELISA), and isolation of *C. difficile* (the culture method was available in March 2008 onward) [7].]. Bacterial culture of the piglet intestinal contents was used to identify other bacterial pathogens, including *Salmonella enterica* serovars, and *Enterococcus durans*. Identification of porcine reproductive and respiratory syndrome virus (PRRSV) infection in lung tissue was accomplished by positive staining of PRRSV antigen by IHC, or by identifying PRRSV using gel-based PCR (before June 2010) or multiplex real-time RT-PCR (from June 2010 onward) [8].

2.3. Data Analysis

The two data sets were merged in a spreadsheet and imported to Stata 10 Intercooled for Windows XP (StataCorp LP, College Station, TX) for statistical analysis. Separate logistic regression models were fitted each for ETEC (Appendix 3.2), *C. perfringens* (Appendix 3.1), and *C. difficile* (Appendix 3.5) to analyze the association between diagnosis of the pathogen and independent variables. Univariable regression was performed and the predictor variables with $P<0.20$ were selected for inclusion in multivariable analyses, respectively for the diagnosis of ETEC (Appendix 3.4), *C. perfringens* (Appendix 3.2), and *C. difficile* (Appendix 3.5). The year for which the cases were diagnosed was included as a fixed effect for ETEC and *C. perfringens*. Univariable logistic regression models were used to analyze the association between the diagnosis of rotavirus and *I. suis*, respectively and the season for which the GIT cases occurred (Appendix 3.5). Pair-wise correlation coefficients were calculated between independent variables, and coefficients with an absolute value greater than 0.8 were considered colinear. A manual stepwise procedure was used to build the models. A variable was identified as a confounder if it changed the coefficient of the main effects by 20% or

more when the potential confounder variable was removed. Interaction was evaluated between the detection of each pathogen. Each interaction term was assessed for statistical significance with the main effects model, and the interaction terms with $P<0.05$ were selected for inclusion in multivariable analysis. Interaction terms that were not significant in the final model were removed if removal of the interaction term did not result in a significant change in the likelihood ratio test. The logistic regression models were assessed for goodness-of-fit, which was indicated by $P>0.05$ in the Hosmer-Lemeshow test or Pearson's chi-square test.

3. RESULTS

A total of 237 GIT cases involving the submission of live or dead piglets, 1 to 7 days of age, from 2001 to 2010 were submitted to the AHL. The number of these GIT cases submitted per year ranged from 10 to 39, with a mean of 23.7 $\pm$ 7.97 cases per year. A successful diagnosis was defined as a gastrointestinal case where the etiological agent was identified. The diagnostic success rate for GIT cases submitted per year is indicated in Figure 1. The annual prevalences for GIT cases diagnosed with single etiological agents are indicated for ETEC, *C. perfringens*, rotavirus, *I. suis*, *C. difficile*, and TGEV (Figure 2).

There were 79 (33%) GIT cases submitted to the AHL where an etiological agent was not identified. There were a total of 51 (22%) cases in the fall, 50 cases in the spring (21%), 46 (19%) cases in the summer, and 90 (38%) cases in the winter. The diagnosis of pathogens involved in each GIT case is given in Table 3.1 (individual cases indicated in Appendix 3.6).

Enterotoxigenic *E. coli* infection was diagnosed as the cause of GIT disease for 63 cases that involved a single etiological agent, and 10 cases that involved multiple etiological agents (31% of total cases) (Table 3.1). ETEC was less likely recovered from a GIT case if *C. difficile*, *C. perfringens*, or rotavirus were detected ($P<0.05$) (Table 3.2). ETEC was more likely diagnosed for GIT cases that occurred in the winter compared to the spring and the summer ($P<0.05$) (Table 3.2). There was a tendency for a GIT case to be more likely diagnosed with ETEC between year 2001 and 2006 than in 2009 ($P<0.1$) (Table 3.2).

C. perfringens was diagnosed as the cause of GIT disease for 19 cases that involved a single etiological agent, and 9 cases that involved multiple etiological agents (12% of total cases). A total of 155 GIT cases were cultured for *C. perfringens*, and the organism was isolated in 133 (86%) of cases. GIT cases were less likely to be diagnosed with *C. perfringens* with increasing age of the pigs ($P<0.05$) and if ETEC was recovered ($P=0.065$) (Table 3.3). *C. perfringens* was more likely diagnosed for GIT cases that occurred in the winter compared to the fall and the spring ($P<0.05$) (Table 3.3), and more likely diagnosed for GIT cases that occurred in the summer compared to the fall (OR=0.13, $P=0.022$) and the spring (OR=0.13, $P=0.029$). The odds of a GIT case being diagnosed with *C. perfringens* was greater in 2010 compared to the years between year 2002-2007 ($P<0.10$). *C. perfringens* isolates from 40 cases were genotyped for major toxin genes and *cpb2*. All isolates from the 40 cases belonged to toxinotype A, and *cpb2* was detected in the *C. perfringens* isolates of 38 (95%) cases. Of the 40 GIT cases where *C. perfringens* isolates were genotyped, 17 cases were diagnosed with *C. perfringens* as

the etiological agent (42.5%). Genotyping was not performed on the isolates from 11 cases diagnosed with *C. perfringens* as the etiological agent.

Rotavirus was diagnosed as the cause of GIT disease for 18 cases that involved a single etiological agent, and 10 cases that involved multiple etiological agents (12% of total cases). Rotavirus was more likely diagnosed for GIT cases that occurred in the fall compared the spring (OR=0.14, *P*=0.012) and the summer (OR=0.15, *P*=0.016).

C. difficile was diagnosed as the cause of GIT disease for 10 cases that involved a single etiological agent, and 11 cases that involved multiple etiological agents (9% of total cases). *C. difficile* was less likely detected if ETEC was recovered (OR=0.1, *P*=0.029), but *C. difficile* was more likely detected if *Salmonella sp.* was detected (OR=11, *P*=0.007).

Isospora suis was diagnosed as the cause of GIT disease for 13 cases that involved a single etiological agent, and 2 cases that involved multiple etiological agents (6% of total cases). Coccidiosis was more likely diagnosed for GIT cases that occurred in the summer compared to the spring (OR=0.26, *P*=0.03), and the winter (OR=0.046, *P*=0.004). TGEV was diagnosed as the cause of GIT disease for 6 cases that involved a single etiological agent and 1 case that involved multiple etiological agents (3% of total cases).

4. DISCUSSION

Neonatal diarrhea cases were most frequent during winter (38%), a well-established finding in countries with harsh winters [9]. The major known pathogens that contributed to neonatal piglet diarrhea from 2001 to 2010 were ETEC, rotavirus, *C.*

perfringens, and *C. difficile.* The combined GIT cases involving these pathogens contribute to more than half of the GIT cases submitted to the AHL. Interestingly, there was marked annual variation in specific etiological diagnoses although the reason for this annual variation is unclear.

Enterotoxigenic *E. coli* was a common cause of neonatal diarrhea from 2001 to 2005 in cases involving single etiological agents, but there was a relative decrease in the number of cases diagnosed with this agent from 2006 onward. ETEC was more frequently diagnosed for GIT cases that occurred in the winter compared to the spring and summer. Post-weaning ETEC enteritis has been documented to occur most frequently in the fall and the winter [15], and another study has reported that neonatal piglet colibacillosis cases were least prevalent in the spring compared to other seasons [17].

Rotavirus was an important pathogen contributing to neonatal piglet diarrhea. The AHL reported that approximately 50% of rotavirus cases were attributed to rotavirus group A [6]. It is possible that rotavirus infection caused by other groups (B or C) were underdiagnosed at the AHL, since PCR methods available for detecting these rotavirus groups were only available from 2010 onward [6]; the introduction of RT-PCR was reported by the AHL to increase the diagnosis of rotavirus B and rotavirus C infection [8]. Studies conducted on the frequency of different swine enteric pathogens have also indicated that rotavirus is a common pathogen isolated from diarrheic neonatal piglets [1,10,11]. Our study reported that rotavirus was more likely to be diagnosed for GIT cases that occurred in the fall compared to the spring and the summer, suggesting that rotaviral infection often occurred in the fall season.

C. perfringens was the second most frequent etiological diagnosis of GI illness in piglets within the first week of their life in this study. It is possible that diarrhea associated with *C. perfringens* type A is misdiagnosed, since the current diagnostic method for this pathogen is not specific. There was a total of 16 undiagnosed GIT cases submitted to the AHL that were suspected to involve *C. perfringens*, but *C. perfringens* was not included in the final diagnosis (data not shown) and the etiology was indicated as unknown. Genotyping confirmed the absence of *C. perfringens* type C infection in Ontario swine. In addition, most isolates were positive for *cpb2*. *C. perfringens* was considered as the etiological diagnosis for only a small proportion of cases with a positive *C. perfringens* in culture, suggesting that *C. perfringens* was often regarded as a normal commensal of neonatal piglets by the pathologist. A negative association was seen in diagnosis of *C. perfringens* and piglet age, and it has been reported that *C. perfringens* associated diarrhea usually occurs within 48 h after birth [2]. Our study indicated that *C. perfringens* was more likely diagnosed for GIT cases that occurred in the winter compared to the fall and the spring. Previous studies have indicated an association with *cpb2*-positive *C. perfringens* type A and neonatal piglet diarrhea [4,5]. However, a recent study indicated that the beta2 toxin CPB2 was detected in both healthy and diarrheic piglets, and counting the numbers of *cpb2*-positive *C. perfringens* type A did not provide a useful diagnosis for *C. perfringens* type A enteritis [11]. Because *cpb2*-positive *C. perfringens* were isolated from diarrheic piglets where an etiological agent was not identified in that study, it was suggested that *C. perfringens* type A enteritis was often misdiagnosed in cases where no other known pathogens except *C. perfringens* was identified [11].

Neonatal diarrhea cases attributed to *C. difficile* appeared sporadic, and approximately half of the total cases associated with *C. difficile* involved a complex of other pathogens. For example, *C. difficile* was more likely to be diagnosed for GIT cases when *Salmonella sp*p. was also isolated. Some studies indicate that *C. difficile* is an emerging pathogen in neonatal diarrhea [1,11]. The difficulty of culturing this anaerobic organism suggest that cases attributed to *C. difficile* may be underdiagnosed [3]. The bacterial culture test for *C. difficile* became available at the AHL in 2008 [7]. There is currently no uniform consensus "gold standard" method for the diagnosis of *C. difficile* enteritis in humans, and guidelines for diagnosing *C. difficile* enteritis in animals are unavailable [16]. A study has reported that the commercial tests available for detection of *C. difficile* toxins have lower sensitivity and specificity in piglets compared to humans [16]. A two-step algorithm has been recommended for the detection of *C. difficile* in pig herds, similar to that used in medical diagnostic laboratories: a first step suggested was the use of a test with high negative predictive value such as real-time PCR, followed by confirmation of the positive results with toxigenic culture as a second step [16]. However, the study was more focused on the presence or absence of the organism in herds rather than specific diagnosis of *C. difficile* enteritis in piglets [16]. *C. difficile* toxins can be detected in healthy piglets and therefore its presence alone does not confirm the diagnosis. However, it is suggested that *C. difficile* may be an important pathogen causing subclinical infection in piglets [12]. Clearly, further work is required to develop a sensitive diagnostic approach for *C. difficile* enteritis in piglets.

Isospora suis was diagnosed as a single etiological agent in neonatal diarrhea cases that occurred mainly from 2001 to 2005. The general lack of diarrheal cases attributed to

I. suis from 2006 onwards suggests that it is currently not a major pathogen of concern for 1-to 7-day-old pigs. This is in agreement with reports that conclude coccidiosis is associated with a diarrhea that begins after 1 or 2 weeks of age [10-14]. Coccidiosis was more likely diagnosed for GIT cases that occurred in the summer compared to the spring and the winter, in agreement with another report that indicated neonatal piglet coccidiosis cases were most frequently identified in the summer [9]. Neonatal diarrhea cases attributed to TGEV were sporadic and absent from 2008 onwards, indicating that it is not currently a concern for the Ontario swine industry.

The number of neonatal pig GIT cases submitted to the AHL generally decreased in the 10-year period, which may reflect the decrease in number of swine operations, an actual decrease in prevalence of neonatal diarrhea due to improved farm management practices, or other factors including poor economic returns from 2007 to 2010. Furthermore, no etiological agent was determined in one third of the GIT cases submitted to the AHL. This suggests that there may be emerging pathogens causing neonatal piglet diarrhea that are not detected by current diagnostic methods. Three prevalence studies on enteric pathogens involved in piglet diarrhea conducted in United States, Germany, and Japan could not identify etiological agents in 22%, 58% and 17%, respectively of the total pigs examined [1,13,10]. A recent study was also conducted in Ontario which identified enteric pathogens in diarrheic suckling pigs, but the etiological agent was not identified in 38% of total pigs [11].

It is possible that some GIT cases had an etiological agent identified, but that it was not recorded. Another reason for the lack of diagnosis in some GIT cases is that the producer may choose not to pursue additional laboratory tests for cost reasons. There may

be other unknown pathogens involved in the undiagnosed GIT cases submitted to the AHL and further investigation is required to determine the current causes of piglet diarrhea in Ontario swine farms.

5. CONCLUSION

This study identified the current pathogens involved in neonatal diarrhea for Ontario swine farms. *C. difficile* appears to be an emerging pathogen, and ETEC and rotavirus remain as pathogens of concern for neonatal piglet diarrhea. Further research in the diagnostic method of these pathogens may be useful in improving the diagnostic rate for GIT cases. The data suggested that *C. perfringens* type A is an important pathogen for neonatal piglet diarrhea, but the lack of specific diagnostic method currently for this pathogen made it difficult to determine the significance of this pathogen.

REFERENCES CITED

1. Yaeger M, Funk N, and Hoffman L: A survey of agents associated with neonatal diarrhea in Iowa swine including *Clostridium difficile* and porcine reproductive and respiratory syndrome virus. *J Vet Diagn Investig* 2002, 14:281-287.

2. Schwartz KJ: Clostridium-associated diseases in swine. *In Proceedings of American Association of Swine Veterinarians: 7-10 March 2009; Dallas.* 415-421.

3. Songer JG, Uzal FA: Clostridial enteric infections in pigs. *J Vet Diag Invest* 2005, 17:528-536.

4. Garmory HS, Chanter N, French NP, Bueschel D, Songer JG, Titball RW: Occurrence of *Clostridium perfringens* b 2-toxin amongst animals, determined using genotyping and subtyping PCR assays. *Epidemiol Infect* 2000, 124:61–67.

5. Bueschel, D M, Jost BH, Billington SJ, Trinh HT, Songer JG: Prevalence of cbp2, encoding beta2 toxin, in *Clostridium perfringens* field isolates: correlation of genotype with phenotype. *Vet Microbiol* 2002, 94:121–129.

6. Carman S: AHL Newsletter, volume 14, number 3. [http://guelphlabservices.com/files/AHL/AHL%20Newsletters/2010/AHLNewsletter%20Sept%202010.pdf]

7. Slavic D: AHL Newsletter, volume 12, number 1. [http://guelphlabservices.com/files/AHL/AHL%20Newsletters/2008/AHLNewsletter%20March%202008.pdf]

8. Carman S, McEwen B, Fairles J: AHL Newsletter, volume 14, number 2. [http://www.guelphlabservices.com.asp1-13.dfw1-

2.websitetestlink.com/files/AHL/AHL%20Newsletters/2010/AHLNewsletter%20June%202010.pdf]

9. Sanford SE, Josephson GKA: Porcine Neonatal Coccidiosis. *Can Vet J* 1981, 22:282-285.

10. Katsuda K, Kohmoto M, Kawashima K, and Tsunemitsu H: Frequency of enteropathogen detection in suckling and weanling pigs with diarrhea in Japan. *J Vet Diagn Investig* 2006, 18:350-354.

11. Farzan A, Kircanki J, DeLay J, Soltes G, Songer GJ, Friendship R, Prescott JF: An investigation into the association between cpb2-encoding C. perfringens type A and diarrhea in neonatal piglets. *Can J Vet Res, in press.*

12. Yaeger MJ, Kinyon JM, Songer GJ: A prospective, case control study evaluating the association between *Clostridium difficile* toxins in the colon of neonatal swine and gross and microscopic lesions. *J Vet Diag Invest* 2007, 19:52.

13. Wieler LH, Ilieff A, Herbst W, Bauer C, Vieler E, Bauerfeind R, Failing K, Klos H, Wengert D, Baljer G, and Zahner H: Prevalence of enteropathogens in suckling and weanling suckling pigs with diarrhea in southern Germany. *J Vet Med B* 2001, 48:151-159.

14. Spinato M, Peregrine S: AHL Newsletter, volume 13, number 1. [http://www.guelphlabservices.com.asp1-13.dfw1-2.websitetestlink.com/files/AHL/AHL%20Newsletters/2009/AHLNewsletter%20March%202009.pdf]

15. Amezcua R, Friendship RM, Dewey CE, Gyles C, Fairbrother JM. Presentation of postweaning Escherichia coli diarrhea in southern Ontario, prevalence of hemolytic E. coli serogroups involved, and their antimicrobial resistance patterns. *Can J Vet Res* 2002, 66:73-78.

16. Keessen EC, Hopman NEM, van Leengoed LAMG, van Asten AJAM, Hermanus C, Kujiper EJ, Lipman LJA: Evaluation of Four Different Diagnostic Tests to Detect *Clostridium difficile* in Piglets. *J Clin Microbiol* 2011, 49:1816–1821.

17. Morin M, Turgeon D, Jolette J, Robinson Y, Phaneuf JB, Sauvageau R, Beauregard M, Teuscher E, Higgins R, Larivière S. Neonatal diarrhea of pigs in Quebec: infectious causes of significant outbreaks. *Can J Comp Med* 1983, 47(1):11–17.

TABLES

Table 3.1: Etiological diagnosis for gastrointestinal disease submissions of 1-to-7-day old piglets to the Animal Health Laboratory from 2001 to 2010

Pathogens involved in GIT case	Number of cases for single etiological diagnosis (% of total GIT cases)	Number of cases for multiple etiological diagnosis (% of total GIT cases)
Enterotoxigenic *Escherichia coli* (n=73)	63 (26.6)	10 (4.2)
Clostridium perfringens (n=28)	19 (8.0)	9 (4.3)
Rotavirus (n=28)	18 (7.6)	10 (4.2)
Clostridium difficile (n=21)	10 (4.2)	11 (4.6)
Coccidia (n=15)	13 (5.5)	2 (0.84)
TGEV (n=7)	6 (2.5)	1 (0.42)
Salmonella sp. (n=6)	2 (0.84)	4 (1.7)
PRRS (n=3)	2 (0.84)	1 (0.42)
Cryptosporidium parvum (n=2)	1 (0.42)	1 (0.42)
Enterococcus durans (n=2)	1 (0.42)	1 (0.42)

Table 3.2: Logistic regression comparing the association of Enterotoxigenic *E. coli* diagnosis in gastrointestinal disease case submissions to the Animal Health Laboratory and independent variables

Parameters		Odds Ratio	Standard Error	95% Confidence Interval	*P*-value
C. difficile detection	No	Referent			
	Yes	0.11	0.125	0.013-.97	0.047
C. perfringens detection	No	Referent			
	Yes	0.20	0.148	0.049-0.85	0.029
Rotavirus detection	No	Referent			
	Yes	0.29	0.170	0.95-0.91	0.034
Season	Winter	Referent			
	Spring	0.38	0.171	0.16-0.92	0.032
	Summer	0.37	0.177	0.15-0.94	0.037
	Fall	0.61	0.273	0.26-1.5	0.271
Year	2009	Referent			
	2001	9.6	7.60	2.0-45	0.004
	2002	9.6	7.56	2.1-45	0.004
	2003	12	8.68	2.7-50	0.001
	2004	5.0	3.95	1.1-24	0.042
	2005	4.0	3.03	0.89-18	0.07
	2007	4.3	3.39	0.93-20	0.062

*Parameters for 2006, 2008, and 2010 are not shown ($P>0.1$).

Table 3.3: Logistic regression comparing the association of *C. perfringens* diagnosis in gastrointestinal disease case submissions to the Animal Health Laboratory and independent variables

Parameters		Odds Ratio	Standard Error	95% Confidence Interval	*P*-value
Average age of submitted piglets (d)		0.69	0.106	0.51-0.93	0.016
Season	Winter	Referent			
	Spring	0.13	0.116	0.024-0.73	0.021
	Fall	0.13	0.108	0.028-0.65	0.013
ETEC detection	No	Referent			
	Yes	0.27	0.192	0.068-1.1	0.065
Year	2010	Referent			
	2002	0.074	0.0967	0.0059-0.94	0.045
	2003	0.089	0.0956	0.011-0.73	0.025
	2004	0.056	0.0713	0.0046-0.68	0.024
	2005	0.19	0.163	0.037-1.0	0.052
	2006	0.040	0.0500	0.0033-0.47	0.011
	2007	0.078	0.0840	0.0095-0.64	0.018
	2009	0.17	0.159	0.029-1.0	0.056

*Parameters for 2001 (no cases of *C. perfringens* diagnosed), 2008, and Summer ($P>0.1$) are not shown.

FIGURES

Figure 3.1: Diagnostic success rate for gastrointestinal disease submissions of 1-to-7-day old piglets to the Animal Health Laboratory from 2001 to 2010

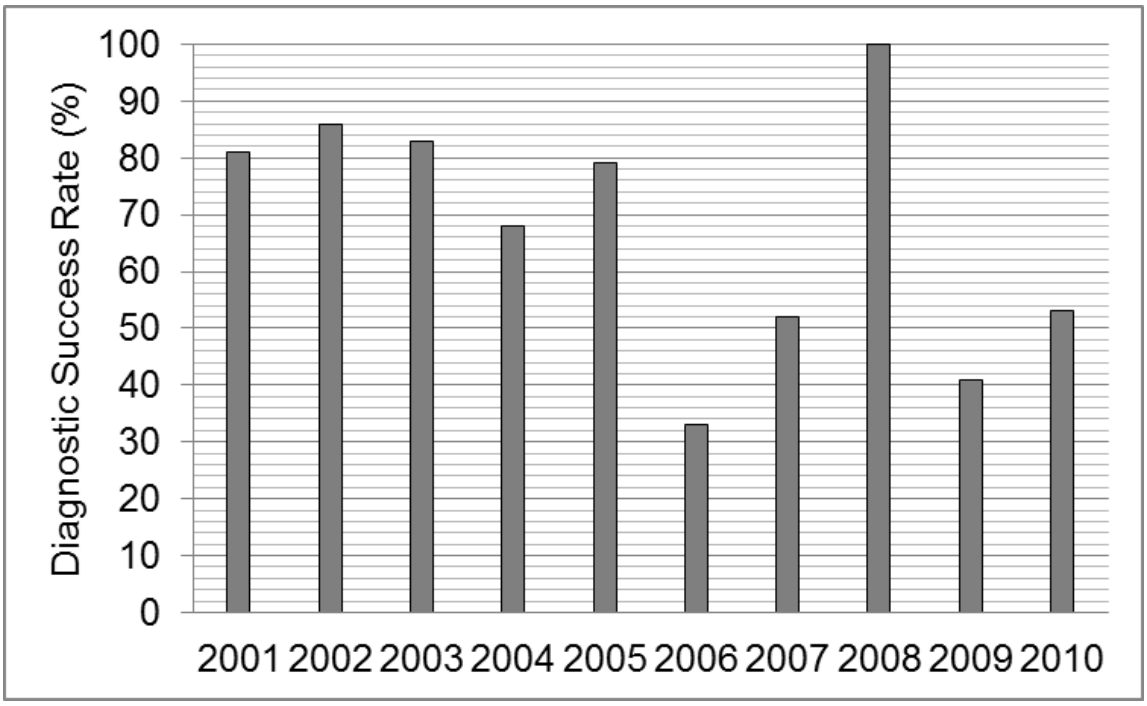
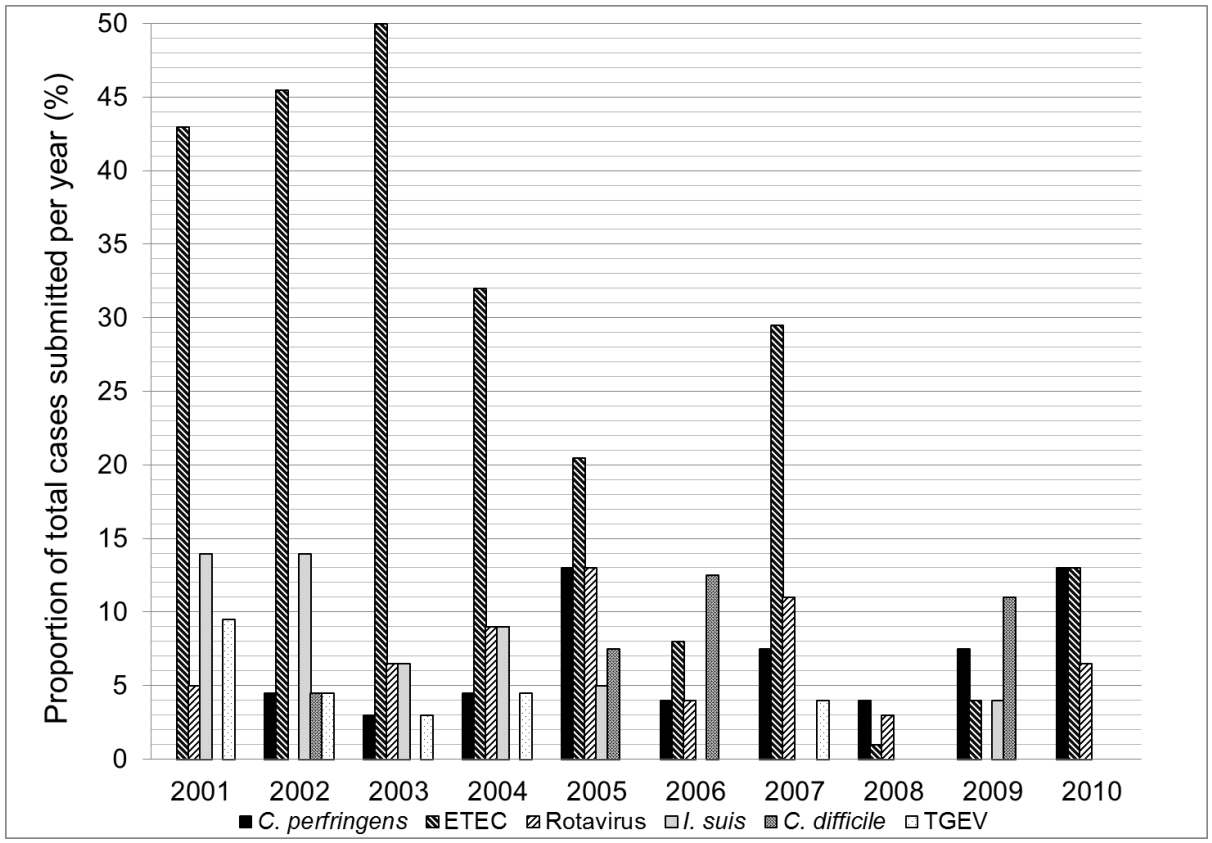


Figure 3.2: Annual prevalence of pathogens in gastrointestinal disease submissions of 1-to-7-day old piglets to the Animal Health Laboratory from 2001 to 2010 with single etiological diagnoses



CHAPTER FOUR

How do veterinary pathologists and swine practitioners arrive at a diagnosis of *Clostridium perfringens* type A enteritis in neonatal piglets?

1. INTRODUCTION

Clostridium perfringens type A are spore-forming, Gram-positive, anaerobic bacteria. They are part of the normal flora of the colon and small intestine of swine and most other warm-blooded species of animal [1]. *C. perfringens* type A populations reach 10^9 CFU/g of intestinal content within 12 h after birth and peak in concentration in non-medicated piglets between 1 and 7 d of age [2]. Large numbers of *C. perfringens* type A can be isolated from healthy piglets, and currently it is not possible to differentiate between disease-causing strains and normal flora. Therefore, the epidemiology of *C. perfringens* type A enteritis is speculative [1]. However, the diagnosis of *C. perfringens* type A enteritis has increased for cases of neonatal piglet diarrhea in the past decade, and is associated with 2-5% of pre-weaning mortality [3]. It has been proposed that diarrhea caused by *C. perfringens* type A involves bacterial proliferation and production of the beta2 (CPB2) toxin, which can develop in piglets between 1 and 7 d of age [1, 2]. The current diagnostic method for *C. perfringens* type A enteritis is not well established. The objective of this study was to investigate how Canadian veterinary practitioners diagnose, treat, and control neonatal piglet diarrhea associated with *C. perfringens* type A, and how veterinary pathologists in selected parts of the world diagnose neonatal piglet enteritis associated with *C. perfringens* type A.

2. METHODS

One questionnaire was created for veterinary practitioners and a second for veterinary pathologists. Questions pertained to the diagnosis of *C. perfringens* type A in neonatal piglet enteritis, and in the case of practitioners additional questions were asked related to treatment and control. Neonatal enteritis was defined as diarrhea or enteritis that occurred in piglets between 1 and 7 d of age. The online survey was made in LimeSurvey. The responses from the survey were entered into a spreadsheet (Microsoft Excel 2007; Microsoft Corp., Redmond, WA).

2.1. Veterinary Practitioner Questionnaire

Canadian veterinary practitioners were conveniently selected to participate in a survey with a total of 7 questions. The survey was administered to practitioners who attended the Ontario Pork Congress event in June 2011, and it was also administered online from July 2011 to March 2012. The survey collected information on the estimated cases of neonatal enteritis that the practitioner investigated within the past 12 months, and the estimated number of cases that were attributed to *C. perfringens* type A. They were asked for the diagnostic method of *C. perfringens* type A, including the specific clinical signs and history used to make a preliminary diagnosis, and how they confirmed the diagnosis. In addition, they rated their level of confidence for the diagnosis from “not confident”, “moderately confident”, or “very confident” (those who were not confident of their diagnosis were asked to provide an explanation). They were asked to list recommendations they would give the producer for treatment and control of enteritis related to *C. perfringens* type A. They were given an optional question to provide any additional comments regarding diarrhea associated with *C. perfringens* type A.

2.2. Veterinary Pathologist Questionnaire

Veterinary pathologists were conveniently selected to participate in an online survey with a total of 4 questions administered from May 2011 to March 2012. The survey for veterinary pathologists collected information on how they confirmed a diagnosis for *C. perfringens* type A. In addition, they were asked to rank their level of confidence for the diagnosis from "not confident", "moderately confident", or "very confident". Those who were not confident of their diagnosis were asked to provide an explanation. They were given an optional question to provide any additional comments regarding enteritis associated with *C. perfringens* type A.

3. RESULTS

3.1. Veterinary Practitioner Questionnaire

A total of 43 Canadian practitioners were invited to participate in the survey, and 22 practitioners completed the survey (51% response rate). The estimated cases of neonatal enteritis that the practitioners investigated in a 12-month period ranged from 0 to 100, with a mean of 21 $\pm$ 22 cases. The estimated cases of enteritis attributed to *C. perfringens* type A ranged from 0 to 70, with a mean of 7 $\pm$ 16 cases. A total of 15 (68%) practitioners had diagnosed at least one case of neonatal enteritis associated with *C. perfringens* type A within a 12-month period. The estimated numbers of cases for neonatal enteritis attributed to *C. perfringens* type A are listed in Table 1. The majority (68%) of practitioners considered the age of onset for *C. perfringens* type A enteritis to be important, which could occur within 24 h to 7 d after birth. A list of clinical signs and history used by practitioners for the preliminary diagnosis of *C. perfringens* type A enteritis is presented in Table 2. One practitioner indicated that piglets affected by *C.*

perfringens type A associated enteritis responded poorly to antibiotics used for treating colibacillosis, and 1 practitioner indicated that piglets affected by *C. perfringens* type A associated enteritis were responsive to treatment with penicillin.

In order to confirm a diagnosis, most (95%) practitioners responded that they would submit samples to the veterinary diagnostic laboratory including: fecal samples, intestinal swabs, fresh or fixed tissues, dead piglets, or live untreated piglets. These practitioners considered the isolation of *C. perfringens* type A through bacterial culture important for confirming the diagnosis. Eight (40%) practitioners indicated that they confirmed that the *C. perfringens* isolated were toxinotype A either through polymerase chain reaction (PCR) or toxin detection through enzyme-linked immunosorbent assay (ELISA). Three (14%) practitioners indicated that it was also necessary to rule out other etiological agents, which included *Escherichia coli* and/or rotavirus.

One (5%) practitioner indicated that they were "not confident" of their diagnosis for *C. perfringens* type A enteritis (the respondent also indicated never diagnosing *C. perfringens* type A). Thirteen (59%) practitioners indicated that they were moderately confident, and 8 (36%) practitioners indicated that they were very confident of their diagnosis. Two (10%) practitioners indicated that it was difficult to distinguish diarrhea caused by *E. coli* and *C. perfringens*, and 2 practitioners believed that diarrhea associated with *C. perfringens* type A was occurring more frequently or being underdiagnosed. On the other hand, 4 (18%) practitioners indicated that they did not believe *C. perfringens* type A was a primary pathogen or that *C. perfringens* type A could be found concurrent to other pathogens, such as *E. coli*, coccidia, or rotavirus.

Practitioners indicated that they believed control of enteritis associated with *C. perfringens* type A was mainly achieved through changes in farm management factors. Hygiene and disinfection of the farrowing area and crates were considered important, in addition to humidity control and temperature control of the creep area. Practitioners reported that sow health should be monitored by reviewing parity, diet, assisted farrowing, biofeedback, and/or cross-fostering piglets that did not receive adequate colostrum. It was recommended that the sow should be brought to full feed in a controlled fashion, and the gestation diet should be fed until after farrowing to avoid shifts in the intestinal flora prior to farrowing. The use of drying agents for piglets (such as corn starch) was considered important. Practitioners differed in the treatment strategy for *C. perfringens* type A, which included: reducing routine medication or using selective treatment for piglets, autogenous or commercial vaccination for sows, addition of protective acids or bacitracin methylene disalicylate (BMD) to the sow pre-farrowing ration, and treatment of piglets with BMD, antibiotics (tylosin, ampicillin, penicillin, or ceftiofur), or probiotics (for prophylaxis).

3.2. Veterinary Pathologist Questionnaire

The veterinary pathologist survey was forwarded to 16 diagnostic laboratories across North America, and forwarded to the European College of Veterinary Pathologists (ECVP), and the Japanese Society of Veterinary Pathology (JSVP). A total of 17 veterinary pathologists representing 15 diagnostic laboratories responded to the survey: 5 (29.5%) were located in Canada, 6 (35%) were located in the United States, 5 (29.5%) were located in Europe and 1 (6%) was located in Japan. All pathologists indicated that the isolation of *C. perfringens* type A through anaerobic culture was important for the

diagnosis. Seven (41%) pathologists used histopathology to rule out other pathogens, including enterotoxigenic *Escherichia coli* (ETEC), rotavirus, transmissible gastroenteritis virus, and/or porcine reproductive and respiratory syndrome virus (PRRSV). Specific gross lesions or histopathological lesions associated with *C. perfringens* type A enteritis were not reported by the respondents. One pathologist reported that the bowel loops could be thin-walled and fluid-filled, and Gram positive rods were present either in proximity to or adhering to the enterocytes with possible erosion and ulcers. A list of criteria for the diagnosis of *C. perfringens* type A associated enteritis is listed in Table 3.

Seven (41%) pathologists indicated that they were not confident in making a diagnosis of *C. perfringens* type A enteritis, including 4 pathologists in Canada, 1 pathologist in the United States, and 2 pathologists in Europe. Five (29.5%) pathologists indicated that they were moderately confident, including 1 pathologist in Canada, 2 pathologists in the United States, and 2 pathologists in Europe. Five (29.5%) pathologists indicated that they were very confident of their diagnosis, including 3 pathologists in the United States, 1 pathologist in Europe, and 1 pathologist in Japan. The pathologists that were not confident of their diagnosis were asked to provide an explanation. The responses given included the lack of published data for the role of *C. perfringens* type A in swine disease, absence of specific lesions in *C. perfringens* type A enteritis, or that detection of the beta2 toxin gene (*cpb2*) in *C. perfringens* type A isolates did not provide a useful diagnosis. Two (12%) pathologists indicated that *C. perfringens* type A was often a diagnosis by default, 2 pathologists indicated that they seldom have cases associated with *C. perfringens* type A, and 1 (6%) pathologist indicated that fresh

laboratory samples were required to make a diagnosis. The level of confidence reported for the diagnosis by practitioners and pathologists are included in Figure 1.

Two (12%) pathologists did not believe that *C. perfringens* type A was a primary pathogen. One of the pathologists suggested that *C. perfringens* type A infection was a syndrome that involved a complex of other pathogens, such as *C. difficile*, uncharacterized clostridial pathogens, or undetected pathogens; the syndrome could occur due to changes in farm management or antibiotic overuse, causing a disruption in the pig microbial flora. One (6%) pathologist reported that the isolation of *C. perfringens* type A was not sufficient to make a diagnosis, but the presence of mild lesions and detection of *cpb2* in the isolates supported the diagnosis. In contrast, 2 (12%) pathologists indicated that detection of *cpb2* in *C. perfringens* type A isolates was not considered in their diagnosis. One pathologist indicated that more knowledge was needed regarding the toxins involved in *C. perfringens* type A enteritis, and suggested that a quantitative test should be developed to detect significant concentrations of the ubiquitous organism. In one diagnostic laboratory, *C. perfringens* type A was the most commonly diagnosed cause of enteritis for pigs in the first wk of life. The pathologists stated that they were confident in their diagnosis when 3 or 4 affected live piglets were submitted and samples were taken immediately after euthanasia. Fecal samples or swabs could be sufficient for detecting other pathogens but were not recommended. One pathologist commented that testing for *C. perfringens* type A was considered time-consuming and required fresh samples.

4. DISCUSSION

The survey for Canadian veterinary practitioners indicated remarkably different responses in that neonatal piglet enteritis associated with *C. perfringens* type A was commonly diagnosed in some practices, but rarely diagnosed in others (Table 1). One surveyed Canadian pathologist indicated that most of the neonatal piglet enteritis cases submitted to their laboratory was diagnosed with *C. perfringens* type A. Similarly, *C. perfringens* type A was the second most commonly diagnosed infectious agent in neonatal piglet diarrhea cases submitted to the Iowa State University Veterinary Diagnostic Laboratory from 2004 to 2009 [2]. In contrast, 2 surveyed pathologists representing different European diagnostic laboratories reported that they rarely diagnosed *C. perfringens* type A in neonatal piglet diarrhea cases, suggesting that the prevalence of *C. perfringens* type A enteritis may differ by geographical location, or that European laboratories do not recognize the involvement of this organism in disease. There is no doubt that large numbers of *C. perfringens* are present in the intestine of piglets in Europe [14]. It is also possible that the respondents who rarely diagnosed cases of *C. perfringens* type A enteritis overlooked the role of the organism in neonatal diarrhea, or on the other hand, neonatal diarrhea cases attributed to *C. perfringens* type A could be overestimated by other respondents. The results of the survey indicate a need to clarify the role of *C. perfringens* type A in enteric disease, as an accurate diagnosis is recommended to successfully control a diarrhea outbreak in the herd [4].

Most (95%) of the practitioners were moderately or very confident of their diagnosis for *C. perfringens* type A enteritis, however, some of the respondents indicated that they did not believe *C. perfringens* type A was a primary pathogen and/or ruled out

other pathogens known to cause enteritis. Similarly in a case report, a practitioner suspected *C. perfringens* type A as the cause of diarrhea outbreak in 3-day-old piglets because the organism was isolated in anaerobic culture, and the tests for rotavirus and TGEV were negative [4]. The survey results of both practitioners and pathologists showed that isolation of *C. perfringens* type A was necessary for the diagnosis, but did not always provide sufficient evidence to support the diagnosis. Almost half (41%) of the pathologists were not confident of their diagnosis possibly due to the lack of gross and histological lesions observed in *C. perfringens* type A enteritis. The difference between levels of confidence of the practitioner and pathologist emphasized that a diagnosis of neonatal diarrhea should not be made based on diagnostic test results alone [2], and samples should be resubmitted to the laboratory if a diagnosis could not be confirmed [4]. Two respondents indicated that they believed other unknown pathogens could be involved in neonatal enteritis. Some studies conducted on the frequency of pathogens causing piglet diarrhea have also indicated that an etiological diagnosis could not be identified for 17%-58% of the piglets [5-8], including the Ontario study that analyzed the presence of *C. perfringens* type A in neonatal piglets [5]. Therefore, it is possible that there are other pathogens involved in neonatal diarrhea that are not detected by current diagnostic methods. Perhaps in the absence of other known enteric pathogens, neonatal diarrhea cases are often misdiagnosed as *C. perfringens* type A enteritis.

Some studies reported an association of *cpb2* positive *C. perfringens* type A and neonatal piglet diarrhea [9-11], but our survey respondents expressed different opinions regarding the importance of *cpb2* in *C. perfringens type* A enteritis. The quantification of *cpb2*-positive *C. perfringens* does not provide a useful diagnosis [5], and there is no

method currently to differentiate pathogenic *C. perfringens* strains from *C. perfringens* strains that are commensals [1, 12]. The survey results indicated that the current diagnostic method of *C. perfringens* type A enteritis was not specific, therefore improved diagnostic tools should be established if the organism is indeed a true pathogen associated with swine enteritis.

The control of *C. perfringens* type A enteritis generally involved changes in farm management, supportive therapy, and treatment with antibiotics. Practitioners appeared to differ in opinion regarding the response to antibiotics in pigs as a clinical sign for *C. perfringens* type A enteritis, but it should not necessarily be regarded as a contradiction because most practitioners did not specify to which antibiotics they were referring. The practitioners also suggested autogenous vaccination or commercial pre-farrowing vaccination for sows. The commercial vaccines currently available contain C. *perfringens* type C toxoid, which may not successfully protect pigs against *C. perfringens* type A infection [3]. It is difficult to evaluate the efficacy of autogenous vaccines and medication, since other changes in farm management are usually made concurrently [13]. It is possible for the diarrhea problem to disappear regardless of whether the herd was treated or not [4].

The practitioners and pathologists were conveniently selected to complete the surveys, and some selected participants did not respond to the survey, therefore the results do not necessarily represent the majority of veterinary practitioners and pathologists. The type of survey questions administered regarding how *C. perfringens* type A was diagnosed were short answer questions, and it is possible that respondents did not record all the diagnostic criteria they used. Overall, the survey respondents gave

remarkably different opinions regarding whether *C. perfringens* type A is a pathogen that causes enteritis in neonatal piglets. Further research is required to determine whether *C. perfringens* type A is a cause of neonatal enteritis in swine, and the basis on which the diagnosis can be made with confidence.

5. CONCLUSION

The survey for the diagnostic method of *C. perfringens* type A enteritis indicated that the current diagnostic method used by practitioners and pathologists are not specific. *C. perfringens* type A enteritis were reported by practitioners to cause mild to severe diarrhea in piglets less than 7 days of age. Both practitioners and pathologists indicated that isolation of *C. perfringens* type A was important for the diagnosis, but specific gross and histopathological lesions were not mentioned. The practitioners were considerably more confident of their diagnosis compared to pathologists. Currently, there is no method to definitively diagnose infection. In view of the importance that is placed by practitioners' on this disease, and the difficulty experienced by some pathologists in diagnosing the infection, further research is required to determine whether *C. perfringens* type A is a cause of neonatal enteritis in swine, and the basis on which the diagnosis can be made with confidence.

REFERENCES CITED

1. Songer JG, Uzal FA: Clostridial enteric infections in pigs. *J Vet Diag Invest* 2005, 17:528-536.

2. Schwartz KJ: Clostridium-associated diseases in swine. *In Proceedings of American Association of Swine Practitioners: 7-10 March 2009; Dallas.*

3. Lehe K: Successful control of clostridial enteritis: A report from the back forty. *In Proceedings of American Association of Swine Practitioners: 7-10 March 2009; Dallas.*

4. Wilson K: Diagnostic approach to enteric diseases of swine. *Swine Health Prod* 2000, 8(5):235-236.

5. Farzan A, Kircanski J, DeLay J, Soltes G, Songer GJ, Friendship R, Prescott JF: An investigation into the association between *cpb2*-encoding C. perfringens type A and diarrhea in neonatal piglets. *Can J Vet Res, in press.*

6. Wieler LH, Ilieff A, Herbst W, Bauer C, Vieler E, Bauerfeind R, Failing K, Klos H, Wengert D, Baljer G, and Zahner H: Prevalence of enteropathogens in suckling and weanling suckling pigs with diarrhea in southern Germany. *J Vet Med B* 2001, 48:151-159.

7. Yaeger M, Funk N, and Hoffman L: A survey of agents associated with neonatal diarrhea in Iowa swine including *Clostridium difficile* and porcine reproductive and respiratory syndrome virus. *J Vet Diagn Invest* 2002, 14:281-287.

8. Katsuda K, Kohmoto M, Kawashima K, and Tsunemitsu H: Frequency of enteropathogen detection in suckling and weanling pigs with diarrhea in Japan. *J Vet Diagn Investig* 2006, 18:350-354.

9. Garmory HS, Chanter N, French NP, Bueschel D, Songer JG, Titball RW: Occurrence of *Clostridium perfringens* b 2-toxin amongst animals, determined using genotyping and subtyping PCR assays. *Epidemiol Infect* 2000, 124:61–67.

10. Bueschel, DM, Jost BH, Billington SJ, Trinh HT, Songer JG: Prevalence of cbp2, encoding beta2 toxin, in *Clostridium perfringens* field isolates: correlation of genotype with phenotype. *Vet Microbiol* 2002, 94:121–129.

11. Waters M, Savoie A, Garmory HS, Bueschel D, Popoff MR, Songer GJ, Titball RW, McClane BA, Sarker MR: Genotyping and phenotyping of beta2-toxigenic *Clostridium perfringens* fecal isolates associated with gastrointestinal diseases in piglets. *J Clin Microbiol* 2003, 41:3584–3591.

12. Baker AA, Davis E, Rehberger T, Rosener D: Prevalence and Diversity of Toxigenic *Clostridium perfringens* and *Clostridium difficile* among Swine Herds in the Midwest. *Appl Environ Microbiol* 2010, 76(9):2961-2967.

13. Songer JG, Glock RD: Enteric infection of swine with *Clostridium perfringens* type A and C. *Swine Health Prod* 1998, 6(5):223-225.

14. Smith WH, Jones JET: Observations on the alimentary tract and its bacterial flora in healthy and diseased pigs. *J Path Bact* 1963, 84:387-412.

TABLES

Table 4.1: Estimated cases of neonatal enteritis for 22 veterinary practices investigated in a 12-month period

Province	No. of Respondents	Estimated mean number of neonatal enteritis cases (range)	Estimated mean number of cases associated with C. perfringens type A (range)
Alberta	4	11 $\pm$ 6.4 (2-30)	2.5 $\pm$ 2.9 (0-5)
British Columbia	1	2	0
Manitoba	3	40 $\pm$ 10 (30-50)	9.7 + 11.7 (1-23)
Ontario	9	23 $\pm$ 29.6 (4-100)	9.9 $\pm$ 22.7 (0-70)
Quebec	3	15.7 $\pm$ 12.9 (5-30)	8.7 $\pm$ 13 (0-24)
Saskatchewan	2	6.5 $\pm$ 9.2 (0-13)	2 $\pm$ 2.8 (0-4)

Table 4.2: Clinical signs and history of *C. perfringens* type A associated neonatal piglet enteritis

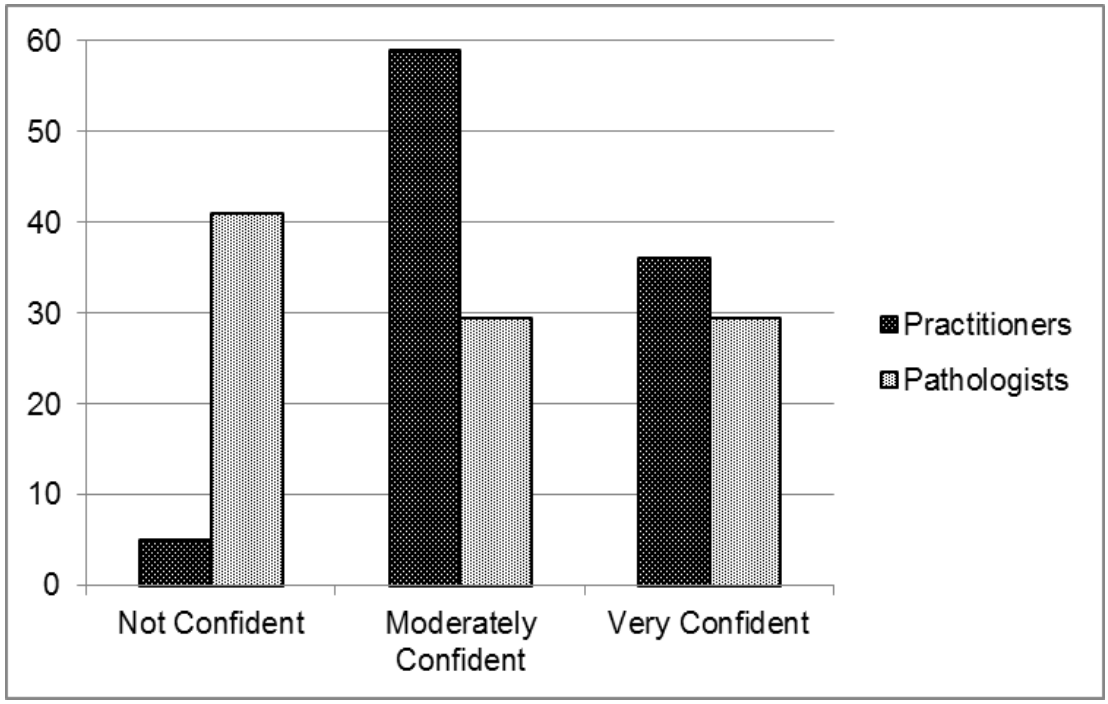
Clinical signs and history	Veterinary practitioners who considered it important in the preliminary diagnosis (%)
Age of onset for diarrhea at ≤7 days old	15 (68)
Sows or piglets were not responsive to antibiotics	6 (27)
Severe diarrhea (yellow watery diarrhea, dehydration, and/or growth retardation)	4 (18)
Mild diarrhea (sticky stool to yellow pasty diarrhea, with little or no dehydration)	3 (14)
Low mortality within a litter	3 (14)
Blood is present in diarrhea	2 (9)
Sows or piglets were responsive to antibiotics	2 (9)
Sows vaccinated for common pathogens other than *C. perfringens*	2 (9)
High morbidity within a litter	1 (4.5)
Sporadic diarrhea within the farrowing room	1 (4.5)
No sow parity bias	1 (4.5)

Table 4.3: Diagnostic criteria used by 17 veterinary pathologists for confirming *C. perfringens* type A associated enteritis.

Criteria for confirming diagnosis of *C. perfringens* type A	Veterinary pathologists who considered the criteria important in confirming their diagnosis (%)
Isolation of *C. perfringens* (anaerobic culture)	17 (100)
Histopathology revealed *C. perfringens*	13 (76)
PCR results indicated *C. perfringens* isolates were toxinotype A	12 (70.5)
Other pathogens causing enteritis ruled out	7 (41)
Histopathology revealed no lesions to minimal lesions, and/or mild inflammation of villus tips	4 (23.5)
PCR results indicated *C. perfringens* isolates were *cpb2*-positive	3 (18)
C. perfringens toxins detected by ELISA	3 (18)

FIGURES

Figure 4.1: Confidence by practitioners and pathologists in the diagnosis of *C. perfringens* type A associated enteritis.



CHAPTER FIVE

Summary and Conclusions

Clostridium perfringens type A is believed by some to be a common cause of neonatal piglet diarrhea [1-3; Chapter 4] However, large numbers of *C. perfringens* can be isolated from clinically normal piglets [1, 2], and it is speculative whether *C. perfringens* is truly a pathogen causing enteric disease in piglets [2]. Previous studies have indicated that *cpb2*-positive *C. perfringens* isolates are associated with diarrhea because *cpb2* was more likely detected in the isolates of diarrheic piglets compared to that of (usually small numbers of) controls [4-6]. The epidemiology of *C. perfringens* in Ontario swine farms is currently unknown, and has not been studied in other jurisdictions in such detail as here. One of the objectives was to compare the distribution of *C. perfringens* and associated toxin genes among pigs at different stages of production and farm factors that may affect the distribution. This objective was met through the development of multivariable models using *C. perfringens* count and genotyping results for fecal samples collected, and survey results collected from 48 Ontario swine farms (Chapter 2).

The results indicated that *cpb2*-positive *C. perfringens* were isolated from clinically normal pigs from all stages of production and *cpb2* could be recovered from *C. perfringens* isolates of half of the farms studied (Chapter 2). However, *cpb2* was most frequently detected in the isolates of suckling pigs compared to other stages of production (Table 2.4). Furthermore, no significant correlation was found between the presence of *cpb2* or *atyp-cpb2* in the isolates from sows and their litters. These results suggest that different *C. perfringens* strains may proliferate in pigs of different age groups and the

manure pits, and that the *cpb2*-positive strains appeared to specifically colonize young pigs [1]. The study also identified that fecal samples from sucking pigs had a higher *C. perfringens* count compared to fecal samples from other stages of production, but an interaction was identified for samples collected from lactating and gestating sows in farms where other agricultural species were present on the farm (Table 2.2). There was an increase in *C. perfringens* count in fecal samples that came from sows compared to suckling pigs in mixed farms, suggesting that the sows were harboring *C. perfringens* isolates that originated from other species.

In the epidemiological study, we surveyed the producers to collect information on various farm management factors, but we did not find a significant association between the likelihood of detecting *cpb2*-positive *C. perfringens* isolates and farm factors in multivariable analysis (Table 2.4). Some variables were associated with the presence of *cpb2* in the *C. perfringens* isolates in univariable analysis, including herd size and presence of other agricultural species (Appendix 2.5). The history of diarrhea on the farm was a survey question that may help us determine the association of C. *perfringens* type A and diarrhea in suckling pigs, but only 30 farms that were surveyed were farrowing operations. If research funds were available for a larger sample size of farms, we may have obtained a better understanding of the association of different farm management factors and the distribution of *C. perfringens* and associated toxin genes. Further large scale studies comparing the epidemiology of *C. perfringens* in diarrheic piglets and healthy piglets are recommended to fully understand the association of *C. perfringens* type A and neonatal enteric disease. However, it was noteworthy that healthy neonatal piglets usually shed large numbers (5.0 ± 1.70 $\log_{10}$ CFU/g) of *cpb2*-positive *C.*

perfringens in their feces, suggesting that the isolation of large numbers of such bacteria from diarrheic pigs may lead to the troubles in diagnostic interpretation experienced by some pathologists (Figure 4.1).

One strength of the study was the use of real-time fluorogenic multiplex PCR assays for genotyping the *C. perfringens* isolates, which has greater sensitivity and is particularly less time consuming compared to conventional gel-based PCR [7] The combination of probe dyes for the real-time assays and the designation of *cpa* as internal positive control allows a highly specific and sensitive method for genotyping *C. perfringens* isolates for major toxin genes, *cpb2,* and *cpe* [7]. The PCR allowed rapid screening of 9 virulence-associated genes in a large number (354) of isolates, including *netB* and *tpeL* that have not previously been examined in pig isolates. The PCR also allowed identification of the atypical *cpb2* gene, and identified its presence in a high proportion of *cpb2* positive isolates (Table 2.1). Another strength of the study was the multivariable models that also provided strong evidence for associations and accounted for clustering and control of confounding bias.

Diarrhea is a major cause of death in neonatal piglets, and prevalence studies have been conducted in different parts of the world to investigate the relative importance of different enteric pathogens that contribute to piglet diarrhea [8-10]. Our study investigated the frequency and trends of different pathogens contributing to neonatal piglet diarrhea in Ontario swine farms from 2001 to 2010. We identified seasonal trends for the etiological diagnosis of ETEC, *C. perfringens* type A, and rotavirus using logistic regression (Chapter 3). The data also confirmed the absence of *C. perfringens* type C infection in Ontario swine. Multivariable analysis identified a decreasing trend in

diagnosis of *E. coli* since 2001 (Table 3.2) and an increasing trend with diagnosis of *C. perfringens* since 2002 (Table 3.3). The study also indicated that 33% of gastrointestinal cases did not have an etiological diagnosis, suggesting that there may be pathogens associated with neonatal diarrhea that are not detected by current diagnostic methods. *C. perfringens* was the second most frequent etiological diagnosis of gastrointestinal illness in neonatal piglets after ETEC (Table 3.1). However, 86% of the total gastrointestinal illness cases were cultured for *C. perfringens*, and only 8% were diagnosed with *C. perfringens* type A for cases involving a single etiological agent. These results suggested that *C. perfringens* isolated from the piglets were often regarded as commensal organisms by the pathologist.

The data for submissions of gastrointestinal cases to the Animal Health Laboratory were available for a 10-year time frame, which lends strength to this research. Since the focus of our study was on neonatal diarrhea, we only analyzed cases for piglets that were between 1 to 7 days of age. However, if we had included the cases for older piglets in the analysis, it could be possible to identify other trends, such as changes in the relative frequency of enteric pathogens with increase of age. The results of our study identified the current pathogens of concern for the swine industry, including ETEC, *C. perfringens* type A, rotavirus, and *C. difficile.* Future studies are recommended to strengthen the diagnostic methods for these pathogens and to identify the bases of neonatal diarrhea cases that did not have an etiological diagnosis. In particular, there is a need to clarify the role of *C. perfringens* type A in enteric disease by determining the pathogenic mechanism.

We surveyed practitioners from Canada and pathologists from selected parts of the world in order to investigate the diagnostic method and control for *C. perfringens* type A enteritis in neonatal piglets (Chapter 4). Some practitioners identified that *C. perfringens* type A enteritis was a common problem seen in their practices (Appendix 4.1). However, survey respondents did not report specific clinical signs, gross lesions, or histopathological lesions associated with *C. perfringens* type A enteritis. It was possible that enteritis cases were often misdiagnosed as *C. perfringens* type A in the absence of other known enteric pathogens. The lack of confidence expressed by almost half of the pathologists in their diagnosis of *C. perfringens* type A (Figure 4.1) indicated that more research is needed to determine whether *C. perfringens* type A is a primary pathogen of swine enteric disease. We also reported the recommendations given by practitioners for controlling *C. perfringens* type A enteritis, which were mainly achieved through farm management factors, supportive therapy, vaccination, and selective treatment of piglets with antibiotics. However, it is unknown whether vaccination or medication successfully controlled *C. perfringens* type A enteritis. Overall, our survey results showed that the current method of diagnosis for *C. perfringens* type A is not specific and respondents differed in their opinion regarding whether *C. perfringens* type A was a primary enteric pathogen in piglets and their confidence in the diagnosis. The response rate of the pathologist survey was low, and having more respondents for this survey may provide more specific information for the diagnostic method. However, the literature has indicated that there are no specific gross or histological lesions associated with *C. perfringens* type A, and the current diagnostic method is generally based on isolating

large numbers of *cpb2*-positive *C. perfringens* type A, which can be found in healthy and diarrheic piglets [1].

Finally, this work suggests that further research is required to determine whether *C. perfringens* type A is a cause of neonatal enteritis in swine, and the basis on which the diagnosis can be made with confidence. Without such basic information, attempts to control the disease must remain highly empirical.

REFERENCES CITED

1. Songer JG, Uzal FA: Clostridial enteric infections in pigs. *J Vet Diag Invest* 2005, 17:528-536.

2. Schwartz KJ: *Clostridium*-associated diseases in swine. *In Proceedings of American Association of Swine Practitioners: 7-10 March 2009; Dallas.*

3. Lehe K: Successful control of clostridial enteritis: A report from the back forty. *In Proceedings of American Association of Swine Practitioners: 7-10 March 2009; Dallas.*

4. Garmory HS, Chanter N, French NP, Bueschel D, Songer JG, Titball RW: Occurrence of *Clostridium perfringens* b 2-toxin amongst animals, determined using genotyping and subtyping PCR assays. *Epidemiol Infect* 2000, 124:61–67.

5. Waters M, Savoie A, Garmory HS, Bueschel D, Popoff MR, Songer GJ, Titball RW, McClane BA, Sarker MR: Genotyping and phenotyping of beta2-toxigenic *Clostridium perfringens* fecal isolates associated with gastrointestinal diseases in piglets. *J Clin Microbiol* 2003, 41:3584–3591.

6. Bueschel, DM, Jost BH, Billington SJ, Trinh HT, Songer JG: Prevalence of cbp2, encoding beta2 toxin, in *Clostridium perfringens* field isolates: correlation of genotype with phenotype. *Vet Microbiol* 2002, 94:121–129.

7. Albini S, Brodard I, Jaussi A, Wollschlaeger N, Frey J, Miserez R, Abril C: Real-time multiplex PCR assays for reliable detection of *Clostridium perfringens* toxin genes in animal isolates. *Vet Microbiol* 2008, 127:179–185.

8. Wieler LH, Ilieff A, Herbst W, Bauer C, Vieler E, Bauerfeind R, Failing K, Klos H, Wengert D, Baljer G, and Zahner H: Prevalence of enteropathogens in suckling and weanling suckling pigs with diarrhea in southern Germany. *J Vet Med B* 2001, 48:151-159.

9. Yaeger M, Funk N, and Hoffman L: A survey of agents associated with neonatal diarrhea in Iowa swine including *Clostridium difficile* and porcine reproductive and respiratory syndrome virus. *J Vet Diagn Invest* 2002, 14:281-287.

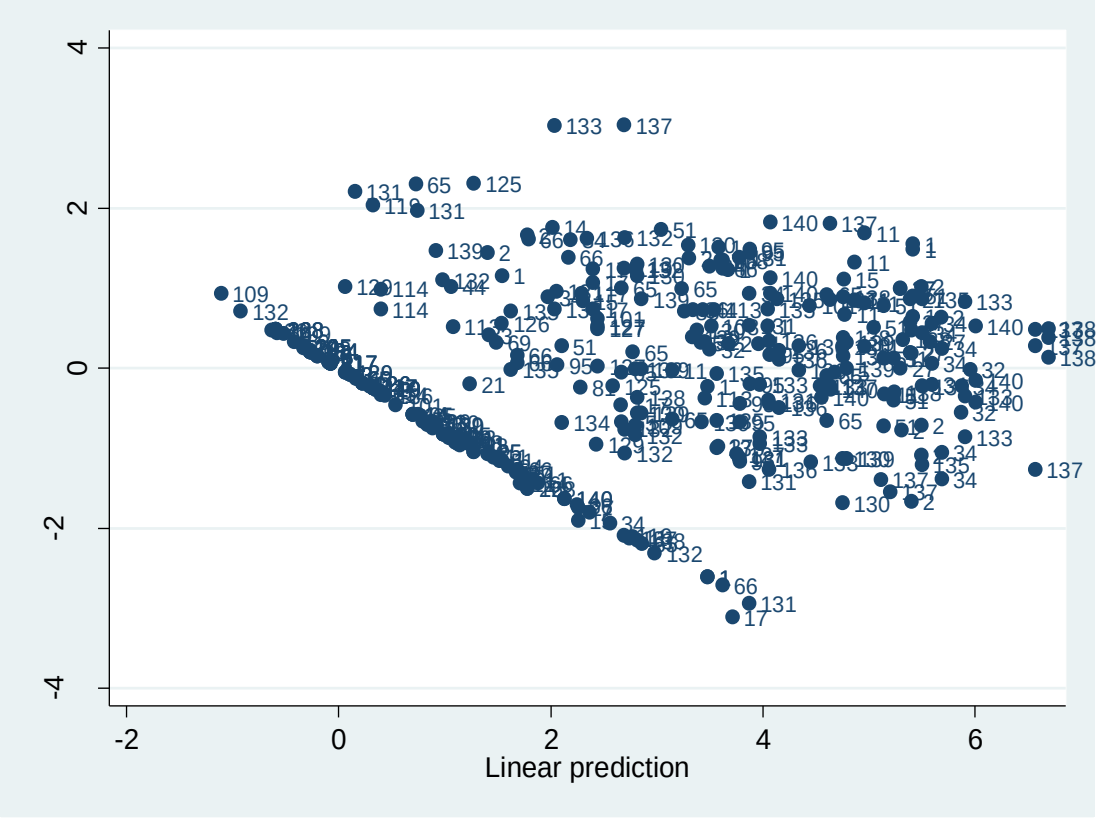
10. Katsuda K, Kohmoto M, Kawashima K, and Tsunemitsu H: Frequency of enteropathogen detection in suckling and weanling pigs with diarrhea in Japan. *J Vet Diagn Investig* 2006, 18:350-354.

APPENDIX

Appendix 2.1: Association of log_{10} *C. perfringens* count (CFU/g) and independent variables in univariable linear regression

Parameters		Coefficient	Standard Error	95% Confidence Interval	*P*-value
Stage	Piglet	Referent			
	Lactation sow	-1.1	0.314	(-1.7)-(-0.45)	0.001
	Gestation sow	-0.67	0.354	(-1.4)-(-0.25)	0.059
	Weanling	-3.7	0.336	(-4.4)-(-3.1)	<0.001
	Grower-finisher	-4.4	0.279	(-5.0)-(-3.9)	<0.001
	Manure pit	-2.3	0.310	(-2.9)-(-1.7)	<0.001
Farrowing operation	No	Referent			
	Yes	2.5	0.268	2.0-3.0	<0.001
Herd size	128-940 (n=9)	Referent			
	1050-3000 (n=28)	0.11	0.310	(-0.50)-0.72	0.732
	3200-7200 (n=9)	-0.67	0.413	(-1.5)-0.15	0.110
Sampling Period	First	Referent			
	Second	1.7	0.253	1.2-2.2	<0.001

Appendix 2.2: Standardized residuals for assessing outliers in the linear multivariate model



Potential outliers were identified for Farms 1, 66, 17, 131, 133, and 137.

Farm 1: Two lactation sow fecal samples did not have any *C. perfringens* count (rstandard=-2.6).

Farm 66: A piglet fecal sample did not have any *C. perfringens* count (rstandard=-2.7).

Farm 17: A manure pit fecal sample did not have any *C. perfringens* count (rstandard=-3.1).

Farm 131: A piglet fecal sample did not have any *C. perfringens* count (rstandard=-2.9).

Farm 133: A weanling pig fecal sample had a similar *C. perfringens* count (6.0 $\log_{10}$ CFU/g) to that of suckling pig fecal samples (mean=5.7 $\log_{10}$ CFU/g) (rstandard=3.0)

Farm 137: A weanling pig fecal sample had a similar *C. perfringens* count (6.7 $\log_{10}$ CFU/g) to that of suckling pig fecal samples (mean=6.3 $\log_{10}$ CFU/g) (rstandard=3.0)

Appendix 2.3: Leverage values for assessing extreme observations in linear multivariate model

Potential extreme observations were identified for Farms 101 and 109.

Farm 101: The weanling fecal sample did not have *C. perfringens* count (leverage=0.29), but the gestation sow sample had a count of 5.4 $\log_{10}$ CFU/g (leverage=0.30).

Farm 109: The weanling fecal sample did not have *C. perfringens* count (leverage=0.29), but the gestation sow sample had a count of 2.0 $\log_{10}$ CFU/g (leverage=0.30).

Appendix 2.4: Linear regression model with farm as a random effect

Stage 1 = Gestation Sow
Stage 2 = Grower-finisher
Stage 3 = Manure pit
Stage 4 = Lactation Sow
Stage 5 = Piglet
Stage 6 = Weanling

Regular linear regression with the main effects model:

```
. xi:reg logcfu i.stage i.visit
i.stage           _Istage_1-6         (_Istage_5 for stage==P omitted)
i.visitall        _Ivisitall_1-2      (naturally coded; _Ivisitall_1 omitted)

      Source |       SS       df       MS              Number of obs =     354
-------------+------------------------------           F(  6,   347) =   61.79
       Model |  1038.44204     6  173.073673           Prob > F      =  0.0000
    Residual |  972.022077   347  2.80121636           R-squared     =  0.5165
-------------+------------------------------           Adj R-squared =  0.5082
       Total |  2010.46412   353  5.69536577           Root MSE      =  1.6737

------------------------------------------------------------------------------
      logcfu |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
   _Istage_1 |  -.5492143   .3657557    -1.50   0.134    -1.268591    .1701627
   _Istage_2 |  -4.286927    .305385   -14.04   0.000    -4.887565   -3.686288
   _Istage_3 |  -2.132467   .3282718    -6.50   0.000     -2.77812   -1.486814
   _Istage_4 |  -1.063012   .3135096    -3.39   0.001     -1.67963   -.4463938
   _Istage_6 |  -3.621377   .3463126   -10.46   0.000    -4.302513   -2.940241
   _Ivisit_2 |   .2816217    .218461     1.29   0.198    -.1480525     .711296
       _cons |   4.732944   .3112384    15.21   0.000     4.120793    5.345095
------------------------------------------------------------------------------
```

Regular linear regression with significant variables *P*<0.20 from univariable analysis:

```
. xi:reg logcfu i.stage i.farrow i.herd i.visitall
i.stage           _Istage_1-6         (_Istage_5 for stage==P omitted)
i.farrow          _Ifarrow_0-1        (naturally coded; _Ifarrow_0 omitted)
i.herd            _Iherd_1-3          (naturally coded; _Iherd_1 omitted)
i.visitall        _Ivisitall_1-2      (naturally coded; _Ivisitall_1 omitted)

      Source |       SS       df       MS              Number of obs =     350
-------------+------------------------------           F(  9,   340) =   44.76
       Model |  1081.88997     9  120.209996           Prob > F      =  0.0000
    Residual |  913.175505   340  2.68581031           R-squared     =  0.5423
-------------+------------------------------           Adj R-squared =  0.5302
       Total |  1995.06547   349  5.71651998           Root MSE      =  1.6388

------------------------------------------------------------------------------
      logcfu |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
   _Istage_1 |  -.5103284   .3605756    -1.42   0.158    -1.219568    .1989114
   _Istage_2 |  -3.682404   .3446659   -10.68   0.000     -4.36035   -3.004458
   _Istage_3 |  -1.766621   .3385441    -5.22   0.000    -2.432526   -1.100716
   _Istage_4 |  -1.063012   .3069836    -3.46   0.001    -1.666838   -.4591858
   _Istage_6 |  -3.539697   .3408742   -10.38   0.000    -4.210185   -2.869209
  _Ifarrow_1 |   .9578233   .2668711     3.59   0.000      .432897     1.48275
    _Iherd_2 |   .2878885   .2149094     1.34   0.181    -.1348309    .7106078
```

```
    _Iherd_3 |  -.0262755     .29344    -0.09   0.929    -.6034619    .5509109
_Ivisitall_2 |   .3648967   .2224654     1.64   0.102     -.072685    .8024785
       _cons |   3.512787   .4636489     7.58   0.000     2.600806    4.424769
------------------------------------------------------------------------------
```

Non-significant variables ($P<0.05$) were removed using a non-significant partial F-test ($P>0.05$) unless it was a confounder. An independent variable that changed the coefficient of the main effects model by 20% upon removal were identified as a confounder and included in multivariate analysis.

Variables removed: herd size, farrow (farrowing operation)
Variable included due to confounding: visit (sampling period)

All possible interactions between production stage and other independent variables were evaluated, and the significant interactions ($P<0.05$) are listed below:

```
. xi:reg logcfu i.stage*i.farrow
i.stage           _Istage_1-6         (_Istage_5 for stage==P omitted)
i.farrow          _Ifarrow_0-1        (naturally coded; _Ifarrow_0 omitted)
i.stage*i.far~w   _IstaXfar_#_#       (coded as above)
note: _IstaXfar_1_1 omitted because of collinearity
note: _IstaXfar_3_1 omitted because of collinearity
note: _IstaXfar_4_1 omitted because of collinearity

      Source |       SS       df       MS              Number of obs =     354
-------------+------------------------------           F(  8,   345) =   49.74
       Model |  1076.78693     8  134.598366           Prob > F      =  0.0000
    Residual |   933.67719   345   2.7063107           R-squared     =  0.5356
-------------+------------------------------           Adj R-squared =  0.5248
       Total |  2010.46412   353  5.69536577           Root MSE      =  1.6451

------------------------------------------------------------------------------
      logcfu |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
   _Istage_1 |  -.6709967   .3473077    -1.93   0.054    -1.354104    .0121104
   _Istage_2 |  -2.908779   .5355531    -5.43   0.000     -3.96214   -1.855419
   _Istage_3 |   -1.66141   .3445255    -4.82   0.000    -2.339045   -.9837755
   _Istage_4 |  -1.063012    .308153    -3.45   0.001    -1.669107   -.4569171
   _Istage_6 |  -3.345836   1.716981    -1.95   0.052    -6.722904    .0312311
  _Ifarrow_1 |   1.668729   .4407185     3.79   0.000     .8018959    2.535563
_IstaXfa~2_1 |  -1.342698   .5556353    -2.42   0.016    -2.435557   -.2498394
_IstaXfa~6_1 |  -.3544795   1.721476    -0.21   0.837    -3.740389     3.03143
       _cons |   3.345836   .4916421     6.81   0.000     2.378843    4.312829
------------------------------------------------------------------------------

. xi:reg logcfu i.stage*i.otherspp
i.stage           _Istage_1-6         (_Istage_5 for stage==P omitted)
i.otherspp        _Iotherspp_0-1      (naturally coded; _Iotherspp_0 omitted)
i.stage*i.oth~p   _IstaXoth_#_#       (coded as above)

      Source |       SS       df       MS              Number of obs =     342
-------------+------------------------------           F( 11,   330) =   36.43
       Model |  1071.00381    11  97.3639828           Prob > F      =  0.0000
    Residual |  881.925212   330  2.67250064           R-squared     =  0.5484
-------------+------------------------------           Adj R-squared =  0.5334
       Total |  1952.92902   341  5.72706458           Root MSE      =  1.6348

------------------------------------------------------------------------------
      logcfu |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
```

```
   _Istage_1 |  -1.509031   .4873968    -3.10   0.002    -2.467828   -.5502347
   _Istage_2 |   -5.01718   .3907868   -12.84   0.000    -5.785928   -4.248433
   _Istage_3 |  -2.086259   .4295693    -4.86   0.000    -2.931298   -1.241219
   _Istage_4 |  -1.935019    .422098    -4.58   0.000    -2.765361   -1.104676
   _Istage_6 |  -3.895812   .4477025    -8.70   0.000    -4.776523   -3.015101
_Iotherspp_1 |   -.778599   .4336645    -1.80   0.074    -1.631694    .0744965
_IstaXot~1_1 |    1.61017   .7096255     2.27   0.024     .2142099     3.00613
_IstaXot~2_1 |   1.089482   .5488047     1.99   0.048      .009885    2.169079
_IstaXot~3_1 |  -.2601261   .6129164    -0.42   0.672    -1.465842    .9455899
_IstaXot~4_1 |   1.840903   .6132942     3.00   0.003     .6344435    3.047362
_IstaXot~6_1 |   .0804645   .6757403     0.12   0.905    -1.248837    1.409766
       _cons |   5.383376   .2984683    18.04   0.000     4.796235    5.970516
------------------------------------------------------------------------------
```

Significant interaction (P<0.05) between independent variables were included in multivariate analysis. The farm was included as a fixed effect to evaluate Bayesian Information Criteria (BIC) for removal of non-significant interaction terms. The farm coefficients are omitted.

```
. xi:reg logcfu i.stage*i.farrow i.stage*i.otherspp i.visitall i.farm
i.stage           _Istage_1-6         (_Istage_5 for stage==P omitted)
i.farrow          _Ifarrow_0-1        (naturally coded; _Ifarrow_0 omitted)
i.stage*i.far~w   _IstaXfar_#_#       (coded as above)
i.otherspp        _Iotherspp_0-1      (naturally coded; _Iotherspp_0 omitted)
i.stage*i.oth~p   _IstaXoth_#_#       (coded as above)
i.visitall        _Ivisitall_1-2      (naturally coded; _Ivisitall_1 omitted)
i.farm            _Ifarm_1-140        (naturally coded; _Ifarm_1 omitted)
note: _IstaXfar_1_1 omitted because of collinearity
note: _IstaXfar_3_1 omitted because of collinearity
note: _IstaXfar_4_1 omitted because of collinearity
note: _Ifarm_75 omitted because of collinearity
note: _Ifarm_82 omitted because of collinearity
note: _Ifarm_101 omitted because of collinearity

      Source |       SS       df       MS              Number of obs =     342
-------------+------------------------------           F( 59,   282) =   12.03
       Model |  1397.63326    59  23.6886994           Prob > F      =  0.0000
    Residual |   555.29576   282   1.9691339           R-squared     =  0.7157
-------------+------------------------------           Adj R-squared =  0.6562
       Total |  1952.92902   341  5.72706458           Root MSE      =  1.4033

------------------------------------------------------------------------------
      logcfu |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
   _Istage_1 |  -1.382827   .4373133    -3.16   0.002     -2.24364   -.5220142
   _Istage_2 |  -3.104295   .5926368    -5.24   0.000    -4.270848   -1.937741
   _Istage_3 |   -1.31032   .4225826    -3.10   0.002    -2.142136   -.4785032
   _Istage_4 |  -1.935019   .3623197    -5.34   0.000    -2.648213   -1.221824
   _Istage_6 |   -2.79862   1.646701    -1.70   0.090    -6.040006    .4427661
  _Ifarrow_1 |   1.896732    1.67888     1.13   0.260    -1.407994    5.201459
_IstaXfa~2_1 |  -.9870057   .5075029    -1.94   0.053    -1.985981    .0119691
_IstaXfa~6_1 |  -1.083354   1.604061    -0.68   0.500    -4.240806    2.074098
_Iotherspp_1 |   -.781655   .9108685    -0.86   0.392    -2.574619    1.011309
_IstaXot~1_1 |   1.564581   .6250778     2.50   0.013     .3341704    2.794992
_IstaXot~2_1 |   .6583064   .6270851     1.05   0.295    -.5760554    1.892668
_IstaXot~3_1 |   -.225942   .5901406    -0.38   0.702    -1.387582    .9356978
_IstaXot~4_1 |   1.840903   .5264384     3.50   0.001     .8046551     2.87715
_IstaXot~6_1 |   .1347494   .6005941     0.22   0.823    -1.047467    1.316966
_Ivisitall_2 |    .360385    .293975     1.23   0.221     -.218279     .939049
       _cons |   3.153059   1.753463     1.80   0.073     -.298479    6.604598
------------------------------------------------------------------------------
```

```
. estat ic

-----------------------------------------------------------------------------
       Model |    Obs    ll(null)   ll(model)     df          AIC         BIC
-------------+---------------------------------------------------------------
           . |    342    -783.206    -568.159     60     1256.318    1486.407
-----------------------------------------------------------------------------
               Note:  N=Obs used in calculating BIC; see [R] BIC note

. xi:reg logcfu i.visitall i.stage*i.otherspp i.farm
i.visitall        _Ivisitall_1-2      (naturally coded; _Ivisitall_1 omitted)
i.otherspp        _Iotherspp_0-1      (naturally coded; _Iotherspp_0 omitted)
i.stage           _Istage_1-6         (_Istage_5 for stage==P omitted)
i.stage*i.oth~p   _IstaXoth_#_#       (coded as above)
i.farm            _Ifarm_1-140        (naturally coded; _Ifarm_1 omitted)
note: _Ifarm_75 omitted because of collinearity
note: _Ifarm_82 omitted because of collinearity
note: _Ifarm_101 omitted because of collinearity

      Source |       SS       df       MS              Number of obs =     342
-------------+------------------------------           F( 56,   285) =   12.52
       Model |  1388.62623    56   24.796897           Prob > F      =  0.0000
    Residual |  564.302789   285  1.98000979           R-squared     =  0.7110
-------------+------------------------------           Adj R-squared =  0.6543
       Total |  1952.92902   341  5.72706458           Root MSE      =  1.4071

------------------------------------------------------------------------------
      logcfu |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
_Ivisitall_2 |   .3736901   .2927669     1.28   0.203    -.2025697    .9499499
_Iotherspp_1 |   -.785603   .9130818    -0.86   0.390    -2.582843    1.011636
   _Istage_1 |  -1.369237    .438429    -3.12   0.002    -2.232206   -.5062672
   _Istage_2 |  -3.827797   .4613427    -8.30   0.000    -4.735869   -2.919726
   _Istage_3 |  -1.455462   .4168031    -3.49   0.001    -2.275865   -.6350594
   _Istage_4 |  -1.935019   .3633189    -5.33   0.000    -2.650148    -1.21989
   _Istage_6 |  -3.874291   .3972562    -9.75   0.000    -4.656219   -3.092362
_IstaXot~1_1 |   1.554695   .6267745     2.48   0.014     .3210009     2.78839
_IstaXot~2_1 |   .6994386   .6249876     1.12   0.264    -.5307386    1.929616
_IstaXot~3_1 |  -.3598603   .5877452    -0.61   0.541    -1.516733    .7970118
_IstaXot~4_1 |   1.840903   .5278902     3.49   0.001     .8018445    2.879961
_IstaXot~6_1 |   .1582936   .5964158     0.27   0.791    -1.015645    1.332232
       _cons |   5.038613   .5062976     9.95   0.000     4.042056     6.03517
------------------------------------------------------------------------------

. estat ic

-----------------------------------------------------------------------------
       Model |    Obs    ll(null)   ll(model)     df          AIC         BIC
-------------+---------------------------------------------------------------
           . |    342    -783.206   -570.9104     57     1255.821    1474.405
-----------------------------------------------------------------------------
               Note:  N=Obs used in calculating BIC; see [R] BIC note
```

Removed the farrow variable because it was not significant in the final model and removal of the variable resulted in a smaller BIC value.

```
. hettest

Breusch-Pagan / Cook-Weisberg test for heteroskedasticity
         Ho: Constant variance
         Variables: fitted values of logcfu

         chi2(1)      =     2.40
         Prob > chi2  =   0.1210

. predict rstandard, rstandard
(220 missing values generated)

. swilk rstandard

                  Shapiro-Wilk W test for normal data

    Variable |    Obs       W           V         z       Prob>z
-------------+-------------------------------------------------
   rstandard |    342    0.99465      1.280     0.584    0.27977
```

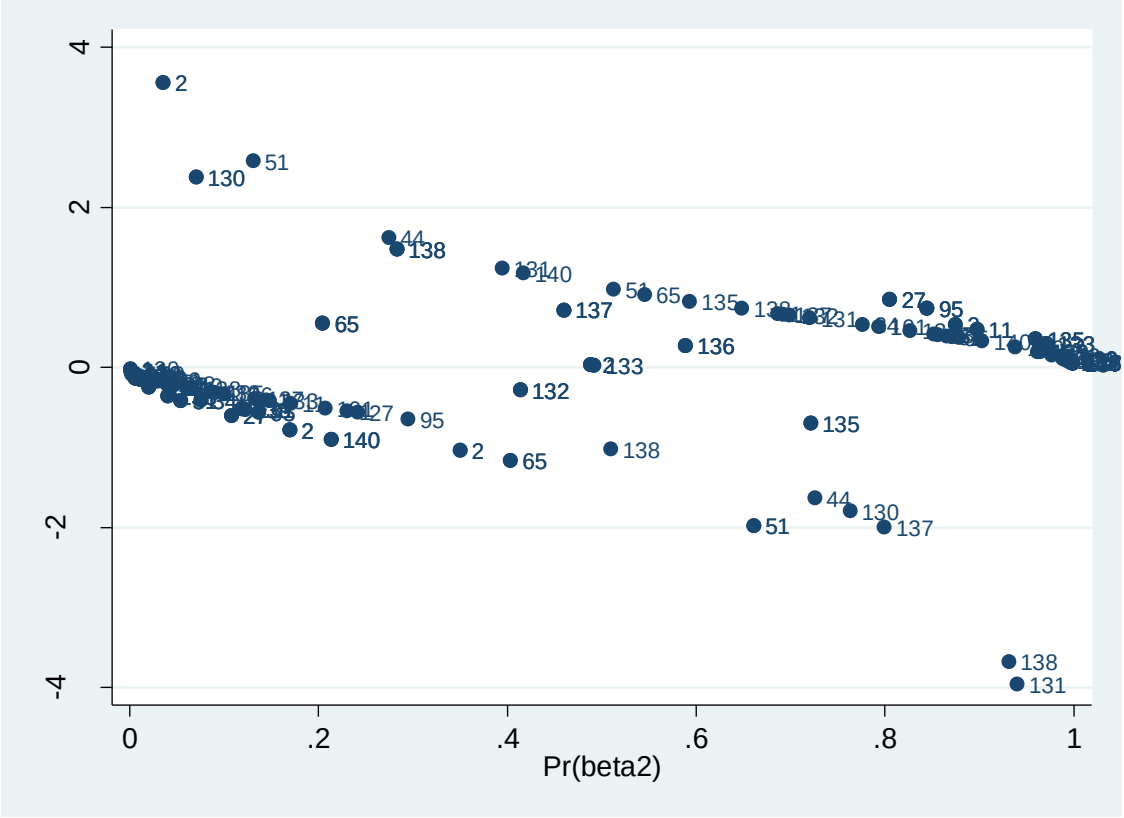
Final model for multivariable linear regression analysis with farm as a random effect

```
. xi:xtreg logcfu i.stage i.visitall i.stage*i.otherspp, i(farm)
i.stage           _Istage_1-6         (_Istage_5 for stage==P omitted)
i.visitall        _Ivisitall_1-2      (naturally coded; _Ivisitall_1 omitted)
i.otherspp        _Iotherspp_0-1      (naturally coded; _Iotherspp_0 omitted)
i.stage*i.oth~p   _IstaXoth_#_#       (coded as above)

Random-effects GLS regression                   Number of obs      =       342
Group variable: farm                            Number of groups   =        46

R-sq:  within  = 0.4961                         Obs per group: min =         4
       between = 0.6463                                        avg =       7.4
       overall = 0.5459                                        max =        14

Random effects u_i ~ Gaussian                   Wald chi2(12)      =    355.31
corr(u_i, X)       = 0 (assumed)                Prob > chi2        =    0.0000

------------------------------------------------------------------------------
      logcfu |      Coef.   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
   _Istage_1 |  -1.354421   .4381951    -3.09   0.002    -2.213267   -.4955743
   _Istage_2 |  -4.382386   .4135223   -10.60   0.000    -5.192875   -3.571897
   _Istage_3 |  -1.720155   .4031459    -4.27   0.000    -2.510306   -.9300034
   _Istage_4 |  -1.935019   .3668672    -5.27   0.000    -2.654065   -1.215972
   _Istage_6 |  -3.841296   .3983586    -9.64   0.000    -4.622064   -3.060527
_Ivisitall_2 |   .3424058   .2341717     1.46   0.144    -.1165623    .8013738
_Iotherspp_1 |  -.9302048   .4844581    -1.92   0.055    -1.879725    .0193157
_IstaXot~1_1 |    1.52306   .6260952     2.43   0.015     .2959357    2.750184
_IstaXot~2_1 |   .9835715   .5577976     1.76   0.078    -.1096916    2.076835
_IstaXot~3_1 |  -.2718716   .5642025    -0.48   0.630    -1.377688    .8339449
_IstaXot~4_1 |   1.840903   .5330456     3.45   0.001     .7961524    2.885653
_IstaXot~6_1 |   .0959167   .5962863     0.16   0.872    -1.072783    1.264616
       _cons |   4.926791   .3887239    12.67   0.000     4.164906    5.688676
-------------+----------------------------------------------------------------
     sigma_u |  .78637027
     sigma_e |  1.4071282
         rho |  .23798532   (fraction of variance due to u_i)
------------------------------------------------------------------------------
```

Appendix 2.5: Association of *cpb2* positive *C. perfringens* isolates and independent variables in univariable logistic regression

Parameters		Odds Ratio	Standard Error	95% Confidence Interval	*P*-value
Stage	Piglet	Referent			
	Lactation sow	0.096	0.0436	0.040-0.23	<0.001
	Gestation sow	0.016	0.0130	0.0033-0.78	<0.001
	Weanling	0.29	0.176	0.085-0.96	0.043
	Grower-finisher	0.031	0.0257	0.0062-0.16	<0.001
	Manure pit	0.14	0.0633	0.056-0.34	<0.001
Atyp-cpb2	Absent	Referent			
	Present	16	6.76	7.0-37	<0.001
Farrowing operation	No	Referent			
	Yes	5.0	0.315	1.4-17	0.012
Other species	Absent	Referent			
	Present	0.44	0.127	0.25-0.78	0.004
Herd size	128-940 (n=9)	Referent			
	1050-3000 (n=28)	1.4	0.484	0.75-2.8	0.272
	3200-7200 (n=9)	3.25	1.62	1.2-8.6	0.018
Sampling Period	First	Referent			
	Second	2.5	0.995	1.2-5.5	0.017

Appendix 2.6: Pearson residuals for assessing outliers in the logistic multivariate model



Potential outliers were identified for Farms 51, 2, 138, and 131.

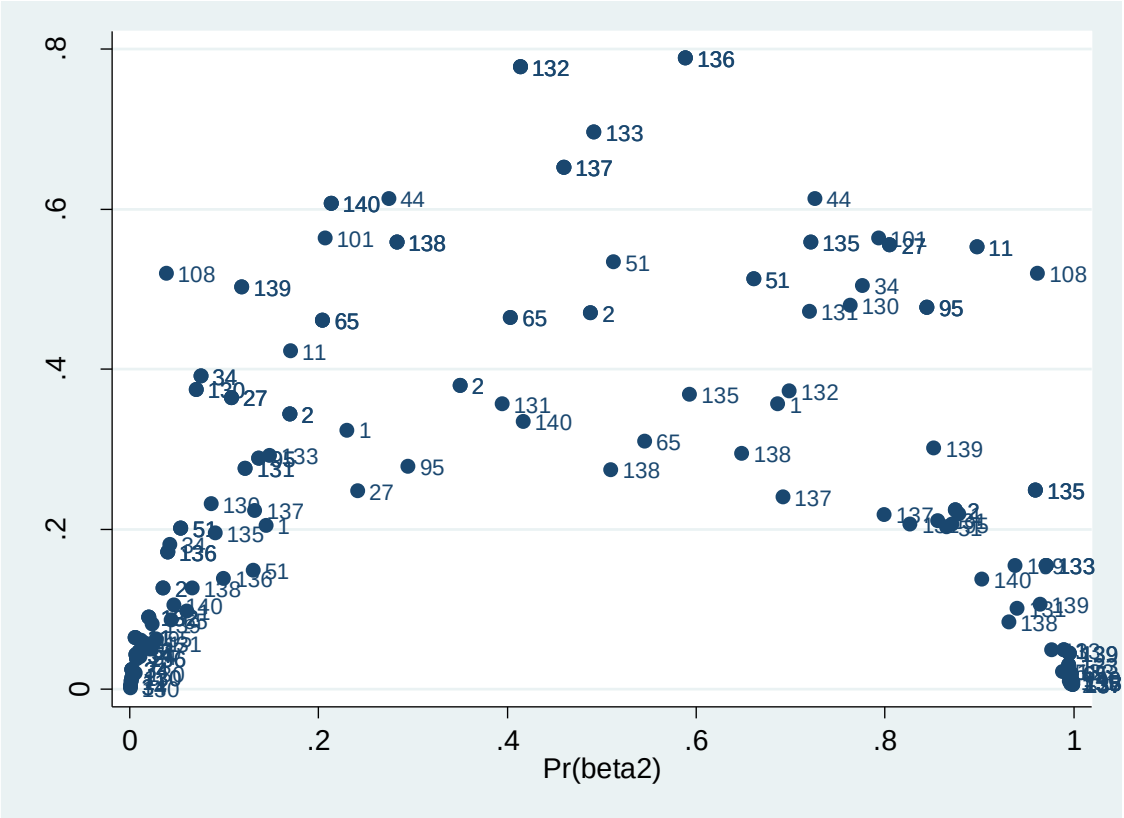
Farm 51: A low count of C. perfringens (3.8 $\log_{10}$ CFU/g) was isolated from the manure pit fecal sample (r=2.6).

Farm 2: *Cpb2* was recovered from the *C. perfringens* isolates of one gestation sow fecal sample, but not from the isolates of another gestation sow fecal sample (r=3.6).

Farm 138: *Cpb2* nor atyp-cpb2 were recovered from the *C. perfringens* isolates of a suckling pig fecal sample (r=3.7).

Farm 131: *Cpb2* was not recovered from the *C. perfringens* isolates of a manure pit fecal sample, but the isolates were positive for *atyp-cpb2* (r=4.0).

Appendix 2.7: Leverage values for assessing extreme observations in the logistic multivariate model

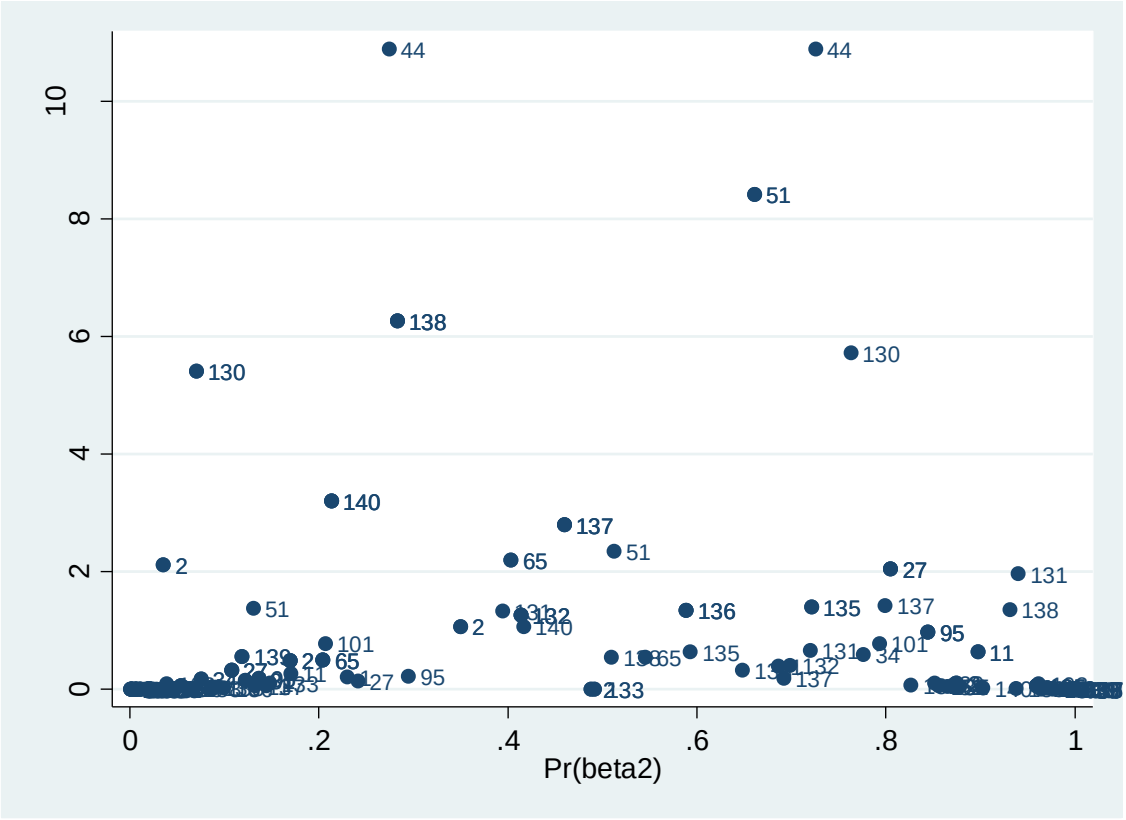


Potential extreme observations were identified for Farms 132 and 136.

Farm 132: Cpb2 was recovered from one of three suckling pig fecal samples (lev=0.78)

Farm 136: *Cpb2* was recovered from two of three suckling pig fecal samples (lev=0.79).

Appendix 2.8: Dbeta values for assessing influential covariate patterns in the logistic multivariate model



A potential influential covariate pattern was identified for Farm 44. *Cpb2* was not recovered from the *C. perfringens* isolates of a grower-finisher fecal sample, but it was recovered from a manure pit fecal sample (lev=11).

Appendix 2.9: Logistic regression models with farm as a random effect

Stage 1 = Gestation Sow
Stage 2 = Grower-finisher
Stage 3 = Manure pit
Stage 4 = Lactation Sow
Stage 5 = Piglet
Stage 6 = Weanling

Regular logistic regression with the main effects model:

```
. xi:logit beta2 i.stage, or
i.stage           _Istage_1-6         (_Istage_5 for stage==P omitted)

Iteration 0:   log likelihood = -147.64762
Iteration 1:   log likelihood = -113.69956
Iteration 2:   log likelihood = -112.91578
Iteration 3:   log likelihood = -112.90213
Iteration 4:   log likelihood = -112.90211
Iteration 5:   log likelihood = -112.90211

Logistic regression                               Number of obs   =        220
                                                  LR chi2(5)      =      69.49
                                                  Prob > chi2     =     0.0000
Log likelihood = -112.90211                       Pseudo R2       =     0.2353

------------------------------------------------------------------------------
       beta2 | Odds Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
   _Istage_1 |    .016129   .0129625    -5.14   0.000     .0033382    .0779288
   _Istage_2 |     .03125   .0256961    -4.21   0.000     .0062363    .1565928
   _Istage_3 |    .137931    .063301    -4.32   0.000     .0561071    .3390831
   _Istage_4 |   .0961538   .0436356    -5.16   0.000     .0395078    .2340189
   _Istage_6 |   .2857143   .1764719    -2.03   0.043     .0851503    .9586895
------------------------------------------------------------------------------
```

Regular logistic regression with significant variables $P<0.20$ from univariable analysis:

```
. xi:logit beta2 i.stage i.atypb2 i.farrow i.otherspp i.herd i.visitall, or
i.stage           _Istage_1-6         (_Istage_5 for stage==P omitted)
i.atypb2          _Iatypb2_0-1        (naturally coded; _Iatypb2_0 omitted)
i.farrow          _Ifarrow_0-1        (naturally coded; _Ifarrow_0 omitted)
i.otherspp        _Iotherspp_0-1      (naturally coded; _Iotherspp_0 omitted)
i.herd            _Iherd_1-3          (naturally coded; _Iherd_1 omitted)
i.visitall        _Ivisitall_1-2      (naturally coded; _Ivisitall_1 omitted)

Iteration 0:   log likelihood = -143.52307
Iteration 1:   log likelihood = -88.312413
Iteration 2:   log likelihood = -87.611521
Iteration 3:   log likelihood = -87.609033
Iteration 4:   log likelihood = -87.609033

Logistic regression                               Number of obs   =        212
                                                  LR chi2(11)     =     111.83
                                                  Prob > chi2     =     0.0000
Log likelihood = -87.609033                       Pseudo R2       =     0.3896

------------------------------------------------------------------------------
       beta2 | Odds Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
```

```
-------------+----------------------------------------------------------------
   _Istage_1 |   .0261313   .0235054    -4.05   0.000     .0044822    .1523443
   _Istage_2 |   .0217442   .0243869    -3.41   0.001     .0024137    .1958839
   _Istage_3 |   .1477834   .0955883    -2.96   0.003     .0415965    .5250425
   _Istage_4 |   .1031786   .0544513    -4.30   0.000     .0366756    .2902695
   _Istage_6 |   .4962464   .3714492    -0.94   0.349     .1144348    2.151973
  _Iatypb2_1 |   13.49156   7.462796     4.70   0.000     4.562722    39.89335
  _Ifarrow_1 |   2.042516   1.658369     0.88   0.379     .4159621    10.02945
_Iotherspp_1 |   .5825495   .2748089    -1.15   0.252     .2310955    1.468501
    _Iherd_2 |   .9691418   .5175201    -0.06   0.953     .3402883    2.760117
    _Iherd_3 |   2.184032   1.767774     0.97   0.334     .4469812    10.67158
_Ivisitall_2 |   .7490764   .4764594    -0.45   0.650     .2153321    2.605815
-------------------------------------------------------------------------------

. est store a
```

There were no confounders. Non-significant variables ($P<0.05$) were removed using a non-significant likelihood ratio test ($P>0.05$). Interaction was evaluated between production stage and independent variables. There were no interactions found.

```
. xi:logit beta2 i.stage i.atypb2 i.farrow i.otherspp i.herd, or
i.stage           _Istage_1-6         (_Istage_5 for stage==P omitted)
i.atypb2          _Iatypb2_0-1        (naturally coded; _Iatypb2_0 omitted)
i.farrow          _Ifarrow_0-1        (naturally coded; _Ifarrow_0 omitted)
i.otherspp        _Iotherspp_0-1      (naturally coded; _Iotherspp_0 omitted)
i.herd            _Iherd_1-3          (naturally coded; _Iherd_1 omitted)

Iteration 0:   log likelihood = -143.52307
Iteration 1:   log likelihood =   -88.3603
Iteration 2:   log likelihood = -87.714024
Iteration 3:   log likelihood =  -87.71211
Iteration 4:   log likelihood =  -87.71211

Logistic regression                               Number of obs   =        212
                                                  LR chi2(10)     =     111.62
                                                  Prob > chi2     =     0.0000
Log likelihood =  -87.71211                       Pseudo R2       =     0.3889

-------------------------------------------------------------------------------
       beta2 | Odds Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+-----------------------------------------------------------------
   _Istage_1 |   .0297205   .0251565    -4.15   0.000     .0056568    .1561508
   _Istage_2 |   .0268276   .0273175    -3.55   0.000     .0036462    .1973906
   _Istage_3 |   .1679865   .0971383    -3.08   0.002     .0540838    .5217735
   _Istage_4 |   .1033827   .0545765    -4.30   0.000      .036736    .2909403
   _Istage_6 |   .5120916   .3822304    -0.90   0.370     .1185775    2.211531
  _Iatypb2_1 |   13.51381   7.487738     4.70   0.000     4.561878    40.03243
  _Ifarrow_1 |   2.131801    1.70883     0.94   0.345     .4430292    10.25796
_Iotherspp_1 |   .5984239   .2792356    -1.10   0.271     .2397854    1.493465
    _Iherd_2 |   1.003432   .5274072     0.01   0.995     .3581738    2.811139
    _Iherd_3 |   2.393629   1.878977     1.11   0.266     .5138917    11.14916
-------------------------------------------------------------------------------

. est store b

. lrtest a b

Likelihood-ratio test                                 LR chi2(1)  =      0.21
(Assumption: b nested in a)                           Prob > chi2 =    0.6498

. xi:logit beta2 i.stage i.atypb2 i.farrow i.otherspp, or
i.stage           _Istage_1-6         (_Istage_5 for stage==P omitted)
```

```
i.atypb2          _Iatypb2_0-1        (naturally coded; _Iatypb2_0 omitted)
i.farrow          _Ifarrow_0-1        (naturally coded; _Ifarrow_0 omitted)
i.otherspp        _Iotherspp_0-1      (naturally coded; _Iotherspp_0 omitted)

Iteration 0:   log likelihood = -143.52307
Iteration 1:   log likelihood = -89.323206
Iteration 2:   log likelihood = -88.676413
Iteration 3:   log likelihood = -88.674956
Iteration 4:   log likelihood = -88.674956

Logistic regression                                     Number of obs   =        212
                                                        LR chi2(8)      =     109.70
                                                        Prob > chi2     =     0.0000
Log likelihood = -88.674956                             Pseudo R2       =     0.3822

------------------------------------------------------------------------------
       beta2 | Odds Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
   _Istage_1 |   .0311037   .0259604    -4.16   0.000     .0060585    .1596836
   _Istage_2 |   .0244107   .0248305    -3.65   0.000     .0033246    .1792345
   _Istage_3 |   .1661144   .0947774    -3.15   0.002     .0542937    .5082351
   _Istage_4 |   .0986908   .0518856    -4.40   0.000     .0352182     .276558
   _Istage_6 |   .4535793   .3339217    -1.07   0.283     .1071531    1.920001
  _Iatypb2_1 |   13.96023   7.603368     4.84   0.000     4.800536    40.59711
  _Ifarrow_1 |   2.073786   1.645032     0.92   0.358     .4380741    9.817033
_Iotherspp_1 |   .5024125   .1962733    -1.76   0.078       .23363    1.080419
------------------------------------------------------------------------------

. est store c

. lrtest b c

Likelihood-ratio test                                 LR chi2(2)  =      1.93
(Assumption: c nested in b)                           Prob > chi2 =    0.3818


. xi:logit beta2 i.stage i.atypb2 i.otherspp, or
i.stage           _Istage_1-6         (_Istage_5 for stage==P omitted)
i.atypb2          _Iatypb2_0-1        (naturally coded; _Iatypb2_0 omitted)
i.otherspp        _Iotherspp_0-1      (naturally coded; _Iotherspp_0 omitted)

Iteration 0:   log likelihood = -143.52307
Iteration 1:   log likelihood = -89.722507
Iteration 2:   log likelihood =  -89.12379
Iteration 3:   log likelihood = -89.122672
Iteration 4:   log likelihood = -89.122672

Logistic regression                                     Number of obs   =        212
                                                        LR chi2(7)      =     108.80
                                                        Prob > chi2     =     0.0000
Log likelihood = -89.122672                             Pseudo R2       =     0.3790

------------------------------------------------------------------------------
       beta2 | Odds Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
   _Istage_1 |     .03123   .0260674    -4.15   0.000     .0060825    .1603477
   _Istage_2 |    .017449   .0168344    -4.20   0.000     .0026336     .115608
   _Istage_3 |   .1371282   .0736447    -3.70   0.000     .0478618    .3928841
   _Istage_4 |   .0983205   .0517977    -4.40   0.000     .0350114    .2761079
   _Istage_6 |   .4544177   .3348059    -1.07   0.284     .1072277    1.925766
  _Iatypb2_1 |   14.54421    7.89396     4.93   0.000     5.019904    42.13908
_Iotherspp_1 |   .5028928   .1957995    -1.77   0.077      .234457    1.078668
------------------------------------------------------------------------------
```

```
. est store d

. lrtest c d

Likelihood-ratio test                                 LR chi2(1)  =      0.90
(Assumption: d nested in c)                           Prob > chi2 =    0.3440
```

Variables removed: herd size, farrow (farrowing operation), and visit (sampling period)

The other species variable could not be tested using a likelihood ratio test due to different number of observations. The other species variable was removed in the random effects model because it was non-significant ($P>0.05$) and removal resulted in a slightly smaller BIC:

```
. xi:xtlogit beta2 i.stage i.atypb2 i.otherspp, i(farm) or nolog
i.stage           _Istage_1-6         (_Istage_5 for stage==P omitted)
i.atypb2          _Iatypb2_0-1        (naturally coded; _Iatypb2_0 omitted)
i.otherspp        _Iotherspp_0-1      (naturally coded; _Iotherspp_0 omitted)

Random-effects logistic regression              Number of obs      =       212
Group variable: farm                            Number of groups   =        40

Random effects u_i ~ Gaussian                   Obs per group: min =         1
                                                               avg =       5.3
                                                               max =        12

                                                Wald chi2(7)       =     47.14
Log likelihood  = -88.123011                    Prob > chi2        =    0.0000

------------------------------------------------------------------------------
       beta2 |         OR   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
   _Istage_1 |   .0229918   .0210046    -4.13   0.000     .0038366    .1377855
   _Istage_2 |   .0177548   .0185538    -3.86   0.000     .0022899    .1376649
   _Istage_3 |   .1160288   .0700502    -3.57   0.000     .0355361    .3788457
   _Istage_4 |   .0733392   .0453198    -4.23   0.000     .0218443     .246226
   _Istage_6 |   .3403867   .2868413    -1.28   0.201     .0652637    1.775308
  _Iatypb2_1 |   16.46196   9.911842     4.65   0.000     5.057875    53.57904
_Iotherspp_1 |   .5143878   .2627352    -1.30   0.193     .1890254    1.399784
-------------+----------------------------------------------------------------
    /lnsig2u |  -.4459202   1.057819                     -2.519208    1.627368
-------------+----------------------------------------------------------------
     sigma_u |   .8001468   .4232054                      .2837664    2.256204
         rho |   .1629054   .1442519                      .0238914    .6074295
------------------------------------------------------------------------------
Likelihood-ratio test of rho=0: chibar2(01) =     2.00 Prob >= chibar2 = 0.079

. estat ic

-----------------------------------------------------------------------------
       Model |    Obs    ll(null)   ll(model)     df          AIC          BIC
-------------+---------------------------------------------------------------
           . |    212           .   -88.12301      9      194.246    224.4553
-----------------------------------------------------------------------------
               Note:  N=Obs used in calculating BIC; see [R] BIC note
```

Final logistic regression model with farm as a random effect for the association of *cpb2* and stage of production:

```
. xi:xtlogit beta2 i.stage i.atypb2, i(farm) or nolog
i.stage           _Istage_1-6         (_Istage_5 for stage==P omitted)
i.atypb2          _Iatypb2_0-1        (naturally coded; _Iatypb2_0 omitted)

Random-effects logistic regression              Number of obs      =       220
Group variable: farm                            Number of groups   =        42

Random effects u_i ~ Gaussian                   Obs per group: min =         1
                                                               avg =       5.2
                                                               max =        12

                                                Wald chi2(6)       =     46.66
Log likelihood  = -90.470784                    Prob > chi2        =    0.0000

------------------------------------------------------------------------------
       beta2 |         OR   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
   _Istage_1 |   .0201962   .0188551    -4.18   0.000     .0032404    .1258776
   _Istage_2 |   .0169425   .0184726    -3.74   0.000     .0019994    .1435661
   _Istage_3 |   .1086548   .0676953    -3.56   0.000     .0320423    .3684459
   _Istage_4 |   .0689516   .0437402    -4.22   0.000     .0198873    .2390634
   _Istage_6 |   .2626721   .2155308    -1.63   0.103     .0525988    1.311752
  _Iatypb2_1 |   19.21433   11.95651     4.75   0.000     5.674765    65.05831
-------------+----------------------------------------------------------------
    /lnsig2u |   .0926864   .8121331                     -1.499065    1.684438
-------------+----------------------------------------------------------------
     sigma_u |   1.047434   .4253279                      .4725874    2.321513
         rho |   .2500846   .1523093                      .0635712    .6209519
------------------------------------------------------------------------------
Likelihood-ratio test of rho=0: chibar2(01) =     4.53 Prob >= chibar2 = 0.017

. estat ic

------------------------------------------------------------------------------
       Model |    Obs    ll(null)   ll(model)     df          AIC         BIC
-------------+----------------------------------------------------------------
           . |    220           .   -90.47078      8     196.9416    224.0906
------------------------------------------------------------------------------
               Note:  N=Obs used in calculating BIC; see [R] BIC note
```

Farm was included as a fixed effect to assess the model's goodness of fit. Farm coefficients were omitted.

```
. xi:logit beta2 i.stage i.atypb2 i.farm, or nolog
i.stage           _Istage_1-6         (_Istage_5 for stage==P omitted)
i.atypb2          _Iatypb2_0-1        (naturally coded; _Iatypb2_0 omitted)
i.farm            _Ifarm_1-140        (naturally coded; _Ifarm_1 omitted)

note: _Ifarm_20 omitted because of collinearity
note: _Ifarm_24 omitted because of collinearity
note: _Ifarm_26 omitted because of collinearity
note: _Ifarm_81 omitted because of collinearity
note: _Ifarm_105 omitted because of collinearity
note: _Ifarm_128 omitted because of collinearity

Logistic regression                             Number of obs   =        173
                                                LR chi2(26)     =     130.15
```

```
                                                Prob > chi2     =     0.0000
Log likelihood =  -54.00159                     Pseudo R2       =     0.5465

------------------------------------------------------------------------------
       beta2 | Odds Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
   _Istage_1 |   .0052347   .0069282    -3.97   0.000     .0003911    .0700594
   _Istage_2 |   .5391434   .8130552    -0.41   0.682     .0280576    10.35998
   _Istage_3 |   .0769973   .0649648    -3.04   0.002     .0147331    .4024001
   _Istage_4 |   .0292072    .023624    -4.37   0.000     .0059841    .1425531
   _Istage_6 |   .1365499   .1417701    -1.92   0.055     .0178463    1.044802
  _Iatypb2_1 |   42.67469   40.13272     3.99   0.000      6.75572    269.5685
------------------------------------------------------------------------------

. lfit, g(10)

Logistic model for beta2, goodness-of-fit test

  (Table collapsed on quantiles of estimated probabilities)

       number of observations =       173
             number of groups =        10
     Hosmer-Lemeshow chi2(8) =         3.53
                 Prob > chi2 =         0.8969

. char stage [omit] W

. xi:xtlogit beta2 i.stage i.atypb2, i(farm) or nolog
i.stage           _Istage_1-6         (_Istage_6 for stage==W omitted)
i.atypb2          _Iatypb2_0-1        (naturally coded; _Iatypb2_0 omitted)

Random-effects logistic regression              Number of obs      =       220
Group variable: farm                            Number of groups   =        42

Random effects u_i ~ Gaussian                   Obs per group: min =         1
                                                               avg =       5.2
                                                               max =        12

                                                Wald chi2(6)       =     46.66
Log likelihood  = -90.470784                    Prob > chi2        =    0.0000

------------------------------------------------------------------------------
       beta2 |         OR   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
   _Istage_1 |   .0768876   .0772117    -2.55   0.011     .0107416     .550358
   _Istage_2 |   .0645006   .0796905    -2.22   0.027     .0057267    .7264794
   _Istage_3 |   .4136519   .3210476    -1.14   0.255     .0903633    1.893555
   _Istage_4 |   .2625008   .2004904    -1.75   0.080       .05875    1.172879
   _Istage_5 |   3.807028   3.123787     1.63   0.103     .7623392    19.01183
  _Iatypb2_1 |   19.21433   11.95651     4.75   0.000     5.674765    65.05831
-------------+----------------------------------------------------------------
    /lnsig2u |   .0926864   .8121331                     -1.499065    1.684438
-------------+----------------------------------------------------------------
     sigma_u |   1.047434   .4253279                      .4725874    2.321513
         rho |   .2500846   .1523093                      .0635712    .6209519
------------------------------------------------------------------------------
Likelihood-ratio test of rho=0: chibar2(01) =     4.53 Prob >= chibar2 = 0.017
```

Logistic regression model with farm as a random effect for the association of *atyp-cpb2* and stage of production:

```
. xi:xtlogit i.atypb2 i.stage, i(farm) or nolog
i.atypb2          _Iatypb2_0-1        (naturally coded; _Iatypb2_0 omitted)
i.stage           _Istage_1-6         (_Istage_5 for stage==P omitted)

Random-effects logistic regression              Number of obs      =       220
Group variable: farm                            Number of groups   =        42

Random effects u_i ~ Gaussian                   Obs per group: min =         1
                                                               avg =       5.2
                                                               max =        12

                                                Wald chi2(5)       =     13.04
Log likelihood  = -101.53216                    Prob > chi2        =    0.0230

------------------------------------------------------------------------------
  _Iatypb2_1 |         OR   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
   _Istage_1 |   9.63e-10   5.25e-06    -0.00   0.997            0           .
   _Istage_2 |   .3955344   .2839199    -1.29   0.196      .096868    1.615058
   _Istage_3 |   .3185786    .158648    -2.30   0.022     .1200409    .8454811
   _Istage_4 |   .1950404   .0977052    -3.26   0.001     .0730662    .5206342
   _Istage_6 |   .1973137   .1553072    -2.06   0.039     .0421862    .9228788
-------------+----------------------------------------------------------------
    /lnsig2u |   -.214314   .7241956                     -1.633711    1.205083
-------------+----------------------------------------------------------------
     sigma_u |   .8983846   .3253031                      .4418187    1.826756
         rho |   .1969983   .1145605                      .0560114    .5035589
------------------------------------------------------------------------------
Likelihood-ratio test of rho=0: chibar2(01) =     5.34 Prob >= chibar2 = 0.010
```

Farm was included as a fixed effect to assess the model's goodness of fit. Farm coefficients were omitted.

```
. xi:logit i.atypb2 i.stage i.farm, or nolog
i.atypb2          _Iatypb2_0-1        (naturally coded; _Iatypb2_0 omitted)
i.stage           _Istage_1-6         (_Istage_5 for stage==P omitted)
i.farm            _Ifarm_1-140        (naturally coded; _Ifarm_1 omitted)

note: _Ifarm_20 omitted because of collinearity
note: _Ifarm_24 omitted because of collinearity
note: _Ifarm_26 omitted because of collinearity
note: _Ifarm_81 omitted because of collinearity
note: _Ifarm_105 omitted because of collinearity
note: _Ifarm_109 omitted because of collinearity
note: _Ifarm_128 omitted because of collinearity

Logistic regression                             Number of obs   =        142
                                                LR chi2(22)     =      37.45
                                                Prob > chi2     =     0.0211
Log likelihood = -71.425278                     Pseudo R2       =     0.2077

------------------------------------------------------------------------------
  _Iatypb2_1 | Odds Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
   _Istage_2 |   .4192017   .5251558    -0.69   0.488     .0359814    4.883909
   _Istage_3 |   .3915814   .2313217    -1.59   0.112     .1230228    1.246403
   _Istage_4 |   .1546127   .0838145    -3.44   0.001     .0534337     .447379
   _Istage_6 |   .1349544   .1149439    -2.35   0.019     .0254213    .7164333
------------------------------------------------------------------------------
```

```
. lfit, g(10) table

Logistic model for _Iatypb2_1, goodness-of-fit test

  (Table collapsed on quantiles of estimated probabilities)
  +--------------------------------------------------------+
  | Group |   Prob | Obs_1 | Exp_1 | Obs_0 | Exp_0 | Total |
  |-------+--------+-------+-------+-------+-------+-------|
  |     1 | 0.0483 |     1 |   0.6 |    14 |  14.4 |    15 |
  |     2 | 0.1139 |     1 |   1.4 |    13 |  12.6 |    14 |
  |     3 | 0.1772 |     1 |   1.9 |    13 |  12.1 |    14 |
  |     4 | 0.2088 |     3 |   3.0 |    12 |  12.0 |    15 |
  |     5 | 0.2532 |     2 |   3.4 |    12 |  10.6 |    14 |
  |-------+--------+-------+-------+-------+-------+-------|
  |     6 | 0.3815 |     5 |   4.5 |    10 |  10.5 |    15 |
  |     7 | 0.4641 |     9 |   7.5 |     8 |   9.5 |    17 |
  |     8 | 0.5355 |     7 |   5.1 |     3 |   4.9 |    10 |
  |     9 | 0.6460 |    11 |   9.1 |     4 |   5.9 |    15 |
  |    10 | 0.9139 |     7 |  10.5 |     6 |   2.5 |    13 |
  +--------------------------------------------------------+

       number of observations =       142
             number of groups =        10
    Hosmer-Lemeshow chi2(8) =        10.80
                Prob > chi2 =         0.2131
```

Appendix 3.1: Association of *Clostridium perfringens* diagnosis and independent variables in univariable logistic regression

Parameters		Odds Ratio	Standard Error	95% Confidence Interval	*P*-value
Average age (d)		0.73	0.0889	0.58-0.93	0.01
Season	Winter	Referent			
	Fall	0.27	0.175	0.075-0.97	0.044
	Spring	0.18	0.138	0.040-0.81	0.025
	Summer	0.64	0.331	0.24-1.8	0.392
Enterotoxigenic *E. coli* detection	No	Referent			
	Yes	0.24	0.150	0.070-0.82	0.022
C. difficile detection	No	Referent			
	Yes	2.6	1.46	0.88-7.8	0.084
Enterococcus detection	Yes	Referent			
	No	7.7	11.0	0.47-127	0.153

Appendix 3.2: Multivariable logistic regression model for the etiological diagnosis of *Clostridium perfringens*

```
. xi:logit cperf ageavg i.ecoli i.cdiff i.ent i.season, or nolog
i.ecoli           _Iecoli_0-1         (naturally coded; _Iecoli_0 omitted)
i.cdiff           _Icdiff_0-1         (naturally coded; _Icdiff_0 omitted)
i.ent             _Ient_0-1           (naturally coded; _Ient_0 omitted)
i.season          _Iseason_1-4        (_Iseason_4 for season==Winter omitted)


Logistic regression                               Number of obs   =        237
                                                  LR chi2(7)      =      27.24
                                                  Prob > chi2     =     0.0003
Log likelihood = -72.463149                       Pseudo R2       =     0.1582

------------------------------------------------------------------------------
       cperf | Odds Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
      ageavg |   .7070865   .0974993    -2.51   0.012     .5396367    .9264963
   _Iecoli_1 |   .2237719   .1476359    -2.27   0.023     .0614068    .8154452
   _Icdiff_1 |   1.474417    .919284     0.62   0.533     .4344153    5.004213
     _Ient_1 |   4.638127   7.538359     0.94   0.345      .191815    112.1509
  _IseasonFA |    .218467    .148749    -2.23   0.025     .0575205    .8297532
  _IseasonSP |   .1592355   .1261333    -2.32   0.020     .0337122    .7521283
  _IseasonSU |   .6384136   .3507428    -0.82   0.414     .2174975    1.873916
------------------------------------------------------------------------------

. xi:logit cperf ageavg i.ecoli i.season, or nolog
i.ecoli           _Iecoli_0-1         (naturally coded; _Iecoli_0 omitted)
i.season          _Iseason_1-4        (_Iseason_4 for season==Winter omitted)

Logistic regression                               Number of obs   =        237
                                                  LR chi2(5)      =      25.75
                                                  Prob > chi2     =     0.0001
Log likelihood = -73.203921                       Pseudo R2       =     0.1496

------------------------------------------------------------------------------
       cperf | Odds Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
      ageavg |   .7117574   .0962343    -2.51   0.012     .5460644    .9277268
   _Iecoli_1 |   .2040786   .1331603    -2.44   0.015     .0568058    .7331662
  _IseasonFA |   .2120943   .1429424    -2.30   0.021     .0566052    .7946978
  _IseasonSP |    .147699   .1164011    -2.43   0.015     .0315172    .6921608
  _IseasonSU |   .5826156   .3156227    -1.00   0.319     .2014918    1.684639
------------------------------------------------------------------------------

. xi:logit cperf ageavg i.ecoli i.season i.year, or
i.ecoli           _Iecoli_0-1         (naturally coded; _Iecoli_0 omitted)
i.season          _Iseason_1-4        (_Iseason_4 for season==Winter omitted)
i.year            _Iyear_2001-2010    (naturally coded; _Iyear_2010 omitted)

Logistic regression                               Number of obs   =        216
                                                  LR chi2(13)     =      43.15
                                                  Prob > chi2     =     0.0000
Log likelihood = -61.731968                       Pseudo R2       =     0.2590

------------------------------------------------------------------------------
       cperf | Odds Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
      ageavg |    .687446   .1064884    -2.42   0.016     .5074389    .9313081
   _Iecoli_1 |   .2712612   .1915359    -1.85   0.065     .0679766     1.08247
  _IseasonFA |   .1343364   .1080188    -2.50   0.013     .0277811    .6495881
```

```
  _IseasonSP |   .1332258    .116341    -2.31   0.021     .0240584    .7377511
  _IseasonSU |   1.068591   .6615469     0.11   0.915     .3175751    3.595641
 _Iyear_2001 |  (omitted)
 _Iyear_2002 |   .0747474   .0966748    -2.01   0.045     .0059251    .9429741
 _Iyear_2003 |    .088724   .0956278    -2.25   0.025     .0107301    .7336287
 _Iyear_2004 |   .0558817   .0713116    -2.26   0.024     .0045818    .6815581
 _Iyear_2005 |   .1936419   .1634265    -1.95   0.052     .0370354    1.012469
 _Iyear_2006 |   .0395178   .0500472    -2.55   0.011     .0033021    .4729286
 _Iyear_2007 |   .0782523   .0840325    -2.37   0.018     .0095372     .642058
 _Iyear_2008 |   1.214818   1.175206     0.20   0.841     .1824169    8.090167
 _Iyear_2009 |   .1736724   .1588985    -1.91   0.056     .0289027    1.043573
------------------------------------------------------------------------------
```

Changed referent category of season:

```
. xi:logit cperf ageavg i.ecoli i.season i.year, or
i.ecoli           _Iecoli_0-1         (naturally coded; _Iecoli_0 omitted)
i.season          _Iseason_1-4        (_Iseason_3 for season==Summer omitted)
i.year            _Iyear_2001-2010    (naturally coded; _Iyear_2010 omitted
Logistic regression                               Number of obs   =        216
                                                  LR chi2(13)     =      43.15
                                                  Prob > chi2     =     0.0000
Log likelihood = -61.731968                       Pseudo R2       =     0.2590

------------------------------------------------------------------------------
       cperf | Odds Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
      ageavg |    .687446   .1064884    -2.42   0.016     .5074389    .9313081
   _Iecoli_1 |   .2712612   .1915359    -1.85   0.065     .0679766     1.08247
  _IseasonFA |   .1257137   .1140385    -2.29   0.022     .0212438    .7439301
  _IseasonSP |   .1246743    .118955    -2.18   0.029     .0192143    .8089662
  _Iseason_W |   .9358121   .5793459    -0.11   0.915     .2781146    3.148862
 _Iyear_2001 |  (omitted)
 _Iyear_2002 |   .0747474   .0966748    -2.01   0.045     .0059251    .9429741
 _Iyear_2003 |    .088724   .0956278    -2.25   0.025     .0107301    .7336287
 _Iyear_2004 |   .0558817   .0713116    -2.26   0.024     .0045818    .6815581
 _Iyear_2005 |   .1936419   .1634265    -1.95   0.052     .0370354    1.012469
 _Iyear_2006 |   .0395178   .0500472    -2.55   0.011     .0033021    .4729286
 _Iyear_2007 |   .0782523   .0840325    -2.37   0.018     .0095372     .642058
 _Iyear_2008 |   1.214818   1.175206     0.20   0.841     .1824169    8.090167
 _Iyear_2009 |   .1736724   .1588985    -1.91   0.056     .0289027    1.043573
------------------------------------------------------------------------------

. lfit, g(10)

Logistic model for cperf, goodness-of-fit test

  (Table collapsed on quantiles of estimated probabilities)

       number of observations =       216
             number of groups =        10
     Hosmer-Lemeshow chi2(8) =      5.52
                 Prob > chi2 =      0.7012
```

Appendix 3.3: Association of Enterotoxigenic *Escherichia coli* diagnosis and independent variables in univariable logistic regression

Parameters		Odds Ratio	Standard Error	95% Confidence Interval	*P*-value
Average age (d)		1.1	0.0799	0.99-1.3	0.079
Season	Winter	Referent			
	Fall	0.79	0.294	0.38-1.6	0.526
	Spring	0.61	0.236	0.28-1.3	0.200
	Summer	0.54	0.222	0.24-1.2	0.135
C. perfringens detection	No	Referent			
	Yes	0.24	0.150	0.070-0.82	0.022
C. difficile detection	No	Referent			
	Yes	0.1	0.103	0.01-0.76	0.026
Rotavirus detection	Yes	Referent			
	No	0.45	0.232	0.16-1.2	0.122

Appendix 3.4: Multivariable logistic regression model for the etiological diagnosis of Enterotoxigenic *Escherichia coli*

```
. xi:logit ecoli ageavg i.season i.cdiff i.cperf i.rota, or nolog
i.season          _Iseason_1-4        (_Iseason_4 for season==Winter omitted)
i.cdiff           _Icdiff_0-1         (naturally coded; _Icdiff_0 omitted)
i.cperf           _Icperf_0-1         (naturally coded; _Icperf_0 omitted)
i.rota            _Irota_0-1          (naturally coded; _Irota_0 omitted)

Logistic regression                               Number of obs   =        237
                                                  LR chi2(7)      =      27.67
                                                  Prob > chi2     =     0.0003
Log likelihood = -132.51549                       Pseudo R2       =     0.0945

------------------------------------------------------------------------------
       ecoli | Odds Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
      ageavg |   1.109209   .0836952     1.37   0.170     .9567229    1.285999
  _Iseason_1 |   .7124472   .2848199    -0.85   0.396     .3254333    1.559708
  _Iseason_2 |     .41332   .1707983    -2.14   0.033     .1838824    .9290362
  _Iseason_3 |   .3701492   .1608965    -2.29   0.022     .1578979    .8677153
   _Icdiff_1 |   .1013643   .1064422    -2.18   0.029     .0129432     .793831
   _Icperf_1 |   .2143473   .1411672    -2.34   0.019     .0589556    .7793122
    _Irota_1 |   .3269054   .1769153    -2.07   0.039     .1131793    .9442284
------------------------------------------------------------------------------

. xi:logit ecoli i.season i.cdiff i.cperf i.rota i.year, or nolog
i.season          _Iseason_1-4        (_Iseason_4 for season==Winter omitted)
i.cdiff           _Icdiff_0-1         (naturally coded; _Icdiff_0 omitted)
i.cperf           _Icperf_0-1         (naturally coded; _Icperf_0 omitted)
i.rota            _Irota_0-1          (naturally coded; _Irota_0 omitted)
i.year            _Iyear_2001-2010    (naturally coded; _Iyear_2009 omitted)

Logistic regression                               Number of obs   =        237
                                                  LR chi2(15)     =      53.63
                                                  Prob > chi2     =     0.0000
Log likelihood =  -119.5329                       Pseudo R2       =     0.1832

------------------------------------------------------------------------------
       ecoli | Odds Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
  _Iseason_1 |   .6125189   .2729642    -1.10   0.271     .2557339     1.46707
  _Iseason_2 |   .3780234   .1713184    -2.15   0.032       .15551    .9189229
  _Iseason_3 |   .3741167   .1767476    -2.08   0.037      .148206    .9443836
   _Icdiff_1 |   .1137981    .124711    -1.98   0.047     .0132833    .9749097
   _Icperf_1 |   .2026628   .1477633    -2.19   0.029     .0485462    .8460439
    _Irota_1 |   .2947558   .1695107    -2.12   0.034     .0954874    .9098686
 _Iyear_2001 |   9.619974   7.595845     2.87   0.004     2.046787    45.21422
 _Iyear_2002 |   9.608336   7.559227     2.88   0.004     2.055782    44.90755
 _Iyear_2003 |   11.57215   8.675445     3.27   0.001     2.662455     50.2974
 _Iyear_2004 |   5.001585   3.953433     2.04   0.042     1.062398    23.54659
 _Iyear_2005 |   3.976006   3.032492     1.81   0.070     .8917381    17.72788
 _Iyear_2006 |   .8808598   .8719292    -0.13   0.898     .1265713    6.130254
 _Iyear_2007 |   4.328694   3.394371     1.87   0.062     .9308552    20.12944
 _Iyear_2008 |   3.898774   4.232098     1.25   0.210     .4644653    32.72674
 _Iyear_2010 |   1.842436   1.880965     0.60   0.549     .2491097    13.62681
------------------------------------------------------------------------------
```

```
. lfit

Logistic model for ecoli, goodness-of-fit test

       number of observations =       237
 number of covariate patterns =        86
             Pearson chi2(70) =        50.12
                  Prob > chi2 =         0.9652
```

Appendix 3.5: Logistic regression models for the etiological diagnosis of rotavirus, *Clostridium difficile*, and *Isospora suis*

Removed least significant pathogen variables in the association of *C. difficile* diagnosis until only significant variables ($P<0.05$) remained in the model.

```
. xi:logit cdiff ageavg i.ecoli i.cperf i.salm i.prrs i.ent, or nolog
i.ecoli           _Iecoli_0-1         (naturally coded; _Iecoli_0 omitted)
i.cperf           _Icperf_0-1         (naturally coded; _Icperf_0 omitted)
i.salm            _Isalm_0-1          (naturally coded; _Isalm_0 omitted)
i.prrs            _Iprrs_0-1          (naturally coded; _Iprrs_0 omitted)
i.ent             _Ient_0-1           (naturally coded; _Ient_0 omitted)


Logistic regression                               Number of obs   =        237
                                                  LR chi2(6)      =      23.98
                                                  Prob > chi2     =     0.0005
Log likelihood = -58.946846                       Pseudo R2       =     0.1690

------------------------------------------------------------------------------
       cdiff | Odds Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
      ageavg |   .7758921   .1203004    -1.64   0.102     .5725645    1.051425
   _Iecoli_1 |     .10916   .1183751    -2.04   0.041     .0130318    .9143695
   _Icperf_1 |   1.698913    1.04337     0.86   0.388     .5098174     5.66145
    _Isalm_1 |   14.28573   12.93468     2.94   0.003     2.422156    84.25636
    _Iprrs_1 |   3.265629   4.210351     0.92   0.359      .260927    40.87094
     _Ient_1 |   11.70163   18.60437     1.55   0.122     .5187103    263.9781
------------------------------------------------------------------------------

. xi:logit cdiff i.ecoli i.salm, or nolog
i.ecoli           _Iecoli_0-1         (naturally coded; _Iecoli_0 omitted)
i.salm            _Isalm_0-1          (naturally coded; _Isalm_0 omitted)

Logistic regression                               Number of obs   =        237
                                                  LR chi2(2)      =      16.18
                                                  Prob > chi2     =     0.0003
Log likelihood = -62.843196                       Pseudo R2       =     0.1141

------------------------------------------------------------------------------
       cdiff | Odds Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
   _Iecoli_1 |   .1023111   .1067122    -2.19   0.029     .0132466     .790206
    _Isalm_1 |   11.23978   10.15007     2.68   0.007     1.914617    65.98328
------------------------------------------------------------------------------

. lfit

Logistic model for cdiff, goodness-of-fit test

       number of observations =       237
 number of covariate patterns =         4
              Pearson chi2(1) =      0.17
                  Prob > chi2 =    0.6810
```

Association of rotavirus diagnosis and season

```
. xi:logit rota i.season, or
i.season          _Iseason_1-4        (_Iseason_1 for season==Fall omitted)

Iteration 0:   log likelihood = -86.080669
Iteration 1:   log likelihood = -80.537414
Iteration 2:   log likelihood =  -79.79205
Iteration 3:   log likelihood = -79.790099
Iteration 4:   log likelihood = -79.790099

Logistic regression                               Number of obs   =        237
                                                  LR chi2(3)      =      12.58
                                                  Prob > chi2     =     0.0056
Log likelihood = -79.790099                       Pseudo R2       =     0.0731

------------------------------------------------------------------------------
        rota | Odds Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
  _IseasonSP |   .1354167   .1074672    -2.52   0.012     .0285861    .6414891
  _IseasonSU |   .1477273   .1174131    -2.41   0.016      .031112    .7014436
  _IseasonWI |         .5   .2264554    -1.53   0.126      .205803    1.214754
------------------------------------------------------------------------------
```

Association of *I. suis* diagnosis and season

```
. xi:logit cocc i.season, or
i.season          _Iseason_1-4        (_Iseason_3 for season==Summer omitted)

Logistic regression                               Number of obs   =        237
                                                  LR chi2(3)      =      15.75
                                                  Prob > chi2     =     0.0013
Log likelihood = -48.039583                       Pseudo R2       =     0.1408

------------------------------------------------------------------------------
        cocc | Odds Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
  _IseasonFA |   .2569444   .1802836    -1.94   0.053     .0649529    1.016436
  _IseasonSP |   .1712963   .1390533    -2.17   0.030     .0348954     .840869
  _IseasonWI |   .0461923   .0495222    -2.87   0.004     .0056494    .3776876
------------------------------------------------------------------------------
```

Appendix 3.6: Etiological diagnosis for 237 gastrointestinal cases with the submission of 1-7- day old piglets to the Animal Health Laboratory, University of Guelph from 2001 to 2010

Pathogen(s) involved in GIT case	Number of cases (%)
No etiological agent identified	79 (33)
ETEC[a]	63 (27)
ETEC[a] and Rotavirus	5 (2)
ETEC[a] and TGEV[b]	1 (0.4)
C. perfringens	19 (8)
C. perfringens and *C. difficile*	3 (1)
C. perfringens and ETEC[a]	2 (0.8)
C. perfringens and *Rotavirus*	1 (0.4)
C. perfringens, *C. difficile*, and *Enterococcus*	1 (0.4)
C. perfringens, *C. difficile*, and Rotavirus	1 (0.4)
C. perfringens, ETEC[a] and *Salmonella*	1 (0.4)
Rotavirus	18 (7.5)
C. difficile	10 (4)
C. difficile and ETEC[a]	1 (0.4)
C. difficile and PRRS[c]	1 (0.4)
C. difficile and Rotavirus	1 (0.4)
C. difficile and *Salmonella*	2 (0.8)
C. difficile, Rotavirus, and *Salmonella*	1 (0.4)
I. suis	13 (5.5)
I. suis and *Cryptosporidium*	1 (0.4)
I. suis and Rotavirus	1 (0.4)
TGEV[b]	6 (2.5)
Salmonella	2 (0.8)
PRRS[c]	2 (0.8)
Cryptosporidium	1 (0.4)
Enterococcus	1 (0.4)
Total	237

[a]Enterotoxigenic *Escherichia coli*
[b]Transmissible Gastroenteritis Virus
[c]Porcine Reproductive and Respiratory Syndrome

Appendix 4.1: Estimated cases of neonatal diarrhea for 22 veterinary practices investigated in a 12-month period

Respondent Location	Estimated neonatal diarrhea cases	Estimated cases attributed to C. perfringens type A (%)
Alberta	30	5 (17)
Alberta	17	5 (29)
Alberta	12	0
Alberta	2	0
British Columbia	2	0
Manitoba	50	1 (2)
Manitoba	40	5 (12.5)
Manitoba	30	23 (77)
Ontario	100	70 (70)
Ontario	27	1 (4)
Ontario	20	2 (10)
Ontario	15	8 (53)
Ontario	15	5 (33)
Ontario	15	0
Ontario	9	2 (22)
Ontario	6	1 (17)
Ontario	4	0
Quebec	30	24 (80)
Quebec	12	2 (17)
Quebec	5	0
Saskatchewan	13	4 (31)
Saskatchewan	0	0