Clostridium difficile in Swine: Zoonotic Transfer and the Potential Consequences

By

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Abstract

Clostridium difficile is an anaerobic, gram-positive, spore-forming bacterium that can infect both animals and humans. *C. difficile* infection (CDI) is a toxin-mediated disease that can result in the production of three main virulence factors: toxin A, toxin B and binary toxin. Newborn piglets are highly susceptible to CDI, however age decreases the chances of colonization. Prevention and treatment strategies are limited, and there is currently no commercially available treatment option for CDI. However, treatment methods and prevention strategies using a nontoxigenic *C. difficile* strain and equine-origin antitoxins have been explored and show preliminary promising results. With evidence of possible zoonotic transfer increasing, agriculture and medical professionals should take action to prevent the spread of *C. difficile*. Consequences including economic loss and decline in consumer confidence could result if a highly virulent resistant strain of *C. difficile* emerged and caused an increase in morbidity and mortality rates among pigs and humans.

Clostridium difficile in Neonatal Piglets

Clostridium difficile is an anaerobic, gram-positive, spore-forming bacterium present in both animals and humans (Squire and Riley 2013). *Clostridium difficile* infection (CDI) occurs in organisms with a compromised immune system and those with disrupted gut floras. *C. difficile* is one of the leading causes of enteritis in newborn piglets and it produces three main virulent toxins: toxin A, toxin B, and binary toxin (Abt, McKenney and Pamer 2016). Colonization can occur within 48 hours of birth in virtually 100% of neonates (Hopman et al. 2011). Within swine herds, *C. difficile* is shown to have the ability to impact, on average, two-thirds of litters, causing

97-100% morbidity and a mortality rate as high as 16% within the litter (Anderson and Songer 2008, Songer 2004). Though a high percentage of neonates are colonized with *C. difficile* shortly after birth, not all piglets will show signs of disease. Some neonates may present as asymptomatic, healthy piglets. However, some diseased piglets may experience watery to pasty yellow diarrhea, while others may experience constipation (Yaeger, Kinyon and Songer 2007). Although these symptoms are not definitive indicators of CDI, developmental and growth impairment is usually a result of CDI (Songer 2004). High colonization rates are observed in newborn piglets; however, as piglets age colonization frequency decreases by as much as 70.3% in 62 day old pigs (Weese et al. 2010). Pig necropsy findings include mesocolonic edema and, although not a clear indicator of CDI, there is a strong correlation with the production of *C. difficile* toxins (Yaeger et al. 2007).

Clostridium difficile Sporulation, Germination and Vegetative Growth

Clostridium difficile has spore-forming capabilities. Sporulation occurs when nutrients become less readily available. In response, a *C. difficile* cell will split into two unequal compartments. The larger compartment, the mother cell, will prepare the forespore, the smaller compartment, for dormancy where it will become a stress-resistant cell (Abt et al. 2016). Once matured, the mother cell releases the forespore into the environment through lysis. These spores are resistant to heat, oxygen, and common disinfectants (Rodriguez-Palacios and LeJeune 2011). The main transcription factor that initiates sporulation is stage 0 sporulation protein A (SpoA), which is activated through sensor histidine kinases in response to extracellular stimuli (Edwards and McBride 2014).

Spores germinate to give rise to vegetative cells under favorable environmental conditions. This occurs in the lower gastrointestinal tract where oxygen is minimal and bile salts are present to induce germination (Koenigsknecht et al. 2015). In particular, taurocholate, a conjugated primary bile acid initiates germination by signaling through the *C. difficile* serine protease bile acid receptor, CspC (Brown and Wilson 2018). After initiation and activation of the spore cortex lytic enzyme, SleC, by serine protease bile acid receptor, CspB, loss of the endospore's three protective layers, the cortex, coat and exosporium, is achieved and growth of a vegetative cell can now occur (Francis et al. 2013, Adams et al. 2013). However, not all bile salts act in the same manner. In the intestine there are primary and secondary bile acids, and primary bile acids can be conjugated into secondary bile acids. Primary bile acids include taurocholate and cholate, and secondary bile acids include deoxycholate and lithocholate (Brown and Wilson 2018). Under normal healthy conditions, secondary bile salts inhibit spore germination and vegetative growth (Brown and Wilson 2018, Winston and Theriot 2016). For example, deoxycholate promotes germination but suppresses vegetative growth, allowing intestinal immune components to kill *C. difficile* cells that lack chemical and physical resistance (Wilson 1983, Sorg and Sonenshein 2008). Chenodeoxycholate, another bile acid, inhibits spore germination by competitive inhibition with taurocholate (Sorg and Sonenshein 2009, Sorg and Sonenshein 2010). Normal, healthy gut flora can inhibit CDI by way of bile salts, however antibiotics can disrupt normal function and alter conjugation of primary bile acids which allows for *C. difficile* cell proliferation and possible antibiotic-resistance (Sun and Hirota 2015, Brown and Wilson 2018).

Virulence Factors: Toxin A, Toxin B, and Binary Toxin

Clostridium difficile is a toxin-mediated disease that produces toxins in response to nutrient deficiency. The main *C. difficile* virulence factors include toxins A and B, which target intestinal epithelial cells and enteritis can occur as a result. The genes responsible for toxins A and B are *tcdA* and *tcdB* respectively, and they are located in the *C. difficile* pathogenic island (Pruitt et al. 2010). The homologous toxins contain four domains: a glycosyltransferase domain (GTD), autoprotease domain (APD), delivery domain, and combined repetitive oligopeptides (CROPs) domain (Alvin and Lacy 2017). Once the toxins have entered the cell via receptor-mediated endocytosis, cytosolic Rho GTPases are inactivated via glycosylation (Abt et al. 2016). Rho GTPases include Rho, Ras and Cdc42 which are responsible for many cellular processes including actin and microtubule dynamics, gene expression, the cell cycle, cell polarity, and membrane transport (Schwartz 2004). Subsequent to inactivation, the cell undergoes necrosis and consequently activates the host inflammatory response.

There is a third highly virulent toxin that is estimated to be in about 20% of *C. difficile* strains known as binary toxin or *C. difficile* transferase (CDT) (Peng et al. 2017). CDT is encoded by two genes, *cdtA* and *cdtB*, which are located on the CDT locus (Popoff et al. 1988, Perelle et al. 1997). CdtA is an ADP-ribosyl transferase that ribosylates actin, and CdtB forms pores in the endosome to allow CdtA to enter the cytosol of the infected cell. Depolymerization of the actin cytoskeleton causes cellular protrusions formed by microtubules that consequently increases bacterial adherence to target cells (Gerding et al. 2014, Abt et al. 2016).

Although not a clear indicator of CDI, there is a strong correlation with the production of *C. difficile* toxins (Songer et al. 2009). Neonatal pigs with *C. difficile* colonization can appear asymptomatic or symptomatic. Clinical features include dyspnea, mild abdominal distention,

mesocolonic edema, scrotal edema, and yellow, pasty diarrhea (Songer 2004, Yaeger et al.

2007). Histological findings can include marked loss of goblet cells, neutrophil infiltration in the

superficial lamina propria, and neutrophil and fibrin exudation form inflamed mucosal

segments in the lumen (Yaeger et al. 2007). Compared to the large intestine, there are usually

no remarkable lesions in the small intestine (Songer 2004).

Immune Response

An immune response is activated via toxin-mediated damage, loss of epithelial integrity, and

the detection of translocating bacteria (Abt et al. 2016). Pro-inflammatory cytokines and

chemokines are released by damaged epithelial cells and resident immune cells which activate

circulating innate and adaptive immune cells (Abt et al. 2016). This leads to neutrophil

accumulation and activation of innate lymphoid cells (Abt et al. 2016). The intensity of the host

inflammatory response correlates with the severity of CDI. For example, in a study by Steele

and others, IL-8 levels were significantly higher in acute diseased pigs compared to chronic

diseased pigs (Steele et al. 2010). IL-8 is a pro-inflammatory chemokine released by resident

innate immune cells and epithelial cells, and it is responsible for neutrophil migration (Abt et al.

2016). Cytokines TGF-β and TNF-α were also increased in acute diseased pigs compared to

chronic diseased pigs but were not statistically significant (Steele et al. 2010).

Prevention and Treatment Strategies

Nontoxigenic Clostridium difficile

There is a need for CDI prevention and treatment options as there is currently no commercially

available products for immunoprophylaxis of CDI (Songer et al. 2007). Past research has shown

that when hamsters are given a nontoxigenic strain of *C. difficile* as a probiotic, disease and death by precolonization is avoided (Sambol et al. 2002). Arruda has demonstrated this in newborn piglets (Arruda et al. 2016, Songer et al. 2007). Arruda gave piglets heat-shocked nontoxigenic *C. difficile* (NTCD) spores within four hours of birth. Mesocolonic edema was observed in 17% of piglets given NTCD then challenged, which was significantly lower than 54% of positive control challenged pigs, and it was not statistically different from control animals (Arruda et al. 2016). In addition, only 5.8% of challenged NTCD piglets tested positive for toxins, compared to approximately 30% of positive control challenged pigs. These results show a potential candidate for the prevention of CDI.

Equine-origin Antitoxins

Antitoxin from equine plasma is also a possible preventative measure that can be administered to protect piglets from CDI. When newborn piglets were given equine-origin antitoxin within the first 72 hours of life and later infected, they obtained lower total microscopic lesion scores compared to challenged piglets who were not given the antitoxin. However, the mean weights of infected and non-infected piglets did not differ. In addition to decreased microscopic lesions, antitoxin can also be given to piglets orally which suggests this method as being both beneficial and practical (Ramirez et al. 2014).

Zoonotic Transfer

Clostridium difficile can infect both pigs and humans. Ribotype 078 (RT078) has been isolated from both species, suggesting transmission between the two can occur. It is also the most common cause of community acquired CDI in humans in the Northern Hemisphere (Knight et al.

2015). In one study by Knetsch and others, 247 RT078 samples were collected from humans and animals (cattle, horses, pigs, poultry) between 1996 and 2012 from North America, Europe, Australia, and Asia. Of the 183 samples from humans and 59 from animals, six total clusters were discovered, and of those six clusters, one was found to share both human and animal isolates from more than one country suggesting that zoonotic transmission is not limited to geographical regions (Knetsch et al. 2018).

In a smaller, more local study by Keessen and others, RT078 was also predominantly found in both pigs and humans. Keessen took a total of 128 samples from pig farmers, their relatives, and their employees from 32 pig farms. Twelve (25%) of the 48 persons who had daily contact with pigs, and 3 (14%) of 22 persons who had weekly contact with pigs had positive *C. difficile* colonization fecal samples. The number of persons willing to submit a fecal sample ranged from 1 to 10 per farm, and of the 32 farms, 16 farms had at least one *C. difficile* positive fecal sample. Thirteen of the 16 farms had genetically related human and pig isolates, including 100% identical multilocus variable number tandem repeat analysis (MLVA) results for human and pig RT078 isolates from 3 farms (Keessen et al. 2013). These results suggest that zoonotic transmission is occurring either through direct contact or the environment, but more studies are needed to determine this.

In addition to animal to human *C. difficile* transmission, it has yet to be determined if spore contaminated animal derived food products can cause CDI in humans because spore germination and vegetation is normally inhibited in individuals with healthy guts (Brown and Wilson 2018). However, retail meat products in North America have tested positive for *C. difficile* spores in both ready-to-eat, cooked meats and uncooked meats (Brown and Wilson

2018). Spore containing meat included uncooked ground pork (43%) from Arizona, ground pork (12.2%) from Canada, uncooked pork sausage (23%) from Arizona, and cooked Braunschweiger pork sausage (63%) from Arizona (Brown and Wilson 2018). In addition, all of the previously stated meat products also tested positive for RT078 but had spore counts as low as 20-60 per gram (Alam et al. 2017, Brown and Wilson 2018, Songer et al. 2009, Weese et al. 2009). It is estimated that 100-1000 spores can cause CDI in susceptible humans but the actual number is unknown (Warriner et al. 2017).

Researchers have suggested that a "One Health" approach is needed when evaluating the impact *C. difficile* infected animals have on humans contracting CDI (Knetsch et al. 2018). In the USA, 80% of all antibiotics are used in agriculture and only 70% are considered 'medically important' (Knetsch et al. 2018). This should be of a concern to the agriculture and medical communities since it is known that the overuse of antibiotics can produce highly resistant strains of bacteria. This needs to be considered when exploring treatment options for CDI in piglets to prevent the production of newly virulent *C. difficile* strains that could have the ability to also infect humans.

Potential Economic Impact

CDI mortality rates in piglets are generally low, but rates as high as 16% have been reported (Anderson and Songer 2008). There is little, if any, literature regarding the economic impact pig CDI can have in the agriculture industry. However, there are reports that document the economic consequences caused by other pig outbreaks that can be recognized as a potential outcome if there was a CDI outbreak in pigs. For example, the 1998-1999 Nipah outbreak in

Malaysia and Singapore resulted in the death of millions of pigs, an estimated loss of $97 million and 80% decrease in local pork consumption (Chua et al. 2000, Khan et al. 2013). During the H1N1 outbreaks in 2009, pork prices declined as low as $0.49 per lb. and it was estimated that pork producers lost an estimated $13.64 per head totaling about $7.2 million in losses daily (Khan et al. 2013). Widespread outbreaks not only cause morbidity and mortality in a large quantity of pigs resulting in a significant financial loss to the agricultural industry, but it also can result in a loss of consumer confidence. Direct evidence of zoonotic transfer could also decrease pork consumption even more. Recovered piglets are approximately 0.5 Kg lighter on average, so there is already a financial loss as a consequence of CDI (Songer 2004). It is not certain that pig CDI could produce such a tremendous economic impact, but as increasing bacterial resistant strains arise, *C. difficile* certainly has the potential.

Conclusion

Clostridium difficile can infect a variety of species, including pigs and humans. *C. difficile* is the number one cause of enteritis in newborn piglets, and for unknown reasons the frequency of CDI decreases with age. To reduce CDI, improved prevention and treatment options need to be explored. This is especially important now with increasing evidence supporting a possible zoonotic transfer and spore contaminated meats. Agricultural and medical officials should be concerned about preventing possible *C. difficile* outbreaks that could cause large economic impacts in both industries.

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