C. difficile in neonatal piglets on 3 Dutch farms; a cohort study

Masterthesis
Student: Klaas Visscher
Supervisors: Dr. L.A.M.G van Leengoed and Dr. A. van Nes

Introduction

Clostridia species play a major role in the intestines of neonatal pigs and are an important part of the anaerobic microbiota during the first days of life (Kubasova et al., 2017; Swords et al., 1993). The most common *Clostridium* species found in piglets are *Clostridium perfringens* and *Clostridium difficile* (Songer et al., 2005). These bacteria are both known for their ability to cause neonatal diarrhea, but can also be present without clinical signs. *C. perfringens* is a common agent of enteric infections in animals (Popoff et al., 2013). Type A and C of *C. perfringens* may cause enteritis in young piglets (Songer et al., 2005). *C. difficile* is known as a cause of neonatal enterocolitis in piglets (Songer et al., 2005). In recent years, several authors have described outbreaks of *C. difficile* associated diarrhea in piglets (Kim et al., 2015; Mcelroy et al., 2016). *C. difficile* may be one the most important uncontrolled causes of neonatal diarrhea in piglets which is difficult to manage (Songer et al., 2006). One virulent strain of *C. difficile*, named ribotype (RT) 078, is found in various animal species and is also known as cause of enterocolitis in humans (Moono et al., 2016).

Pathogenesis of C. difficile

C. difficile is a Gram positive, anaerobic bacterium able to form highly resistant spores. These spores are resistant to heat and many chemical disinfectants. The spores can also pass the stomach, but they are not able to produce toxins. These spores have to germinate before they can grow. The induction of germination of spores in the intestines is caused by a combination of several factors, such as bile salts and L-glycine. These factors start a cascade to hydrolyse the cortex of the spore resulting in development of the spore to a vegetative cell (Awad et al., 2015; Daniel Paredes-Sabja et al., 2014). After colonization in the intestinal tract of a piglet, *C. difficile* is able to produce toxins that cause damage to the epithelial cells. Two toxins are involved in the pathogenesis of *C. difficile*, toxin A (TcdA) and toxin B (TcdB) (Songer et al., 2005). Some strains of *C. difficile* also produce the so-called binary toxin. The exact role of this toxin remains unclear (Awad et al., 2015; Paredes-Sabja et al., 2014). TcdA and TcdB both cause damage to the cytoskeleton of the epithelial cells in the intestines. During this process TcdA attaches itself to the apical side of the cell and TcdB to the basolateral side of the cells. Both toxins cause damage to the actin filaments in the cell. Following this damage, the tight junctions between the epithelial cells are disrupted. The absorptive function of the gut decreases which results in diarrhea. In addition to their effect on the cell skeleton, TcdA and TcdB also activate inflammation pathways by triggering the production of inflammation factors (Awad et al., 2015; Rupnik et al., 2009).

Clinical signs of C. difficile infection

The clinical manifestation of an infection with *C. difficile* varies (Songer et al., 2006; Steele et al., 2010). Experiments with gnotobiotic piglets showed that infection can lead to systemic disease and sudden death. In these cases also respiratory distress was seen (Steele et al., 2010). In neonatal piglets, housed under conventional circumstances, the characteristic clinical signs are mild to severe diarrhea. After necropsy mesocolonic edema is found and content of the colon is yellowish and

pasty. Histologically typical volcano lesions, with exudation of neutrophils and fibrin into the lumen, are seen in the colonic wall (Songer et al., 2006).

In the Netherlands *C. difficile,* ribotype 078, was determined in piglets suffering from diarrhea (Debast et al., 2009). Another study found a positive correlation between the presence of *C. difficile* in young piglets and clinical signs of diarrhea (Keessen et al., 2010). Moreover, *C. difficile* can be found in fecal samples of piglets within one hour after birth and all piglets in this study (n=71) were positive on *C. difficile* within 32-48 hours after birth (Hopman et al., 2011). The aim of this study is to investigate presence of *C. difficile* on successive moments in the early life of piglets on Dutch farms and the relationship between clinical signs and the presence of *C. difficile* and between clinical signs and the presence toxins of *C. difficile* in the feces of piglets.

Materials and methods

Farms

This experiment was an observational cohort study, a group of animals was followed during a longer period and retrospectively animals were appointed to a group with exposed and non-exposed animals to either *C. difficile* or toxins of *C. difficile* (Petrie et al., 2006). The study was conducted in November 2015 on three Dutch pig breeding farms. Two of the three farms (farm 1 and 2) had a history with clinical *C. difficile* problems. Farm 3 was a farm with a higher health status and did not have known problems with *C. difficile*. At farm 1 there was a clinical manifestation of neonatal diarrhea. These occurred especially during the first few days of life and in litters of first parity sows, but in some cases the clinical signs occurred after a week. Farm 2 was also a farm with diarrhea in neonatal piglets. However, at this farm there was no clear linkage with the parity of the sow. Farm 3 served as a control farm to make it possible to relate the results of farm 1 and 2 to the findings on a farm with a higher health status. On this farm no problems with neonatal diarrhea were reported before the start of the study.

Animals

For the study, litters on farm 1 were selected from first and second parity sows because of the history with neonatal diarrhea in piglets from first parity sows. On farm 2 and 3 litters were selected random because on farm 2 clinical problems were not related to the parity of the sows and on farm 3 no problems with neonatal diarrhea were reported. On each farm 10 male piglets from 5 different litters were selected to participate in the study.

Sampling

The feces of the neonatal piglets (n=30) and their dams (n=15) was collected by taking samples. Fecal samples of the piglets were taken by manual stimulation of the piglets' abdomen. The sows were sampled rectally with a glove. The feces was collected in 10 ml sterile test tubes and immediately stored in a coolbox. On farm 1 and 2 samples were taken by the researchers. Because of the high health status on farm 3, the employees of this farm took the samples. On each farm, 10 piglets were sampled at 1, 4, 7 and 11 days of age. The sows were also sampled; on farm 1 and 2 on the same moments as the piglets and on farm 3 only at day 1 because the employees on this farm did not sample the sows each day. After the samples were taken, they were transported to the laboratory and stored in a freezer.

Clinical scoring/ examination

Each sample moment a diarrhea score was performed on individual basis using a scale from 1 to 3: (1) no diarrhea, normal feces; (2) slight diarrhea, with a pasty consistence of feces; (3) watery diarrhea. Score 3 was considered as clinical diarrhea. Diarrhea score was performed by researchers on farm 1 and 2. On farm 3 scores were performed by employees of the farm.

DNA isolation

After taking the feces samples from the freezer, the samples were thawed and DNA was isolated by the method as described by Crielaard et al., 2011. With sterile loops some feces from each test tube was brought into wells and mixed with lysis buffer, zirconium beads and phenol (Crielaard et al., 2011). Because of the high levels of impurities in the feces, phenol extraction was carried out twice (Ladirat et al., 2014).

qPCR

After isolation of the DNA, a quantitative PCR (qPCR) was used to measure the total amount of *C. difficile* and *C. perfringens* respectively. The qPCR was carried out by TNO (Zeist, Netherlands), using their own primers for *C. difficile* and *C. perfringens*. The qPCR was performed on the Applied Biosystems 7500 Fast Real-Time PCR System. The specific genes detected by using by this primers were not provided by this laboratory and also no information about detection level, specificity and sensitivity of the test was provided due to confidential company information.

Toxin ELISA

All fecal samples were tested with an ELISA kit for presence of toxins of *C. difficile*. Because of the limited availability of ELISA kits, one half of all samples that were taken was tested in a kit for Toxin A that only turned positive when Toxin A was present in the sample. The other half of all samples was tested in a combi-kit with toxin A and B that demonstrates the presence of toxin A, toxin B or a combination of these toxins. These tests only provide qualitative information in giving a positive or negative result. This analysis was also carried out by TNO (Zeist, Netherlands).

Data analysis and statistics

Crosstabs of the data were analyzed by Fisher's exact test because of the low numbers of tested samples. Results were considered significant only when $P < 0,05$. Statistical analyses were carried out with the statistical analysis program SPSS v.25 (SPSS Inc.).

Results

Clinical scores

The results of clinical scores of the diarrhea of the piglets are shown in table 1. Score 3 was considered as clinical diarrhea. On farm 3 no clinical score was performed for one piglet on day 7 and 11.

Farm		Day			
		1	4	7	11
Farm 1	Piglets	1/10	0/10	2/10	3/10
Farm 2	Piglets	6/10	0/10	0/10	0/10
Farm 3	Piglets	5/10	5/10	0/9	0/9

Table 1 *Number of piglets with a diarrhea score of 3.*

qPCR

All fecal samples were examined on the presence of genetic material of *C. difficile.* Figure 1 shows the development of the amount bacterial DNA which was found in the samples in which the PCR test turned out positive. There is a huge variation in the amount of bacterial DNA found in the feces of piglets.

The numbers of samples in which *C. difficile* was found during the first days of life of the piglets are displayed in table 2. Even on farm 3, the reference farm, the feces samples from both piglets and sows tested positive on *C. difficile*. On day 1, 63% of the piglets in the study tested positive on *C. difficile.* On day 4 most piglets were positive (29/30). On day 11 only half of the piglets tested positive on *C. difficile*. All samples of piglets were positive at all moments for *C. perfringens*. Only three samples of sows from farm 2 tested negative for C. *perfringens*. All these sows tested negative only once; one sow (fifth parity) on day 1 and the other two on day 11 (second and fifth parity).

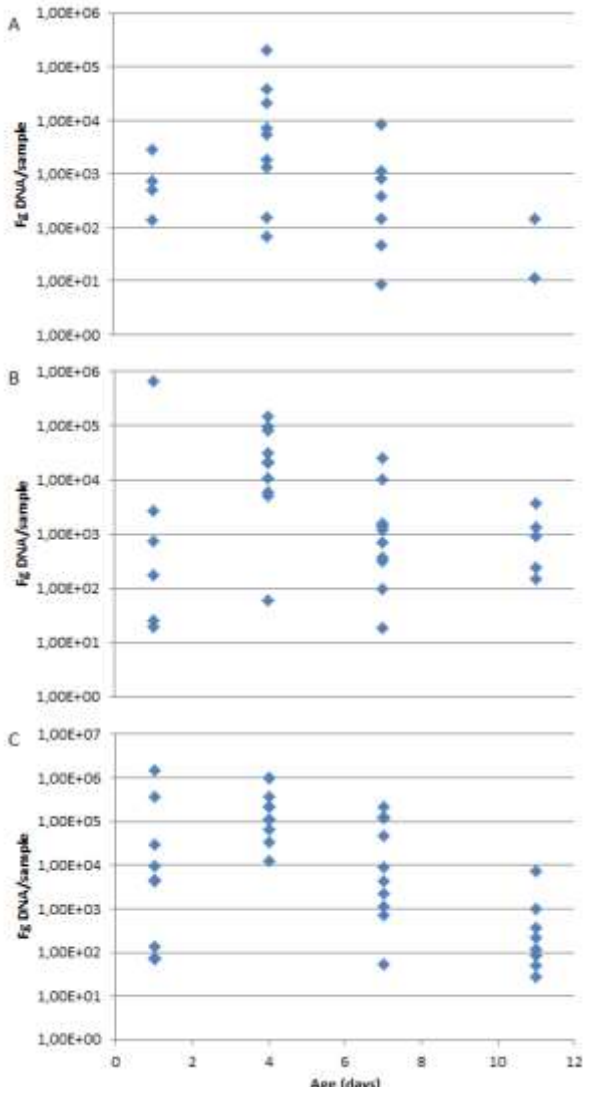

Figure 1 *Development of amount of DNA of C. difficile in positive fecal samples of piglets on Farm 1 (A), Farm 2 (B) and Farm 3 (C) expressed in femtogram (fg) DNA per sample.*

Farm		Day			
		1	4	7	11
Farm 1	Piglets	4/10	9/10	7/10	2/10
	Sows	2/5	0/5	0/5	1/5
Farm 2	Piglets	6/10	10/10	10/10	5/10
	Sows	2/5	1/5	1/5	0/5
Farm 3	Piglets	9/10	10/10	10/10	8/10
	sows	3/5	-	-	-

Table 2 *Number of animals positive for C. difficile tested by PCR*

Toxin ELISA

On day 1 only 1/15 samples of piglets tested positive on toxin A and 2/15 on the toxin A and B ELISA. From the sows 2/8 tested positive in the toxin A and B ELISA and 0/7 in the toxin A ELISA. On day 4 the number of positive piglets was highest. After day 4 the presence of toxins decreases with the increase of age of the piglets. On day 11 only 3/16 and 0/14 tested positive in the toxin A and the toxin A/B ELISA respectively (table 3 and 4).

Farm		Day			
ToxA		1	4	7	11
Farm 1	Piglets	0/3	2/4	0/4	0/7
	Sows	0/3	0/2	0/2	0/3
Farm 2	Piglets	1/7	6/7	1/7	2/4
	Sows	0/2	0/3	0/3	0/3
Farm 3	Piglets	0/5	6/7	3/6	1/5
	sows	0/2	-	-	-

Table 3 *Number of samples positive on toxin ELISA toxin A.*

Farm		Day			
ToxA/B		1	4	7	11
Farm 1	Piglets	1/7	2/6	0/6	0/3
	Sows	1/2	3/3	2/3	2/2
Farm 2	Piglets	0/3	0/3	0/3	0/6
	Sows	1/3	1/2	2/2	2/2
Farm 3	Piglets	1/5	3/3	3/4	0/5
	sows	1/3		-	-

Table 4 *Number of samples positive on toxin ELISA toxin A and B*

There was no relationship between the clinical score of the piglets and the presence of *C. difficile* (Table 5) and between the clinical scores and the presence of toxins of *C. difficile* (Table 6). Only 1 sample tested positive on toxins of *C. difficile* and negative in the qPCR at the same moment (Table 7). In this crosstab P<0.05 which means that when *C. difficile* is not detected by the qPCR, it is less likely that toxins of *C. difficile* are present in the sample.

Clinical score	qPCR *C. difficile*	
	Positive	Negative
1	49	14
2	25	8
3	15	7

Table 5 Clinical score and the presence of *C. difficile* demonstrated by qPCR in fecal samples of piglets (n=118) Clinical score 3 was considered to be clinical diarrhea. (P>0.05)

Clinical score	Toxin ELISA	
	Positive	Negative
1	18	45
2	8	25
3	5	17

Table 6 Clinical score and the presence of toxins of *C. difficile* in fecal samples of piglets (n=118) Clinical score 3 was considered to be clinical diarrhea (P>0.05)

qPCR *C. difficile*	Toxin ELISA	
	Positive	Negative
Positive	31	59
Negative	1	29

Table 7 Presence of *C. difficile* demonstrated by qPCR and the presence of toxins of *C. difficile* in fecal samples of piglets (n=120) (P<0.001)

Discussion

In this study the presence of *C. difficile* is evaluated in the first days of life of piglets. On day 1 after birth *C. difficile* was present in 63% of the piglets which increased till 97% on day 4. On day 11 only 50% of the piglets tested positive. The presence of toxins of *C. difficile* showed the same pattern as the presence of *C. difficile.* Only 12/30 piglets developed diarrhea during the first day. There was no link between presence of *C. difficile* and clinical diarrhea and neither between the presence of toxins of *C. difficile* and clinical diarrhea. Besides *C. difficile* also the presence of *C. perfringens* was evaluated which was present in all piglets on all sample moments.

Several studies showed that *Clostridia spp.* are the most common bacteria during the first days of life of piglets (Bian et al., 2016; Everaert et al., 2017; Kubasova et al., 2017). The main bacterium that is mentioned in this context is *C. perfringens* which amount decreases rapidly during the first days of life of piglets (Kubasova et al., 2017; Swords et al., 1993). *C. perfringens* is known for the ability to cause (enteric) disease in many species (Diab, 2016; Theoret et al., 2016; Uzal, 2016). Non virulent strains of *C. perfringens* play also a role in the gut microbiome in mammals (Sawires et al., 2006). In the current study the pathogenicity of *C. perfringens* is not reviewed, so it is difficult to relate *C. perfringens* to the clinical scores of the piglets.

The bacterium *C. difficile* is frequently found in pigs in many countries (Janezic et al., 2014). According to Hopman et al., fecal samples of piglets can test positive in *C. difficile* culture within 1 hour after birth (Hopman et al., 2011). In the current study a remarkable part of the piglets tested positive on day 1, but this increased to all piglets testing positive on day 4. After that, the prevalence of *C. difficile* decreased. This finding is in agreement with the findings of Weese et al., 2010 who did a longitudinal study on *C. difficile* and found the highest prevalence on day 2 (74%). The next sample moment in the study of Weese et al., 2010 was day 7 on which the prevalence was lower than on day 2 (Weese et al., 2010). Grzeskowiak et al., 2016 did a quantitative examination on *C. difficile* in feces of piglets in Poland. The highest concentration of *C. difficile* was found on day 6 after birth and decreased after that. *C. difficile* was undetectable on 21 days of age (Grzeskowiak et al., 2016).
In the current study 7/15 sows tested positive on *C. difficile* on day 1 after parturition. This result is in agreement with other studies who examined feces samples of sows around farrowing (Grzeskowiak et al., 2016; Weese et al., 2010).

Although detection of toxins is considered to be the standard to diagnose *C. difficile* infection in pigs (Songer et al., 2005), in the current study there was no statistical prove that the presence of toxins was related to the observed clinical signs. Besides that, even the presence of *C. difficile,* tested by qPCR, was not correlated with clinical diarrhea. This finding disagrees with findings of other studies who found a relationship between presence of *C. difficile* and clinical signs of diarrhea (Keessen et al., 2010; Kim et al., 2018). However, another study also has not found a clear relationship between clinical diarrhea and presence of *C. difficile* (Alvarez-Perez et al., 2009). Yaeger et al., 2007 have even found that piglets which tested positive on *C. difficile* toxins could have normal feces and no histopathological signs of typical *C. difficile* infection as mesocolonic edema (Yaeger et al., 2007). Therefore, diagnosing *C. difficile* infection is not only testing toxins of *C. difficile* as stated by Songer et al., 2005. According to the latest diagnostic guideline of the European Society of Clinical Microbiology and Infectious Diseases, it is difficult to diagnose *C. difficile* infection in humans. Because of that, it is advised to use a two-step algorithm to diagnose infection with *C. difficile*, first

with a high sensitive test followed by a more specific test in case of a positive result from the first test (Crobach et al., 2016). The current study used tests that targeted two different characteristics of *C. difficile* as the qPCR demonstrates the presence of genetic material of the bacterium and the ELISA the presence of toxins produced by *C. difficile.* The qPCR test can be considered as the high sensitive test because it detects genetic material of *C. difficile* and the ELISA test as a more specific test as this test only detects toxins which could potentially cause damage to the epithelial cells in the gut (Awad et al., 2015; Crobach et al., 2016). In the current study there was a significant difference in the result of the ELISA test between the group that tested positive using the qPCR and the group that had a negative result in the qPCR. Samples that tested negative in the qPCR, had a significant lower chance to test positive in the ELISA test for toxins which means that the ELISA tests were more specific as they only give an indication of the presence of toxins of *C. difficile* which could possibly cause damage to the gut cells. However, this study did not show a relation between the presence of toxins of *C. difficile* and clinical signs of diarrhea, the most obvious clinical sign of *C. difficile* infection (Songer et al., 2000). So it is not clear if *C. difficile* caused the clinical signs or if there were other causes of diarrhea that were not studied. Other diagnostic methods as necropsy with histological evaluation of the colon could give more information about the cause of the clinical signs as *C. difficile* gives typical lesions in the colonic wall (Songer et al., 2006) and histopathological evaluation of tissues makes it possible to link tissue damage to the presence of one or more pathogens as some pathogens could be present without causing clinical signs (Cooper, 2000).

There were few limitations in the practical execution of the current study that eventually could influence the results and therefore should be discussed. Because of the high health status of farm 3 in this study was it not possible for the researchers to visit this farm to collect samples and perform the clinical scores of the animals. Especially in case of the clinical scores there may be an observer bias in the results of the current study which influences the results that relates to the clinical signs. However, there were no relevant differences in the results between the farms and separate analysis of the results per farm did not reveal other outcomes.

Another influencing factor could be the low number of animals per farm in this study which made it difficult to perform statistical analysis of the results in order to find correlations between the presence of *C. difficile* and clinical signs and between the presence of toxins of *C. difficile* and clinical signs. To improve the power of a following study regarding *C. difficile*, more animals need to be used.

In the current study a quantitative PCR test was used, because of the used method for DNA isolation in the laboratory no exact amount of sample was used for this test. This made it impossible to analyze these results as the amount of bacterial DNA was expressed as femtogram DNA per sample without correction for the amount of analyzed feces. Therefore the test results were only considered to be positive or negative when DNA of *C. difficile* was present or when no DNA of *C. difficile* was demonstrated by the PCR. To quantify the amount of *C. difficile* in the feces it is necessary to use a standardized amount of research material and a test that expresses the amount of bacterial content in the sample. Nevertheless, although the test gave no reliable quantitative information about *C. difficile* it did provide qualitative information about the presence of *C. difficile* which made possible to analyze the link between the presence of *C. difficile* and clinical signs of diarrhea.
With respect to the toxin detection, two different ELISA test kits were used due to limited availability of kits at the laboratory. The first test only showed the presence of TcdA and the other test showed

the presence of TcdA and/or TcdB. Some strains of *C. difficile* only produce TcdB (Songer et al., 2005). Toxins of these strains would not be found with the first test used in this study as that test only demonstrates the presence of TcdA. This could give an underestimation of the number of toxin-positive samples compared to the group which were tested with the second test. However, most studies done to *C. difficile* in pigs which looked for toxins only found strains that could produce both TcdA and TcdB (Avbersek et al., 2009; Janezic et al., 2014; Kim et al., 2018; Weese et al., 2010). Therefore it is less likely that the use of a test that only demonstrates TcdA gives an underestimation of the toxin-positive samples.

The farms that were involved in this study are representative for the pig industry in the Netherlands. Two farms were conventional pig-breeding farms and the third farm was a farm with a high health status with extensive hygienic procedures to prevent introduction of pathogens on this farm. In this study no obvious differences were observed between these farms. The findings of this study in combination with other Dutch studies make it plausible that *C. difficile* is widespread in the Dutch pig industry (Debast et al., 2009; Hopman et al., 2011; Keessen et al., 2010). However, the clinical relevance of *C. difficile* in this study remains unclear as there was no obvious relation between the presence of *C. difficile* and clinical signs. Therefore, further study of *C. difficile* in pigs is necessary to clarify the role of this bacterium which is possibly zoonotic (Debast et al., 2009; Keessen et al., 2013).

Conclusion

C. difficile is still a pathogen that is present on Dutch pig farms, with the highest prevalence in 4-day old piglets. There is no clear relation between clinical signs and the presence of *C. difficile* or between clinical signs and the presence of toxins of *C. difficile* in this study which makes it plausible that *C. difficile* does not always cause disease. The mechanism what makes *C. difficile* a disease-causing pathogen in piglets need further study to clarify the role of this bacterium in the Dutch pig industry.

References

Alvarez-Perez, S., Blanco, J. L., Bouza, E., Alba, P., Gibert, X., Maldonado, J., & Garcia, M. E. (2009). Prevalence of Clostridium difficile in diarrhoeic and non-diarrhoeic piglets. *Veterinary Microbiology*, *137*(3–4), 302–305. https://doi.org/10.1016/j.vetmic.2009.01.015

Avbersek, J., Janezic, S., Pate, M., Rupnik, M., Zidaric, V., Logar, K., Vengust, M., Zemljic, M., Pirs, T., Ocepek, M. (2009). Diversity of Clostridium difficile in pigs and other animals in Slovenia. *Anaerobe*, *15*(6), 252–255.

Awad, M. M., Johanesen, P. A., Carter, G. P., Rose, E., & Lyras, D. (2015). Clostridium difficile virulence factors: Insights into an anaerobic spore-forming pathogen. *Gut Microbes*, *5*(5), 579–593. https://doi.org/10.4161/19490976.2014.969632

Bian, G., Ma, S., Zhu, Z., Su, Y., Zoetendal, E. G., Mackie, R., Liu, J., Mu, C., Huang, R., Smidt, H., Zhu, W. (2016). Age, introduction of solid feed and weaning are more important determinants of gut bacterial succession in piglets than breed and nursing mother as revealed by a reciprocal cross-fostering model. *Environmental Microbiology*, *18*(5), 1566–1577. https://doi.org/10.1111/1462-2920.13272

Cooper, V. L. (2000). Diagnosis of neonatal pig diarrhea. *The Veterinary Clinics of North America. Food Animal Practice*, *16*(1), 117–133. https://doi.org/10.1016/S0749-0720(15)30139-0

Crielaard, W., Zaura, E., Schuller, A. A., Huse, S. M., Montijn, R. C., & Keijser, B. J. (2011). Exploring the oral microbiota of children at various developmental stages of their dentition in the relation to their oral health. *BMC Medical Genomics*, *4*, 22. https://doi.org/10.1186/1755-8794-4-22 [doi]

Crobach, M.J.T., Planche, T., Eckert, C., Barbut, F., Terveer, E.M., Dekkers, O.M., Wilcox, M.H. Kuijper, E.J. (2016). European Society of Clinical Microbiology and Infectious Diseases: update of the diagnostic guidance document for Clostridium difficile infection. *Clinical Microbiology and Infection*, *22*, S63–S81. https://doi.org/10.1016/j.cmi.2016.03.010

Debast, S. B., Leengoed, L. A. M. G. Van, Goorhuis, A., Harmanus, C., Kuijper, E. J., & Bergwerff, A. A. (2009). Clostridium difficile PCR ribotype 078 toxinotype V found in diarrhoeal pigs identical to isolates from affected humans. *Environmental Microbiology*, *11*(2), 505–511.

Diab, S. S. (2016). Diseases Produced by *Clostridium perfringens* Type C. In *Clostridial Diseases of Animals* (pp. 143–155). Hoboken, NJ: John Wiley & Sons, Inc. https://doi.org/10.1002/9781118728291.ch12

Everaert, N., Cruchten, S. Van, Westrm, B., Bailey, M., Ginneken, C. Van, Thymann, T., & Pieper, R. (2017). A review on early gut maturation and colonization in pigs, including biological and dietary factors affecting gut homeostasis. *Animal Feed Science and Technology*. https://doi.org/10.1016/j.anifeedsci.2017.06.011

Grzeskowiak, L., Zentek, J., & Vahjen, W. (2016). Anaerobe Determination of the extent of Clostridium difficile colonisation and toxin accumulation in sows and neonatal piglets. *Anaerobe*, *40*, 5–9. https://doi.org/10.1016/j.anaerobe.2016.04.012

Hopman, N. E. M., Keessen, E. C., Harmanus, C., Sanders, I. M. J. G., van Leengoed, L. A. M. G., Kuijper, E. J., & Lipman, L. J. A. (2011). Acquisition of Clostridium difficile by piglets. *Veterinary Microbiology*. https://doi.org///dx.doi.org/10.1016/j.vetmic.2010.10.013

Janezic, S., Zidaric, V., Pardon, B., Indra, A., Kokotovic, B., Blanco, J.L., Seyboldt, C., Diaz, C.R., Poxton, I.R., Perreten, V., Drigo, I., Jiraskova, A., Ocepek, M., Weese, J.S., Songer, J.G., Wilcox, M.H., Rupnik, M. (2014). International Clostridium difficile animal strain collection and large diversity of animal associated strains. *BMC Microbiology*, *14*, 173. https://doi.org/10.1186/1471-2180-14-173 [doi]

Keessen, E. C., Harmanus, C., Dohmen, W., Kuijper, E. J., & Lipman, L. J. A. (2013). Clostridium difficile infection associated with pig farms. *Emerging Infectious Diseases*, *19*(6), 1032. https://doi.org/10.3201/eid1906.121645

Keessen, E. C., van Leengoed, L., Bakker, D., den Brink, K. Van, Kuijper, E. J., & Lipman, L. J. A. (2010). Aanwezigheid van Clostridium difficile in biggen verdacht van CDI op elf varkensbedrijven in Nederland. *Tijdschrift Voor Diergeneeskunde*, *135*, 134–137.

Kim, H. Y., Jung, B. Y., Byun, J.-W., Lee, K. H., Cho, A., Lee, M. H., Songer, J. G., Kim, B. (2015). Fatal case of Clostridium difficile infection in a neonatal piglet in Korea. *Pakistani Veterinary Journal*, *35*(2), 264–266. https://doi.org/10.1097/QCO.0b013e3283638104

Kim, H. Y., Cho, A., Kim, J. W., Kim, H., & Kim, B. (2018). High prevalence of Clostridium difficile PCR ribotype 078 in pigs in Korea. *Anaerobe*, *51*, 42–46. https://doi.org/10.1016/j.anaerobe.2018.03.012

Kubasova, T., Davidova-Gerzova, L., Merlot, E., Medvecky, M., Polansky, O., Gardan-Salmon, D., Quesnel, H., Rychlik, I. (2017). Housing systems influence gut microbiota composition of sows but not of their piglets. *PLoS ONE*, *12*(1). https://doi.org/10.1371/journal.pone.0170051

Ladirat, S. E., Schuren, F. H. J., Schoterman, M. H. C., Nauta, A., Gruppen, H., & Schols, H. A. (2014). Impact of galacto-oligosaccharides on the gut microbiota composition and metabolic activity upon antibiotic treatment during in vitro fermentation. *FEMS Microbiology Ecology*, *87*(1), 41–51. https://doi.org/10.1111/1574-6941.12187

Mcelroy, M. C., Hill, M., Moloney, G., Aogáin, M. Mac, Mcgettrick, S., Doherty, Á. O., & Rogers, T. R. (2016). Typhlocolitis associated with Clostridium difficile ribotypes 078 and 110 in neonatal piglets from a commercial Irish pig herd. *Irish Veterinary Journal*, 4–7. https://doi.org/10.1186/s13620-016-0070-9

Moono, P., Foster, N. F., Hampson, D. J., Knight, D. R., Bloomfield, L. E., & Riley, T. V. (2016). Clostridium difficile Infection in Production Animals and Avian Species :, *13*(12), 647–655. https://doi.org/10.1089/fpd.2016.2181

Paredes-Sabja, D., Shen, A., & Sorg, J. A. (2014). Clostridium difficile spore biology: sporulation, germination, and spore structural proteins. *Trends in Microbiology*. https://doi.org///dx.doi.org/10.1016/j.tim.2014.04.003

Paredes-Sabja, D., Shen, A., & Sorg, J. A. (2014). Clostridium difficile spore biology: sporulation, germination, and spore structural proteins. *Trends in Microbiology*, *22*(7), 406–416. https://doi.org/10.1016/j.tim.2014.04.003 [doi]

Petrie, A., & Watson, P. (2006). *Statistics for Veterinary and Animal Science*. *Wiley-Blackwell: Oxford*: 56-57

Popoff, M. R., & Bouvet, P. (2013). Genetic characteristics of toxigenic Clostridia and toxin gene evolution. *Toxicon*, *75*, 63–89. https://doi.org/10.1016/j.toxicon.2013.05.003

Rupnik, M., Wilcox, M. H., & Gerding, D. N. (2009). Clostridium difficile infection: new developments in epidemiology and pathogenesis. *Nature Reviews Microbiology*, *7*(7), 526–536.

Sawires, Y. S., & Songer, J. G. (2006). Clostridium perfringens: Insight into virulence evolution and population structure. *Anaerobe*, *12*(1), 23–43. https://doi.org/10.1016/J.ANAEROBE.2005.10.002

Songer, J. G., & Anderson, M. A. (2006). Clostridium difficile: An important pathogen of food animals. *Anaerobe*. https://doi.org///dx.doi.org/10.1016/j.anaerobe.2005.09.001

Songer, J. G., Post, K. W., Larson, D. J., Jost, B. H., & Glock, R. D. (2000). Infection of neonatal swine with Clostridium difficile. *Journal of Swine Health and Production*, *8*(4), 185–189.

Songer, J. G., & Uzal, F. A. (2005). Clostridial enteric infections in pigs. *Journal of Veterinary Diagnostic Investigation*, *17*(6), 528–536. https://doi.org/10.1177/104063870501700602

Steele, J., Feng, H., Parry, N., & Tzipori, S. (2010). Piglet models of acute or chronic Clostridium difficile illness. *The Journal of Infectious Diseases*, *201*(3), 428–434. https://doi.org/10.1086/649799 [doi]

Swords, W. E., Wu, C.-C., Champlin, F. R., & Buddington, R. K. (1993). Postnatal changes in selected bacterial groups of the pig colonic microflora. *Neonatology*, *63*(3), 191–200.

Theoret, J. R., & McClane, B. A. (2016). Toxins of *Clostridium perfringens*. In *Clostridial Diseases of Animals* (pp. 45–60). Hoboken, NJ: John Wiley & Sons, Inc. https://doi.org/10.1002/9781118728291.ch5

Uzal, F. A. (2016). Diseases Produced by *Clostridium perfringens* Type A in Mammalian Species. In *Clostridial Diseases of Animals* (pp. 107–116). Hoboken, NJ: John Wiley & Sons, Inc. https://doi.org/10.1002/9781118728291.ch8

Weese, J. S., Wakeford, T., Reid-Smith, R., Rousseau, J., & Friendship, R. (2010). Longitudinal investigation of Clostridium difficile shedding in piglets. *Anaerobe*, *16*(5), 501–504. https://doi.org/10.1016/j.anaerobe.2010.08.001

Yaeger, M. J., Kinyon, J. M., & Songer, J. G. (2007). A prospective, case control study evaluating the association between Clostridium difficile toxins in the colon of neonatal swine and gross and microscopic lesions. *Journal of Veterinary Diagnostic Investigation*, *19*(1), 52–59. https://doi.org/10.1177/104063870701900108