ADDENDUM

Impact of early-life events on the susceptibility to *Clostridium difficile* colonisation and infection in the offspring of the pig

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ABSTRACT

*Clostridium difficile* has been documented as a major cause of uncontrolled outbreaks of enteritis in neonatal pigs and antibiotic-associated infections in clinical settings. It belongs to the natural cohort of early colonisers of the gastrointestinal tract of pigs and can be detected in faeces up to two weeks post-partum. In older pigs, it often remains under the detection limit. Most neonatal pigs show no clinical signs of disease although *C. difficile* and its toxins can be detected at high levels in faeces. Increased mortality rates associated with *C. difficile* on pig farms are, so far, considered “spontaneous” and the predisposing factors are mostly not defined. The infection caused by *C. difficile* is multifactorial and it is likely that the repertoire of maternal factors, host physiology, the individually developing gut microbiota, co-infections and environmental stress define the conditions for disease development. In this addendum to our recently published work on CDI in neonatal piglets, we discuss the “early-life events” that influence *C. difficile* spread and infection in neonatal piglets.

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Introduction

For more than a decade *Clostridium difficile* has been documented as a major cause of uncontrolled enteritis outbreaks in neonatal pigs.¹,² Nowadays, it is known that different farm animal species can be affected, making them also a potential reservoir for *C. difficile* infection (CDI) in humans.³ Besides the historic ribotype 027, a new type of *C. difficile*, ribotype 078, originating from pigs, has been found to transmit to farmworkers and cause CDI.⁴ In the western industrialised countries, in hospitalised patients, *C. difficile* is a leading cause of nosocomial infection, morbidity, and mortality where the latter is typically evident in the elderly (> 80 years). Besides nosocomial infections, community acquired CDI is increasingly important and the newly-reported ribotype such as 078 has linked disease in humans and pigs.⁵ Current treatment of CDI in pigs and humans includes the use of antibiotics, however treatment failure and infection relapse can occur.⁶ A promising solution in current clinical practice is faecal microbiome transplantation (FMT) which supports the concept of “colonisation resistance”.⁷ The outcomes of clinical trials with the use of FMT are characterised by high cure rates (up to 95%) and the method is gaining interest among both health practitioners and patients.⁷–⁹

Spores of *C. difficile* facilitate a rapid spread of the bacterium between animals and in the environment.¹⁰,¹¹ The diagnosis of CDI in pigs and humans usually includes diarrhoea and colitis as well as the identification of virulent *C. difficile* and detection of toxins. However, clinical symptoms often do not correlate with *C. difficile* and their toxins, making the diagnosis of CDI extremely difficult.¹²,¹³ Interestingly, *C. difficile* belongs to the natural early colonisers of the gastrointestinal tract of pigs and up to 100% of piglets...
test positive (and so increasing the probability of being colonised by toxigenic ribotypes) within two days after birth followed by a rapid decline with age. 

Increased pre-weaning mortality rates associated with *C. difficile* on pig farms are so far termed “spontaneous” and the predisposing factors are largely not known. It is very likely that maternal factors, host physiology, the individually developing gut microbiota, co-infections and environmental stress are important determinants.

In this Addendum to our recently published work on CDI in neonatal piglets, we aim at discussing the “early-life events” that influence the spread of *C. difficile* infection in neonatal piglets.

**C. difficile infection in pigs**

*C. difficile* colonises the piglet gut at birth and can be detected in faeces up to two weeks post-partum. Even more, most neonatal pigs show no clinical signs of disease although *C. difficile* and its toxins are present at high levels in the faeces. *C. difficile* is also found in adult pigs though often at a very low level. Although neonatal piglets are normally asymptomatic carriers of *C. difficile* (including toxigenic ribotypes), more severely infected animals may be underweight by 10–15% and exhibit decreased growth. The mortality rate among diarrhoeic neonatal piglets can be up to 14%. Pathogenic *C. difficile* have the ability to produce several toxins which may be associated with CDI symptoms in neonatal piglets. The action of the two major exotoxins secreted by the bacterium, toxin A (TcdA) and toxin B (TcdB) is related to the modulation of the intestinal epithelial cell physiology and disruption of barrier function. The toxins can inactivate Rho proteins involved in the formation of the cell cytoskeleton, leading to disruption of tight junctions (TJ) and finally epithelial integrity. Thus, in the last phase of *C. difficile* infection, intestinal pathology develops due to a fluent inflammatory response induced by the toxins, virulence factors and additionally by translocation of the gut microbiota. The loss of epithelial integrity and accompanying reduction of transepithelial resistance can also be demonstrated in an IPEC-J2 cell culture model and it seems to be toxin dose-dependent (Figure 1).

The induction of pro-inflammatory cytokines by the toxins leads to the migration of neutrophils and macrophages into the site of infection and formation of a fluent inflammatory response. The eventual subsequent loss of epithelial integrity and accompanying reduction of transepithelial resistance can also be demonstrated in an IPEC-J2 cell culture model and it seems to be toxin dose-dependent (Figure 1).

**Figure 1.** Response of the IPEC-J2 to different concentrations of toxin A (TcdA) and toxin B (TcdB) as measured by transepithelial electrical resistance (TEER) in an *in vitro* assay up to 20 h of incubation. Control: growth media (Dulbecco’s Modified Eagle’s Medium – DME, DMEM | Sigma-Aldrich). Spent supernatant containing TcdA (1291 ng/ml) and TcdB (829 ng/ml) diluted 1:2, 1:10, 1:100 and 1:1000. Methods in Supplementary file S1.
Typical clinical symptoms in infected piglets include pasty-to-watery diarrhoea, anorexia, growth retardation and dehydration. These manifestations may finally lead to animal death. The inflammation leads to harmful epithelial damage that is responsible for the clinical course of the infection and it triggers an adaptive immune reaction that is essential as a long-lasting specific defence. Besides the influence of the inflammatory response and microbiome shift on the course of the disease, CDI is a risk factor for resistome expansion in pigs and humans; in humans treated in intensive care units or suffering from CDI, shifts in the intestinal microbiome were recently linked with resistome changes.

The susceptibility to CDI in pigs decreases with age but the reasons for this are not clear. Differences in the susceptibility to CDI in neonatal piglets could result from the presence of toxin receptors or intestinal concentration of bile salts which are critical for the germination of C. difficile spores in the intestine. In addition, the synthesis of C. difficile toxins (major virulence factors) have been found to depend on the presence of certain amino acids (e.g., cysteine or proline as inhibitors) and short chain fatty acids (e.g. butyric acid as inducer) and are thus related to the intestinal micro-environment.

Microbial dysbiosis in C. difficile infection

Still little is known about the phenomenon of resistance and susceptibility to CDI in piglets. It is unclear why and under which conditions piglets become sick or are asymptomatic carriers of toxigenic ribotypes. Interestingly, the natural colonisation of porcine and human neonates with C. difficile is more prominent when they are formula fed. The pathogenesis of CDI is most likely multifactorial and thus accompanied by co-infections with other pathogens or a predisposing intestinal dysbiosis. There is increasing evidence that certain diseases are not only associated and due to a single causative pathogen but rather, are associated (and may be due to) a collection of different microbes (i.e., the “pathobiome hypothesis”). Thus, any disruption of the natural colonisation process or perturbances of the intestinal ecosystem could enhance the susceptibility to CDI. In line with this hypothesis, our recent data on the CDI model show a high abundance of Proteobacteria including putative pathogens such as Escherichia spp. or Shigella spp.

In humans, C. difficile is probably the best-known pathogen that follows antibiotic-mediated changes in the gut microbiome. Whether this is also true in the pig is yet not clear. However, we showed in a previous study that antibiotic treatment in a sow was associated with an increased concentrations of C. difficile and toxins in her piglets as compared to non-treated sows. Antibiotics are known to alter the structure and metabolism of the gut microbiota allowing the expansion of opportunistic pathogens including C. difficile. However, non-antibiotic treated piglets may also develop CDI. A deeper knowledge regarding the influence of feeding on microbiome signatures, resistome, and C. difficile pathology could assist the development of protective strategies to combat CDI in piglets.

Neonatal microbial programming through mother-offspring association

The association between mother and offspring gut microbiota during early life is a critical factor for the subsequent succession of intestinal commensal bacteria and immune development later on. The newborn piglet is continuously exposed to microbes from its mother and the environment, which enter the gut together with the mother’s milk. The early gut colonisers in neonatal piglets between 1 and 3 days of age include clostridia, enterobacteria, enterococci, streptococci and peppotrepococci, whereas lactobacilli and other species become predominant afterwards. This early neonatal phase also seems to determine the microbial profile and intestinal health later in life. In contrast to early-life colonisation patterns, changes in the intestinal microbial ecosystem during the abrupt weaning process in pigs have been studied intensively during the past decades and are accompanied with functional adaptations related to diet complexity. Compared to humans, the aspect of mother-offspring association and its effect on early microbial programming in pigs has not been studied in detail. Still, little is known about the impact of diet on the microbial
association between the mother sows and their offspring as well as the establishment of the infant gut microbiota early in life. Few studies suggest a positive impact of sow milk on the intestinal microbiota and immune system of the piglets and that certain probiotics given to sows may alter the microbiota composition and immune status of their offspring.

The only source of nutrients for the new-born piglet is milk, which contains numerous growth factors, microbial antigens and host antibodies e.g., directed against certain pathogens and contributing to passive immunisation in the offspring. Interestingly, antibodies against TcdA have been identified in human blood serum which may protect against CDI. We could demonstrate that the IgG antibodies against TcdA can be found in sows’ blood serum and milk (Figure 2). Recent studies have shown that the administration of TcdB-specific bovine colostrum could prevent and treat CDI in mice and reduced disease recurrence by 67%. Thus, immunisation offers promising tools to actively protect the individuals against CDI. The above data suggest that manipulating the mother’s antibody repertoire in milk could protect neonatal piglets from CDI. In several studies it has been demonstrated that the amount of immunoglobulins targeting toxin A and B are decisive for asymptomatic carriage or recurrent courses.

In addition to the humoral reaction of the host itself, also intravenously administered human monoclonal antibodies that bind to TcdB show evidence of protection. Although one must suppose that serum titres of antibodies only reflect the amount of mucosal antibody production that is a prerequisite for a direct defence of the mucosal surface, nothing is known about the mucosal secretion of immunoglobulins specific for toxins. In addition, the mechanism of how intravenous administered antibody can protect mucosal surfaces from toxin-mediated damage has to be elucidated. Interestingly, human milk oligosaccharides were found to adhere to TcdA and TcdB and slightly inactivate their toxicity in in vitro cell culture and such an effect might also be demonstrated using porcine milk oligosaccharides but this has yet to be studied. In addition, porcine milk oligosaccharides could provide an important selective advantage to some bacteria, thereby modulating C. difficile colonisation.

Figure 2. Estimation of the strength of the relationship between the antibody titres (anti-IgG-anti-toxinA) in serum and milk from four lactating sows, as assessed by enzymatic immunoassay method and measured by spectrophotometry. Methods in Supplementary file S2. Animals and study approval were described previously.
Mechanisms by which other bacteria could prevent colonisation by virulent *C. difficile* have not been clarified in pigs. The diversity of the gut microbiome may influence on the complete recovery from CDI or recurrent disease: patients with severe disease harbour a significantly less diverse microbiome as compared to patients with non-recurrent infections. Therefore, a phenomenon termed “colonisation resistance”, where *C. difficile* is replaced by other bacteria in the developing ecosystem could thereby contribute to protection of the host from CDI. For example, it has been shown in humans that *Clostridium scindens* can successfully outcompete *C. difficile* and prevent or ameliorate CDI. Even more, non-pathogenic or less virulent *C. difficile* ribotypes (natural colonisers of neonatal piglets) may successfully outcompete the toxigenic ribotypes, which we have recently observed in co-culture, *in vitro* (Figure 3). Similar effects have been previously demonstrated in neonatal pigs and hamsters. These observations indicate that colonisation with the commensal microbiota including non-toxigenic *C. difficile* could provide protection to CDI. However, the horizontal transfer of toxin genes between toxigenic and non-toxigenic *C. difficile* cannot be overruled, especially when using this bacterium as a probiotic.

Among the abundant commensal microorganisms, lactic acid bacteria are generally considered as beneficial due to their antagonistic properties against putative pathogens. Despite (lactic) acid production, they can modulate the intestinal environment and host metabolism through bile salt deconjugation and dehydroxylation, which in turn may affect the growth and activity of *C. difficile*. In fact, primary bile salts (e.g. taurocholate) act as germinants for *C. difficile*, while secondary bile salts (e.g. deoxycholate) inhibit its growth *in vitro*. Changing the activity of lactic acid bacteria could therefore change the susceptibility of the host to CDI.

Finally, the normal proteolytic activity of the developing gut microbiota could also have some advantage in suppressing CDI by contributing to the biological inactivation of the clostridial toxins TcdA and TcdB (N-terminal glucosyltransferase domain, responsible for the initiation of infection). Such mechanisms could in part explain the lack of clinical manifestation of infection in piglets, although the toxins are still

![Figure 3](image-url). Percentage distribution of three *C. difficile* ribotypes (078, 014/020, 005) co-incubated all together at equal concentrations in BHIS media, plated and identified from a mixed culture by PCR-ribotyping coupled with Agilent 2100 Bioanalyzer (Agilent; Santa Clara, CA-USA). Methods in Supplementary file S3.
detectable using immunochemical or cell culture tools.\textsuperscript{15}

Taken together, the above-mentioned factors point towards the importance of mother-offspring interaction early in life and that maternal nutrition may play a role in CDI in neonatal pigs.

**C. difficile and diet**

To date, only a few animal studies have focused directly on the influence of diet on *C. difficile* colonisation and susceptibility to CDI in animals. It has been reported that hamsters and mice fed either atherogenic, axenic or elemental diets demonstrate higher *C. difficile* and toxin concentrations in their gut and their survival rates are lower as compared to animals fed normal diets.\textsuperscript{59-62} The results highlight the potential to directly manipulate the susceptibility to CDI by dietary means, at least in these animal models. However, direct or indirect dietary effects on *C. difficile* colonisation and infection in pigs still need to be clarified.

A negative impact of high levels of SCFA and low pH on *C. difficile* growth and toxin production has been shown in a previous *in vitro* study.\textsuperscript{63} A higher concentration of SCFA and low pH could inhibit or stimulate toxin production by *C. difficile*, *in vitro*.\textsuperscript{25,63} Therefore, this approach could offer an attractive way to control the colonisation patterns in the offspring via modulation of the mother sow diet and thereby protect against *C. difficile* expansion in suckling piglets.

**Conclusions**

*C. difficile* is still one of the most important emerging pathogens in clinical settings and has been shown to be relevant for pig farming. The disease is multifactorial and the repertoire of different maternal and environmental factors seems to set the conditions for *C. difficile* expansion and development of infection. There are fundamental knowledge gaps that define an urgent need to substantially expand research into the conditions and factors that contribute to the transmission and development of CDI. One relevant gap is a better insight into a very short “window of opportunity” for *C. difficile* to outgrow in the colon of piglets which is up to two weeks of age only, while beyond this the bacterium remains under current detection limits. Such early colonisation of *C. difficile* in neonatal piglets only up to two weeks post-partum could raise questions about when the piglet starts to contact the environmental microbiota. Next is an explanation of how healthy carriers of toxigenic *C. difficile* can remain free of symptoms since asymptomatic carriage of this bacterium in piglets and humans is well recognised. In addition, the zoonotic potential of *C. difficile* and resistant pathogens as a consequence of antibiotic treatment is of major importance for human health. Thus, the long-term goal would be to develop strategies to modulate the resistance patterns to CDI under normal conditions. Finally, the emerging presence of *C. difficile* in animals and foods and the need for science-based prevention strategies of serious CDI necessitates a deeper knowledge about the protective potential of diet, microbiota, immunoglobulins, mucosal immune reactions following distinct host factors that determine the course of infection in piglets and humans.

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