

Variety of *Clostridium difficile* PCR ribotypes in pigs arriving at the slaughterhouse

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Abstract

Food products of animal origin might play a role in interspecies transmission of *C. difficile*. In pigs, *Clostridium difficile* can cause neonatal enteritis and can be isolated from faeces from both diseased and healthy animals. To determine the prevalence of *C. difficile* in Dutch pigs arriving at the slaughterhouse a pilot study was conducted at one slaughterhouse in the Netherlands. Rectal faecal samples were taken from fifty slaughtering pigs from ten farms just after the pigs were sedated. These samples were examined using a real time PCR (BD GeneOhm™ Cdiff Assay), in combination with culturing after enrichment. Using real time PCR, none of the faecal samples were found to be positive for *C. difficile* while after culturing 14 samples (coming from pigs from nine different farms) were found to be positive for *C. difficile*. The positive samples derived from 9 different farms and encompassed seven different ribotypes.

Introduction

Clostridium difficile has been found in meat samples destined for human consumption. This suggests that food products may play a role in interspecies transmission of *C. difficile* (11, 12, 13).

In Canada, 60 retail ground meat samples were analyzed for the presence of *C. difficile* using an enrichment broth. *C. difficile* was isolated from 20% (12) of these samples purchased over a 10-month period in 2005. Eleven isolated strains were toxigenic (10). In Tucson, Arizona, 88 retail meat samples were collected and analyzed for the presence of *C. difficile*. Forty-two percent (37) of these samples yielded *C. difficile* i.e. 41.3 % of the analyzed porcine meat samples. (12) In Austria, three percent of ground meat samples tested positive for *C. difficile*, two of the isolates were identified as Austrian Isolate-57 and one isolate was identified as ribotype 053. These isolates only came from mixed beef and pork samples. (5) De Boer et al. (2011) could not isolate *C. difficile* from 63 pork samples in the Netherlands in 2011 (2).

Little is known about the route of contamination of pork or the risk of foodborne transmission. Metcalf et al. (2010) suggested different routes like contamination of pork originating from pigs arriving at the slaughterhouse or through humans working in food production (8).

The prevalence of *C. difficile* has been investigated among 165 pigs arriving at the slaughterhouse in Switzerland. None of these pigs were positive for *C. difficile* (3). There seems to be an age dependent decline in colonization rate of pigs with *C. difficile* as was reported by Weese et al. (2010). At day 2, 74% of the piglets were colonized while on day 62 only 3,7 % were positive for *C. difficile* (15). Contamination of pork originating from pigs arriving at the slaughterhouse is only possible if pigs at that age are colonized with *C. difficile*. Therefore, the colonization rates of pigs at the time of slaughter are more relevant to human exposure than those earlier in life (15). To determine the colonization rate of pigs with *C. difficile* arriving at the slaughterhouse a pilot study was carried out at one slaughterhouse in the Netherlands.

Materials and methods

Fifty rectal faecal samples taken of Dutch pigs, just after they had been electrically stunned and bled at a slaughterhouse situated in the southern part of the Netherlands, were tested for the presence of *C. difficile*. All animals arriving at the slaughterhouse were processed in their original peer group. Therefore it was possible to collect five rectal samples randomly within one herd. Five samples per pig farm were collected resulting in the acceptance of ten Dutch farms in this study. The number of 50 pigs from 10 farms was chosen because this project was a pilot study to determine an estimate of the colonization rate of pigs with *C. difficile*.

These rectal faecal samples were examined using a real-time PCR (BD GeneOhm™ Cdiff Assay) directly on faecal samples, in combination with culturing after enrichment.

BD GeneOhm™ Cdiff Assay

BD GeneOhm™ Cdiff Assay is an in vitro diagnostic test for direct and qualitative detection of *C. difficile* toxin B gene in human stool specimens from patients suspected of having *C. difficile* infection (CDI). This assay has not yet been validated for use in faeces from animals. The test is based on real-time PCR and performed directly on stool specimens, and in case of faeces from pigs, directly on faeces.

Faeces was collected and transported directly to the laboratory (under cooled conditions) where the manufacturer's instructions for human samples were followed.

Culturing, identification and confirmation of *C. difficile*

All faecal samples were cultured for *C. difficile* using an enrichment broth as described by Rodriguez-Palacios et al. (2007) (10). The culturing protocol was followed as described by Hopman et al. (2010) (4). Approximately 1 g of faeces was placed into 9 ml of Clostridium difficile moxalactam norfloxacin (CDMN) broth (broth produced by Mediaproducts, the Netherlands) and incubated anaerobically at 37°C for 24 hours. After broth incubation, the broth was homogenized and 2 ml was mixed with 2 ml 96% ethanol in a sterile tube and left at room temperature for a minimum of 60 minutes (alcohol shock to select for bacterial spores). After centrifugation (4000 x g for 10 min), the supernatant was discarded and the sediment was plated onto commercially prepared *C. difficile* agar (CLO-agar, Biomérieux). These CLO-plates were incubated anaerobically at 37 °C for 48 hours.

Colonies characteristic for *C. difficile* were identified by their morphological shape, their characteristic horse-manure odour and positive Gram-staining. Only when culturing revealed colonies characteristic for *C. difficile*, a colony was sent for confirmation of identification at the National Reference Laboratory for *C. difficile* at Leiden University Medical Center. Genetic identification of *C. difficile* was done by an in-house PCR for the presence of the gene encoding glutamate dehydrogenase (gluD) specific for *C. difficile* (9). All strains were further investigated by PCR ribotyping based upon Bidet et al. (2000) (1). Detection of toxin genes was done as described by Van den Berg et al. (2007) (13)

Results

C. difficile was cultured from 14 (28%) of 50 faecal samples, derived from pigs originating from nine different farms (table 1). Seven different ribotypes were found, which all were positive for TcdA and TcdB. The real-time PCR (BD GeneOhm™ Cdiff Assay) performed directly on faecal samples gave no positive results.

Discussion and conclusion

Present results demonstrate that *C. difficile* can be found in Dutch pigs at the moment of slaughtering. In total, 14 out of 50 samples were positive for *C. difficile* (28%). Pigs, from nine of ten examined farms, proved to have *C. difficile* in their faeces. Seven different ribotypes were found: *C. difficile* ribotype 005, 013, 015, 035, 062, 078 and a *C. difficile* belonging to an unknown ribotype. This variety of *C. difficile* PCR ribotypes in pigs is a remarkable finding since rarely other PCR ribotypes than PCR ribotype 078 have been isolated from piglets in the Netherlands (6). PCR ribotype 045 has been isolated from a few piglets on farms that housed predominantly PCR ribotype 078 positive piglets (results not shown). Except for PCR ribotype 078, the other PCR ribotypes found in this study are not belonging to the seven most frequently found PCR ribotypes in human *C. difficile* infections (data from the National Reference Laboratory for *C. difficile*). Data on the prevalence of found PCR ribotypes among other animals in the Netherlands are limited, but a recently completed study indicated that ribotype 005 was present in horses and pigs, ribotype 015 in sheep and ribotype 035 in horses (7). It is possible that pigs acquire *C. difficile* from the environment during transport to the slaughterhouse. More research is needed to find out whether this is route of contamination plays a role in the acquisition of *C. difficile* by pigs.

Our results are in great contrast with the findings of Hoffer et al. (2010) who found no positive pigs among 165 pigs sampled at the slaughterhouse (3). Colonization percentages are of course influenced by the methodology used to detect *C. difficile*. Hoffer et al. did not use the same enrichment broth as was used in present study (3). In present study a specific *C. difficile* enrichment broth containing moxalactam norfloxacin and 5% horse blood as described by Rodriguez-Palacios et al. (2007) was used (10). Hoffer et al. used brain heart infusion broth (Difco, Becton Dickinson, Sparks, MD) which is a general-purpose liquid medium used in the cultivation of fastidious and non-fastidious microorganisms, including aerobic and anaerobic bacteria.

The real-time PCR used direct on faecal samples was not sensitive enough to determine the presence of *C. difficile* in pigs because no positive samples were found, while 14 cultures after enrichment were positive for *C. difficile* (culturing without enrichment was not performed). The real-time PCR has not yet been validated for use in pigs and is predominantly used in stool specimens of human patients already suspected of having CDI. Possibly the number of *C. difficile* bacteria in porcine faecal samples is low explaining why the real-time PCR gave no positive signals but also inhibiting factors in pig faeces could influence the results of this test. Culturing after enrichment, although it is time-consuming, seems to be the best technique to check for *C. difficile* positive slaughtering pigs.

Finding *C. difficile* in faeces of pigs raises the question whether contamination pre-, during or post-processing can explain contaminated retail products. Explanations can be found by doing further research at slaughter and processing.

Present data indicate that pigs arriving at the slaughterhouse in the Netherlands are positive for *C. difficile* in their faeces. Found variety of ribotypes is remarkable because at farm level only a limited number of ribotypes has been found.

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