The presence of *C. diffcile* in finisher pigs at slaughter age, are there variables of influence?

Abstract

A total of 677 faecal samples from finisher pigs at slaughter age was collected from 52 herds that originated from an equal number of farms. The samples were collected at a Vion slaughterhouse in Groenlo right after stunning and bleeding. The identification of *C. difficile* was done by PCR analysis. The overall prevalence of *C. difficile* at herd level was 8,6%. In total 16 different ribotypes were found, all of these have been previously found in human stool samples. From each of the farms a set of possible risk factors for the presence of *C. difficile* was obtained. Geographical location of a farm, hygiene status or slaughter age appeared to have an influence on the presence of *C. difficile*. Not enough data for were available to determine if the difference in prevalence among these variables was significant. To determine if these variables are truly of influence on the presence of *C. difficile* in finisher pigs further research is necessary.

Introduction

Clostridium difficile infection (CDI) is the most frequently diagnosed cause of hospital-acquired diarrhoea in humans (Weese JS, 2009). Hospitalization and treatment with antimicrobial agents are identified as the major risk factors (Kuijper EJ, 2008). The incidence and severity of infection increased over the last two decades. Highly virulent ribotype s017, 027 and 078 seem to be the cause of this increased incidence and severity (Kuijper EJ, 2008) (Indra A., 2009). National surveillance in the Netherlands from February 2005 till November 2006 on patients with a severe course of C. *difficile* associated diarrhoea (CDAD) showed that in 25.3% ribotype 027 was the cause of CDAD and 74.7% had CDAD due to other ribotypes, mainly 001 (17.8%) and 014 (7.2%) (Goorhuis A, 2007). A recent study in the Netherlands suggests that the incidence of ribotype 027 is decreasing and that the incidence of ribotype 078 is increasing. Ribotype 078 is now the third strain in the Netherlands (Goorhuis, 2008). In several other European countries type 078 is also increasingly observed (Kuijper EJ, 2008). There is evidence that ribotype 078 may be overrepresented in community associated infections (Jhung MA, 2008).

Clostridium *difficile* has also been found in many animals, including food animals. In calves and piglets 078 is the predominant strain (Keel K, 2007). The 078 strain found in piglets is highly genetically related to that found in humans suggesting a common origin (Debast SB, 2009) (Bakker D, 2010). The colonization with C. *difficile* varies among different age groups. Norman et al. (2009) report a 50% colonization in suckling pigs, 8,4% in weaned pigs and 3.7% in finisher pigs. In a recent pilot study conducted by our study group 28% (14/50) of the samples obtained from finisher pigs at slaughter age contained *C. difficile*, the results of this study were not published. The effect of age needs to be taken in to consideration, as the main risk in terms of foodborne disease is shedding from animals around the time of slaughter which could lead to contamination of meat products .

There is still little knowledge with respect to possible transmission routes from animals to humans. Investigation of retail meat, including pork report contamination of the meat with *C. difficile* (Weese JS, 2009) (Rodriguez-Palacios A, 2007) (Songer GL, 2008). One study in Canada reports the majority of toxigenic isolates from meat samples to be C. difficile type 027. (Rodriguez-Palacios A, 2007) Contamination of meat could be a result of the presence of *C. difficile* in the guts of finisher pig at slaughter age, but since *C. difficile* is a bacteria that can be found in the environment, this is unclear. Contamination could occur at the slaughterhouse or at any other stage in between slaughtering the animal and the packaging of the meat. Other than age, risk factors for the prevalence of *C. difficile* in animals are not well documented.

Although *C. difficile* is now a days recognised as an important cause of severe enteritis in neonatal piglets asymptomatic carriers of *C. difficile* have also been reported in piglets and in other animals (Songer JG, 2006) (Arroyo LG, 2005) (Alvarez-Perez, 2009).There are a few reports on the prevalence of *C. difficile* in finisher pigs at slaughter age. Prevalence's for *C. difficile* found in older pigs are 3,9% (15/382) for samples from grower finisher pigs in the USA (Norman KN, 2009), 6,9% (30/435)in a study in Canada on grower-finisher pigs close to the time of slaughter (Weese JS, 2010) and 3,3% 2/61)for pigs sampled at an abattoir in Austria (Indra A., 2009). Gathering more data on the prevalence of *C. difficile* in finisher pigs and on possible risk factors for infection is important to gain more insight in the possibility of food as a possible route of transmission and in identifying a source of common origin for human and porcine *C. difficile* strains .

Hypothesis/Purpose of the research:

The aim of this study was:

- To determine the prevalence of *C. difficile* in the gut of grower-finisher pigs at slaughter age.
- To determine if the ribotypes found in samples are similar to those found in humans with Clostridium Difficile Associated Disease.
- To identify a list of possible risk factors on the presence of *C. difficile* in finisher pigs.

Material and methods

In this cross sectional (observational) study the results of the microbiological research were combined with information on factors obtained from the farms the sampled herds originated from. The information was used for an epidemiologic study after risk factors for colonization of *C. difficile* in slaughter pigs. The data were analysed using SPSS statistical software.

Sampling

A total of 677 faecal samples from grower finisher pigs at slaughter age was collected. In total 52 farms were included in the study, both conventional and biological farms. The samples were collected at the slaughter line, right after stunning and bleeding. All of the samples were collected at the same Vion slaughterhouse in Groenlo, the Netherlands.

Over a period of five consecutive weeks samples were collected on 5 different days, from Monday till Friday. Sterile gloves were used to obtain the samples from the rectum. After tying, the gloves were placed in a cool box with ice packs. Within a couple hours the samples were transported to the laboratory and processed there the same day.

A farm was considered an observational unit that possessed a set of variables. These variables are characteristics of the observational unit and are similar for all individual animals on the farm. To be able to report on possible risk factors for *C. difficile* presence we calculated the required sample size per herd to determine if *C. difficile* was present or not. Based on literature we expected a relatively low prevalence of approximately 3,5% (Norman KN, 2009) (Indra A., 2009) .However in our pilot study a prevalence of 28% was found (Keessen et al., 2010). Based on literature and the pilot study we decided to calculate the samples size with a prevalence of 20% per observational unit. Win Episcope 2.0 was used to calculate the sample size. With a *C. difficile* prevalence of 20% and a herd size of 120 animals, a minimum of 13 animals had to be sampled (Level of Confidence 95. If one sample tested positive we considered the observational unit to be positive. The initial goal was to obtain a minimum of 15 samples per farm. In reality the rate at which the samples could be processed was limited due to the logistics at the slaughtering facility, workload and workspace in the laboratory and costs of material.

Culturing

C. difficile was isolated using the procedure as described by Rodriguez-Palacios et al, for the detection of *C. difficile* on meat samples (Rodriguez-Palacios A, 2007). *C. difficile* culture agar(CM0601) supplemented with *C. difficile* moxalactam norfloxacin(CDMN=SR0173E) and 5% horse blood (SR0048C)was used to culture *C. difficile*(CLO agar plate, by . Biomérieux). *C. difficile* broth was prepared by mixing the ingredients of CM0601, except for the agar, with 0.1% sodium taurocholate (Clostridium difficile enrichment broth, Mediaproducts). This is a specific growth medium for *C. difficile*

Approximately 1 gram of faeces was mixed with 9 ml of the CDMN broth and incubated anaerobically at 37°C for 7 days. The broth was alcohol-shocked for spore selection by mixing 2 ml homogenized broth with 96% ethanol in a sterile tube and leaving it for 1 hour at room temperature. After centrifuging this mixture(4000 x g for 10 minutes) the supernatant was discarded, and the pellet was streaked onto a CLO agar plate and incubated anaerobically for 4 days at 37°C. Suspicious colonies were subcultured on blood agar and identified as *C. difficile* by Gram stain appearance, colony morphology (swarming, non-haemolytic, greyish, rough) and characteristic odour. The subcultured colonies were Gram stained again and the positive isolates sent to the University Medical Center in Leiden for PCR ribotyping. With an in-house PCR the presence of the GluD gene, encoding the glutamate dehydrogenase specific for *C. difficile*, was identified (Paltansing, 2007), the PCR ribotyping was performed as described by (Bidet, 2000).

To identify risk factors for *Clostridium difficile* infection in pigs a set of variables from each observational units was gathered. These data were gathered by employees of Vion, after the sampling took place.

The following data were obtained from the farming facilities:

- Farrow-to-finisher farms versus grower -finisher farms: in the latter weaners are sourced from other farms, this might lead to introduction of *C. difficile* on these farms more easily than in farrow-to-finisher farms.
- The number of animals: with more animals present the risk of one animal getting contaminated could be higher.
- Possible other animal species on the farm: other animal species are known to be carriers of *C. difficile*, these animals are more likely to be outdoors frequently and ingest *C. difficile* from the environment. They could be shedding *C. difficile* and thereby increase the risk for finisher pigs to ingest *C. difficile*.
- Salmonella status: the Salmonella status could be an indicator of the overall hygiene and presence of other bacteria.
- Hygiene status: in farming facilities with appropriate hygienic measures a lower presence of *C. difficile* was expected.
- Slaughter age: although there is little difference in slaughter ages between herds, age might affect the presence of *C. difficile* in the faeces of finisher pigs at slaughter age.

Results

A total of 677 rectal faecal samples of individual finisher pig was collected at the slaughterhouse, the pigs belonged to a total of 52 different herds. The number of individual animals found positive was 56, at individual pig level the prevalence was therefore (56/677) 8,6%.

Number of different Ribotypes	Number of farms
1	14 (55%)
2	7 (32%)
3	2 (9%)
4	1 (5%)

Table 1 ribotypical diversity

A herd was determined to be positive if at least one sample tested positive; all herds that tested positive were included. If all the samples in a herd tested negative and the sample size was less than 13 the herd was excluded from further study since the sample size was too small to identify the herd as either positive or negative. This was the case with 13 herds.

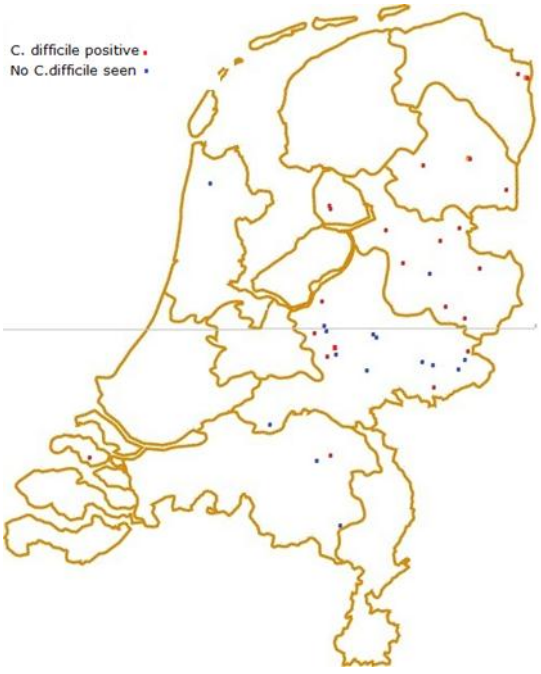

Figure 1 *C. difficile* geographical location

At herd level the prevalence was 61,5% (24/39). In 41,7% (10/24) herds more than one ribotype was identified, in one of the herds up to 4 different types were identified. In the other herds only one type was identified. See table 1. However, since the sample size was limited in some cases this doesn't mean that there were no other ribotypes present in the herd or on the farm. A total of 16 different ribotypes was identified. The predominant ribotype found was 078. All 16 of the in finisher pigs identified ribotypes have been found in human stool samples.

From two herds where *C. difficile* was present no additional information, like geographical origin, was available. These 2 herds were excluded from further

Province	Number of herds	Positive herds	different ribotypes identified
Drenthe	3	3 (100%)	078, 13, 15, 45, 62, 26
Flevoland	2	2 (100%)	078(2)
Groningen	2	3 (100%)	078, 14, 54
Overijssel	8	7 (87%)	14 (2), 11(2), 23 (2), 45, 50,1,5,19,15, 26
Gelderland	17	6 (35%)	078 (3), 014(2), 1, 3, 103
Brabant	3	1 (33%)	78
Noordholland	1	0 (0%)	
Zeeland	1	1 (100%)	14, 2

Table 2 Ribotypical diversity *C. difficile*

research. Out of the remaining 37 herds 45% (17/37) originated from farms located in the province of Gelderland, Overijssel was the second largest province of origin with 21% (8/37). Other regions of origin were Drenthe (3), Brabant (3), Flevoland (2), Groningen (2), Zeeland (1), Noord Holland (1).

When the country is divided in North and South, with the same number of farms included in this study below and above the line the percentage of positive farms in the North is 83%(15/18) and the number of positive farms in the South is 36%(7/19). For the location of the farming facilities see figure 1.

In Gelderland *C. difficile* was present in 35%(6/17) of the sampled herds, in Overijssel *C. difficile* was present in 87% (7/8) of the herds in all sampled herds from Drenthe (3/3), Flevoland (2/2) and Groningen (2/2) C. difficile was present. Two important human ribotypes 078 and 014 were found in samples from herds that originated from respectively 5 and 4 different provinces. For an overview of the different ribotypes per province see table 2.

Factor	Groups	Number of farms C. difficile +	Number of farms C. difficile -
farmtype	Finisher	**9** (56%)	**7** (44%)
	Farrow to finisher	**12** (57%)	**9** (43%)
farmsize	< 1000 animals	**13** (56%)	**10** (44%)
	> 1000 animals	**9** (69%)	**4** (29%)
other animal species	no	**13** (62%)	**8** (38%)
present	yes	**9** (60%)	**6** (40%)
salmonellastatus	1	**6** (67%)	**3** (33%)
	2	**3** (43%)	**4** (57%)
hygiene status	other	**7** (50%)	**7** (50%)
	excellent/good	**14** (64%)	**8** (34%)
slaughter age	≥ 6 months	**15** (51%)	**14** (49%)
	< 6 months	**7** (88%)	**1** (12%)

Table 3 Variables per farm in combination with *C. difficile* presence

Besides the geographical location of the herds a set of variables was gathered from the farming facilities that the herds originated from. For the variables see table 2. Not on all variables information was available. For all 37 farms included was determined if they were farrow-to- finisher farms or grower-finisher farms, the herds that tested positive or negative were distributed evenly between the two farm types. The farm size (number of pigs on the farm) was available for 36 farms, 71% of herds originating from farms with over 1000 pigs were positive for *C. difficile* opposed to 59% of herds from farms that housed less than 1000 animals. The Salmonella status was estimated by the Vion worker that gathered the information . Salmonella status was estimated for 16 farms. *C. difficile* was present in 67% of the farms with Salmonella status 1, opposed to 43% of the farms with Salmonella status 2. *C. difficile* was found more often in herds that came from farms with a good or excellent hygiene status than in farms with a lesser hygiene, respectively 64% and 50%. Herds that were delivered to the slaughterhouse at a relative early age tested positive for *C. difficile* more often than herds delivered at other ages, respectively 88% versus 15%.

Discussion

Identifying ribotype 078 as the predominant type is consistent with data from other studies in pigs (Rupnik, 2008). PCR ribotype 078 is currently the most frequently isolated strain from pigs and calves and is also increasingly reported in human population particularly in those with community associated infections (Keel K, 2007). In the Netherlands 078 is currently the third most common PCR ribotype (Hensgens MP, 2009). Together with the finding that there is a lot of similarity between the human and pig isolates this could suggest a common source of origin for the human and porcine 078 strains (D. Bakker 2010). Ribotype 027, which is associated with CDI in hospitalised patients, was not found in pigs in this study whilst other ribotypes that are 'common' in hospitalised humans, 001 and 014, were among the ribotypes found in the finisher pigs in this study. One study found an overlap in the occurrence of CDI due to type 078 in humans and the concentration of pig farms in the eastern part of the Netherlandsthis same study they also report that outbreaks of CDI in humans due to type 027 are concentrated in an part of the Netherlands were pig farms are not as abundant (Goorhuis 2008). In our study *C. difficile 078* was present in herds originating from 5 different provinces in the eastern and northern. To further investigate an overlap in the occurrence of CDI due to type 078 and the presence of this same type in finisher pigs further research on farms from different regions needs to be done.

In total 16 different ribotypes were identified in the faeces of finisher pigs, all these types have previously been identified in humans. To our knowledge this ribotypical diversity in pigs is not described in other reports. In a study where the diversity of ribotypes in different animal species is subject of research , 4 different ribotypes in pigs are described (Keel K, 2007). In a recent study in the Netherlands on samples of rectal contents from piglets 3 different ribotypes were identified. Out of 45 farms 43 tested positive. At 2 farms types 078 and 45 were found, at one farm 033 was found and in the other 40 farms 078 was the only ribotype identified. (Unpublished data Keessen et al, 2010). Our results show that there are many different ribotypes present in the guts of finisher pigs at slaughter age. It is not known if these human and porcine ribotypes possess the same similarity as is the case with the 078 strain. Transmission from pigs to humans or vice versa has not been described, but a common origin of human and porcine strains is still subject of research.

Another interesting finding is the geographical distribution of farms where *C. difficile* is present. These appeared to be concentrated in the North East of the Netherlands. Over 90% of pig farms is located in the Eastern part of the Netherlands; most sampled herds originated from farms located in Gelderland (17) and Overijssel (8). From the other provinces only a few herds were sampled Flevoland (2), Groningen (2), Drenthe (3). All herds originating from the last three provinces and over 80% of the herds from Overijssel tested positive, this suggests a certain geographical distribution for *C. difficile*. This is consistent with the results of a recent study in Canada where a significance difference in farm level prevalence between provinces was found (Weese JS et al. , 2010). A limiting factor in our study is that the true prevalence of *C. difficile* in finisher pigs is unknown and the sample size per herd in this study could have been too small, this could have led to false negative herds. The number of positive farms could be higher and in that case the distribution of positive farms could change. Further epidemiological research is needed to confirm if there is a difference in geographical distribution of *C. difficile*.

In addition to the finding of risk factors for *C. difficile* presence in humans we used a set of data from each farm to try to determine if these could be identified as risk factors for *C. difficile* presence in pigs at slaughter- age. For none of the individual data a significant difference was found. An interesting finding was the effect of hygiene on the presence of *C. difficile* in a herd. *C. difficile* was present more often in herds that originated from farms with good or excellent hygiene status. *C.difficile* is a spore forming bacteria, the spores are able to survive for long periods of time on surfaces (Gerding DN, 2008)and are resistant against many disinfectants, such as quaternary ammonium compounds (Settle CD, 2008) (Shapey S, 2008). One study reports that spore formation by *C. difficile* strains can even be increased by exposure to low concentrations of detergents (Warren, 2000). Chlorine based disinfectants are known to be effective against *C. difficile* spores (Perez J, 2005). Resistance might lead to selection of C. difficile and enhance the risk of colonisation of the intestines of pigs. The detergents used on the sampled farms are unknown therefore it is not possible to determine if there is a relationship between the type of disinfectant used and presence of *C. difficile*. To confirm if the use of specific detergents has an effect on the presence of *C. difficile* in pigs at slaughter age more research is needed.

Slaughter age appears to be a risk factor as well. Although there are not enough data in this study for the results to be significant. *C. difficile* prevalence in has been reported to decrease with age (Norman KN, 2009) but there are no reports that compare finisher pigs slaughtered at a relatively young age with other age groups delivered to the slaughter house.

Limitations, conclusions & possibilities for future research

The different variables between farms were collected after sampling and therefore the herds were not evenly distributed among the different groups, furthermore the number of data was insufficient to determine if a variable truly is of influence on the presence of *C. difficile*.

In addition to the characteristics compared in this study other data might be equally interesting, for example the use of different types of antibiotics and if all pigs in a herd were the same age (all in all out or continuous production). These data were not readily accessible for the farms included in this study and were therefore left out.

As stated earlier the prevalence used to calculate the sample size was 20%, the prevalence found was 8,6% and therefore false negatives are possible.

It seems that there is a geographical distribution for *C. difficile* in finisher pigs and that hygiene status and slaughter age might be of importance. To confirm this further research is necessary .

The finding of similar ribotypes in human stool samples and rectal samples of finisher pigs might suggest a common source of origin, but there is no proof of this in current literature.

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