

## **Presence of *Clostridium difficile* on pig carcasses during the slaughter process**

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### **Abstract**

*Clostridium difficile* can be isolated from the intestines of healthy pigs (10%) at slaughter age. Contamination of meat during the slaughter process has been described for many intestinal bacteria, i.e. *Salmonella*. Since *C. difficile* spores could survive the recommended cooking temperatures for meat and infect the consumer, *C. difficile* contaminated meat could be a potential public health risk. The aim of this study was to determine to which level contamination of the carcasses occurs and whether the presence of *C. difficile* on carcasses of pigs changes during the slaughter process. Rectal samples, cork bore samples and swab samples were taken from carcasses at the beginning and the end of the slaughter process. To determine whether the prevalence of *C. difficile* on carcasses correlates with its presence in meat, pork trimmings were also collected. The samples were cultured with both quantitative and qualitative culture methods. *C. difficile* was not isolated from any of the carcasses or the pork trimmings with the quantitative culture method. Even with qualitative culture methods cork bore samples of a carcass from a pig that intestinally carried *C. difficile* were negative. With the qualitative culture method *C. difficile* was isolated from 28 (18%) carcasses that were sampled with electrostatic cloths directly after stunning, but from none of the 163 carcasses that were sampled after chilling. *C. difficile* was cultured from two of the eight pork trimmings. The results of this study indicate that carcasses can be contaminated at the beginning of the slaughter process with *C. difficile*, although with very low numbers of spores, since the bacterium was only recovered from samples when qualitative culture methods were used. The procedures along the slaughterline are sufficient to remove *C. difficile* from the carcasses, because spores were not found on any of the carcasses that were sampled with electrostatic cloths after chilling. Further research is required to investigate the source of the contamination of the pork trimmings.

### **Introduction**

*Clostridium difficile* can be isolated from many animal species including food animals (Keel et al., 2007). The finding of overlapping ribotypes and identical isolates in humans with *C. difficile* Infection (CDI) and food animals has led to the hypothesis that interspecies transmission is likely to occur (Arroyo et al., 2005; Rupnik. 2007; Jhung et al., 2008; Debast et al., 2009; Indra et al., 2009; Songer. 2010). A well known transmission route for intestinal bacteria from animals to humans is via food of animal origin. Research on contamination of meat by intestinal bacteria, e.g. *Salmonella* , shows that

carcasses can become contaminated either because of direct contamination when *Salmonella*-positive pigs are slaughtered or through cross-contamination via environmental sites in the slaughterhouse. Likewise, pork retail meat has been reported to be contaminated with *Clostridium difficile*. Frequently isolated *C. difficile* ribotypes from retail meat are also implicated in human CDI, and considered hypervirulent, e.g. type 027 and 078, albeit the number of spores on the meat was low (Songer et al., 2009; Weese et al., 2009; Metcalf et al., 2010;). Contaminated meat could possess a risk for humans because of the spore-forming capacity of *C. difficile*. Spores are tolerant for heat treatment, so even after heating meat according to recommended temperatures, viable spores can be consumed (Rodriguez-Palacios et al., 2007). It is not clear, however, whether contamination of the retail meat occurs in the slaughterhouse or afterwards in the retail process, since during food processing the meat can become contaminated with the bacterium either via healthy food handlers colonized with *C. difficile* or via environmental contamination in the retail plant. To determine the level of contamination with *C. difficile* on pig carcasses in the slaughterhouse and whether the prevalence of *C. difficile* changes during the slaughter process, carcasses were sampled at the beginning and the end of the slaughtering process. Additionally, samples were taken from pork trimmings to investigate whether the prevalence of *C. difficile* on carcasses correlates with its presence in the pork trimmings.

## **Material and methods**

### Sampling procedure:

Rectal samples and cork bore samples were taken from nine carcasses at the start of the slaughtering process, directly after stunning and bleeding. From each carcass that was used for rectal sampling, four round slices of skin each with a surface of 5 cm<sup>2</sup> were taken with a sterile cork bore, scalpel, and tweezer. The sampling sites on the carcasses, were similar to the standard sampled sites by the slaughterhouse to detect the presence of *Salmonella* according to EU legislation, e.g. the jowl, belly and back close to the cutting edge, and ham close to the anus. The pigs that were sampled were marked to make it possible to take cork bore samples from the same carcasses again after chilling. A maximum of two carcasses per farm were sampled, to prevent sampling bias. Five carcasses were marked and sampled before and after scalding, and after chilling. Electrostatic cloths were used to sample the ham of 152 randomly chosen carcasses directly after stunning and bleeding, and the ham of 163 randomly chosen carcasses after chilling. The farm number from the ear tag of the pig was given to the swab. A maximum of 16 carcasses per farm were sampled, to avoid confounding of the results by farm-effects.

Eight pork trimmings were collected in the meat processing department, adjacent to the slaughterhouse.

#### Sample handling:

##### *Rectum samples*

Rectum samples of randomly selected pigs were collected at the slaughter line, directly after stunning and bleeding. Sterile swabs were used to obtain the samples from the rectum. Each swab was given a unique number correlating with the unique number from the ear tag of the pig. After sampling, the swabs were stored in a cooler for transport to the laboratory. Subsequently, the swabs were immersed in 9 ml *Clostridium difficile* moxalactam norfloxacin broth with sodium taurocholate (CDMN, broth produced by Mediaproducts, The Netherlands).

##### *Cork bore samples*

Cork bore samples of one carcass were placed together in a stomacher bag and then stored in a cooler for transport to the laboratory. At the laboratory, the samples were immersed in 60 ml CDMN broth and mixed in the stomacher for 180 seconds.

##### *Pork trimmings*

The pork trimmings were placed in stomacher bags and then stored in a cooler for transport to the laboratory. At the laboratory, 15 grams of meat was immersed with 50 ml of CDMN broth and mixed in the stomacher for 180 seconds.

##### *Electrostatic cloths*

The electrostatic cloths were handled with sterile gloves and directly after sampling stored in a cooler for transport to the laboratory. Subsequently, the electrostatic cloths were immersed in 36 ml CDMN broth and mixed in the stomacher for 180 seconds.

#### Quantitative culture method:

Quantitative analysis was used for the cork bore samples and the pieces of meat. A similar procedure for both types of samples was followed. One ml broth was transferred into a sterile tube directly after adding to- and mixing with the samples, and mixed with one ml 96% ethanol. This mixture was alcohol shocked at room temperature for 60 minutes. After vortexing the mixture, 0,1 ml was plated directly onto commercially-prepared *Clostridium difficile* agar (CLO agar, Biomérieux) and incubated anaerobically at 37 °C for 48 hours.

#### Qualitative culture method:

Rectum samples, cork bore samples, electrostatic cloths and pork trimmings were used for qualitative analysis. After mixing the broth with the samples, it was incubated anaerobically, using gaspaks (GasPak EZ Anaerobe Container System Sachets, BD) and anaerobic jars, at 37°C for seven days. Subsequently, the broth was homogenized and two ml was transferred into a sterile tube. The broth was mixed with two ml 96% ethanol and left at room temperature for 60 minutes. 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 culture plates were incubated anaerobically at 37 °C for 48 hours.

#### Characterization of *C. difficile*:

Colonies characteristic for *C. difficile* were identified based on morphological criteria, the characteristic horse-manure odour and Gram-staining. Genetic identification of *C. difficile* by the presence of the gene encoding glutamate dehydrogenase (gluD), was done by following the protocol of Paltansing et al. (2007) in the reference center for *C. difficile* in The Netherlands. All strains positive for gluD were further investigated with PCR-ribotyping based upon the protocol of Bidet et al. (2000).

### **Results**

*Clostridium difficile* was not isolated from any of the cork bore samples of the carcasses after stunning, before and after scalding, and after chilling, with neither the quantitative, nor the qualitative culture method.

With qualitative culture methods one of the nine rectum swabs was positive for *C. difficile*, indicating that one pig carried the bacterium in its intestines. Carcass contamination due to intestinal carriage was not found, since all the cork bore samples from the corresponding carcass were negative for *C. difficile*.

None of the eight pieces of pork trim yielded *C. difficile* after culturing with quantitative methods, however two of the eight pieces of pork trim were positive after culturing with enrichment strategies.

From the 152 carcasses that were sampled with electrostatic cloths directly after stunning, 28 samples were positive, thus the prevalence of *C. difficile* on carcasses at the beginning of the slaughter process was 18.4 percent. The prevalence of the bacterium at the end of the slaughter process was 0 percent, since *C. difficile* was not found in any of the samples of the 163 carcasses that were sampled with electrostatic cloths after chilling. An overview of the results is given in table 1,2,3, and 4.

## Discussion

The aim of this study was to determine whether the presence of *C. difficile* on carcasses changes during the slaughtering process. To examine this question, carcasses were followed along the slaughter line and samples were taken from the same pigs at the start and at the end of the line. Although the infectious dose of *C. difficile* is unknown, knowledge on the level of contamination of carcasses is important before a risk assessment on the presence of *C. difficile* on carcasses can be made. Therefore, both quantitative and qualitative culture methods were used to analyze the samples. Since there is no ISO method to sample carcasses a destructive method, i.e. the cork boar method was first chosen and used to sample the same carcasses at various moments along the slaughterline. The cork bore method has been described as a more efficient method to recover bacteria from the skin than non-destructive methods, i.e. swabs, because of the attachment of bacteria to the skin (chapter X from the PhD thesis of Swanenburg et al.). Furthermore, since the total surface of a carcass that is sampled with a cork boar is very precise, it is an excellent method for quantitative analysis. However, the number of *C. difficile* spores on the carcass was either too low, or the spores and, or the dispersion of the spores on the carcass was very in-equal, since none of the cork bore samples of the carcasses were positive, while *C. difficile* was found in 28% of the carcasses after stunning when swabs, that have the advantage that a larger surface can be sampled, were used. Another advantage of the use of electrostatic cloths versus a cork boar is that it is much less time-consuming and therefore, to sample a larger number of carcasses to assess the prevalence of *C. difficile* at the beginning and the end of the slaughterline, electrostatic cloths were used.

Since a prevalence of 10% (Keessen et al., 2011) of *C. difficile* in the intestines of slaughter pigs has been reported, it was expected that the prevalence of the bacterium on the carcasses would be higher, because of fecal contamination during transport, waiting, and during the slaughtering process. Although *C. difficile* was isolated from one of the nine rectal samples, this did not result in carcass contamination since none of the cork bore samples was positive for the bacterium. All the cork bore samples that were taken at 4 locations in the slaughterhouse, e.g. directly after stunning, before and after scalding, and after chilling were negative for *C. difficile* with both quantitative and qualitative culture methods.

The finding that 28 (18.4%) of the 152 carcasses were found positive for *C. difficile* directly after stunning, indicates that carcasses at the start of the slaughter process are frequently contaminated with *C. difficile*. However, during the slaughter process *C. difficile* is efficiently removed from the carcasses, since the bacterium was not isolated from any of the 163 carcasses after chilling when

electrostatic cloths were used. From the results of this study it is not clear at which moment during the slaughter process the bacteria are removed from the carcass. Furthermore, the presence of *C. difficile* on the carcasses after chilling cannot entirely be excluded, since it has been described for other bacteria, such as *Salmonella* spp. that chilling the carcasses can lead to withdrawing of the bacteria in the hair follicles (Berends et al., 1998).

*C. difficile* was not isolated from any of the pork trimmings with the quantitative method, but two pork trimmings were positive with the qualitative method, indicating that the meat can become contaminated, although with low levels. The level of contamination may have been below 20 spores per gram, since the detection threshold of *C. difficile* in meat with a similar quantitative culture method was 20 spores per gram meat, while the threshold of a similar qualitative culture method was reported to be  $\leq 10$  spores per gram (Weese et al. 2009).

Although high prevalences, varying from 12 to 41.3% of *C. difficile* in retail pork meat were found in North America, *C. difficile* was not found in any of the pork samples in studies conducted in Austria, The Netherlands, Switzerland, Sweden, and France, even though in all these studies enrichment methods were used to culture the bacterium (Indra et al., 2009; Von Abercron et al., 2009; Bouttier et al., 2010; Hoffer et al., 2010; Jobstl et al., 2010; de Boer et al., 2011). Therefore, the isolation of *C. difficile* from two of the eight pork trimmings was unexpected. However, the source of the contamination of the pork trimmings is not yet elucidated. Since the bacterium was not cultured from any of the samples of the carcasses at the end of the slaughter process, with both the destructive and non-destructive sample methods, it is unlikely that the source of the contamination comes from the carcasses themselves. On the other hand site, the detection threshold for isolation *C. difficile* from carcasses is probably higher than from meat. It has also been suggested that contamination of muscle tissue with *C. difficile* spores can occur preharvest, since spores have been recovered from muscle tissue in healthy horses and cows (Vengust et al., 2003; Rodriguez-Palacios et al., 2009). It has to be taken in consideration though that pork trimmings are small pieces of meat originating from the trimming of cuts of meat and are therefore considered by the slaughter house as the meat products with the highest microbiological risks. Contamination of the pork trimmings could easily occur because they were cut off at various places within the meat cutting areas and collected afterwards, and there has been contact of the pork trimmings with the hands of the employees and slaughter equipment.

## **Conclusion**

Results from this research implies that although *C. difficile* can be found in the intestines and on the carcasses of slaughter pigs at the beginning of the slaughter process, the procedures on the slaughter line are sufficient to remove the bacterium from the carcasses. However, pork trimmings were found to be contaminated with low numbers of spores when qualitative culture methods were used. Further research is required to investigate the source of this contamination.

**Table 1**

Cork bore samples after stunning					
Quantitative	1 a	Negative	Qualitative	1 a	Negative
Method	1 b	Negative	Method	1 b	Negative
	1 c	Negative		1 c	Negative
	2 a	Negative		2 a	Negative
	2 b	Negative		2 b	Negative
	3 a	Negative		3 a	Negative
	3 b	Negative		3 b	Negative
	4 a	Negative		4 a	Negative
	4 b	Negative		4 b	Negative
Rectum swab samples after stunning					
Qualitative	1 a	Negative			
Method	1 b	Negative			
	1 c	Negative			
	2 a	Negative			
	2 b	Negative			
	3 a	Negative			
	3 b	Negative			
	4 a	Negative			
	4 b	Positive			
Cork bore samples before fire oven					
Quantitative	5 a	Negative	Qualitative	5 a	Negative
Method	5b	Negative	Method	5 b	Negative
	6 a	Negative		6 a	Negative
	6 b	Negative		6 b	Negative
	6 c	Negative		6 c	Negative
Cork bore samples after fire oven					



Quantitative	5 a	Negative	Qualitative	5 a	Negative
Method	5b	Negative	Method	5 b	Negative
	6 a	Negative		6 a	Negative
	6 b	Negative		6 b	Negative
	6 c	Negative		6 c	Negative
Cork bore samples after chilling					
Quantitative	1 a	Negative	Qualitative	1 a	Negative
Method	1 b	Negative	Method	1 b	Negative
	1 c	Negative		1 c	Negative
	2 a	Negative		2 a	Negative
	2 b	Negative		2 b	Negative
	3 a	Negative		3 a	Negative
	3 b	Negative		3 b	Negative
	4 a	Negative		4 a	Negative
	4 b	Negative		4 b	Negative
	5 a	Negative		5 a	Negative
	5b	Negative		5 b	Negative
	6 a	Negative		6 a	Negative
	6 b	Negative		6 b	Negative
	6 c	Negative		6 c	Negative

**Table 2**

Pork trimmings					
Quantitative	1	Negative	Qualitative	1	Positive
Method	2	Negative	Method	2	Negative
	3	Negative		3	Negative
	4	Negative		4	Negative
	5	Negative		5	Positive
	6	Negative		6	Negative
	7	Negative		7	Negative
	8	Negative		8	Negative

**Table 3**

Electrostatic cloth samples after stunning, Qualitative Method					
Farm 1	A	Negative	3	L	Negative
	B	Negative		M	
	C	Positive		N	Negative
	D	Negative		O	Positive
	E	Negative	4	A	Positive
	F	Negative		B	Positive
	G	Negative		C	Negative
	H	Negative		D	Negative
	I	Negative		E	Negative
	J	Positive		F	Negative
	K	Negative		G	Positive
	L	Negative		H	Negative
	M	Negative		I	Positive
	N	Negative		J	Negative
	O	Negative		K	Negative
Farm 2	A	Negative		L	Negative
	B	Negative		M	Negative

	C	Positive		N	Negative
	D	Negative	5	A	Negative
	E	Negative		B	Negative
	F	Negative		C	Positive
	G	Negative		D	Negative
	H	Negative		E	Positive
	I	Negative		F	Negative
	J	Negative		G	Negative
	K	Negative		H	Negative
	L	Positive		I	Positive
	M	Negative		J	Negative
Farm 3	A	Negative		K	Positive
	B	Negative		L	Negative
	C	Negative		M	Negative
	D	Negative		N	Negative
	E	Negative		O	Negative
	F	Negative	6	A	Positive
	G	Negative		B	Negative
	H	Negative		C	Negative
	I	Negative		D	Positive
	J	Negative		E	Negative
	K	Negative		F	Negative
Farm 6	G	Negative	10	L	Negative
	H	Negative		M	Negative
	I	Negative		N	Negative
	J	Negative		O	Negative
	K	Negative	11	A	Negative
	L	Positive		B	Negative
	M	Negative		C	Positive
	N	Negative		D	Positive

	O	Positive		E	Negative
	P	Negative		F	Positive
Farm 7	A	Negative		G	Negative
	B	Negative		H	Negative
	C	Negative		I	Negative
	D	Negative		J	Negative
	E	Negative		K	Negative
	F	Negative		L	Positive
	G	Negative		M	Negative
	H	Negative		N	Positive
	I	Negative		O	Negative
	J	Negative	12	A	Positive
	K	Negative		B	Positive
Farm 8	A	Negative		C	Negative
	B	Negative	13	A	Negative
	C	Negative			
	D	Negative			
	E	Positive			
	F	Negative			
	G	Negative			
	H	Negative			
	I	Negative			
	J	Negative			
	K	Negative			
	L	Negative			
	M	Negative			
	N	Negative			
	O	Negative			
9	A	Negative			
	B	Positive			

	C	Negative
	D	Positive
10	A	Negative
	B	Negative
	C	Negative
	D	Negative
	E	Negative
	F	Negative
	G	Negative
	H	Negative
	I	Negative
	J	Negative
	K	Positive

**Table 4**

Electrostatic cloth samples after chilling, Qualitative Method					
1	A	Negative	7	G	Negative
	B	Negative		H	Negative
	C	Negative		I	Negative
	D	Negative		J	Negative
2	A	Negative		K	Negative
	B	Negative		L	Negative
	C	Negative		M	Negative
	D	Negative		N	Negative
	E	Negative		O	Negative
	F	Negative		P	Negative
	G	Negative	8	A	Negative
	H	Negative		B	Negative
	I	Negative		C	Negative
	J	Negative		D	Negative

	K	Negative		E	Negative
	L	Negative	9	A	Negative
	M	Negative		B	Negative
	N	Negative		C	Negative
	O	Negative		D	Negative
3	A	Negative		E	Negative
	B	Negative		F	Negative
	C	Negative		G	Negative
	D	Negative		H	Negative
	E	Negative		I	Negative
	F	Negative		J	Negative
4	A	Negative		K	Negative
	B	Negative		L	Negative
	C	Negative		M	Negative
	D	Negative		N	Negative
	E	Negative		O	Negative
	F	Negative	10	A	Negative
5	A	Negative		B	Negative
	B	Negative		C	Negative
	C	Negative		D	Negative
	D	Negative		E	Negative
	E	Negative		F	Negative
	F	Negative		G	Negative
6	A	Negative		H	Negative
	B	Negative		I	Negative
	C	Negative		J	Negative
	D	Negative		K	Negative
	E	Negative		L	Negative
	F	Negative	11	A	Negative
	G	Negative		B	Negative

7	A	Negative		C	Negative
	B	Negative		D	Negative
	C	Negative		E	Negative
	D	Negative		F	Negative
	E	Negative		G	Negative
	F	Negative		H	Negative
12	A	Negative	14	G	Negative
	B	Negative		H	Negative
	C	Negative		I	Negative
	D	Negative	15	A	Negative
	E	Negative		B	Negative
	F	Negative		C	Negative
	G	Negative		D	Negative
	H	Negative		E	Negative
	I	Negative		F	Negative
	J	Negative		G	Negative
	K	Negative		H	Negative
	L	Negative		I	Negative
	M	Negative		J	Negative
	N	Negative		K	Negative
	O	Negative		L	Negative
13	A	Negative		M	Negative
	B	Negative		N	Negative
	C	Negative		O	Negative
	D	Negative			
	E	Negative			
	F	Negative			
	G	Negative			
	H	Negative			
	I	Negative			

	J	Negative
	K	Negative
	L	Negative
	M	Negative
	N	Negative
	O	Negative
13	A	Negative
	B	Negative
	C	Negative
	D	Negative
	E	Negative
	F	Negative
	G	Negative
	H	Negative
	I	Negative
	J	Negative
	K	Negative
	L	Negative
	M	Negative
	N	Negative
	O	Negative
14	A	Negative
	B	Negative
	C	Negative
	D	Negative
	E	Negative
	F	Negative



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