International Journal of Food Microbiology 166 (2013) 256–262

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Presence of *Clostridium difficile* in pigs and cattle intestinal contents and carcass contamination at the slaughterhouse in Belgium

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ARTICLE INFO

Article history:
Received 2 January 2013
Received in revised form 17 July 2013
Accepted 19 July 2013
Available online 29 July 2013

Keywords:
Clostridium difficile
Slaughter pigs and cattle
Carcass contamination

ABSTRACT

The objective of this study was to evaluate the presence of *Clostridium difficile* in intestinal and carcass samples collected from pigs and cattle at a single slaughterhouse. *C. difficile* was isolated in 1% and 9.9% of the pig and cattle intestinal contents and in 7.9% and 7% of cattle and pig carcass samples respectively. A total of 19 different PCR-ribotypes were identified, among them types 078 and 014. Seven of 19 ribotypes correlated with the PCR-ribotypes involved in human *C. difficile* infections in Belgium. This study confirms that animals are carriers of *C. difficile* at slaughter and ribotypes are identical than those in humans, and that carcass contamination occurs inside the slaughterhouse.

1. Introduction

Clostridium difficile is an anaerobic, spore-forming bacterium that remains the main cause of nosocomial diarrhoea in humans after use of antibiotics. *C. difficile* has also been described in other environments outside of hospitals, such as soil, river and seawater samples (Al Saif and Brazier, 1996; Pasquale et al., 2011; Zidaric et al., 2010) and in animals, in which it can also cause enteric disease (Rodriguez-Palacios et al., 2006; Songer and Anderson, 2006). The possibility of transmission of *C. difficile* pathogenic isolates between animals, environments and humans has been suggested (Janezic et al., 2012).

In recent years, the interest in *C. difficile* in food and in food animals has increased, leading to studying animals as a possible reservoir and a potential risk for food borne infections linked to *C. difficile*. Studies in various countries have determined differences in the prevalence of *C. difficile* in animals just before slaughter (Baker et al., 2010; Hoffer et al., 2010; Houser et al., 2012; Keeseen et al., 2011; Rodriguez et al., 2012). In addition, many types, including PCR-ribotype 078, are present in humans, animals (Debast et al., 2009; Janezic et al., 2012) and meat (Boer et al., 2011; Curry et al., 2012; Weese et al., 2009). The PCR-ribotype 078 was among the three most prevalent ribotypes of *C. difficile* isolated from humans in Europe in 2009 (Bauer et al., 2011), and it also appears to be associated with increased virulence (Goorhuis et al., 2008) as the highly virulent epidemic strain *C. difficile* 027 (Kuijper et al., 2006). However, there is not much data describing *C. difficile* on carcasses at the slaughterhouse, and studies have failed to establish the importance of the faecal contamination of the carcass on the slaughter line.

Differences in prevalence have been observed between studies. These differences between continents may be due to geographical differences in occurrence, seasonality or methodological variations (Hensgens et al., 2012; Rodriguez-Palacios et al., 2009; Weese, 2010). In most cases, isolation of *C. difficile* from animals requires an enrichment stage, and the methods recently used to detect *C. difficile* in animal samples have varied greatly. The influence of different factors such as enrichment time can affect the recovery rates of *C. difficile* in faecal samples or carcasses (Limbago et al., 2012).

The primary objective of this study was to determine the presence of *C. difficile* in intestinal contents and on carcasses in full-grown animals at the slaughterhouse. Additionally, the influence of the enrichment duration was evaluated with a method of 3 enrichment days and an increased enrichment step to a maximum of 30 days. *C. difficile* isolates were characterized and compared to the main PCR-ribotypes found in humans in Belgium.

2. Materials and methods

2.1. Sampling

Sampling was carried out between September 2011 and May 2012, and a total of 402 cattle and pig samples, including intestinal contents and carcass samples were collected. Intestinal contents and carcass samples were collected from a single slaughterhouse. This local slaughterhouse has a mean daily production of 154 cattle and 985 pigs with a work schedule of four days a week. Intestinal and carcass samples were collected between September and November 2011 on nine different

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http://dx.doi.org/10.1016/j.ijfoodmicro.2013.07.017

rotatory days at 1 to 2 week intervals. Between 4 and 20 consecutively slaughtered animals were sampled. Cattle samples were recovered from animals coming from 57 different herds. Most of the samples were from animals between 15 and 56 months old. Only two animals sampled were younger than 12 months, and 6 were older than 7 years. Pig samples were collected from animals between 5 and 6 months of age coming from 14 different herds with an average weight of 96 kg per carcass.

2.1.1. Intestinal contents

A total of 101 intestinal samples from cattle and 100 from pigs were recovered from the large intestine of each animal at the slaughter line, directly from the viscera processing area. Approximately 50 g of intestinal contents were collected by making an incision of approximately 3 cm on the top of the cecum. The sample content was extracted by applying pressure on the surface and after discarding the first outgoing content. All collected samples were kept in individual, identified, sterile 50 ml tubes at room temperature for a maximum of 5 h. They were processed the same day, immediately upon arrival at the laboratory.

2.1.2. Carcass samples

In total, 101 carcasses from cattle and 100 from pigs were sampled 2 h after slaughter, just after fast chilling in the chilling room. For cattle, 80.1% of carcass samples (81 animals) were taken from the same animal from which the intestinal content had been collected. In those cases when it was not possible to take the intestinal content and the carcass swabs from the same animal, another carcass in the chilling room was randomly selected for swabbing. In the case of pig samples, intestinal contents and carcass swabs were always taken from different animals.

Samples were taken from half carcasses according to the Belgian Royal Decree of 20 August 2002 from four different places. Briefly, cotton cosmetic pads were first moistened in sterile buffered peptone water with cysteine 0.5% (Oxoid, Dardilly, France). A 400 cm^2 area of rump, flank, brisket and posterior face of the anterior limb were swabbed with the wet side of the cotton, representing in total an area of 1600 cm^2 on each cattle carcass. On pig carcasses, swabs were taken from ham (100 cm^2), basin (100 cm^2), sternum (300 cm^2) and forelimb (100 cm^2), covering a total area of 600 cm^2. After using the wet side of the cotton, the same procedure was repeated in the same areas with the dry cotton face. For each carcass swab, an effort was made to exert the maximum pressure possible. Swabs from each half carcass were placed together in sterile, identified 100 ml tubes. They were kept at room temperature for a maximum of 2 h until arrival at the laboratory, where they were immediately processed.

2.2. C. difficile isolation and characterization

Culture of all samples was performed with an enrichment step. The enrichment broth used, cycloserine cefoxitin fructose taurocholate (CCFT), was freshly prepared in the laboratory as described by Delmée et al. (1987) but without agar. One gram of intestinal sample was inoculated into 9 ml of CCFT as previously described (Rodriguez et al., 2012). Carcass swabs were put into 50 ml of CCFT. Subsequently, the enrichment broth of each sample was incubated in an anaerobic workstation (Led Techno, Heusden-Zolder, Belgium) at 37 °C for 3 and 30 days. After each phase of enrichment, approximately 10 μl of the broth was spread on home-made cycloserine cefoxitin fructose agar taurocholate agar plates (CCFAT) (Delmée et al., 1987) and incubated anaerobically for 48 h at 37 °C. Colonies of *C. difficile* were identified from culture plates by morphological criteria as yellowish colonies with an appearance of ground glass and a characteristic horse manure odour. One morphological suspected colony per plate was subcultured onto blood agar (5% Sheep Blood; Biorad, Nazareth, Belgium) and checked using a *C. difficile* latex agglutination rapid test Kit DR 1107A (Oxoid, Dardilly, France). Multiple colonies where taken only when presumptive colonies were too small to ensure isolation on the blood agar or when morphologies suggested more than one type of colony (4 samples). Confirmation of *C. difficile* by detection of a species-specific internal fragment of *tpi* and detection of genes for toxin B and binary toxin (*cdtA*) were performed according to a specific multiplex PCR as described previously (Rodriguez et al., 2012). A second simplex PCR for the detection of the toxin A encoding gene was performed according to the primers of Antikainen et al. (2009) and the protocol of Lemée et al. (2004). Monolayer MRC-5 cells were used in order to confirm the cytotoxic activity as described previously (Rodriguez et al., 2012).

2.3. GenoType CDiff test and PCR-ribotyping

In addition, all of the isolates were tested using the Genotype CDiff test system (HainLifescience, Nehren, DE). The test detects specific internal fragments of *tpi* and all the toxin genes (*tcdA*, *tcdB*, *cdtA* and *cdtB*) and also deletion in the regulator gene *tcdC* (18 bp and 39 bp deletions or single base deletion at position 117). Procedures and reagents were accomplished following the manufacturer's instructions. PCR-ribotyping was performed with primers used for amplification of 16S–23S intergenic spacer regions previously described by Bidet et al. (1999). DNA was extracted using a chelex 100 solution 5% (Biorad, Nazareth, Belgium) as described by O'Neill et al. (1996). PCR amplification was performed following a previously described protocol (Rodriguez et al., 2012). Amplicon size was analysed by capillary electrophoresis using the ABI 3100 Automated Capillary DNA Sequencer and GeneScan Analysis (Applied Biosystems, California, USA). As an internal marker, 35–500 bp ROX ladder (Applied Biosystems, California, USA) was used for each sample. The isolates with an international number had presented a PCR-ribotype profile corresponding to one of the 23 reference Cardiff ribotypes from the strain collection available in our laboratory. If a strain had a profile that did not correspond to any of the 23, an arbitrary internal number beginning with UCL was given.

3. Results

3.1. Prevalence of C. difficile in intestinal contents and carcass samples

A total of 202 cattle samples were analysed. *C. difficile* was isolated from 10/101 (9.9%) intestinal samples from slaughter cattle. Most of the positive samples (8/10) were already isolated after 3 days of enrichment, but 2 positive samples were only isolated after 30 enrichment days. In cattle carcass samples, *C. difficile* was isolated from 8/101 samples (7.9%). Seven positive samples were detected after 3 days of enrichment, and only 1 sample was negative after 3 enrichment days but positive after 30 enrichment days. Positive samples were from animals aged between 11 months and 6 years. Two animals with positive carcasses came from the same herd, while the remaining positive samples (intestinal contents and carcasses) were from animals coming from different herds. Only in one case was *C. difficile* detected in the intestinal content and on the carcass sample of the same animal (Table 1).

From pigs, a total of 200 samples were analysed. *C. difficile* was isolated from 1/100 (1%) intestinal samples from slaughter pigs. This sample was already detected after 3 days of enrichment, and it was also positive after 30 enrichment days. On pig carcass samples *C. difficile* was isolated from 7/100 samples (7%) on the same sampling day. Six positives were detected after 3 and 30 days of enrichment, while 1 sample was only positive after 30 days of enrichment (Table 2).

All of the positive samples (intestinal and carcass samples from pigs and cattle) detected after 3 days of enrichment were also positive after 30 enrichment days.

3.2. PCR-ribotyping, toxin activity, toxin genes detection and Genotype CDiff

From the total of 26 positive samples, 19 different PCR-ribotypes were identified. Six of these PCR-ribotypes have a ribotype profile

Table 1
Clostridium difficile in intestinal and carcass samples obtained from slaughter cattle per sampling day and herd after 3 and 30 days of enrichment.

Date of sampling	Positive animals (total animals)	Positive herds (total herds)	Positive herd identification (animal sampled by herd)	Positive animals in each herd	Age of positive animals (months)	Positive Intestinal contents		PCR-ribotypes isolated from Intestinal contents		Positive Carcasses		PCR-ribotypes isolated from Carcasses	
						3 days[a]	30 days[b]	3 days[a]	30 days[b]	3 days[a]	30 days[b]	3 days[a]	30 days[b]
15/09/11	0 (7)	0 (7)											
23/09/11	3 (11)	3 (4)	A (7)	1	26	+	+	UCL118	UCL118				
			B (1)	1[c]	17	+	+	UCL16R	UCL16L				
			C (3)	1	76	+	+	UCL270	UCL5a				
30/09/11	2 (12)	2 (8)	D (3)	1	24	+	+	UCL273/029[d]	029				
			E (1)	1	30	+	+	UCL254/078[d]	UCL254				
07/10/11	2 (7)	1 (6)	F (5)	2[c]	19					+	+	UCL5a	UCL5a
					18					+	+	015	015
21/10/11	1 (12)	1 (8)	G (1)	1	11	−	+		014				
04/11/11	2 (12)	2 (7)	H (1)	1	17	+	+	078	078				
			I (1)	1	18	−	+		078				
18/11/11	3 (11)	3 (8)	J (1)	1	22	+	+	UCL103	UCL103				
			K (1)	1	20					+	+	UCL16u	UCL16u
			L (1)	1	23					+	+	UCL16u	UCL16u
22/11/11	3 (6)	3 (5)	M (1)	1	20					+	+	023	023
			N (1)	1	20	+	+	078	078	+	+	UCL11	UCL11
			O (1)	1	18						+		UCL23d
29/11/11	1 (4)	1 (4)	P (1)	1	22					+	+	UCL5a	UCL5a

Shadowed parts of the table mean that no positives were found in the samples.
[a] Positive results detected after 3 days of enrichment.
[b] Positive results detected after 30 days of enrichment.
[c] Intestinal and carcass samples were not taken from the same animal.
[d] Two different PCR-ribotypes isolated from the same sample.

corresponding to the international collection (078, 014, 029, 023, 015, 081), while the rest of the strains were not associated with any reference Cardiff ribotypes (Table 3). The same PCR-ribotype was isolated from cattle intestinal contents and cattle carcass samples (PCR-ribotype UCL5a) (Table 3). PCR-ribotype 078 was isolated from pig and cattle intestinal contents. PCR-ribotype 014 was isolated from cattle intestinal contents and from pig carcasses (Table 3).

From cattle, in two intestinal contents more than one PCR-ribotype (n = 2) was isolated from each sample after 3 days of enrichment, while after 30 enrichment days only one PCR-ribotype was detected (Table 1). For the other positive samples, the same PCR-ribotype was detected after 3 and 30 days of enrichment except in two intestinal samples (positive herd B: 3 days with PCR-ribotype UCL16R and 30 days with PCR-ribotype UCL16L; positive herd C: 3 days with PCR-ribotype UCL270 and 30 days with PCR-ribotype UCL5a). Two animals with positive carcasses came from the same herd (herd F), but different PCR-ribotypes were detected on each carcass (Table 1). Regarding the only animal testing positive in both intestinal content and carcass samples, different PCR-ribotypes were detected from each sample (intestinal content with PCR-ribotype 078; carcass with PCR-ribotype UCL11) (Table 1). Isolates from intestinal contents presented the widest variety in PCR-ribotypes (11 different PCR-ribotypes among the 19 identified) followed by the isolates from carcasses (6 different PCR-ribotypes identified) (Table 3).

From pigs, the same PCR-ribotype was detected after 3 and 30 days of enrichment, except in the positive intestinal content where two different PCR-ribotypes were isolated (3 days with PCR-ribotype 078; 30 days with PCR-ribotype UCL46) (Table 2). All positive carcasses (n = 7) came from the same sampling day, and 3 different PCR-ribotypes were identified (014, 081 and UCL36) (Table 2).

Forty-two of the total isolates (n = 50) had a toxic activity confirmed by the cytotoxicity assay. In cattle intestinal contents a total of 8

Table 2
Clostridium difficile in intestinal and carcass samples obtained from slaughter pigs per sampling day after 3 and 30 days of enrichment.

Date of sampling	Positive animals (total animals)		Positive Intestinal contents		PCR-ribotypes isolated from Intestinal contents		Positive Carcasses		PCR-ribotypes isolated from Carcasses	
	Intestinal contents	Carcass samples	3 days[a]	30 days[b]	3 days[a]	30 days[b]	3 days[a]	30 days[b]	3 days[a]	30 days[b]
23/09/11	0 (20)	0 (20)								
30/09/11	0 (12)	0 (12)								
07/10/11	0 (12)	0 (12)								
21/10/11	0 (12)	0 (12)								
04/11/11	1 (12)	0 (12)	+	+	078	UCL46				
22/11/11	0 (12)	0 (12)								
29/11/11	0 (20)	7 (20)					+	+	UCL36	UCL36
							+	+	014	014
							+	+	014	014
							+	+	081	081
							+	+	UCL36	UCL36
								+		014
							+	+	014	014

Shadowed parts of the table mean that no positives were found in the samples.
[a] Positive results detected after 3 days of enrichment.
[b] Positive results detected after 30 days of enrichment.

Table 3
PCR-ribotypes, *tcdC* and toxin gene profiles of *C. difficile* isolated from cattle and pigs intestinal contents, carcass and meat samples.

Animal group	Sample type	PCR-ribotype	No. isolates	Toxin activity	Detection of toxin genes by PCR			Genotype CDiff test system						
					tcdA	*tcdB*	*cdtA*	*tcdA*	*tcdB*	*cdtA*	*cdtB*	*tcdC*18 bp	*tcdC*39 bp	*tcdC*117 bp
Cattle	Intestinal contents	078	6	+	+	+	+	+	+	+	+	−	+	−
		UCL5a	1	+	+	+	+	+	+	+	+	−	+	−
		014	1	+	+	+	−	+	+	−	−	−	−	−
		UCL16L	1	+	+	+	−	+	+	−	−	−	−	−
		029	2	+	+	+	−	+	+	−	−	−	−	−
		UCL118	2	+	+	+	−	+	+	−	−	−	−	−
		UCL16R	1	+	+	+	−	+	+	−	−	−	−	−
		UCL254	2	+	+	+	−	+	+	−	−	−	−	−
		UCL270	1	−	−	−	−	−	−	−	−	−	−	−
		UCL273	1	−	−	−	−	−	−	−	−	−	−	−
		UCL103	2	−	−	−	−	−	−	−	−	−	−	−
	Carcass samples	023	2	+	+	+	+	+	+	+	+	+	+	−
		UCL5a	4	+	+	+	+	+	+	+	+	−	+	−
		UCL11	2	+	+	+	+	+	+	+	+	−	+	−
		015	2	+	+	+	−	+	+	−	−	+	−	−
		UCL16u	4	+	+	+	−	+	+	−	−	−	−	−
		UCL23d	1	+	+	+	−	+	+	−	−	−	−	−
Pork	Intestinal contents	078	1	+	+	+	+	+	+	+	+	−	+	−
		UCL46	1	+	+	+	−	+	+	−	−	−	−	−
	Carcass samples	014	7	+	+	+	−	+	+	−	−	−	−	−
		081	2	+	+	+	−	+	+	−	−	−	−	−
		UCL36	4	−	−	−	−	−	−	−	−	−	−	−

PCR-ribotypes had toxic activity, and only three were identified as non-toxigenic, while all cattle carcass PCR-ribotypes identified were toxigenic. Among PCR-ribotypes recovered from pigs, only one strain from carcass was non-toxigenic. These results are obtained at the same time by the PCR results targeting *tpi*, *tcdA*, *tcdB* and *cdtA* and by the results of the GenoType CDiff test system. All the toxigenic identified isolates contained *tcdA* and *tcdB* genes. All isolates of PCR-ribotypes 078, 023, UCL5a and UCL11 also contained *cdtA* and *cdtB* gene coding for the binary toxin and had a 39 bp deletion in the regulator gene *tcdC*. An 18 bp deletion in *tcdC* was only detected in all the isolates of PCR-ribotypes 015 and 023. All the isolates of PCR-ribotypes UCL 270, UCL 273, UCL 103 and UCL 36 were negative for all toxin genes (Table 3).

4. Discussion

The present study determined the prevalence of *C. difficile* in intestinal contents and carcasses in slaughter pigs and cattle in Belgium and also includes toxin activity, toxin gene detection and detection of deletion in *tcdC* gene of all isolates.

C. difficile was isolated most frequently from intestinal contents (9.9%) of cattle at the slaughterhouse. In our previous study in Belgium (Rodriguez et al., 2012) and in two other studies in The United States (Houser et al., 2012; Thitaram et al., 2011) the prevalence reported ranges between 6.3% and 12% in cattle just before slaughter. This prevalence is much lower in other studies conducted in slaughter cattle in Europe (Hoffer et al., 2010; Koene et al., 2011). However, the difference in prevalence among studies may be due to geographical, seasonal or methodological variations as previously described (Hensgens et al., 2012; Weese, 2010). The condition that the sampling size between studies is not identical should also be considered. Limited information is available for the prevalence of *C. difficile* on cattle carcasses at the slaughterhouse. Rodriguez-Palacios et al. (2011) reported 0 positive carcasses from a total of 168 samples analysed. Houser et al. (2012) detected the *tpi* housekeeping gene by PCR in 4 of 100 carcass swabs. One of these carcasses was also positive for the *tcdA* gene, but *C. difficile* was not isolated using culture techniques. This present study is the first to target *C. difficile* isolated from cattle carcasses at the slaughterhouse with an observed prevalence of 7.9% (8/101). Positive samples were detected on three different sampling days. Despite the high prevalence of *C. difficile* in intestinal contents and on carcasses, only one animal was positive for both samples but ribotypes were not identical. These results suggest that carcass contamination during processing might occur.

C. difficile was recovered from only one intestinal content (1%) from pigs at the slaughterhouse. Reported prevalence rates of *C. difficile* vary widely among other studies conducted in different countries. A prevalence ranging between 3.3 and 8.6% was reported in The Netherlands (Keeseen et al., 2011), Austria (Indra et al., 2009), The United States (Norman et al., 2009) and Canada (Weese et al., 2011). In other studies the prevalence described is much lower: in Switzerland (0%) (Hoffer et al., 2010), in The United States (0.3%) (Susick et al., 2012) and in our previous study in Belgium (0%) (Rodriguez et al., 2012). However, as in the case of intestinal contents from cattle, the condition that the sampling size or methodologies between studies are not identical must be considered. Carcasses from 7% (7/100) of the slaughter pigs were positive for *C. difficile*. There are only a few studies describing *C. difficile* on pig carcasses at the slaughterhouse. In The United States Susick et al. (2012) reported a prevalence of 2.2% (4/182) and 2.5% on post-evisceration and post-chill swabs respectively in antimicrobial free pigs. Harvey et al. (2011) detected 3 positive *C. difficile* samples from a total of 10 sponge swabs collected from carcass hide, post-excision hide and ears in a processing plant in Texas. Another recent study conducted in Canada (Hawken et al., 2013) reported a total of 3 out of 20 positive carcasses (15%) sampled at post-bleed and a further 3 out of 20 (15%) at post-evisceration. To the authors' knowledge, this current study is the first study to isolate *C. difficile* from pig carcasses at the slaughterhouse in Europe. However, the 80 negative carcasses previously collected in 6 different sampling days and the sole positive sample of intestinal content detected corroborate that the prevalence of *C. difficile* in slaughter pigs is very low. The higher prevalence of *C. difficile* in pig carcasses reported in the present study (7%) could be explained by an unusually high contamination in the slaughtered herd or a previous faecal contamination through at the slaughter line. In the sampling plan developed for pigs, first, intestinal contents were collected from consecutively slaughtered animals coming from the same herd. Next, carcasses from another herd were sampled in the chilling room. These carcasses were from animals

Table 4
C. difficile PCR-ribotypes isolated from pig at cattle at slaughter age and comparison with the most frequent PCR-ribotypes isolated from humans.

Cardiff PCR-Ribotypes	Cattle						Pigs						Humans	
	Faecal samples			Carcass samples			Faecal samples			Carcass samples			No. Isolates (%)	
	No. Isolates (%)	Country	Reference	No. Isolates (%)	Country	Reference	No. Isolates (%)	Country	Reference	No. Isolates (%)	Country	Reference	Belgium 2011[2]	Europe 2008[3]
014	1 (7.1)	Belgium	Present study[1]	–	–	–	9 (15.5)	Netherlands	Keeseen et al., 2011	7 (50)	Belgium	Present study[1]	56 (12.1)	61 (16)
	1 (7.1)	Belgium	Rodriguez et al., 2012											
	1 (33.3)	Austria	Indra et al., 2009											
002	1 (7.1)	Belgium	Rodriguez et al., 2012	–	–	–	1 (1.7)	Netherlands	Keeseen et al., 2011	–	–	–	37 (8)	18 (5)
027	–	–	–	–	–	–	–	–	–	–	–	–	36 (7.8)	19 (5)
078	4 (28.6)	Belgium	Present study[1]	–	–	–	1 (50)	Belgium	Present study[1]	6 (100)	Canada	Hawken et al., 2013	35 (7.6)	31 (8)
	1 (100)	Switzerland	Hoffer et al., 2010				20 (67)	Canada	Weese et al., 2011	3 (100)	USA	Harvey et al., 2011		
	4 (100)	Canada	Costa et al., 2011				18 (31)	Netherlands	Keeseen et al., 2011	–	–	–		
020	–	–	–	–	–	–	–	–	–	–	–	–	31 (6.7)	61 (16)
001	–	–	–	–	–	–	2 (3.4)	Netherlands	Keeseen et al., 2011	–	–	–	10 (2.1)	37 (10)
023	–	–	–	1 (12.5)	Belgium	Present study[1]	2 (3.4)	Netherlands	Keeseen et al., 2011	–	–	–	10 (2.1)	10 (3)
012	6 (85.7)	Netherlands	Koene et al., 2011	–	–	–	–	–	–	–	–	–	9 (2)	17 (4)
015	–	–	–	1 (12.5)	Belgium	Present study[1]	2 (3.4)	Netherlands	Keeseen et al., 2011	–	–	–	4 (0.87)	13 (3)
081	1 (7.1)	Belgium	Rodriguez et al., 2012	–	–	–	–	–	–	1 (16.7)	Belgium	Present study[1]	–	
087	1 (7.1)	Belgium	Rodriguez et al., 2012	–	–	–	–	–	–	–	–	–	–	
033	1 (14.3)	Netherlands	Koene et al., 2011	–	–	–	–	–	–	–	–	–	–	
013	–	–	–	–	–	–	5 (8.6)	Netherlands	Keeseen et al., 2011	–	–	–	–	
018	–	–	–	–	–	–	–	–	–	–	–	–	–	23 (6)
106	–	–	–	–	–	–	–	–	–	–	–	–	–	20 (5)
017	–	–	–	–	–	–	–	–	–	–	–	–	–	14 (4)
126	–	–	–	–	–	–	–	–	–	–	–	–	–	12 (3)

(1) PCR-ribotypes of isolates after 30 days of enrichment were only taken into account in the case of being different of the previously found after 3 days of enrichment.
(2) Unpublished data from the National *Reference Laboratory* for *Clostridium difficile* in Belgium.
(3) Data from the study of Bauer et al. (2011).

slaughtered before the animals from which the intestinal contents had been taken. Therefore no conclusion about the focus of the contamination can be stated.

The detection method that was used in this study was performed without ethanol-shock treatment. A previously pilot study in our laboratory demonstrated that the detection of the *C. difficile* colonies improved without pre-treatment of the samples (unpublished data). Furthermore, the medium used (CCFT) is an excellent selective and differential medium for *C. difficile*, as described previously (Delmée et al., 1987; George et al., 1979). This detection method has two enrichment phases. In our first study the enrichment step was prolonged to a maximum duration of 30 days in 15 pig intestinal samples that tested negative after 3 days of enrichment. A total of three new positive samples resulted (Rodriguez et al., 2012). As the sample size (n = 15) was small for drawing conclusions, in the present study the enrichment step of 30 days was applied to all samples. Additional positives were found in 20% of cattle intestinal samples (n = 2), 12.5% of cattle carcasses (n = 1) and 14.3% of pig carcasses (n = 1). It seems that the increase in the time of enrichment improves the sensitivity of the method. However 30 days of enrichment is a long technique for laboratory purposes for the slight increase of the sensitivity observed. Moreover, a bacterial competition or a low level of *C. difficile* in the enrichment broth could explain the differences between 3 and 30 days of enrichment, which can have a direct impact on the results (Weese et al., 2009). After 30 days of enrichment, rarely other colonies than *C. difficile* were found. The presence of other bacteria in the plate was more relevant after 3 days of enrichment. The finding of different PCR-ribotypes in some of the samples after 3 and 30 enrichment days reinforced this hypothesis.

Six PCR-ribotypes out of the total of 19 found could be assigned to international Brazier types. Intestinal contents and carcass samples from slaughter cattle showed the greatest variety of PCR-ribotypes. Intestinal contents also showed a considerable percentage of non-toxigenic PCR-ribotypes. Some of these strains, like PCR-ribotype UCL273 had not been isolated before in humans in Belgium. Moreover, several different PCR-ribotypes were obtained in some single intestinal samples from cattle. This finding is in accordance with a previous study describing the presence of more than one different type of isolate in rectal samples of calves (Zidaric et al., 2012).

Other studies in various countries have also identified *C. difficile* PCR-ribotypes from slaughter animals closely related to human PCR-ribotypes (Table 4). In 2011 in Belgium the most prevalent PCR-ribotypes in hospitals were 014, 002, 027, 078, 020, UCL46, UCL16l, UCL26, 001, 023, UCL23f, 012, UCL16b, 015, UCL5a, UCL20a and UCL49 sorted by decreasing values in number of isolates (unpublished data). A total of 7 of these 17 ribotypes were isolated in the present study.

In conclusion, this study shows that toxigenic *C. difficile* is present in the slaughterhouse in Belgium, among them PCR-ribotypes 078, 029 and 014. Carcasses were contaminated with a variety of PCR-ribotypes that were not found in the intestinal samples for the same animals, suggesting a slaughterhouse environmental contamination. This study further documented that animals are carriers of *C. difficile* at slaughter, and carcass contamination occurs inside the slaughterhouse.

Acknowledgements

The authors would like to thank Frédéric Brassinne for technical support and the public slaughterhouse of Liège-Waremme for permission to sample.

Our most sincere thanks to Eng. Alberto Rufi for the critical reading of the manuscript and Phyllis Smith and Ginna Vogt of the Institut Supérieur des Langues Vivantes of the University of Liège for the support on editing the manuscript.

This study was funded by the Federal Public Service of Health, Food Chain Safety and Environment (contract RF09/6226).

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