International Journal of Food Microbiology 144 (2011) 561–564

Contents lists available at ScienceDirect

International Journal of Food Microbiology

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

Short Communication

Prevalence of *Clostridium difficile* in retailed meat in The Netherlands

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ARTICLE INFO

Article history:
Received 26 July 2010
Received in revised form 1 November 2010
Accepted 4 November 2010

Keywords:
Clostridium difficile
Meat
Typing

ABSTRACT

Recent reports indicate that a large proportion of community-acquired *Clostridium difficile* infections (CA-CDI) are not linked to recent antibiotic therapy, older age, significant comorbidity or previous hospitalization. Possible community sources for CA-CDI include animals and food, and therefore a surveillance study on the prevalence of *C. difficile* in meat was performed. Samples of different meat species were collected from the retail trade and analyzed for the presence of *C. difficile* using a method that included selective enrichment in *C. difficile* broth, subsequent alcohol shock-treatment and plating onto *C. difficile* selective medium. *C. difficile* isolates were tested for the presence of toxin genes and were typed using PCR ribotyping. Of 500 samples tested, 8 (1.6%) were positive for the presence of *C. difficile*: 1 from lamb (6.3%) and 7 from chicken meat (2.7%). The isolated strains belonged to PCR ribotypes different from those that are currently most frequently found in patients with CDI in the Netherlands, except for *C. difficile* PCR ribotype 001 which was found in one chicken meat sample. This observation suggests that other matrices than meat may serve as a source for CA-CDI.

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1. Introduction

Clostridium difficile is an important cause of infectious diarrhoea that usually develops in patients after hospitalization and antibiotic treatment. The symptoms of *Clostridium difficile* infection (CDI) range from asymptomatic colonization to mild diarrhoea and severe life threatening pseudomembranous colitis (Kuijper and van Dissel, 2008).

CDI has also been described in non-hospitalized patients without underlying disease or predisposing risk factor (Chernak et al., 2005). Reports from Canada, USA and Europe indicate that a large proportion of these cases of community-acquired *Clostridium difficile* infection (CA-CDI) are not linked to recent antibiotic therapy, older age, significant comorbidity or previous hospitalization (Bauer et al., 2009; Dial et al., 2008; Wilcox et al., 2008).

C. difficile also appears to be an important cause of enteric disease in a variety of animal species, suggesting that animals and humans may share a common source (Rupnik et al., 2009). In accordance herewith, recent reports show a remarkable overlap between isolates from animals and humans (Goorhuis et al., 2008a).

Possible community sources for CDI include soil, water, animals, meats and vegetables (Weese et al., 2009). The mechanisms of transmission of *C. difficile* between animals and humans are not yet fully understood (Jhung et al., 2008). If animals are a potential source of *C. difficile*, food could be one of the transmission routes from animals to humans. Though there is currently no clear evidence that *C. difficile* contamination of food can cause CDI in humans, it is important to collect information on the possible exposure of humans by *C. difficile*-contaminated food.

2. Materials and methods

2.1. Samples

A total of 500 samples, including raw beef, pork, calf, lamb and chicken meat, were collected from the retail trade in the period October 2008 to March 2009. The samples were transported and stored at a temperature between 1 °C and 8 °C and analyzed within 48 hours from the time of sampling. Sampled meat was without addition of spices or other additives and included a wide variety of samples of fresh meat (small and larger pieces), minced meat and meat preparations according to the legal definitions.

2.2. Detection method

The detection and isolation method used was based on the method described by Rodriguez-Palacios et al. (2007). Briefly, 5 g of each sample was added to 20 ml of *C. difficile* broth (CDB). The composition of CDB (per litre of medium) was as follows: proteose peptone (40.0 g), disodium hydrogen phosphate (5.0 g), potassium dihydrogen phosphate (1.0 g), magnesium sulphate (0.1 g), sodium chloride (2.0 g), fructose (6.0 g), sodium taurocholate (1.0 g), laked horse blood (Oxoid SR0048C) (50 ml), *C. difficile* selective supplement (Oxoid SR0173E) (2 vials). Incubation of CDB took place for 10 to

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doi:10.1016/j.ijfoodmicro.2010.11.007

15 days at 37 °C, under anaerobic conditions. From the enriched culture 2 ml was added to 2 ml of 96% ethanol in a centrifuge tube and homogenized during 50 min on a shaker. After centrifugation (3800×*g* for 10 min), a loopful material from the sediment was streaked onto *Clostridium difficile* moxolactam norfloxacin (CDMN) agar (Mediaproducts bv, Groningen, The Netherlands). The plates were incubated for 24 to 48 h at 37 °C, under anaerobic conditions and up to 5 suspected colonies were subcultured on tryptone soya agar (Oxoid CM0131). Presumptive identification of suspect colonies was done by testing for agglutination using the *C. difficile* test kit (Oxoid DR1107A). Spiking experiments showed that the detection limit of this method for minced meat was in the range of 2 CFU per 5 g.

Further identification and toxigenic type characterization of *C. difficile* isolates was done by real-time PCR targeting the triose phosphate isomerase (*tpi*) housekeeping gene, the toxin A (*tcdA*) and toxin B (*tcdB*) genes. The primers and probes used are listed in Table 1. The isolates to be tested were grown on tryptone soya agar (overnight at 37 °C). One colony was suspended in 500 µl distilled water and after heating for 10 min at 95 °C, the suspension was centrifuged (5 min, 10,000×*g*) and 2.5 µl of the supernatant was used as template in the PCR assays. Amplification and detection were carried out in a LightCycler Instrument (version 2.0, Roche Diagnostics) in a final volume of 20 µl containing 2.5 µl of the sample DNA, 500 nM of each primer, 250 nM of the probe, and the LightCycler TaqMan Master mix kit (Roche Diagnostics). Samples were amplified with an initial denaturation step at 95 °C for 10 min to activate the FastStart *Taq* DNA polymerase and 40 cycles of denaturation at 95 °C for 10 s and annealing and extension at 60 °C for 15 s. The temperature transition rate was 20 °C per s. Samples positive for the target gene were identified by the instrument at the cycle number where the fluorescence attributable to the target sequences exceeded that measured for background. Additionally, the isolates were tested for the glutamate dehydrogenase gene and the genes *cdtA* and *cdtB*, encoding the enzymatic component and the binding domain, respectively, of the actin-specific ADP-ribosyltransferase (CDT) (binary toxin) as described previously (Paltansing et al., 2007). Further characterization by PCR ribotyping was performed as described by Bidet et al. (2000).

3. Results and discussion

The results of the prevalence testing are summarized in Table 2. *C. difficile* strains were isolated from a sample of lamb and from samples of chicken meat, but not from any of the samples of beef, pork and calf meat. Recently published studies report the isolation of *C. difficile* from raw beef in relatively high percentages, ranging from 2.4 to 42.4% of samples collected at retail (Rodriguez-Palacios et al., 2009; Songer et al., 2009; Von Abercron et al., 2009; Weese et al., 2009). *C. difficile* has also been found in samples of pork, ranging from 12 to 41.3% of retail samples (Songer et al., 2009; Weese et al., 2009). However, Von Abercron et al. (2009) did not detect *C. difficile* in meat samples other than beef. Similar as in France (Bouttier et al, 2010), an Austrian study reported a low prevalence of 3% for *C. difficile* in retail ground meat samples; positive samples could only be found among mixed beef and pork samples, but not among ground beef (Jöbstl et al., 2010). *C. difficile* spores were detected in 3 (7.5%) of 40 samples of ready-to-eat salads (Bakri et al., 2009) and were isolated from 29% of 100 chicken faeces collected at market places (Simango and Mwakurudza, 2008). A recently completed pilot study in Switzerland revealed only one positive faecal culture of 204 calves and 165 pigs (Hoffer et al. 2010).

Table 2
Prevalence of *Clostridium difficile* in raw meat.

Meat type	n	Number (%) positive
Beef	145	0
Pork	63	0
Calf	19	0
Lamb	16	1 (6.3)
Chicken	257	7 (2.7)
Total	500	8 (1.6)

The differences in isolation frequencies of *C. difficile* from meat reported by different countries will be mainly caused by the use of different detection methods. Further improvement of detection methods for *C. difficile* in foods is needed. Till now no associations were found with country, meat processors, storage or other factors. A possible seasonality has been observed, with a highest prevalence in winter (Rodriguez-Palacios et al., 2009). The source of *C. difficile* in meat is not clear. Carcasses may become contaminated with faecal material or from the environment during the slaughtering process. Contamination at retail level may also occur from the environment or through transmission by food handlers. The excellent survival of *C. difficile* spores in the environment increases the possibilities for contamination of animals and foods.

Quantification of *C. difficile* contamination in retail meat showed that generally low numbers, ranging from 60 to 240 spores/g, can be found (Weese et al., 2009). The meaning of this is not clear, as the infectious dose for *C. difficile* is not known.

The results of the identification and characterization of the isolates are shown in Table 3. For typing of *C. difficile* strains the preferred technique is PCR ribotyping, because of its discriminatory power, reproducibility and simplicity (Bidet et al., 2000). In recent years, a particularly virulent strain, PCR ribotype (RT) 027, has emerged in several countries, in relation with hospital outbreaks (Kuijper et al., 2007, 2008). Strains of RT 027 were not found in our study. Another recent emerging strain belonging to RT 078 has been found in animals and humans in the United States and in Canada. RT 078 strains have mainly been isolated from pigs and calves (Keel et al., 2007; Weese et al., 2009) although a recent study revealed the presence of RT 078 in 12.8% of chicken samples in Canada (Weese et al., 2010). RT 078 strains have also been found on pig farms in the Netherlands (Debast et al., 2009). The animal RT 078 isolates share a large genetic

Table 1
Primers and probes used for the identification and toxigenic type characterization of *Clostridium difficile* isolates by real-time PCR.

Target gene	Designation[a]	Sequence (5′→3′)	Position	GenBank accession no.
tpi	C.diff-TPI-F	TGAATGTCCTATTACAACATAGTCC	3707412–3707436	AM180355
	C.diff-TPI-R	ATAAAGATAGGTGCTCAAAATATGC	3707525–3707501	
	C.diff-TPI-P	AGAGGTGAAACTTCTCCTGTAAATGCTCCT	3707458–3707487	
tcdA	C.diff-TCDA-F	AAATAGCACCATACTTACAAGTAGG	797185–797209	AM180355
	C.diff-TCDA-R	GCATAAGCTCCTGGACCAC	797263–797245	
	C.diff-TCDA-P	ATGCCAGAAGCTCGCTCCACAATAAGTT	797214–797241	
tcdB	C.diff-TCDB-F	GCACCATCAATAACATATAGAGAGC	790960–790984	AM180355
	C.diff-TCDB-R	GTTTTGTGCCATCATTTTCTAAGC	791140–791117	
	C.diff-TCDB-P	TGTCCATCCTGTTTCCCAAGCAAATACTCT	791103–791074	

[a] F = Forward primer; R = Reverse primer; P = Probe, labelled at the 5′-end with the reporter dye 6-carboxyfluorescein (FAM) and at the 3′-end with the black-hole quencher (BHQ).

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