CHAPTER 3

Clostridium difficile: Its Potential as a Source of Foodborne Disease

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Contents

I. Introduction 54
II. Methods for Detection of *C. difficile* in Food: We Lack a Standard Approach 55
III. *C. difficile* in Meat and Meat Products: Isolation in Multiple Locations in the United States and Europe Establish this as a Widespread Phenomenon 57
IV. *C. difficile* in Other Foods: Possible Association with Environmental Strains or Organisms from Animal Feces 57
V. *C. difficile* in Companion Animal Feed: Animal Exposure may have Far-Reaching Effects on Human Disease 60
VI. Possible Sources of Food Contamination: *C. difficile* is Widespread in Animals, Humans, and the Environment 60
VII. Occurrence of Common Genotypes in Animals, Humans, and Foods: Crossover is Very Common 61
VIII. *C. difficile* in Food as a Source of Human Colonization: Contaminated Food may be a Source of the Organism in the Hospital and the Community 62
IX. Conclusions: We have Much to Learn 63
References 63

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Abstract

Clostridium difficile has been recognized as an important human pathogen for several decades, but its importance as an agent of animal disease was established only recently. The number of reports on *C. difficile* in food is rising, but the findings vary among studies. In North America, the prevalence of contamination in retail meat and meat products ranges from 4.6% to 50%. In European countries, the percentage of *C. difficile* positive samples is much lower (0–3%). This chapter summarizes current data on association of *C. difficile* with different foods and the difficulties associated with isolation of the organism, and discusses the potential of *C. difficile* as a food-transmitted pathogen.

I. INTRODUCTION

Clostridium difficile has been traditionally regarded as a nosocomial human pathogen (Rupnik *et al.*, 2009). Hospitals were the main reservoir for infection that occurred either in outbreaks or as isolated cases. Since 2003, the severity and mortality of *C. difficile* infection (CDI) have risen dramatically in North America and several European countries (Kuijper *et al.*, 2006; Loo *et al.*, 2005). The epidemiology has changed in parallel. Community-associated (CA)-CDI has, in the past, been considered a minor issue (in comparison to healthcare facility-associated (HA)-CDI), due to the mild nature of these infections. However, both incidence and severity of CA-CDI are changing, and new populations at risk are emerging (Chernak *et al.* 2005; Limbago *et al.*, 2009).

C. difficile has been isolated from many domestic and wild animals, including camels, horses, donkeys (Hafiz and Oakley, 1976), dogs and cats (O'Neill *et al.*, 1993; Riley *et al.*, 1991; Weber *et al.*, 1989), domestic fowl, seals, and snakes (Levett, 1986). It has been found rarely in septicemias and pyogenic infections in domestic animals (Hirsh *et al.*, 1979). There have been sporadic reports of disease in wild species, including cases in a Kodiak bear (Orchard *et al.*, 1983), a rabbit (Rehg and Shoung, 1981), a penguin (Hines and Dickerson, 1993), and captive ostriches (Frazier *et al.*, 1993). The organism is an important cause of diarrhea and fatal necrotizing enterocolitis in foals (Jones *et al.*, 1988a,b; Traub-Dargatz and Jones, 1993) and nosocomial diarrhea in adult horses (Baverud, 2002).

Isolation of *C. difficile* from substantial numbers of normal or diseased food animals was reported relatively recently. Species infected most commonly include piglets (Songer and Anderson, 2006), calves (Hammitt *et al.*, 2008; Rodriguez-Palacios *et al.*, 2006), and poultry (Shimango and Mwakurudza, 2008; Zidaric *et al.*, 2008).

This observation has logically led to studies on *C. difficile* in meat and meat products. First reports on possible foodborne transmission of the organism date to almost thirty years ago (Borriello *et al.*, 1983; Gurian *et al.*, 1982), but the first of the very few published studies of *C. difficile* in meats came from Canada (Rodriguez-Palacios *et al.*, 2006). However, interest in *C. difficile* in animal-derived and other foods is growing. Our aim here is to give an overview of current knowledge of the association of *C. difficile* with foods and to comment on isolation methods and interpretation of the public health relevance of the results.

II. METHODS FOR DETECTION OF *C. DIFFICILE* IN FOOD: WE LACK A STANDARD APPROACH

Sample preparation may be by maceration (e.g., in a Stomacher (Seward, Bohemia, NY, USA) or similar apparatus), grinding in a blender, or equivalent.

Direct bacteriologic culture for *C. difficile* has been accomplished by plating on commercial *C. difficile* plates with cefoxitin and cycloserine (CCFA; Indra *et al.*, 2009) or moxalactam and norfloxacin (CDMN; Weese *et al.*, 2009) and usually supplemented with 5% blood (horse or sheep).

Enrichment broths have consisted of brain heart infusion (BHI) with cysteine and yeast extracts (Songer *et al.*, 2009) and Oxoid *C. difficile* medium without agar (Rodriguez-Palacios *et al.*, 2009; Weese *et al.*, 2010; Table 3.1). These enrichment media may be supplemented with cefoxitin (~16 μg/ml) and cycloserine (~500 μg/ml) or moxalactam (32 μg/ml) and norfloxacin (12 μg/ml). Alcohol shock and subculture on commercial solid media described above follow incubation under anaerobic conditions for 2–12 days.

Duplicate cultures (e.g., heat-shocked (80 °C, 10 min) and non-heat-shocked) can improve recovery rates (Rodriguez-Palacios *et al.*, 2009; Songer *et al.*, 2009). In work to date, specimens were culture positive in enrichments subjected to one treatment or the other, but no specimen was positive with both. The same method used on two duplicate samples had culture sensitivities of 39% and 23%, respectively (Rodriguez-Palacios *et al.*, 2009).

Comparison of recovery rates from 214 samples examined by three methods (differing in meat sample preparation, selective supplement in enrichment broth, and agar media for subculturing of enrichments), one of them in duplicate to document reproducibility, revealed *C. difficile* recovery rates from 1.4% to 2.3%. However, there was no correlation of culture results among methods, and results varied from one repetition to the next (Rodriguez-Palacios *et al.*, 2009). This is clearly a potential growth

TABLE 3.1 Comparison of isolation methods

Medium	Selective supplement in enrichment	Meat sample/medium	Incubation	Reference
Ingredients of Oxoid selective agar (except agar), 0.1% sodium taurocholate	CDMN supplement (Oxoid)-moxalactam, norfoxacin	4–5 g meat 20 ml prereduced CDMN broth	2–15 days EtOH shock on 2 ml enrichment broth Sediment on selective agar	Rodriguez-Palacios *et al.* (2007), Weese *et al.* (2010)
Ingredients of Oxoid selective agar (except agar), 0.1% sodium taurocholate	Cefoxitine, cycloserine	2 g meat	7 days EtOH shock on 2 ml enrichment broth Sediment on selective agar	Rodriguez-Palacios *et al.* (2009)
BHI with 0.5% yeast extract, 0.05% cysteine, 0.1% taurocholate	None reported (selection as heat shock at 80 °C for 10 min)	1 g meat in 10 ml medium	1–3 days	Songer *et al.* (2009)
BHI	CDMN supplement (Oxoid)-moxalactam, norfoxacin	25 g in 50 ml medium	10–12 days	von Abercron *et al.* (2009)
Thioglycolate broth	None reported	5 g in 20 ml medium	12 days EtOH shock Inoculation on *C. difficile* selective agar (bioMerieux)	Indra *et al.* (2009)

area in study of *C. difficile* biology. Improved selective isolation methods, as well as media for enrichment, direct culture, and subculture, would facilitate study of the organism in CDI, the carrier state, the environment, and in foods.

III. *C. DIFFICILE* IN MEAT AND MEAT PRODUCTS: ISOLATION IN MULTIPLE LOCATIONS IN THE UNITED STATES AND EUROPE ESTABLISH THIS AS A WIDESPREAD PHENOMENON

C. difficile has been reported in meat and meat products in Canada, the United States, France, Austria, and Sweden (Table 3.2). The first publication from Canada reported a high percentage of culture positive samples (Rodriguez-Palacios *et al.*, 2007), although the same group reported only 6% of samples to be culture positive in a subsequent study in 2008 (Rodriguez-Palacios *et al.*, 2009). Yet, other Canadian studies reported that 12% of samples of beef and pork ground meat were culture positive (Weese *et al.*, 2010) and the prevalence in chicken meat was 12.8% (Weese *et al.*, 2010). The prevalence of *C. difficile* in meat and meat products was much higher (>40%) in a U.S. study geographically limited to Arizona (Songer *et al.*, 2009). There are few data for *C. difficile* presence in retail meats in EU countries, but the percentage of positive samples has generally been <5% (Table 3.2) (Bouttier *et al.*, 2007; Indra *et al.*, 2009; Jöbstl *et al.*, 2010; von Abercron *et al.*, 2009).

Ground beef is the meat specimen most likely to contain *C. difficile*, followed by other types of ground meat (pork, turkey, chicken). Seasonality in occurrence of *C. difficile* in meat has been observed, with 11.5% of samples culture positive in January and February, but only 4% positive from March to August (Rodriguez-Palacios *et al.*, 2009).

IV. *C. DIFFICILE* IN OTHER FOODS: POSSIBLE ASSOCIATION WITH ENVIRONMENTAL STRAINS OR ORGANISMS FROM ANIMAL FECES

Al Saif and Brazier (1996) reported *C. difficile* in different nonhospital, nonhuman sources, such as soil and water. Three hundred raw vegetable samples (on sale in retail premises) were examined by impression of unwashed surfaces onto plates of selective medium. Seven culture positive raw vegetable samples included cucumber ($n = 1$), onion ($n = 1$), potato ($n = 2$), mushroom ($n = 1$), carrot ($n = 1$), and radish ($n = 1$). Tomato, cabbage, and lettuce did not contain *C. difficile* on their surfaces. A very recent study of ready-to-eat salads, in Scotland (none of which

TABLE 3.2 Detection of *C. difficile* in meat and meat products

Country	Sampling time interval	Type of meat/meat product	Number of tested samples/number of positive samples	Genotype (PCR ribotypes or PFGE type or toxinotype)	Reference
Canada	Jan to Oct 2005	Retail ground meat samples	12 of 60 (20%)	Ribotype/toxinotype (M26/tox-, 077/0, M31/III, 014/0)	Rodriguez-Palacios *et al.* (2006)
Canada	Jan to Aug 2008	Ground beef Veal chop	10/149 (6.7%) 3/65 (4.6%)	Ribotypes/toxinotype (M26/tox-, 077/0, J/III, 014/0, C/nd, F/VIII, H/0, K/III)	Rodriguez-Palacios *et al.* (2009)
Canada	Aug 2008 to Nov 2008	 Ground beef Ground pork	All 28/230 (12%) 14/115 (12%) 14/115 (12%)	Ribotype 078, 027	Weese *et al.* (2010)
Canada	Nov 2008 to Jun 2009	Chicken meat	26/203 (12.8%)	Ribotype 078	Weese *et al.* (2010)
United States (Tucson, Arizona)	Jan to Apr 2007	Ground beef Ground pork Ground turkey Meat products[a]	13/26 (50%) 3/7 (42.9%) 4/9 (44.4%) 17/46 (36.9%)	PFGE types/toxinotypes (NAP1/III, NAP1-related/III, NAP7/V, NAP8/V)	Songer *et al.* (2009)
Austria	Feb to Apr 2008	Beef, pork, chicken	0/84		Indra *et al.* (2009)

Austria	Jul 2007 to Feb 2008	Ground meat Beef ($n = 30$) Beef and pork ($n = 70$)	 Beef only 0% Beef and pork 3%	Ribotypes AI-57, 053	Jöbstl *et al.* (2010)
France	(2006 no precise data given)	Vacuum packed ground beef pork sausage	2 of 60 (3%) 0/50	Toxinotype 0	Bouttier *et al.* (2007)
Sweden	Apr to Sep 2008	Ground beef	2.4%	Not done	von Abercron *et al.* (2009)

[a] Meat products included summer sausage (ready to eat (RTE)), braunschweiger (RTE), chorizo (uncooked), and pork sausage (uncooked).

originated in the United Kingdom), revealed that 3 of 40 (7.5%) were culture positive (Bakri *et al.*, 2009). Salad mixes (~10%) and bagged spinach (~7%) in the United States have also been culture positive (M. Lloyd and J.G. Songer, unpublished). Analysis of raw milk samples did not reveal contamination with *C. difficile* (Jöbstl *et al.*, 2010).

V. *C. DIFFICILE* IN COMPANION ANIMAL FEED: ANIMAL EXPOSURE MAY HAVE FAR-REACHING EFFECTS ON HUMAN DISEASE

C. difficile was isolated from two samples of vacuum-packed meat intended for dogs (Broda *et al.*, 1996). An additional report on *C. difficile* in raw turkey dog food was published more recently (Weese *et al.*, 2005). No *C. difficile* was found in 10 samples of feline raw diet (Bouttier *et al.*, 2007).

VI. POSSIBLE SOURCES OF FOOD CONTAMINATION: *C. DIFFICILE* IS WIDESPREAD IN ANIMALS, HUMANS, AND THE ENVIRONMENT

Sources of *C. difficile* food contamination might include transfer of spores from the gut to the musculature of healthy or diseased animals, contamination at slaughter, contamination at processing and packaging, or contamination in the local retail market. In fact, each of these sources may contribute to a greater or lesser extent to the final contamination level in meat and meat products.

Muscle tissue from healthy horses (Vengust *et al.*, 2003) and cows (unpublished data reported in Rodriguez-Palacios *et al.*, 2009) can contain low numbers of clostridial spores. The high rate of contamination of pork braunschweiger (Songer *et al.*, 2009) suggests that *C. difficile* spores may localize in Kupffer cells in liver antemortem. Clostridia are members of the gut microbiome in animals and comprise one of the main groups participating in natural carcass degradation. Colonization of muscle tissue in the form of dormant spores could provide a selective advantage, both by augmenting the success of transmission to new hosts (via consumption of contaminated meat) and by precolonization of tissues to facilitate their eventual degradation.

Carcass contamination by gut contents at slaughter probably contributes most to the presence of *C. difficile* in meat and meat products. Slaughter techniques differ from country-to-country, with those in the United States being more of the 'quick and dirty' variety than in the EU. This coincides with the high percentage of positive meat samples (Songer *et al.*, 2009).

However, data on *C. difficile* in animals prior to slaughter are scarce. An Austrian study that examined cows, pigs, and broilers in the time interval from March to July, 2008, found *C. difficile* in feces or gut contents of cows (4.5%), pigs (3.3%), and broilers (5%) (Indra *et al.*, 2009).

Feedlot calves are commonly infected with *C. difficile* as neonates, and even normal cattle can be culture positive through the end of the feeding period. Hammitt *et al.* (2008) investigated the possible role of *C. difficile* in calf enteritis. *C. difficile* and toxins were found in 25.3% and 22.9%, respectively, of stool samples from diarrheic calves. These findings agree with those of others (Porter *et al.*, 2002; Rodriguez-Palacios *et al.*, 2006) and strengthen the hypothesis that CDI can manifest as calfhood colitis and diarrhea and that calves are, at the very least, multiplying hosts for this organism.

Shimango and Mwakurudza (2008) collected feces from live broilers sold in food markets in Zimbabwe and examined them by bacteriologic culture without enrichment. *C. difficile* was isolated from samples (and was, hence, present in birds) in five of six markets. Interestingly, 40% of chickens reared at homesteads in Zimbabwe were culture positive and 20% of samples from chickens reared on commercial farms were positive.

There is, to date, no published study on presence of *C. difficile* in slaughter house or packing plant environments, or on equipment or the hands of the workers.

VII. OCCURRENCE OF COMMON GENOTYPES IN ANIMALS, HUMANS, AND FOODS: CROSSOVER IS VERY COMMON

C. difficile genotypes are currently determined mostly by PCR ribotyping or pulsed-field gel electrophoresis (PFGE) (Rupnik *et al.*, 2009). Most strains isolated from food sources have a genotype identical to those of human and animal isolates from the same geographic area or worldwide. Two Canadian studies (Rodriguez-Palacios *et al.*, 2006, 2009) reported substantial heterogeneity in strains recovered from meats. In the more recent study, 28 isolated strains belonged to 8 genotypes. Of these, seven were toxinogenic and identical to well-known human and previous animal isolates by PFGE and ribotyping (North American pulsed-field type (NAP) 1/ribotype 027, NAP2/ribotype 077, and NAP4/ribotype 014). These were reported in CA-CDI in the United States (Limbago *et al.*, 2009) and were among the predominant types in the 2005 EU prevalence study (Barbut *et al.*, 2007). Type 014 is the most prevalent type in the current EU surveillance study (Bauer *et al.*, 2010). Ribotypes 077 and 014 were isolated from calves in Canada (Avbersek *et al.*, 2009; Rodriguez-Palacios *et al.*, 2006) and type 077 from dogs in the United States (Keel *et al.*, 2007).

The American study reported high percentages of culture positives among retail meats, but genotypes were more homogenous (Songer *et al.*, 2009). Thirty-seven strains belonged to two ribotypes only (027 and 078), although both were further divided by PFGE typing (027/NAP1; 027/NAP1-like; 078/NAP7; 078/NAP8). All of these, with the exception of the NAP1-like strains, are frequently found in animals, especially pigs and cows (Keel *et al.*, 2007; Rodriguez-Palacios *et al.*, 2006). Comparison of human and animal strains via PFGE and MLVA indicates that strains from both sources are indistinguishable (Debast *et al.*, 2009; Jhung *et al.*, 2008).

Genotypes found in salad mixes (ribotypes 017 and 001) (Bakri *et al.*, 2009) are very common among human isolates in the United Kingdom (Brazier *et al.*, 2008) and elsewhere (Barbut *et al.*, 2007). They are rare, but nonetheless present, in animals and were detected in calves (017; Rodriguez-Palacios *et al.*, 2006) and horses (001; Keel *et al.*, 2007).

VIII. *C. DIFFICILE* IN FOOD AS A SOURCE OF HUMAN COLONIZATION: CONTAMINATED FOOD MAY BE A SOURCE OF THE ORGANISM IN THE HOSPITAL AND THE COMMUNITY

The human infectious dose for *C. difficile* is not known. However, given the biology of this organism, the dose of *C. difficile* ingested may have little to do with the likelihood of developing CDI. Ingested spores emerge as vegetative cells upon exposure to germinants in the intestine and multiply to a greater or lesser extent, depending upon conditions. Furthermore, evidence strongly suggests that these altered conditions are necessary for development of disease. Thus, a large dose of spores, ingested under normal intestinal conditions, might have no effect. A small dose, ingested under altered conditions, might be the primary inciting factor in a case of CDI. Thus, the greatest risk imposed by *C. difficile* contamination of foods is that people may be exposed continuously to larger or smaller numbers of the organism. This would have the effect of maintaining colonization for times when intestinal conditions are amenable to establishment of the organism and production of clinical signs and lesions.

The numbers of *C. difficile* spores in food are low, and have been detected mainly by enrichment and subculture. A recent study (Weese *et al.*, 2009) enumerated *C. difficile* in retail ground beef and ground pork in four Canadian provinces. The organism was isolated by direct culture from 28% of ground beef samples (20 spores/g in two samples and 120 and 240 spores/g in one sample each). Twenty-eight percent of ground pork samples were also positive by direct culture (20 spores/g in three samples and 60 spores/g in one). Ribotype 078 was predominant, but

ribotype 027/NAP1 strains were also identified in both beef and pork (Weese *et al.*, 2009). Additional work with traditional or molecular methods may allow increased sensitivity and specificity, and better estimation of contamination levels.

Most meats are cooked before eating, but the recommended internal temperature is 70 °C. Results of work by Rodriguez-Palacios *et al.* (2007), albeit in buffer rather than in meat, suggest that *C. difficile* strains from meat would survive cooking. This is to say nothing of ready-to-eat meat products, which are also contaminated with *C. difficile* (Songer *et al.*, 2009), and salad products (Bakri *et al.*, 2009), most of which are consumed raw.

IX. CONCLUSIONS: WE HAVE MUCH TO LEARN

Observation of food contamination with *C. difficile* and consequent discussion of the contribution of food transmission to the increase of *C. difficile* infection rates is only recent. The percentages of contaminated food samples differ greatly among studies between and within countries and with different sampling intervals. However, detection methods still need optimization and standardization. The number of spores is low but the infectious dose and the role of low dose exposure in establishing infection are unknown. The large overlap of genotypes from food, food animals, and humans suggests a contribution of food to the infection process.

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