Clostridium difficile food transmission and its possible role in AMR

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Abstract

Clostridium difficile is a major cause of illness in healthcare environments and immunocompromised patients with thousands of severe cases per year in the EU. *C. difficile* has been shown to occur in foods, particularly meats, but also in vegetables and shellfish. Furthermore, it has been demonstrated that *C. difficile* produce heat resistance spores so the possibility for spores to survive low temperature food processing practises is clear. It is therefore imperative that this possible route of infection is investigated, and this proposal will investigate the hypothesis that food transmission of *C. difficile* may be responsible for non-healthcare associated cases. The epidemiology of *C. difficile* is changing, with the emergence of highly virulent types accompanying more severe infections, higher rates of recurrences and higher mortalities. One of the main reasons for these changes to epidemiology is antibiotic resistance. Several important mechanisms for *C. difficile* antibiotic resistance have been described, including the acquisition of antibiotic resistance genes via the transfer of mobile genetic elements. Resistance of *C. difficile* to multiple antibiotics, such as aminoglycosides, lincomycin, tetracyclines, erythromycin, clindamycin, penicillin's, cephalosporins, and fluoroquinolones has already been demonstrated. Animal transmission (including food products) should be considered as a major part of antimicrobial resistance (AMR) control. There are programs in place to monitor animal transmission for other bacteria-antimicrobial combinations, but *C. difficile* transmission through the food chain has not yet been evaluated. In order to establish a possible foodborne route and its impact on AMR, appropriate mathematical models are essential. They can then be implemented in a quantitative microbial risk assessment approach (QMRA). Only if the adverse effect on the population of the foodborne route is correctly characterized, measures to reduce its incidence will be considered by regulatory bodies and industry.

Mini-Review

Introduction

Clostridium difficile is a spore-forming Gram-positive bacterium and is the most common cause of antibiotic-associated diarrhoea that can cause pseudomembranous colitis, toxic megacolon and fatal colitis (1). *C. difficile* was reclassified as *Peptoclostridium difficile* (2), but more recently has again been reclassified as *Clostridioides difficile* (3). *C. difficile* produces the two potent exotoxins, TcdA and TcdB and are both members of the large clostridial toxins (LCT) family. These exotoxins glycosylate Rho family GTPases in host cells, resulting in the disruption of the actin cytoskeleton, cell death and a strong inflammatory response (4).

Despite health practice guidelines for the prevention, diagnosis, and treatment of *C. difficile* infection (CDI), the rate of CDI continues to rise. In 2016 a report by the European Centre for Disease Prevention and Control (ECDC) revealed that 20 countries reported 7711 CDI cases, 5756 of which (74.6%) were healthcare-associated CDI; 1955 CDI cases (25.4%) were either community-associated (CA) or of unknown origin. The CDI cases reported in 2016 contributed to significant morbidity and case fatality. In the United States The Centre for Disease Control and Prevention (CDC) reports that CDI is associated with over 14,000 deaths each year and resulting in more than $1 billion in excess medical costs (1).

Until relatively recently, it was believed that *C. difficile* was principally a clinically associated infection. However, due to increased surveillance and the more frequent use of whole genome sequencing (WGS), the percentage of CDI acquired from clinical settings is significantly lower (35%) of the total cases reported and therefore the rest are considered community acquired (5). The actual source of *C. difficile* implicated in community acquired CDI is undefined although a foodborne link has been proposed (5-8). In this mini-review we will discuss the current evidence with regards to *C. difficile* in foods and if this may be a possible route of infection. We will also discuss how antimicrobial resistance is having a major impact and how mathematical modelling and a quantitative risk assessment approach could be used to help control this important pathogen. The review is a 'snapshot' of previous *C. difficile* in foods reviews (5, 6, 8, 9)

Clostridioides difficile in food and the environment

Sequencing of strains of *C. difficile* have shown that in a number of regions, animals and humans are colonised with undistinguishable *C. difficile* clones (8). Furthermore, given that other clostridia such as *Clostridium perfringens* and *Clostridium botulinum* are foodborne pathogens, this may indicate that *C. difficile* may follow similar routes.

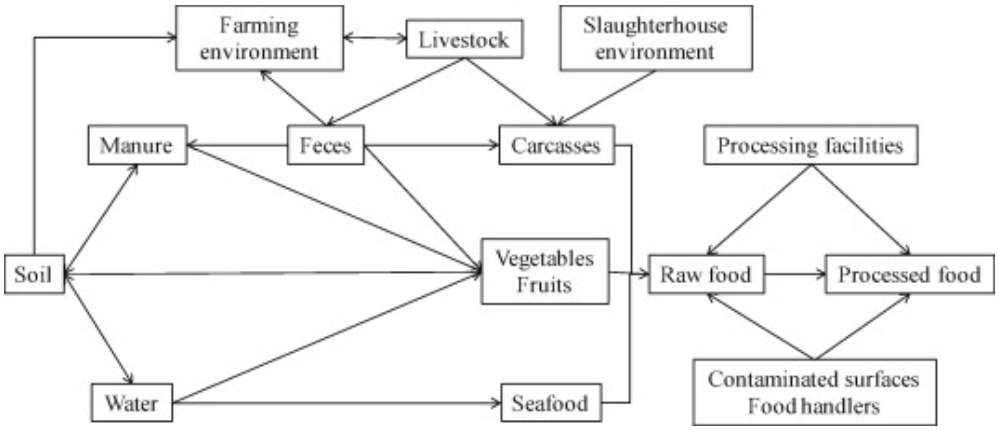
C. difficile has been isolated from the intestinal tract of many types of food animals, including cattle, pigs, sheep, and poultry, as well as domesticated animals, including dogs and cats (10). Although there is currently no definitive cost estimate on treating infection in production animals, it is considered to be high as *C. difficile* can cause mortality in breeding, weight loss, and delayed weight gain in animals. *C. difficile* has been commonly described in both healthy pigs and pigs with diarrhoea and is the most common cause of diarrhoea (8). As in the case of

pigs, the prevalence of *C. difficile* in cattle can vary considerably from one study to another depending on the geographical location studied. Furthermore, the pathogenicity in cattle is not understood. However, similar to swine *C. difficile* infection has been associated with diarrhoea. Finally, other production animals such as chickens, lambs, sheep and goats have been also described as carriers of the bacterium, with a prevalence varying between 0.6 and 10.1 % (8).

The high prevalence of *C. difficile* in animals means this then translates into carriage of the pathogen on meat through cross-contamination events during the slaughter process or manures. Traditional organic fertilizers for crops, can also contain spores of the bacterium. This may result in *C. difficile* transfer to the food chain by contamination of vegetables and fruits directly from manures or as a result of irrigation or washing with contaminated water (6). Indeed there are numerous ways *C. difficile* contamination may occur (figure 1)

The contamination by *C. difficile* spores has been discovered in a variety of food products, including seafood, vegetables and meats (table 1). There is a large discrepancy in reported prevalence however. In North America relatively high prevalence rates (42% of beef, 41% of pork and 44% of turkey samples) have been reported in uncooked meat products. Whereas lower prevalence rates, of up to 4.3% and 2.7% in ground beef/pork and chicken meat, respectively, have been reported in Europe. In Canada, toxigenic *C. difficile* was isolated from 28 of 230 (12%) of samples of retail ground beef and ground pork. These differences in reported prevalence may be due in part to the use of different methodology (5).

Fig. 1. Flow diagram of the main routes of *C. difficile* spore contamination of foods (6)



Given the prevalence of *C. difficile* in food animals, the environment and isolation from a variety food types, it is probable that this may be the source of community acquired CDI. However, its survival during food processing practices is presently un-clear. Lund and Peck (table 2) compared the thermal resistance of *C. difficile* spores with those of *C. perfringens* spores and stated that the spores of both organisms could survive the typical thermal processing temperatures used in cooking of meat and poultry products, potentially resulting in foodborne illness.

Table 1. Presence of *C. difficile* in meats (in processing plants or the retail trade) and other foods (at farms or markets). Adapted from Rodriguez 2016 (8).

Country	Year	Sample type	Prevalence %	T % toxigenic strains	Main PCR-ribotypes (%)
Belgium	2012	Ground and burger beef	3/133 (2.3)	T (100)	078 (33.3)/014 (66.7)
		Ground and sausage pork	5/107 (4.7)	T (80)	078 (20)/014 (40)
Netherlands	2008-2009	Beef meat	0/145 (0	-	-
		Pork meat	0/63 (0)	-	-
		Calf meat	0/19 (0)	-	-
		Lamb meat	1/16 (6.3)	T (100)	045 (100)
		Chicken meat	7/257 (2.7)	T (2.7)	001/003/087/071
Switzerland	2010	Minced meat products	0/46 (0)	-	-
France	20017-2008	Ground beef	2/105	T (100)	012 (100)
		Pork sausage	0/59	-	-
Austria	2007-2008	Ground meat	3/100	T (66.7)	053 (33.3)
	2008	Beef meat	0/51 (0)	-	-
		Pork meat	0/27 (0)	-	-
		Chicken meat	0/6 (0)	-	-
Sweden	2008	Ground meat	2/82 (2.4)	T (100)	-

Table 2. Heat Resistance of Spores of *C. difficile* and *C. perfringens*. Adapted from Lund & Peck (10).

Heating medium	Strains tested	D value at specified temperature
C. difficile		
Phosphate buffer	108 strains	D100°C=2.5–33 min
Distilled water	4 strains	D100°C=∼4–6 min
Phosphate-buffered saline	20 strains	D71°C=∼30 min
Phosphate –buffered saline	22 strains	D85°C=6.0–8.5 min
Gravy, 0% fat; lean ground beef, 3% fat; ground beef 30% fat.	4 strains	D96°C=0.59–1.19 min
Gravy, 0% fat; lean ground beef, 3% fat; ground beef 30% fat.	4 strains	D85°C=2.5–3.3 min
Gravy, 0% fat; lean ground beef, 3% fat; ground beef 30% fat.	4 strains	D71°C=47–71 min
C. perfringens		
Culture medium	5 strains; chromosomal cpe gene	D100°C=30–124 min
Culture medium	7 strains; plasmid cpe gene	D100°C=0.5–1.9 min
Culture medium	14 strains; chromosomal cpe gene	D100°C=30–170 min
Phosphate buffer	10 strains; chromosomal (plus one plasmid) cpe gene	D95°C=>7.5 min
Phosphate buffer	5 strains; plasmid cpe gene	D95°C=<7.5 min

D value, time at specified temperature for 10-fold reduction in viable numbers.

Characterization of heat resistance of bacteria and bacterial spores requires a precise methodology to efficiently describe its inactivation process. Commercial heat processes have complex profiles, therefore equipment that can simulate dynamic heating conditions can more accurately describe the inactivation kinetics of bacteria and spores (11). Such equipment has been extensively used to establish heat resistance of bacteria and food components (12). The first models used in predictive microbiology assumed that the bacterial population reduction followed a log-linear relationship with time during an isothermal treatment (13). Mathematical models to describe dynamic inactivation profiles have been developed by extending the ones used for isothermal treatments (14, 15). Increase in heat resistance has been related to adaptation/acclimation and can justify survival of pathogenic bacteria. Recently, a mathematical model to describe dynamic thermal inactivation of microorganisms, taking into account the acclimation to thermal stress, has been developed (16). Although there are data describing heat inactivation of *C. difficile*, they are based on classical D and z values, so presence of non-linear kinetics or development of acclimation have not been evaluated up to date.

Furthermore, for *C. perfringens*, there is information on the temperatures permitting germination and growth and on rates of growth, enabling evidence-based determination of the rate of growth during cooling of cooked foods; this in turn allows the determination of temperature controls and a cooling regimen to prevent growth of this bacterium Moreover, more Information is required on conditions in which surviving spores of *C. difficile* would germinate and vegetative bacteria would multiply in cooked meat dishes, or whether the spores would persist, and whether the spores or vegetative bacteria would result in asymptomatic or symptomatic infection after consumption of the meat (10). This information requires advanced modelling to integrate data in a risk assessment of foodborne CDI.

Clostridioides difficile Anti-microbial resistance

Antimicrobial resistance (AMR) is responsible for an estimated 25,000 deaths per year in the EU and costs the EU 1.5 billion euros per year in healthcare costs and productivity losses .In June 2017 the Commission adopted the EU One Health Action Plan against AMR. Antibiotic use is considered to be the most significant risk factor for CDI. Furthermore, *C. difficile* is a spore-forming organism and can survive antimicrobial therapy and therefore following germination, relapse of CDI can occur after the cessation of therapy. Antibiotics for instance metronidazole, vancomycin and fidaxomicin are therapies of choice for *C. difficile* infection. Numerous mechanisms for *C. difficile* antibiotic resistance have been described, including the acquisition of antibiotic resistance genes via the transfer of mobile genetic elements, selective pressure in vivo resulting in gene mutations, altered expression of redox-active proteins, iron metabolism, and DNA repair, as well as via biofilm formation (9). The emergence and spread of *C. difficile* isolates resistant to multiple antibiotics, especially among the hypervirulent *C. difficile* ribotype 027 strains, is an increasing problem for the treatment of CDI.

Diverse antibiotic resistances have been noted from isolates originating from animal/food production (Table 3). For instance, clindamycin resistance has been observed in cattle and linezolid resistance has been observed in pigs (7). Perhaps the most concerning and of relevance is the emergence of metronidazole resistance in *C. difficile* as it has been found occurring in both animal and human *C. difficile* isolates (17). In American lettuce *C. difficile*

isolates were identified as having antibiotic resistance to metronidazole, vancomycin, and erythromycin. In Canada two *C. difficile* strains from ginger had different antimicrobial reactions to levofloxacin and clindamycin (18). Furthermore, Isolates from ready-to-eat salads were susceptible to vancomycin and metronidazole but variably resistant to other antimicrobial drugs (19). Regardless of species, multiple drug resistance is most often observed to combinations of clindamycin and levofloxacin and ampicillin, clindamycin and levofloxacin.

CDI remains a significant and increasing healthcare-associated infections, with antibiotic exposure being a well-established risk factor for CDI and recurrence. Moreover, the role of CDI in the community should be further evaluated.

Table 3. Antimicrobial resistance profile of 376 multiple resistant *C. difficile* isolates. A total of 376 *C. difficile* strains (94 each from swine and dairy faeces, and 188 from beef cattle faeces) were isolated from healthy food animals on farms during studies conducted by the National Animal Health Monitoring System. Using the Etest (AB Biodisk, Solna, Sweden), samples were tested for susceptibility to nine antimicrobials implicated as risk factors for *C. difficile* associated disease (9).

	Number of isolates by species			
Resistance groups[1]	Swine feces	Dairy feces	Beef cattle	Total
AM, CM, EM, LE	6	0	2	8
AM, CM, RI, LE	0	0	2	2
CM, MZ, RI, LE	0	0	1	1
AM, CM, XL, LE	0	0	1	1
CM, EM, LZ, LE	1	0	0	1
AM, CM, LE	4	13	24	41
CM, RI, LE	1	0	18	19
CM, EM, LE	11	2	3	16
CM, LZ, LE	4	2	1	7
AM, EM, LE	1	1	0	2
AM, LZ, LE	1	0	0	1
CM, LE	26	58	111	195
RI, LE	1	0	4	5
AM, LE	2	2	0	4
EM, LE	3	0	1	4
LZ, LE	2	0	1	3
CM, RI	0	0	2	2
LE	31	16	12	59
CM	0	0	5	5
Total	94	94	188	376

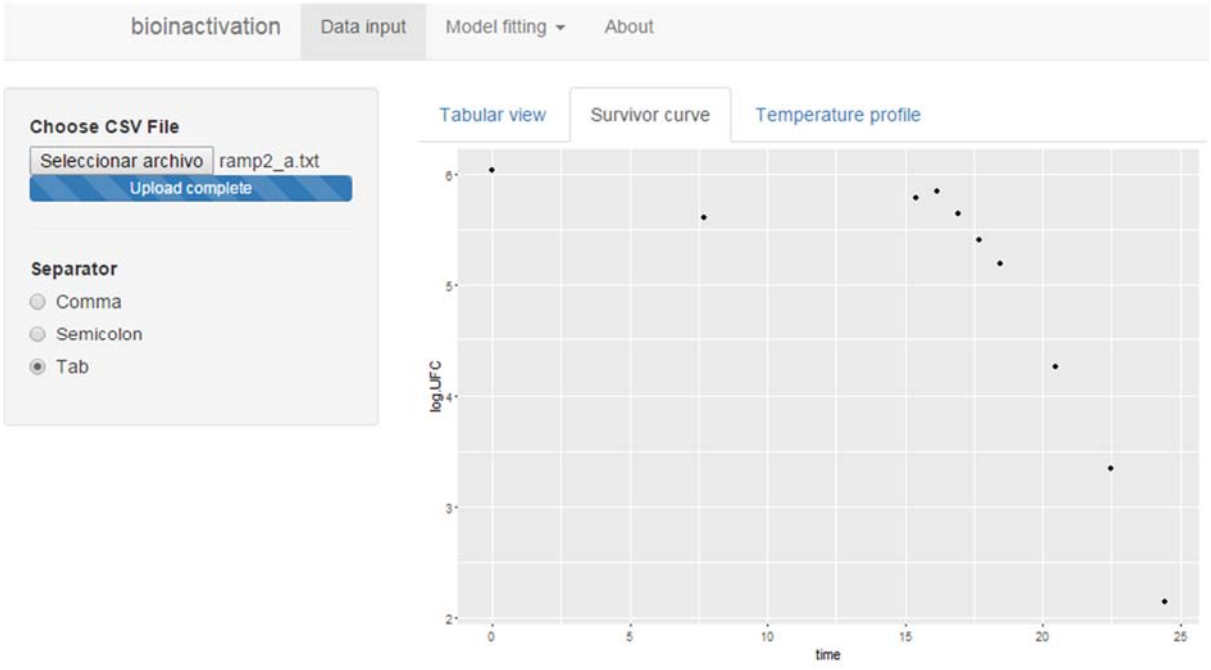
LZ = Linezolid, XL = Amoxicillin-clavulanic acid, AM = Ampicillin, CM = Clindamycin, EM = Erythromycin, MZ = Metronidazole, LE = Levofloxacin, RI = Rifampicin, VA = Vancomycin.

Application of predictive modelling and integration in a foodborne CDI risk assessment.

Mathematical modelling is a key tool in predictive microbiology to describe and quantify processes, establish relationships and optimize conditions that can be integrated in microbial risk assessment (20). Modelling is in iterative process that includes different steps, where model structure and parameter estimates are outstanding aspects. Parameter estimation requires experimental data, that should provide as much information as possible of the process evaluated. In this regard, optimal experimental design (21) is a big step forward in the modelling scheme. Sometimes models include parameters with small significant influence on the effects analysed or presenting high correlation to other model parameters. Sensitivity and identifiability analysis allow to detect these problems, designing informative models that avoid over-parametrisation (22). The complexity of these tasks increases significantly in the case of non-linear dynamic models, as those required for germination or heat inactivation studies.

In order to establish a foodborne CDI risk assessment, adequate models should be used to describe all the steps involved in the onset of disease (23). Open access tools, specially software with a hands-on format, that can run "R" code in the background, enable the use by scientists without programming background. Bio-inactivation (figure 2) is an example to estimate inactivation parameters using different models available (24).

Fig 2. Overview of Bioinactivation Shiny R-Studio (from Garre et al., 2017).



Microbial risk assessment consists of four steps, according to CODEX: hazard identification, exposure assessment, hazard characterization and risk characterization. Hazard identification involves the definition of the problem, i.e. identification of the relevant pathogen(s) and food product. Then, exposure assessment is performed, identifying the pathways that can lead to human consumption of pathogens or toxins. Hazard characterization, relates the probability and severity of contracting an illness when a given dose of the pathogenic microorganism (or

toxin) is consumed. Finally, risk characterization integrates the previous three steps to estimate the probability and severity of an outcome (e.g. illness or death) for a given scenario. Sensitivity analysis is also a crucial tool in quantitative microbial risk assessment, to identify parameters or steps in the food chain that are critical in relation to a risk for the consumer. Methods based on Monte Carlo simulations have been widely used in risk assessment, although they present limitations for sensitivity analysis due to the computational cost associated and their uni-directional without retro-feeding among model parameters. An alternative is the use of Bayesian Belief Networks (25, 26) Once a model has been validated, the next step would be its use in optimization tasks. The presence of multiple opposed objectives is frequent in processes such as germination or microbial inactivation (some factors will increase some of them, whereas others will reduce them). Multi-objective techniques (27) can be applied to obtain a Pareto front, that provides a group of optimal solutions considering all the objectives and is a powerful decision tool. For nonlinear, dynamic models, such as those describing process in predictive microbiology, the most efficient techniques of multi objective optimization are those based on evolutive algorithms (28).

Therefore, in order to perform a foodborne CDI QMRA, it is essential to collect information available from literature and to generate high quality data where a lack of knowledge exists. Then, the most up-to-date models available (or new developments) should be used to analyze results and implement them in a quantitative microbiological risk assessment. Only then, sufficient evidence will be provided to establish the impact of the foodborne transmission routeon the consumer health. Providing scenarios will allow health authorities and regulatory bodies to consider mitigation measures in order to reduce the incidence of unexplained *C. difficile* infections in the EU.

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