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Review

REVIEW

Clostridium difficile colitis and zoonotic origins—a narrative review

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Abstract

Clostridium difficile is a major cause of hospital-associated diarrhoea, and in severe cases leads to pseudomembranous colitis and toxic megacolon. The frequency of *C. difficile* infection (CDI) has increased in recent decades, with 453 000 cases identified in 2011 in the USA. This is related to antibiotic-selection pressure, disruption of normal host intestinal microbiota and emergence of antibiotic-resistant *C. difficile* strains. The burden of community-acquired CDI has been increasingly appreciated, with disease identified in patients previously considered low-risk, such as young women or patients with no prior antibiotic exposure. *C. difficile* has been identified in livestock animals, meat products, seafood and salads. It has been postulated that the pool of *C. difficile* in the agricultural industry may contribute to human CDI. There is widespread environmental dispersal of *C. difficile* spores. Domestic households, turf lawns and public spaces are extensively contaminated, providing a potential reservoir for community-acquired CDI. In Australia, this is particularly associated with porcine-derived *C. difficile* UK PCR ribotype 014/020. In this article, the epidemiological differences between hospital- and community-acquired CDI are discussed, including some emerging evidence for community-acquired CDI being a possible zoonosis.

Key words: *Clostridium difficile*; healthcare-associated infections; zoonoses

Introduction

Clostridium difficile is a major cause of healthcare-related diarrhoea, in severe cases leading to sepsis, pseudomembranous colitis, toxic megacolon and multiorgan failure. Frequency of *C. difficile* infection (CDI) has increased, with 453 000 US cases identified in 2011 [1, 2]. Community-acquired CDI (CACDI) is increasingly recognized, with severe disease in low-risk groups, including younger women or patients with no prior antibiotic exposure [3–6]. *Clostridium difficile* has been identified in livestock animals, meat products, seafood and salads. It has been postulated that this pool of *C. difficile* in the food and agricultural industries contributes to human CDI [7]. For this review, clinical guidelines and microbiology data were sourced from both national and international societies and regulatory bodies. The emergence of CACDI and the evidence for *C. difficile* being a zoonotic pathogen were derived from a literature review using the National Center for Biotechnology Information (NCBI) database of articles published in English since 1975.

Normal gut function inhibits CDI

In healthy adults, gastric acid and commensal intestinal flora are protective against pathogenic organisms. *Clostridium difficile* are endospore-forming, obligate anaerobic bacteria. Their vegetative forms do not survive prolonged exposure to oxygen outside the body [8]. Gastric acid (pH = 1–2) kills ingested vegetative *C. difficile* cells but not endospores [9, 10]. *Clostridium difficile* spores begin germination in the duodenum and vegetate in the terminal ileum

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and colon due to activation of the *C. difficile* serine protease bile acid receptor (CspC) by primary bile acids, particularly taurocholate [11–13]. Spore germination is also enhanced by less-acidic conditions (pH = 6), presence of phosphate, KCl ions and amino acid nutrients (L-glycine) [12]. Conjugated primary bile acids (taurocholate, glycocholate) are normally deconjugated by bacterial bile salt hydrolases (BSHs) in the *small intestine* by three main phyla: *Firmicutes* (30%), *Bacteroidetes* (14.4%) and *Actinobacteria* (8.9%). These include *Clostridium*, *Bacteroides*, *Lactobacillus*, *Bifidobacterium* and *Enterococcus* genera [14, 15].

Normal colonic microbiota convert the resulting unconjugated primary bile acids (cholic and chenodeoxycholic acids) to secondary bile salts (deoxycholic and lithocholic acids). This occurs by dehydroxylation and epimerization of primary bile acid 3-, 7- and 12-hydroxyl groups by bacterial bile acid hydroxysteroid dehydrogenases (HSDHs). The most important process in humans is 7α-dehydroxylation under anaerobic conditions [16]. Epimerization requires the action of both α- and β-HSDHs, which can be present in a single bacterial species or shared by two different species. Many colonic bacteria dehydrogenate unconjugated primary bile acids, but very few species perform 7α-dehydroxylation of primary bile acids, including anaerobic *Clostridium* and *Eubacterium* spp. from the *Firmicutes* phylum [15]. Faecal Clostridial species also epimerize chenodeoxycholic acid to ursodeoxycholic acid (UDCA). UDCA makes up 3% of the total human bile acid pool [16].

Secondary bile acids effectively inhibit spore germination and vegetation of *C. difficile* cells [14, 15]. Broad-spectrum antibiotics suppress normal gut flora and prevent the deconjugation of primary bile acids, changing cholate/chenodeoxycholate ratios and decreasing secondary bile acid formation [17]. This permits *C. difficile* proliferation and provides a survival advantage to antibiotic-resistant *C. difficile* strains. Use of clindamycin, fluoroquinolones, third-generation cephalosporins and penicillins predispose patients to CDI [18, 19]. CDI can occur after single-dose antibiotic prophylaxis in surgical patients [20, 21]. CDI usually starts during or shortly after antibiotic administration, but can occur up to 3 months later. Impairment of colonization resistance can still occur after narrow-spectrum antibiotic treatment for CDI, including oral vancomycin, metronidazole or fidaxomicin, contributing to persistent intestinal dysbiota, altered bile acid metabolism and recurrent CDI. This is because non-toxigenic *C. difficile* spp. and other beneficial anaerobes are suppressed by CDI antibiotic treatment [22]. Treatment with subinhibitory concentrations of antibiotics, particularly clindamycin and ampicillin, increase colonization factors of *C. difficile*. Such factors include three adhesins, Cwp66, the S-layer protein P47 and Fbp68 (2- to 10-fold increase), and a cysteine protease, Cwp84 (2- to 41-fold increase). Co-amoxiclav induces spore germination and toxin production in RT027 CD strains. Cefotaxime use is associated with increased *C. difficile* toxin production, as compared to piperacillin/tazobactam [23].

Different *Clostridium difficile* toxins affect virulence of CDI

Clostridium difficile causes diarrhoea and pseudomembranous colitis via enterotoxin A (TcdA) and cytotoxin B (TcdB) production by vegetative cells. The genes that encode for toxin A (tcdA) and toxin B (tcdB) are part of the pathogenicity locus (PaLoc), which also includes tcdR (positive regulator) and tcdC (negative regulator) genes [24]. Binding of TcdA and TcdB toxins to enterocyte receptors leads to glucosylation and inactivation of the Rho family GTPases Rho, Rac and Cdc42 [25]. This results in disruption of colonic mucosal integrity, secretory diarrhoea and acute colitis. More virulent strains of *C. difficile* include UK PCR ribotypes (RT) 001, 018, 027, 078 and 126 [26]. The hypervirulent RT027 strain (North American pulsed-field-type NAP1) was implicated in a severe outbreak in Quebec in 2003 during which incidence and mortality increased 5- and 3-fold, respectively [27]. The most important risk factor in the RT027 CDI epidemics in North American hospitals in 2002–06 was administration of fluoroquinolones in hospital patients. RT027 strain has higher sporulation rates, fluoroquinolone resistance, increased secretion of toxins A and B (via loss of tcdC regulator gene) and produces binary toxin (*C. difficile* transferase, CDT). Binary toxin production in RT027 strains is controlled by the orphan response regulator CdtR, which also up-regulates TcdA and TcdB production [28].

Other virulence factors, which are shared with epidemic or outbreak ribotype strains, allow *C. difficile* to adhere to host enterocytes, germinate in the presence of primary bile acids, sporulate when stressed, burrow under intestinal mucus, form biofilms, survive and adapt to host defences and adverse environmental conditions. Important virulence factors include adhesion molecules (S-layer subunits, Cwp66 protein, fibronectin-binding protein Fbp68, collagen-binding protein CbpA, lipoprotein CD0873), spore-germinant CspC, flagellar proteins (fliC, fliD), heat-shock protein GroEL, type IV fimbriae, sporulation initiator spo0A and proteases such as Cwp84 or Zmp1 [25, 29–32]. Heat-shock protein GroEL is part of the heat-shock protein (HSP) 60 family and enhances *C. difficile* adhesion to enterocytes in response to heat shock, acidic pH or low iron levels [33].

CDT belongs to the binary ADP-ribosylating toxin family, including *C. botulinum* C2 toxin, *C. perfringens* iota toxin, *C. spiroforme* toxin and the *B. cereus/thuringiensis* vegetative insecticidal proteins. CDT is an iota-like toxin with two components: CDTb binds to the LSR cell surface receptor on enterocytes and interacts with the enzyme component CDTa. The CDT-LSR complex is then endocytosed and the CDTa component induces depolymerization of actin tubules and destruction of the enterocyte actin cytoskeleton [34]. Translocation of CDTa into the enterocyte is dependent on intracellular helper proteins, including HSP90 [34].

Clostridium difficile subtypes have varying pathogenicity

C. difficile bacteria are divided into five clades and each is prevalent in specific continents. These include clade 1 (Europe), clade 2 (North America), clade 3 (Africa), clade 4 (Asia) and clade 5 (Australia). Human CDI mortality is closely related to clade type and binary toxin production. In a large study of human CDI from Oxfordshire, UK, the 14-day mortality was 25% in clade 5 (PCR RT078), 20% in clade 2 (PCR RT027) and 12% in clade 1 CDI ($P < 0.001$) [35]. Several techniques have been developed to identify different strains of *C. difficile* in order to study its epidemiology [36]. These include multilocus sequence typing (MLST), multilocus variable number tandem repeat analysis (MLVA), restriction endonuclease analysis (REA), pulsed-field gel electrophoresis (PFGE), repetitive-element PCR typing, toxinotyping and UK PCR ribotyping [21].

PCR ribotyping identifies ribosomal RNA genes using primers complimentary to the 16 s and 23s RNA regions. There have been 116 distinct ribotypes of *C. difficile* identified. This technique has been used to track CDI in the UK since 1995. PCR detection of CDT genotypes (cdtA+ and cdtB+) allows identification of binary toxin producing *C. difficile* strains [34]. These include UK PCR RT027, 078, 244, 126/127, 033 and 251 [37].

In contrast, PFGE analyses the whole genome using specific restriction enzymes, but is more expensive and time-consuming [36]. Toxinotyping analyses changes in the pathogenicity locus (PaLoc), with 11 toxinotypes identified (0, I–X) [38].

The epidemiology of non-RT027 binary toxin producing strains and CDI has changed dramatically since 1990. Binary toxin producing isolates were not identified before 1990 in an Italian study of human CDI, but comprised 24% (1991–99) and 45% (2000–01) in later analyses [39]. Similarly, in 2005, CDT positive strains made up 17.2% of all toxinogenic strains in 14 EU countries and, in 2008, 23% of all strains from 34 EU countries [40]. Only 5% of the isolates were RT027. Binary toxin production was associated with an increased 30-day all-cause mortality compared to patients infected with *C. difficile* isolates without binary toxin genes (31% vs 14%, $P = 0.02$), in a study from a London NHS trust in 2011 [41]. In this study, only 8% of the isolates were RT027, but binary toxin genes were detected in 28% of isolates. A 2011 Danish study found the 30-day CDI mortality was 28% for RT027 isolates, 27.8% for binary toxin non-RT027 strains and 17% for TcdA+, TcdB+, CDT-strains [42]. Non-RT027 binary toxin positive isolates included RT 078 (33%), RT 066 (36%), RT 023 and nine other ribotypes (31%) [34].

Clostridium difficile subtype strains in Australia differ

Australia has different distributions of CDI ribotypes compared to the USA and Europe. Australian hospital surveillance of CDI was mandated in 2010, which improved source tracing and epidemiology. In 2012, the ribotype proportions of CDI from Australian hospitals and private laboratories were RT014/020 (25.5%), RT002 (10.5%), RT056 (5.9%), RT070 (4.2%), RT244 (2.4%), RT027 (1.6%) and RT078 (0.9%) [37]. Ribotype 244 shares clade 2 with RT027 and produces CDT, but is fluoroquinolone-sensitive. The Australasian outbreak of RT244 in 2011–12 was predominantly community-acquired and associated with severe disease, with a 42% 30-day mortality [43, 44]. Ribotype 078 has not been found in Australian livestock, but similar CDT-producing clade-5 ribotypes 126/127, 237 and 033 have been isolated. For example, a study of Australian neonatal veal carcass contamination by *C. difficile* identified binary toxin positive RTs in 70.3% (71/101) of isolates; 127 (A+, B+, CDT+, 32.7%), 288 (A–, B–, CDT+, 28.7%), 033 (A–, B–, CDT+, 6.9%) and 126 (A+, B+, CDT+, 2.0%). Degree of *C. difficile* contamination included 66.7% (10/15) of faecal subset samples (range 2.0×10^3 to 2.3×10^6 CFU/mL, median count 2.5×10^4 CFU/mL) and in 16.7% (25/150) of carcass samples (range 3–33 CFU/cm^2, median count 7 CFU/cm^2) [45]. Together with clade 1 RT014 and RT056, these livestock-associated *Clostridia* were also isolated in Australian human CDI [37, 46, 47].

CDI is increasingly recognized in the community

Up to 75% of CDI begins in patients who are not hospitalized, including recently discharged patients, outpatients and nursing-home residents [48]. CACDI is defined as CDI in persons with no overnight stay in an inpatient healthcare facility in the 12 weeks prior to symptom onset [48]. Hospital-acquired CDI (HACDI) in contrast is defined as a positive stool CD culture result >72 hours after admission or earlier with hospital contact in the previous 4 weeks [5].

The reported relative incidence of CACDI/overall CDI varies between countries, including Singapore (13.6%), Australia (26%), Canada (27%) and the USA (20–32%) [24]. Variability may be related to underdiagnosis or differences in public health reporting. The reported 30-day mortality of CACDI is 1.3% (USA), 3.2% (Finland) and 4% (Sweden). This is lower than the observed HACDI 30-day mortality of 6.9% in Quebec before 2003 [49], 13.8% in the Quebec outbreak in 2003 [27], 9.3% in the USA in 2003 [50] and 12.7% in Finland in 2013 [51, 52]. This compares to 11% for CACDI and 8% for HACDI in a retrospective 2014 Australian study [5].

Additional risk factors for CDI and recurrent CDI susceptibility include advanced age (>65 years), severe comorbidity, immunosuppression, chemotherapy, inflammatory bowel disease or renal failure [53].

Hospital contamination with spores, symptomatic inpatient 'super shedders', host susceptibility and antibiotic use are recognized risk factors for HACDI [54, 55]. The majority of community-onset CDI is also related to nosocomial acquisition (onset after discharge or through frequent hospital visits/contacts) or antibiotic treatment. Up to 25% of CDI patients have no traditionally recognized risk factors [56, 57]. Patients with HACDI are usually elderly (median age 72), immunosuppressed or recently received antibiotics. When compared to HACDI, CACDI patients are more often younger (median age 50 years), likely to be female (72 vs 60%) and 27% have had no exposure to antibiotics 180 days prior to CDI diagnosis [37, 56, 58]. Antibiotic treatment is still, however, seven times more likely to produce CACDI than no prior antibiotic treatment. Clindamycin (OR 20.43), fluoroquinolones (OR 5.65), cephalosporins (OR 4.47), penicillins (OR 3.25), macrolides (OR 2.55) and sulphonamides/trimethoprim (OR 1.84) were the most-implicated antibiotic agents in a 2013 meta-analysis of eight studies and 30 184 patients with CACDI [19]. Gastric acid suppression is associated with 18% of CACDI cases, more so with proton pump inhibitors (PPIs) than H_2 antagonists [24, 56]. Antibiotics and PPIs may be synergistic risk factors for CACDI, particularly with widespread community use of PPIs [59, 60]. PPIs change the gut microbiome by decreasing *Bacteroidetes* and increasing *Firmicutes* species, leading to favourable conditions for *C. difficile* spores to germinate and vegetate [61–63]. PPIs may also interfere with intestinal neutrophil phagocytosis and lysosome killing of *C. difficile* [31, 64].

Clostridium difficile spore exposure from asymptomatic carriers, food sources or the environment may be important community sources of CDI. Both symptomatic patients and asymptomatic carriers excrete *C. difficile* spores in high numbers. Carriage is common in neonates and infants, with up to 70% colonized with *C. difficile* [65] and 13% harbouring toxigenic strains [66]. Neonatal acquisition appears to be from environmental rather than maternal sources. Human infants rarely develop pseudomembranous colitis. This may be related to cellular membrane toxin receptor expression, protective factors in colostrum or neonatal gut flora [67]. Females are twice as likely to develop CACDI than males, particularly aged 15–44 years [51]. CACDI is increasingly described in peripartum women, who now comprise 1% of all cases [68]. Human infants may be an important reservoir for *C. difficile* excretion [66]. Contact with infants younger than 2 years of age is a significant risk factor for CACDI, which may explain the female preponderance [24, 57].

Clostridium difficile is prevalent in the environment and the food chain

The number of *C. difficile* spores required to cause CDI in susceptible humans is unknown, but estimated to be low (100–1000 spores) [61]. Several animal species have been used to assess

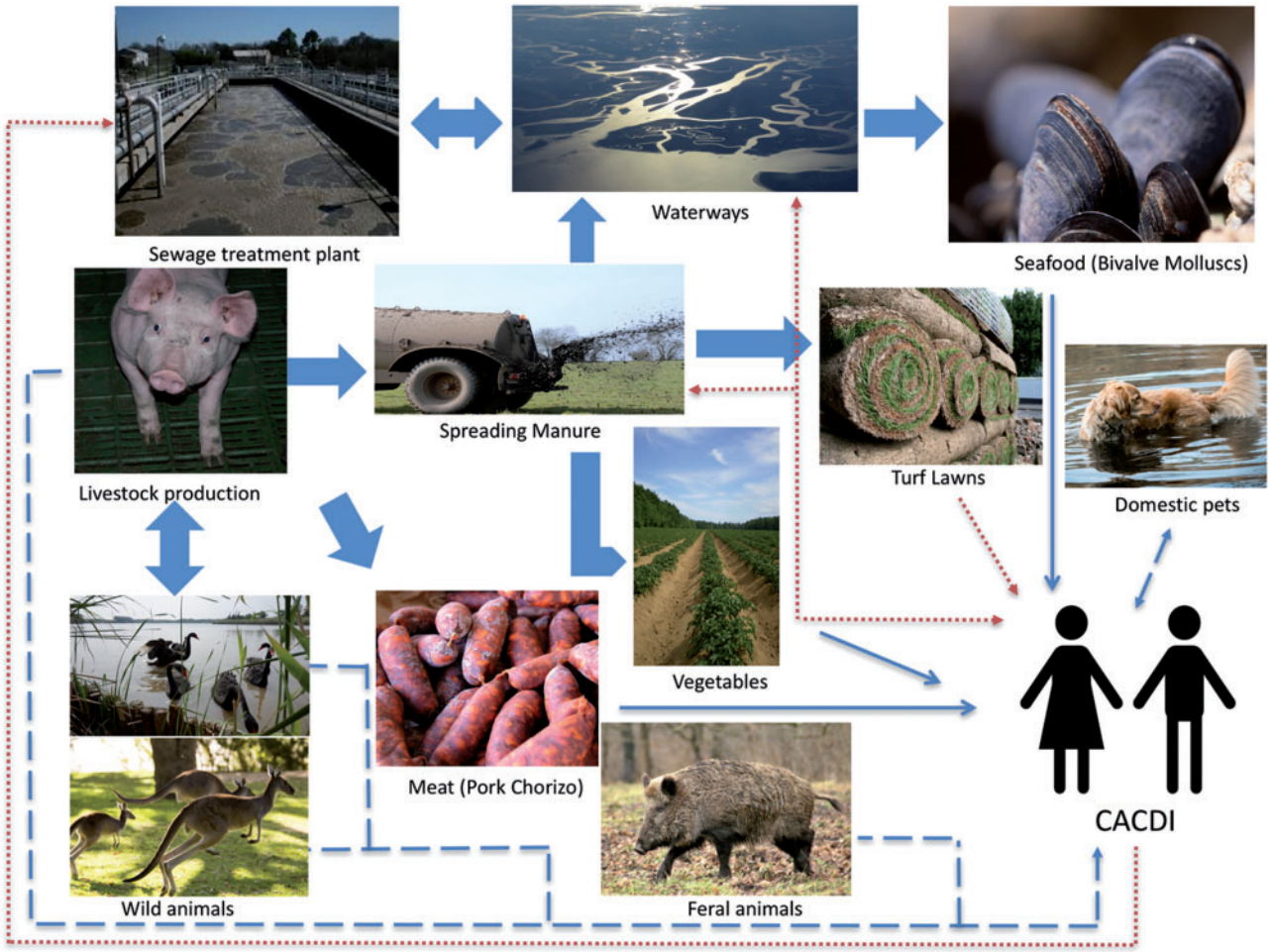

Figure 1. The cycling and recycling of *C. difficile* from zoonotic (- - - -), environmental (.....) or food-borne (_____) sources implicated in community-associated *C. difficile* infection (CACDI). Adapted from Warriner *et al* [61].

C. difficile susceptibility, including rabbit, rat, guinea pig, Syrian Golden hamster, conventional and germ-free mouse and germ-free piglet models [69]. Hamsters are extremely susceptible to CDI after antibiotic administration [70, 71] with as few as 1–2 CFUs sufficient to cause enterocolitis and death [72]. Mouse and piglet models have been developed to more closely match the disease course in humans, with susceptibility to CD spores induced by antibiotic treatment or by a lack of intestinal microbiome in gnotobiotic animals. The severity of the disease in conventional mouse models is related to the size of the spore inoculum. Using RT 027 spores in mice pretreated with a single dose of clindamycin, Sun *et al.* showed a dose of 10^4 CFUs of spores caused 0% mortality and 30% diarrhoea, 10^5 CFUs a 30% mortality and 70% diarrhoea, and 10^6 CFUs a 50% mortality and 100% diarrhoea [73]. Mice which were immunosuppressed with dexamethasone were more susceptible to severe and fulminant CDI. Using a conventional mouse model with 5 days of cefoperazone pretreatment, it was shown that only 100 *C. difficile* spores were sufficient to consistently cause CDI in mice. The spores were heat-treated for 20 minutes at 65°C prior to oral gavage, which is designed to kill any vegetative cells but also stimulates spore germination [74].

Transmission of spores between healthy asymptomatic carriers in CACDI was confirmed by PCR ribotyping and pulse field gel electrophoresis [75]. Potential modes of long-range environmental *C. difficile* spore dissemination include treated piggery waste water, bioeffluent, interstate stock transportation, reclaimed irrigation water, composting, biosolids, manure, turf lawns, estuarine sludge, river sediments and slaughtering of colonized pigs [61] (Figure 1). *Clostridium difficile* spores have been identified in soil samples from Swedish horse farms, rural parks and gardens [76], Zimbabwean farmers' markets [77], suburban soil in South Wales [78] and Australian municipal lawns [79]. *Clostridium difficile* spores were identified in 59% of lawn soils in Perth, Western Australia, 39% of which were RT014. The highest viable count was 1200 CFU/g. Contamination with RT014 spores was thought to be due to turf lawns being grown with pig manure [79]. The frequency of toxigenic *C. difficile* spore contamination of community environs in Houston, Texas, was highest in parks (24.6%), followed by homes (17.1%), commercial shops (8.1%) and fast-food restaurants (6.5%), as compared to 16.5% positive isolates from hospitals. Spores isolated from community environmental sources were more frequently ribotypes 014/020 (21%), 002 (12%) and 078/126 (7%), as compared to RT027 (4.5%). A similar distribution of ribotypes between environmental isolates and clinical cases was found, with the exception of RT027. Hospital wards (patient bathrooms and tables) were more likely to be colonized with RT027 (32% of *C. difficile*-positive hospital-environment isolates) and clinical CDI cases were more likely to be caused by RT027 (24.1%). Clinical cases

Table 1. Incidence and ribotypes of environmental *C. difficile*

Source	Location	Proportion contaminated (%)	Main ribotypes (%)	Reference
Ground beef (uncooked)	Arizona, USA	13/26 (50)	027 (11.5)078 (30.8)	[102]
Beef Sausage (cooked)	Arizona, USA	1/7 (14.3)	027 (14.3)	[102]
Ground beef	Ontario/Quebec, Canada	11/53 (20.8)	077, M31, 014, M26	[103]
Ground beef	Canada	10/149 (6.7)	M26, 077, J, 014, C, F, H	[104]
Ground beef	Canada	14/115 (12.2)	027, 078, C	[105]
Ground pork (uncooked)	Arizona, USA	3/7 (42.9)	027 (14.3)078 (28.6)	[80]
Braunschweiger (cooked)	Arizona, USA	10/16 (62.5)	027 (18.8)078 (43.8)	[102]
Ground pork	Canada	14/115 (12.2)	027, 078, C, E, Y	[105]
Pork sausage (uncooked)	Arizona, USA	3/13 (23.1)	027 (7.7) 078 (15.4)	[102]
Chorizo (uncooked)	Arizona, USA	3/10 (30)	027 (10)078 (20)	[102]
Chicken	Ontario, Canada	26/203 (12.8)	078 (12.8)	[106]
Ground Turkey (uncooked)	Arizona, USA	4/9 (44.4)	078 (44.4)	[102]
Molluscs	Italy	36/925 (3.9)	078/126 (22.2, 8/36)010 (19.4, 7/36)001 (8.3, 3/36)	[100]
Oysters	Louisiana, USA	9/19 (47.4)	tcdB positive (100, 9/9)	[101]
Raw vegetables	Canada	5/111 (4.5)	078	[96]
Raw vegetables	Australia	14/71 (19.7)	–	[94]
Salads	Scotland	3/40 (7.5)	017, 001	[97]
Shoe soles	Texas, USA	25/63 (39.7)	001, 002, UM-8	[88]
Lawns	Australia, Perth	182/311 (58.5)	014/020 (39)	[79]

were patients who were hospitalized with CDI, but no data on CACDI versus HACDI was provided [80].

Clostridium difficile spores are highly resistant to extreme physical or biochemical environments. They exist on hard surfaces for up to 6 months and even longer on concrete, wooden or dirt surfaces [81]. Freezing (to –80°C), heating (to 85°C), drying, ultraviolet radiation, alcohol gel and most disinfectants have proved ineffective in eradication [82–84]. Vaporized hydrogen peroxide or chlorine-based disinfectants are sporicidal. Quaternary ammonium/surfactant-based detergents are not sporicidal and may actually *increase* sporulation in virulent outbreak strains such as RT027 and RT001 [85]. Spores are easily spread by the faecal–oral route, contaminated hands or airborne dispersal in hospitals and nursing homes. These facilities are rapidly and extensively colonized, with spores being deposited on most surfaces and fomites [85–87].

Few studies have analysed the prevalence of *C. difficile* in household or community environments. Toxigenic *C. difficile* spores were detected in 32% of samples collected from 25/30 households in Houston, Texas. The most frequently contaminated surfaces were soles of shoes (39.7%), floor dust (33.3%) and bathroom/toilet surfaces (33.3%). Spore transfer on the soles of shoes was considered important in domestic contamination [88]. *Clostridium difficile* ribotypes from HACDI and CACDI were compared at two tertiary hospitals in Australia and 79% of hospital isolates had matching ribotypes in the community, suggesting transmission between the two reservoirs [89]. Whole-genome sequencing of 1250 CDI cases between 2007 and 2011 in healthcare settings and the community in Oxfordshire, UK, found 45% of cases were *not* due to contact exposure with symptomatic patients. Acquisition was more likely from asymptomatic carriers or non-hospital environmental sources [90]. In Europe and North America, HACDI is more likely to be associated with *C. difficile* RT027 and RT001, and CACDI more diverse ribotypes, including livestock-associated RT078 strain [51, 54, 91, 92]. It has been postulated that, in Australia, the emergence of livestock-associated *C. difficile* CDT producing ribotypes 127, 126 and 033 may parallel that of Northern Hemisphere RT078 strains in the pathogenesis of human CACDI [93].

In Australia, 20% of vegetables grown in enriched soils are contaminated with *C. difficile* spores. Vegetable spore contamination rates include carrots (5%), onions (6%), beetroots (22%) and potatoes (50%) [94]. Prevalence was lower in the USA (0% root vegetables, 2.4% other vegetables) [95] and Canada (4.5%) [96]. *Clostridium difficile* has also been identified in 7.5% of ready-to-eat salads in Scotland [97]. Of *C. difficile* spores isolated from river sediment in Ontario, Canada, 92% were toxigenic [98]. RT078 spores survived municipal water treatment for domestic housing in Ontario [99]. River and estuarine sediments containing *C. difficile* spores have been implicated in seafood contamination, including bottom-dwelling molluscs harbouring RT078/126 (22.2%), 010 (19.4%) and 001 (8.3%) in Italy. Filter feeders such as oysters, mussels and clams bioaccumulate pathogens of animal and human origin. However, in the Troiano and Montazeri studies, *C. difficile* contamination of molluscs was not correlated with indicators of human faecal pollution [100, 101] (Table 1).

The contribution of *C. difficile* spore contamination of food in the development of human CDI remains to be established. This is because, after ingestion, spore germination and vegetation are normally inhibited by an intact gut microbiome, with CDI occurring only in susceptible individuals. *Clostridium difficile* spores have been identified in North American retail meat products, including chicken (12.5%), turkey (44.4%), ground beef (50%), ground pork (43%) and Braunschweiger pork sausage (63%) [61, 106, 107] (Table 1). In the USA, these were predominantly RT078 (75%), but some were RT027 strain. Viable spores were found in both ready-to-eat, cooked meats and uncooked meats, although absolute spore counts were low. Twenty to 60 spores per gram were identified by direct culture of ground pork, 20–240 spores per gram from ground beef [105] and <100 CFU/g in chicken meat [106]—counts sufficient to cause disease in susceptible hosts [61, 74, 108].

The growth of *C. difficile* bacteria (as opposed to spores) is rare in foods because germination and vegetation of spores require primary bile salts and neutral to alkaline conditions (pH = 5.5–9.0) [12]. No germination of RT027 or RT078 spores occurred in beef or fish extracts without the addition of sodium taurocholate [61]. *Clostridium difficile* bacteria are heterotrophic

obligate anaerobes, and require intestinal fermentation of organic substrates such as amino acids to produce ATP. The presence of amino acids such as glycine or histidine enhance cholate-induced germination after spore ingestion [11, 12, 109]. This differs from microaerophilic *Campylobacter jejuni* or facultative anaerobic *Salmonella enterica* bacteria, which can grow in contaminated food, making source tracing of CDI challenging.

Clostridium difficile spores are resilient

The persistence of *C. difficile* spores in cooked meat demonstrates their survival ability in adverse environments [82, 110]. *Clostridium difficile* spores have several lamellations of the spore coat contributing to resilience [109]. RT078 is particularly heat-resistant compared to other ribotypes, including RT027 [111]. *Clostridium difficile* spores can survive cooking to a core temperature of 74°C and RT078 up to 96°C. Heat selection may explain the recent emergence of RT078 strain CDI in humans in the USA and Europe. Heating meat to 63 and 71°C (minimal recommended temperatures for cooking seafood and hamburgers, respectively) eliminates all vegetative microbiota but increases subsequent *C. difficile* spore germination by 30%. Sublethal cooking temperatures in modern food preparation instead of traditional methods such as pressure cooking or boiling may induce heat-shock protein expression in *C. difficile* RT078 and thence antibiotic resistance and virulence pathogenicity genes [111].

Clostridium difficile carriage and antibiotic resistance in domestic farm animals

Domestic farm animals have varying levels of symptomatic and asymptomatic *C. difficile* carriage. Neonatal animals are much more likely to be affected than adult animals. Carriage rates in a Texas, USA, piggery were 50.0% (61/122) in suckling pigs, followed by 23.8% (34/143) in lactating sows [112]. All isolates were positive for binary toxin gene and 93% were tcdA+ and tcdB+. In a 2009 Spanish study, 26% (140/541) of newborn piglets were found to have *C. difficile* on rectal swabs and 94% (132/140) were toxigenic strains (tcdA+, tcdB+) [113]. This included animals with and without diarrhoea, and animals from control farms without diarrhoea. Older piglets (1–2 months old) did not show any carriage of *C. difficile*. Seemingly healthy piglets that tested positive for *C. difficile* still had classic features of acute colitis on histology, suggesting a subclinical course in some animals [114]. In a Dutch study of *C. difficile* acquisition, all caesarean-section-derived piglets were *C. difficile*-negative, but were rapidly colonized with RT078 strain within 48 hours [115].

Transmission was thought to be from lactating sows or from the farm environment. In a Belgian study of *C. difficile* prevalence in beef cattle farms, there was a higher colonization rate of calves less than 6 months of age versus older calves >11 months old [116]. Some studies have suggested that intensive farming of pigs and cattle increases the carriage of toxigenic *C. difficile*, particularly with antibiotic use in lactating animals with mastitis or in suckling neonatal calves and piglets [61, 113, 114, 117]. However, other studies of *C. difficile* in pig herds have found no differences in overall carriage rates in pigs from conventional or organic farms [118]. Farm system, size or the presence of other animal species on the farm did not result in statistically significant differences in the *C. difficile* carriage rate.

Agricultural antibiotic use may influence *Clostridium difficile* strains and pathogenicity in humans

The emergence of CACDI may be related to zoonotic transmission. *Clostridium difficile* colonizes many domestic and wild animals, including cats, dogs, horses, pigs, calves, poultry, goats, rats, rabbits, raccoons, kangaroos, feral swine, birds, elephants, ostriches and Kodiak bears [61, 119–121] (Figure 1). Juvenile animals are most commonly affected, including 20% of beef calves and 90% of piglets [122]. Ribotype 027 has been isolated in livestock in the USA, but RT078 appears to predominate, particularly in poultry, calves and piglets [123]. Ribotype 078 is the most common cause of CACDI in the Northern Hemisphere [120].

Antibiotics are widely used in the agricultural industry as 'growth promoters'. In the USA, 80% of all antibiotics are used in agriculture, 70% of which are considered 'medically important', i.e. also used in human medical therapy. In 2011, over 26 tons of cephalosporins were used in the US agricultural industry [124]. Because of the risk of antibiotic-selection pressure leading to emergence of multiresistant organisms and vertical transmission, attempts have been made to improve antibiotic stewardship in the agricultural industry. In 2012, the US Food and Drug Administration (FDA) issued an order to prohibit 'extra-label' use of cephalosporins. However, 'approved' indications allow continued widespread use. The 2006 ban on antibiotic use in EU countries for livestock growth promotion did not lead to a decrease in antibiotic consumption [125].

Toxigenic *C. difficile* have developed high levels of resistance to beta lactams, macrolides, tetracyclines and aminoglycosides. This is due to antibiotic selection pressure, gene mutations and acquisition from other gut bacteria via conjugative and mobilizable transposons and bacteriophages [31]. The off-label use of ceftiofur, a third-generation cephalosporin, for the treatment and prevention of post-weaning diarrhoea in pigs caused by enterotoxigenic *Escherichia coli*, represented the biggest risk to public health faced by the Australian pork industry in 2014 [126]. This is because ceftiofur is analogous to ceftriaxone and cefotaxime, which are used in human medicine. Ceftiofur use can drive the amplification and transmission of multiresistant organisms such as *C. difficile* in intensive animal farming. Up to 25% of large piggeries in Australia use off-label ceftiofur in their herds. Ceftiofur is also registered for use for respiratory infections in cattle in Australia [127]. High carriage levels of binary toxin producing *C. difficile* ribotypes have been isolated in neonatal piglets and veal calves in Australia, which may provide a reservoir for vertical transmission to humans. In veal calves, CD was isolated in 53% and, of these CDI strains, 76% were CDT producing, including ribotypes 127, 126 and 033 [93].

The One Health Commission began in 2007 as a collaboration between the American Medical and Veterinary Associations. It aims to achieve optimal health outcomes, recognizing the interconnection between people, animals, plants and their shared environment. This has led to further initiatives to improve antibiotic stewardship [128].

Many human enteric infections have a zoonotic origin linked to agricultural antibiotic use. The emergence of fluoroquinolone resistance in human *Campylobacter jejuni* and *Salmonella typhimurium*-definitive phage type104 gastroenteritis was associated with the use of fluoroquinolones (enrofloxacin) in the poultry industry since 1993. The US FDA withdrew fluoroquinolones in poultry production in 2005. Their use in poultry production is banned in Nordic European countries and Australia. The UK and

most EU countries continue to allow their use in turkey, duck, geese and chicken meat production, with ciprofloxacin resistance rates in human *Campylobacter* infections as high as 97.9% in Portugal and 84.7% in Spain and Lithuania in 2014 [129]. The average rate of ciprofloxacin resistance in human *Campylobacter* infections in EU countries was 60% (2014 data), compared to 22.3% in the USA (2013 data), 14% in Sweden (2014 data), 11.6% in Finland (2012 data) and 2% in Australia (2001–02 data) [130]. Treatment failures, higher hospitalization rates and a 2-fold increase in mortality were observed in patients with fluoroquinolone-resistant *S. typhimurium* DT104 infections. Fluoroquinolones have never been approved in Australia for use in the meat and livestock industry [129–131]. The conservative use of fluoroquinolones in agriculture and clinical medicine may explain the lack of domestic emergence of fluoroquinolone-resistant *C. difficile* RT027 in Australia [37].

Antibiotic selection of resistant organisms and their transfer from livestock to farm workers have been widely demonstrated. A 1997 Netherlands study reported higher carriage of vancomycin resistant *enterococci* in turkeys (50%) treated with avoparcin (a vancomycin-like growth promoter) and their farmers (39%) compared to local residents (14%) [131, 132]. Avoparcin was banned as a growth promoter in animal feeds in Europe in 1999. Tetracycline-resistant *E. coli* was identified in both chickens and chicken farmers in 1975, when tetracyclines were used for prophylaxis and growth promotion [133]. Tetracycline was banned as a growth promoter in food animal production in EU countries in 2006, due to the emergence of tetracycline-resistant *S. typhimurium* and *E. coli*. Whole-genome analysis of *C. difficile* RT078 strains in the Netherlands from 2002 to 2011 found identical strains were shared between pigs and pig farmers, indicating transmission between the two groups [91]. These included identical streptomycin- and tetracycline-resistance determinants. Other *non-clonal* strains suggested alternative reservoirs for the *community spread* of RT078, including wild animals and environmental sources [91]. There is genomic evidence that RT078 and 027 porcine strains are similar to strains isolated from human CDI, indicating interspecies transmission [120].

Whole-genome and proteome analysis by Knight *et al.* demonstrated substantial similarities between human and porcine strains of *C. difficile* (RT014/NAP4) in Australia [94]. Specific CDI strains can also be traced through pan-genome analysis and characterization of antibiotic resistance, prophage content and *in silico* virulence potential [134]. This enables source origin, vector patterns, CDI risk factors and modes of environmental contamination to be established [120]. RT014/020 is the most prevalent ribotype in Australia, accounting for 24% of human CDI cases. It is also the most common ribotype in neonatal pigs, found in 23% of isolates collected thousands of kilometres and many months apart in Australia. Interspecies transmission was substantiated in 42% of human strains and 37.5% of porcine strains based on single nucleotide variant analysis. Of these interspecies clonal groups, 50% of human strains were classified as CACDI. This suggests that, in Australia, porcine *C. difficile* can be transferred to the human population and cause CACDI.

Conclusion

There is accumulating evidence of a persistent community reservoir of *C. difficile*. This involves spore contamination of soil, water, food, households, shoes, lawns and public spaces. The mechanism of this reservoir leading to disease in susceptible hosts requires further exploration. *Clostridium difficile* genome analysis and differing demographic patterns between CACDI and HACDI suggest a zoonotic origin in Australian CACDI, particularly porcine-derived RT014/020. This may be driven by antibiotic use in the agricultural industry. The importance of One Health initiatives, antibiotic stewardship and source control in human and veterinarian medicine as well as the agricultural industry is emphasized.

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