Clostridium difficile – A possible zoonotic link

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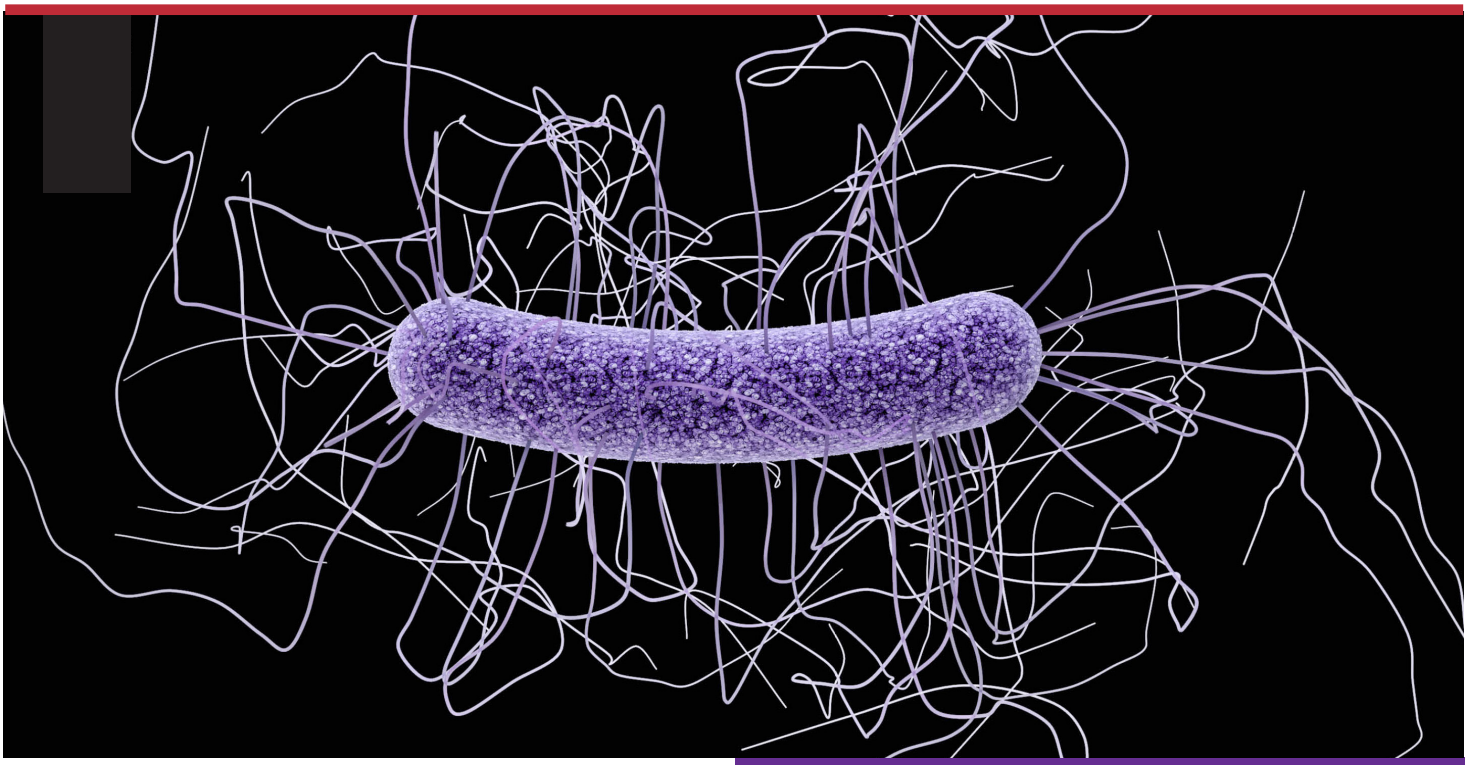

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Clostridium difficile – A possible zoonotic link

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Contents

Introduction

In the Danish Integrated Antimicrobial Resistance Monitoring and Research Program DANMAP 2012 it is stated that the *Clostridium difficile* types containing the toxin genes *tcdA* and *tcdB* may contribute to human infections, and the zoonotic importance of these types should be further investigated.

To evaluate the zoonotic importance of *C. difficile* from an animal reservoir, the National Food Institute, Technical University of Denmark has at the request of the Danish Veterinary and Food Administration, Ministry of Food, Agriculture and Fisheries conducted this report.

The Danish Veterinary and Food Administration requests an assessment of the overall importance of a zoonotic reservoir of *C. difficile* and the risk on human health associated with this reservoir. This report focuses on answering:

- If the toxins TcdA and TcdB can cause infections in humans?
- What is the incidence of cases in Denmark?
- If there is evidence, that the presence of *C. difficile* presences in meat represents a risk to the consumers?
  - Under what circumstances and conditions is there a possible risk?
  - How likely is the risk?
- If there is evidence, that the presence of *C. difficile* in production animals represents a risk to persons working with infected animals?
  - Under what circumstances and conditions is there a possible risk?
  - How likely is the risk?
- If there is known future changes in the production systems or antibiotic consumption pattern in animals that may influence the risk of humans regarding *C. difficile* infections?

The assessment should include international per reviewed literature from the last 10 years and available Danish data. The report is not a systematic review. This report was conducted between April and December 2014.

Search parameters: *C. difficile* combined with: Zoonotic, zoonoses, food (including meat and ready-to-eat food), antibiotic resistance, surveillance, epidemiology, community acquired infections, risk factors, production animals and environment.

Abbreviations:

CDI = *Clostridium difficile* infection
MLVA = Multi Locus Variable-number tandem repeat Analysis
PCR = Polymerase Chain Reaction
PFGE = Pulsed-field gel electrophoresis
PMC = Pseudomembranous colitis
WGS = Whole genome sequencing

Dansk resumé

Clostridium difficile (*C. difficile*) er en sporedannende strikt anaerob bakterie. Bakterien kan forekomme naturligt i tarmen hos mennesker og forskellige dyr, som heste, svin, kvæg, fjerkræ, geder, får, gnavere, hunde og katte. *C. difficile* smitter direkte via afføring, via person-til-person smitte eller indirekte via sporer efterladt på overflader eller hud. Sporerne er et dvalestadie, hvor bakterien er inaktiv. Sporer kan overleve i årevis i jord og støv eller på arbejdstøj og redskaber. De er varmetolerante og modstandsdygtige overfor alkoholbaserede desinfektionsmidler (i stedet anbefales sæbe håndvask). Sporerne kan under de rette betingelser aktiveres til levedygtige bakterier, der kan fremkalde sygdom. *C. difficile* producerer forskellige toksiner, som er sygdomsfremkaldende hos mennesker. Dels toksinerne TcdA og TcdB, men også et binært toksin CDT. Det binære toksin er forbundet med en højere dødelighed hos hospitalsindlagte patienter sammenlignet med infektioner forårsaget af *C. difficile*, der kun bærer toksinerne TcdA og TcdB. Nogle stammer af *C. difficile* er højvirulente og producerer en større mængde af de enkelte toksiner. *C. difficile* er naturligt resistent overfor cefalosporiner og kan derudover bære resistens overfor en lang række af antibiotika, der anvendes til behandling af mennesker, herunder fluoroquinoloner, makrolider, tetracyklin og vancomycin. Den infektive dosis for *C. difficile* kendes ikke, men varierer sandsynligvis blandt individer, og er afhængig af forudgående behandling med antibiotika, bagvedliggende sygdom samt påvirkninger af den normale tarmflora.

Infektioner med *C. difficile* er en vigtig årsag til diarré, særligt hos hospitalsindlagte ældre patienter samt immunkompromitterede patienter. Infektionen kan spænde fra en asymptomatisk infektion, til mild diarré, livstruende pseudomembranøs colitis (PMC), toksisk megacolon, tarm perforering, sepsis og død. *C. difficile* infektion (CDI) opstår typisk i forbindelse med antibiotika behandling, som forstyrrer eller fjerner den normale tarmflora og dermed giver *C. difficile* optimale betingelser for at formere sig og producere de toksiner der forårsager sygdom. *C. difficile* er resistent overfor en lang række antibiotika, hvilket kan medføre en forværret infektion, som er sværere at behandle. I flere lande, inklusive Danmark, har en særlig højvirulent resistent variant af *C. difficile* med navnet ribotype 027 forårsaget sygdomsudbrud på flere hospitaler. Flere andre ribotyper er højvirulente og associeret med sygdom. I Danmark blev der i årene 2009 – 2013 rapporteret i alt 4.347 *C. difficile* ribotype 027 patienter med virulens toksinerne TcdA og TcdB og det binære toksin.

De seneste år er CDI i højere grad set blandt personer, som ikke har været i kontakt med sundhedsvæsenet. Disse patienter ofte yngre, og infektionen er ikke i samme grad som den hospitalserhvervede udløst af forudgående antibiotika behandling. Årsagen til stigningen i de samfundserhvervede CDI er ukendt. Da der er sammenfald i ribotyper og antibiotika resistens i det humane- og veterinære reservoir, og da der er fundet genetisk identiske stammer ved fuld genom-sekventering (WGS) i begge reservoirer har forskere over hele verden undersøgt, om forekomsten af *C. difficile* i dyr kan være årsag til stigningen i de samfundserhvervede CDI og om *C. difficile* kan smitte via fødevarer. En række lande i Europa, heri blandt Holland har fundet genetisk identiske ribotype 078 hos både smågrise og mennesker. *C. difficile* ribotype 078 ses i højere grad hos de samfundserhvervede CDI end ribotype 027, som primært findes i hospitalsmiljøet. I Danmark har man ligeledes fundet overlap mellem det humane- og veterinære reservoir. En række undersøgelser i Europa, Canada og USA dokumenterer positive fund af *C. difficile* i svinekød, kalvekød, kyllingekød, grøntsager, vand og jord.

Det har ikke været muligt at estimere, hvorvidt forekomsten af *C. difficile* i fødevarer er årsag til human sygdom, eller om forekomsten udgør en reel risiko for den almindelige forbruger. Modtagelighed hos det enkelte individ spiller formentlig en højere rolle end ved andre fødevarebårne mave-tarm infektioner. Der rejses dog videnskabelig bekymring overfor sammenfaldet mellem stigningen i de samfundserhvervede CDI og fund i fødevarer og produktionsdyr.

Ligesom der ikke er bevis for, at *C. difficile* i fødevarer udgør en risiko for den almindelige forbruger, er der heller ikke bevis for at en arbejdsmæssig kontakt til produktionsdyr udgør en risiko for personer med almen god sundhedstilstand, hvis man følger de almindelige hygiejneregler for området.

Selvom der ikke er bevis for, at *C. difficile* i fødevarer eller produktionsdyr udgør en risiko, anbefales det dog, at følgende områder inden for fødevareproduktionen bør have særlig opmærksomhed: A) Ændringer, der begunstiger anaerobe forhold, som muliggør overlevelse af *C. difficile* i slutproduktet, B) brug af antibiotika i produktionsdyr, som kan påvirke forekomsten af *C. difficile* i dyrene, C) ændringer i hygiejneprocedurer og varmebehandlingsparametre i produktionskæden, som kan påvirke overlevelsen af *C. difficile* sporer.

For at kunne forstå epidemiologien bag de samfundserhvervede CDI samt udbredelsen af *C. difficile* i dyr og mennesker er der behov for standardiserede mikrobiologiske metoder og overvågning. Overvågningsprogrammer af *C. difficile* bør fokusere på alle ribotyper, der er i stand til at producerer toksiner.

Sammenlignende mikrobiologiske undersøgelser, der anvender genetisk diskriminative metoder af *C. difficile* stammer fundet i mennesker, dyr, fødevarer og miljø (herunder vand) kombineret med epidemiologiske metoder, kan potentielt bidrage til en bedre forståelse af *C. difficile,* og hvorvidt det veterinære reservoir udgør en human risiko.

1. Background

Clostridium difficile (C. difficile) is a motile spore-forming bacteria and strict anaerobic. The spores of C. difficile enable the organism to survive in adverse conditions, for example in soil, dust and on skin, and can persist on environmental surfaces for months and even years. Alcohol, chlorhexidine, iodophors, and other antiseptic agents are ineffective at inactivating C. difficile spores. The occurrence of C. difficile suggests a seasonal variation. C. difficile is the most commonly diagnosed cause of antimicrobial-associated and hospital-acquired diarrhea.

1.1 Clostridium difficile characteristics

C. difficile is a motile gram-positive spore-forming bacteria classified as strict anaerobe. The normal location for *C. difficile* is in the intestinal tract of humans and various animal species as production animals, horses and pets (Weese 2010). The bacteria are spread by the faecal–oral route, indirectly through spores left on surfaces and by person-to-person transmission (Hookman & Barkin 2009; DePestel & Aronoff 2013).

In Europe, agarose gel-based polymerase chain reaction (PCR[1]) ribotyping is the most common used typing method of *C. difficile*. Although PCR-ribotyping has proved to be useful to study epidemiology on local, national and European level, efforts are made to replace it with the capillary electrophoresis PCR-ribotyping in order to increase pattern recognition, reproducibility and interpretation. However, this method lacks sufficient discriminatory power to study outbreaks and therefore the typing method multilocus variable-number tandem repeat analysis (MLVA) has been developed to study transmission between humans, animals and food. In North-America, pulsed-field gel electrophoresis (PFGE) is commonly used and the obtained banding patterns are referred to as NAP-field types. Various other typing methods for *C. difficile* are used: Restriction endonuclease analysis (REA), multilocus sequence typing (MLST), tandem repeat sequence typing (TRST) and whole genome sequencing (WGS) (Knetsch et al. 2013). TRST is a new method for genotyping of *C. difficile* and is used in Denmark for both human, veterinary and food isolates. *C. difficile* has more than 150 PCR ribotypes and 24 toxinotypes (Kuijper et al. 2006).

1.2 C. difficile spores and survival

C. difficile spores frequently contaminate the environment around infected patients and hospitalization provides not only a reservoir, but also a vector for transmission (Stanley et al. 2013). The epidemic strains of *C. difficile* (e.g. ribotype 027) are believed to have a greater sporulation capacity in vitro than non-outbreak strains (Rupnik 2007). The spores of *C. difficile* enable the organism to survive in adverse conditions, for example in soil, dust and on skin. The spores, as well as the vegetative form, can persist on fomites (e.g. cloths, utensils) and environmental surfaces for months (Hookman & Barkin 2009; DePestel & Aronoff 2013) and even years (Rupnik et al. 2009).

Alcohol, chlorhexidine, iodophors, and other antiseptic agents are ineffective at inactivating *C. difficile* spores. The most effective to prevent the transmission of spores is frequent hand wash with soap and water, rather than with alcohol-based waterless hand hygiene, especially when

[1] Biochemical technology in molecular typing

directly or indirectly taken care of patients with a *C. difficile* infection (CDI) in the health care system or having other contact to CDI patients (Hookman & Barkin 2009; Epi-Nyt Statens Serum Institut week 13; 2009World Health Organization 2009[2];). *C. difficile* spores have been shown to survive the temperatures and disinfectant treatment of typical hospital laundering cycles and to cross-contaminate bed linens during a wash cycle (Rupnik 2007). A study testing the survival of *C. difficile* at cooking temperature 71°C showed that all 20 tested isolates had viable spores after extended heating for 2 hours[3] (120 min) (Rodriguez-Palacios et al. 2010). A later study conducted by the same researcher found that cooking aliquots containing less than 4 $\log_{10}$ at 85°C in liquid media yielded no cultivable spores after 15 min heat treatment. Considering a concentration of *C. difficile* spores in naturally contaminated retail raw meat products may be less than 3.3 $\log_{10}$/g of product, cooking at 85°C could according to the researchers markedly reduce human exposure to *C. difficile* (Rodriguez-Palacios and LeJeune 2011).

1.3 Seasonality

A Canadian study performed by Rodriguez-Palacios et al. in 2009 suggests a seasonal variation in the prevalence of *C. difficile* in the reservoir. Meat samples from retail were systematic sampled over an 8-month period. Isolation of *C. difficile* was more common in February than in the other months, although the reason for this apparent seasonality was unclear (Rodriguez-Palacios et al. 2009). In foods and food animals, according to Rodriguez-Palacios et al. 2013, three independent studies document higher prevalence in winter in North America. Also the number of cases of CDI in humans appears to be higher during winter months, at least in northern latitudes. This seasonal increase has been partly attributed to a larger number of cases associated with seasonal respiratory and enteric viral infections that require antimicrobial administration or hospitalization (Rodriguez-Palacios et al. 2013), which both are known risk factors for CDI.

1.4 C. difficile infection in humans

C. difficile causes human infection, CDI. It is assumed that disruption of the normal protective gastrointestinal microbiota, as a result of antimicrobial therapy, is an important factor for a colonization by *C. difficile* in the intestine and involves overgrowth by toxigenic strains of *C. difficile*, followed by the production of toxins (Hookman and Barkin 2009; Weese 2010). The infection can range from an asymptomatic colonization, to a mild self-limiting diarrheal illness, to the life-threatening antibiotic-associated diarrhea pseudomembranous colitis (PMC), toxic megacolon, intestinal perforation, sepsis and death (Hookman and Barkin 2009; Keessen et al. 2011; DePestel and Aronoff 2013). Pseudomembranous colitis (PMC) is an intestinal disease which is characterized by offensive-smelling diarrhea, abdominal pain, and fever, particularly following antibiotic treatment. Although the severe form of *C. difficile* disease, PMC, was described back in 1893, *C. difficile* was not linked to PMC, and little was known about the organism except that it was considered to be part of the normal intestinal ecology of infants. Notably

[2] Hygienic precautions described in Statens Serum Institut Epi-Nyt week 13 2009 and WHO Guidelines on hand hygiene in health care, Guide to appropriate hand hygiene in connection with Clostridium difficile spread, World Health Organization 2009, pp242-245

[3] The Danish Food and Veterinary Administration recommends that carved meat, minced meat and mechanically tenderized meat is thoroughly cooked to at least a temperature of 75°C. Fakta om Fødevarehygiejne, Fødevarestyrelsen 2005 ISBN: 87-91569-97-4

PMC was rare before the widespread use of antibiotics in the late 1940s and early 1950s (Rupnik et al. 2009).
C. difficile was first identified in the 1970s as the predominate bacterial cause of antibiotic-associated diarrhea and in 1978 reported as the cause of antibiotic-associated colitis by criteria of Koch (Stanley et al. 2013). *C. difficile* is now the most commonly diagnosed cause of antimicrobial-associated and hospital-diarrhea, and the most cases of PMC (Weese 2010; DePestel and Aronoff 2013). The risk of developing CDI increases if *C. difficile* is resistant to the antimicrobial agents used, which enables the bacteria to colonize the intestine faster (Huang et al. 2009).

Individual genetic differences regarding the sensibility of CDI have been reported. Individuals that lack a host response to produce sufficient quantities of antibodies directed against toxin A and those individuals with a common single-nucleoside polymorphism in the -251 region of the interleukin-8 gene promoter may have an increased risk of recurrences. The risk of recurrences increases with advanced age, with patients' ≥65 years of age at highest risk. Disruption of the gut microbiota and loss of colonization resistance have been investigated as a risk factor for recurrence and provide the biological basis for a successful use of faecal microbiota transplantation. Other risk factors for recurrence include concurrent use of antibiotics for non-CDI, acid suppressing agents, exposure to the health care environment, and underlying chronic comorbidities (DePestel & Aronoff 2013).

The infective dose of *C. difficile* for humans is not known and probably varies among individuals (Weese 2010). Different studies emphasize that the pathophysiology of CDI is not yet fully understood and in the recent years an animal reservoir has been discussed as a possible source of CDI.

2. The virulence genes of *C. difficile*

C. difficile produces several toxins capable of introducing human disease. Toxinotyping is a PCR-based method and toxin A and toxin B were the first toxins to be discovered. Some strains of C. difficile also produce the binary toxin CDT. The role of the binary toxin CDT in human disease is not yet fully understood, but there is information suggesting that this toxin may be clinically relevant. Strains possessing the binary toxin genes and genes encoding for tcdA and tcdB, seem to have a higher case-fatality risk, irrespective of ribotype.

2.1 Toxin A and toxin B

C. difficile produces several toxins capable of introducing human disease. The enterotoxin toxin A (TcdA) and a cytotoxin toxin B (TcdB), both belong to a group of large clostridial toxins. Because of their unusual pattern of toxin production, the TcdA and TcdB strains were the first variant strains to be discovered (Weese 2010). The two toxins have different behaviour affecting the cells; TcdA binds more effectively on the apical side of the host cell and TcdB binds to an unknown receptor on the basolateral side of the host cell. Studies have shown that the main clinical symptoms and signs of CDI largely can be explained by TcdA and TcdB (Rupnik et al. 2009). In the intestinal tract the toxins cause disruption of the actin cytoskeleton and tight junctions, and resulting in decreased transepithelial resistance, fluid accumulation and destruction of the intestinal epithelium (Rupnik et al. 2009). Most of the toxigenic *C. difficile*

strains produce both TcdA and TcdB. A small percentage of clinically relevant strains produce TcdB but not TcdA (Weese 2010).

2.2 Binary toxin CDT and hypervirulent strains of *C. difficile*

C. difficile produces also a binary toxin CDT. The role of the binary toxin CDT in human disease is though not yet fully understood (Rupnik et al. 2009; Carter et al. 2012). Previously, the prevalence of binary toxin-producing strains was low (<10%); however, this has increased in the recent years, and binary toxin producing strains in some studies now represent more than 30% of isolates from humans (Weese 2010). A Danish study has shown that patients with the binary toxin had higher case-fatality rates (28.0%) 30 days after diagnosis than patients without the binary toxin adjusted for age, sex, and geographic region (RR 1.6, 95% [CI 1.01–2.4]). Similar case-fatality rates were observed for patients infected with ribotype 027 or ribotype non-027 (28.0% and 27.8 % respectively). The study concludes, that the binary toxin either is a marker for more virulent *C. difficile* strains or contributes directly to strain virulence and efforts to control CDI should target all virulent strains irrespective of PCR ribotype (Bacci et al. 2011).

Some strains of *C. difficile* are hypervirulent and several microbial characteristics have been attributed to possible hypervirulence, including mutations in a regulatory gene *tcdC* causing hyperproduction of *tcdA* and *tcdB*, production of the binary toxin CDT, antibiotic resistance and improved toxin binding to target cells, increased sporulation, and mutations in surface layer proteins that increase its adherence to intestinal epithelium (Coia 2009; DePestel & Aronoff 2013).

3. Serotypes involved in *C. difficile* infections

During the last decade, human infections due to C. difficile have become more frequent, more severe, more refractory to standard therapy, and more likely to relapse than in previous years. This pattern has been seen throughout the United States, Canada and Europe, and has mainly been attributed to the strain of C. difficile ribotype 027. This ribotype has both in North America and Europe been implicated in CDI hospitals outbreaks. Also the emerging binary toxin-positive ribotype 078 strain, which has similar mechanisms for hyper production of toxins as ribotype 027, has the last years received more scientific attention. Ribotype 078 is the predominant type found in production animals and their immediate environment, and is now also emerging in both hospital- and community-acquired CDI. In some European countries ribotype 078 are more frequently reported than ribotype 027.

3.1 *C. difficile ribotype 027*

The name of the strain BI, NAP1/027 reflects its characteristics, demonstrated by different typing methods: Pulsed-field gel electrophoresis (NAP1), restriction endonuclease analysis (BI) and polymerase chain reaction (027, PCR 027) (Hookman & Barkin 2009). Ribotype 027 was first assigned in 1988 and originated from a 28-year-old woman with severe pseudomembranous colitis. Until March 2004, it was considered to be an unimportant and very rare ribotype (Kuijper et al. 2006). Ribotype 027 contains genes for the binary toxin CDT and has a genetic mutation in *tcdC*, which results in deregulated expression of TcdA and TcdB, and can produce up to 16 times more TcdA and 23 times more TcdB in vitro than other strains (Kuijper et al. 2008; Hookman & Barkin 2009).

Ribotype 027 has both in North America and Europe been implicated in CDI hospitals outbreaks. The outbreaks have been characterized by an increased incidence and severity, refractory to traditional therapy, greater risk of relapse and associated with increased morbidity and a significant mortality (Kuijper et al. 2008; DePestel & Aronoff 2013). The strain also tends to infect hospitalised and elderly who are more vulnerable in terms of acquiring CDI, but also have more difficulties to recover from illness in general (DePestel & Aronoff 2013). Ribotype 027 has been detected in meat; ground beef and veal, ground pork, uncooked pork sausages and other food sources[4] (Rodriguez-Palacios et al. 2009; Songer et al. 2009). Finally the strain has s universally high-level resistant to fluoroquinolones in vitro, which was infrequent prior to 2001 (Hookman & Barkin 2009).

In Denmark in 2006, Statens Serum Institut (SSI) detected a cluster of eight patients with *C. difficile* ribotype 027 in a retrospective survey covering a county in South Denmark. The isolates were recovered from 22 faecal samples that had been collected between November 2006 and March 2007. All eight cases were hospitalized. Subsequently, active surveillance was initiated in the same region for the period June-August 2007, which resulted in five additional ribotype 027 cases among 22 *C. difficile* isolates tested. Interestingly, all 13 isolates were resistant to newer fluoroquinolones and cephalosporins, but susceptible to erythromycin and clindamycin (Kuijper et al. 2008).

3.2 *C. difficile ribotype 078*

The focus in surveillance of *C. difficile* strains in Europe has been on ribotype 027 (Kuijper et al. 2008). The virulence factors are however not unique for ribotype 027, but are also present in other ribotypes such as the emerging binary toxin-positive ribotype 078 strain (also known as PCR 078), which has similar mechanisms for hyper production of toxins as ribotype 027 (Kuijper et al. 2008). Since 2005, ribotype 078 has increased in occurrence both regarding the hospital- and community-acquired CDI (Jones et al. 2013). Noteworthy is, that ribotype 078 is more commonly reported than ribotype 027. In a pan-European surveillance study performed in 2008 in 34 European countries, ribotype 078 was reported as the third most prevalent ribotype (8%) whereas ribotype 027 was reported in 5% of the cases (Freeman et al. 2010). The same pattern was seen in The Netherlands in the period May 2013 – May 2014, where 20 hospitals participated in a sentinel surveillance: During this period among 911 *C. difficile* isolates, ribotype 078 was more frequently detected (13.4%) than ribotype 027, which was found in 28 isolates (3%) (NethMap 2014; Eighth Annual Report 2014).

Compared with patients infected with ribotype 027, patients with ribotype 078 tend to be younger with fewer co-morbidities, have community-acquired CDI and similar to 027 patients have been more likely to have received fluoroquinolones in antimicrobial therapy. However, according to a review conducted by Jones et al. 2013; a significant proportion of patients included in a study of ribotype 078 had not received any antibiotic therapy in the 6 weeks prior to developing CDI in the community, leading to the hypothesis that there is a yet unknown selection mechanism that favours the emergence of these strains (Jones et al. 2013). Although

[4] See section 8.2

similar ribotypes are found in the animal and human reservoir direct transmission of *C. difficile* from animals, food or the environment to humans has not been proven. Conversely, emerging *C. difficile* ribotype 078 is the predominant found in piglets, calves, and their immediate environment. Circumstantial evidence points towards a zoonotic potential of this type (Indra et al. 2009; Rupnik et al. 2009; Jones et al. 2013)

3.3 Other *C. difficile* ribotypes

Other *C. difficile* ribotypes, such as ribotype 0126, 056, and 018 have also been reported to be associated with more complicated disease outcomes carrying the same virulence genes as ribotype 027 and 078 (Rupnik et al. 2009). Ribotype 018 was one of the more frequently (6%) reported ribotypes from humans in the pan-European survey (Jones et al. 2013).

3.4 Geographical distribution of ribotypes in Europe and Denmark

A variation in ribotypes within the European Union (EU) should be expected. Different studies has shown that the ribotypes seem to vary between geographical areas and animal species (Rodriguez-palacios et al. 2007; Keessen et el. 2011; Koene et al. 2012). In figure 1, the regional variation within the EU in human ribotypes is shown. The number of typed isolates per country is though limited and various in numbers, and might not be representative due to different reporting systems. According to the figure, ribotype 014-020 have a higher prevalence in the Scandinavian countries than reported in Central Europe and on the contrary to ribotype 001; which seems more prevalent in Central Europe (Jones et al. 2013).

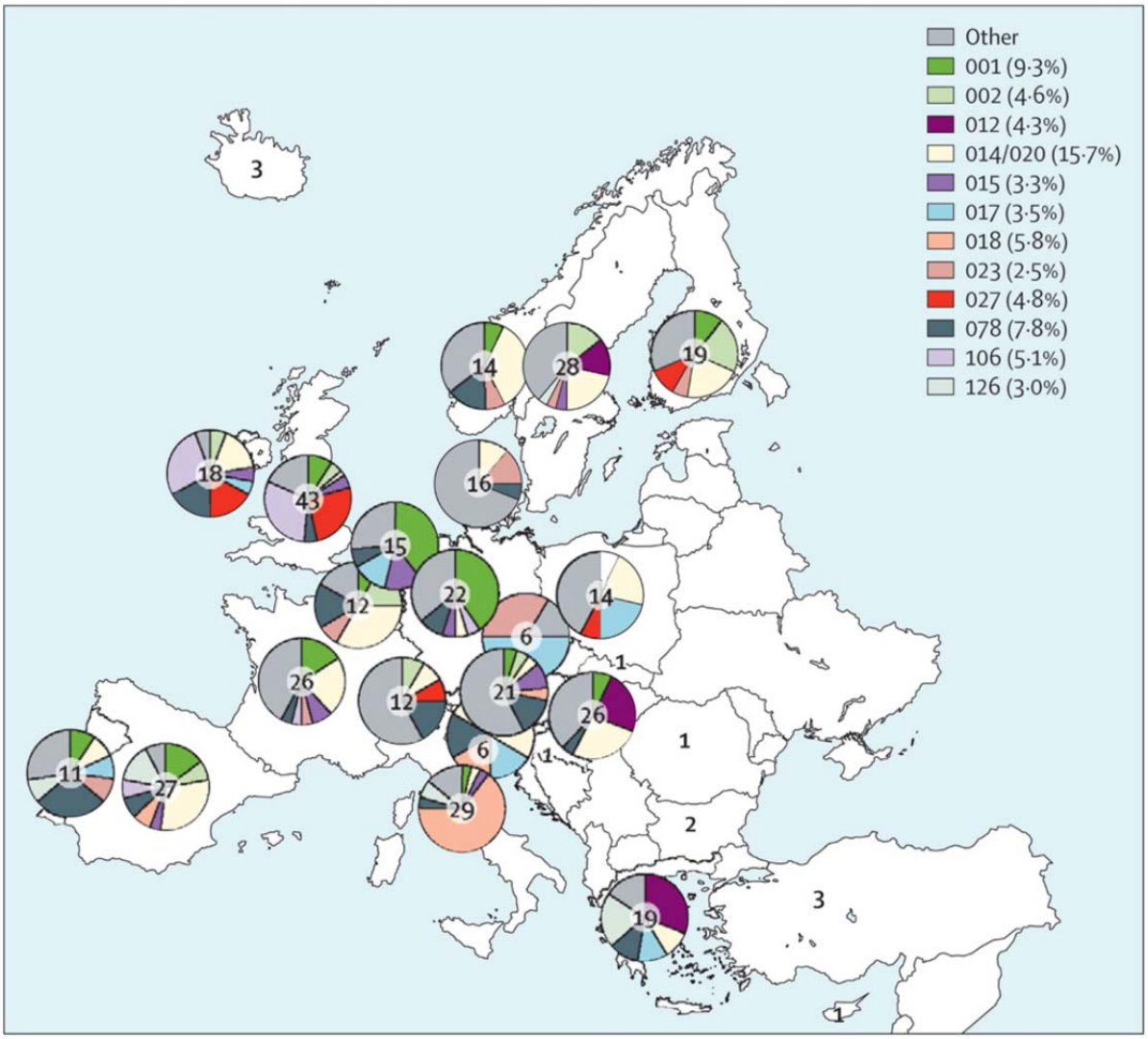

Figure 1 European distribution of ribotypes (Jones et al. 2013). Geographical distribution of *C. difficile* PCR-ribotypes in European countries with more than five typable isolates, November 2008. Pie charts show proportion of most common PCR-ribotypes per country. The number in the centre of the pie charts is the number of typed isolates within the country. No data from Greenland and Iceland.

The ribotype distribution in Denmark is diverse. A Danish cohort investigation of the incidence of CDI in Danish patients attending general practice from August 2009 – February 2011, found 69 different ribotypes among 257 toxigenic *C. difficile* strains accessible for PCR-ribotyping. Ribotype 014-020-077 was the predominant type found in 28% (72) of the patients and were mainly community-acquired CDI (Table 1).

Table 1 Distribution of ribotypes (PCR) related to origin of infection and age, Denmark 2009 - 2011, Søes et al. 2013 (n=259)

PCR ribotype	No. of *C. difficile* strains	Binary toxin-positive[a]	Origin of *C. difficile* infection						Age groups			
			Community-associated CDI		Healthcare-associated CDI		Unknown origin or no data		<2 years of age		≥2 years of age	
	n	*n*	*n*	(%)	*n*	(%)	*n*	(%)	*n*	(%)	*n*	(%)
014-020-077	72	0	64	(32)	4	(13)	4	(14)	43	(60)	29	(40)
106-117	16	0	9	(5)	3	(10)	4	(14)	7	(44)	9	(56)
027	11	11	2	(1)	7	(23)	2	(7)	1	(9)	10	(91)
001	10	0	8	(4)	1	(3)	1	(4)	7	(70)	3	(30)
015	10	0	9	(5)	1	(3)	0	(0)	5	(50)	5	(50)
066	9	9	6	(3)	0	(0)	3	(11)	5	(56)	4	(44)
DK0086	9	0	7	(4)	0	(0)	2	(7)	4	(44)	5	(56)
012	8	0	7	(4)	0	(0)	1	(4)	6	(75)	2	(25)
023	6	6	6	(3)	0	(0)	0	(0)	2	(33)	4	(67)
070	6	0	4	(2)	2	(6)	0	(0)	1	(17)	5	(83)
078	6	6	4	(2)	2	(6)	0	(0)	1	(17)	5	(83)
DK0020	6	0	5	(3)	1	(3)	0	(0)	2	(33)	4	(67)
029	5	0	3	(2)	1	(3)	1	(4)	3	(60)	2	(40)
DK0053	5	5	2	(1)	1	(3)	2	(7)	0	(0)	5	(100)
DK0061	5	0	5	(3)	0	(0)	0	(0)	1	(20)	4	(80)
Minor PCR ribotypes[b]	73	15	57	(29)	8	(26)	8	(29)	–	–	–	–
Unknown PCR ribotype	2	0	–	–	–	–	–	–	2	–	0	–

[a] *C. difficile* strains with the toxin gene profile: *tcdA+*, *tcdB+*, *cdtA+/cdtB+*

[b] 54 different PCR ribotypes with four or fewer patients represented in each

3.5 Summary ribotypes

Due to the number of hospital outbreaks and hospital associated cases the focus in the previous and current surveillance programs of *C. difficile* strains has been on ribotype 027. The virulence factors are, however, not unique for ribotype 027, but are also present in other ribotypes such as the emerging binary toxin-positive ribotype 078 strain, which has similar mechanisms for hyperproduction of toxins as ribotype 027. Ribotype 078 is the predominant type found in pigs, calves and their immediate environment, and has also increased in occurrence for both the hospital- and community-acquired CDI. Ribotype 078 is now more commonly reported than ribotype 027 in humans. Other ribotypes have also been reported to cause human disease, therefore *C. difficile* ribotype 027 can no longer be considered the only ribotype associated with severe disease, and efforts to control CDI should target all virulent strains of *C. difficile* and not only ribotype 027. The importance of the different virulence genes regardless of ribotypes should be taking into consideration when designing surveillance programs of *C. difficile*.

4. Resistance in *C. difficile*

C. difficile are not yet significant resistant to the antibiotics used in treatment. The risk of developing CDI increases though if the C. difficile strain is resistant to the antimicrobial agents used in antimicrobial therapy. The occurrence of CDI is indirectly linked to the use of antibiotics as CDI develops in connection with or after the consumption of antibiotics.

C. difficile are naturally resistant to cephalosporins and display reduced susceptibility to a range of different antimicrobials used for clinical treatment of human infections (Table 2). The European Committee on Antimicrobial Susceptibility Testing (EUCAST)[5] has set epidemiological break-points for *C. difficile* for some of these antimicrobials, and resistance to fluoroquinolones (moxifloxacin), lincosamides (clindamycin), macrolides (erythromycin) and tetracyclines are common (EUCAST 2014, includes isolates from humans, animals, food and environment).

Fluoroquinolones and 3rd- or 4th-generation cephalosporins constitute the first-line therapy for invasive Gram-negative bacterial infections such as *Salmonella* and *E. coli.* Similarly, macrolides, fluoroquinolones and on occasion tetracyclines are used to treat enteric *Campylobacter* species infections in humans when treatment is considered necessary. A review conducted by Dhara Shah et al. from the University of Houston College of Pharmacy, Texas, provides an update on the in vitro susceptibility and new antibiotic treatment options for CDI and points out that a number of *C. difficile* strains with decreased susceptibility to metronidazole have been reported (Shah et al. 2011). Metronidazole is a nitroimidazole antibiotic used particularly for anaerobic bacteria and protozoa, and is used for first episodes of mild-to-moderate CDI.

Occurrence of fluoroquinolone resistance in *C. difficile* has been increasing for more than a decade, mainly due to emergent spread of the fluoroquinolone resistant *C. difficile* ribotype 027 clones at hospitals (Mcdonald et al. 2005; Rupnik et al. 2009). The use of fluoroquinolones in treatment of patients with general infections has been implicated in outbreaks caused by the ribotype 027 strain (Hookman & Barkin 2009).

C. difficile strains can also carry resistance to vancomycin. Vancomycin is used in the treatment of a number of bacterial infections including serious infections caused by Gram-positive bacteria known or suspected to be resistant to other antibiotics. Vancomycin is recommended as treatment for CDI. In Denmark, it is believed that there is a connection between an increased usage of vancomycin in treatment of serious CDI and an increased occurrence of vancomycin resistant enterococcus (VRE), mainly in hospitals on Zealand where the number of CDI is higher than in the rest of the country (Epi-Nyt Statens Serum Institut week 16/17 2014).

It has been speculated if and how antibiotic resistance is a driver of the epidemic of CDI. One explanation can be that the antimicrobial resistant strain of *C. difficile* will be able to colonize and multiply in the gut and initiate CDI in a case as soon as the normal microbiota is sufficiently disrupted, whereas the sensitive strain will be inhibited until such time as treatment with the drug ceased and the gut levels have fallen below the inhibitory threshold. Furthermore if the first case develops CDI, the environment becomes contaminated with spores of the re-

[5] EUCAST is a standing committee jointly organized by the European Society of Clinical Microbiology and Infectious Diseases (ESCMID), the European Centre for Disease Prevention and Control (ECDC) and the European national breakpoint committees. EUCAST deals with breakpoints and technical aspects of phenotypic in vitro antimicrobial susceptibility testing and functions as the breakpoint committee of the European Medicines Agency (EMA) and ECDC. Most antimicrobial MIC (the minimum inhibitory concentration) breakpoints in Europe have been harmonised by EUCAST.

sistant *C. difficile* strain. This, in turn, increases the likelihood that subsequent cases will ingest spores of an antibiotic resistant strain, rather than a susceptible strain. The epidemiology of CDI is therefore complex and there are likely to be a range of other drivers, as other characteristics of the epidemic strains themselves (e.g. ability to stimulate toxin production, sporulation, and ability to disrupt the normal gut microbiota), host population factors, hospital hygiene and infection-control practices, and the use of other drug therapies (Coia 2009). It is important to keep in mind that the relative risk of a particular antibiotic and its association with CDI depends on the local prevalence of strains and the resistance pattern to a given antimicrobial agent (DePestel & Aronoff 2013).

Table 2 Resistance *C. difficile*, EUCAST 2014

	MIC[1] range EUCAST	ECOFF[2]	Observations	No. Resistant	% Resistant EUCAST
Cephalosporins	-	-	-	-	Natural resistance
Benzylpenicillin	0,016-32	No EOFF	2238	-	-
Piperacillin/	0,016-256/	No EOFF	930/	-	-
Piperacillin-tazobactam	0,25-64	No EOFF	3089		
Amoxicillin	0,032-64	No EOFF	675	-	-
Ertapenem	2-16	No EOFF	276	-	-
Meropenem	0,125-32	No EOFF	584	-	-
Ciprofloxacine	8-256	No EOFF	528	-	-
Linezolid	0,125-16	No EOFF	215	-	-
Fidaxomicin	0,004-2	No EOFF	1183	-	-
Teicoplanin	0,016-0,5	No EOFF	401	-	-
Moxifloxacine	0,25-128	4.0	5225	2234	42,76%
Clindamycin	0,016->512	16.0	5217	947	18,20%
Rifampicin	0,002-128	0.0040	3393	1986	58,53%
Erythromycin	0,032->512	2.0	4281	2664	62,20%
Tetracyclines	0,008-256	0.25	1098	194	17,70%
Tigecycline	0,016-2	0.25	3579	16	0,40%
Metronidazole	0,016-64	2.0	7842	56	0,71%
Vancomycin	0,032-32	2.0	7955	34	0,43%
Fusidic acid	0,032-256	2.0	1102	5	0,45%
Daptomycin	0,032-4	4.0	206	0	Susceptible

Note: The distributions and % resistant should never be referred to in any epidemiological context since data from many sources (isolates from human, animals, food and environment), time periods and countries have been aggregated. Table from http://mic.eucast.org, accessed December 2014.

1) MIC: The minimum inhibitory concentration (the basis of phenotypic methods).
2) Epidemiological cut-offs (ECOFFs) have been used in preference to clinical breakpoints in veterinary resistance surveillance studies to describe the upper MIC limit of the susceptible peak in an MIC distribution, the wild-type (WT) population. An ECOFF value is not defined if too few isolates are available or if the data are not considered sufficiently reproducible or clear enough to set an ECOFF. Additional distributions are continually being added to the database and distributions are reviewed in the light of new data.

In a risk prioritization of bacteria conducted by the Centers for Disease Control and Prevention (CDC) in 2013, the agents were divided in three categories: urgent, serious, and cause for concern. *C. difficile* was categorised as an urgent threat due to the high numbers of Americans that are affected each year. In total, 250,000 cases of CDI are hospitalised each year in the United States. In most of these cases, the use of antibiotics was a major contributing factor leading to infection. At least 14,000 of the CDI cases were fatal. The cases were mainly hospital associated infections, but with the potential risk that infections can be spread from the hospital environment to the community. Although *C. difficile*, at this stage, does not have significant resistance to the antibiotics used to treat CDI, antibiotic resistance was included in the threat assessment because of *C. difficiles* unique relationship with resistance issues, antibiotic use, and the high morbidity and mortality (Center for Disease Control and Prevention 2013).

The high risk category is due to the high number of cases, the high fatality rate, the fact that *C. difficile* can spread rapidly, and that it is of critical importance to public health to identify and reduce infection.

4.1 Resistant *C. difficile* in animals in Denmark

In Denmark, an investigation from 2010 showed high levels of resistance towards clindamycin in *C. difficile* from broilers (100 %, n=6), pigs (81 %, n=14) and cattle (93 %, n=26). A few isolates from pigs and cattle were also resistant to fluoroquinolones (moxifloxacin, n=1 and n=2) and one pig isolate were resistant to macrolides (erythromycin). None of the Danish isolates were resistant to metromidazole, which is the drug of choice for first episodes of mild-to-moderate CDI (DANMAP 2010). In 2011, the occurrence of *C. difficile* in Danish pig farms decreased from 15% in 2010 to 2.8% in 2011 when the voluntary ban of cephalosporins in pig production was effectuated. This could indicate that the use of cephalosporins can influence the occurrence of *C. difficile*. In the same period the occurrence of *C. difficile* in cattle at slaughter was tested, here no major changes in the antimicrobial usage (including cephalosporins) had been made and notable no changes in the occurrence of *C. difficile* were observed (15% in both years) (DANMAP 2012). The data are though at present limited and the effect of a reduced use of specific antibiotics on the occurrence of *C. difficile* in livestock is unknown.

4.2 Summary resistance in *C. difficile*

C. difficile are naturally resistant to cephalosporins and according to EUCAST display reduced susceptibility to a range of different antimicrobials used for clinical treatment of human infections: Fluoroquinolones, macrolides, tetracyclines and vancomycin. The occurrence of CDI is indirectly linked to the use of antibiotics, as CDI develops in connection with or after the consumption of antibiotics. There are though most likely a range of other drivers, as the characteristics of the *C. difficile* strains themselves (e.g. the ability to stimulate toxin production, sporulation, and ability to disrupt the normal gut microbiota) also host population factors, hospital hygiene and infection-control practices, and the use of other drug therapies are important factors in order to develop CDI. Although *C. difficile*, at this stage, does not have significant resistance to the antibiotics used to treat CDI, antibiotic resistance was included in the threat assessment conducted by CDC in 2013, because of *C. difficiles* unique relationship with resistance issues, antibiotic use, and the high morbidity. In Denmark, an investigation in animals showed high levels of resistance towards clindamycin in *C. difficile* isolates from broilers, pigs and cattle. A few isolates from pigs and cattle were also resistant to fluoroquinolones. When the voluntary ban of cephalosporins in pig production was effectuated the occurrence of *C. difficile* in Danish pig farms decreased. This could indicate that the use of cephalosporins can influence the occurrence of *C. difficile*. The effect of a reduced use of antibiotics on the occurrence of *C. difficile* in livestock is unknown.

5. Surveillance of *C. difficile* in humans in Europe and Denmark

Surveillance of C. difficile is at this stage not mandatory in the EU, and focus has mainly been on ribotype 027 in those countries that have established surveillance programs. Therefore, the prevalence and the distribution of other ribotypes are not known at the European level. In Denmark, the monitoring of ribotype 027 has been intensified after several hospital outbreaks.

5.1 Surveillance of *C. difficile* in humans in Europe

Consensus recommendations for surveillance, including case definitions, were published in 2006 by the European Centre for Disease Prevention and Control (ECDC) and in 2007 by the CDC (DePestel and Aronoff 2013). Since 2005, individual countries in Europe have developed surveillance studies of the spread of *C. difficile* ribotype 027 and by 2008, the ribotype had been detected in 16 European countries[6] including Denmark (Kuijper et al. 2008). In Europe, the incidence of hospital-acquired CDI per 10,000 patient days ranges from 0 in Luxembourg and Turkey to 19.1 in Finland. In Austria an increase of 255% between 2003 and 2007 was recorded by the Austrian National Reference Centre for *C. difficile*. A Spanish study reported a significant increase in CDI between 1999 and 2007 from 3.9 to 12.2 cases per 10,000 hospitalized patients (Jones et al. 2013). In contrast, England and Wales have seen a significant reduction in the overall number of CDI cases; by >50% from 2008 - 2010. The reduction in cases was associated with a substantial decrease in the proportion of CDI cases caused by ribotype 027. The reductions in infection rate and prevalence of ribotype 027 were manifested by a reversal of the trend of increasing reports of *C. difficile* related deaths up until 2007, where the number of death certificates in England mentioning *C. difficile* decreased between 2007 and 2010 by 70%. While multiple interventions in hospitals and healthcare institutions likely contributed to these changes, providing timely information on which ribotypes were causing CDI cases, and especially clusters, probably helped infection control teams to focus prevention measures in human healthcare more effectively (Jones et al. 2013).

In a pan-European surveillance study performed in 2008 in 34 European countries, the most frequently reported *C. difficile* toxigenic strains were ribotype 117, 014 and 020 (16%), 001 (10%), 078 (8%), 018 (6%) and 106, 027 and 002 (5%) (Jones et al. 2013). In a Dutch hospital survey, the most frequent ribotypes among the 911 *C. difficile* isolates were ribotypes 014 (16%), 001 (14%), 078 (12%), 002 (6%) and 005 (5%) (NethMap 2014). A recent survey in the United States[7] revealed that 94.0% of persons with CDI received health care; of these, 75.0% had onset among persons not currently hospitalized, including recently discharged patients, outpatients, and nursing home residents. The same kind of data are not available for Europe, but according to the author these data emphasize the importance of obtaining a complete patient history to correctly diagnose community-onset, healthcare associated CDI (Jones et al. 2013).

5.2 Surveillance of *C. difficile* in humans in Denmark

In the recent years, there have been several hospitals outbreaks of *C. difficile* ribotype 027 in Denmark, and ribotype 027 is considered endemic in the hospitals in the Capital Region and on Zealand. As a result of these current hospitals outbreaks the Danish Health and Medicines Authority[8] has intensified the monitoring of ribotype 027.

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[6] Austria, Belgium, Denmark, Finland, France, Germany, Ireland, Luxembourg, The Netherlands, Norway, Poland, Spain, Sweden, Switzerland and the United Kingdom (England, Wales, Northern Ireland and Scotland)

[7] Conducted by the Centers for Disease Control and Prevention (CDC) and identified in Emerging Infections Program data in 2010

[8] Sundhedsstyrelsen

5.2.1 Criteria for the surveillance of *C. difficile*

The national Registry of Enteric Pathogens includes weekly case-based notifications of cultures positive for *C. difficile* from all departments of clinical microbiology of regional hospitals in the country. A second case-based database, the *C. difficile* Microbiological Database, which is separate from the Registry of Enteric Pathogens, contains information on isolates that undergo genotypic toxin detection and PCR-ribotyping at the National Reference Laboratory at SSI. Isolates are forwarded by the departments of clinical microbiology if they are: 1) resistant to fluoroquinolones, 2) if severe clinical cause is observed, 3) if an outbreak is suspected or 4) if the genes encoding for toxin A, toxin B and the binary toxin are detected (Figure 2) (Bacci et al. 2011).

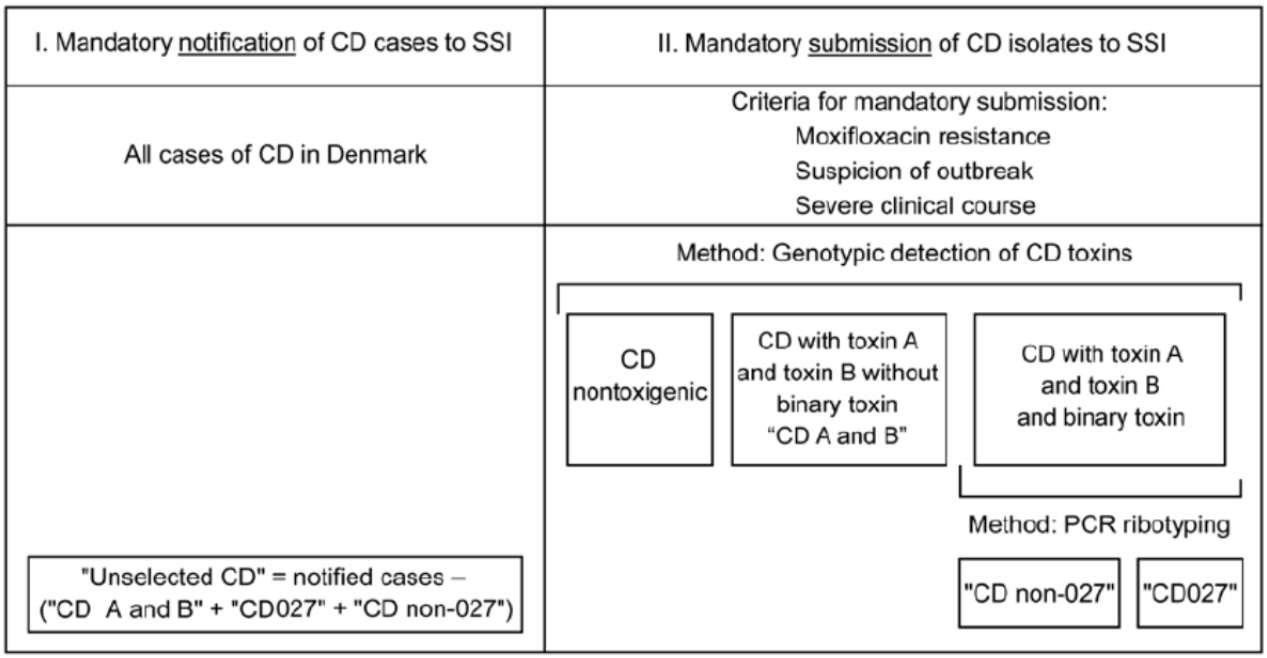

Figure 2 Description of *C. difficile* infections surveillance in Denmark, with the 4 groups of *C. difficile*–infected patients included in the study, week 1, 2008–week 22, 2009. SSI (Bacci et al. 2011)

These criteria were established in 2007, when sporadic cases of *C. difficile* ribotype 027 were found for the first time in Denmark. They were reinforced in 2009, after the first large ribotype 027 outbreak involving different hospitals of the Copenhagen Capital Region (Bacci et al. 2011). For laboratories performing diagnostics that includes markers for ribotype 027 it is not required to submit these isolates, unless *C. difficile* with toxin TcdA, TcdB and the binary toxin without a marker for 027 is detected. These non-ribotype 027 coding for the virulence genes (*tcd*A, *tcd*B and the binary toxin) will then be serotyped at SSI (Epi-Nyt Statens Serum Institut week 7/8 2012).

5.2.2 Surveillance of *C. difficile* ribotype 027

In the Danish surveillance from 2009 – 2013 of *C. difficile* ribotype 027 with the virulence toxin TcdA, TcdB and the binary toxin, a total of 4,347 patients were reported (table 3). In 2012 and

2013, the incidence decreased compared to 2011. The reason for the high number of cases in 2011 is unknown.

Table 3 C. *difficile* ribotype 027 with toxin A, B and the binary toxin in Danish regions, number of patients 2009 - 2013

Year	Copenhagen	Zealand	South Denmark	Mid Denmark	North Denmark	Denmark total
2009	492	98	4	3	1	598
2010	703	152	1	7	3	866
2011	657	438	9	12	42	1,158
2012	429	304	25	13	42	813
2013	722	136	18	25	11	912
I alt	**3,003**	**1,128**	**57**	**60**	**99**	**4,347**

(Source: Statens Serum Institut overvågningsdata 2009 - 2013)

In 2011, PCR ribotyping of 677 *C. difficile* isolates received as part of the surveillance at SSI showed that the predominant ribotype was 027 (81%), ribotype 078 accounted for 7% and ribotype 066 for 3%. Beside these three ribotypes over 30 different ribotypes were detected, all strains were positive for TcdA, TcdB and the binary toxin (Epi-Nyt Statens Serum Institut week 7/8 2012). It must be noted, that these results not necessarily are representative for the ribotypes prevalent in Denmark due to the reporting criteria 1-4 (See 5.2.1).

In a Danish cohort study of CDI patients attending general practice 2009 – 2011, the mean annual incidence was 34/100,000 persons. For CDI patients ≥60 years of age, the annual incidence was 46/100,000 (Søes et al. 2013). In comparison, the incidence for infections due to *Salmonella* and *Campylobacter* in 2011 was 21/100,000 and 73/100,000 respectively.

Most patients 2009 – 2013 were reported from the Copenhagen area (69.1%) and Zealand (25.9%). This trend seems to be the same for 2014 as patients reported until July this year are from the Copenhagen area, corresponding to 89.5% (data not shown). The reasons for this geographical difference might be explained by differences in local typing methods and reporting systems, and a higher incidence in the capital area and Zealand (personal message Søren Persson, Statens Serum Institut).

5.2.3 Clinical manifestation from the surveillance of ribotype 027

In a three quarter period from 2008 – 2009[9], SSI registered 60 *C. difficile* cases as ribotype 027 defined by the resistance and toxin pattern. A total of 32 (54%) were women, and the median age was 81 years. During the two months up to diagnosis 54 (92%) had been hospitalized at least once, and 55 (93%) had, according to their medical journal received antibiotics. Diarrhea without other symptoms was reported by 32 (53%), while the other had severe manifestations as clinical sepsis and PMC. In eight cases, CDI might have been contributing to the cause of death.

[9] From week 29 of 2008 to week 10, 2009

5.3 Summary surveillance

Surveillance of *C. difficile* is at this stage not mandatory in the EU and the distribution of the different ribotypes beside 027 is not known at the European level. In Denmark, ribotype 027 is endemic in the hospitals in the Capital Region and on Zealand. The Danish monitoring of *C. difficile* is based on four criteria: 1) resistant to fluoroquinolones, 2) if severe clinical cause is observed, 3) if an outbreak is suspected or 4) if the genes encoding for toxin A, toxin B and the binary toxin are detected. Due to differences in prioritizing, typing methods and national reporting systems, the data are incomparable within both the EU and even within countries, including Denmark. Some investigations have been conducted; showing an increase of community-acquired CDI. Ribotype 027 is mainly associated to healthcare-acquired CDI, as a more diverse prevalence of ribotypes are seen in the community-acquired CDI. Though an increase has been reported from most of the EU countries England and Wales have due to different intervention in hospitals and healthcare institutions seen a notable decrease in the number of CDI and CDI associated deaths. The importance of the different virulence genes regardless of ribotypes should be taking into consideration when designing surveillance programs for *C. difficile*.

6. Use of antibiotics in human therapy and the development of *C. difficile infection*

C. difficile is recognized as the main cause of infectious diarrhea that develops in patients after hospitalization and exposure to antibiotic treatment. The association between antimicrobial therapy and CDI has been almost universal, as C. difficile capability to colonize the gut only is possible if the normal intestinal microbiota is disrupted by e.g. the use of antibiotics or is absent.

All antibiotics have been associated with subsequent CDI, but some antibiotics are associated with higher risk than others, including penicillin, cephalosporin and clindamycin (Mcdonald et al. 2005; Hookman and Barkin 2009; Rupnik et al. 2009). In general, expanded-spectrum and broad-spectrum cephalosporins and clindamycin are the most frequently implicated antibiotics in CDI (Freeman et al. 2010).

Figure 3 shows the relationship between antibiotic and *C. difficile*, and how the presence of antibiotic enables the bacteria to colonize the intestine after the antibiotic has disrupted the normal gut flora.

Clindamycin has been shown to have a large negative impact on the intestinal microbiota as seen by reduced resistance to colonization by pathogens, leading to a high risk for PMC due to *C. difficile* overgrowth. *C. difficile* may increase in number as a consequence of antibiotic-induced disturbances, in particular following suppression of the normal beneficial members of the anaerobic microbiota in the intestine (Rupnik et al. 2009; Jernberg et al. 2010). During the last decade particular, fluoroquinolones have been an increasing problem, and all fluoroquinolones have been implicated in CDI, including levofloxacin, moxifloxacin, gatifloxacin and ciprofloxacin (Mcdonald et al. 2005; Hookman and Barkin 2009; Rupnik et al. 2009). The rise in the fluoroquinolone-associated risk has been coincided with the rising incidence of *C. difficile* ribotype 027 and other strains that carry high-level fluoroquinolone resistance (Rupnik et al. 2009). The excessive use of these agents is probably responsible for the worldwide epidemic of fluoroquinolone-resistant ribotype 027 infections (Rupnik et al. 2009).

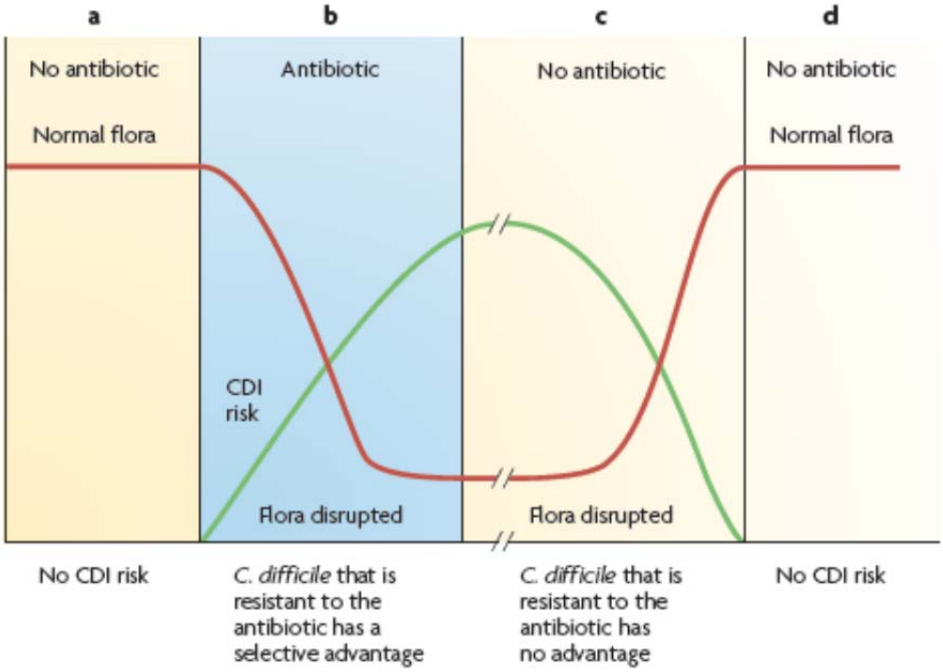

Figure 3 The effect of antibiotics on the normal gut flora and the risk of CDI (Rupnik et al. 2009).
Patients are resistant to CDI if their normal gut flora is not disrupted by antibiotics (a). Once antibiotic treatment starts, infection with a *C. difficile* strain that is resistant to the antibiotic is more likely while the antibiotic is being administered owing to the presence of the antibiotic in the gut (b). When the antibiotic treatment stops, the levels of the antibiotic in the gut diminish rapidly, but the microflora remains disturbed for a variable period of time (indicated by the break in the graph), depending on the antibiotic given (c). During this time, patients can be infected with either resistant or susceptible *C. difficile*. Finally, after the microflora recovers, colonization resistance to *C. difficile* is restored (d) (Rupnik et al. 2009).

7. Epidemiology of *C. difficile* infection in humans

Antimicrobial therapy is the most widely reported risk factor for CDI in humans, and traditionally, elderly and hospitalized patients who had received antibiotic therapy were considered to be the most vulnerable to CDI. Because these high-risk patients are primarily located in healthcare facilities, CDI was regarded as a primarily nosocomial disease for many years. This disease pattern has changed, as persons outside hospitals and healthcare facilities increasingly are developing CDI.

7.1 Community acquired *C. difficile*

According to the scientific literature there have been two main changes in the epidemiology of CDI over the past 10 – 15 years. First an international increase in the incidence and severity of hospital-acquired CDI, with large outbreaks, high mortality rates, and poorer response to treatment. This has been largely attributed to the emergence and dissemination of the virulent ribotype 027 but other factors, such as the increasing use of fluoroquinolones, may also be involved. The second change has been the increasing incidence of community-acquired CDI among the younger populations in the community, who were historically considered to be at low risk, such as healthy pregnant women, children, patients without an antibiotic history, and those with minimal or no recent health care exposure (Rupnik et al. 2009; Weese 2010; DePestel and Aronoff 2013). Data from North America and Europe suggest that approximately

20% to 27% of all CDI cases are community-acquired, with an incidence of 20 to 30 per 100,000 population (DePestel & Aronoff 2013). No outbreaks of CDI have been reported among humans in the community, which might suggest that host factors vulnerability to CDI are of more importance than an increased exposure to *C. difficile* (Hensgens et al. 2012; Jones et al. 2013).

The reason for the increased incidence of community-acquired CDI among the populations in the community without an antibiotic history, and those with minimal or no recent health care exposure is unknown. In The Netherlands, an overlap between the location of pig farms and the occurrence of human *C. difficile* ribotype 078 infections was observed. The fact that infections with ribotype 078 in humans occurred in a younger population and were more frequently community-acquired than infections with ribotype 027 strains, together with the fact that 078 is the predominant ribotype in piglets, suggest a common source (Hensgens et al. 2012). Elderly hospitalized patients receiving antibiotics are though still the main group at risk of infection caused by *C. difficile* (Rupnik et al. 2009).

7.2 Investigation of community acquired *C. difficile infections* in Denmark

SSI and Odense University Hospital have investigated the incidence of community-acquired CDI among patients in general practice in Denmark. Faecal samples from the patients were examined for both common pathogenic intestinal bacteria and for *C. difficile*. A survey was conducted among the *C. difficile* positive patients to identify the symptoms and risk factors for community-acquired CDI. In total, 259 CDI cases and 455 non-CDI cases were included in the study. The study divided cases in healthcare-acquired and community-acquired CDI[10]. Overall, 69% of cases aged ≥ 2 years were found to be community-acquired CDI. Cases in the community, who were admitted directly to hospital because of severe diarrhea, are missing in the study, leading to a possible underestimation of community-acquired cases. Toxin A and B and genes encoding for the binary toxin were present in 35 (25 %) of cases over 2 years of age. The genes for the binary toxin were found more often in cases with healthcare-acquired CDI [12 out of 31 cases (39 %)] compared to community-acquired CDI [27 out of 200 cases (14 %)] (OR 4.0; 95 % CI 1.8–9.3). In total, 69 different ribotypes were identified among the 257 toxigenic *C. difficile* strains. Ribotype 014-020-077 was the predominant type found in 72 (28 %) of the CDI patients, ribotype 027 was present in 11 (4 %) cases. The most prevalent ribotypes among the binary toxin-positive *C. difficile* strains were ribotype 027, 066, 078, 023 and DK0053[11]. Cases with community-acquired CDI were found to be infected by a more heterogeneous spectrum of ribotypes than cases with healthcare-acquired CDI. Comparing ribotype 014-020-077 to 027 in terms of origin of infection, the latter was statistically significantly more often present in patients with healthcare-acquired CDI compared to patients with community-acquired CDI (OR 56; 95 % [CI 8.6–362]) (Søes et al. 2014).

[10] Community-acquired CDI cases had neither been admitted to hospital in the 6 months prior to onset of infection nor did they have any antibiotics in the 3 months prior to onset of symptoms.
[11] Strains with a unique PCR-ribotype profile not matching any strain in the reference strain collection were assigned a DK number.

It is interesting that the cases with community-acquired CDI have a more heterogeneous spectrum of ribotypes than the cases with healthcare-acquired CDI. This could suggest a more diverse transmission and exposure in the cases of community-acquired CDI than the CDI acquired in a healthcare setting. The ribotype data presented in this Danish study differs from what was reported from both the pan-European surveillance study (2008) and the sentinel surveillance in The Netherlands (2013-2014)[12], where ribotype 078 was reported more often than ribotype 027 (Freeman et al., 2010; NethMap 2014; Eighth Annual Report 2014). This could suggest another prevalence of ribotypes in Denmark compared to The Netherlands and the average reported in the pan-European surveillance study. Though only a low number of ribotype 078 were detected in the Danish study, they were more frequently associated with community-acquired CDI (2%) than ribotype 027 (1%), which also were the case for ribotype 014-020-077 of which 32% were community-acquired CDI and 13% associated to a healthcare-acquired CDI. Ribotype 027 was the most often involved ribotype in the healthcare-acquired CDI (23%) (Søes et al. 2014). All strains were positive for TcdA, TcdB and the binary toxin and considered to be hypervirulent.

7.3 Risk factors for *C. difficile* infection in humans

Antimicrobial therapy is the most widely reported risk factor for CDI in humans (Hookman and Barkin 2009; Weese 2010). It is scientifically accepted, that an exposure to antibiotics disrupting the colonic microbiota in the intestine is leading to an overgrowth by *C. difficile*. Many patients are asymptomatic carriers of *C. difficile* on hospital admission, and they may develop CDI after they are treated with antibiotics (Jernberg et al. 2010). Exposure to antibiotics not only increases the risk of CDI during receipt of antibacterial therapy but also increases the risk of CDI in the 3 months after end of treatment, with the highest risk during the first month. Importantly, receipt of antibiotics during and after treatment of CDI has been associated with lower cure rates, prolonged time to diarrhea resolution, and a trend toward recurrent CDI (DePestel & Aronoff 2013). A study concerning ripotype specific risk factors for CDI in The Netherlands found that prior use of fluoroquinolones (mainly ciprofloxacin) was associated with CDI due to ribotype 078 (Keessen et al. 2013).

A Danish case-control study of CDI in the community (both rural and urban area) conducted in 2009 – 2011 confirmed hospitalization and the use of antibiotics as risk factors for CDI for patients aged ≥2 years. The study also found that beef consumption was associated with CDI in cases ≥ 2 years of age (OR=5.5, 95% [2.0-15]). The research group behind the Danish study does not refer to similar findings (beef being a risk factor for CDI) in other studies, nor could it be confirmed by the literature examined by this report. Ribotype 078 was detected in four out of the five cases reporting beef consumption (Søes et al. 2013), but many other *C. difficile* ribotypes were also found to be present in the cases consuming beef, and so the findings of ribotype O78 could not exclusively indicate beef as a source of infection. In 2010 in connection with the DANMAP[13] program, both cattle and pigs were investigated for the presence of *C. difficile*.

[12] See chapter 3.2

[13] DANMAP - Use of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals, food and humans in Denmark

Three isolates isolated from cattle all belonged to the same ribotype named DK136. One human case with ribotype DK136 has been reported in Denmark (DANMAP 2010).
This does not confirm that cattle are the source of human CDI, but indicate an overlap between an animal and human reservoir.

For a subgroup of cases aged <2 years, including only cases and controls without other generally accepted enteropathogens than *C. difficile*, contact with animals was significantly associated with CDI (Søes et al. 2013). However infants under the age of 2 years have been known to have *C. difficile* as part of the normal intestinal ecology. Also many different animals species (dog, cat, guinea pig, rabbit, horse, cattle and pig) were included in the animal category, so this result may not reflect a risk of having contact with production animals, but having contact with animals in general. It has been suggested that exposure to household contacts with CDI and children less than 2 years of age, could serve as a potential reservoir for transmission (DePestel & Aronoff 2013), but this was not confirmed by the Danish study.

Findings by Hookman and Barkin (2009) suggest that asymptomatic carriers of epidemic and non-epidemic *C. difficile* strains have the potential to contribute significantly to disease transmission in long-term care facilities. Spores on the skin of asymptomatic patients were transferred easily to investigators' hands. It is not known if this transmission of *C. difficile* spores play a role in community-acquired CDI, but *C. difficile* spores from human or environmental sources are presumably a common sources of transmission to people (Stanley et al. 2013).

7.4 Summary of epidemiology and risk factors for *C. difficile* infection in humans

Over the past 10 – 15 years there has been an increased incidence of community-acquired CDI among the younger populations, who were historically considered to be at low risk, such as healthy pregnant women, children, patients without an antibiotic history, and those with minimal or no recent health care exposure. The reason for this increase is unknown. Antimicrobial therapy is the most widely reported risk factor for CDI in humans. A study concerning type specific risk factors for human CDI in The Netherlands found that prior use of fluoroquinolones (mainly ciprofloxacin) was associated with CDI caused by ribotype 078. No outbreaks of CDI have been reported among humans in the community, which might suggest that host factors vulnerability and the use of antibiotics are of more importance than the exposure to *C. difficile*.
A Danish case-control study for CDI in the community confirmed hospitalization and the use of antibiotics as risk factors for CDI for patients aged ≥2 years. The study also found that beef consumption was significantly associated with CDI in cases ≥ 2 years, but this could not be confirmed by the references in this report. The same study found that contact with animals for children under 2 years of age was significantly associated with CDI. However many different species were included in the animal category (including pets and wild life animals), so it may not reflect a risk of having contact to production animals, but having contact to animals in general.
It has been suggested that asymptomatic carriers of epidemic and non-epidemic *C. difficile* strains have the potential to contribute significantly to disease transmission in long-term care facilities. It is not known if this transmission of *C. difficile* spores plays a role in community-acquired CDI, but *C. difficile* spores from human or environmental sources are presumably a common source of transmission.

8. Investigations of *C. difficile* in animals and food

Several studies have examined if there could be a possible zoonotic link between the increased incidence in humans and the animal reservoir. It is therefore reasonable to investigate transmissions routes known from other zoonotic bacterial agents e.g. Salmonella, Campylobacter and Listeria. In the recent years several studies in Europe, Canada, United States, Australia and Africa have detected and confirmed the findings of C. difficile in different food, production animals and meat in retail outlets, vegetables (including ready-to-eat salads) and in different types of water. In The Netherlands investigators found that human isolates and porcine ribotype 078 isolates were indistinguishable evaluated by pulsed-field gel electrophoresis (PFGE), multilocus variable-number tandem repeat analysis (MLVA) and whole genome analysis (WGS). The different findings indicate an established reservoir for C. difficile in the global food production chain, but the role of C. difficile as a zoonotic agent is not fully understood. Data are though limited as few investigations have been reported and the impact on CDI are unknown.

8.1 *C. difficile* in animals

In animals, *C. difficile* was mainly known as an important pathogen in horses, although it has been reported to infect numerous wild and production animals; poultry, sheep, pigs, chickens, goats, cattle and calves and *C. difficile* are now a major cause of neonatal enteritis in piglets (Songer & Anderson 2006; Indra et al. 2009). Numerous studies have been conducted as reviewed by Keesen et al. 2011 (table 4). Investigators in The Netherlands found that ribotype 078 strain of *C. difficile* is the second most common strain causing human infections and the same strain is commonly found in pigs. A Canadian study has demonstrated that cattle can harbour toxigenic strains of *C. difficile*, including the hypervirulent 027 strain (Indra et al. 2009).

Disease in animals is, as CDI in humans, associated with non-protective normal gut flora, due to either antibiotics or young age. The heterogeneity of the genotypes that have been isolated from animals (approximately 30–50 different ribotypes) is lower than the heterogeneity of human isolates (approximately 190 ribotypes). This could be caused by the limited number of animal typing studies that have been performed in comparison with human typing studies. The most prevalent ribotypes differ between animal and human populations, but a substantial number of ribotypes have been isolated from both populations (Rupnik et al. 2009; Indra et al. 2009). This may suggest that the animal reservoirs and a transmission via food are possible sources for human illness, especially for community-acquired human infections. Nevertheless, the exact role of animals as source of CDI is not yet known (Indra et al. 2009).

In a Dutch study published in 2013 (Keessen et al. 2013), the investigators compared the antimicrobial susceptibility profiles of human and piglet *C. difficile* strains ribotype 078 and found that besides considerable overlap in susceptibility profiles, the human and porcine strains also had great genetic similarity when evaluated by PFGE and WGS. The methods showed that numerous of the human isolates and porcine ribotype 078 isolates were indistinguishable (49 and 50 respectively). Also the resistance patterns found in piglets and in human isolates highly overlapped despite a different antimicrobial pressure; the use of antimicrobials in human therapy in The Netherlands is among the lowest in the EU, while veterinary use of antimicrobials is among the highest. The investigators concluded, that the increased incidence of ribotype 078 in association with community-acquired CDI and the presence of ribotype 078 in more than 90% of the *C. difficile* positive piglets, strengthen the hypothesis that human and piglet ribotype 078 had a common origin (Keessen et al. 2013). Similar studies, with the same result, have been conducted in Austria and Germany (Indra et al. 2009; Schneeberg et al. 2013).

Table 4 Prevalence and ribotypes of *C. difficile* in animals (Keesen et al. 2011)

Animal species	Prevalence (in faecal samples of animals with diarrhea)	Prevalence (in faecal samples of animals without diarrhea)	Ribotype
Pigs	United States: 58.4% piglets (Songer and Anderson, 2006).	Slovenia: 50.9% piglets less then 10 days old (Avbersek et al., 2009). Austria: 3.3% Pigs (Indra et al., 2009) United States: 50% Piglets, 3.9% Grow/Finish pigs, 3.9% Breeding sows, boars (Norman et al., 2009)	Predominant: 078 (Keel et al., 2007; Debast et al., 2009). 011, 029, 066 (Avbersek et al., 2009)
Cows	Canada: 7.6% calves (Rodriguez-Palacios et al., 2006) United States: 25.3% calves (Hammitt et al., 2008)	Canada: 14.9% calves (Rodriguez-Palacios et al., 2006) United States: 13.2% calves (Hammitt et al., 2008) Slovenia: 9.5% calves (Avbersek et al., 2009) Austria: 4.5% cows (Indra et al., 2009).	Predominant: 078 (Keel et al., 2007; Avbersek et al., 2009). 002, 014, 017, 027, 033, 077 (Rodriguez-Palacios et al., 2006; Keel et al., 2007; Avbersek et al., 2009; Indra et al., 2009)
Poultry		South Wales: 1.6% (al Saif and Brazier, 1996) Zimbabwe: 17% (Simango, 2006). Zimbabwe: 29% (Simango and Mwakurudza, 2008) Slovenia: 62% (Zidaric et al., 2008)[a] Austria: 3.4% (Indra et al., 2009)	No predominant ribotype, 023 (Zidaric et al., 2008)
Dogs	Germany: 2.7% (Weber et al., 1989) US: 16.7% (Struble et al., 1994) Canada: 7% (Weese et al., 2001b) US: 16.1% (Marks et al., 2002)	UK: 21% (Borriello et al., 1983)/10% (al Saif and Brazier, 1996) Germany: 9.3% (Weber et al., 1989) Australia: 40% (Riley et al., 1991) Switzerland: 3.1–67.1% puppies/1.4% dogs older then 3 months (Perrin et al., 1993) US: 18.4% (Struble et al., 1994)/10.5–14.3% (Marks et al., 2002) Canada: 58% (Lefebvre et al., 2006)[b]/19% (Clooten et al., 2008)/10% (Weese et al., 2010a)	Predominant: 010 (Keel et al., 2007), 001 (Weese et al., 2010a)
Cats	Germany: 6.7% (Weber et al., 1989)	UK: 30% (Borriello et al., 1983)/2% (al Saif and Brazier, 1996) Australia: 38.1% (Riley et al., 1991) Germany: 9% (Weber et al., 1989) US: 9.4% (Madewell et al., 1999) Canada: 7.1% (Clooten et al., 2008)/21% (Weese et al., 2010b)	001 (Weese et al., 2010a)
Horses	Canada: 12.7% horses/35.5% foals (Weese et al., 2001a) Sweden: 42% Horses with antibiotic treatment (Båverud et al., 2003) Sweden: 6% Horses without treatment with antibiotics (Båverud et al., 2003)	Canada: 1.2% horses/0% foals (Weese et al., 2001a) Sweden: 1% (Båverud et al., 2003)	Predominant: 015 (Keel et al., 2007). 033, 078, 001 (Keel et al., 2007; Avbersek et al., 2009)

[a] Samples were taken on four occasions on a single poultry farm producing replacement laying hens.
[b] Samples were taken from hospital visitation dogs.

8.1.1 *C. difficile* in animals in Denmark

In Denmark in 2010, the presence of *C. difficile* including the toxins TcdA and TcdB and the binary toxin was investigated in production animals. *C. difficile* was isolated from 15 of 99 pig farms (15%). All of the isolates were tested for toxin genes and 73% had all three toxin genes whereas 27% had *tcd*A and *tcd*B. In samples from cattle at slaughter 29 of 192 samples (15%) was found *C. difficile* positive of which 24% contained all three genes, 69% contained *tcd*A and *tcd*B, and 7% contained only *tcd*A. The isolates from cattle and pigs were found to be more virulent as some, in addition to *tcd*A and *tcd*B, also contained the genes encoding for the binary toxin. The isolates with the three toxin genes present were ribotyped, and ribotype 078 was detected in 6 out of the 18 isolates from pigs (4) and cattle (2). The rest belonged to ribotypes rarely or not previously found in humans in Denmark (DANMAP 2010). Findings of ribotype 078 in Danish pigs are not surprising since this type is known to be common among pigs and corresponds with findings in other countries.

High levels of resistance were detected. Most isolates were resistant to clindamycin (87%). However, all isolates were susceptible to vancomycin and metronidazole. Both vancomycin and metronidazole are used in antibiotic treatment of CDI. One isolate from pigs and one isolate from cattle were resistant to erythromycin. Moreover, one isolate from pigs was resistant to moxifloxacin. This isolate contained all three toxin genes and belonged to a ribotype DK135 not previously found in humans (DANMAP 2010). A follow-up study in 2012, investigated the occurrence of *C. difficile* in pig farms, cattle at slaughter and in meat samples collected from retail and outlets in Denmark to determine if humans via meat are exposed to virulent *C. difficile* isolates originating from production animals. The study showed a decrease of *C. difficile* in pig farms compared to the previous years, which may be explained by a reduction close to zero of cephalosporin consumption in the same period (DANMAP 2012). The occurrence of *C. difficile* was generally low in meat and none of the most virulent types containing the binary toxin were observed in the meat although present in cattle and pigs. In broiler flocks at slaughter; 6 of 197 (3%) were found positive and all six isolates contained TcdA and TcdB (DANMAP 2012).

8.1.1 *C. difficile* in pets

Since we live in close contact with our pets, it is plausible to hypothesize that they could both be a reservoir and a transmission route for *C. difficile*. Several studies have investigated samples from dogs and cats, but mainly non-toxigenic strains of *C. difficile* have been isolated. This was also the conclusion of a Canadian study in 2009, where investigation of dogs in the household as the source of CDI showed no evidence that dogs were a significant source of household *C. difficile* contamination (Weese et al. 2010). There have though been positive findings of *C. difficile* in pets. A large study in South Wales in the mid-90s investigated 100 dogs and 100 cats and found *C. difficile* strains in 10.0% of the dogs and in 2.0% of the cats. Three of these strains (2 dogs and 1 cat) were found positive for TcdA (Saif and Brazier 1996; Freeman et al. 2010). The findings suggest that the pet reservoir should not be neglected, but the impact is unknown as are the routes of transmission.

8.2 *C. difficile* in food of animal origin

The first specific investigation of *C. difficile* contamination of retail meat intended for human consumption was a study from Canada in 2007 by Rodriguez-Palacios et al. The study involved

a convenience sample of ground beef (n = 53) and veal (n = 7). *C. difficile* was isolated from 12 of 60 (20%) samples (21% ground beef and 14% ground veal).
The most common strain, accounting for 67% of the isolates, was a toxigenic strain that possessed genes encoding for the toxins TcdA, TcdB and the binary toxin CDT (Rodriguez-palacios et al. 2007). Since the first findings, several studies in Africa, Australia, Canada, Europe and the United States have confirmed the ability of *C. difficile* to be present in different food, production animals, vegetables and meat at retail (table 5).

Table 5 Prevalence of isolation and ribotype distribution of *C. difficile* from food animals and retail meat (Weese 2010)

Country	Sample type	Prevalence (%)	Ribotype 027/ toxinotype III (%)	Ribotype 078/ toxinotype V (%)
Canada [34]	Calves	15	12	26
USA [33]	Calves	25	0	94
Canada [40]	Veal calves	49	0/1	65
Slovenia [61]	Calves	1.8	0	0
Austria [49]	Cows	4.5	0	0
Slovenia [36]	Chickens	62	0	0
Austria [49]	Chickens	5	0	0
Zimbabwe [62]	Chickens	29	NT	NT
Slovenia [61]	Piglets	52	0	0/77
USA [32]	Piglets	79	NT	NT
USA [63]	Piglets	NA	0	83
Austria [49]	Pigs	3.3	0	0/50
Canada [37]	Piglets	95	0	94
Canada [44]	Beef, veal	20	0/67	0
USA [23]	Various	42	27	73
Canada [45]	Beef, veal	6.1	0/27	0
Canada [46]	Pork	1.8	43/57	0
Canada [47]	Chicken	15	0	96
Canada [48]	Pork	12	7.1/14	71
	Beef	12	7.1	86

NT, typing was not performed; NA, not applicable, as the study was an evaluation of previously collected isolates.

In Europe, studies from: Slovenia (Pirs et al. 2008), Sweden (Von Abercron et al. 2009), Austria (Jöbstl et al. 2010), France (Bouttier et al. 2010), The Netherlands (Koene et al. 2012; De Boer et al. 2011) and Germany (Schneeberg et al. 2013) have investigated the occurrence of *C. difficile* in the food production chain. In all the studies mentioned, different levels of toxigenic *C. difficile* strains have been detected in both animals and food of animal origin. This raises scientific concern regarding the possible risk of human exposure to *C. difficile* from food. This concern is supported by other studies (Rodriguez-palacios et al. 2007; Indra et al. 2009; Keessen et al. 2011), but no clear association has been found between the occurrence in the animal reservoir and in humans. The source of *C. difficile* in meat is not clear, but carcasses may become contaminated with faecal material or from the environment during the slaughtering process.

Contamination at retail level may also occur from the processing 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 (De Boer et al. 2011).

In Canada in 2006, a total of 28 *C. difficile* isolates were cultured from 13 meat packages (22 from ground beef, 6 from veal). PCR-ribotyping showed 8 distinct genotypes, 7 of which were toxigenic and present in 10 (77%) meat packages. Genotypes resembling human ribotype 027 were found in 30.8% (n=4) of positive samples, and ribotypes 077 and 014, formerly reported in cattle and retail meats, were identified in 23.1% (n=3) and 15.4% (n=2) of the samples, respectively. Multiple genotype contamination was also documented. PFGE confirmed that selected meat and human ribotypes were identical. Fluoroquinolone and clindamycin resistance was common among the tested isolates (41.6%–58.3%). Although ingestion of spores does not necessarily imply infection, this study supports the potential for foodborne transmission (Rodriguez-Palacios et al. 2009). Even though the data represents few samples, the presence of *C. difficile* ribotype 077 and 014, and the high level of resistance in the meat isolates are of concern as these specific ribotypes are among the most common in community-acquired CDI in the EU including Denmark.

Different *C. difficile* ribotypes involved in human disease have been detected in both production animals and in food products. A study from the United States, using convenience sampling from stores reported isolation of *C. difficile* from different types of meat and ready-to-eat sausages in 37 of 88 (42%) samples. Ribotype 078 was the most common strain, accounting for 73% of the isolates, with the remaining isolates belonging to ribotype 027 (Songer et al. 2009). Recent studies have identified *C. difficile* in retail meat, including pork, beef and turkey, with a predominance of ribotype 027 and ribotype 078 strains (Weese et al. 2010). A study by Indra et al. from 2009 indicates together with other reports that the rate of *C. difficile* contaminated meat in Europe is lower than reported from the United States, but this might have changed during the past five years (Indra et al. 2009).

In Sweden, 82 meat samples were evaluated to estimate the occurrence of *C. difficile* in retail ground meat. *C. difficile* was isolated from two ground beef samples from two different retail shops collected on two different sampling occasions (May and September 2008). The meat was of Swedish origin and both *C. difficile* isolates were found positive for TcdA and TcdB. No *C. difficile* was isolated from pork, sheep, hamburger, poultry, or other type of meat. (Von Abercron et al. 2009). Even though the Swedish findings were few, they did raise some concern in Sweden as both isolates were considered toxigenic for humans.

In Denmark, besides pigs and cattle, poultry meat has also been investigated in the DANMAP program. Here a higher prevalence was observed in broiler meat (7%) compared with pigs (2.8% in 2011). Whether the findings were due to differences in the slaughter processes for broilers compared to pigs or differences in occurrence in the animals is not known. The occurrence of *C. difficile* in cattle at slaughter was 15% (DANMAP 2012).

8.3 *C. difficile* in other food products, water and environment

Although contamination of retail meat has received the most attention, contamination of other food products may be equally important, particularly for those that are eaten after little cleaning or cooking. There are various possible sources of contamination with *C. difficile* spores,

which both can be of human or animal origin, such as soil, fertilizer (manure), contaminated water, processing environments, or from human hands. Few investigations of other food products have though been reported (Weese 2010).

Two studies of *C. difficile* in vegetables (in South Wales and Canada) reported isolation of *C. difficile* from different types of vegetables (root vegetables[14], mushrooms, cucumber and ginger) (Weese 2010; Metcalf et al. 2010). A Scottish study described isolation of *C. difficile* between May – June 2008 from three of 40 (7.5%) ready-to-eat salads from seven different supermarket brands. The salads were not of UK origin. Isolates were identified as toxigenic; positive for both TcdA and TcdB, and resistant to clindamycin and cefotaxime, one isolate was also resistant to moxifloxacin and erythromycin (Bakri et al. 2009; Weese 2010). Though the numbers of studies are limited, the findings of *C. difficile* strains in vegetables and ready-to-eat salads indicate a possible transmission routes for CDI. Findings of resistant *C. difficile* are not only restricted to food of animal origin. Resistant strains have also been isolated from vegetables and ready-to-eat salads (Bakri et al. 2009; Weese 2010).

In a large study of the distribution of *C. difficile* in the environment of South Wales (UK) from the mid-90s, the highest occurrence of *C. difficile* was obtained from river and seawater samples (87.5% and 44.0% respectively). *C. difficile* was also isolated from swimming pools (50.0%) and main tap water (5.5%). In private residences, the organism was present in 12 (2.2%) of 550 samples, whereas 2.4% of 300 raw-vegetable samples were positive (Saif and Brazier 1996; Freeman et al. 2010).

The presence of *C. difficile* in vegetables, salads and different types of water could be the result of contamination from the environment or from infected food handlers, as both scenarios are known from foodborne outbreak investigations of other pathogens. The findings suggest that food of non-animal origin could present a risk of CDI in humans. However, data are limited and the impact on human disease unknown.

8.4 Limitation in data

Only a limited number of studies have been published, and these have typically involved a small number of geographical regions, with different sampling methods and culture techniques. These methodological variations preclude the comparison of prevalence data from different studies. Furthermore, it is apparent that there is significant variation in *C. difficile* colonization among different age groups in calves, piglets and chickens, with *C. difficile* rates decreasing substantially over time (Weese 2010). The use of different methods in the different European countries requires further improvement of *C. difficile* detection in foods (De Boer et al. 2011), and there are currently no standard methods for isolation of *C. difficile* from retail meat (Weese 2010).

8.5 Summary *C. difficile* in animals and food

Direct transmission of *C. difficile* from animals, food or the environment to humans has not been proven, although similar ribotypes are found. In The Netherlands, ribotype 078 isolates

[14] Potato, onion, carrot, radish and eddo

from humans and pigs have been found to be indistinguishable by PFGE, MLVA and WGS. As no outbreaks of CDI have been reported among humans in the community, host factors that increase vulnerability to CDI might be of more importance than increased exposure to *C. difficile*. Conversely, the emerging *C. difficile* ribotype 078 is found in high numbers in piglets, calves, and their immediate environment. As the ribotype also is present in humans, circumstantial evidence points towards a zoonotic potential of this type. There is scientific concern regarding the possible risk of human exposure to *C. difficile* from food, but there is no clear association between the occurrence in the animal reservoir and in humans. It is important to keep in mind that only a limited number of studies have been published with different sampling methods and culture techniques. The presence of *C. difficile* in vegetables, salads and different types of water could be the result of contamination from the environment (including manure) or from infected food handlers, as both scenarios are known from foodborne outbreak investigation of other pathogens. The findings suggest that food of non-animal origin could be a cause and present a risk of CDI in humans. Data are though limited and the impact on human disease is unknown.

Even though the role of *C. difficile* as a zoonotic agent is not fully understood and the role of food products and a possible foodborne transmission in association to CDI is either unclear or unknown, the detection of genes encoding for the virulent toxins TcdA, TcdB and the binary toxin in food intended for human consumption should be considered as problematic and a risk for *C. difficile* transmission to humans. Also a substantial number of ribotypes have been isolated from both humans and animals suggesting that animal reservoirs and transmission via foods of animal origin or via the environment are possible sources for human illness, especially for community-acquired human infections. Nevertheless, the exact role of animals as source of human CDI is not yet known.

9. The risk of *C. difficile* in different meat types estimated by BIOHAZ and CONTAM

Following a request from the European Commission to EFSA[15], the Panel on Biological Hazards (BIOHAZ) and the Panel on Contaminants in the Food Chain (CONTAM) were asked to deliver a Scientific Opinion on the public health hazards to be covered by inspection of different types of meat. C. difficile was a part the public health hazards discussed by the two panels.

9.1. The risk from *C. difficile* in poultry meat estimated by BIOHAZ and CONTAM

Based on the risk ranking in the Scientific Opinion on the public health hazards to be covered by inspection of poultry meat, the hazards from poultry meat regarding *C. difficile* were classified as follows: Data for ranking *C. difficile* were insufficient, but based on the limited information available, the risk at the present time was considered to be low. Data on reported cases of *C. difficile* were not available at the EU level. No data on the occurrence of *C. difficile* in poultry flocks or carcasses were available from the EU monitoring data, and the proportion of cases caused by poultry meat was unknown. Given the scarcity of data in both humans and animals,

[15] European Food Safety Authority

it was not currently possible to determine the role, if any that poultry meat plays in the epidemiology of human infections with *C. difficile*, but based on the limited available evidence the BIOHAZ Panel concluded that the risk at the present time is low (EFSA Journal 2012;10(6):2741) .

9.2 *C. difficile* risk from bovine meat estimated by the Panel of BIOHAZ

Following a request from the European Commission, the EFSA Panel on Biological Hazards (BIOHAZ) was asked to deliver a Scientific Opinion on the public health hazards to be covered by inspection of bovine meat. The Panel notes that *C. difficile* are present in meat, but as there was no evidence of meat-borne transmission and that CDI according to the Panel are a nosocomial infection the Panel considers no risk from *C. difficile* from bovine meat (EFSA Journal 2013;11(6):3266)

9.3 *C. difficile* risk from pig meat estimated by the Panel of BIOHAZ

Following a request from the European Commission, the Panel on Biological Hazards (BIOHAZ) and the Panel on Contaminants in the Food Chain (CONTAM) were asked to deliver a Scientific Opinion on the public health hazards (biological and chemical respectively) to be covered by inspection of pigs (swine). *C. difficile* has been isolated from fresh pork but there is currently no evidence of human disease attributable to this source (unknown because of lack of data). The Panel classifies *C. difficile* in pigs to be at preliminary low risk (EFSA Journal 2011;9(10):2351 2011).

9.4 *C. difficile* risk from sheep and goats meat estimated by the Panel of BIOHAZ

Following a request from the European Commission, the EFSA Panel on Biological Hazards (BIOHAZ) was asked to deliver a Scientific Opinion on the public health hazards to be covered by inspection of meat from several animal species including sheep and goats' meat. The EFSA Panel concludes; that there are reports of *C. difficile* being isolated from small ruminants. However, there was to date no indication of meat-borne transmission to humans (EFSA Journal 2013;11(6):3265)

9.5 Summary of the risk of *C. difficile* in meat estimated by BIOHAZ and CONTAM

In general, the scientific reports conducted by the Panels of BIOHAZ and CONTAM regarding the hazards from food regarding CDI confirm the insufficient knowledge in the area, both regarding lack of data and knowledge of the transmission routes. Regarding the risk from poultry, the BIOHAZ Panel conclude that the risk at the present is low based on the limited available evidence. *C. difficile* are present in pig meat, but there is no evidence of meat-borne transmission and *C. difficile* in pigs is classified to be at preliminary low risk. According to the BIOHAZ Panel on bovine meat, CDI is exclusively a nosocomial infection and therefore the Panel considers no hazard from bovine meat. *C. difficile* has been isolated from small ruminants, but there is to date no indication of meat-borne transmission to humans from goats or sheep.

10. *C. difficile* transmission from production animals to humans

In contrast to healthcare-acquired CDI, where patient-to-patient transmission is more likely, direct or indirect animal contact (including contact with food of animal origin) is a more plausible means of transmission for community-acquired CDI. Other possible community sources for CDI include soil, water, pets and vegetables. Nevertheless, the exact role of animals as source of hu-

man CDI is not yet known. No direct transmission from animals to humans has to our knowledge been described.

10.1 *C. difficile* in production animals

The levels and trends of *C. difficile* in production animals are not known or are uncertain. Investigation in The Netherlands showed isolation rates in samples from food animals (3.4% in cattle, 5.8% in poultry and 6.6% in pigs), which were found in agreement with other recent European reports, with isolation frequencies up to 3% in meat samples and 5% in samples taken from animals prior to slaughter (Indra et al. 2009). Studies performed in the United States and Canada reported the presence of *C. difficile* in food animals and meat with rates up to 42%, (Rodriguez-Palacios et al. 2009; Weese et al. 2009; Songer et al. 2009). This may reflect differences in geographical and/or temporal variation in *C. difficile* prevalence, although other aspects, such as age of the sample animals, could play a role. The numbers of animals carrying *C. difficile* may also differ between individual animals, and from farm to farm even within the same region (Koene et al. 2012). The gastrointestinal tract in animals is however most likely an important source of *C. difficile* contamination and various comparative genotyping studies suggest that the predominant strains causing CDI in humans and various animal species are identical.

10.2 *C. difficile* transmission to humans

Besides considering a foodborne transmission of *C. difficile* other routes of transmissions between production animals and humans should be considered; animals' hides, the slaughterhouse environment, the processing facility environment, processing equipment, the hands of personnel handling meat, and any other environment where meat is handled or processed prior to sale. *C. difficile* spores are highly resistant to most disinfectants, and can survive common cleaning and disinfection practices, and persist or accumulate in the environment. Retail meat might be contaminated during processing. Personnel in the food production and food handlers might be exposed to *C. difficile* spores. The risk and the health impact from these possible transmission routes are unknown. The high sporulation rate of *C. difficile* ribotype 027 is speculated to cause a persisting presence of the strain in the hospital environment. It is hypothetical plausible, that this high sporulation rate also could be an important component of environmental persistence in slaughterhouses and meat-processing environments (Weese 2010). This is so far only a theory raised in literature.

The different studies suggest an epidemiological connection between the reservoir of production animals and the human reservoir, though the connection is yet unknown or not fully understood.

10.2 *C. difficile* - a possible risk for people who work with production animals

It is known that *C. difficile* spores can be transferred between persons by their hands, and that persons can be infected with *C. difficile* spores from hospital environment (Stanley et al. 2013). Presumably these routes of transmission could also happen from the skin of *C. difficile* positive animals, (which often are contaminated with manure) and outdoor environment infected with *C. difficile* spores. Few investigations of the impact on CDI of *C. difficile* from the environment have though been published. In The Netherlands two herds of piglets suffering from diarrhea proved to be excreting *C. difficile* toxinotype V, ribotype 078. Investigation at the farm showed

that none of the farm workers and none of the family members of the farm owners, even though probably exposed, had developed CDI (Freeman et al. 2010). This might highlight the importance of individually sensibility and host factors.

Keeping in mind that the exposure to *C. difficile,* the infective dose of *C. difficile* and the individually sensibility are unknown, it is very difficult to give a qualified estimation of a possible risk of CDI for persons working with production animals or persons having contact to known infected animals. As mentioned earlier in this report, it is assumed that a disruption of the normal protective gastrointestinal microbiota, as a result of antimicrobial therapy is an important factor for colonization by *C. difficile* and that the risk of CDI depends on individual circumstances (e.g. underlying illness, antibiotic therapy, microbiota resistance), and on the resistance pattern of the *C. difficile* strain. It could be assumed that a person with high sensibility having contact to animals carrying toxigenic strains of *C. difficile* could be at risk of CDI, if personal hygiene precautions were not taken (e.g. hand wash, change of working clothes). The risk would assumable be higher if the animal is excreting *C. difficile*.

10.3 Summary *C. difficile* transmission from production animals to humans

The literature suggests an epidemiological association between production animals and humans though the association is not fully understood. No direct transmission from animals to humans has to our knowledge been described. Comparative genotyping studies suggest though that the predominant strains causing CDI in humans and various animal species are identical. Other routes than foodborne transmission should be considered; e.g. animals' hides, the environment of slaughterhouses and processing facilities, and the hands of personnel. *C. difficile* spores are highly resistant to most disinfectants, and can survive common cleaning and disinfection practices, and persist for long in the environment. It should be kept in mind, that the exposure of *C. difficile* is unknown, the infective dose of *C. difficile* is unknown and the individually sensibility is unknown, therefore it is not possible to estimate a risk of CDI for persons working with production animals or persons having contact to known infected production animals. It could though be assumed; that a person with high sensibility having contact to animals carrying toxigenic strains of *C. difficile* could be at risk of CDI, if personal hygiene precautions were not taken.

11. Future changes in the food production system which may influence the risk to humans.

The knowledge of C. difficile as a cause of human disease and the presence of C. difficile in animals and food products has increased during the last decades, but many factors in the risk of CDI and the background for the increase in the community-acquired CDI remains unknown. It can though be assumed that a change in the veterinary antibiotic consumption would lead to a change in the resistance pattern and in the occurrence of C. difficile. Also changes in the production system that favour anaerobic conditions could influence the growth of C. difficile. Changes in the hygiene- and heat treatment procedures could allow spores to survive in the production chain.

11.1 Changes in the food production system

Changes in the food production system or changes in the occurrence of *C. difficile* in production animals and food are not the only factors which may influence the human incidence of CDI. The proportion of elder citizens will rise globally, and therefore more CDIs are expected to occur in

the future, as elderly in general are at higher risk (Rodriguez-Palacios et al. 2013). The change in the population demographics will put a higher pressure on the healthcare system in general, but also in requirements to the food production networks delivering food to vulnerable persons in the healthcare system.

Though little is known of *C. difficile* in the production system, the capability of survival of *C. difficile* spores, the abilities of the spores to persist on fomites and the resistance profile of *C. difficile* allow us to believe that four areas are considered as important:
A) Changes that favour anaerobic conditions
B) Antimicrobial treatment
C) Changes in hygiene procedures
D) Changes in heat treatment.

11.1.1 Anaerobic conditions

The strains of *C. difficile* are strictly anaerobic, which means that the bacteria are killed at normal concentration of oxygen, which prevents the bacteria from growing and establishing in an aerobe environment. Changes in food production which favour anaerobic growth could though influence the occurrence of *C. difficile* and other anaerobic bacteria in the food. This should be taken into consideration, especially in regards to ready-to-eat products (without heath treatment) produced for consumers that are vulnerable to CDI (e.g. elderly, hospitalised, history of antibiotic treatment).

11.1.2 Veterinary antibiotic consumption pattern

In Denmark in 2011, an investigation showed that a reduction close to zero in the consumption of cephalosporins in the pig production may have decreased the occurrence of *C. difficile* in pigs[16] in the same period (DANMAP 2012). The use of fluoroquinolones in the early 2000s led to an increase in the fluoroquinolone resistance in *C. difficile* ribotype 027 (Hookman & Barkin 2009). *C.* difficile has reduced susceptibility to a range of different antimicrobials used for clinical treatment of human infections. There seem to be an association between the antibiotic consumption and the resistance pattern of *C. difficile*. It is also likely that the occurrence of *C. difficile* in livestock increases as a result of antimicrobial usage especially the use of cephalosporins which *C. difficile* is intrinsic resistant to, but this is not well studied.

11.1.3 Use of recycled hot water and lack in heating and hygiene procedures

C. difficile spores are besides being highly resistant to desiccation and chemicals also resistant to extreme temperatures (Rupnik et al. 2009). If changes are made in these parameters it might influence the occurrence of *C. difficile* spores in the food production and in the community.

Some concern has been raised in a Scientific Opinion done by EFSA regarding the use of recycled hot water at slaughterhouses and the risk of contamination from heat-resistant spores among others *C. difficile*. For carcass decontamination purposes, only use of potable water is currently allowed in the EU. However, recycling of water (i.e. reusing after reheating) used for

[16] See chapter 4.1

carcass decontamination has been practiced in some countries (e.g. Canada, Denmark). The Scientific Opinion points at different potential microbiological risks for carcasses associated with recycled hot water decontamination.
The microbiological risks in the recycled water of main potential concern derive from heat-resistant bacterial spores such as: *B. cereus, C. botulinum, C. perfringens and C. difficile*, however there is a lack of data on the extent of carcass contamination with spores, their germination and inactivation during the recycling process, and the potential for accumulation, during the operations. According to the Scientific Panel, the spores of *C. difficile* generally can survive the 79.5°C temperature of the recycled hot water operation described for the Danish system. Although a certain proportion of the spores may die if exposed for several hours to these temperatures (e.g., spores entering the water at start of the day), additional spores are likely to be introduced in the water throughout the day as new carcasses are processed, and dissolved/suspended proteins and fat may provide protection and enhance their survival (EFSA Journal 2010). If the prevalence of *C. difficile* spores on carcasses is increasing it could be theoretically assumed, that also the prevalence of the spores in the recycling water would increase with the risk of contaminating the production.

*C difficile s*pores have, as previously mentioned, been shown to survive the temperatures and disinfectant treatment of typical hospital laundering and to cross-contaminate other linen during the laundry procedure (Rupnik 2007). Theoretically, spores (if present) can be spread in the same way in a laundry system of a food production (e.g. washing of clothing and towels), if the water is not adequately heated. A study found that cooking aliquots containing less than 4 $\log_{10}$ at 85°C in liquid media yielded no cultivable spores after 15 min heat treatment (Rodriguez-Palacios and LeJeune 2011).

Alcohol, chlorhexidine, iodophors, and other antiseptic agents are shown to be ineffective at inactivating the spores of *C. difficile*. The most effective to prevent the transmission of spores is frequent hand wash with soap and water, rather than with alcohol-based waterless hand hygiene (Epi-Nyt Statens Serum Institut week 13 2009; Hookman & Barkin 2009; World Health Organization 2009[17]). We assume that during the last years, the usage of alcohol-based hand hygiene in the general population has increased, maybe also in food production facilities and institutions (e.g. schools and kindergartens). If hand washing with soap and water in general has been replaced and not combined with alcohol-based hand hygiene this could imply a survival of *C. difficile* spores, which could lead to a transmission of spores in the general population, in the food production and institutions. The Danish Veterinary and Food Administration and the Danish Health and Medicines Authority already recommend hand wash in their hygiene information.

11.2 Summary future changes in the food production chain

When the epidemiology and hazards concerning *C. difficile* and CDI is not fully understood, it is very difficult to make predictions of the impact of future changes in the food production sys-

[17] Hygienic precautions described in SSI, Epi-Nyt, week 13, 2009 and WHO Guidelines on hand hygiene in health care, Guide to appropriate hand hygiene in connection with *Clostridium difficile* spread, World Health Organization 2009, pp242-245

tem. It is though believed that the following areas should have special attention: Changes that favour anaerobic conditions in the food production which enables survival of *C. difficile* in the final product, antimicrobial treatment in production animals which might influence the prevalence of *C. difficile* in the animals, changes in hygiene procedures and heat treatment in the production chain which might influence the survival of *C. difficile* spores.

12. Preventive measures to reduce *C. difficile*

CDI is mainly recognised as a problem in hospitals, and outbreaks due to C. difficile have only been seen in hospitals. The recommendations for the prevention and control of CDI are therefore focused on health care settings. Some papers have though given recommendation regarding measures to reduce spread from animals and food.

12.1 Prevention in hospital settings and nursing homes

On behalf of the European *C. difficile* Infection Control Group and ECDC the following evidence-based guidance and recommendations for the prevention and control of CDI and *C. difficile* outbreaks has been published (Vonberg et al. 2008) in order to limit the spread of *C. difficile* in healthcare settings:

- Early diagnosis of CDI
- Surveillance of CDI cases
- Education of the hospital and cleaning personnel as well as patients and visitors
- Appropriate use of isolation precautions
- Hand hygiene (hand wash with soap and water)
- Protective clothing
- Environmental cleaning and cleaning of the medical equipment
- Responsible antibiotic therapy, and
- Specific measures during outbreaks.

Because alcohol is ineffective at killing *C. difficile* spores, health care workers must frequently wash their hands with soap and water, rather than with alcohol-based waterless hand sanitizers, especially when taking care of CDI patients. In hospitals and nursing homes in Denmark, patients with ribotype 027 are already isolated in a private room with private toilet, to prevent the infection from spreading. Staff must wear gloves and cover suit coat during care and treatment of the patient. To reduce the transmission of *C. difficile* spores, environmental disinfection with 10% sodium hypochlorite and hand washing with soap and water can be effective at removing the spores from hands and surfaces (Hookman & Barkin 2009; World Health Organization 2009[18]; Epi Nyt week 13, 2009[19]).

[18] WHO Guidelines on hand hygiene in health care, Guide to appropriate hand hygiene in connection with *Clostridium difficile spread*, World Health Organization 2009, pp242-245

[19] Hygienic precautions described in SSI, Epi-Nyt, week 13, 2009

12.2 Prevention in animals and food

The advice to healthcare institutions regarding hand washing with soap and water in order to reduce the spread and transmission of *C. difficile* spores could also be beneficial in the food production, in the general population, kinder gardens, schools and other places where spores can be transmitted. Where food is prepared to vulnerable persons in the community or in healthcare settings; food should be ensured to be heated to more than 85°C as a simple and important intervention to reduce the risk of inadvertent ingestion of *C. difficile* spores (Rodriguez-Palacios and LeJeune 2011).

In supplement to the recommendations in healthcare settings, Alex Rodriguez-Palacios et al. (2013) has raised the need for new recommendations concerning animals and food to reduce the exposure to *C. difficile*:

- Contact precautions regarding human and animals with CDI, healthy pets and wild animals
- Cleaning and disinfection – Addressing food, home, kitchen and laundry environments
- Thorough cooking – Current food safety guidelines are ineffective against *C. difficile*
- As *C. difficile* could still survive cooking temperatures and multiply in heated foods, it is also recommended that foods should be properly chilled and stored as indicated for other clostridia foodborne pathogens.

Some extra hygienic consideration should be taken for persons in antibiotic therapy in order to minimize the direct contact to production animals, or improved hygienic standards in order to prevent the transmissions from *C. difficile* spores in the environment.

Conclusion

C. difficile produces the enterotoxin A (TcdA) and a cytotoxin B (TcdB) both capable of introducing human disease. Some *C. difficile* strains also produce the binary toxin CDT. The binary toxin is either a marker for more virulent *C. difficile* strains or contributes directly to strain virulence. The infective dose of *C. difficile* for humans is unknown and probably varies among individuals, depending on health status and individual susceptibility. Antimicrobial therapy is the most widely reported risk factor for CDI in humans. Antibiotic consumption may disrupt the colonic microbiota, which enables the toxigenic *C. difficile* strains to colonize the gut. *C. difficile* can either be present in the intestine or be introduced. If the strains are resistant to the antibiotics used in therapy the overgrowth by *C. difficile* may be more rapid and more severe.

There is no conclusive evidence that the presence of *C. difficile* in meat represents a risk to the consumer. There is though scientific concern regarding the possible risk of human exposure to *C. difficile* from food (including vegetables). An increase of community-acquired CDI in a younger population without antibiotic history and recent health care exposure has been observed, and CDI with ribotype 078 is found to be more often community-acquired than infections with ribotype 027. Considerable overlap between strains found in the animal and human reservoir have been documented, especially ribotype 078 found in animals have been shown to have genetic similarity to strains found in humans. In a Dutch study of piglets, ribotype 078 isolates were indistinguishable on MLVA and WGS from those found in humans, but no clear association has been found and the transmission routes are unknown.

It is, at this stage, not possible to estimate the consumers' risk of CDI from food. Even exposed to *C. difficile,* the risk of CDI depends on individual circumstances and sensibility (e.g. underlying illness, antibiotic therapy, microbiota resistance), and on the resistance pattern of the *C. difficile* strain.

To our knowledge no direct transmission from animals to humans has been described. The risk for working with production animals should be considered as low if respecting general hygienic precautions described for the area. The spores of *C. difficile* enable the organism to survive in adverse conditions and they are resistant to most disinfectants. Hazards could be indirect contact with animals via the environment (manure), the animals' hides, the environment of slaughterhouses and processing facilities. It could be assumed, that a person with high sensibility having contact with animals carrying toxigenic strains of *C. difficile* could be at risk of CDI if personal hygiene precautions were not taken.

When the epidemiology and hazards concerning *C. difficile* and CDI is not fully understood, it is very difficult to draw conclusions about the impact of future changes in the food production system. It is though believed that 4 areas should have special attention: A) Changes that favour anaerobic conditions in the food production which enables survival of *C. difficile* in the final product. B) Antimicrobial treatment in production animals which might influence the prevalence and resistance pattern of *C. difficile* in the animals. C) Changes in hygiene procedures and D) Heat treatment in the production chain which may influence the survival of *C. difficile* spores.
The use of antibiotics in livestock seem to influence the occurrence of *C. difficile*, but to what extent the usage affects the occurrence of *C. difficile* in livestock and in meat from these animals is not well studied.

Recommendations

In order to understand the epidemiology of community-acquired CDI, to understand the spread of *C. difficile* both within the animal and human reservoir and to compare cross countries, there is a need for standardized definitions and surveillance methods to assess disease trends. In order to implement intervention to reduce and control CDI, national surveillance programs are vital to monitor the incidence both in the healthcare system and in the community, to identify populations at risk and characterize the molecular epidemiology of strains causing CDI. Experience from England and Wales shows that it is possible to reduce the number of severe CDI cases by implementing intervention in hospitals and nursing homes. Surveillance programs of *C. difficile* should focus on all types able to produce toxin regardless of ribotypes.

Comparative microbiological studies, using discriminatory methods as MLVA and WGS of *C. difficile* strains found in humans, animals, food and environment (including water) combined with epidemiological methods, could potentially contribute to a better understanding of *C. difficile* and its zoonotic potential. Focus should not only be on the highly virulent types but on all types able to produce toxin. It is expected that more studies in the future will be published from other countries.

As *C. difficile* may arise as a result of treatment with cephalosporins in production animals, testing for the occurrence of *C. difficile* when testing for ESBL[20] bacteria should be considered, and especially in cattle where cephalosporins are still used, though phased out from 2014. Another focus area could be the use of antibiotics in other animals as pets and horses, which could have an impact on the occurrence and spread of *C. difficile* in the community.

In food production, four areas should have special attention: A) Changes that favour anaerobic conditions in the food production, B) antimicrobial treatment in animals, C) changes in food production and hygiene procedures and D) changes of heat treatment in the food production chain. In these four areas, awareness on possible effects on the occurrence *C. difficile* and the survival of spores should be kept in mind.

New food hygiene measures could be beneficial if implemented together with recommendations of cooking at higher temperatures to reduce the prevalence of *C. difficile* and spores in food from productions facilities producing food for vulnerable persons and patients in risk of CDI in e.g. nursing homes and hospitals.

Even though the role of *C. difficile* as a zoonotic agent is not fully understood and the role of food products and a possible foodborne transmission in association with CDI is unknown the detection of genes encoding for the virulent toxins TcdA, TcdB and the binary toxin in food intended for human consumption should be considered as problematic and a risk for *C. difficile* transmission to humans.

[20] ESBL: Extended-spectrum beta-lactamase producing bacteria

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