Ecology and Epidemiology of Human Pathogen \textit{Clostridium difficile} in Foods, Food Animals and Wildlife

\textbf{DISSEPTION}

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By

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Abstract

The world is experiencing an increase in illness and deaths associated with one of the most common nosocomial pathogens, *Clostridium difficile*. However, the sources of infection for people at risk, especially in the community which is also increasingly affected, are often unknown. In 2006 a study reported new hypervirulent and multidrug resistant epidemic *C. difficile* strains of relevance in human disease were present in livestock and retail meats highlighting the potential for foodborne transmission. The main goal of this PhD dissertation was to investigate and identify factors associated with *C. difficile* shedding in food animals, food contamination at harvest and its relation with wildlife and their environment with particular attention to inter/intra-species transmission and dissemination potential. Knowing how epidemic strains of bacteria disseminate among food animals and reach the food supply will help to identify strategies to mitigate the risk of food contamination and therefore transmissibility to humans.

By using observational, experimental and applied microbiological studies this dissertation documented: 1) the low prevalence of epidemic *C. difficile* in mature livestock at harvest, 2) the potential emergence of multidrug resistant strains that are unlikely to originate from food animal production systems, 3) fecal supershedding status relevant for infectious disease dynamics and environmental contamination, and 4) transient intestinal colonization and unaffected shedding dynamics despite enhanced use
of antimicrobials in naturally infected cattle. Geographical clustering of captive white-tailed deer harboring emerging *C. difficile* PCR ribotype 078 was also documented. Nontoxigenic *C. difficile* strains, which can prevent the virulent effect of toxigenic strains, were unexpectedly prevalent in food animals at the time of harvest. Thermal studies with recovered spores conclusively documented the actual food safety risk highlighted in former publications, mechanistically showed that temperature affects cell division but not spore germination, and that hypervirulent strains are significantly favored with sublethal heating during cooking. Here it was also determined that wild birds are not clinically affected by human epidemic *C. difficile* strains and that dissemination at a regional scale is possible in anthropogenic and food animal ecosystems. The identification of which food animal species and to what extent wild birds are active disseminators of emerging *C. difficile* strains is relevant to advance our ecological understanding of human diseases and to further develop strategies to reduce the risk of food contamination. These studies are expected to help increase global public and environmental health initiatives.
To the ones I love, friends and family.

My Grandma, mama and tata.

To Cata who still forgives me with love and patience for captivating long working sessions

And from the deepest of my liver …to my inspiring and cheering children

Gabi, Dani and beba Anna, my most precious non-repeatable experiments.

Thanks to them heartening butterflies still dance in celebration
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Publications

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 Loerch, S, JT LeJeune. Transient fecal shedding and limited animal-to-animal
 transmission of Clostridium difficile in naturally infected feedlot cattle.


Clostridium difficile PCR-ribotypes in Calves (and Relatedness Between Bovine and Human PCR Ribotypes), Canada. [Google cites: 78 times, including CDC -MMRW weekly, April 4, 2008 / 57(13);340-343].

Boermans HJ, Karrow NA. Characterization of ovine hepatic gene expression profiles in response to Escherichia coli lipopolysaccharide using a bovine cDNA microarray. [Google cites: 12 times].

Quinton M, Boermans H, Karrow NA. Assessment of the ovine acute phase response and hepatic gene expression in response to Escherichia coli endotoxin. [Google cites: 8 times]

Journal Articles related to Teaching:


Book Chapter

Fields of Study

Major Field: Veterinary Preventive Medicine
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Chapter 1 — PhD Program Project Description and Study Objectives

Abstract

*Clostridium difficile* has been estimated to be responsible for at least half million cases of serious enteric disease in humans in the USA every year, which is just a fraction of all probable cases. Although *C. difficile* was in the past considered a pathogen of hospital environments, increasing numbers of cases occur now in the community. This changing epidemiology has been partly attributed to the emergence and global dissemination of more virulent strains. Since 2006, we know that such epidemic strains can be isolated from neonatal dairy calves, piglets and most importantly, from up to 20% of retail ground meat, which highlights foodborne transmission potential. At the time this proposal was presented, it was unknown 1) to what extent food animals at harvest were a source for food contamination, and 2) the ecological reasons for the rapid dissemination of such virulent strains across distant agricultural regions in North America, and to 3) what extent moist heat (*i.e.*, routine cooking) affected the risk of ingesting foodborne spores. Addressing these unknowns will have a profound impact on our understanding of the ecology of this pathogen which is constantly re-emerging in the community.
Introduction

The frequency and severity of *Clostridium difficile* infections (CDI) have been steadily increasing in North America since 2000. In Ohio, the number of annual cases present only in health care centers is over 17,000 individuals, for a state of 11.5 million inhabitants. Traditionally, CDI has been regarded as a disease of nosocomial origin, but now it is well known that cases of more severe disease have emerged in the community, with the inclusion of children and pregnant women who were previously deemed to be at low risk. This increased severity has been largely attributed to emerging hyper-virulent multi-drug resistant strains, especially of strains of an emerging genotype, designated PCR-ribotype 027/North American Pulse-Field type 1. In 2006, I led a study reporting for the first time the presence of emerging *C. difficile* strains from dairy calves raising concerns about zoonotic transmissibility. A year later, I led another study reporting the identification of emerging genotypes from retail ground beef indicating *C. difficile* might induce foodborne infections.

Knowing how epidemic strains reach the food chain and how they disseminate among food-producing animals will help to identify strategies to mitigate the ultimate risk of food contamination and therefore transmissibility to humans. Today, it is unknown 1) if cattle and other food-producing animal species are sources of *C. difficile* at the time of harvest, 2) to what extent intestinal carriage is related to carcass contamination, 3) if the apparent dissemination of such strains among regions and farms is due in part to wild birds, and 4) if cooking effectively inhibits epidemic *C. difficile* spores. The lack of
understanding of these basic aspects of disease ecology prevents us from studying and implementing preharvest, harvest and postharvest strategies to reduce the risk of food and environmental contamination.

**Hypothesis**

Our central hypothesis is that food animals at the time of harvest and wild birds can be sources of toxigenic *C. difficile* of current epidemiological relevance for disease in humans and be effective disseminators.

**Specific Aims**

The long-term goal of the research program in Dr LeJeune’s laboratory and mine as well is to identify and validate strategies to reduce the risk of foodborne transmissibility of human pathogens from their source in animals or foods. Thus the main objective of this project was to determine the role of food animals and wild birds in the epidemiology of *C. difficile* from an ecological perspective, and to determine to what extent moist heat is inhibitory when used as recommended.

The ‘Gaps in knowledge’ we deemed necessary to address during this PhD program to enhance global food safety against *C. difficile* included:

- Understanding dissemination and reducing the risk of food contamination
- Understanding the effect of cooking on spores’ survival.
- Promoting educational programs
Goals and Experimental Approach

**Goal 1 – Food Animals at harvest and food contamination**

To assess *C. difficile* shedding in food animals prior to harvest, as it is important to infer the potential for carcass and food contamination. *The working hypothesis* of this aim was that the intestinal carriage of *C. difficile* varies among livestock species, and that in cattle such carriage represents a risk for carcass contamination at harvest.

*The experimental approach:*

**Study 1.** Single-cohort (longitudinal) study of *C. difficile* in a group of beef steers from fattening to harvest, (Appl Env, Micro. Published; Chapter 3).

**Study 2.** Cross-sectional study of *C. difficile* prevalence in various food animal species and horses in Ohio during summer. (For submission; Chapter 4).

**Study 3.** Two-group cross-sectional study of fecal prevalence of *C. difficile* at harvest in large-scale processing plants for beef and culled dairy cattle in the USA. (J of Food Prot. In Press Chapter 5).

**Study 4.** Prevalence study of *C. difficile* in retail fresh vegetables (For submission; Chapter 6).

**Goal 2 – Ecology of C. difficile in wildlife and mechanisms of spore dispersion**

To understand the ecology of *C. difficile* and explore mechanisms of spore dispersal, as it is important to identify critical control points to prevent spore dissemination if applicable. The *working hypothesis* of this aim was that wild birds can
harbor and disseminate *C. difficile* strains with molecular virulence factors of relevance for enteric disease.

*The experimental approach:*

**Study 1.** Cross-sectional study of *C. difficile* prevalence in captive white tailed deer intended for food production and recreational hunting in designated reserves in Ohio. (Foodborne Path and Dis, Published: Chapter 7).

**Study 2a.** Cross-sectional study of *C. difficile* prevalence in three wild bird populations to identify common reservoirs, (For submission: Chapter 8).

**Study 2b.** Experimental infection study where selected wild birds were orally inoculated with regular or hyper-virulent *C. difficile* strains to determine their fecal shedding patterns, (For submission: Chapter 8).

**Study 2c.** Single-cohort (longitudinal) study of *C. difficile* from wild birds in a public park (in rural Ohio) to determine the prevalence dynamics of *C. difficile* overtime. (For submission; Chapter 8).

**Goal 3 – Thermal resistance of *C. difficile* spores and spore superdormancy**

To quantify the inhibitory effect of various time-temperature combinations and determine the patterns of survivability and spore germination with recommended minimum cooking temperatures. This is important because it will determine if current guidelines are ineffective, and if revised recommendations to target educate susceptible people are needed.
The *working hypothesis* of this aim is that *C. difficile* survives and cannot be inhibited at temperatures widely publicized in most international cooking guidelines.

*The experimental approach:*

**Study 1.** Experimental thermal studies to determine spore resistance to heat, and mechanistic studies of spore resistance. ([Appl. Env. Micro. Published](#): Chapter 9).

**Study 2.** Selection thermal studies to determine if cooking favors the selection of emerging hypervirulent *C. difficile* strains ([Submitted](#), Chapter 10).

**Goal 4 – Risk of ingestion of *C. difficile* and food safety education**

The final most comprehensive goal of this project was associated with the production of two documents, one for general medical practitioners who can have access to communities at risk (elderly, children, etc) and the other prepared for communities at risk. This is important because revised food safety guidelines are more effective when they target the populations at risk.

The *working theory* of this aim is that if susceptible individuals were targeted for education, the risk of inadvertent ingestion of spores would decrease, and so theoretically the incidence of CDI in the community.

*The approach:*

**Perspective article.** An manuscript written for a medical journal to target general practitioners. It will inform medical and public health practitioners about the risk of foodborne infection (as *C. difficile* can be isolated from foods) and the need to “prescribe” recommendations to improve cooking and hygiene to minimize the
exposure to sources of *C. difficile* spores for communities at risk (Submitted; Chapter 11).

**White Paper.** An encyclopedic white paper (literature review with high readability index) to provide policy makers and average internet users with information regarding *C. difficile* biology, infections and food safety research facts. (For Submission; Chapter 1).

**Invited Review.** An editor-invited review paper is under preparation. This will target the community of food and animal scientists

The identification of which food animal species and to what extent wild birds are active disseminators of emerging *C. difficile* strains is relevant to further develop strategies to increase our Nation’s food safety and health. Results are expected to contribute to the understanding of the disease epidemiology in humans, and to the development of intervention strategies to reduce the risk of transmission in the future.
Chapter 2 — Literature Review on *Clostridium difficile* in animals and retail foods: Understanding and reducing risks

Written as an encyclopedic White Paper. Structured according to guidelines to achieve the readability index of average internet users. (For Submission)

Summary

With no doubt, *Clostridium difficile* is the most important infectious agent associated with intestinal diseases acquired in health-care centers, but now it is increasingly linked with more cases in the community worldwide. *C. difficile*-associated diseases represent a complex of enteric diseases ranging from transient diarrhea, sporadic cases of bacteremia (bacteria in the blood), to often fatal cases of pseudo-membranous colitis (severe inflammation of the colon). Although *C. difficile* have been traditionally associated with hospital environments, recent molecular studies indicate that it is no longer the case. Livestock and retail foods might be involved in the current changing epidemiology of this international pathogenic bacterium. In 2006, the isolation of *C. difficile* strains (responsible for disease in humans) in dairy calves and retail foods ntended for humans, raised concern for its potential zoonotic (animal-human-animal) and foodborne (food-human) transmissibility. Though this issue remains unsettled—due to the absence of conclusive reports linking human disease with exposure to infected animals or contaminated foods, the fact is that *C. difficile* can be found in ready-to-eat
foods. Thus, it is imperative to advance our understanding of how spores from *C. difficile* of relevance for humans reach animals and foods, and/or vice versa. This paper highlights disease facts to contextualize the relevance of potential health risks and future research needs.

**Introduction - why is this relevant today?**

With the potential for inadvertent exposure of animals and humans to infective *C. difficile*, there is need to enhance prevention and food safety measures at various levels. Compared to other enteric pathogens—say the infamous *Escherichia coli* O157:H7, *C. difficile* has been largely unexplored in livestock and food production.

Many different foods and animals can carry *C. difficile*. But it does *not* mean we always get sick after every exposure. There is a chance of getting sick however. But the magnitude of such chance is largely uncertain as it depends on interactions with other—often unknown—risk factors. For example we have differences understanding concepts related to such modifying factors and current research needs will allow citizens to make informed/responsible decisions regarding prevention of *C. difficile*.

**History and Burden**

*Major events in the history of C. difficile*

The bacterium we now know as *Clostridium difficile* was first isolated from healthy infants in 1935 (Bartlett 2008). Then, it was shown that those first isolates were fatal to laboratory hamsters. However, despite that observation, no attention was directed
to study the potential health risks of *C. difficile* in older people until three decades later (Bartlett 2008). During those years, it was assumed that *C. difficile* was a normal inhabitant of the intestinal tract in children. In 1962, another study documented the presence of *C. difficile* in various types of non-intestinal infections of adult people (Smith and King 1962). That study examined eight *C. difficile* isolates obtained from human patients, and found that such isolates were fatal to hamsters as shown in 1935. Despite this finding, the authors’ conclusion was that in those human cases “*C. difficile* was not pathogenic for man”. This bacterium continued being considered as a normal part of our gut flora until it was associated with a life-threatening form of inflammation of the human colon.

In the 1970s several studies in humans and hamsters finally establishd a connecting link between *C. difficile* and a severe form of colitis (colon inflammation) in people. Called pseudo-membranous colitis, that form of disease had been described since 1893 as a disease induced by antibiotic consumption (Bartlett 2008). Usually, pseudo-membranous colitis would occur after the use of widely used clindamycin. In subsequent years, other antibiotics increasingly prescribed to humans, mainly cephalosporins, were also causing such pseudo-membranous colitis and other forms of *C. difficile* infections (CDI) (Bartlett 2008). Currently, it is well accepted that almost every case of pseudo-membranous colitis has as a culprit *C. difficile*. But not always *C. difficile* results in that type of colitis. Infections were traditionally seen as sporadic cases, particularly among people residing in hospitals or health-care centers. However, things changed in the 1990s
as highlighted in a medical journal article entitled “C. difficile: A pathogen of the nineties” (Riley 1998; Pepin et al. 2004).

Compared to the early 1980s, the frequency of C. difficile infections in hospitals in developed countries doubled during the 1990s. By the end of the 1990s, it was considered that humans were the most important reservoirs for infection to other humans (Riley 1998). At the same time, the first studies in domestic animals and the environment demonstrated that C. difficile could also be found almost “everywhere” including root vegetables and household pets (al Saif and Brazier 1996). However, the increasing number of cases in hospitals maintained the attention placed on the role of human-to-human and to environment-to-human transmission (Kaatz et al. 1988). Infections originated in the community, where patients acquire the diseases outside hospitals, received little consideration for disease prevention.

Since the identification as a pathogen, C. difficile was thought to induce disease via the concurrent production of two major toxins (A and B) inside the gut. Toxin A was initially considered the initiating virulence factor. Toxin B was then thought as an accompanying toxin worsening the injury path created by toxin A. Almost always, strains capable fo causing disease carry both toxins, A+B+, and strains that cannot produce either toxins (A+B−) are clinically deemed nontoxigenic and nonpathogenic. However, since 1999, new naturally-occuring C. difficile mutant strains with only toxin B (in short, A−B+) have been identified as the cause of large outbreaks in hospitals worldwide (al-Barrak et al. 1999; Loo et al. 2005). In 2009, a study showing that toxin B, and not toxin A, was essential for disease induction in hamsters (Lyras et al. 2009) created controversy.
However, subsequent studies have shown that both toxins are important for disease and can act independently, locally in the gut lumen, and systemically in various organs when toxins reach the blood stream. In the past, \textit{C. difficile} A\textsuperscript{+}B\textsuperscript{+} mutants were uncommon (often less than 5 percent of cases where \textit{C. difficile} caused disease). Nowadays, A\textsuperscript{+}B\textsuperscript{+} strains are becoming predominant in some regions, for instance, Korea (Figure 1) (Kim \textit{et al.} 2008; Shin \textit{et al.} 2008; Shin \textit{et al.} 2008). In addition to toxins A and B, a minority of strains produce another toxin called binary toxin (CdtA, CdtB), but its role in disease remains unclear (Geric \textit{et al.} 2004; Geric \textit{et al.} 2006; Stare \textit{et al.} 2007). Some consider that the production of binary toxins alone cannot explain \textit{C. difficile} pathogenesis (Geric \textit{et al.} 2006), although it may be a contributing virulence factor (Terhes \textit{et al.} 2004; Schwan \textit{et al.} 2009). Together, the three toxins through slightly different mechanisms produce disruption of the cytoskeleton and cell death.

Since 2005, additional parallel discoveries indicated that unusual pathogenic strains were associated with more outbreaks and disease severity in various countries. Compared to isolates from before 2000, current \textit{C. difficile} isolates from people are more resistant to antibiotics (Muto \textit{et al.} 2005). Further, some strains have been shown that can produce up to 16-to-20 times more toxins A or B compared to regular strains (often called hyper-virulent strains) (Loo \textit{et al.} 2005; Warny \textit{et al.} 2005).

Of public health relevance for communities at risk, identical hyper-virulent high toxin-producing \textit{C. difficile} strains associated with severe disease in humans have been increasingly isolated from food animals and retail foods since 2006 (Rodriguez-Palacios \textit{et al.} 2006; Rodriguez-Palacios \textit{et al.} 2007; Rodriguez-Palacios \textit{et al.} 2009; Songer \textit{et al.} 2009).
Since the publication of those first reports, there is increased awareness about the potential for animal-to-human (zoonotic) and food-to-human (foodborne) transmission (Rupnik 2007). Although, there are no proven cases of zoonotic or foodborne-associated 

*C. difficile* infections, there are disease facts and research findings that highlight the potential risk for transmission.

**More severity and cases of *C. difficile* infections internationally**

Testing of patients for *C. difficile* has increased since 2000, and with it, the number of people diagnosed with *C. difficile* associated infections and the severity of such infections have also increased (Dallal *et al.* 2002). Despite increased awareness of *C. difficile* infections and potential reporting bias, studies have confirmed that the increasing prevalence of *C. difficile* in disease is real, and not simply a reflection of more testing.

For example, in Germany between 2000 and 2007, thousands of stool samples from people in hospitals and the community tested for *C. difficile* confirmed a parallel increase in the number of cases in hospitals and the community (Figure 2) (Borgmann *et al.* 2008). At its peak in 2003, that study showed that the percentage of affected outpatients (community) had increased almost 7 times compared to the year 2000—in other words, went from one case every 40 outpatients in 2000 to one in every 6 in 2003. Despite a subsequent decline observed, it was still one in every 10 outpatients by 2007.

**The numbers**

This increase frequency of CDI has also resulted in a financial burden to individuals and national health systems. It is estimated that there are over 500,000 cases of CDI every year in the USA in health care settings alone (Rupnik *et al.* 2009). This
estimate is based on data obtained during a state wide mandatory reporting system adopted in Ohio during 2006 (Campbell et al. 2009). Few states in USA has adopted mandatory report since, but that effort was critical to better grasp the magnitude of the problem and raise awareness among health-care workers. During that reporting, health care officials also kept track of recurrent cases of CDI. Considering that CDI re-occurrences is currently a growing problem (Pepin et al. 2005), and that CDI is increasingly reported in the community (Limbago et al. 2009), the quoted national estimates are likely low.

The cost for medical treatment for every CDI case hospitalized could be in excess of $3,000 in North America (Dubberke and Wertheimer 2009). Overall, the cost to the USA finances may represent over 3.2 billion dollars every year (O'Brien et al. 2007), the amount equivalent to collecting yearly taxes from approximately 300,000 individuals in USA (Bachman 2008).

With an increase in the number of human infections, there has also been an increase of social concerns. Today there is more disease severity, with more patients requiring surgical removal of the inflamed colon (one every ten CDI cases—10 percent), higher mortality rates, and of relative concern more liability (Pepin et al. 2005; Sailhamer et al. 2009). The catastrophic effect that such medical consequences have at the individual level and their families cannot be quantified. Therein, infection control and surveillance initiatives have been reinforced internationally to reduce the raising incidence of *C difficile* infections (Pepin et al. 2004); unfortunately, in the majority of cases with limited success.
Given that *C. difficile* also infects animals, the disease can have impact on companion animals and livestock too. Financial loss estimates associated with *C. difficile* infections in animals are not available. But in horses and other companion pets where *C. difficile* causes enteric disease the costs associated with veterinary medical treatment are assumed by the owners. In livestock production, no estimates are yet available either, largely due to absent evidence that *C. difficile* causes disease and/or growth delays in production animals. But the farmer will finally carry the financial burden for treatment and loss of production (Songer 2004; Kiss and Bilkei 2005). Gaining further understanding about the disease in animals would allow us to implement preventive strategies to reduce this burden on agriculture and animal’s owners.

**New patterns of disease — In brief**

Overall, *C. difficile* infections (CDI) can be classified based on the time of acquisition and the onset of clinical signs as associated with health-care settings or with the community when no attribution can be made to health-care settings. Often due to the opportunistic nature of *C. difficile* and the variable number of days that pass between exposure to a risk factor (for instance, antimicrobials or antacid use) and the recognition of signs of disease, the distinction between hospital and community acquisition is often difficult. Hence, definitions often vary from study to study. Health-care-facility onset refers to cases when signs of disease start after 48 hours of admission, and community-onset applies when the complex definition of health-care onset/acquisition does not apply.
Considering that elderly over 65 year-old are often affected in health-care centers, two new patterns of *C. difficile* infections have internationally emerged since 2000:

- More severe and frequent cases of CDI among people in **health-care centers** (Gastmeier *et al.* 2009).
- More severe and frequent cases of CDI among people in the **community**, including those previously thought to be at low risk; specifically pregnant women, children and young healthy adults (Limbago *et al.* 2009).

Comparatively, studies have highlighted the remarkable parallel in disease occurrence between hospital and community-acquired infections (Dubberke *et al.* 2009). In hospital settings, the admission of patients with community-acquired CDI represents sources of new *C. difficile* strains for other patients. Thus, understanding the factors associated with *C. difficile* infections in the community would in theory reduce the incidence of *C. difficile* in hospitals (Figure 3).

**Clostridium difficile has also changed as a pathogen**

There is conclusive evidence that *C. difficile* has also genetically and physiologically changed over the past decades. New *C. difficile* strains are often mutants, produce more toxins and are more resistant to antimicrobials that would have otherwise been effective to them before 2000. Recent microarray-based data indicate that food animals might have been the original sources of epidemic strains of *C. difficile*, particularly newly emerging *C. difficile* (Stabler *et al.* 2006; Goorhuis *et al.* 2008), but no conclusive studies are yet available to determine if animal shedding or food
contamination are associated with changing patterns of disease in humans. Has the pathogen moved from humans to animals and foods or viceversa? Given the spore forming nature of *C. difficile*, it is possible that inter-species transmission occurs from widespread environmental sources and that some level of host adaptation (Janvilisri *et al.* 2009) and clonality has also ensued in paralell over millions of years (He *et al.* ; Stabler *et al.* 2006). Horizontal gene transfer and homologous recombinations (i.e., the exchange of pieces of DNA among *C. difficile* strains) are very frequent processes in *C. difficile* (He *et al.*).

*Not everyone knows where or why they became sick with C. difficile*

Only a fraction of all individuals affected with *C. difficile* can be linked to known risk factors for disease. Some individuals are more likely than others to become ill with *C. difficile*. Those include individuals that:

- are over 65 years old
- have direct or indirect contact with hospital/health-care centers, or
- have consumed antibiotics or antacids.(Kuijper and van Dissel 2008)
- among racial ethnicities, whites appear to be at higher risk (Redelings MD *et al.* 2007)

However, a large proportion of (approximately one third) CDI patients do not have any of the above mentioned risk factors, and the source of their infection cannot often be traced (Wilcox *et al.* 2008). Less clear are the sources of *C difficile* spores for people in the community that have no exposure to hospital settings. Moleculart studies
identifying pathogenic strains in rivers, animal manure and retail foods indicate those could be source of spores which are needed for the induction of disease (Rodriguez-Palacios et al. 2006; Rodriguez-Palacios et al. 2007; Rodriguez-Palacios et al. 2009; Songer et al. 2009; Von Abercron et al. 2009; Weese et al. 2009; Weese 2010; Zidaric et al. 2010). However, no direct evidence is available confirming zoonotic or foodborne transmission.

Pathogen and facilitator factors

**Strains today are more resistant to antibiotics than before 2000**

In addition to high amounts of toxin production in some genetically mutated strains, *C. difficile* strains isolated today are more frequently resistant to antimicrobials than before 2000. Today, the increase resistance to fluroquinolones, metronidazole and vancomycin has become an emerging global health issue (Spigaglia et al. 2008). Although, the use of antimicrobial in animal production is often blamed for the increase resistance among pathogens derived from infected humans, the same assumption is difficult to justify in the case of emerging *C. difficile*. Fluoroquinolones, metronidazole and vancomycin are prohibited drugs for use in food animals in most developed countries, where *C. difficile* is a problem. More recently, high MIC values (intermediate resistance) were identified in a *C. difficile* isolate derived from cattle at the time of harvest (Rodriguez-Palacios et al. 2011). Linezolid is a new antimicrobial drug (prototype of new drug class) that has not been reportedly used in the veterinary literature. Horizontal gene transfer of antimicrobial resistance from naturally resistant
microorganisms and spontaneous mutations (Holzel et al. 2010) with natural selection are possible options.

**Clostridium difficile is an environmental-resistant microorganism**

*Clostridium difficile* is a bacterium that grows in the absence of oxygen (anaerobic environments), including the intestinal tract of animals and humans. When ideal growing conditions turn into adverse scenarios the bacteria produce spores—this is, cells that are dormant and very resistant to environmental temperature changes and the presence of sanitizers (Lawley et al. 2009). When the conditions become more favourable, these dormant spores awake and germinate to produce a new offspring of actively dividing bacterial cells. These growing cells have the capacity to produce virulent toxins and escape the action of antimicrobials to become opportunistic pathogens of the gut.

In susceptible animals and people, antimicrobials (among other factors) apart from facilitating the induction of enteric disease, they also stimulate the rapid production of spores by growing *C. difficile* in the intestinal tract which favours the survival of antimicrobial-resistant strains (Baverud 2004; Rupnik et al. 2009). In the environment, spores from infected animals can survive for months or even years in feces or soil (Baverud et al. 2003) and could be dispersed via air, waters and animals, at least locally. There, spores can remain dormant until they are ingested by a new host.

**Fate of Clostridium difficile spores following ingestion**

Like other enteric pathogens, activation of *C. difficile* starts with inadvertent ingestion of spores. Ingested spores may have two fates, remain dormant and be expelled
in the feces or wake up, produce offspring, and induce intestinal disease. The latter only results in disease if the immune status of the individual and the integrity of his/her microbial gut flora are impaired. However, since the mid 2000s, more cases of disease seem to be occurring in otherwise healthy individuals (Rupnik et al. 2009).

Ingested spores or insulated delicate vegetative cells can survive gastric acids and germinate and/or proliferate in the gut owing to its adequate nutritional conditions, optimal host temperature and low oxygen. Under ideal laboratory conditions, a spore can awake from dormancy to produce active *C. difficile* cells within one hour. But how long it takes inside the gut of humans or animals is largely unknown.

The likelihood of becoming sick following ingestion of spores is uncertain to date. Due to ethical limitations and the opportunistic multi-factorial nature of CDI it is difficult to determine how many spores are needed to cause disease (*i.e.*, the infective dose) in people. Experimental infection studies in neonatal calves (Rodriguez-Palacios et al. 2007) and foals (Arroyo et al. 2004) have shown that spore ingestion alone is not sufficient to induce disease. Predisposing factors are almost always needed. Treatment with antimicrobials is one of these factors.

In farm animals, *C. difficile* may persist in the intestinal tract of animals (Norman et al. 2009) or in the soil (Baverud et al. 2003) at the farm for months if not years. One study, conducted in a single swine (pig) operation indicates that at least a strain of human relevance can be prevalent among animals through most of the production cycle, although it becomes less common close to the time of harvest compared to newborn piglets (Norman et al. 2009). However, the ability of *C. difficile* to complete a life cycle
outside animated hosts has not been elucidated. The ecological component of *C. difficile* in the patterns of human and animal disease remains largely unexplored. The relevance of such understanding lies in the fact that susceptible individuals often acquire infective spores from environments formerly inhabited by shedding individuals (Figure 4).

_If you get sick once, you can get sick twice, or more_

Of additional concern is that following recovery from a *C. difficile* infection, re-infections in the same individual are occurring with increasing frequency. Although it is happening more often, not everyone suffers *C. difficile* re-infections. There are however overly sensitive individuals that can be seriously affected; the reasons of such susceptibility is currently under intense investigation. Low antibody titters effective against the *C. difficile* toxins (Wilcox 2004), and disrupted intestinal flora due to antimicrobials (Rupnik et al. 2009) are among the risk factors that may enhance susceptibility to re-infections.

Reported rates of re-infections vary between 15 and 30 percent, with recurrences being more common in elderly patients (over 65 years old)(Kyne 2010). Further, the more recurrences a person has, the more likely he/she is to have a recurrence again. In most cases, subsequent infections are caused by a new *C. difficile* strain that is molecularly different from that of the first CDI episode.

Prevention measures in re-infected individuals are particularly important considering that most CDI that require medical attention are treated with antibiotics that disrupt the gut flora beyond the time of recovery from the CDI symptoms.(Rupnik et al. 2009) Unfortunately, preventive medicine reports are not yet available. Understanding
the interaction dynamics between the antimicrobials, their impact on the gut flora, and the windows of opportunity for *C. difficile* to proliferate and re-induce disease might reduce the risk of exposure to infective spores during periods of higher risk for CDI.

**Differentiating *C. difficile* from various potential origins**

*Molecular fingerprinting (“bar-coding”) of *C. difficile*

To differentiate *C. difficile* strains (or lineage) and thus to study their possible origin, scientists use molecular methods to create genetic fingerprints. These fingerprints are largely unique and identical among highly related isolates, but different among different distantly related strains. Genetic fingerprints allow tracking routes of contamination and study the potential for pathogen transmission, and the vehicles of dissemination that could be involved. To enhance accuracy, scientists use a variety of molecular tests to classify strains of *C. difficile*, often in combination. The most common methods for studying *C. difficile* isolates include:

- PCR ribotyping
- Pulse field gel electrophoresis (PFGE)
- Restricted fragment length polymorphism (widely known as toxinotyping)
- Multilocus variable tandem repeat analysis (MLVA)

The most common typing methods cited in current studies are PCR-ribotyping (mostly used in Europe), followed by Toxinotyping (international), and Pulse Field Gel Electrophoresis (PFGE) and Restriction Endonuclease typing (REA) mostly used in the
USA. A search of studies about *C. difficile* published in PubMed search in 2009 showed that PCR ribotyping was more used (48 studies) compared to PFGE and REA (7 and 6 studies, respectively) (Rodriguez-Palacios, A, unpublished data). PCR ribotyping is widely used because it allows for the contextualization of results at a global scale. Toxinotyping is widely used because it helps group the strains by their virulence profile *(i.e.,* toxins A and B gene molecular characteristics).

**There are several *C. difficile* strains (lineages) but often few are the bad ones**

Molecular typing methods help understand how strains are genetically related and to source-track and infer potential routes of transmissibility locally or across large geographical distances. However, each technique has pros and cons that are considered depending on the purpose of the typing (Tanner *et al.* 2010).

Depending on the typing method used, the number of *C. difficile* strain types can vary largely. According to an internationally cited Reference Laboratory in the United Kingdom—where the strain collection begun in the early 1990s (O'Neill *et al.* 1996), there are now over 300 *C. difficile* strain types *(i.e.,* molecularly distinct with a fingerprinting method)(Balassiano *et al.* 2009).

To date, many studies are using the UK PCR-ribotyping nomenclature for international comparisons (Rodriguez-Palacios *et al.* 2006; Balassiano *et al.* 2009). The nomenclature designation for that system is numeric and started with PCR-ribotype 001 in the 1980s and continued in order as new strains were identified. The use of such international naming system has allowed scientist to compare isolates across landscapes and over time, studying the dynamics and emergence of epidemic *C. difficile* strains.
(Figure 4). Today, PCR ribotypes 001, 014, 017, 027, 077 and 078 are among the most common strains in certain regions. Of public health relevance, all these strains have been isolated from animals and foods (Rodriguez-Palacios et al. 2006; Rodriguez-Palacios et al. 2007).

**About the Disease**

*How does C. difficile ingestion result in intestinal disease?*

Whether an individual who is exposed to *C. difficile* spores becomes sick depends upon many factors. First, it depends on the age of the host that is exposed and their immune status (elderly patients with cancer, or debilitating medical conditions are at higher risk), the concurrent use of medications that are disruptive of the intestinal flora (*i.e.*, antimicrobials), and the ability of the spores to grow and produce damaging toxins in the gut. Ingested *C. difficile* spores are not capable of inducing disease, they need to “wake up” from dormancy (germinate) and actively produce rapidly multiplying daughter cells which produce the toxins responsible for intestinal lesions and disease. With few exceptions, *C. difficile* strains that induce disease need to be able to produce at least one of three currently recognized virulent toxins—A, B, and CDT. Strains that cannot produce toxins do not cause disease, and rather may play a protective role (Songer et al. 2007).

Toxins A and B may act independently *in vitro*; however, a recent study showed that toxin B alone is essential to induce disease and mortality in experimental animal models. Although most strains carry both toxins A and B, more cases of strains producing
only toxin B have been associated with disease worldwide since the first outbreak of serious disease in hospitalized people was identified in Canada in 1999 (Alfa et al. 2000). The role of the third toxin, referred as CDT, is under investigation.

Functionally, the toxins induce cell roundening and death, which creates microscopic gaps or craters on the lining that cover the intestinal tract. Disruption of this protective layer is responsible for the clinical signs observed in humans and animals when disease occurs. Inflammation in the colon, with destruction of the gut barrier, renders hosts susceptible to other opportunistic bacteria and complications associated with initial intestinal damage. Bacterial invasion of deeper tissues and blood originating from the intestine can occasionally occur in some individuals (Smith and King 1962; Gérard et al. 1989; Thomas et al. 2011). This complication however seem to occur in the absence of intestinal signs of disease and in immunocompetent patients (Cid et al. 1998; Libby and Bearman 2009).

While the effect of toxigenic strains has been well studied, the effect of ingesting non-toxigenic strains (*i.e.*, strains unable to produce toxins due to lack of functional toxin genes; A`B`CDT”) on intestinal health is largely unclear. Preliminary studies in animals and humans indicate however that exposure to non-toxigenic strains might have a protective role against disease by preventing the proliferation of toxigenic strains if subsequently ingested. Ongoing clinical trials are underway to test related hypothesis.

**New C. difficile strains can produce much more toxins - why?**

While humans required years to reach reproductive age, bacteria—including *C. difficile*—can have a new offspring every 20-60 minutes (Goulding et al. 2009). With rapid generation turnover, genetic mistakes (mutations) can result during bacterial
replication. Often those genetic mistakes can be fatal to the new daughter cells, but at times, mutations may boost the chances for a bacterial cell to thrive in challenging environments facilitating adaptation.

The production of toxins A and B is controlled by protein TcdC, which is a product of the regulatory gene tcdC. Natural mutations in this gene have resulted in C. difficile strains with increased toxin production. In 2005, a study of the common strain (epidemic PCR ribotype 027/NAP1) from human origin showed that genetic machinery that control toxin production in that strain was disrupted increasing the amount of toxin production. Strains since about the early 2000s are likely to produce more toxins compared to regular non-epidemic strains (Warny et al. 2005). Although some studies indicate that such strains have increased the incidence of disease and associated mortality outside health care centers (Kuijper and van Dissel 2008), few studies have reported no direct associations with disease severity in hospitals (Morgan et al. 2008). Irrespecive of the finding, all studies demonstrate that this and other emerging mutated strains are on the rise as responsible for disease in humans.

In animals, recent studies have shown this and other types of mutations in the same toxin regulatory mechanism, including strains designated PCR ribotype 078/Toxinotype V. This strain increasingly isolated in humans, is a major predominant strain in livestock, namely young cattle and piglets.

**C. difficile exposure vs. risk factors for CDI — Two different things**

One thing is identifying the sources where spores can be ingested from, but the other is the settings where risk factors are likely to make subjects prone to developing enteric disease. Risk factors such as antibiotic or antacid ingestion can occur at home and
in health care centers, where spores may or may not be present in the environment. In humans and animals, CDI is a diagnosis primarily based on the combined presence of clinical signs of enteric disease and the identification of toxins in the stools (feces). (Russmann et al. 2007) In practice, the simultaneous association of both—the risk factor and the source of spores—largely depend on diagnostic criteria used to confirm CDI and the clarity of definitions used to call something a risk.

Where can we get both the spores and the risk for CDI? — Onset vs. Acquisition

To date most studies have divided all forms of CDI in two majors groups, but the epidemiology of it is more complicated than that. One thing is the onset (when clinical signs start) and the other is acquisition (where the spores were ingested). To enhance our understanding in this area, scientists have long collaborated to achieve global scientific standards. Now, there is international consensus definitions for community associated cases: (Kuijper and van Dissel 2008)

“The European Centre for Disease Prevention and Control and the US Centers for Disease Control and Prevention have proposed similar definitions for community-acquired C. difficile infection:

[A] the onset of symptoms occurred while the patient was outside a health care facility and the patient had not been discharged from a health care facility within 12 weeks before symptom onset (community onset, community acquired);

[B] the onset of symptoms occurred within 48 hours after admission to a health care facility and the patient had no prior stay in a health care facility within the 12 weeks before symptom onset (health-care-facility onset, community acquired)”
“Community-acquired C. difficile infection is almost certainly under diagnosed”

(Kuijper and van Dissel 2008)

In the past, CDI were a disease of mostly elderly over 65 years of age. Now things appear to be changing, affecting younger populations. The unanswered question is where these affected individuals are picking up the virulent C. difficile strains. Among many possible alternatives, the following excerpt further illustrates the absence of traditionally known risk factors among people that get sick in the community:

“Of 241 patients, 36 percent had no history of antibiotic use within 3 months before symptom onset, and 25 percent had no underlying medical condition or recent hospital admission and, moreover, were younger than 45. ...”

About Animals, the Environment and Foods

In animals, the first studies reporting the isolation of C. difficile from fecal samples companion animals and pigs were published in the early 1980s. However, it was early in the 2000s when association with enteric disease started to be confirmed in animals. Molecular (i.e., genetic) fingerprinting studies comparing animal and human isolates began to be reported in 2005. (Arroyo et al. 2005) These started to indicate the potential for identical strains to share human and animal habitats. With it the risk of cross-transmission began to be considered.

*Clostridium difficile* in the environment and animals
As an environmental microorganism, it is possible to think that an obvious source of infective spores for people is unnoticed contaminated environments. However, such observations been documented since the 1980s by the same groups of scientists who started the UK collection of *C. difficile* that is now reference landmark for molecular epidemiology studies internationally (Borriello *et al.* 1983).

*Clostridium difficile* was then (1982) isolated by Borriello *et al.* from rivers draining a city, and nearby ocean waters, soils and a few root vegetables. Concurrently studies in companion animals showed that *C. difficile* was present in dogs, especially puppies but no linking studies were conclusive to establish a strong connection. Other species assessed included horses, in which *C. difficile* has been now recognized as an established cause of serious enteric diseases in adult horses (Weese *et al.* 2006). In young foals the situation is not completely clear, but antimicrobials are deemed a major predisposing factor (Arroyo *et al.* 2004). Although the possibility of animals being reservoirs of disease was suggested by the researchers at that time, no subsequent studies were conducted to validate such observation.

In 2005, a study from Staempfli and Weese’s group, at the University of Guelph, Canada, reported the first molecular studies comparing human and strains derived from companion animals indicated that no strains were common (Arroyo *et al.* 2005). However in 2006, during studies conducted with calves in an attempt to determine the role of *C. difficile* in enteric disease in young calves the majority isolates matched now known as epidemic human PCR genotypes, namely PCR ribotypes 078, 027, 014 and 017 (Rodriguez-Palacios *et al.* 2006).
**Companion Animals: Household pets - Dogs, cats and other creatures**

Dogs and cats can carry toxigenic strains of *C. difficile* in their feces. This was first identified in the 1980s (Borriello *et al.* 1983). But in the majority of cases, *C. difficile* does not cause disease (Weese *et al.* 2010). If risk factors, that parallel that of humans (antimicrobials) animals may have diarrhea (Weese *et al.* 2001). No pseudomembranous colitis or bacteraemia have being documented in dogs.

Screening studies indicated recently that up to 10 percent of household pets may carry *C. difficile* in the community representing a risk for owners (Weese *et al.* 2010). Although no direct transmissibility from pets has been documented to humans, reports of the virulent strains of *C. difficile* (including PCR ribotype 027) in visitation dogs indicate that hospital visiting animals might carry strains within and outside health-care facilities (Lefebvre *et al.* 2006). Of epidemiological relevance, pets owned by an immune-compromised person are more likely to be colonized by *C. difficile* (Weese *et al.* 2010).

In veterinary hospitals, outbreaks of severe diarrhea associated *C. difficile* have been reported in small animal clinics (Weese and Armstrong 2003), therefore pets that visit animal hospitals may be inadvertent carriers of *C. difficile* spores. In households, a report in 2009 found *C. difficile* in dogs of immune-compromised people and in the environment of a few households, but molecular typing (fingerprinting) of the strains did not support that dogs were the source for household contamination (Weese *et al.* 2010). Those dogs, however, had strains identical to the ones that had affected people in the region of the study a year earlier. No studies have assessed the potential of dogs and cats to be vehicles of *C. difficile* strains in food or livestock production systems.
Companion Animals: Horses - Pleasure and working animals

*Clostridium difficile* has been studied in horses since the mid 1980s (Ehrich *et al.* 1984). Today, it is known that horses can carry *C. difficile*, but the proportion of animal shedding the pathogen varies across studies as it depends on culture methods and the animals’ age. Adult horses are less likely to carry the bacterium compared to neonatal foals. Overall, between 2 and 30 percent of horses could be found carrying spores at any given time without showing signs of disease (Baverud *et al.* 2003). But like other species, horses also can develop diarrhea and other forms of serious disease. In equine hospitals, strict isolation and infection control measures are widely recommended to avoid outbreaks. (Baverud 2004). As in human hospitals, antimicrobials increase the risk of horses being affected with CDI (Weese *et al.* 2006).

Currently, horses provide pleasure for some, but historically, horses have been companions for work purposes since the time of wars. And although they have been large displaced by modern mechanized equipment, some cultures still rely on horse power to work on their agricultural lands to produce foods. Indeed, regulated production and slaughter of horse meat sustain at least partially local economies in some regions (USDA 1997). Since horse manure can contain living *C. difficile* spores for years (Baverud *et al.* 2003) and it has been used as fertilizer for decades (Randall 2003), the potential for crop contamination (Pell 1997) is an additional alternative to currently documented food contamination with *C. difficile*. In this regard, no studies are yet available in horses to further address the potential risk for zoonotic and foodborne transmissibility.

Food Animals: Pigs
As in horses, the first studies reporting the presence of \textit{C. difficile} in pigs date to the early 1980s (Jones and Hunter 1983). Since then, over 50 published studies have served to now recognize \textit{C. difficile} as an enteric pathogen in this domesticated species. And among pigs, the major clinical relevance resides in piglets (Post et al. 2002). Mortality and morbidity rates are largely uncertain, but some estimates indicate that up to 100 percent of litters and individual piglets can be affected in infected farrowing facilities (Songer 2004). In non-fatal cases, weaning weights of diseased pigs can be 10 percent below the expected average weight (Songer 2004). In older animals, \textit{C. difficile} has been associated with increased mortality in sows that received antimicrobial treatment against a post-parturient syndrome characterized by lack of milk production, and uterine inflammation (Kiss and Bilkei 2005). Sows that received antimicrobial treatment were more likely to die if they concurrently had \textit{C. difficile} compared to sows that were not treated. Apart from this report in an outdoor production system in Croatia (Kiss and Bilkei 2005), no other studies have described the syndrome in swine.

On the other hand, \textit{C. difficile} can also be isolated from up to 4 percent of healthy pigs close to the harvest time, which has further raised concerns about potential for carcass contamination (Norman et al. 2009). As with other species, in the harvest stages, \textit{C. difficile} infections goes unnoticed as no obvious signs of disease differentiate shedders from non-shedders.

Molecularly, swine-derived \textit{C. difficile} isolates have called the largest attention from public health officers as among all possible PCR ribotypes, PCR ribotype 078—the increasingly documented emerging human ribotype in the community—have accounted
for up to 80 percent of all swine isolates in most studies conducted involving pigs in
North America and Europe (Keel et al. 2007; Debast et al. 2009; Songer 2009).

Regarding the type of production system, a study in Europe found no difference between
organic and conventional swine operations (Keessen et al. 2011).

**Food Animals: Cattle**

Despite all efforts to document the presence of *C. difficile* in animals since the
1980s, little attention has been directed to study ruminants and bovine animals in
particular. The first study that described *C. difficile* in calves indicated the presence of *C.
difficile* in veal calves in 2002 (Porter MC et al. 2002). However, the first peer-reviewed
study assessing the impact of *C. difficile* in the bovine industry aimed to determine—in
2004—the role of the pathogen as a cause of diarrhea in young calves (Rodriguez-
Palacios et al. 2006). In that study, Rodriguez-Palacios and his colleagues conducted a
case–control study of calves less than 28 days of age—from 102 dairy farms in Canada—
and showed that significantly more diarrheic calves than non-diarrheic controls were
positive to *C. difficile* toxins, suggesting its association with disease (Rodriguez-Palacios
et al. 2006). However, in further experimental studies the same group could not induce
disease when calves fed colostrums were given orally high numbers of *C. difficile*
(Rodriguez-Palacios et al. 2007). Further, *C. difficile* spores were more common in
healthy calves, clearly showing the potential for inadvertent dissemination. Subsequent
studies conducted in calf ranches conducted by others indicated that the lesions observed
are compatible with *C. difficile* toxin action (Hammitt et al. 2008). Other unpublished
studies indicate that veal calves can increase the rate of *C. difficile* shedding as they are treated with antibiotics upon entry to finishing operations (Weese 2010).

In older cattle, *C. difficile* shedding has been observed to decrease overtime during the finishing period. However, *C. difficile* can be found in healthy feedlot steers and culled dairy cattle at the time of harvest highlighting the risk for carcass contamination.

Regardless of its association with enteric disease, *C. difficile* isolates derived from cattle were the first to call the attention for the potential for foodborne transmissibility involving current epidemic human strains.(Rodriguez-Palacios *et al.* 2006) Calf isolates that were identified in 2006 include strains of PCR ribotypes that occur in humans worldwide and that have been later found in retail foods, particularly PCR ribotypes 017, 027, 077, 014 and 078 (Rodriguez-Palacios *et al.* 2006; Keel *et al.* 2007; Hammitt *et al.* 2008; Rodriguez-Palacios *et al.* 2009). Together, the molecular characteristics of bovine and swine *C. difficile* isolates represent an epidemiological link with human disease that is under intense research. However, much more need to be done to enhance prevention. This area of science is relatively new.

**Food Animals: Poultry**

This is probably the livestock species that has been studied the least. But perhaps the study that highlight the potential relevance of this species are from Africa (Simango 2006; Simango and Mwakurudza 2008) and Slovenia (Zidaric *et al.* 2008). In Zimbabwe, Simango and colleagues have showed that up to 30 percent of free range chickens carried toxigenic *C. difficile* strains and antimicrobial resistance patterns of relevance for humans.
(Simango 2006; Simango and Mwakurudza 2008). Although the presence of \textit{C. difficile} in cultures where marketing free range living animals is well rooted, results from a study in confined poultry operations in Slovenia indicate that in confined farming environments the percentage of birds colonized could be higher. In that study, over 60 percent of animals carried \textit{C. difficile}, but it was less frequent as animals approached harvest time. In a study conducted in operations in intensive rearing facilities in Ohio, USA, the percentage of contamination approached zero (Rodriguez-Palacios, \textit{et al.} 2011). In a published report from Austria, 5 percent of poultry tested had \textit{C. difficile} (Indra \textit{et al.} 2009). Clearly much more work needs to be done in this field also.

\textbf{Waters and the environment}

In general, spore-forming bacteria including clostridia can be considered long lasting microorganisms inhabiting the environment. With very few exceptions, \textit{C. difficile} produce spores that can survive for months in the environment (Baverud \textit{et al.} 2003), but not many publications are available in this regard. Perhaps the most relevant of them is a British publication that in 1996 described the presence of toxigenic \textit{C. difficile} in different environmental samples (al Saif and Brazier 1996). In that study, scientists found \textit{C. difficile} in soils, well and recreational waters, and veterinary clinics and in households; they even suggested that those places could be potential sources of infection (al Saif and Brazier 1996). Another relevant study in Africa showed that soils in rural areas of Zimbabwe had toxigenic spores (Simango 2006). However, despite these publications, little attention has been placed in this very relevant aspect of the disease epidemiology—this is the environment as a source of infectious spores but also its mediating effect in
spore dissemination. *C. difficile* spores have been shown to be able to disseminate via air in indoor environments (Roberts *et al.* 2008). At the farm level, this area of research remains largely unexplored. But sewage should be expected to be a way how farming may be impacting the environment.

**C. difficile in Foods**

Great efforts have been accomplished by various governments around the world to monitor the patterns of antimicrobial resistance in bacterial isolates from foods which cause disease in humans. Although countries in North America and Europe are constantly monitoring trends, such surveillance programs are important initiatives (Jones and Masterton 2001) that have assisted in the study of food products as carriers of *C. difficile*.

Before summarizing this interesting growing body of literature, it is important to mention that as of August 16, 2010 there were no reports confirming that people have gotten *C. difficile* infections via consumption of contaminated foods. Also it is important to highlight that at least three reports published in the 1980s warning about the potential foodborne (Gurian *et al.* 1982; Borriello *et al.* 1983) and zoonotic (Borriello *et al.* 1983) transmissibility of *C. difficile* remain largely overlooked until the mid 2000s. (Rupnik 2007) Now is the time to further enhance our knowledge in this regard.

**Raw meat products**

Raw ground beef and pork were the first products to be found contaminated with *C. difficile*, and as usual with major discoveries it was an incidental finding. In 1994, Broda and her colleagues studying which microorganisms caused gas blown-packed spoilage in meats (indeed they were ready-to-eat products) found *C. difficile*, which did
not produce gas. So the relevance of such findings seemed as disregarded by food scientists in the field that could have read the article. The next study, unaware of Broda’s findings—and therefore truly unbiased—was conducted by veterinarians in Raw meat products for dogs also found \textit{C. difficile} but little was thought about the validity of that observation in human health by others in veterinary medicine or related fields that might have also read the study in both human and veterinary medicine-in retrospect- also overlooked that observation.

In 2006, Rodriguez-Palacios and his colleagues, for the first time, found identical strains of \textit{C. difficile} in ground meats in Canada that matched strains of relevance for humans locally but also in the UK. Subsequent studies have confirmed that this pathogen can be found elsewhere. Now, scientific reports describe toxigenic \textit{C. difficile} in meats in several countries. Although, the percentages of meat packages that have showed contamination with \textit{C. difficile} have ranged from 3 to 42 percent, the overall expected real prevalence of \textit{C. difficile} contamination under natural conditions might be around 6 percent. Poultry has been the type of meats least studied; at least one published study found no \textit{C. difficile} in retail poultry,(Indra \textit{et al.} 2009) but a recent review indicate that poultry meats can also carry toxigenic strains at about 7\% frequency (Weese 2010)

\textbf{Ready-to-eat foods}

In modern times when life style allows us a few spare minutes to buy, cook and eat our own foods, convenient ready-to-eat products are gaining market share. And with it food scientists have been accompanying the change with processing methods that seek to eliminate most unwanted microorganisms, while marinating the flavoury nutritional
value. Among this group, processed meats and ready to eat fruits and vegetables are common ingredients recommended for healthy lives. However, like other infamous bacteria- including *E. coli* O157:H7, *C. difficile* has also been found in these products. But this is not something new; we just missed its relevance in the past.

In part due to lack of molecular studies which have now help to evaluate the potential risk by showing that strains derived from ready to eat salads in Scotland were identical to the most common PCR ribotypes affecting people (Bakri et al. 2009). In the past; we overlooked initial reports. *C. difficile* was first isolated in the UK in 1996, from root vegetables (al Saif and Brazier 1996). Their finding allowed scientists then to warn about the potential food safety concern might have been a potential problem. However, only recently studies have confirmed that between 1 and 6 percent of vegetables can be sources of *C. difficile*, including PCR ribotype 078 (Metcalf et al. 2010).

**Potential for control of *C. difficile* in foods and food animals**

Because several aspects of ecology and epidemiology are still unknown regarding the ability of this pathogen to survive in different environments and to effectively amplify, it is necessary to determine survival and persistence factors at preharvest and postharvest level. The main objective of the present thesis was to determine factors associated with fecal shedding dynamics, seasonality and prevalence in food producing animals and wildlife, and to determine factors of thermal resistance that would be relevant for the enhancement of food safety through cooking and education. This is the particular objective of this White Paper. It will be modified and adjusted for free online
publication. Appropriate quantitative data is also needed to assist in the implementation of risk assessment programs, food safety policies and recommendations, and to propose intervention strategies for the future.
Figure 1. Increase of toxigenic *C. difficile* strains A-B+ in a multi-center study in hospitals of Korea compared to A+B+ strains, 2000-2005 (Shin et al. 2008a).
Figure 2. Study testing *C. difficile* toxins in fecal samples from patients visiting 40 hospitals and over 2,000 physicians in southern Germany.

Despite the increased number of samples submitted for *C. difficile* testing, these illustration depicts the rising percentage of people affected by this pathogen over the years, in both hospitals and the community. Similar trends occur in other regions of Europe and North America. (Archibald *et al.* 2004; Ricciardi *et al.* 2007; Kuijper *et al.* 2008; Limbago *et al.* 2009) (*Reproduced with permission from Borgmann *et al.* Borgmann *et al.* 2008) Copyright Eurosurveillance, 2008)
Figure 3. Possible routes of transmission of *C. difficile* spores.

Representation of confirmed and unconfirmed routes of transmission of *C. difficile* spores to susceptible individuals. Elucidating new routes will help with prevention.
Clostridium difficile isolates from human patients that were molecularly characterized with an international PCR ribotyping method in the Netherlands. Note the increased number of samples obtained and tested since 2005. Most importantly note that only 5—of all possible 180 PCR ribotypes known—account for almost half of all human cases. Those PCR ribotypes are very common among diseased people and have been isolated from foods and food animals (see Table 1). This study represents the first report where the emerging PCR ribotype 027 started to decrease; this highlights the changing epidemiology of this pathogen. (Reproduced with permission, Copyright Eurosurveillance, 2009) (Hensgens et al. 2009)
Table 1. *Clostridium difficile* types with increasing relevance to humans and animals

<table>
<thead>
<tr>
<th>PCR/PFGE</th>
<th>Toxinotype</th>
<th>Toxins</th>
<th>Toxin Regulator</th>
<th>International</th>
<th>Foods/animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>027/NAP1</td>
<td>III</td>
<td>A B CDT</td>
<td>Mutated-18bp</td>
<td>NA, Europe</td>
<td>Yes/yes</td>
</tr>
<tr>
<td>078/NAP7-8</td>
<td>V</td>
<td>A B CDT</td>
<td>Mutated-39bp</td>
<td>NA, Europe</td>
<td>Yes/yes</td>
</tr>
<tr>
<td>001/NAP</td>
<td>0</td>
<td>A B CDT</td>
<td>Normal</td>
<td>Europe</td>
<td>Yes/unknown</td>
</tr>
<tr>
<td>014/NAP</td>
<td></td>
<td>A B CDT</td>
<td>Normal</td>
<td>NA, Europe</td>
<td>Yes/yes</td>
</tr>
<tr>
<td>077/NAP</td>
<td></td>
<td>A B</td>
<td>Normal</td>
<td>NA, Europe</td>
<td>Yes/yes</td>
</tr>
<tr>
<td>017/NAP</td>
<td></td>
<td>B CDT</td>
<td>Normal</td>
<td>NA, Europe</td>
<td>Yes/yes</td>
</tr>
</tbody>
</table>

Other strains of PFGE-NAP1 differ from PCR-type 027 but have identical virulent genes.

PCR, PCR ribotype; PFGE, pulse field gel electrophoresis nomenclature in North America; AB and CDT, names of toxins; NA, North America.
References


Chapter 3 — Transient Fecal Shedding and Limited Animal-to-Animal Transmission of *Clostridium difficile* by Naturally Infected Finishing Feedlot Cattle

As Published in:


Abstract

To longitudinally assess fecal shedding and animal-to-animal transmission of *Clostridium difficile* among finishing feedlot cattle as a risk for beef carcass contamination, we tested 186±12 steers (1,369 samples) in an experimental feedlot facility during the finishing period and at harvest. *Clostridium difficile* was isolated from 12.9% of steers on arrival (24/186; 0 to 33% among 5 suppliers). Shedding decreased to undetectable levels a week later (0%, *P* < 0.001); and remained low (<3.6%) until immediately prior to shipment for harvest (1.2%). Antimicrobial use did not increase fecal shedding despite treatment of 53% of animals for signs of respiratory disease. Animals shedding *C. difficile* on arrival, however, had 4.6 times higher odds of receiving antimicrobials for respiratory signs than nonsheddners (95% CI OR = 1.4, 14.8; *P* = 0.01). Most isolates (39/42) were deemed nontoxigenic based on two complementary multiplex
PCRs and ELISA testing. Two linezolid- and clindamycin-resistant PCR ribotype 078 (tcdA\textsuperscript{+} tcdB\textsuperscript{+} cdtB\textsuperscript{+}/39bp-type deletion in tcdC) isolates were identified from two steers (at arrival and on week 20), but these did not become endemic. The other toxigenic isolate (tcdA\textsuperscript{+} tcdB\textsuperscript{+} cdtB\textsuperscript{+}/classic tcdC; PCR ribotype 078-like) was identified in the cecum of one steer at harvest. Spatio-temporal analysis indicated transient shedding and failed to identify direct animal-to-animal transmission. The association between C. difficile shedding upon arrival and the subsequent need of antimicrobials for respiratory disease might indicate common predisposing factors. The isolation of toxigenic C. difficile from bovine intestines at harvest highlights the potential for food contamination in meat processing plants.

**Introduction**

_Clostridium difficile_ is the leading cause of infectious diarrhea in hospitals worldwide, with more than double the incidence observed today compared to the early 2000s (McFarland 2008). In the United States, _C. difficile_ is estimated to be associated with over 300,000 clinical cases each year resulting in a major financial burden to the health care system (Dubberke and Wertheimer 2009). Moreover, the incidence of severe infections and deaths in the community, particularly in children and pregnant women, is also increasing (Centers for Disease Control and Prevention (CDC) 2008). This change in the epidemiology of _C. difficile_ is coincidental with the emergence of hypervirulent antimicrobial resistant strains, namely, epidemic PCR ribotypes 027 and 078 (Jhung _et al._ 2008). The presence of these strains in livestock and up to 42% of retail meats (Rupnik
indicates that livestock may serve as a reservoir for contamination of the food supply, making foods a plausible source for community-acquired *C. difficile* infections. Resistance to fluoroquinolones and clindamycin, antibiotics used in human medicine, is also common among *C. difficile* isolates of animal and food origin (Rodriguez-Palacios et al. 2006).

Most pathogens that contaminate meat originate from the bacterial flora harbored by the live animal (Gill 2007). As such, preharvest interventions to control foodborne pathogens on the farm are predicted to decrease the frequency of carcass contamination therefore enhancing food safety (Oliver et al. 2009). *Clostridium difficile* spores of epidemic genotypes have been isolated from retail meats (Rodriguez-Palacios et al. 2007; Rodriguez-Palacios et al. 2009; Songer et al. 2009) and vegetables (al Saif and Brazier 1996; Bakri et al. 2009; Metcalf et al. 2010), and from neonatal and young cattle and pigs (Rodriguez-Palacios et al. 2006; Rupnik et al. 2008; Norman et al. 2009). However, the patterns of fecal *C. difficile* shedding among cattle during the finishing period, and most importantly, information regarding the frequency of bovine carcass contamination at the time of harvest is limited.

A better understanding of the epidemiology of *C. difficile* in food-producing animal populations will help to elucidate the sources of food contamination and determine if preharvest interventions for this pathogen may be beneficial. The goal of this study was to determine the prevalence of *C. difficile* in cattle upon entry to a feedlot, the incidence of new infections (assessed as new fecal shedders) and the spatiotemporal
distribution of *C. difficile* subtypes in a group of beef cattle up until and at the time of harvest.

**Materials and Methods**

*Experimental unit and source of animals.*

This study was conducted as part of a study evaluating body weight and dietary supplementation in a finishing feedlot facility at The Ohio State University, Wooster, Ohio, USA. Purchased Angus-cross steers were maintained as a closed population; animals entered the feedlot in early fall (October, 2007) and were harvested between 7 and 8.5 months later in spring. Prior to animal arrival, disinfection of the facility included pressure washing of floors, feeders, waterers, and fresh painting of pen divisions and fences. The waterers were stainless-steel, automatically refillable, and shared between two pens. The pens used had concrete slatted floors and an underground pit to accumulate fecal matter.

The steers originated from four university-owned operations and one commercial livestock auction. University-owned cattle originated from cow-calf farms that had the steers (~7-8 months old) alongside their dams in permanent pasture with no transitional grain based diets offered prior to shipment. Animals from the auction had unknown origin and management history. Multiple trucks were used for transporting animals from the source to the feedlot facility. Farms A, B, C, D and the auction market were located at 151, 283, 70, 155, and 265 km away, respectively (transport time 1-4 hours).

*Animal allocation, sampling and antimicrobial therapy.*

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Upon arrival to the feedlot, fecal samples were collected from the rectum using individual plastic sleeves (occasionally, some animals had no feces available for testing). Animals were also weighed, ear tagged, and systematically assigned to 24 pens based on order of arrival (8-10 animals/pen). Fecal samples were also collected from each animal on weeks 1, 4, 12, 20, and the day prior to shipment to the meat processing plant. During the first month, the steers remained in the originally assigned pens while they were fed a transitional diet. This diet consisted of a stepwise increase from corn silage, soy hulls and high moisture corn (45, 20, and 10% of the diet, respectively) to a finishing diet of mostly high moisture corn (65%) and corn silage (10%). The remaining 25% of the diet was a pelleted supplement containing protein (soybean meal and urea), vitamins, and minerals to meet the requirements of growing cattle (Council 2000). No grass or hay was offered to the animals during the study.

Signs of bovine respiratory disease complex (ranging from fever to fatal pneumonia) are common in beef steers entering feedlots. A protocol based on antiinflammatory and antimicrobial drugs approved for use in cattle in the USA was used to treat affected animals. Flunixin meglumine (1.1 mg/kg, IV, single dose, Banamine, Schering-Plough) and tulathromycin (2.5 mg/kg, subcutaneous, one dose, Draxxin, Pfizer) were initially administered to animals with fever (>39.7°C), anorexia, and depression. If no clinical response was observed within 24-48 hours indicated by persistently elevated rectal temperature, florfenicol was given (40 mg/kg, subcutaneous, one dose, Nuflor, Schering-Plough). Ceftiofur (1mg/kg, subcutaneous, one dose a day, for 3 days, Excenel, Pharmacia & Upjohn) and oxytetracycline (20 mg/kg, subcutaneous,
one dose, Liquamycin LA-200, Pfizer) were the third and last antimicrobial options, respectively, used in nonresponsive cases. Due to removals associated with respiratory disease, sudden death, or injury, animals were systematically reallocated on weeks 4 and 6 to ensure a final animal density of 7 steers per pen (of comparable body weights) providing the space needed at the end of the finishing period.

Animals were harvested at two commercial meat processing plants in groups of 26-28 animals (4 pens per week) over a period of 6 weeks. Following evisceration, two additional samples were collected: cecal content (2-4 ml by transmural puncture of cecal apex with 16 gauge needle), and a composite external carcass swab using a single Hydrated-Sponge (3M™, HS10BPW, 10 ml of buffered peptone water broth) per animal. Swabbing occurred after carcass trimming on both front quarters and the thoracic area (40 x 40 cm).

Laboratory analysis.

Eight samples per animal (six fecal samples at the feedlot, and one cecal sample and one carcass swab at harvest) were cultured for C. difficile. Bacterial isolation was based on a described enrichment protocol (Arroyo et al. 2005; Rodriguez-Palacios et al. 2007). Briefly, one gram of feces, cecal contents, and the carcass swabs were inoculated into 10, 10, and 35 ml of broth, respectively. This broth was prepared and supplemented with sodium thaurocholate (0.1%, Sigma Aldrich, St Louis, MO, USA) and antimicrobials D-cycloserine (250 mg/L) and cefoxitin (8 mg/L) (SR0096, Oxoid, MD, USA) as previously described (Arroyo et al. 2005; Rodriguez-Palacios et al. 2007). The broths were anaerobically incubated (CO₂/H₂/N₂, 10/10/80%) at 37°C for 10 days and
then centrifuged (7,000 x g, 10 min). The sediments were treated with 96% ethanol (1:1, v/v, 30 min) to reduce contaminants, centrifuged again and stored at -80°C without supernatant. Thawed sediments were inoculated onto pre-reduced cycloserine cefoxitin fructose \textit{C. difficile} agar supplemented with 7% defibrinated horse blood. Agar plates were inspected after 5 days of anaerobic incubation at 37°C for suspect colonies (Arroyo \textit{et al.} 2005). Up to five \textit{C. difficile}-suspect (i.e., morphology and 365-nm fluorescence) colonies from each plate were subcultured onto tryptic soy agar (Acumedia, Lansing, MI) supplemented with 5% defibrinated sheep blood. Nonhemolytic colonies were biochemically confirmed as \textit{C. difficile} via L-proline aminopeptidase activity (Fedorko and Williams 1997). Five days later, spores were harvested and stored in selective broth with 30% glycerol at -80°C. All samples were re-coded and blindly processed in composite batches. Positive controls included \textit{C. difficile} strain ATCC 9569.

\textit{Molecular subtyping.}

Thawed \textit{C. difficile} isolates were revived on blood agar for crude DNA extraction. Single colonies resuspended in 200 µL of distilled water were boiled for 15 min and centrifuged (16,000 x g, 1 min) to harvest the supernatant which was stored at -20°C. DNA concentrations ranged between 17 and 70 ng · µl⁻¹ (mean, 40.7; Nanodrop ND-1000, Wilmington, DE). Recovered isolates were molecularly assessed using three multiplex PCRs (Spigaglia and Mastrantonio 2002; Lemee \textit{et al.} 2004; Persson \textit{et al.} 2008). The gene combinations tested were \textit{tpi/tcdA/tcdB} for multiplex 1, \textit{16S rDNA/tcdA/tcdB/cdtA/cdtB} for multiplex 2, and \textit{tcdE/tcdC} for multiplex 3 (Spigaglia and Mastrantonio 2002; Lemee \textit{et al.} 2004; Persson \textit{et al.} 2008). PCR ribotyping was also
conducted to assess strain similarity (Bidet et al. 1999). Cluster analysis of fingerprint patterns was conducted using the unweighted pair group method with median and Pearson coefficients (Bionumerics, v5.1, Applied Maths Inc, Austin, TX). International PCR ribotype designations were given when available; otherwise, descriptive nomenclature for the first representative isolate was used for its respective cluster.

**Verification of toxin production.**

Two commercial ELISAs were used to verify toxin production in the first two isolates recovered from each PCR ribotype clusters (Thonnard et al. 1996; Garcia et al. 2000) using 72-hour-old colonies grown on blood agar. One ELISA detected toxins A and B (Wampole-C. difficile TOX A/B II, TechLab, Blacksburg, VA) and the other detected toxin A only (Clearview C.diff A, Inverness Medical Innovations, Princeton, NJ). ELISA testing was performed in duplicate. A strain was defined: *i*) A`B` if no toxins were detected with the ELISA TOX A/B kit, *ii*) A`B` if the ELISA TOX A/B was positive but the ELISA C diff A was negative, and *iii*) A`B` if both ELISAs were positive. *Clostridium difficile* ATCC 9569 was used as control. Binary toxin production was not evaluated.

**Toxinotyping and antimicrobial susceptibility.**

Restriction fragment length polymorphism of the pathogenicity locus (toxinotyping) was conducted on select isolates (one per major phylogenetic cluster) as described by Rupnik (toxin gene fragments A3 and B1) ([http://www.mf.unimb.si/mikro/tox/](http://www.mf.unimb.si/mikro/tox/)). Minimum inhibitory concentrations against six antimicrobial classes relevant for treatment or induction of *C. difficile* infections in humans were also
determined. Metronidazole, vancomycin, moxifloxacin, clindamycin, linezolid (first compound of oxazolidinones) and tigecycline (first compound of glycylcyclines) were assessed using the E-test method as described in a previous study (AB biodisk, bioMérieux, France) using Brucella agar (Acumedia, Lansing, MI) (Rodriguez-Palacios et al. 2009). Reference breakpoints from the Clinical and Laboratory Standards Institute or reported ranges for vancomycin, linezolid and tigecycline were used for interpretation (Ackermann et al. 2003; Hecht and Citron 2007; Jones et al. 2009; Nagy and Dowzicky 2010).

Statistical analysis.

Based on reported C. difficile prevalences in calves (Rodriguez-Palacios et al. 2006), a one-sample size estimation indicated that 171 animals would be sufficient to test a hypothetical shedding prevalence of 7% (alternate 13%, power=0.8, α=0.05). As a closed population cohort study, variables were assessed using a risk based cumulative incidence analysis (Dohoo et al. 2003). Binary and continuous data on arrival were analyzed using chi-square, Fisher’s exact, t-test or ANOVA statistics, or their nonparametric options, accordingly (Petrie and Watson 1999). Adjusting for events with probability close to zero, 95% confidence intervals were calculated using binomial exact statistics (Clopper and Pearson 1934; Brown et al. 2001). Analyses were conducted using STATA software package (v 10.1, College Station, TX). To quantify the risk of animal-to-animal transmission for C. difficile, the effective reproduction number (‘R(t)’, number of secondary shedders produced by each primary shedder during the first week following arrival) was estimated (Chowell et al. 2009). No reproduction numbers were calculated
after week one due to larger sampling intervals (3-8 weeks). We also compared the cumulative risk of becoming a shedder when exposed to shedders and nonshedders (Dohoo et al. 2003). For binary data at pen level, Join-Count analysis using the Rook’s case was computed as an index of spatial autocorrelation (Bell et al. 2008) to differentiate clustered from disperse and random distributions of C. difficile-positive pens.

**Results**

In total, 197 steers arrived to the feedlot, 116 directly from cow-calf farms and 81 from the livestock auction. Upon arrival, fecal samples were obtained from 186 animals (95.4% of 197; 105 from farms and 81 from auction; 11 steers had no feces in the rectum at arrival). A total of 1,369 samples were tested for C. difficile.

*Shedding at arrival.*

*Clostridium difficile* was isolated from 12.9% of steers on arrival (24/186; 95% CI = 8.4, 18.5). At the source level, the prevalence of *C. difficile* ranged from 0 to 33% (Fig. 5A), but there was no statistical difference between animals from farms and the livestock auction (Chi-square $P > 0.5$). Univariate and logistic regression analysis controlling by farm effect showed no association between body weight and *C. difficile* shedding at arrival ($P > 0.2$; odds ratio = 1); *C. difficile* was isolated from steers of all body weight ranges (Fig. 5B) indicating no effect of body weight (and possibly age) on shedding in young finishing steers.

*Shedding over time and at harvest.*
The proportion of animals shedding *C. difficile* at the feedlot facility decreased significantly from 12.9% (24/186) at arrival to 0% (0/176; 95% CI = 0, 2.1) a week later and to 1.7% (3/176; 95% CI = 0.4, 4.9) by week four (Fisher’s exact \( P < 0.0001 \)). *Clostridium difficile* was not detected on week 12 (0%; 0/168; 95% CI = 0, 2.2) but the prevalence at week 20 was 3.6% (5/168; 95% CI = 1.3, 7.6). *Clostridium difficile* was also isolated from the feces of two animals (1.2%, 2/167; 95% CI = 0.1, 4.2) prior to shipment for harvest, and from the intestinal content of two other animals at the time of harvest (cecum, 1.2%; 2/168; 95% CI = 0.1, 4.2). One of these two steers that was *C. difficile* positive prior to shipment for harvest was also positive when it entered the feedlot. Likewise, one of the two steers that had *C. difficile* at harvest was positive on week four. Neither of these two animals shed the same strain twice. All carcass swabs were negative (0/168; 95% CI = 0, 2.2; Fig. 6).

**Antimicrobial use and *C. difficile* shedding.**

Clinical signs of respiratory disease (*i.e.* fever and increased respiratory rate) prompted the use of antimicrobials in 53% (99/186) of animals; most of them were treated between weeks one and five. One, two, three and four courses of the antibiotic choices were necessary in 63, 24, 8, and 7% of the 99 animals treated, respectively. Tulathromycin was the most commonly used antibiotic, followed by ceftiofur, florfenicol and oxytetracycline (60, 22, 17 and 1%, of 184 total single doses, respectively). The use of antimicrobials during the first month of the study (3 single doses on day 6, plus 156 doses between days 8 and 31) did not enhance *C. difficile* shedding (Fig. 6). By the end of the study, cumulative data analysis of the 12 steers that became new shedders at the...
feedlot indicated that antimicrobial use remained not associated with \( C. \text{ difficile} \) shedding (7/12 treated vs. 5/12 nontreated, \( P > 0.1 \)). Although no signs of enteric disease (i.e., watery diarrhea) were observed, logistic regression analysis controlling for farm effect indicated that animals shedding \( C. \text{ difficile} \) on arrival had higher odds of subsequently receiving antimicrobials at the feedlot (odds ratio 4.6; 95% CI = 1.4, 14.8; \( P = 0.01 \)) compared to nonshedders. Compared to the source of origin, this effect was driven by farm B (\( P = 0.003 \)) whose steers also had the longest transport distance to the feedlot facility (283 km, \~{}3.2 h, directly from cow-calf farm).

\textit{Reallocation and animal-to-animal transmission.}

A total of 29 steers were removed (25 had moderate respiratory signs, 2 had leg injuries, and 2 died) early in the study leaving 168 animals by week 6. The body weight of animals removed was not different from that of the remaining animals (t-test \( P = 0.132 \)). Three of the 29 removed steers (10.3%) were positive for \( C. \text{ difficile} \) on arrival. Adjusting for withdrawals associated with disease, injuries, and to balance body weights in each pen; animals were reallocated after having completed the dietary adjustment, on weeks 4 and 6. At the animal level, spatial statistics showed that the ordered allocation of incoming steers resulted in a random distribution of shedders within pens (Join-Count, \( P \geq 0.4 \)) (Fig. 7). Regarding animal-to-animal transmission, the reproduction number derived from data obtained within one week of arrival was zero (i.e., no new shedders arose from contact with shedding animals detected at arrival). No spatial statistics were done at individual sampling points after week one due to limited number of shedders detected at each point (n<5).
**Molecular subtyping.**

In total, 42 isolates recovered from 34 steers were examined. Molecular typing with one of the multiplex PCR methods (Lemee et al. 2004) showed most strains were \( tcdA^{+}tcdB^{+} \) but the second multiplex method used (Persson et al. 2008) indicated they were \( tcdA^{-}tcdB^{+} \) (Table 2). A variant genotype (\( tcdA^{+}tcdB^{+}cdtB^{+/39bp-} \)type deletion in \( tcdC \)) identical to human and animal epidemic PCR ribotype 078 was also identified in two steers (arrival, \( n=1 \); week 20, \( n=1 \)) using the two multiplex methods. At harvest, one of the two cecal isolates identified was a toxigenic \( tcdA^{+}tcdB^{+}cdtB^{+/} \)classic \( tcdC \) strain. PCR ribotyping clearly resulted in distinguishable ribotypes recovered from the same animal on different dates. In addition, some animals shed distinct ribotypes on the same date (Fig. 8). The two animals that shed PCR ribotype 078 in this feedlot never had direct nose-to-nose contact, shared a common pen, or have indirect contact via their pen mates.

**Toxin production, toxinotyping and antibiotic sensitivity.**

ELISA testing of 20 isolates confirmed that \( tcdA^{+}tcdB^{+}cdtB^{+} \) strains produced no toxins and that \( tcdA^{+}tcdB^{+}cdtB^{+} \) toxigenic strains produced both toxins. The multiplex-discrepant (\( tcdA^{+}tcdB^{+}; tcdA^{-}tcdB^{-} \)) strains had no detectable toxin. Cytotoxicity neutralization assays were not conducted. Toxinotyping and antimicrobial susceptibility testing showed that the two PCR-ribotype 078 strains, identified at arrival and on week 20, were toxinotype V and resistant to clindamycin and linezolid (MICs were 256 \( \mu g/ml \) and 8-12 \( \mu g/ml \), respectively). All isolates were susceptible to metronidazole, vancomycin, tigecycline and moxifloxacin.
Discussion

This study provides new insights to the on-farm epidemiology of \textit{C. difficile} in food-producing animals, particularly during the final stages of beef production as well as the associated potential for food contamination. Although young steers entering the feedlot harbored \textit{C. difficile}, fecal carriage of this bacterium, irrespective of toxigenic genotype, was transient and the transmission to other animals within the feedlot was neither temporally nor spatially clustered. The prevalence of \textit{C. difficile} in purchased steers arriving from the suppliers (\textit{i.e.,} cow-calf farms) was comparable to the reported prevalence for young calves (\textit{i.e.,} 10-13\%), however most isolates were nontoxigenic (Rodriguez-Palacios \textit{et al.} 2006). The reasons for the discrepancy observed between the multiplex PCRs performance regarding the detection of gene \textit{tcdB}, which made the difference between the preliminary identification of some isolates as toxigenic by multiplex 1 and as nontoxigenic by multiplex 2, are unclear but may be in part due to differences in primer design and specificity.

Irrespective of toxin genotype, we did not observe molecular or temporal evidence to indicate that shedding persisted over time, either when animals were sampled one week apart after arrival to the feedlot or when testing occurred 24 hours apart prior to harvest. These two short sampling intervals were considered critical to assess shedding persistence upon entry and prior to leaving the feedlot, but sparser sampling was chosen during the rest of the study (every 4 and 8 weeks) to reflect transition periods typical of feedlots (\textit{i.e.,} dietary adjustment, susceptibility to bovine respiratory disease, and
reallocations). More frequent sampling could provide information about *C. difficile* shedding at a more precise timescale, but may yield limited information with regards to food safety risks.

In the feedlot, the cause of the marked reduction of *C. difficile* during the first week was not determined but may be attributed to dietary changes (i.e., animals shifted from grass and milk based diets to grain based diets in the feedlot). In humans, diet appears to modulate the growth of *C. difficile* in the intestinal tract (Iizuka *et al.* 2004), but similar effects are unknown for food-producing animals. Alternatively, the move of cattle to the feedlot may have eliminated exposure to potential sources of *C. difficile* spores present on the farms of origin. At the feedlot, the slatted floors could have also reduced the number of spores to which the animals were exposed.

In humans and other animal species, antimicrobials enhance shedding, transmission of *C. difficile*, and the induction of *C. difficile* associated disease (Clooten *et al.* 2008; McFarland 2008). For example, under experimental conditions, antimicrobials given to asymptomatic shedding mice resulted in “super-shedder” states and increased transmission to previously *C. difficile*-negative immune-competent co-housed cohorts (Lawley *et al.* 2009). In contrast, in this study, we conclude that antimicrobial use was not the cause of the decrease in *C. difficile* shedding because most animals were treated only after the significant shedding reduction to 0.6% was identified on week one. Only 1.6% of all antimicrobial doses were given (i.e., two animals) prior to sampling the feedlot on week one.
The effect of transportation as a source of stress and contamination is also unknown. In feedlot steers, bovine respiratory disease complex often develops as indicator of increased stress-related immune suppression following transportation and entry to feedlots (Yates 1982). In our study, the association of *C. difficile* shedding at arrival (primarily nontoxigenic isolates) with higher odds of receiving antimicrobials for respiratory signs in the feedlot indicates that *C. difficile* shedding and the bovine respiratory disease complex may share common predisposing factors at the farm of origin.

In neonatal calves, *C. difficile* shedding has been documented to last for at least 6 days (Rodriguez-Palacios *et al.* 2007). Fecal excretion of *C. difficile* was detected on only one occasion in most shedding steers, and those animals with two positive samples were shedding distinct *C. difficile* genotypes at non-consecutive sampling points. The fecal shedding pattern was therefore considered transient. It is uncertain if detectable *C. difficile* shedding resulted from short-term successful bacterial colonization and proliferation or from intestinal passage of dormant spores ingested from the environment in food or water.

Despite the low incidence of new shedders in this study, we identified two animals with *C. difficile* immediately prior to shipment for slaughter, and two different animals carrying *C. difficile* in the cecum at harvest. One of the cecal isolates was fully toxigenic (*tcdA*⁺*tcdB*⁺*cdtB*⁺/classic *tcdC*) and had 83% fingerprint pattern similarity to PCR ribotype 078, but it was clearly different as it had additional bands of lower molecular weight (Fig. 4). Epidemic PCR ribotype 078 (*tcdA*⁺*tcdB*⁺*cdtB*⁺/39bp deletion
in putative toxin negative regulator \( tcdC \)/toxinotype V) was identified on two occasions in this cattle population (one animal at arrival and one on week 20) but did not become endemic. PCR ribotype 078/toxinotype V is emerging as a cause of disease in hospitalized people (Debast \textit{et al.} 2009) and the community (Limbago \textit{et al.} 2009), and is a predominant type in swine operations (Keel \textit{et al.} 2007), retail meats (Songer \textit{et al.} 2009), and young dairy cattle (Rodriguez-Palacios \textit{et al.} 2006). It was also recently isolated from clustered white-tailed deer farms in Ohio, USA (French \textit{et al.} 2010), in geographical proximity to where this study was conducted.

The presence of toxigenic \textit{C. difficile} in feces and intestinal contents of cattle at the time of harvest indicates that contamination of transport vehicles, lariage areas, and meat processing plants is possible. For other foodborne pathogens, for instance \textit{Escherichia coli} O157:H7, the prevalence of carcass contamination is strongly associated with the prevalence and load of fecal shedding by live animals at the time of harvest (Elder \textit{et al.} 2000). The extent of such an association for \textit{C. difficile} remains unknown. In this study, \textit{C difficile} was not identified in sponges of carcass swabs. To date, there are no conclusive reports of foodborne \textit{C. difficile} infections. However, the increasing isolation of human epidemic strains from retail meats and food animals, including finishing beef cattle in the present study, and the thermal resistance of \textit{C. difficile} to minimum recommended cooking temperatures (Rodriguez-Palacios \textit{et al.} 2010) underscore the potential for foodborne transmission.

In contrast to recent studies where PCR ribotype 078 was clearly prevalent in young food animals (up to 94% in calves) and retail meats (73% of identified genotypes)
(Keel et al. 2007; Rupnik et al. 2008; Debast et al. 2009; Songer et al. 2009), this longitudinal study showed that such epidemic genotype did not become endemic in our facility. The transient shedding observed in this feedlot indicates that cattle are unlikely persistent reservoirs of *C. difficile* during the finishing period. However, the isolation of one tcdA<sup>+</sup>tcdB<sup>+</sup>cdtB<sup>+</sup> *C. difficile* strain from the cecum at harvest (although all carcass swabs were negative) demonstrates that meat contamination with clinically relevant isolates could occur at harvest (43, 47). In addition, as with other clostridia (Turcs et al. 2001; Vengust et al. 2003; Sathish and Swaminathan 2008; Rodriguez-Palacios et al. 2009), spore intestinal translocation to deeper muscle tissues could also occur in vivo. Because bacterial proliferation during processing or in the final products is plausible, identifying and applying multiple barrier approaches including those that emphasize control of cross-contamination, and decontamination during processing and food preparation, could reduce the risk of food contamination with this emerging pathogen.
Acknowledgements

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Figure 5. *Clostridium difficile* in steers at arrival to the feedlot.

A) Prevalence of fecal shedding of *C. difficile* by source. The combined prevalence was 12.9%.  

B) *C. difficile* shedding by body weight. Controlling for farm, there was no association between shedding status and body weight (*F*-test, *P* > 0.1). Lines with solid squares represent group means.
Figure 6. Longitudinal study — Prevalence of *C. difficile* shedding in cattle in the feedlot, and at harvest.

Cecum and carcass samples were collected at harvest, 24 hours after the last on-farm sampling (shipment).
Figure 7. Spatio-temporal distribution of steers shedding *C. difficile* at the feedlot over time (*n* = 1,369 samples tested). Panels represent two separate rows of 12 contiguous pens (6-7 animals/pen).

Spatial analysis indicated that pens holding incoming shedders followed a random distribution (Join Count, *P* > 0.4). Note the absence of new infections in association with PCR ribotype 078. Arrows and capital letters in left panel indicate order of arrival and farm of origin.
Figure 8. PCR ribotypes of *C. difficile* from beef cattle in the feedlot.

**A)** Cluster analysis of representative isolates with at least one toxin gene reaction positive. Note similarity at arrival. The upper shaded rectangle highlights the similarity of three toxigenic isolates to PCR ribotype 078. ELISA tested for toxins A and B. -, not tested. Type, arbitrary letter designation; subscripts indicate the extra bands were observed compared to other isolates in cluster. See Table 2 for toxin gene profile of the PCR types (similarity >80%). Steers 86 and 169 carried two different strains.

**B)** PCR ribotypes highlighted in cluster type D illustrating similarity to PCR ribotype 078.
Table 2. Toxin gene profiles of *C. difficile* PCR ribotypes from beef cattle in the feedlot.

<table>
<thead>
<tr>
<th>PCR ribotype</th>
<th>Multiplex 1</th>
<th>Multiplex 2</th>
<th>tcdE/tcdC</th>
<th>ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td>A, A1, A2</td>
<td>tpi⁻ tcdA⁻ tcdB⁺</td>
<td>16S⁻ tcdA⁻ tcdB⁺ cdtA¹ cdtB⁻</td>
<td>+/-</td>
<td>Negative</td>
</tr>
<tr>
<td>B</td>
<td>tpi⁺ tcdA⁺ tcdB⁺</td>
<td>16S⁺ tcdA⁺ tcdB⁺ cdtA⁺ cdtB⁻</td>
<td>+/-</td>
<td>Negative</td>
</tr>
<tr>
<td>C, C1</td>
<td>tpi⁺ tcdA⁺ tcdB⁺</td>
<td>16S⁺ tcdA⁺ tcdB⁺ cdtA⁺ cdtB⁺</td>
<td>+/-</td>
<td>Negative</td>
</tr>
<tr>
<td>D (PCR 078)</td>
<td>tpi⁻ tcdA⁻ tcdB⁺</td>
<td>16S⁻ tcdA⁻ tcdB⁻ cdtA⁻ cdtB⁻</td>
<td>+/-39bp del</td>
<td>A'B⁺</td>
</tr>
<tr>
<td>D₁</td>
<td>tpi⁺ tcdA⁺ tcdB⁺</td>
<td>16S⁺ tcdA⁺ tcdB⁻ cdtA⁺ cdtB⁺</td>
<td>+/-classic</td>
<td>ND</td>
</tr>
<tr>
<td>E</td>
<td>tpi⁺ tcdA⁺ tcdB⁺</td>
<td>16S⁺ tcdA⁺ tcdB⁺ cdtA⁺ cdtB⁻</td>
<td>+/-</td>
<td>Negative</td>
</tr>
<tr>
<td>F-L</td>
<td>tpi⁺ tcdA⁺ tcdB⁺</td>
<td>16S⁺ tcdA⁺ tcdB⁺ cdtA⁺ cdtB⁻</td>
<td>+/-</td>
<td>Negative</td>
</tr>
<tr>
<td>Others</td>
<td>tpi⁻ tcdA⁻ tcdB⁻</td>
<td>16S⁻ tcdA⁻ tcdB⁻ cdtA⁻ cdtB⁻</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Subscripts in PCR ribotype letters indicate extra bands were observed compared to representative isolates in cluster (Fig 4). Isolates were tested with two complementary PCR multiplex methods for *C. difficile* (Lemee *et al.* 2004; Persson *et al.* 2008). The discrepancy between tcdB in both multiplexes for some PCR ribotypes (*e.g.*, PCR ribotype A) indicates differential performance for tcdB gene. Isolate type D₁ was not available for further testing. ND, not determined. Nontoxigenic *C. difficile* are unlikely to induce enteric disease.
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Chapter 4 — Summer prevalence of Clostridium difficile in pigs, cattle, poultry, and horses: finishing food animals are not naturally reservoirs

(For Submission)

Abstract

Enteric human pathogen Clostridium difficile has been isolated from food and companion animals and from retail foods raising concerns about foodborne transmission. To comparatively assess the potential for food or environmental contamination at the time of animal harvest, we conducted a cross-sectional fecal prevalence study in animals intensively raised for food production (swine, beef and dairy cattle, poultry and turkey) within 24 hours of harvest, and compared it to the prevalence of a group of intensively managed horses at a racetrack. Animals were sampled in the same region (Ohio, USA) during one month period in summer. Clostridium difficile was isolated from 5 out of 875 (0.6% prevalence) animals. One isolate was from a commercial beef steer at a feedlot (1/180), while the remaining isolates were from racing horses (7.2%; 4/55). Hence, horses had the highest prevalence of C. difficile compared to the food animal groups (2 x 6, chi-square, P < 0.001). The low prevalence of C. difficile in finishing food animals during summer, in relation to the reproducible prevalence of C. difficile in horses, indicate commercial finishing food animals are not naturally successful reservoirs for this
enteric pathogen, and that the risk of food contamination at harvest is relatively low in the warmth of summer. With this low level of *C. difficile* prevalence (summer baseline), it is now necessary to determine the shedding dynamics in winter, when *C difficile* in people, animals and foods seem to occur with higher frequency.

**Introduction**

The enteric pathogen *Clostridium difficile* associated with infections (CDI) in humans has been repeatedly isolated from retail foods since 2006, raising concerns about foodborne transmission to communities at risk (Rodriguez-Palacios *et al.* 2006; Rodriguez-Palacios *et al.* 2007). However, little is known about how food gets contaminated. Initial studies documented the presence of emerging hypervirulent strains of *C difficile* in foods and young livestock (Rodriguez-Palacios *et al.* 2006; Keel *et al.* 2007; Pirš *et al.* 2008; Norman *et al.* 2009), but there is limited research regarding the presence of such strains in food animas at the time of harvest, and when they enter the food chain. Some studies have been conducted at single commercial operations and largely on single animal species. The resistant nature of this pathogen indicates that contamination of various animal species within the same region could be possible. This is particularly possible, especially because there are reports of long survival (years) of spores in soil and horse manure (Baverud *et al.* 2003), presence of toxigenic spores in bodies of water (Zidaric *et al.* 2010), and indications of aerial dissemination of spores within commercial swine operations and towards their surrounding environment (Keessen *et al.* 2010) in Europe.
Independent studies indicate that regional predominance of *C. difficile* hypervirulent subtypes is possible. A recent study on *C. difficile* conducted on farmed deer, identified the presence of *C. difficile* PCR ribotype 078 in a cluster of farms in a relatively small region of Ohio, USA (French et al. 2010). The lack of apparent social or food/water network connectivity between those farms indicated that other mechanisms might facilitate spore dissemination at a local scale (French et al. 2010). In the same region, the PCR ribotype 078 was concurrently isolated from a feedlot cattle operation, although the pathogen was not persistently shed until the time of harvest (Rodriguez-Palacios et al. 2011).

In other regions of North America, *C. difficile* have been isolated from pleasure healthy horses (Weese et al. 2006), but little is known about the prevalence in horses in Ohio, and no studies are available for poultry in the USA. In Africa and Europe, single studies indicate that the prevalence of *C. difficile* in poultry can be high (18-29%) towards the end of the production system (Simango 2006; Simango and Mwakurudza 2008), but the production conditions differ from the ones encountered in commercial operations in the USA.

Considering that fecal shedding at harvest is a major risk factor for carcass contamination with foodborne pathogens (Elder et al. 2000), understanding if the risk of carriage of a given pathogen is higher in any given farm animal species would be important to target intervention efforts to reduce the risk of food contamination at the time of harvest and to prevent the potential for environmental contamination via animal excrements, which may vary with the type of operation.

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Because the fecal shedding of *C. difficile* in young livestock (at their farms) has been reported to be high in winter and low in summer in calves and piglets (Rodriguez-Palacios et al. 2006; Norman et al. 2009), the present study was designed to quantify the lowest baseline prevalence of fecal shedding of *C. difficile* in farm animals (mature, or ready for harvest) during the least favorable reported season for fecal shedding at the farm, summer. In a cross sectional study we determined if common farm animal species, particularly food animals and horses, which have different management production systems, have the same probability of carrying *C. difficile* in a relatively well defined geographical area and temporal frame (Ohio, USA), i.e., during a one month sampling in the warmest months of summer.

**Materials and Methods**

*Food animal farms, processing plants, and horse racetrack.*

We conducted a cross-sectional study of *C. difficile* in fecal or hind gut intestinal samples from finishing swine, dairy calves and mature dairy and feedlot cattle, chicken and turkey, and stabled horses at the end of a racing season in Ohio, USA. The facilities visited and sampled were located within a radius of 290 ± 32 kilometers from our research center. Establishments sampled corresponded to commercial farms intended for food production, with the exception of the racing horses. Samples were collected during one month in summer (August–September), 2007. Farms were sampled based on convenience, but it corresponded to producers with moderate or large market share in the region and who kindly volunteered to participate in the study. Importantly, it was
considered that all animals and operations here sampled had in common that they were intensively managed, have relatively high animal production densities, and were assumed to have an average high level of stress for the type of production system (i.e., prior to or at harvest for food animals, racing conditions for horses).

**Sampling.**

Within each farm or production unit, the median number of animals sampled was thirty. Animals were sampled using a systematic sampling approach. This is the sample size divided by the number of finishing lots or animals of interest (i.e., immediately prior to harvest as in the case of feedlot cattle or finishing pigs, or at harvest as in the case of poultry). Animals were sampled at regular intervals to minimize potential clustering associated with animal groupings or lots. The intervals were calculated by dividing the number of animals available by the estimated sample size. For every production system five farms were sampled for a total of 150 animals per production system. Horses were one exception; 55 animals (~25% of all possible horses) were sampled from one single available racetrack facility, where animal traffic on a weekly basis was inherently high. None of the facilities simultaneously housed more than one animal species of interest.

Cattle samples were collected from the rectum while swine and horse samples were collected from the pen floor. For poultry, an inspector directed the collection of the viscera and internal organs from selected animals at the time of evisceration; the viscera was packed in individual plastic bags, refrigerated and sent to the laboratory for sample collection (composite two grams of cecal and rectal-cloacal content). In all cases, fecal
and intestinal samples were transported refrigerated to the Ohio State University for *C. difficile* analysis and processed within 24 hours of collection.

**C. difficile prevalence and molecular confirmation.**

We used an enrichment protocol for *C. difficile* as in previous studies (Arroyo et al. 2005; Rodriguez-Palacios et al. 2006; Rodriguez-Palacios et al. 2007). In brief, homogenized fecal or intestinal samples (1 gram) were enriched in 15 ml of selective *C. difficile* broth (Arroyo et al. 2005) supplemented with 0.1% sodium taurocholate (Sigma Aldrich, St Louis, MO) and *C. difficile* supplement SR0096 (Oxoid, Columbia, MD, cycloserine and cefoxitin). The following steps included incubation (37°C, 7 days), centrifugation of the broths and treatment of sediments with 96% ethanol, and plating onto *C. difficile* fructose agar supplemented with 7% defibrinated horse blood (Quad Five, Ryegate, MT) and the cycloserine/cefoxitin supplement. After 5 days of anaerobic incubation, suspect nonhemolitic fluorescent (UV\(_{365}\)) colonies on tryptic soy (plus 5% defibrinated sheep blood) agar were biochemically (L-proline aminopeptidase activity; Pro Disc, Remel, Lenexa, KS, USA) and molecularly confirmed (Fedorko and Williams 1997). These steps are described in more detail in one of our recent enumeration studies (Rodriguez-Palacios et al. 2011).

Pure single colonies revived on blood agar (48 hours of anaerobic incubation) were suspended in double-deionized sterile water and boiled for 8 minutes to obtain crude DNA, which was used as template in the PCR reactions. Isolates were confirmed for the presence of the triose-phosphate isomerase (*tpi*) gene; toxin genes *tcdA* and *tcdB* were also assessed (Lemee et al. 2004). PCR ribotyping was conducted as described
(Bidet et al. 1999) to determine strain diversity. Isolates were deemed toxigenic if they had one of the toxin genes tcdA, tcdB, cdtA/B and toxins (Rupnik et al. 2005).

**Toxin ELISAs and dialysis cytotoxicity assay in Vero cells.**

Representative isolates were tested with two complementary commercial ELISAs (C. difficile TOX A/B II, TechLab, Blacksburg, VA, and Clearview C. diff A (Wampole Laboratories, Inverness Medical Professional Diagnostics, Princeton, NJ) as described (Thonnard et al. 1996; Garcia et al. 2000) using 72-hour culture colonies. Toxigenic strain (tcdA+/tcdB+/cdtA+/B/toxinotype 0) ATCC 9689 was used as a positive control. Binary toxin production was not determined. Dialysis tubing and citotoxicity assay was conducted on ELISA and toxin-gene negative isolates to verify the lack of toxin production as described (Rodriguez-Palacios et al. 2011).

**Statistics.**

In a previous study, we estimated based on simple-random sampling calculations a sample size of 120 animals to verify a hypothetical prevalence of C. difficile of 5.6 ± 6% in cattle at harvest (one-sample, α = 0.05, power = 0.8). Our target here was to sample 5 farms for each animal species based on convenience. To empirically control for clustering due to animal-animal transmissibility (within farms previously shown to be very low in mature finishing cattle) (Rodriguez-Palacios et al. 2011), we arbitrarily increase the sample size to 150 individuals per animal species. Based on a previous study where farm level prevalence was documented to be 23% for cattle based on testing of 2-6 calves per farm (Rodriguez-Palacios et al. 2006), it was deemed sufficient to sample 30 animals per farm to detect at least one clustered group of finishing animals (farm).
exposed to *C. difficile*, as recently shown for pig farms (Thakur *et al*. 2010). Culture binary data was analyzed using Fisher’s exact or chi-square statistics (Petrie and Watson 1999). Confidence intervals were estimated using exact statistics (Clopper and Pearson 1934). (STATA, v10.1, College Station, TX). Although this was not a clinical trial, we considered the ‘REFLECT’ guidelines for study reporting (Sargeant *et al*. 2010).

**Results**

*Clostridium difficile* was isolated from 5 out of 875 (0.57% prevalence) animals in summer. One isolate was derived from one commercial beef steer at a feedlot (0.55%; 1/180), while the remaining were racing horses (7.3%; 4/55). In this study, the population of clinically healthy race horses (with no evidence of diarrhea at the individual basis not at the facility as a collective; visited by A.R.P) was the one with the highest rate of *C. difficile* isolation (2 x 6, chi-square, P < 0.001). Despite the isolation of *C. difficile* from one cattle, the shedding prevalences were comparably low across the food animal operations (2 x 5, chi-square, P < 0.469; Table 3).

Available ELISA and molecular tests identified that the PCR ribotype found in the horse population was indistinguishable by PCR ribotyping to the most common nontoxigenic strain identified in harvested cattle a year later. Dialysis citotoxicity assay on vero cells confirmed that the nontoxigenic isolate did not produce toxins even after 72 hours of incubation and despite heavy noticeable production of cell biomass. No toxin testing is yet available for the remaining isolates.
Discussion

As the incidence of *Clostridium difficile* infections (CDI) in humans have increased, the prevalence and clinical relevance of this enteric pathogen in domestic animals has also been increasingly documented since the mid 2000s. Comprehensive studies in humans and food animals have indicated that there is a seasonal component which modifies the prevalence (and incidence) dynamics of this pathogen, with the lowest estimates observed during summer (Rodriguez-Palacios *et al.* 2006; Norman *et al.* 2009). This observation, in addition to the identification of emerging hypervirulent strains from young livestock (Rodriguez-Palacios *et al.* 2006; Goorhuis *et al.* 2008), indicated that there is great similarity between the epidemiology and seasonal dynamics of *C. difficile* in animals and humans. Weather conditions are an obvious factor that can independently produce seasonal transformation in animals and humans (e.g., disease susceptibility to debilitating diseases, pathogen persistence). However, the documented evidence for seasonality of food contamination with *C. difficile* indicates that tainted foods could theoretically serve as a complement link between the two species seasonality patterns (Rodriguez-Palacios *et al.* 2009). Here we determined the shedding of *C. difficile* in food animals ready to enter the food chain during summer when the probability of shedding is the lowest to determine if they could be successful natural reservoirs, and to infer the risk for food contamination at harvest.

By sampling various farm animal species we confirmed a very low prevalence of *C. difficile* in food animals in a region where *C. difficile* is a growing problem for the
health care system since the 2000s (Campbell et al. 2009). This low shedding prevalence indicates that the potential for food contamination during the first steps of food production in summer is low, compared to the prevalence of pathogens such as *Campylobacter* spp (2-8%, cattle and pigs) (Boes et al. 2005; Dodson and LeJeune 2005). However, we have shown that even a small number of positive shedders might have enough spores in the feces to consider environmental contamination of processing facilities a risk for post-harvest contamination of the food supply (Rodriguez-Palacios et al. 2011). Especially, because regular disinfection eliminate most vegetative fecal contaminants, but spores are resistant to most commercial disinfection products (Wilcox and Fawley 2000).

Here we recovered *C. difficile* from 7.2% of racing horses at the racetrack facilities. Compared to food animals, horse shed at a higher proportion, but the proportion observed was reproducible as it was comparable to reported values in horses from other racetracks (5-8%) (Medina-Torres et al. 2011). Thus, the isolation of *C. difficile* from horses documents the appropriate performance of our culture method, which has been recently shown to be adequate to comparatively screen for this pathogen in beef and dairy cattle, with some limitations for pigs (Thitaram et al. 2011). Although no methods have been validated for poultry, the isolation principles of the method used here (Arroyo et al. 2005) are also comparable to methods used in previous poultry studies. Comparative considerations regarding culture methodology thus indicate that food animals are indeed uncommon *C. difficile* shedders, at least during summer. A study of pigs, cattle and broiler sampled at abattoirs in Austria (March-July, 2008) found prevalence of *C. difficile*
between 3.3 and 5% (total n=187) (Indra et al. 2009), suggesting that weather conditions, and/or regional differences, definitely play a substantial role in the epidemiology of *C. difficile* in food animals.

The low shedding prevalence observed in our finishing food animals is in agreement with the low prevalence observed in longitudinal studies conducted independently for finishing cattle, pigs and poultry in diverse regions (Zidaric et al. 2008; Norman et al. 2009; Rodriguez-Palacios et al. 2011; Rodriguez-Palacios et al. 2011). Regarding poultry species, no studies are yet available characterizing the shedding dynamics of this pathogen in intensive production systems in the USA. As a referent, studies in Slovenia found in a single farm an age-dependent reduction of *C. difficile* shedding in laying hens, with decreasing shedding prevalence from 100% in chicks to a final 30% in mature laying hens (Zidaric et al. 2008). Here we did not sample hens, but the high prevalence (7%) of *C. difficile* in retail chicken meats in Canada (Weese et al. 2010) indicate that there must be very relevant management factors that could modulate the dynamics of the pathogen in broilers in intensive systems. If our results were applicable to other farms, alternatively, there could be factors at harvest or postharvest to explain the rise in the rate of retail chicken contamination (7%) with *C. difficile*. Of food safety reassurance, none of the commercially raised poultry and the finishing swine tested (450 animals), which are part of a single commercial network (farms span a radius of about 40 kilometers), had *C. difficile* at the time of harvest.

Taken together, the low prevalence of *C. difficile* in finishing food animals, in relation to the reproducible prevalence of *C. difficile* shedding in intensively managed
racing horses during summer, indicate that commercially raised food animals are not naturally successful reservoirs for this enteric pathogen, and that the risk of food contamination at harvest is relatively low in summer. With this low baseline prevalence, it is now necessary to explore the dynamics and prevalence of shedding in winter months, in developed temperate nations when reportedly higher prevalence of \textit{C. difficile} in people, animals and foods seem to occur.

\textbf{Acknowledgements}

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Table 3. Comparative summer prevalence of *C. difficile* fecal shedding in farm animals, Ohio, USA.

<table>
<thead>
<tr>
<th>Animal production</th>
<th>Relation to food chain/sampling</th>
<th>Primary Diet</th>
<th>Animals/operation (Operations, (n))</th>
<th><em>C. difficile</em> prevalence</th>
<th>95% CI of prevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cattle, beef feedlot</td>
<td>Meat - Prior harvest</td>
<td>Grain-based</td>
<td>30 (6)</td>
<td>0.6% (1/180)</td>
<td>0.14 – 3%</td>
</tr>
<tr>
<td>Cattle, mature dairy</td>
<td>Milk Production</td>
<td>Total mixed ratio</td>
<td>30 (5)</td>
<td>0% (0/150)</td>
<td>0 – 2%</td>
</tr>
<tr>
<td>Swine, finishing</td>
<td>Meat - Prior harvest</td>
<td>Concentrate</td>
<td>30 (5)</td>
<td>0% (0/150)</td>
<td>0 – 2%</td>
</tr>
<tr>
<td>Broilers, finishing</td>
<td>Meat - At harvest</td>
<td>Concentrate</td>
<td>30 (5)</td>
<td>0% (0/150)</td>
<td>0 – 2%</td>
</tr>
<tr>
<td>Chickens, local</td>
<td>Meat - At market</td>
<td>Unspecified</td>
<td>40 (1)</td>
<td>0% (0/40)</td>
<td>0 – 9%</td>
</tr>
<tr>
<td>Turkeys, finishing</td>
<td>Meat - At harvest</td>
<td>Concentrate</td>
<td>30 (5)</td>
<td>0% (0/150)</td>
<td>0 – 2%</td>
</tr>
<tr>
<td>Horses, racing</td>
<td>None - End of season</td>
<td>Hay, concentrate</td>
<td>55 (1)</td>
<td>7.2% (4/55)*</td>
<td>2 – 17%</td>
</tr>
</tbody>
</table>

| Total | n/a | n/a | 875 (28) |

*Statistically different from the rest, Chi square, \(P < 0.001\).*
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Chapter 5 — Prevalence, enumeration and antimicrobial resistance in *Clostridium difficile* in cattle at harvest, USA

As Accepted in:


**Abstract**

To assess the potential for food contamination with *Clostridium difficile* from food animals, we conducted a cross-sectional fecal prevalence study in 944 randomly selected cattle harvested at seven commercial meat processing plants, representing four distant regions (median distance 1,500 km) of the United States. In total 944 animals were sampled in summer 2008. *C. difficile* was isolated from 1.8% (17/944) of cattle, with median fecal shedding concentration of 2.2 log$_{10}$ CFU·g$^{-1}$ (range = 1.6 to 4.8; 95% CI = 1.6, 4.3). Toxigenic *C. difficile* isolates were recovered from only four cattle (0.4%). One of these isolates was emerging PCR ribotype 078/toxinotype V. The remaining toxigenic isolates were toxinotype 0, one of which was an isolate with resistance to linezolid, clindamycin and moxifloxacin (E-test). All isolates were susceptible to vancomycin, metronidazole and tigecycline, but the minimum inhibitory concentrations against linezolid were as high as the highest reported values for human-derived isolates.
The source of the linezolid-clindamycin-moxifloxacin resistance in a toxigenic *C. difficile* isolate from cattle is uncertain. However, since the use of these three antimicrobials in cattle is not allowed in North America, it is possible that resistance originated from an environmental source, from other species where those antimicrobials are used, or transferred from other intestinal bacteria. This study confirms that commercial cattle can carry epidemiologically relevant *C. difficile* strains at the time of harvest, but the prevalence at the time they enter the food chain is low.

**Introduction**

*Clostridium difficile* has been isolated from food animals and retail meats (including ground beef) since 2006, raising concerns about potential zoonotic and foodborne transmission of this pathogen (Rodriguez-Palacios *et al.* 2006; Rodriguez-Palacios *et al.* 2007). Despite the increasing number of published studies on the subject, most research about *C. difficile* in food producing animals has focused on determining the prevalence of fecal shedding in very young stock (Rodriguez-Palacios *et al.* 2006; Keel *et al.* 2007; Pirs *et al.* 2008; Norman *et al.* 2009), an animal population that contributes minimally (~0.5%) to the millions of pounds of meat processed for human consumption in the United States every year (USDA 2010).

During processing of ground beef, carcass trimmings from mature animals are mixed to achieve target fat contents between 3 and 30% in final products. While fat trimmings often originate from cattle intentionally raised for beef production (hereinafter referred to as fed cattle), the sources of leaner trimmings are frequently mature cattle...
primarily used for breeding purposes or milk production (hereinafter referred to as mature cattle) which can be domestic or imported (NCBA 2002). Both types of cattle represent distinct production systems, may have different risks and prevalence of pathogens, and therefore could differentially contribute to microbial contamination of ground beef products.

At the time of harvest, fecal contamination of the hides and processing facilities contaminate carcasses (Elder et al. 2000). Establishing the fecal prevalence of *C. difficile* in cattle at harvest is thus a valuable step to assess the potential risk for food contamination (carcass or meat products) in the early stages of food processing. This study determined the prevalence and magnitude of fecal shedding of *C. difficile* in commercial fed and mature cattle at harvest in the USA. Furthermore, we assessed the public health relevance of recovered isolates by characterizing their toxin genes and antimicrobial resistance patterns to six drugs of relevance in human medicine.

**Materials and Methods**

*Meat processing plants.*

We conducted a cross-sectional study of *C. difficile* in fed and mature cattle at the time of harvest at seven commercial beef processing plants in four regions of the United States. Establishments inspected by the United States Department of Agriculture that were harvesting between 400 and 1,500 cattle per day were visited and sampled once during summer (July–September) in 2008. Five of these participants specialized in harvesting either fed or mature cattle, whereas the other two processed both animal types.
**Sampling of animals.**

Animals sampled were part of commercial lots scheduled for harvest the day of sampling and were sampled during the normal harvest process. The geographical distribution of the plants was deemed adequate to represent the diversity of cattle production systems that ship animals for slaughter in the USA, including eastern, central, western and southern regions. Three of the seven meat processing plants indicated that animals arriving from Canada were also being harvested on the day of sampling. In the processing line, sampling was conducted by dedicated personal working at the conveyor belt immediately after each evisceration station. In plants where fed and mature cattle were both harvested, the plant supervisor guided the sampling by indicating the respective viscera to the sampling personnel. Based on systematic sampling principles, animals were sampled at regular animal intervals to minimize potential clustering associated with cattle groupings or lots. The intervals were calculated by dividing the number of animals available for harvest the day of sampling over the estimated sample size.

Starting with the first harvested animals, and continuing for 5-6 consecutive hours (50-80% of required time to process all animals), 10 grams of fecal contents were aseptically obtained through a 10-15 cm incision made on the dorsal wall of the rectum about 20 cm from the anus. Fecal samples were stored in WhirlPak bags (Nasco, Fort Atkinson, WI) and shipped refrigerated to The Ohio State University for *C. difficile* testing. Within 24 h of collection, samples were coded and blindly processed. Aliquots
from the first two processing plants (west region) were frozen at \(-80^\circ\text{C}\) to determine the magnitude of fecal shedding (i.e., CFU enumeration) at a later date.

**Enrichment culture and molecular typing.**

*Clostridium difficile* prevalence was based on an enrichment protocol used in previous studies (Arroyo *et al.* 2005; Rodriguez-Palacios *et al.* 2006; Rodriguez-Palacios *et al.* 2007). Briefly, one gram of manually homogenized feces was enriched in 15 ml of selective *C. difficile* broth (Arroyo *et al.* 2005) supplemented with 0.1% sodium taurocholate (Sigma Aldrich, St Louis, MO) and *Clostridium difficile* supplement SR0096 (Oxoid, Columbia, MD; broth concentrations of D-cycloserine and cefoxitin are 250 and 8 mg · L\(^{-1}\), respectively). Following anaerobic incubation at 37\(^\circ\)C for 7 days (Anaerobic chamber; CO\(_2\):H: N gas ratio of 10:10:80) and centrifugation of the broths (7,000 x g, 10 min), the sediments were treated with 96% ethanol (1:1, v/v, 30 min), centrifuged again to remove the supernatants, and plated onto 3-hour prereduced *C. difficile* fructose agar (CM0601, Oxoid, Columbia, MD) supplemented with 7% defibrinated horse blood (Quad Five, Ryegate, MT) and the cycloserine/cefoxitin supplement. After 5 days of anaerobic incubation at 37\(^\circ\)C, up to three suspect yellow-green fluorescent (UV\(_{365}\)) colonies were subcultured onto tryptic soy agar (Acumedia, Lansing, MI) supplemented with 5% defibrinated sheep blood (Quad Five, Ryegate, MT). Nonhemolytic isolates, positive for L-proline aminopeptidase activity (Pro Disc, Remel, Lenexa, KS, USA) (Fedorko and Williams 1997) were stored at \(-80^\circ\text{C}\) for molecular confirmation.
Frozen isolates were revived on blood agar and after 48 hours of anaerobic incubation single colonies were processed for DNA extraction using a commercial chelex resin (InstaGene Matrix, Bio-Rad, Hercules, CA). Clostridium difficile was molecularly characterized by using three multiplex PCR reactions. One multiplex confirmed C. difficile by detecting the triose-phosphate isomerase (tpi) gene and toxin genes tcdA and tcdB (Lemee et al. 2004), the second by detecting a typical 16S rRNA gene amplicon and toxin genes tcdA, tcdB, cdtA and cdtB (Persson et al. 2008); the third multiplex assessed the presence of the putative toxin regulator gene tcdC using tcdE as a referent amplicon (Spigaglia and Mastrantonio 2002; French et al. 2010). PCR ribotyping and visual comparison of resulting fingerprint patterns were used to assess strain similarity among cattle types and regions (Bidet et al. 1999). In addition, toxinotyping (i.e., restriction fragment length polymorphism of the pathogenicity locus) was conducted on toxin gene fragments A3 and B1 as described by Rupnik (http://www.mf.uni-mb.si/mikro/tox/). Isolates were deemed toxigenic if they had at least one of the tcdA, tcdB, cdtA/B toxin genes and associated toxins (Rupnik et al. 2005).

**Toxin production.**

In addition to identifying the toxin genes, representative isolates were tested with two complementary commercial ELISAs to assess their toxigenic potential as previously described (Thonnard et al. 1996; Garcia et al. 2000) using 72-hour culture colonies. Toxin gene testing was conducted at least in duplicate using reference toxigenic strain ATCC 9569 (tcdA⁺/tcdB⁺/cdtAB⁻/toxinotype 0) as positive control. The production of C. difficile toxins A and B was confirmed by interpreting in parallel ELISA results from
commercial kits *C. difficile TOX A/B II* (TechLab, Blacksburg, VA) and Clearview *C. diff*

A (Wampole Laboratories, Inverness Medical Professional Diagnostics, Princeton, NJ). Binary toxin production was not evaluated.

**Dialysis tubing and toxin cytotoxicity assay on Vero cells.**

To confirm the lack of toxin production by strains identified as toxin-negative by PCR and ELISA, selected isolates were tested in dialysis assays (Sullivan *et al.* 1982) using toxigenic strain ATCC 9569 and nontoxigenic *C. difficile* PCR ribotype M26 (Rodriguez-Palacios *et al.* 2007) as controls. In brief, 48h *C. difficile* cultures, grown on blood agar were anaerobically resuspended in antibiotic-free brain heart infusion (BHI) broth supplemented with 0.5% yeast extract (Acumedia, Lansing, MI) at an OD$_{600}$ of 0.3. Ten milliliters of those suspensions were placed inside 6 mm diameter pretreated dialysis tubing (Spectra/Por, 12-14,000 MWCO, Spectrum Laboratories, Rancho Dominguez, CA), which were submerged in 500 ml of BHI-yeast broth and anaerobically incubated at 37°C for 72 hours. At the end of the assay, the broths within the tubing were harvested, centrifuged (10,000 x g, 5 minutes) and filtered (0.2 µm) for sterile testing on 90% confluent monolayer cultures of Vero cells line. Vero cells were grown using D-MEM high glucose medium (Gibco, Carlsbad, CA) supplemented with penicillin and streptomycin (100U and 0.1mg ml$^{-1}$, respectively; Sigma Aldrich, St Louis, MO) and 10% fetal bovine serum (Atlas Biologicals, Fort Collins, CO), but tested for cytotoxin activity only with the medium (D-MEM with antibiotics; 80 µl), 1% fetal bovine serum and 20 µL of the toxin filtrate. Cytotoxicity (cell roundening) of two-fold serial dilutions was quantified 24h after toxin inoculation in duplicate using 96-well microtiter plates.
A virologist blindly assessed the plates using a scoring system with determined kappa agreement between ratings greater than 0.95.

**Antimicrobial susceptibility.**

Commercial E-test strips (AB Biodisk, Solna, Sweden) were used to determine the minimum inhibitory concentrations (MICs) against six antimicrobial classes of clinical relevance in humans: metronidazole and vancomycin (first choice treatments for *C. difficile* infections, CDI) (Cohen *et al.*), moxifloxacin and clindamycin (widely associated with CDI induction) (Biller *et al.* 2007; Cohen *et al.*), and linezolid and tigecycline (newer therapeutic classes against CDI) (Rello 2005; Jones *et al.* 2009).

Single isolates from 7 fed and 7 mature cattle representing all PCR ribotypes were tested. Briefly, 24 hour old *C. difficile* colonies grown on blood agar and resuspended (inside anaerobic chamber) in *Brucella* broth (Oxoid, Columbia, MD) were used at an OD$_{600}$ 0.23 ± 0.01 to lawn 6-hour prerduced *Brucella* agar, where the six E-test strips were seeded. Plates were read after 48 hours of anaerobic incubation at 37°C following the manufacturer’s recommendations. Minimal inhibitory concentrations and interpretive categories for metronidazole, clindamycin, and moxifloxacin derived from the Clinical and Laboratory Standards Institute, while the values for vancomycin, linezolid and tigecycline were from previously published studies (Rodriguez-Palacios *et al.* 2006; Hecht and Citron 2007; Jones *et al.* 2009; Nagy and Dowzicky 2010).

**Magnitude of fecal shedding.**

The quantification of *C. difficile* shedding (log$_{10}$ CFU·g$^{-1}$ of feces) was determined using the standard spread plate method. Six available frozen samples that
were *C. difficile* positive by the enrichment method and three negative samples were enumerated. In brief, duplicate 2 g aliquots of feces homogenized with phosphate buffered saline (PBS, 1:1, vol/vol) were split in two subsamples, one for direct plating and one for ethanol shock. Ethanol was used to inactivate vegetative fecal contaminants to facilitate enumeration of *C. difficile* spores (Arroyo *et al.* 2005). Then, $10^0$ to $10^{-6}$ dilutions were spread plated onto blood and *C. difficile* selective agars. After 72 h of incubation, *C. difficile*-like colonies were enumerated, subcultured onto blood agar, and confirmed with a multiplex PCR (Persson *et al.* 2008) and PCR ribotyping (Bidet *et al.* 1999). A reference strain of *C. difficile* PCR ribotype 078 was used as control to estimate the detection thresholds of our enrichment and spread plate methods. For this purpose, duplicates from three samples were spiked with spores of PCR ribotype 078 ($3.4 \log_{10}$ CFU·g$^{-1}$) that had been aged for four weeks in PBS.

**Statistics.**

Assuming simple random sampling, 120 animals were deemed adequate to test a hypothesized *C. difficile* prevalence of 5.5 (±6%) for each cattle type at harvest (one-sample, $\alpha = 0.05$, power = 0.8). *Clostridium difficile* culture binary data was analyzed using logistic regression with cattle type (fed vs. mature) and region as predictors. Fisher’s exact statistics tested proportions with zero counts (Petrie and Watson 1999). Confidence intervals of binomials were estimated using exact statistics (Clopper and Pearson 1934). Nonparametric statistics and significance values for $\log_{10}$ CFU·g$^{-1}$ data were adjusted for ties (Minitab-15, State College, PA; STATA-10.1, College Station,
Results

Prevalence.

In total, single fecal samples from 944 animals were tested for *C. difficile* (118±12 in each processing plant). Using the enrichment method, 29 *C. difficile* isolates were recovered from 17 fecal samples (1.8%); 10 from fed cattle and 7 from mature cattle (Fig. 9). The proportion of *C. difficile* shedders was comparable for all plants and regions, and no differences were observed between fed and mature cattle controlling for region (odds ratio of shedding in mature cattle = 0.8; 95% CI = 0.03, 2.1; Logistic *P* = 0.596). Toxigenic *C. difficile* was recovered from four (24%) of the 17 shedding animals; although these four animals were all fed cattle, there was not statistically difference when compared to the shedding prevalence in mature cattle (Fisher’s exact, *P* = 0.125; Table 4).

Genotypes.

PCR ribotyping identified most isolates had unique fingerprints except for a *tcdA*/*tcdB*/*cdtAB* PCR ribotype (toxins A and B not detectable) that was common in the west and central regions (superscript a in Fig. 9). Dialysis tubing assay confirmed this strain was nontoxigenic (control ATCC strain cytotoxic titer was 1:1024). Emerging PCR ribotype 078/toxinotype V was identified in the east in one of the four fed cattle shedding toxigenic *C. difficile*. The other three toxigenic isolates were toxinotype 0, each had unique PCR ribotype fingerprints. Toxin fragments A3 and B1 did not amplified in the
remaining isolates which were \textit{tcdA/tcdB/cdtAB}. Simultaneous shedding of two \textit{C. difficile} PCR ribotypes was identified in 3 of 17 shedders (18%). Two of these three animals shed simultaneously two PCR ribotypes which were negative to the toxins and toxin genes tested. In the remaining animal that shed two PCR ribotypes, one isolate was toxigenic, the other had no toxin genes or toxin detectable.

\textit{Antimicrobial susceptibility.}

All isolates tested were susceptible to metronidazole and vancomycin but many-fold more susceptible to tigecycline (Fig. 10, Table 5). In contrast, 93% of isolates had intermediate or resistant MICs for clindamycin. Only one isolate (7%) was resistant to moxifloxacin. The MICs for linezolid were comparable to moxifloxacin and metronidazole but were among the highest values reported for \textit{C. difficile} isolates of human origin (Ackermann \textit{et al.} 2003; Jones \textit{et al.} 2009). One of the toxigenic isolates (toxinotype 0) was multidrug resistant and had the highest measurable MICs for clindamycin and moxifloxacin and among the highest MICs reported for linezolid (Fig. 10). Using the actual MIC values, the cut offs for resistance, and the geometrical means as previously reported (Wilcox \textit{et al.} 2000), \textit{C. difficile} isolates from fed and mature cattle had comparable levels of susceptibility to the drugs tested \((P > 0.05)\). However, \textit{C. difficile} from fed cattle had more variability, due to high MICs in toxigenic isolates (Bartlett’s test controlling for cattle type and strain toxinotype, \(P < 0.001\); Fig. 10).

\textit{Magnitude of shedding.}

Analysis of enumerated plate series where \textit{C. difficile} was confirmed \((n = 28)\) indicated that the median of shedding of \textit{C. difficile} at the time of harvest was \(2.2 \log_{10}\)
CFU·g\(^{-1}\) of feces, but in some cases it reached almost 5 log\(_{10}\) units (range = 1.6 to 4.8; 95% CI = 1.6, 4.3). When duplicate samples were spiked with spores from strain PCR ribotype 078, our spread plate-serial dilution method recovered a slightly lower number of spores than inoculated (median = 0.45 log\(_{10}\) CFU·g\(^{-1}\) lower; 95.7% CI = 0.076, 1.283; Mann-Whitney, \(P = 0.083\); Fig. 11). In contrast, direct plating of PBS suspensions containing PCR ribotype 078 yielded 1.2 log\(_{10}\) units more \(C.\) difficile than matched samples treated with ethanol shock (95% CI = 0.9-1.5; Mann-Whitney, \(P = 0.011\); Fig. 11).

**Discussion**

This enrichment-based study indicate that the prevalence of fecal shedding of toxigenic \(C.\) difficile in cattle at harvest (0.4%; 4/944) is in agreement with a recent longitudinal study conducted in a naturally infected feedlot (0.6% at harvest) (Rodriguez-Palacios et al. 2011), but it is much lower than the prevalence reported in calves at the farm (7-13%) and upon entry to veal calf operations (33-36%) (Costa et al. 2009). It is however important to note that the present study was conducted in summer when \(C.\) difficile shedding in neonatal calves (and possible the extent and susceptibility to environmental contamination) is the lowest (Rodriguez-Palacios et al. 2006).

The toxigenic \(C.\) difficile isolates here identified correspond to important toxinotypes associated with CDI in humans, toxinotype 0 and V. PCR ribotype 078/toxinotype V is an emerging \(C.\) difficile genotype increasing its participation in human disease and its presence in food animals and foods (Keel et al. 2007; Rupnik et al.)
2008; Songer et al. 2009). The isolation of emerging PCR ribotype 078 from one of our fed cattle in one of the meat processing plants in the east is of epidemiological interest. This genotype has also been isolated from dairy cattle in Ontario, Canada (Rodriguez-Palacios et al. 2006), and from finishing steers (Rodriguez-Palacios et al. 2011) and farmed deer in Ohio, USA (French et al. 2010), indicating that PCR ribotype 078 might have the potential for geographical distribution across farming operations in the east. Of complementary interest, the most common genotype identified in meat plants of the west and central regions was a nontoxigenic PCR ribotype (superscript ‘a’ in Fig 1A) that has not been reported in our studies on farm animals in the east. Understanding the factors associated with the dissemination of \textit{C. difficile} in livestock and the shedding prevalence (toxigenic:nontoxigenic), which can vary along the production system, is important because nontoxigenic \textit{C. difficile} has been proposed as a probiotic strategy to prevent \textit{C. difficile} colonization in animals and people (Shim et al. 1998; Songer et al. 2007).

The magnitude of \textit{C. difficile} shedding ranged from about 1 to 5 log\textsubscript{10} CFU·g\textsuperscript{-1} of feces. This shedding variability could represent actual temporal variation \textit{in vivo}, local heterogeneity of bacterial concentration within the fecal samples (Pearce et al. 2004), or simply the variability of enumeration methods. In our study, ethanol shock of control strain PCR ribotype 078 spores yielded fewer spores than with direct plating. This indicates that our enumeration of spores in fecal samples is likely an underestimation of the real \textit{C. difficile} shedding in cattle. Recently, a study of culture methods by Thitaram et al. (2011) comparing single versus double alcohol shock treatments as part of an enrichment protocol similar to ours demonstrated differential performance across food
animal production systems, and that single alcohol shock (used in our study) was best to concurrently assess the prevalence of *C. difficile* in dairy and beef cattle feces (Thitaram *et al.* 2011). Comparing the observed prevalence of *C. difficile* in both studies for single alcohol shock and plating onto TCCFA agar results, there were no differences for mature cattle (7/462 [1.5%] vs. 19/1,325 [1.4%]; Chi square *P* = 0.9). However, *C. difficile* was significantly more prevalent in beef cattle in Thitaram’s study (10/482 [2.1%] vs. 157/2965 [5.3%]; Chi square *P* = 0.002). Collectively, both studies indicate that fecal shedding of *C. difficile* in beef feedlot cattle may be more subject to significant regional or production variability than in mature cattle.

It is uncertain if fecal shedding of large numbers of *C. difficile* persists over time or if it is an oscillating pattern (Chase-Topping *et al.* 2008). Periods of fecal shedding of greater than 3 or 4 log\(_{10}\) units have been referred by some as “supershedding” status, a determinant for infectiousness (Chase-Topping *et al.* 2008). Supershedding has recently been described for *C. difficile* in response to antimicrobial therapy in mice (Lawley *et al.* 2009), but the factors associated with the magnitude of fecal shedding in cattle and its causal role in food contamination are unknown. The identification of up to 4-5 log\(_{10}\) CFU·g\(^{-1}\) of feces indicates that small amounts of feces could be sufficient to contaminate meat processing facilities, equipment, beef carcasses, and processed foods.

There is an important contrast between this and other studies conducted at harvest (Rodriguez-Palacios *et al.* 2011) and studies with similar culture methods conducted on retail meat products: the low prevalence of *C. difficile* shedding at harvest (<1.2%) seems remarkably different from the much higher proportion of *C. difficile* contamination
observed in retail products (6-42%) (Rodriguez-Palacios et al. 2009; Songer et al. 2009). Identifying post-harvest factors that could contribute to product contamination, and proliferation, of C. difficile within foods is important, especially because emerging strains can survive minimum cooking recommendations for ground meats (Rodriguez-Palacios et al. 2010; Rodriguez-Palacios and LeJeune 2011).

In contrast to other reports of cattle- and meat-derived C difficile isolates where 100% of fluoroquinolone resistance was observed (Rodriguez-Palacios et al. 2006; Rodriguez-Palacios et al. 2007), all our isolates, except one, were susceptible to moxifloxacin. The regulations in the USA restrict fluoroquinolone usage in food animals (FDA 2009; FDA 2009; FARAD 2011). Enrofloxacin, the only fluoroquinolone permitted, is restricted to treatment of nonresponsive respiratory disease in i) beef cattle and ii) nonlactating replacement dairy heifers under 20 months of age. Explicitly, enrofloxacin is prohibited for dairy cattle or calves intended for production of beef (FDA 2002), although noncompliance might occur (FDA 2009; FDA 2009).

Purposely, we chose tigecycline and linezolid, which are newer drug classes with emerging potential for treatment of CDI, to contribute to the general understanding of susceptibility in C. difficile. These drugs exhibit marked inhibitory activity against bacterial strains resistant to other antimicrobial classes, including fluoroquinolones. Because these antimicrobials are relatively new, and not used in cattle in North America, it would be unlikely that resistance in cattle-derived isolates could be attributable to the use of such medications on farms. The isolates recovered in this study displayed the same high susceptibility documented for tigecycline in global surveillance programs (Jones et
But high MICs of linezolid were required to inhibit most C. difficile isolates. The MICs found here are comparable to the highest MICs reported for human-derived isolates (Wise et al. 1998). Of public health relevance, the isolate that exhibited the highest MIC for linezolid (8 µg · ml⁻¹) was also highly resistant to clindamycin and moxifloxacin and was toxigenic. Although clindamycin is not approved for use in food animals (FDA 2007), most isolates had intermediate or resistant MICs to clindamycin.

The source of the linezolid-clindamycin-moxifloxacin resistance in the abovementioned toxigenic C difficile isolate is uncertain. However, since the use of these antimicrobials, particularly linezolid, in commercial cattle for harvest is unlikely in North America, it is possible that this resistant strain originated from an environmental source, or indirectly from species where those antimicrobials are more commonly used, including humans. Transfer of resistance from other enteric bacteria (e.g., Enterococcus spp.) where intrinsic resistance may occur (particularly linezolid) (Fluckey et al. 2009; Zhang et al. 2010) is another possibility.

In summary, this study indicates that live cattle may be a source of C. difficile contamination of meat processing facilities at the time of harvest, but the prevalence of cattle harboring this pathogen at the time they enter the food chain is low. Of epidemiological concern, two common toxigenic genotypes of C. difficile (toxinotype 0 that in addition was linezolid-clindamycin-moxifloxacin resistant and emerging toxinotype V/PCR ribotype 078 which has been increasingly isolated from animals and foods) were identified in cattle at harvest. Understanding how nontoxigenic C. difficile
modulate fecal shedding of toxigenic strains in various steps of food production might provide insight into prevention strategies to reduce the risk of food contamination.

Acknowledgements

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Authors are grateful to Mike Kauffman, Jennifer Schrock, Laura Harpster, and Pamela Schlegel for invaluable technical assistance during sample collection, and Michele Williams for editorial assistance during final preparation of the manuscript. Special thanks go to the participating meat processing plants and Maria Murgia for her splendid collaboration during dialysis and cytotoxicity assays.
Figure 9. *Clostridium difficile* in bovine feces collected from the colon of cattle processed at meat processing/packing plants, USA. A) Sequential distribution of positive animals (vertical bars) at harvest. Only isolates sharing identical PCR ribotypes are indicated (superscript letter a), the remaining isolates had unique ribotypes. Asterisks indicate toxigenic *C. difficile*. B) Driving distances between plants among and within (black cells) regions.
Figure 10. Minimum inhibitory concentrations (MICs) of cattle-derived *C. difficile* isolates to six antimicrobial classes of relevance for human *C. difficile* infections (CDI). Susceptibility zones within antibiotic ranges tested are illustrated. Vertical bars indicate published linezolid MICs ranges for human isolates; superscript ‘a’ for ref (Ackermann *et al.* 2003) and ‘b’ for ref (Wise *et al.* 1998).
Figure 11. Magnitude of *C. difficile* fecal shedding in cattle at harvest.

Bars and 95% Bonferroni confidence intervals of the means; M-W, Mann-Whitney statistics.
Table 4. Estimated prevalence of cattle carrying *C. difficile* at harvest, USA, summer 2008.a,b

<table>
<thead>
<tr>
<th>Cattle tested</th>
<th>95% CI of estimated shedding prevalence by strain type (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Toxigenic strains</td>
</tr>
<tr>
<td>Mature</td>
<td>n=462</td>
</tr>
<tr>
<td>Fed</td>
<td>n=482</td>
</tr>
<tr>
<td>Total</td>
<td>n=944</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fed vs. Mature  

|               | P = 0.12 | P = 1.0 | P = 0.6 |

<sup>a</sup>Two-sided 95% exact confidence intervals (CI); one-sided 97.5% CI reported for zero counts.

<sup>b</sup>Relevance: toxigenic strains cause CDI in humans and animals (Rodriguez-Palacios *et al.* 2006; Hammitt *et al.* 2008; Rupnik *et al.* 2008), but nontoxigenic strains might prevent it (Shim *et al.* 1998; Songer *et al.* 2007).

<sup>c</sup>One fed animal simultaneously shed a toxigenic and a toxin-gene negative strain.
Table 5. Antimicrobial susceptibility of C. difficile from cattle at harvest.a

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>MIC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>MIC&lt;sub&gt;90&lt;/sub&gt;</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metronidazole</td>
<td>1.0</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>0.75</td>
<td>0.75</td>
<td>1.5</td>
</tr>
<tr>
<td>Linezolid</td>
<td>1.5</td>
<td>2.0</td>
<td>8</td>
</tr>
<tr>
<td>Tigecycline</td>
<td>0.036</td>
<td>0.064</td>
<td>0.5</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>6</td>
<td>12</td>
<td>256</td>
</tr>
<tr>
<td>Moxifloxacin</td>
<td>1.0</td>
<td>1.5</td>
<td>32</td>
</tr>
</tbody>
</table>

*a determined with the E-test method (µg · ml⁻¹)
References


Clopper, C. J. and E. S. Pearson (1934). "The use of confidence or fiducial limits illustrated in the case of the binomial " Biometrika 26: 404-413


Chapter 6 — *Clostridium difficile* in retail fresh vegetables, Ohio, USA

**Abstract**

A prevalence study of *C. difficile* in retail fresh vegetables was conducted in Ohio, USA to determine the prevalence of toxigenic *C. difficile*. Retail products were sampled from various retailers during summer. A toxigenic strain of *Clostridium difficile* was isolated from the outer leaves of a head of lettuce.

**Introduction**

More frequent outbreaks of severe *Clostridium difficile* infections and the appearance of more virulent strains have emerged since 2000 as a public health problem worldwide, yet the major sources of community acquired infections remain enigmatic (McFarland 2008). Since the isolation of hyper-virulent *C. difficile* strains from livestock in 2006 (Rodriguez-Palacios et al. 2006) and retail meats in 2007 (Rodriguez-Palacios et al. 2007), concerns about food contamination and foodborne transmissibility are now being increasingly documented (Bakri et al. 2009; Rodriguez-Palacios et al. 2009; Songer et al. 2009).

The ability of infectious *C. difficile* spores to survive on indoor surfaces, in feces and soil (Baverud et al. 2003) for several years, and the current use of livestock manure
as fertilizer during vegetable production with increasingly green technologies, led us to determine the period prevalence of *C. difficile* contamination in a variety of retail fresh vegetables. We hypothesized that fresh vegetables including root products (onions, potatoes, etc.) could be contaminated with *C. difficile* serving as a source of infection as recently reported for ready-to-eat salads in Scotland (Bakri *et al.* 2009).

**Materials and Methods**

Aiming to achieve sufficient study power (p=0.8), in 2007, we collected 125 various vegetable products from 4 different retailers (2 chain, 1 auction, and 1 in-farm market) in Ohio, USA, during July and August. Purchased products corresponded to single units systematically selected for most classes of vegetables available. Assuming the possible presence of toxigenic *C. difficile* in surface soils, as previously reported in the United Kingdom (al Saif and Brazier 1996) and Zimbabwe (Simango 2006), we purposively included product units having soil material on the surface. All products were from US (CA, PA, OH, MI, WI, NY and FL) and Mexican origin, but some produce lacked origin description.

In the laboratory, 10-15 g portions of edible parts of the produce, including surface cuts (<3mm thick) and/or the outermost leaves, were aseptically cultured for *C. difficile* by enrichment-based culture methods. The enrichment (50 mL of selective broth with cycloserine and cefoxitin) and subsequent isolation steps were performed as previously described for retail meats (Rodriguez-Palacios *et al.* 2007; Rodriguez-Palacios *et al.* 2009). UV-fluorescence was used to screen selective agar plates for suspect
colonies. Biochemical confirmation was based on L-proline aminopeptidase activity (ProDisc, Remel, Lenexa, KS, USA). Identification bias was minimized by re-coding and blindly processing all samples. Positive and negative controls included *C. difficile* strain ATCC 9689. For quality control purposes, the countertop used for sample processing was swabbed at regular intervals and cultured for *C. difficile* to rule out cross-contamination.

**Results and Discussion**

*Clostridium difficile* was isolated from the outer leaves of a single conventionally-grown iceberg lettuce head, for which the origin could not be determined (Table 1). In contrast to our expectations, none of the other vegetables tested positive for *C. difficile*. The overall prevalence of contamination of all vegetables tested was 0.8% (1 of 125); but the relative proportion of contamination for the leafy vegetable category was 2.4% (1 of 41; table 1). With a single isolate, no differences were detected when comparing aerial- (1/65) and the remaining soil/root-associated (0/60) vegetables (Fisher’s exact p>0.1). No molecular typing of the *C. difficile* isolate was possible as it could not be revived after storage.

Since the product was offered for sale wrapped in plastic, contamination might have occurred anywhere from production to post-harvest processing and handling. The identification of *C difficile* from this retail lettuce head, from minimally processed ready-to-eat salads in Scotland (Bakri *et al.* 2009), and also from bagged spinach in the USA (PCR-ribotype 078; per. com. Dr J. G. Songer, University of Arizona, Tucson, USA, unpublished data), indicate that there are possible critical points during the farm-to-fork
continuum that have resulted in the contamination of some retail vegetables with \textit{C. difficile}.

To date, \textit{Clostridium difficile} has been isolated from retail leafy (0.8-7.5\%) and root (2.3\%) vegetables, and from retail meats (6.1-42\%)(al Saif and Brazier 1996; Rodriguez-Palacios \textit{et al.} 2007; Bakri \textit{et al.} 2009; Songer \textit{et al.} 2009); but the epidemiological role of foods as sources of infection in the community remains uncertain. \textit{C. difficile} is an opportunistic pathogen that especially affects elderly and immune-compromised people; however, other individuals taking antimicrobial and/or antacid medications are also at risk (McFarland 2008). Food contamination thus raises concern as it may serve as a direct or indirect source of infectious spores.

Although \textit{C. difficile} has traditionally been considered to be ubiquitous, its distribution across food production and processing environments is unlikely to be homogeneous. Therefore, studies are needed to identify factors and intervening strategies to minimize vegetable contamination, especially of freshly-eaten leafy green vegetables and herbs, which have large-scale global markets and a complex array of production and post-harvest processing systems that greatly vary around the world (Organization 2008).

\textbf{Acknowledgements}

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Table 6. Retail fresh vegetables tested for *Clostridium difficile* in Ohio, USA, 2007

<table>
<thead>
<tr>
<th>Arbitrary groups</th>
<th>Category</th>
<th>WHO/FAO Priority*</th>
<th>n=†</th>
<th>Organic‡</th>
<th>C. difficile</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aerial parts of plant</td>
<td>Leafy (lettuce/spinach/chard/herbs)</td>
<td>1</td>
<td>41</td>
<td>4</td>
<td>1§</td>
</tr>
<tr>
<td>(n=65)</td>
<td>Tomato/pepper</td>
<td>2</td>
<td>13</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Berries</td>
<td>2</td>
<td>5</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Broccoli</td>
<td>-</td>
<td>3</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Green beans</td>
<td>-</td>
<td>3</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Common contact with soil surface</td>
<td>Melons</td>
<td>2</td>
<td>3</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>(n=13)</td>
<td>Cucurbits</td>
<td>3</td>
<td>10</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Root vegetables</td>
<td>Onions</td>
<td>2-3</td>
<td>20</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>(n=39)</td>
<td>Roots</td>
<td>3</td>
<td>19</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>(carrots/potatoes/beets/parsnip)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>Sprouts</td>
<td>2</td>
<td>2</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>(n=8)</td>
<td>Mushrooms</td>
<td>-</td>
<td>6</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>-</td>
<td>125</td>
<td>14</td>
<td>1 (0.8%)</td>
<td></td>
</tr>
</tbody>
</table>

*Priority levels in terms of fresh produce safety assigned by the Food and Agriculture Organization of the United Nations and the World Health Organization, 2008 (Organization 2008). Leafy green vegetables are global top priority; priorities 2 and 3 depend on regional relevance.

† Total of samples tested per category; retailers sampled (n=4) were located at 0, 26, 174 and 201 km from research center.

‡ Products with organic claims or from local communities that use manure as fertilizer. Farm practices were unknown for most products.

§ Toxigenic *C difficile* was isolated from conventional iceberg lettuce.
References


Chapter 7 — Enteric bacterial pathogens with zoonotic potential isolated from farm-raised white-tailed deer

As Published in:
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Abstract
The raising of captive white-tailed deer (Odocoileus virginianus) is a growing agricultural industry in Ohio as it is in several other areas of the United States and around the world. Pooled fecal samples were collected from 30 white-tailed deer confinement facilities. Samples were cultured for five enteric bacterial pathogens. Premise prevalence rates were as follows: Escherichia coli O157, 3.3%; Listeria monocytogenes, 3.3%; Salmonella enterica, 0%; and Yersinia enterocolitica, 30%; Clostridium difficile, 36.7%. The ail virulence gene could not be amplified from any of the Y. enterocolitica isolates recovered. Toxigenic strains of C. difficile PCR-ribotype 078, an emerging C. difficile genotype of humans and food animals, were recovered from four (36.4%) of 11 C. difficile positive deer farms. Venison from farm-raised deer might become contaminated with foodborne pathogens, deer farmers may have occupational exposure to these zoonotic agents, and farm raised deer could be a reservoir from which the
environment and other livestock may become contaminated with a number of pathogenic bacteria.

**Introduction**

The farming of white-tailed deer is a growing industry of agriculture across the United States. According to the National Agricultural Statistical Service (US), there were approximately 5,654 farms raising a collective total of 269,537 deer, up 20% from the previous census. Ohio reported a total of 303 farms in 2007, 4900 deer farms in the US, with Ohio reporting 224 deer farms in 2002 (Service 2009). Commercial farming of white-tailed deer is conducted for the production of venison, harvest of antlers, urine collection, and for the population of hunting preserves. As ruminants, these animals are subject to colonization of many of the same bacterial agents as domestic livestock. It is estimated that annually the industry of rearing cervids contributes $2.3 billion dollars into the US economy through the purchase of feeds, veterinary services, fuel and other purchases (Anderson et al. 2007).

As in other ruminants, bacterial infections in deer can cause illness or remain as asymptomatic infections. Several zoonotic pathogens, notably *E. coli O157:H7, Listeria monocytogenes, Yersinia enterocolitica* and *Salmonella enterica* have been reported in wild deer populations (Branham et al. 2005, Lillehaug et al. 2005, Renter et al. 2006, Garcia-Sanchez et al. 2007, Lyautey et al. 2007). The prevalence of *Clostridium difficile* in white-tailed deer has not been reported. The presence of these zoonotic organisms poses food and environmental safety risks if carcass contamination occurs at
the time of harvest or pathogens are released into the environment during the pre-harvest stages of production. The objective of this study was to determine the farm-level prevalence of the aforementioned five pathogens among confined and intensively managed white-tailed deer in Ohio, USA.

**Materials and Methods**

**Sampling frame**

All 301 individuals licensed through Ohio Department of Natural Resources, Division of Wildlife in 2006 to commercially and non-commercially propagate deer in Ohio were sent an invitation to participate in this study (Figure 1). The mailing contained a letter describing the study, sampling materials, and a postage-prepaid response envelope. Reminder letters were sent to non-respondents. In addition, non-respondents farms, in geographic proximity to the laboratory were visited by investigators as an additional recruitment strategy. Using the clean latex gloves provided, participants were requested to collect one to two tablespoons of freshly deposited feces from 5 or 6 different locations in the confinement area. Samples were submitted in the sterile containers provided. No financial incentives were offered. Upon receipt in the laboratory, samples were stored refrigerated for up to 5 days so that they could be processed in batches. All samples were collected and assayed between November 2006 and May 2007.

**Microbiological and Molecular Analysis**
For bacterial isolation, 20g of each composite sample was homogenized with 180 ml of Universal Pre-enrichment broth (UPB, Acumedia 7510A, Lansing, Michigan) using a peristaltic-type mixer (120 rpm, 2 min). Two 15 ml aliquots of this homogenate was removed and used for *Clostridium difficile* detection (see details below). The remainder of the sample was incubated for 14-18h at 35°C. Testing for *Escherichia coli* O157, *Listeria monocytogenes*, *Yersinia enterocolitica* and *Salmonella enterica* was conducted on aliquots of the pre-enrichment broth as previously described (Dodson and LeJeune 2005, Bowman et al. 2007, Kersting et al. 2009) and outlined below.

*Escherichia coli* O157:H7 was screened in single 1 ml aliquots of UPB using immunomagnetic separation and a commercial automated bead retrieval protocol (BeadRetriever™, Dynal, Oslo, Norway). Briefly, retrieved beads were then plated on Sorbitol MacConkey agar plates containing cefixime (50µg/ml) and tellurite (100µg/ml). Up to five suspect sorbitol-negative colonies from each plate were further screened for lactose fermentation, absence of D-umbeliferyl-Beta-glucoronide cleavage, and latex agglutination for O157 antigens (E. coli O157 Latex, Oxoid Ltd, Basingstoke, UK). Isolates were confirmed as *E. coli* O157:H7 using a multiplex PCR protocol (Hu et al. 1999)

For *Listeria monocytogenes* screening, one ml of the UPB enrichment was transferred to 9 ml of Fraser broth and incubated for 48h at 35°C. Samples that darkened the broth were streaked onto polymyxin acriflavin LiCl ceftazadime esculine manitol agar (PALCAM, Oxoid) and incubated for 14-18h at 35°C. Suspect *Listeria* colonies were tested for β-hemolysis on Columbia Blood agar and chromogenic characteristics on
Rapid’L mono agar (Bio-Rad Laboratories, Hercules, CA). Isolates were confirmed using a multiplex PCR assay (Zhang and Knabel 2005).

For *Salmonella enterica*, 10 μl of the UPB enrichment was incubated into 10 ml of Rappaport-Vassiliadi broth at 37°C. After 14-18h of incubation, a loopful of this enrichment was streaked onto Xylose-lysine-tergitol-4 (XLT4, Neogen Acumedia, MI) agar and incubated 18 hours at 37°C. From each agar plate, up to three suspect colonies were selected and plated on Triple Sugar Iron, Citrate, and Urea agars (Neogen Acumedia, MI). One colony from each fecal sample that produced an acid butt and alkaline slant with H₂S production on Triple Sugar Iron, urea-negative, and citrate-positive isolates were serogrouped using group specific antisera (Becton-Dickinson, Sparks, MD).

For *Yersinia enterocolitica*, one ml of the UPB enrichment was incubated into 9 ml peptone sorbitol bile broth at 4°C (Neogen Acumedia). After 10 days of incubation, 1 ml of this broth was mixed with 9 ml of a 0.5%-KOH 0.5%-NaCl solution; from this KOH/NaCl mixture 100 μl was streaked onto celfsulodin-irgasan-novobiocin agar (CIN, Hardy Diagnostics, Santa Maria, CA) and incubated overnight at 30°C. Suspect colonies (clear zone surrounding a red center) were transferred to lysine-arginine-iron, urea, and bile-esculin agar slants and incubated for 48 h at 30 °C (Neogen Acumedia). Colonies that produced a purple color change with no gas on lysine-arginine-iron, pink color change on urea, and blackening of bile-esculin agars were transferred to 2 tubes with of methyl red-Vogues Proskauer broth. One tube was incubated overnight at 30 °C and the other for 48 h at 37 °C. Isolates that exhibited turbidity at 30 °C and agglutination at 37 °C.
°C were considered presumptive *Y. enterocolitica* and were stored at -80°C for PCR analysis. Confirmation was based on detection of a 16s DNA amplicon and the *ail* virulence gene (Bowman et al. 2007).

For *Clostridium difficile*, duplicate analysis was conducted on sediments of the 15 ml UPB/fecal aliquots (8200 x g, 10 min). Sediments were re-suspended with 15ml of an enrichment broth prepared with the ingredients from a commercial agar base (Oxoid Ltd) as previously described (Rodriguez-Palacios et al. 2007). One aliquot was supplemented with commercial cycloserine/cefoxitine (Oxoid, Ltd) and the other with moxalactam/norfloxacin (CDMN, Oxoid, Ltd). Broths were anaerobically incubated for 7-10 days at 37°C to favor sporulation. To reduce fecal contaminants and select for spores, 2 ml of these homogenized broths were then mixed with 96% ethanol (1:1, v/v, 50min), centrifuged, and streaked onto *C. difficile* agar supplemented with the corresponding antibiotics (cycloserine/cefoxitine or CDMN). Plates were examined for up to 5 days under anaerobic incubation for suspect colonies (non-hemolytic, swarming, p-cresol smell). Up to 3 colonies per plate were sub-cultured onto 5% sheep blood agar. Preliminary confirmation was based on L-proline aminopeptidase activity. Crudely extracted DNA (boiling, 10 min) was used for PCR confirmation (housekeeping *tpi* gene detection), toxin gene profiling (*tcdA, tcdB, cdtA, cdtB, tcdC* deletions, *tcdE, cdu*) and fingerprinting (PCR-ribotyping) of isolates as performed in previous studies (Rodriguez-Palacios et al. 2006 & 2009). Toxin production was confirmed in bacterial suspensions (72 hour old colonies, and PBS) for up to two isolates for each toxin profile using
commercial ELISA Wampole Tox A/B™ (TechLab, Blacksburg, Va) following the manufacturer’s recommendations.

**Farm practices**

Questionnaires (see supplementary material) were completed by participants to ascertain commercial purpose, herd demographic factors (presence of other animals), and management factors (density, grazing area, health, etc).

**Statistics**

Proportions of binary data were compared with Chi-square or Fishers’ exact statistics. Adjusting for rare events, all reported 95% confidence intervals for proportions used exact binomial Clopper-Pearson statistics (Clopper and Pearson 1934). Univariate analysis of continuous data was conducted using 2-sample t-test or Mann-Whitney statistics where appropriate (Sheskin 2000). STATA was the statistical software used (v10, College Station, TX).

**Results**

Thirty of 301 (10%) possible responders collected samples within the time frame specified (Figure 1). Twenty samples were mailed in and an additional 10 samples were collected during farm visits by the investigators. Twenty-two of 30 (73%) of the questionnaires were fully completed. Regarding animal facility, participants reported to have between 4 and 364 deer (median 31; mean 57.9±86.4), and between 0.8 and 40 hectares (median 2; mean 4.9±8.6) available for grazing at the time of sampling. Calculated stocking density ranged from 0.5 to 11.5 deer/hectare (median 5; mean
4.7±2.7); which was similar for pathogen-positive and pathogen-negative farms (t-test $P = 0.94$).

Among the 30 farms, pathogens were isolated from 15 (50%) of the premises; 8 (27%) and 7 (23%) farms yielded one and two pathogens, respectively. Five of the farms, three from which pathogens were isolated, had free access to open waters. Sharing pasture with other livestock or free-range poultry was reported in 5 (22.7%) of the 22 farms submitting fully-answered questionnaires. Analysis of the 22 survey responders, indicate that sharing pastures with other livestock was more common among pathogen-positive farms (4/7) compared to pathogen-negative (1/15) farms (Fisher’s exact $P = 0.021$). None of the respondents reported recent problems with infections or disease in their animals.

*Escherichia coli* O157 was isolated from one of the 30 premises (3.3%) (Table 7). It was noted that the farm from which this sample was collected allowed co-grazing of deer and other animals (cattle, bison, horses, small ruminants, and poultry). *Listeria monocytogenes* was recovered from one specimen. *Salmonella enterica* was not recovered from any specimen in this study.

A total of 14 (46.7%) of all 30 specimens yielded bacterial colonies that were phenotypically indistinguishable and antigenically cross-reactive with the anti-*Yersinia* antisera used. However, the 16S rDNA fragment specific to *Yersinia enterocolitica* could be amplified from only nine (9/30, 30%) specimens, including the specimen from which *E. coli* O157 was recovered. The gene *ail*, could not be amplified from any of these
isolates despite positive control assays for this gene consistently demonstrating the assay was functioning properly.

*Clostridium difficile* was isolated from 11/30 (36.7%) of farms. Seven of the 11 (63.6%) locations yielded isolates with one or more of the toxin genes assayed (Table 8). Among these toxigenic isolates there was a common variant isolates ($tcdA^+ tcdB^+ cdtB^+$) with a 39-bp type A deletion in putative negative toxin regulator $tcdC$ ($n=4$) (Fig. 13A). PCR-ribotyping, an international genomic fingerprinting based on 16S-23S rRNA gene intergenic spacer regions, identified all four strains (*i.e.*, $tcdC$ 39-bp deletion) as human epidemic PCR-ribotype 078 (57% of toxigenic *C. difficile*-positive farms, Fig. 13B, Table 1). Specimens with PCR-ribotype 078 were collected in March 2007 from farms that were clustered within 30 km of distance (Fig. 12). No spatial statistics were conducted to differentiate clustered from random data due to limited computational power, *i.e.*, only 4 farms were PCR ribotype 078.

**Discussion**

In this study, one or more bacterial pathogens were isolated from 50% of farmed white-tailed deer facilities following a single farm visit. This is the first report of *Clostridium difficile* in deer, captive or wild. Although the specific within-farm animal prevalence was not determined, this study demonstrates that enteric pathogens are present among operations that raise captive cervids and that there is the potential to produce food products that are contaminated with dangerous microorganisms, entails possible occupational exposure to zoonosis, can contribute to pathogen loading in the
environment, and may be involved in the cycling of pathogenic organisms between wildlife and domestic animals.

All the human enteric pathogens studied herein, except \textit{C. difficile}, have previously been reported among healthy and ill wild white-tailed deer populations (Lillehaug et al. 2005, Kemper et al. 2006, Renter et al. 2006, Garcia-Sanchez et al. 2007, Lyautey et al. 2007). Although deer may become colonized with \textit{Escherichia coli} O157 and shed the organism in their feces for periods similar to cattle (Fischer et al. 2001), the prevalence of the organism is relatively low in the wild deer populations, with occasional sharing of indistinguishable molecular subtypes between cattle and deer (Rice et al. 1995, Renter et al. 2001). A longitudinal study of a single captive deer population in the state of Louisiana, USA, identified one positive animal out of 226 samples tested (Dunn et al. 2004). In contrast, a very small study of 10 captive deer in the UK demonstrated 3/10 positive for \textit{E. coli} O157 (Chapman and Ackroyd 1997). Like commercial livestock production, the factors that influence the occurrence of \textit{E. coli} O157 on farms and the prevalence within a herd of deer are not fully understood. The single farm testing positive in this study also raised cattle in the deer pen. Samples were not collected from the cattle nor the environment outside the deer pen to determine if other animals shared similar subtypes of \textit{E. coli} O157 or if other environmental niches were at this location contaminated with indistinguishable \textit{E. coli} O157 strains.

Unlike \textit{E. coli} O157, \textit{Salmonella enterica} in addition to being asymptomatic may cause disease in cervids (Robinson et al. 1970, Sato et al. 2000, Clark et al. 2004). Several studies have been conducted to assess the prevalence of \textit{Salmonella enterica} in wild and
semi-domesticated deer populations with the prevalence of *Salmonella* spp. generally found to be extremely low, if detectable at all (Kemper et al. 2006, Renter et al. 2006). However, a prevalence of 7% was reported in deer grazing rangeland shared by cattle (Branham et al. 2005). It has been hypothesized that deer are somewhat refractory to long-term carriage of *Salmonellae* because of their lack of a gall bladder (Henderson and Hemmingsen 1983). In support of this theory, a recent study in humans and mice showed gallstones playing a major role in prolonged fecal shedding due to enhanced colonization of gallbladder tissue and bile with *Salmonella enterica* spp. compared to *E. coli* infections (Crawford et al. 2010). From a public health perspective, prevalence-based cross sectional studies indicate that deer are seemingly less frequent carriers of *Salmonella* spp. compared to other animal species. Nevertheless, assumptions regarding a lower prevalence for salmonella should not overrule the need to exercise preventive measures due to the frequent presence of other relevant pathogens.

*Listeria monocytogenes* is a cause of meningo-encephalitis and septicemia in cervids (Kemenes et al. 1983, Eriksen et al. 1988, Tham et al. 1999), but it has been recently isolated from feces of clinically healthy deer (6%) and moose (5%) in the wild (Lyautey et al. 2007). Likewise, *Yersinia enterocolitica* has been reported in both wild and captive deer populations (Henderson 1983, Henderson 1984). In addition to other virulence genes, most pathogenic *Yersinia enterocolitica* strains harbour the chromosomal *ail* marker (Miller et al. 1989, Zheng et al. 2008). Given that the majority of isolates obtained in this study were likely of low pathogenicity and minimal risk for food
or environmental safety, additional serological or molecular analyses were not performed on these isolates.

This is the first report documenting the presence of *C. difficile* in deer, and specifically, a geographic cluster of farms with highly-relevant human emerging *C. difficile* PCR ribotype 078 (Figure 1). Considering that there are more than 160 recognized *C. difficile* PCR ribotypes to date (Rodriguez-Palacios et al. 2006), the molecular characteristics of the *C. difficile* strain 078 identified in four (36.4%) of 11 *C. difficile* positive farms provides molecular evidence of a cluster. Although no spatial statistics were computed due to low power to distinguish clustered from random distribution, the joint probability of the combined events (culture-yes/no vs. molecular vs. spatial localization-3 of 4 farms were located with 30 km) indicate that the cluster observed occurring due to chance alone is very unlikely. Since the first identification of this human *C. difficile* genotype in cattle in 2004 (Rodriguez-Palacios et al. 2006), and the documented presence of emerging *C. difficile* strains in retail meat products intended for human consumption since 2005 (Rodriguez-Palacios et al. 2006 & 2009, Songer et al. 2009), it has become increasingly evident that PCR ribotype 078 is predominant among calves and swine, and is an emerging strain among people in hospital and community settings in North America and Europe (Rupnik et al. 2008, Debast et al. 2009, Limbago et al. 2009). This region on Ohio where the PCR ribotypes 078 were identified is home to a high density of animal agriculture, particularly dairy production (Bender 2001). Although it is uncertain whether the PCR ribotype 078 strains isolated from deer in this study represent inter-species or only deer-adapted strains, the presence of this genotype,
previously isolated from swine, cattle, and humans indicate the potential of regional dissemination of pathogens among farming operations.

Venison derived from farm raised deer is used for personal consumption, and sold commercially in restaurants in metropolitan areas. Like other cervids, the frequency that hair is shed during post-slaughter handling is of particular concern as it may contaminate the carcass (Gracey et al. 1999). Slaughter hygiene, in addition to pathogen prevalence of incoming animals may impact final microbial loads (Gill 2007). Incidence of microbial contamination of carcasses at approved deer slaughter facilities in New Zealand reported plant-to-plant and lot-to-lot variability in contamination of carcasses with coliforms and *E. coli* (Sumner et al. 1977b, Sumner et al. 1977a). Generally, the microbial counts were lower on carcasses from farm raised deer, but 16/30 samples collected from one plant yielded *Salmonella* St. Paul (Sumner et al. 1977a).

Oftentimes the removal of viscera is performed by individuals who may not be aware of food safety threats posed by contamination events that are not grossly obvious, and performed in the field, lacking appropriate hygienic and temperature controls. One might speculate that the rate on contamination under such more primitive conditions is considerably higher. Subsequent processing of contaminated primal cuts in the processing environment may serve as a source of contamination to other products. These deficiencies can lead to food contamination and public health threats. Of particular concern and direct relevance to food safety is the fact that the slaughter, dressing and processing of deer and other game meats in the US falls outside the regulatory jurisdiction of any federal food safety authority. Current regulations simply require the
processing of game meats to be separated from the processing of meat and poultry. In the US, there is no federal requirement for subsequent professional food safety inspection, although some states may have specific regulations governing this activity. Processing oversight falls outside of the United States Department of Agriculture (USDA) Food Safety and Inspection Service. When intended for human consumption, venison will fall under the charge of the Food and Drug Administration (FDA) only when it enters interstate commerce. FDA does not have regulatory authority if the venison is in intra-state commerce, but the USDA Agricultural Marketing Act of 1946 does provide service for a voluntary USDA inspection (fee for service) of game animals at slaughter.

In addition to food safety hazards, there is a zoonosis hazard via occupational exposure to the approximately 30,000 individuals that are employed in the deer farming industry (Anderson et al. 2007). Many of these individuals may not be aware of the potential hazards to which they are exposed or the possibility that they may bring pathogens from farm sites back to their home or alternate work site (Kersting et al. 2009). Contact between a child with an elevated risk of opportunistic infections and a deer was followed by the child developing diarrhea, septicemia, and multiple organ abscessation (Grigull et al. 2005).

Another concern of production agriculture that has implications to pathogen carriage among farm raised deer is waste management. The bacterial pathogens evaluated in this study, particularly *C. difficile* (al Saif and Brazier 1996), can survive for extended periods and contribute to pathogen loading in the environment, waterways, and serve as a reservoir for bi-directional cycling of pathogens to other wildlife, domestic livestock (Pell
1997, Vercauteren et al. 2006) and even humans. However, it is not possible from this study to determine the extent that deer farms might be a source reservoir for pathogen transfer to livestock, or if the deer were incidental secondary host as a result of the intensive livestock production in the surrounding area. In our study, management was such that deer on 5 of the 22 farms had free access to open waters. More than one pathogen was isolated from 3 of these five farms.

In conclusion, this study demonstrates that fecal samples of deer raised in confinement can harbor enteric human pathogens. Notably, the emerging epidemic C. difficile PCR-ribotype 078 was identified in farmed deer. Although this study was based on voluntary participation of deer framers and therefore there is potential for biased estimates (Table 9), the presence of the pathogens tested in farm-raised deer indicates there are threats to food safety, direct contact zoonoses, and environmental contamination. As culture-based diagnostic methods often yield false negative results (i.e., sensitivity is not 100%), it is possible that the actual prevalence of such pathogens among deer farms might also be slightly higher. As deer farming continues to grow, more individuals will become involved in farming of deer and the processing of venison. It is critical that those individuals currently working in the industry, as well as those who start in the future, are made aware of these hazards and educated in a way to minimize environmental, carcass and direct-contact transmission of these organisms.
Acknowledgements

All authors were supported by state and federal funds allocated to the Ohio Agricultural Research and Development Center, Wooster, Ohio, USA. This work was also financed in part by a grant to The College of Wooster from the Howard Hughes Medical Institute through the Undergraduate Science Education Program to support co-author EF. A special thanks to Dr. Amber McCoig, FDA, for valuable comments on our interpretation of FDA regulations and to the anonymous reviewers for their thoughtful comments and time.
Figure 12. Distribution of 301 contacted premises with captive white-tailed deer, study participants, and positive premises to *C. difficile* and other human enteric pathogens in Ohio, USA.

Small grey irregular areas represent urban centers. Note the close-up map of clustered *C. difficile* positive; four of the *C. difficile* farms shown had epidemic PCR-ribotype 078 (solid black stars). No water sources were common to all farms. Geographical coordinate system, NAD, datum 1927. Geocoding accuracy was modified for illustration.
Figure 13. Multiplex PCR and PCR-ribotyping. A) Multiplex PCR for \( tcdC \), \( tcdE \) and \( cdu-2 \) within the Pathogenicity Locus (PaLoc).

\( C.\ difficile \) isolates from 3 white-tailed deer farms had deletion type A (39bp) in \( tcdC \) gene. Note that \( cdu-2 \), a downstream flanking gene in PaLoc did not amplify with this method indicating a variant gene in PCR-ribotype 078 strains. B) PCR-ribotyping using isolates from duplicate testing in 3 farms have identical pattern to the first PCR-ribotype 078 isolate identified in food animals, Ontario, Canada, 2004 (label 078 in both gels). OH561, control strain verifying typing reproducibility; L, 100-bp molecular marker.
Table 7. Human enteric pathogens among 30 captive-deer operations in Ohio, November 2006-May 2007, USA.

<table>
<thead>
<tr>
<th>Pathogen combination</th>
<th>Farms (%)</th>
<th>Estimated 95%CI</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. enterica</em></td>
<td>0/30 (0)</td>
<td>0, 11.6</td>
</tr>
<tr>
<td><em>E. coli</em> and <em>Y. enterocolitica</em></td>
<td>1/30 (3.3)</td>
<td>0.08, 17.2</td>
</tr>
<tr>
<td><em>L. monocytogenes</em> &amp; <em>C. difficile</em></td>
<td>1/30 (3.3)</td>
<td>0.08, 17.2</td>
</tr>
<tr>
<td><em>C difficile</em> &amp; <em>Y. enterocolitica</em></td>
<td>5/30 (16.7)</td>
<td>5.6, 34.7</td>
</tr>
<tr>
<td><em>Total Y. enterocolitica</em></td>
<td>9/30 (30.3)</td>
<td>14.7, 49.4</td>
</tr>
<tr>
<td><em>Total C. difficile</em></td>
<td>11/30 (36.7)</td>
<td>19.9, 56.1</td>
</tr>
<tr>
<td>Total (Any pathogen)</td>
<td>15/30 (50)</td>
<td>31.3, 68.7</td>
</tr>
</tbody>
</table>

\(^a\)None of the 9 *Y. enterocolitica* farms had the ail virulence marker.

\(^b\)Seven of the 11 *C. difficile* –positive farms had toxigenic strains. Four (57%) of these 7 farms had epidemic *C. difficile* PCR-ribotype 078 (Table 2). CI, confidence interval.
Table 8. Culture recovery and toxin gene profiles of 37 *C. difficile* isolates from 11 deer farms, USA, 2007\(^a\)

<table>
<thead>
<tr>
<th>Toxin gene profile</th>
<th>Isolates tested, (n=)</th>
<th>Farms, (n=) (ID)</th>
<th>Culture media (isolates,(n=))</th>
</tr>
</thead>
<tbody>
<tr>
<td>(tcdA^+tcdB^+cdtB^+); (tcdC) with 39-bp deletion(^b)</td>
<td>13</td>
<td>4 (12, 23, 29, 30)</td>
<td>CCFB (4), CDMN (9)</td>
</tr>
<tr>
<td>(tcdA^+tcdB^+cdtB^+); (tcdC) and (tcdA) did not amplify</td>
<td>9</td>
<td>6 (14,16,20,23,24,28)</td>
<td>CCFB (7), CDMN (2)</td>
</tr>
<tr>
<td>(tcdA^+tcdB^+cdtB^+); (tcdC) did not amplify</td>
<td>8</td>
<td>5 (12,13,20,24,28)</td>
<td>CCFB (7), CDMN (1)</td>
</tr>
<tr>
<td>(tcdA^+tcdB^+cdtB^+); (tcdC) had no deletion (classical)</td>
<td>6</td>
<td>2 (16, 26)</td>
<td>CCFB (4), CDMN (2)</td>
</tr>
<tr>
<td>(tcdA^+tcdB^+cdtB^+); (tcdC) 18-bp (type B) deletion</td>
<td>1</td>
<td>1 (26)</td>
<td>CCFB (0), CDMN (1)</td>
</tr>
</tbody>
</table>

\(^a\) All culture and molecular analysis were conducted in a blinded fashion. *C. difficile* was confirmed with *tpi* gene. Toxin production was confirmed with commercial ELISA Wampole Tox A/B™ (TechLab, Blacksburg, Va).

\(^b\)PCR-ribotype 078 (n=3).

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Chapter 8 — *Clostridium difficile* in anthropogenic wild bird ecosystems and dissemination potential

(For submission)

Abstract

Here we examined at the effect of wild birds in the dissemination of this pathogen and identify ecological aspect that are relevant to the epidemiology of *C. difficile* infections at the interface between a rural and an urban area in a region with high density of food animal productivity. A systematic review of literature and cross-sectional, longitudinal and experimental infection studies were conducted along with radiotelemetry analysis were conducted to determine the dissemination potential of *C. difficile* by wild birds. Toxigenic strains of *C. difficile* were significantly more frequently identified in wild birds inhabiting aquatic environments (Geese and ducks), than invasive terrestrial birds inhabiting dairy farms (European starlings); no toxigenic *C difficile* was identified from terrestrial small passerine birds inhabiting forest. The dissemination potential of *C. difficile* via wild birds was documented with molecular epidemiology methods and telemetry.
Introduction

Concerns about emerging strains of enteric human pathogen have spread globally, as the pathogen continues to reach distant and isolated regions (e.g., Australia) (Clements et al. 2010; Gerding 2010). *Clostridium difficile* has been associated with severe enteric disease since its first description in 1935 but little is known about the ecological factors that might explain increased persistence and global dissemination (Clements et al. 2010; Gerding 2010). Because, emerging strains have been found in food animals and foods of regions where *C. difficile* is public health burden (Clements et al. 2010), some have naturally looked at international trade as a reasonable route for disease expansion. Likewise, the high rapid traffic of people from endemic regions in international flights has been obviously considered (Clements et al. 2010). Here we examined at the effect of wild birds in the dissemination of this pathogen and indentify ecological aspect that are relevant to the epidemiology of *C difficile* infections at the interface between a rural and an urban area in a region with high density of food animal productivity.

In the USA, *C. difficile* infections cause over half a million cases documented per annum, with over 25,000 deaths (Campbell et al. 2009). In this context, *C. difficile* infections are often more serious and frequent than the infamous methicillin-resistant *Staphylococcus aureus* (MRSA) infections. Like other clostridia, most *C. difficile* strains produce spores that survive for years in the environment (Baverud et al. 2003). Disease occurs, in humans and some animals, when spores are ingested and activated in the gut. Rapidly growing generations of daughter cells that produce toxins (A, B, and/or binary) then destroy the gut lining. After active multiplication, induced by unfavorable conditions
within the gut, decaying generations turn back into spores to be excreted in the feces. Fecal contamination thus contributes to environmental dissemination and spore persistence (Baverud et al. 2003).

Due to the natural tendency of some birds to have regular contact with excrements from food animals, mostly due to the presence of easy to get undigested grains that were undigested and come with the feces, interspecies transmission of *C. difficile* strains is plausible. Considered by some producers as “farm pests” pigeons, European Starlings, grackles and House sparrows are among the most frequent species seen in association with cattle. In winter, the comingling between cattle and birds increases. Determining to which extent birds contribute to spore dissemination is important because emerging *C. difficile* strains (namely PCR ribotype 078) is increasingly common in food animals, and foods. These strains now cause more human infections in the community. Recent studies have documented that the farm level prevalence of strains PCR ribotype 078 can reach 100% (Debast et al. 2009) in North America and Europe. But it is uncertain if it is less frequent in more distant regions. Here, we first describe the basis of our hypothesis that wild birds are effective disseminators of *C difficile*; then we tested it using observational studies and animal experimentation.

**The study**

*Retrospective spatial analysis of emerging *C. difficile* in cattle*

To test the basis of our hypothesis we first conducted a retrospective spatial analysis of the first known emerging *C. difficile* strains reported in food animals in North
America to determine the distribution pattern of farming operations affected with emerging epidemic strains (Rodriguez-Palacios et al. 2006). When looking at the three most common genotypes in a study of calves sampled in 102 farms in 2004 in a 150,000 km² region surrounded by the Great Lakes, two apparent patterns of spatial distribution were observed. One was represented by a then newly variant _C. difficile_ strain (functional mutation in gene for toxin A production) found first in severe outbreaks of human disease in Central Canada at 4,000 km from the closest farm, was widely disseminated among the farms, i.e., PCR ribotype 017 (Figure 14). The second pattern corresponded to the remaining common ribotypes (including PCR ribotypes 078 and 027) were seemingly present in clusters. The fact that the first isolate from PCR type 017 reported in humans had been identified 5 years prior to the time of sampling and the location was geographically divided by large bodies of water suggested that terrestrial compared to aerial distribution was less likely. However earlier dissemination and parallel independent evolution of the strains in the two regions is also possible.

**Considerations about aerial dissemination**

Recent reports indicate that aerial dissemination of _C. difficile_ in indoor environments in hospitals is possible. This might suggest that airborne spores could also explain long range dissemination of spores from patients in Manitoba to calves farmed in a vast region of Ontario. Is it that the calves acquired the PCR 017 variant strain from humans, or was it the opposite? A connection between the calves and those patients might be at attribute to global meat markets because meats can harbor cattle derived emerging _C. difficile_ strains (Rodriguez-Palacios et al. 2007; Rodriguez-Palacios et al.
2009). But the inverse connection between the patients and those calves would be more difficult to explain. Airborne dissemination of hospital-associated spores (or from other hypothetical distant source) through large-scale eastbound winds could be a possibility. This is because the major winds blow almost all year round moving particles often in the same direction, as revealed by wild forest fire smoke (NASA 2010). However, that region have had the lowest average values of airborne fine particulate matter pollution (<2.5 μm in diameter) compared to the rest of the world (van Donkelaar et al. 2010). Also, it is important to note that central Canada is very cold (often frozen) during at least six of 12 months, which lowers the values of air pollution, and the carrying potential.

Supporting the difficulty to explain long-range aerial dissemination of spores, a recent study in Europe (with inherent high values of air pollution) showed that indeed spores can disseminate out of intense swine farming facilities with the wind; but the detection decreased to undetectable levels with 30 meters of the facility (Keessen et al. 2010). Aerial dilution of spores as they radiate further apart from farm and spore precipitation or atmospheric elevation could be alternate outcomes that would evade detection. Although aerial dissemination might contribute to spore dissemination, other more effective vehicles of transmission might play a role in disease dynamics. Since invasive wild birds are common infamous inhabitants of farms in North America, especially during winter when we previously documented a higher rate of *C. difficile* in dairy calves, we further considered that birds could be involved in seasonal and continued patterns of spore dissemination.

*Clostridium spp. in wild birds over the past 40 years*
To assess the potential for birds to carry pathogenic *C. difficile* in their digestive tract a systematic review of peer-reviewed literature since 1970 was conducted. In total, over 720 hits were obtained. No reports describing any form of testing for *C. difficile* fecal shedding in any type of wild birds were available. Only one study, published in the 1980 (Borriello *et al.* 1983), tested and identified *C. difficile* in domestic pet ducks (n=2) and geese (n=2) and a parakeet (n=1) and found toxigenic *C. difficile* in two birds.

To explore the potential risk of wild bird exposure to any other pathogenic clostridia we expanded the search to investigate the spatial distribution of reports describing toxicoinfections associated with *C. botulinum* (largely lethal in birds and thought associated with the presence of spores in aquatic sediments and carcasses) and *C. perfringens* enteritis. Extrapolating the obtained data indicated that the risk of ingestion of *C difficile* spores should be widely spread. A large body of spatial evidence exist documenting *C. botulinum* associated with disease of almost exclusively aquatic wild birds, in primarily in coastal lands associated with both sea and fresh water bodies. Increasing description of this problem has emerged from various parts of the world but particularly in Korea. In the USA, cases have been reported sporadically through the past century. Reports of enteritis associated to *C. perfringens* are significantly less, and they are often associated with sporadic cases. A few other species of *Clostridium* spp. have also been reported in association with enteric disease. Collectively, these results indicated that it is very likely to find *C. difficile* spores in the feces of wild birds, especially if they are present in aquatic environments such as rivers and oceans (al Saif and Brazier 1996; Zidaric *et al.* 2010). For most cases of avian botulism, spores are often isolated from
aquatic sediments. We thus tested this hypothesis with a cross sectional observational study.

*Prevalence of C. difficile in wild birds from anthropogenic and forest ecosystems*

To quantify the relative risk of *C. difficile* carriage by wild birds we conducted a cross-sectional study of *C. difficile* shedding in birds representing three distinct ecosystems, one aquatic, and two terrestrials (cattle farms, and urban and rural forests). Because the most common aquatic wild bird identified in North America are mallards and Canada geese, which can be resident in public parks or water bodies used for agricultural purposes, or truly large-range migrants, we decided to test this birds to represent aquatic ecosystems. Among the most common terrestrial invasive bird in North America are European starlings, which are short range migrants and at large residents of farming operations, we decided to include these birds as a representative population that have direct contact with food animals but minimal exposure to aquatic environments. Lastly, to control for exposure to known sources of *C. difficile* spores (i.e., aquatic environments, and food animals), we sampled small terrestrial birds in urban forests and strategic migratory landmarks.

Results from this cross-sectional study, conducted with individual samples from 540 birds, in an area of squared kilometers in Ohio, USA, showed a significantly higher rate of *C. difficile* shedding in waterfowl, followed by the European starlings sampled in the cattle farms. The group of smallest birds was the less likely to yield a positive sample (figure 15). PCR analysis showed that toxigenic strains were more likely to be present in waterfowl. This study confirmed that waterfowl sampled from public places where a
small body of water was present are healthy acrriers of toxigenic *C. difficile*. This finding is of major significance because public parks commonly inhabited by geese or ducks are commonly heavily contaminated with bird excrements that are difficult to avoid. The inadvertent exposure of elderly people (Rodriguez-palacios 2011 unpublished data) who commonly visit or fish in those parks could be another source of *C difficile* for the community; especially, because food and drink consumption is a common practice during leisure times.

**Verification of low shedding prevalence in small migratory birds**

To further validate the results from the previous cross sectional study, and additional historic collection of fecal specimens from small migratory birds were tested. The purpose was to increase the study power to identify a low shedding prevalence. Thus two types of study samples were screened; this is historic and prospective sampling. The historic samples corresponded to fecal samples from birds trapped during migration in forest reserves in the south coast of Lake Erie (a mandatory migration hallmark for many species that reach the lake at this point) in 2005 that had been either frozen or stored on 70% ethanol. The prospectively collected samples corresponded to samples that were subsequently collected during 2008 and 2009 in the preserved marsh area where ~50% of the samples from 2007 had been collected. This time, composite and individual small fresh droppings were collected from perch areas near the research station. Together, testing of those samples (n=310) confirmed that *C. difficile* is not common in small migratory birds, in agreement with date from 400 more recent samples from Eastern Europe (Bandelj *et al.* 2011).
Experimental infection of wild birds with *C. difficile*

Information derived from a cross sectional study only provides information relevant to a single time point but it does not provide dynamical information. To assess the dynamics of fecal shedding in wild birds and therefore of dissemination potential across farms, we documented the dynamics of fecal shedding in European starlings following experimental inoculation with *C. difficile*. Following infection of 28 wild starlings (trapped at cattle farms) with three different strains of *C. difficile* it was possible to document that starlings are also healthy carriers. Under experimental conditions, those birds shed *C. difficile* for up to 5-8 days, but showed no signs of disease. At the end of the study, the score of fat tissue deposits present in the mesentery and associated viscera was similar for all infected groups and controls. Results indicate that infected birds were able to maintain their appetite and body mass in captivity and after infection. Of interest, *C. difficile* was isolated from the liver of some birds indicating translocation from the intestine was possible.

Telemetry study of bird movements and *C. difficile* typing

To determine whether birds could move from farm to farm within those 5 days and disseminate *C. difficile* to cattle in dairy farms we visited farms to trapped birds and concurrently sample cattle. We thus determined the risk of dissemination and transmission of *C. difficile* spores among wild birds and from birds to cattle by combining PCR ribotyping data (a fingerprinting genetic technique) and telemetric-assessed movement data. Starlings captured were tagged and released in strategically selected farms over time, in 2008. Proving the dissemination principle, culture analysis
and PCR ribotyping showed that strains of *C. difficile* collected from birds accompanying tracked birds had identical molecular to identify strains obtained from cattle (figure 16). Further, we documented that during the period of the study that the *C. difficile* positive farms had recent telemetric records indicating that at least one tagged bird (and likely her companions) had moved repetitively between three farms during that time frame (figure 17).

**Discussion**

In summary, we confirmed that *C. difficile* can be carried and disseminated by wild birds that commonly inhabit anthropogenic habitats such as cattle farms and public parks. The most common wild bird associated with *C. difficile* shedding were waterfowls, as we had predicted from the disease ecology from *Clostridium botulinum* in avian species over the past 40 years. In contrast, small migratory and resident forest birds are not natural reservoirs of *C. difficile*. This disproves the somewhat general view that *C. difficile* is a normal inhabitant of the intestinal tract of animals and that spores are widely present in the environment. Lastly, our studies indicate that wild birds that shed *C. difficile* are healthy, even after experimental infection (and stressful housing/handling conditions) with high doses of emerging hypervirulent strains of *C. difficile* relevant for disease in humans.

Understanding the role of birds in the epidemiology and its relationship with anthropogenic landscape ecology is relevant because global warming promotes more residence, and less migration, in wild birds. It is expected that more of these prolific (invasive) bird species be in close contact with an increasing ageing population, who is
already at higher risk for CDI and who frequently visit parks inhabited with wildlife, and often in company of their grandchildren. The inadvertent exposure of humans to unwanted microorganisms via wild bird droppings is not a novel concept, what is to highlight from this study however is that this is the first clostridium organism identified in wild birds that actually causes severe economic and social costs to several health care systems in the world. *Clostridium* spp produce spores which have inherent different disease dynamics, and which resist common disinfectants. Major infection control efforts have not been able to control the ongoing CDI epidemic indicating that it necessary to consider sources of *C difficile* exposure outside of hospitals. Although not all, some public parks inhabited by *C. difficile* carrying wild birds could be a new unrecognized source for spores for communities at risk. The role of transcontinental dissemination of emerging strain between *C. difficile* regional hotspots also needs further consideration.
Acknowledgements

This study was supported by federal funds allocated to the Ohio Agricultural Research and Development Center, The Ohio State University. A.R.P. is a Public Health Preparedness for Infectious Diseases Program Research Fellow. Thanks to M. Kauffman, J. Schrock and P. Schlegel, M. Morash for technical assistance. A.R.P conceived the study, conducted the experiments, and wrote the first manuscript draft. In this thesis, other collaborating individuals have not been acknowledged.
Figure 14. Retrospective spatial analysis of data from a study of *C. difficile* in calves, Canada, 2004 (Rodriguez-Palacios *et al.* 2006). Note the distribution of farms with PCR ribotype 017 (variant without gene for toxin A; tcdA-/tcdB+cdtAB-). PCR ribotype 017 is responsible for severe disease in people in many countries.
Figure 15. Cross sectional study of fecal prevalence of *C. difficile* in wild birds in Ohio, USA, 2007.
Figure 16. PCR ribotyping of isolates from wild waterfowl (geese or ducks), European starlings and dairy cattle sampled within the same period and region. Strains subtypes A-C.
Figure 17. PCR ribotyping and telemetry data indicate that *C. difficile* (Subtype A in this example) can be isolated from several birds (E. starlings) captured over time from farms that had network connectivity through repetitive bird movements.
References


NASA (2010). "NASA Video Shows Global Reach of Pollution from Fires."


Chapter 9 — Moist heat resistance, spore aging, and superdormancy in *Clostridium difficile*

As published in:

**Abstract**

*Clostridium difficile* spores can survive extended heating at 71°C (160°F), a minimum temperature commonly recommended for adequate cooking of meats. To determine the extent to which higher temperatures would be more effective at killing *C. difficile*, we quantified (D-values) the effect of moist heat at 85°C (145°F, for 0 to 30 minutes) on *C. difficile* spores, and compared it to 71 and 63°C. Fresh (1-week-old) and aged (≥20-week-old) *C. difficile* spores from food and food animals were tested in multiple experiments. Heating at 85°C markedly reduced spore recovery in all experiments (5-6 log$_{10}$ with 15 minutes, $P < 0.001$), regardless of spore age. In ground beef, the inhibitory effect of 85°C was also reproducible ($P < 0.001$), but heating at 96°C reduced 6 log$_{10}$ within 1-2 minutes. Mechanistically, optical density and enumeration experiments indicated that 85°C inhibits cell division but not germination, but the effect
was reversible in some spores. Heating at 63°C reduced counts for fresh spores (1 log<sub>10</sub>, 30 minutes, P< 0.04), but increased counts of 20-week-old spores by 30% (15 minutes, P < 0.02) indicating sublethal heat treatment reactivates superdormant spores.

Superdormancy is an increasingly recognized characteristic in Bacillus sp., and it is likely to occur in C. difficile as spores age. The potential for reactivation of (super)dormant spores with sub-lethal temperatures may be a food safety concern, but it has potential diagnostic value. Ensuring food is heated to >85°C would be a simple and important intervention to reduce the risk of inadvertent ingestion of C. difficile spores.

**Introduction**

*Clostridium difficile* is a spore-forming enteric pathogen associated with increasing outbreak frequency and disease severity in humans worldwide (Pepin *et al.* 2005; Warny *et al.* 2005; McFarland 2008). Since 2006, molecular studies have shown that subtypes of *C. difficile* isolates recovered from food animals and retail foods are genetically indistinguishable from human-derived strains (namely, PCR ribotypes 027, 077 and 078) indicating that foods could be a source of inadvertent infection for humans (Rodriguez-Palacios *et al.* 2006; Rodriguez-Palacios *et al.* 2007; Rupnik 2007; Bakri *et al.* 2009; Rodriguez-Palacios *et al.* 2009; Songer *et al.* 2009). Although ingestion of spores may not necessarily result in disease induction, the presence of *C. difficile* spores in a large proportion of raw and ready-to-eat meat products in North America (6-42%) highlights the need to consider this potential risk factor in disease epidemiology (Rodriguez-Palacios *et al.* 2009; Songer *et al.* 2009). Identification of effective measures
to reduce *C. difficile* exposure, especially among higher risk (e.g., elderly, or individuals consuming antimicrobials or antacids) individuals, would further reduce the likelihood of this organism becoming an important foodborne pathogen.

Although interventions to control food contamination with *C. difficile* may be applied both during production and at harvest, little is known about this pathogen in food animals and their environment. Thus, until additional information about the epizootiology and ecology of this pathogen is better understood, post-harvest strategies, such as proper cooking and handling, may be among the most effective measures to control foodborne exposure to *C. difficile* in the short-term. Minimal internal meat cooking temperatures ranging from 63 to 85°C (145 to 185°F) have been promoted by government and industry organizations to control other foodborne pathogens (e.g., *Salmonella* spp., *Escherichia coli* O157) (USDA; FSA 2007; CFIA 2010). For ground meats, a frequent target is to reach 71°C and maintain that temperature for a few seconds to reduce between 6 and 7 \( \log_{10} \) units of most recognized non-spore forming pathogens (USDA 1999; FSA 2007). But *C. difficile* produce spores which are more thermotolerant than vegetative cells. Recent thermo-resistance studies on *C. difficile* where spores were heated at 71°C for two hours (Rodriguez-Palacios *et al.* 2010), and studies with other clostridia (Sanchez-Plata *et al.* 2005), indicate that *C. difficile* spores survive cooking temperatures recommended for ground meats, and if heated spores survive, they could possibly proliferate during the post-cooking chilling phase (Sanchez-Plata *et al.* 2005). Previous thermal studies with *C. difficile* have been single-time point studies and conducted largely with human derived strains (Meisel-Mikolajczyk *et al.* 1995; Wultanska *et al.* 2004). However, effective
comparisons often required multiple time point experiments and the determination of D-values (Juneja et al. 2001). The main objective of the present study was to quantify and compare the inhibitory effect of moist-heat at 63, 71, 85 and 96°C on *C. difficile* spores, from food animal and retail foods, in liquid media and in ground beef. In addition, we tested similar thermal effect on spores aged under different conditions, and preliminarily determined whether the mechanism of thermal inhibition was by impairing spore germination or by impairing cell division after germination.

**Materials and Methods**

**Clostridium difficile strains**

Progressively, three experiment types: *i)* Thermal inhibition in liquid culture media, *ii)* Mechanism of thermal inhibition, and, *iii)* Thermal inhibition on ground beef and gravy were conducted with multiple *C. difficile* strains (*n* = 22; 13 genotypes). Genotypes tested included toxigenic PCR ribotypes 014, 027, 033, 077, and 078, and toxigenic strain ATCC 9569 (Fig. 23). All strains, except the ATCC strain, were isolated from food animals and foods and represent genotypes of public health relevance in North America (Rodriguez-Palacios et al. 2006; Rodriguez-Palacios et al. 2007).

**Spore production and aging**

Prior testing, spores were produced by using the nutrient exhaustion method (Young and Mandelstam 1979). To enhance heterogeneity and thus external validity (Richter et al. 2009), spores were produced in two nutritious broths. In brief, frozen *C. difficile* isolates were revived and purified on tryptic soya agar (Acumedia, Lansing, MI) supplemented with 5% defibrinated sheep blood (Hemostat Laboratories, Dixon, CA)
(blood agar). Single, 72-hour-old colonies were used to inoculate broth supplemented with sodium taurocholate and antibiotics for *C. difficile* as in previous studies (Wilson *et al.* 1982; Levett and Margaritis-Bassoulis 1985; Aspinall and Hutchinson 1992; Rodriguez-Palacios *et al.* 2007). Alternatively for some experiments, spores were produced in antibiotic-free brain heart infusion broth supplemented with 5% yeast extract (Acumedia, Lansing, MI) (BHI broth). Inoculated broths were incubated anaerobically at 37°C for 10 days to allow *C. difficile* to grow and produce spores following nutrient exhaustion of the media. In all cases spores were harvested (7,000 x g for 10 minutes) after 10 days of incubation and re-suspended in plastic transparent tubes using phosphate buffered saline (PBS), and left on a bench top to age at room temperature (23 ± 3°C). If the spores were heat tested within 2 days of production, these spores were considered fresh spores. But to simulate aging conditions likely to occur in food, feces, or the environment, some spores were left at this temperature an additional 20 or 52 weeks: these are referred as aged spores.

**Thermal inhibition in liquid media**

Thermal testing was conducted in PBS containing the spore suspensions using conventional PCR microtubes (210 ± 10 µl) which were submerged in automatic water baths synchronized to the desired temperatures using the average of two external thermometers (EU620-0918, WVN International). Temperatures tested were 63 and 85°C, which encompass the thermal spectrum of widely publicized food safety guidelines for household minimal internal cooking of meat meals (USDA ; FSA 2007; CFIA 2010). Based on pilot trials, timing started 10-15 seconds after the PCR tubes were immersed in
the water bath, when the temperature inside tested tubes reached target temperatures. Then, at specific time intervals (0, 15 and 30 minutes for 63°C; and 0, 3, 6, 9, 12, 15 and 30 minutes for 85°C) the heated aliquots were removed, cooled in icy water and enumerated using the spread plating method on blood agar. Colonies were plated in duplicate and counted after 72 h of anaerobic incubation at 37°C. To confirm that survivor colonies corresponded to the strains tested, PCR ribotyping (Bidet et al. 1999; Rodriguez-Palacios et al. 2006; Rodriguez-Palacios et al. 2007) was used to verify the genotypes of the inoculum and heated recovered organisms were identical. The D-values (decimal reduction times; i.e., time needed to inhibit 90% of spores) were determined using at least two linear equation fits of the inhibition curves excluding the graph shoulders, i.e., the initial and final time points (Juneja et al. 2001). Because precedent enumeration studies of C. difficile in feces indicate that spore recoverability during storage decreases over time (Weese et al. 2000), we also determined if refrigeration (4±2°C) would reduce the recoverability of heated and nonheated spores over time. Thus, replicate aliquots of all non heated strains, and replicate aliquots of heated PCR ribotype 078 strain (85°C) at multiple time points (3, 6, 9, 12 and 15 minutes) were enumerated after 14 and 18 days of refrigeration. Paired analysis and D-values derived from PCR ribotype 078 aliquots were used to assess variability.

**Mechanism of thermal inhibition**

To determine if heat inactivation of C. difficile spores was due to impaired germination, or inhibition of cell division following germination as it has been reported for Bacillus sp. (Ghosh and Setlow 2009; Ghosh et al. 2009), we first used germination
studies to determine if heated spores could germinate in a nutritious broth, and then, followed it with outgrowth studies to determine if these (germinated) spores would then be able to divide to produce visible colonies on blood agar. Normally, during early stages of germination, often within 30 minutes of incubation, most spores release dipicolinic acid and calcium from their core, changing its refractancy and reducing the optical density of the liquid medium that contains them (Paredes-Sabja et al. 2008; Paredes-Sabja et al. 2008). If germination pathways are affected with heat, no germination-related optical density (OD$_{600}$) changes would occur in the incubation broth. If germination occurred normally, and the cell had normal division, the formation of colonies in agar surfaces would also be as expected. However, if heat impaired cell division mechanisms, a reduced number of colonies would then be visible or not at all apparent on the agar.

In brief, the complementary germination-outgrowth experiments were conducted as follows. First, aged spores from C. difficile PCR ribotypes 027, 078 and 077, and toxigenic ATCC 9569 strain were resuspended and heat shocked individually in BHI broth at 63, 71 and 85°C for 15 minutes. To prevent inadvertent spore germination, spores suspensions were maintained in icy water in a cold room set at 4°C. Spore suspensions were seeded in replicates (210 µl; OD$_{600}$ 0.78 ± 0.03) in microtitre plate (Corning Incorporated, Corning, NY) wells to monitor the OD$_{600}$ during subsequent anaerobic incubation in the BHI at 37°C. Although germination of C. difficile spores is not impaired with room air (Jump et al. 2007), the wells were layered with 40 µl of 0.2 µm-filtered pre-reduced heavy mineral oil (Rafii and Park 2005) to minimize potential confounding effects due to air diffusion into the BHI during readings outside of the
anaerobic chamber (<4 minutes). An ELISA reader (Spectra max 340PC, Molecular Devices, Sunnyvale, CA) was used to assess the OD$_{600}$ changes of the spore suspensions after heat shock (time 0) and thereafter at 0.5, 1, 1.5, 2, 3, 4, 5, 6 and 7 hours of anaerobic incubation. Nonheated spores (controls) were concurrently tested to document normal germination OD$_{600}$ changes. The mean OD$_{600}$ time point values were derived from at least 4 replicates. To quantify the ability of heated spores to divide and produce countable colonies (outgrowth study), spores were plated and enumerated on blood agar before and after incubation in BHI. However, because the production of daughter vegetative cells from spores could have been possible during the 7 h of incubation, the BHI broths were treated with 96% ethanol (1:1, v/v, 30 min) (Clabots et al. 1989), washed and resuspended in PBS prior to enumeration to eliminate potential vegetative cells and infer the number of dormant spores capable of normal division.

**Thermal inhibition in ground beef and gravy**

Thermal resistance data from PBS or culture broth studies should not be directly extrapolated to food matrices due to the physicochemical complexity of foods (Juneja et al. 2001). Therefore, we also quantified the extent of spore inhibition using retail ground beef (2-kg packages purchased at a local retailer) and a beef gravy model (5% beef extract, 1.7% soluble starch, 1.5% proteose peptone and 0.5% yeast extract) (Juneja et al. 1998). In these experiments, the strains studied in the abovementioned germination-outgrowth study were tested following a protocol for *E. coli* O157 reported by Juneja *et al.* (Juneja *et al.* 1998) with minor modifications.
In brief, Juneja et al. (Juneja et al. 1998) used a mixture of four strains of *E. coli* (a “cocktail”), experimentally inoculated single random aliquots of ground beef (3 grams), and individually heated those aliquots using Whirlpack™ bags and a water bath. Here, we used the same Whirlpack bag technique, but we assessed the effect of heating for 1:1:1:1 ($10^7$ spores · ml$^{-1}$) mixture of four *C. difficile* genotypes (PCR ribotype 027, 078 and 077, and an ATCC toxigenic strain). We determined the D-values in a complete block design using three food matrices (*i.e.*, a nutritious fat-free gravy model, retail ‘lean’ 7% fat ground beef, and retail 30% fat ground beef) (Juneja et al. 2001) and four heating temperatures (63, 71, 85, and purportedly more effective 96°C). The 96°C was chosen as a sub-boiling water temperature for the city of Wooster where the altitude adjusted boiling temperature is about 98.8°C. These experiments were conducted in duplicate with spore enumeration conducted in triplicate; the spores used here had been aged for 52 weeks at the time of testing. We used Juneja’s gravy model to control and quantify the effect of spore entrapment within the beef, which shrinks with heating. Following experimental inoculation, the 3-gram beef aliquots were heated for 0, 2, 5, 7.5, 10, 15, 20, and 30 minutes, and then mixed with 3 ml of PBS (1:1, v/w), stomached for one minute and enumerated as abovementioned for D-value determination. To control for the natural level of contamination of retail meats with *C. difficile*, we also quantified the spore concentration of noninoculated nonheated aliquots of both ground beef types purchased.
Statistics

Univariate and multivariable analysis of repeated measures of transformed ($\log_{10}$) and normalized enumeration data was conducted as indicated by Davis (Davis 2002), using STATA (College Station, TX, v.10) and Minitab 15 (Upper Saddle River, NJ). Comparisons among D-values were analyzed with generalized linear regression; relevant descriptive statistics and 95% confidence intervals (95% CI) with pooled errors are reported. Percentages of effect were computed as $10^\text{exponentiated to the log}_{10}$ unit of interest.

Results

Thermal inhibition in liquid media

Controlling for strain variability, heating *C. difficile* spores in PBS at 63°C for 30 minutes was not as effective as heating at 85°C in reducing the spore counts (Fig. 18). However, the effect of heating at 63°C depended on whether the spores were fresh or aged. While heating fresh spores at 63°C resulted in a steady significant inhibitory effect overtime (median reduction of 0.37 log$_{10}$, *i.e.*, 57.3% of spores after 30 minutes, 95% CI = 0.13, 0.67 log$_{10}$; GLM, $P = 0.002$), heat treatment of aged spores resulted in a 30% increased recovery compared to baseline counts within 15 minutes of heating (GLM, $P = 0.049$). Heating at 85°C resulted in much faster inhibition for all strains (99.9998% of spores after 15 minutes) with estimated D-value at 85°C ranging from 6.0 to 8.5 minutes, irrespective of spore aging (median reduction of 5.6 log$_{10}$, 95% CI = 4.48, 5.94 log$_{10}$; GLM, $P < 0.0001$; Fig. 18).
When the effect of refrigeration on the recovery of nonheated aged spores was assessed, storage significantly reduced the spore counts by day 18 (median reduction of 0.28 log$_{10}$, i.e., 42.5% of spores, 95% CI = 0.045, 0.915 log$_{10}$; Wilcoxon signed, $P = 0.014$; Fig. 19A). At the strain level, two isolates had a 2-log$_{10}$ reduction by days 14 and 18. Complementarily, repeated quantification of heated aliquots of PCR-ribotype 078 at 85°C showed that refrigeration did not affect spore enumeration and the D-values therein derived (Fig. 19B).

**Heat treatment at 85°C affects cell division but not spore germination**

Assessment of heat-shocked spores (15 minutes) in BHI during 7 hours of incubation demonstrated that the OD$_{600}$ germination curves of all heated strains were normal as they were similar to the germination curves of nonheated controls (GLM, $P > 0.1$; Fig. 20A). However, when we assessed the effect of heat shock on cell division, determined as the number of countable colonies on agar after normal germination (curves), multivariable analysis indicated that 15 minutes of heat shock at 85°C was the only temperature that significantly reduced the number of countable colonies. On average, 85°C induced a 4.2 log$_{10}$ reduction (99.994% of spores) compared to nonheated controls (95% CI= 3.9, 4.5; GLM, $P < 0.0001$; Fig. 20B).

**Thermal inhibition at 85°C can revert during incubation in broth**

Paired analysis (before vs. after 7 hours of incubation in BHI) of ethanol-resistant spores showed that 85°C resulted in complete inhibition of most isolates immediately after 15 minutes of heat shock (Fig 21A, panel 85°C, 0h). However, when those heat shocked spores (85°C) were left incubating in BHI, the number of countable colonies
after 7 hours of incubation increased significantly (Fig. 21A, panel 85°C, 7h) indicating that the thermal inhibitory effect observed immediately after heat shock can be reverted during subsequent incubation. In contrast, heat shock with 63 and 71°C increased the rate of spore recovery compared to nonheated controls immediately after heat shock. These two temperatures also enhanced the rate of spore germination (assessed as the number of ethanol susceptible cells) during the subsequent 7 h of incubation in BHI. The highest rate of germination was observed with 63°C (0.4 log<sub>10</sub> increase, 95% CI = 0.1, 0.9; GLM, P < 0.01; Fig. 21B).

**Thermal inhibition in ground beef and gravy**

Quantification of *C. difficile* contamination in the ground beef packages indicated that natural contamination was present in both products (different processing plants), varied within the packages, and ranged from 0 to 0.5-3.3 log<sub>10</sub> · gr<sup>-1</sup>. Mathematically, the highest level of natural contamination corresponded to less than 0.02% of the spore concentration achieved with experimental contamination and was deemed minimal to bias the estimation of the D-values. Multivariable analysis of D-value data indicated that 85 and 96°C have the highest inhibitory effect against *C. difficile* experimentally inoculated in beef matrices (GLM, P< 0.001; Fig 22). In contrast, 63 and 71°C had comparable and a minimal effect in inhibiting the *C. difficile* mixture (see D-values in supplementary table S2). Although significant differences were observed among the matrices tested (which varied in up to 30% in fat content), the direction of such effects varied with temperature indicating matrix-temperature interactions (Fig 22).
Discussion

This study quantified the inhibitory effect of moist heat at 63, 71, 85 and 96°C on *C. difficile* spores in liquid media and in ground beef. Our quantitative report also evaluated for the first time fresh and aged spores, the latter which are likely to occur naturally in the environment and within contaminated foods. To contribute to food safety validation protocols, temperatures tested included widely publicized food safety guidelines of minimal internal cooking temperatures for meat meals (USDA; FSA 2007; CFIA 2010). Briefly, publicized temperatures range from 63°C (for 30-minute batch milk pasteurization, and for preparation of fish, and steaks/roasts of beef, veal and lamb as “medium-rare”) to 85°C for whole poultry and turkey meals. However, cooking at 71°C is often cited in the “safe handling instructions” of most retail meat labels, and is indicated for “well-done” purposes of beef, veal and lamb steaks, for ground beef, and for pork products (USDA; CIPHI 2003; Silvestre *et al.* 2008; CFIA 2010; FDA 2010). Of concern, recent data indicate that 71°C results only in a 2 log_{10} reduction after heating *C. difficile* spores for 2 hours (Rodriguez-Palacios *et al.* 2010).

Under the conditions of our experiments, moist heat treatment at 85°C, irrespective of spore aging and test media, markedly inhibited (up to 6 log_{10}) spores from most *C. difficile* strains within 15 minutes of treatment. Considering that the concentration of *C. difficile* spores in naturally contaminated retail raw meat products may be of less than 3.3 log_{10} · g^{-1} of product (Weese *et al.* 2009), cooking at least 85°C could markedly reduce human exposure to *C. difficile*. Here we found that heating
aliquots containing less than 4 log_{10} at 85°C in liquid media always yielded no cultivable spores after 15 minutes of heating. For other foodborne pathogens such as *Salmonella* spp., it is known that there is a greater risk of pathogen persistence in the final ready-to-eat meat products after heat treatment if the initial pathogen load in the raw products is high (USDA 1999). However, it is unknown whether *C. difficile* proliferates in retail meals and if bacterial proliferation reaches more than 4 log_{10} g^{-1}. Likewise, it is uncertain to what extent spore aging within food matrices parallels the aging process induced in culture broths in our study and the subsequent magnitude of thermal tolerance.

To date most studies assessing *C. difficile* germination and sporulation have used freshly prepared spores leaving aged spores largely uncharacterized. As aging of *C. difficile* spores could occur in the environment or in foods, studying the effect of heat is critical for understanding spore survivability patterns. Here, we showed that 85°C was markedly inhibitory for aged and fresh spores; however, of food safety relevance was the finding that 63 and 71°C enhanced germination in aged spores. The food safety concern resides in that heat treatment is a well known germination factor that triggers the synchronic activation of most spore-forming organisms associated with food, and that spore germination during the subsequent chilling period is possible in other clostridia (Sanchez-Plata *et al.* 2005). Furthermore, sublethal heat shock of spores in other clostridia is known to significantly enhance the thermal tolerance of surviving spores (Juneja *et al.* 2003).

Storage under refrigeration resulted in lower spore counts over time. Although there are no comparable studies assessing aging of *C. difficile* in pure suspensions under
refrigeration, our finding is comparable to that of a study assessing the *C. difficile* recoverability in inoculated feces (Weese *et al.* 2000). In that study, the rate of isolation of *C. difficile* decreased over time (Weese *et al.* 2000). Although, another similar study stated there was little effect (Freeman and Wilcox 2003), our findings indicate that factors associated with decreased recovery (*e.g.*, super dormancy) might be enhanced during storage. Superdormancy is a recently described characteristic in *Bacillus* species (Ghosh and Setlow 2009), and a rising concern for the food industry as superdormant *Bacillus* spores have higher moist-heat resistance, lower water core content than regular spores, and can recover from heat-stress injuries during refrigeration (Ghosh *et al.* 2009; Ghosh and Setlow 2010; Wei *et al.* 2010). Understanding the properties of superdormancy in *Bacillus spp.* could also indicate ways to reduce the potential impact of *C. difficile* on food safety.

To date, some epidemiological studies assessing *C. difficile* contamination of foods have used thermal treatment (80°C for 10 minutes) to reduce contaminants and facilitate spore recovery (Songer *et al.* 2009). However, the observed inhibition with 85°C in our study indicates that culture-based studies based on thermal methods for spore selection that use between 71 and 85°C might introduce selection bias, favoring more thermo-resistant spores. Hence, it is advisable to use temperatures below 71°C, being 63°C a pasteurization temperature potentially beneficial for spore selection in diagnostic settings.

Thermal inhibition at 85°C is a promising preventive measure since cooking guidelines at this temperature are readily available for preparation of whole turkey meals,
and could be easily adopted. However, this study indicates that more than a few seconds are required to lower the number of \( C \) difficile spores to the desired \( 5-6 \log_{10} \) reduction. It is expected that extended cooking at 85°C could maintain the nutritional quality of the cooked foods, but it is uncertain how extended heating would impact the nutritional and palatability characteristics of non-turkey meals. A balance between food safety risks, nutritional value and food quality needs to be taken into consideration. The marked inhibitory effect of 96°C here documented, further highlight the potential benefits of revising cooking time-temperature recommendations, at least for individuals at risk. This study generated the first D-values for \( C. \) difficile in a food matrix. These data can be used for policy and management decision making relevant to food safety and infection control strategies to disinfect hospital reusable materials (Alfa et al. 2008).

Comparing our estimated D-values at 85°C (<8 minutes) to those reported for other clostridia, it becomes evident that \( C. \) difficile appears to be more susceptible to heat. For instance, the D-values at 100°C for \( C. \) perfringens, the most common spore-forming foodborne pathogen in the USA (Scallan et al. 2011), in beef gravy varies from 16 to 21 minutes (Juneja et al. 2003), and in distilled water the D-value at 85°C was 55 minutes (Heredia et al. 1997). Thus, cooking guidelines for industrial purposes against \( C. \) perfringens (Crouch and Golden 2005) may also be a good basis for the refinement of household guidelines against \( C. \) difficile.

In summary, we quantified the inhibitory effect of heat on \( C. \) difficile spores, estimated D-values (63-96°C) with confidence intervals for ground beef and gravy, and mechanistically determined that the inhibitory effect of heat shock at 85°C, the highest
cooking recommended internal minimal temperature, is due to inhibition of cell division but not germination, and that it can be reversible. Spore germination and recoverability was enhanced with 63°C, especially in aged spores. However, with concern, but as expected, we documented the sub-lethal effect of 71°C and its moderate effect to enhance germination. Thermoresistance experimentation also indicated that spore superdormancy, an increasingly recognized characteristic in *Bacillus* sp., is likely to occur in *C. difficile* as spores age. Ensuring food is heated to more than 85°C would be a simple and important intervention to reduce the risk of inadvertent ingestion of *C. difficile* spores.

**Acknowledgements**

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Figure 18. Effect of moist heat in phosphate buffered saline at 63 and 85°C on viability of fresh (1-week-old) and aged (20-week-old) spores of *C. difficile*. Open symbols represent isolate means; grey squares with interval bars represent 95% CI of the means.
Figure 19. Effect of storage on cultivability of aged *C. difficile* spores during 18 days of refrigeration at 4°C.

A) Nonheated spores illustrate reduced cultivability over time (12 strains; triangles and interval bars represent means and 95% CI). B) Heated spores at 85°C indicate reproducible estimation and cultivability (PCR ribotype 078 strain; Regression fits shown for 0, 3, 14 and 18 days).
Germination and outgrowth studies on *C. difficile* to determine if 15 minutes of heat shock impairs spore germination or the subsequent cell division. A) Germination study. Optical density (OD$_{600}$) reduction of heavily inoculated BHI spore suspensions, incubated at 37°C for 7h, is similar for heated and nonheated spores indicating normal germination of heated spores. Only PCR ribotype 078 and the ATCC strain are shown; means ± SD.

B) Outgrowth study. Enumeration of ethanol resistant cells from aliquots in Figure 3A after 7h of incubation. Countable colonies on blood agar indicate adequate cell division. Reduced counts after heat shock with 85°C (and normal germination in Fig 3A) indicate impaired cell division. Dots represent replica averages; means±95% CI (solid grey bars).
Figure 21. Effect of heat shock on immediate cultivability and subsequent germination of *C. difficile* spores determined by enumeration of ethanol-resistant cells before and after 7h of incubation in BHI.

A) Line plots represent paired enumeration of heat-shocked and nonheated individual spore replicas; four genotypes. Linear equations and extended dashed lines represent average effects (95% CI of means). The highest and steepest dashed line (63°C) indicates immediate enhanced cultivability and subsequently increased spore germination. 85°C shows immediate inhibition and subsequent increased cultivability.

B) Spore germination relative to baseline. Normalized paired differences (before and after BHI incubation). Heat shock with 63 and 71°C enhanced germination in spores that otherwise would have remained dormant after adding 7h of incubation in BHI and 72 h in blood agar at 37°C (here referred to as superdormant spores). Dots represent average difference per replica; means±95% CI (See Table 10).
Figure 22. Minutes of heat needed to inhibit a mixture of aged *C. difficile* spores heated in three food matrices.

Dots indicate individual D-value estimates; means±95% CI. Identical superscripts connect variables with nonsignificant differences (*i.e.*, GLM, *P* > 0.05), whereas different superscripts indicate significance. See also supplementary table S2 for coefficient estimates.
Supplementary material

Figure 23. Genetic diversity of representative isolates tested.

All strains were toxigenic except PCR ribotype M26 which lacks pathogenicity locus (Rodriguez-Palacios et al. 2006; Rodriguez-Palacios et al. 2007).
Table 9. Coefficient estimates of heat-shock enhancement of spore germination after 7h of incubation in nutritious BHI broth

<table>
<thead>
<tr>
<th>Heat-shock treatment</th>
<th>Median difference $\log_{10}$ CFU · ml$^{-1}$</th>
<th>95% CI of median $\log_{10}$ CFU · ml$^{-1}$</th>
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<td>1.04</td>
<td>0.84, 1.28</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>71°C</td>
<td>0.52</td>
<td>0.13, 0.80</td>
<td>0.021*</td>
</tr>
<tr>
<td>85°C</td>
<td>-0.12</td>
<td>-0.62, 0.00</td>
<td>0.272</td>
</tr>
<tr>
<td>Pair-wise differences</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>63 minus 4°C</td>
<td>1.12</td>
<td>0.88, 1.34</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>71 minus 4°C</td>
<td>0.49</td>
<td>0.17, 0.81</td>
<td>0.027*</td>
</tr>
<tr>
<td>85 minus 4°C</td>
<td>-0.17</td>
<td>-0.51, 0.18</td>
<td>1.000</td>
</tr>
<tr>
<td>71 minus 63°C</td>
<td>-0.63</td>
<td>-0.99, -0.26</td>
<td>0.005*</td>
</tr>
<tr>
<td>85 minus 63°C</td>
<td>-1.29</td>
<td>-1.68, -0.89</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>85 minus 71°C</td>
<td>-0.65</td>
<td>-1.14, -0.18</td>
<td>0.004*</td>
</tr>
</tbody>
</table>

* Null hypothesis: median effect difference equals zero; significance held at $P < 0.05$

derived from one-sample Signed Wilcoxon Rank and pair-wise Bonferroni significance tests. Univariate analysis compares value to referent control, i.e., 4°C.
Table 10. Minutes of heat needed to inhibit 90% of spores (D-values) estimated for a 1:1:1:1 mixture of *C. difficile* of PCR ribotypes 027, 078, 077 and ATCC 9689 strains in three food matrices

<table>
<thead>
<tr>
<th>D-value means from two experiments ± SD (95% confidence intervals)</th>
<th>63°C (145°F)</th>
<th>71°C (160°F)</th>
<th>85°C (185°F)</th>
<th>96°C (205°F)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>D-value means</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gravy, 0% fat</td>
<td>55.9±21.8 (21.3 to 90.6)</td>
<td>56.8±30.5 (18.9 to 94.7)</td>
<td>2.51±0.43 (1.83 to 3.2)</td>
<td>0.59±0.32 (0.07 to 1.1)</td>
</tr>
<tr>
<td>Lean ground beef, 3% fat</td>
<td>99.4±62.3 (22 to 176)</td>
<td>71.2±38.7 (9.6 to 132)</td>
<td>3.0±1.1 (1.83 to 4.17)</td>
<td>0.62±0.29 (0.15 to 1.08)</td>
</tr>
<tr>
<td>Ground beef, 30% fat</td>
<td>45.8±10.8 (28.7 to 63)</td>
<td>46.8±10.9 (33.1 to 60.4)</td>
<td>3.27±1.02 (2.2 to 4.33)</td>
<td>1.19±0.67 (0.49 to 1.89)</td>
</tr>
<tr>
<td><strong>Cumulative D-value</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean</td>
<td>69.6±45.4 (42.1 to 96.9)</td>
<td>57.3±27.8 (41.3 to 73.4)</td>
<td>2.97±0.94 (2.48 to 3.48)</td>
<td>0.85±0.55 (0.53 to 1.17)</td>
</tr>
<tr>
<td>95% CI</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Linear function, statistics</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Slope</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean</td>
<td>-0.018515</td>
<td>-0.025223</td>
<td>-0.36619</td>
<td>-1.6678</td>
</tr>
<tr>
<td>S.D.</td>
<td>0.008362</td>
<td>0.018412</td>
<td>0.10826</td>
<td>1.0015</td>
</tr>
<tr>
<td>95% CI, lower limit</td>
<td>-0.023568</td>
<td>-0.036349</td>
<td>-0.42388</td>
<td>-2.2460</td>
</tr>
<tr>
<td>95% CI, upper limit</td>
<td>-0.013462</td>
<td>-0.014097</td>
<td>-0.30850</td>
<td>-1.0896</td>
</tr>
<tr>
<td><strong>r², mean ± SD</strong></td>
<td>0.65±0.23</td>
<td>0.67±0.28</td>
<td>0.86±0.14</td>
<td>0.89±0.15</td>
</tr>
<tr>
<td>n= estimated D-values</td>
<td>13</td>
<td>13</td>
<td>16</td>
<td>14</td>
</tr>
</tbody>
</table>

*r²* is a measure of goodness-of-fit of the linear regressions.


Jump, R. L. P., M. J. Pultz and C. J. Donskey (2007). "Vegetative Clostridium difficile Survives in Room Air on Moist Surfaces and in Gastric Contents with Reduced Acidity: a Potential Mechanism To Explain the Association between Proton Pump


Chapter 10 — Cooking and the Selection of Emerging Pathogens: Clostridium difficile

(Submitted for Publication)

Abstract

Despite our growing understanding of the role of cooking on human evolution, little is known about the role of that practice on the selection (and evolution) of emerging pathogens. Using prototype strains of internationally known enteric pathogen Clostridium difficile, we experimentally demonstrated that cooking at recommended minimum temperatures do not modify the population structure of the pathogen in foods. However, higher temperatures, likely used in routine cooking, strikingly favored the selection of newly emerging hypervirulent C. difficile strains (namely PCR ribotype 078). Cooking, a unique trait of humans, may certainly contribute to the selection (and possibly evolution) of emerging enteric pathogens.

Introduction

Cooking favored the evolution of hominids after the invention of fire by promoting social transformations, maximizing food digestibility, and possibly by reducing energetic expenditures associated with foodborne infections (Carmody and
Despite our growing understanding of the role of cooking on human evolution, little is known about the role of that practice on the selection (and evolution) of emerging pathogens. Here, we experimentally demonstrate that cooking favors the selection of spores of a variant genotype of spore-forming enteric pathogen *Clostridium difficile* (PCR ribotype 078) which emerged in the mid 2000s as a hypervirulent pathogen of humans and animals, and a predominant food contaminant, internationally (Table 11).

Since the 1970s, *C. difficile*-associated diseases have been linked to the ingestion and germination of pathogenic spores with toxin production in the gut. Because spores of emerging hypervirulent *C. difficile* (PCR ribotypes 027 and 078) have been isolated from foods since 2006 (Rodriguez-Palacios *et al.* 2007; Songer *et al.* 2009), there are concerns about foodborne transmission. Although cooking at minimum recommended temperatures controls most foodborne pathogens (60-74°C; Table 12), it is unknown if (and how) cooking modifies the ecology of *C. difficile* in fully and partially heated foods. The resistance of *C. difficile* to recommended cooking temperatures, the presence of emerging *C. difficile* strains in ready-to-eat meats, the ability of spores to recover from heat-injuries and increase germination when heated, and constant outbreaks of foodborne diseases linked to partially cooked foods (Songer *et al.* 2009; Rodriguez-Palacios *et al.* 2010; Alfano-Sobsey *et al.* 2011; Rodriguez-Palacios and LeJeune 2011), led us to hypothesize that cooking could systematically favor the selection of emerging enteric pathogens.
Materials and Methods

We tested our hypothesis by heating and comparing the survival probabilities of four relevant prototypes of *C. difficile*: Emerging PCR ribotypes 027 and 078 (hypervirulent toxinotypes III and V), and PCR ribotype 077 and strain ATCC-9689 (regular toxinotypes 0). Using one-year-old spores, strains were inoculated into retail ground beef, heat tested individually, and then collectively, at minimum and exceeding recommended household cooking temperatures.

To quantify the resistance of *C. difficile* to heat, spores of each strain were first heated individually at 85°C, for 0-30 minutes. Surviving spores were then quantified on blood agar. Multivariable generalized linear analysis indicated that 85°C strongly inhibited *C. difficile* within 10 minutes (~5 log reduction, *P* < 0.001), but the effect significantly varied between strains. Strain ATCC-9689 was the most susceptible (reduction of 10 million to 1-10 spores/gram, within 10 minutes), and PCR ribotype 027 the most resistant, with lower but comparable inhibition for the other ribotypes (Fig. 25).

To determine how cooking influences the ecology of *C. difficile* in fully and partially heated foods, we then used four temperatures (63-96°C) and two types of retail ground beef to quantify the relative individual survival probability for a mixture of three *C. difficile* strains (resistant:intermediate:susceptible, 1:1:1 ratio). In duplicated randomized complete block design, beef aliquots inoculated with the spores were heated at various time-temperatures. Surviving spores, which grew as colonies on agar were systematic and randomly selected, and PCR ribotyped to quantify surviving subtypes. We
also quantified naturally occurring *C. difficile* PCR ribotypes in the retail beef used for experiments, with and without treatment at 63°C (Fig. 26).

**Results**

Microbial analysis confirmed natural contamination of the beef used with various *C. difficile* genotypes (including ribotype-078), which survived 63°C for 30 minutes (pasteurization). Subtyping analysis of beef experimentally contaminated with the spore mixture confirmed that neither cooking at 63 nor 71°C affected the population structure of *C. difficile*. However, heating at 85 and 96°C had a major selective effect, depending on the length of cooking; favoring the selection of PCR ribotype 078 as cooking times increased (Fig. 24A). Multivariable multinomial logistic regression showed the survival probability for PCR ribotype 078 increased as the lethality of cooking increased (Fig. 24B).

**Discussion**

In the past, cooking with boiling water (90-100°C) or direct fire (>100°C) was the norm. But in modern societies, there is increasing dependence on quick-to-cook foods. Protecting public health, food safety guidelines recommend minimum cooking temperatures between 63-74°C (Rodriguez-Palacios and LeJeune 2011). Here we showed that those recommendations did not affect the population structure of *C. difficile*, but importantly, that cooking at higher temperatures (likely in routine cooking) favored the selection of emerging strains. Is this why hypervirulent strains predominate in ready-to-
eat meats? (Songer et al. 2009). The extent to which our findings apply to other strains/pathogens is uncertain, but the ability of enteric pathogens to survive cooking is noteworthy (6).

We might be selecting emerging enteric pathogens without noticing. As we lower the cooking temperatures used by our ancestors, to attain desirable food texture and palatability, we may ingest more heat-injured microorganisms. Does heat-injury promote microbial mutagenesis or transformations within the gut, or foods? Our results, and the remarkable association between heat-shock proteins and the potentiation of rapid evolution of pathogenic traits in fungi (Cowen and Lindquist 2005), indicate that heat-driven evolution might occur with cooking (and possibly disinfection), and be clinically relevant for humans and animals consuming heated foods.

Acknowledgements

Supported by federal funds allocated to the Ohio Agricultural Research and Development Center, The Ohio State University. A.R.P. is a Public Health Preparedness for Infectious Diseases Program Research Fellow. Thanks to M. Kauffman, J. Schrock and P. Schlegel for technical assistance. A.R.P conceived the study; both authors analyzed the data and wrote the manuscript. No conflict of interests.
Figure 24. Cooking favored the selection of *C. difficile* PCR ribotype 078 (and 027 to a lesser extent) as time-temperatures increased.

(A) *C. difficile* single-colony PCR ribotyping summary data (PCR-legend and associated pie charts; N=152 independent-random beef aliquots); and reduction of background beef flora with pasteurization temperature 63°C (dashed lines used as referent in both plots, mean ± SD, 3-5 replicas/point). (B) Predicted survival probability derived from multivariable multinomial logistic regression. Distinct superscripts on PCR types indicate differences (adjusted *P* < 0.05).
Figure 25. Experiment 1 – Individual thermal testing of prototype *C. difficile* PCR ribotypes in lean ground beef at the highest reported minimal cooking temperature recommended for poultry meals in Canada.

Asterisks indicate significantly different effect estimates (Generalized linear model, *P* < 0.01)
Figure 26. PCR ribotyping (Bidet et al. 1999) was used to differentiate between the inoculated, the naturally present, and the heat-survivor *C. difficile* strains recovered on blood agar.

(A) The prototype strains tested in this study were easily distinguishable by PCR ribotyping. (B). Two distinguishable *C. difficile* PCR ribotypes, not previously identified in our laboratory, were naturally present in the retail beef used in this study. They were detected in two single beef aliquots after 5 and 30 minutes of heating at 63°C. ‘ref.’ indicates prototype inoculated strain.
Table 11. Evidence that *C. difficile* PCR ribotype 078 is increasing internationally.

<table>
<thead>
<tr>
<th>Country</th>
<th>Comment or actual quotes</th>
<th>Year (ref.)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Relative occurrence in humans with CDI</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Germany</td>
<td>Third most frequent PCR ribotype (4.7%) in humans with CDI after type 001 (70%) and 027 (4.8%).</td>
<td>(Reil et al. 2011)</td>
</tr>
<tr>
<td>Scotland</td>
<td>November 2007-December 2009. Three PCR ribotypes predominated amongst the Scottish isolates of <em>C. difficile</em>: ribotype 106 (29.4%), ribotype 001 (22%) and ribotype 027 (12.6%) followed by the less prevalent ribotypes including 002, 015, 014, 078, 005, 023 and 020.</td>
<td>(Wiuff et al. 2011)</td>
</tr>
<tr>
<td>Netherlands</td>
<td>34-month prospective case-control study: hospitalized CDI patients, patients without diarrhea, and patients with non-CDI diarrhea. The incidence of CDI was 17.6 per 10,000 hospital admissions. PCR ribotype 078 was the second most frequent type (12.7%).</td>
<td>(Hensgens et al. 2011)</td>
</tr>
<tr>
<td>USA</td>
<td>PCR ribotype 078 (Toxinotype V) was the third most prevalent type (10%) in community-associated <em>C. difficile</em> disease.</td>
<td>(Limbago et al. 2009)</td>
</tr>
<tr>
<td><strong>Increased incidence in people</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Europe</td>
<td>The incidence of <em>C. difficile</em> infection in hospitals was 4·1 per 10,000 patient-days per hospital (range 0·0-36·3). Isolates available from 389 cases corresponded to 65 different PCR ribotypes. Ribotype 078 was the third most frequent (8%) after ribotypes 014/020 (16%) and 001 (9%).</td>
<td>(Bauer et al. 2011)</td>
</tr>
<tr>
<td>Netherlands</td>
<td>Between 2005 and 2009 (2,788 samples): PCR ribotype 027 decreased by 2006. An increase of CDI detected in patients with diarrhea in the community coincided with emergent new ribotypes. Type 078 increased (9.1%) since the end of 2006, and became the third most frequent type in the Netherlands, after ribotypes 001 (27.5%) and 014 (9.3%).</td>
<td>(Hensgens et al. 2009)</td>
</tr>
<tr>
<td>Canada</td>
<td>Data Canadian Nosocomial Infections Surveillance Program CDI surveillance 2004-2008. The incidence of CDI associated with ribotype 078 increased from 0.5% (2004) to 1.6% (2008).</td>
<td>(Mulvey et al. 2010)</td>
</tr>
<tr>
<td>France</td>
<td>Data National Reference Laboratory. PCR ribotype 078 significantly increased in France from 3.25% (8/246) in July to December 2006 to 11.1% (16/144) in July to December 2007.</td>
<td>(Rupnik et al. 2008)</td>
</tr>
<tr>
<td><strong>Recent ‘first reports’ of PCR ribotype 078</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>New Zealand</td>
<td>1/97 (1%) patients with CDI. First PCR ribotype 078 identified in the country.</td>
<td>(Roberts et al. 2011)</td>
</tr>
<tr>
<td>Ireland</td>
<td>1/133 (0.75%) patients with CDI. First clindamycin-resistant PCR ribotype 078.</td>
<td>(Solomon et al. 2011)</td>
</tr>
<tr>
<td>Ireland</td>
<td>First report of PCR ribotype 078 (clindamycin susceptible).</td>
<td>(Burns et al. 2010)</td>
</tr>
<tr>
<td>China</td>
<td>First report. 1/110 (0.91%) isolates from humans with CDI were PCR ribotype 078.</td>
<td>(Huang et al. 2010)</td>
</tr>
<tr>
<td><strong>Relative occurrence in food animals</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Canada</td>
<td>Most common ribotype in commercial young dairy calves in farms (25%), and in veal calves (53%) in a commercial feedlot cattle operation.</td>
<td>(Costa et al. 2011)</td>
</tr>
<tr>
<td>France</td>
<td>CDI rates have increased in the community and new genotypes (e.g. PCR ribotype 078) are emerging in both humans and animals- Update.</td>
<td>(Barbut et al. 2011)</td>
</tr>
<tr>
<td>USA</td>
<td>Predominant type in calves, piglets, and present in finishing cattle and farmed deer.</td>
<td>(Rodriguez-Palacios et al. 2011)</td>
</tr>
<tr>
<td><strong>Relative occurrence in foods</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Canada</td>
<td>All isolates from chicken meats contaminated with <em>C. difficile</em> were PCR ribotype 078.</td>
<td>(Weese et al. 2010)</td>
</tr>
<tr>
<td>Canada</td>
<td>3/5 isolates from retail vegetables were PCR ribotype 078.</td>
<td>(Metcalfe et al. 2010)</td>
</tr>
<tr>
<td>USA</td>
<td>Ribotype 078 was predominant (75%) in ready-to-eat meats contaminated.</td>
<td>(Songer et al. 2009)</td>
</tr>
</tbody>
</table>
Table 12. Recommended safe minimum cooking temperatures and holding times to reduce exposure to foodborne pathogens.

<table>
<thead>
<tr>
<th>Food category</th>
<th>Example Items</th>
<th>Temperature</th>
<th>Holding time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leftovers &amp; Casseroles</td>
<td></td>
<td>74°C (165°F)</td>
<td>None</td>
</tr>
<tr>
<td>Poultry</td>
<td>Chicken, Turkey, Duck, Goose; whole or parts</td>
<td>74°C (165°F)</td>
<td>None</td>
</tr>
<tr>
<td>Ground Meats</td>
<td>Turkey, Chicken</td>
<td>74°C (165°F)</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>Beef, Pork, Veal, Lamb</td>
<td>71°C (160°F)</td>
<td>None</td>
</tr>
<tr>
<td>Fresh Beef, Veal, Lamb</td>
<td>Steaks, roasts, chops</td>
<td>63°C (145°F)</td>
<td>3 minutes</td>
</tr>
<tr>
<td>Seafood</td>
<td>Fin Fish, Shrimp, lobster, and crabs, Clams, oysters, and mussels; Scallops</td>
<td>63°C (145°F) or cook until flesh is opaque and separates easily with a fork.</td>
<td>None</td>
</tr>
<tr>
<td>Pork and Ham</td>
<td>Fresh pork /raw ham</td>
<td>63°C (145°F)</td>
<td>3 minutes</td>
</tr>
<tr>
<td></td>
<td>Precooked ham (to reheat)</td>
<td>60°C (140°F)</td>
<td>None</td>
</tr>
</tbody>
</table>

Adapted from chart publicly available at [www.foodsafety.gov/keep/charts/mintemp.html](http://www.foodsafety.gov/keep/charts/mintemp.html).

In Canada, recommendations state that whole (and stuffed) poultry should be cooked to at least 85°C (185°F), available at [http://www.hc-sc.gc.ca/fn-an/securit/ill-intox/info/poultry-volaille-questions-eng.php](http://www.hc-sc.gc.ca/fn-an/securit/ill-intox/info/poultry-volaille-questions-eng.php). Holding time refers to the number of minutes needed at the recommended temperature to inhibit at least 6 log_{10} units of major foodborne pathogens (e.g., *Salmonella* spp.). Current reports indicate that some seafood will need to be heated to 90°C to inactivate foodborne human viruses (6).
Supplementary Materials and Methods

Rationale for temperatures tested and hypothesis

In order to achieve 5-6 Log_{10} reductions in *C. difficile* spore counts (to meet food safety standards), we recently reported (Rodriguez-Palacios and LeJeune 2011) the need to heat foods at temperatures higher than 71°C/160°F—the temperature typically recommended as safe for cooking most meals (Table S2) (USDA). Heat treatment of meat experimentally inoculated with *C. difficile* indicated that heating at 96°C/205°F (sub-boiling temperature) was effective against 99.9% of spores within 1 minute (95% CI of minutes to destroy 90% of spores [D-value_{96}] = 0.8, 1.4) (Rodriguez-Palacios and LeJeune 2011). However, many spores survived several minutes of heating at 96°C (Rodriguez-Palacios and LeJeune 2011). The presence of thermoresistant spores after cooking indicated that heat applied to foods could destroy some *C. difficile* strains while favoring the selection of others. Here we tested that hypothesis.

*Clostridium difficile* strains

We experimentally quantified the survival probability to moist-heat cooking of toxigenic *C. difficile* PCR ribotypes 078/toxinotype-V, 027/toxinotype-III, PCR ribotype 077/toxinotype-0 of bovine origin isolated from calves in dairy farms of Canada in 2004 (Rodriguez-Palacios *et al.* 2006) and prototypic human-origin strain ATCC-9569/toxinotype-0 isolated from a human affected with *C. difficile* infection in the 1990s. Since spores likely persist in the environment before coming into contact with foods,
spores from all strains tested were aged on phosphate buffered saline for 52 weeks as previously described (Rodriguez-Palacios and LeJeune 2011).

**Experiment 1 – Individual thermal testing**

For individual testing, duplicate samples of retail beef (6.6 g, prepared from a package of 97% lean ground beef) were individually inoculated with PBS suspensions containing single *C. difficile* strains (6.9 ± 0.6 Log_{10} CFU/0.1 g) and manually mixed. Inoculated samples were then split into three pairs of smaller aliquots (1.1 g) for duplicate testing and 3 time points. These aliquots, placed inside 4-ounce Whirlpack bags, were pressed to thin layers (∼64 cm², without air) and heated in a water bath at 85°C/185°F (highest minimal cooking temperature recommended for difficult-to-cook meats) following a protocol used for *Escherichia coli* O157:H7 reported by Juneja et al. (USDA) with slight modifications for *C. difficile* (Rodriguez-Palacios and LeJeune 2011). After 10, 20 and 30 minutes of heating, survivor spores were enumerated from two 100-µL aliquots of beef extract (leakage from heated beef) per aliquot, using the spread plate method on tryptic soy agar supplemented with 5% defibrinated sheep blood.

**Experiment 2 – Collective thermal testing of *C. difficile* mixture**

With observed thermo-resistance differences between the strains, another experiment was conducted in duplicate to determine the concurrent relative probability of survival for the three *C. difficile* strains in a spore mixture (1:1:1 ratio). This time, we assessed the effect of four cooking temperatures (63, 71, 85, and 96°C) in two types of ground beef (3 and 30% fat content). In brief, random 3-g aliquots (n=128) of ground beef were inoculated with 7-8 Log_{10} of *C. difficile*. Inoculated and non-inoculated control
(n=24) aliquots were heated in water baths and survivor spores were enumerated on blood agar after 0, 2.5, 5, 7.5, 10, 15, 20 and 30 minutes of heating. To determine the probability of survival for each individual strain after exposure to various time-temperatures, we randomly selected survivor *C. difficile* colonies from representative blood agar plates and generated a genetic fingerprint, PCR ribotyping (Bidet *et al.* 1999), for each colony selected.

**Systematic random sampling of survivor *C. difficile***

Sampling of survivor *C. difficile* colonies was conducted using a systematic random scheme to prevent selection bias and to represent the effectiveness of the thermal treatment. Starting with the most effective temperature (e.g., the ones with the lowest colony counts), and the last two-fold dilutions yielding *C. difficile*, all countable colonies were subculture for PCR ribotyping if survivor colonies were less than 10 per dilution. When countable colonies were between 10 and 40, all colonies within a random quadrant of the plate were subcultured and typed. No plates were sampled if the colonies were not separated from each other to ensure proper single colony testing.

**Natural contamination in beef with *C difficile* and background microbial flora as controls***

As a control, the natural level of *C. difficile* contamination was quantified in random aliquots of the ground beef and determined if ‘naturally occurring’ spores, sampled as above, could be differentially selected with pasteurization temperature at 63°C. Further the effect of thermal treatments on the total number of non-*C. difficile* colonies (background beef flora) was determined on 10-fold serial dilutions inoculated
onto blood agar after 48 hours of anaerobic incubation to monitor the effectiveness of thermal treatments.

**Statistical Analysis**

Experiment 1 – The effect of heat on the $\log_{10}$ CFU of *C. difficile* data was analyzed using a generalized linear model with minutes of heat, replicate, and strain type as predictors.

Experiment 2 – The relative frequency of isolation data for each PCR ribotype identified in beef was analyzed as continuous variables using generalized linear regression. Predictors tested included minutes of heating, temperature, type of beef, $\log_{10}$ CFU of *C. difficile*, number of isolates PCR ribotyped per aliquot, and replica. To determine the survival probability of each PCR ribotype within a given beef aliquot, the frequencies of isolation data were converted into a categorical variable (yes/no) for each ribotype. Multivariable multinomial logistic regression was then used to compare and estimate predicted survival probabilities as a function of the variables described above (UCLA). Intercooled Stata software (v10.1, College Station, TX) was used. Adjusted p-values are reported.
References


Chapter 11 — *Clostridium difficile* and higher cooking temperatures for communities at risk

(Submitted for Publication)

*Clostridium difficile* infection (CDI) in hospitals and the community continues to be a growing international public health concern and a major financial burden despite strengthened control actions. Evolving antimicrobial prescription and hygiene policies have been widely publicized to protect hospitalized individuals from developing severe CDI. However, communities that were previously unaffected are becoming increasingly susceptible to severe CDI, and these groups have not been targeted for prevention. The elderly, children, pregnant women, and young adults are at high risk for CDI within the community, especially after receiving antimicrobials or antacids, undergoing surgery, or suffering chronic medical conditions. Community-associated CDI have also been associated with emergent antimicrobial-resistant and hypervirulent *C. difficile* strains, but the source for infection is often undetermined.

The fecal-oral transmission of *C. difficile* has been linked to environments contaminated with spores (*e.g.*, health-care centers), a problem potentiated by improper hand washing. In the past, CDI were exclusively attributed to exposure to health-care
settings, and control actions have primarily targeted health professionals to reduce spore dissemination between patients. However, since 2006, the frequent isolation of emergent *C. difficile* strains from raw and ready-to-eat foods (6-42%) in North America (Gould and Limbago 2010) indicate that exposure to foodborne spores is possibly a risk factor, although causality ties with CDI have not been confirmed. Although it is uncertain how many ingested spores are needed to cause disease in people, the presence of <9,000 contaminating spores of *C difficile* per gram of food (Weese et al. 2009; Rodriguez-Palacios and LeJeune 2011), indicate that reducing food contamination may be beneficial.

Infection control recommendations against *C. difficile* are not readily available for patients or household settings. Here we propose a simple intervention to reduce the qualitative risk of ingesting foodborne spores based on results from recent thermal studies that assessed the sensitivity of epidemic *C. difficile* strains to various cooking time-temperature combinations (Rodriguez-Palacios et al. 2010; Rodriguez-Palacios and LeJeune 2011). We propose accompanying medical prescriptions, treatments, or diagnoses that predispose to CDI, with recommendations to: *i*) improve kitchen hygiene, *ii*) increase cooking temperatures, and *iii*) minimize exposure to documented sources of *C difficile* spores (Box 1). Because spores are necessary for disease, these measures might mitigate the incidence of CDI among susceptible individuals.

Based on PCR ribotyping (a molecular classification method) there are over 200 *C. difficile* subtypes isolated from humans, animals and the environment. However, the frequent isolation of only a few specific highly epidemic (Goorhuis et al. 2008) *C.
*difficile* subtypes (PCR ribotypes 001, 078, 027) from foods (Gould and Limbago 2010) is concerning. With the presence of *C. difficile* spores in raw and ready-to-eat foods and people’s preference for rare/medium cooked meats, an obvious question is whether cooking reduces the foodborne exposure risk.

At present, *C. difficile* is not considered a foodborne pathogen (Gould and Limbago 2010). Thus, recommendations for its control are not widely publicized. There are however cooking recommendations for less heat-resistant pathogens. Most food safety guidelines recommend achieving a minimum internal temperature of 71°C (160°F) for a few seconds to make meals safe. (Rodriguez-Palacios *et al.* 2010) For more difficult-to-cook whole-poultry meals, some recommendations state that at least 85°C (185°F) is needed. (Rodriguez-Palacios and LeJeune 2011) At recommended cooking temperatures, most vegetative pathogens (e.g., *E. coli*) can be eliminated within seconds, but clostridia produce resistant spores. Although there is abundant information on thermal inhibition of *Clostridium perfringens* and *Clostridium botulinum* (worldwide foodborne pathogens), species differences prevent extrapolating data to *C. difficile*.

Recently, complementary thermal studies on *C. difficile* have expanded our understanding on this pathogen. (Rodriguez-Palacios *et al.* 2010; Rodriguez-Palacios and LeJeune 2011) The first report documented that heating *C. difficile* spores of epidemic PCR ribotypes from food and food animals at minimum recommendations for ground meats (71°C, <2 minutes) causes no spore inhibition. (Rodriguez-Palacios *et al.* 2010) Not surprisingly, extending the cooking time to two hours only reduced the number of spores by 2-3 Log_{10} units (from 10 million to 100 thousand spores/gram), which is far less than
the 6 Log\(_{10}\) units (from 10 million to 1-10 spores/gram) reduction desired by processing technologies. Hence, given the level and frequency of meat contamination, the risk of ingesting \textit{C. difficile} in foods is real even if current cooking recommendations are followed.

To further assess the effects of cooking \textit{C. difficile} at higher temperatures, we assessed their spore survival in ground beef and gravy at different fat contents (0-30%). (Rodriguez-Palacios and LeJeune 2011) Assessed temperatures included 63 (145°F), 71, 85 and 96°C (205°F). Cooking at 85°C markedly reduced \textit{C. difficile} spores within 10-15 minutes, but the inhibition was more pronounced at sub-boiling temperature (96°C). It approached the desired 6 Log\(_{10}\) spore reduction within 1-2 minutes of heating (Table). Since foods can be contaminated with up to 4 Log\(_{10}\) (<9,000) of spores per gram, (Weese \textit{et al.} 2009; Rodriguez-Palacios and LeJeune 2011) cooking at >96°C could markedly reduce the risk of ingesting \textit{C. difficile}.

Of concern, the same experiments also showed that cooking at 63 and 71°C (widely recommended) enhanced the rate of spore germination and total \textit{C. difficile} counts in most strains. (Rodriguez-Palacios and LeJeune 2011) Furthermore, experiments illustrated that a fraction of the spores that were inhibited with 85°C, recovered within 7 hours if left incubating in nutrient-rich media. Inadequate cooking followed with improper cooling and storage could therefore favor the proliferation of \textit{C difficile} within foods, which is precisely the factor responsible for \textit{C. perfringens} food poisoning in humans. The importance of thorough cooking needs to be emphasized, especially, because recent studies indicated that sublethal heating favors the selection of

In addition to ‘better-cooking-practices’, other preventive measures could be publicized for susceptible communities (Box 2). Hand washing should be reinforced when preparing foods, after visiting bathrooms, and especially after having contact with animals (or their environment) because hypervirulent \textit{C. difficile} strains are often found in excrements (Gould and Limbago 2010). Susceptible communities should also avoid direct exposure to farm/companion animals. Particular attention should be placed on dogs fed raw meats or that are used as ‘therapy animals’ in health-care settings as they may carry \textit{C. difficile} (Gould and Limbago 2010). Public health authorities, physicians and those persons cooking for susceptible communities, should consider these factors when making recommendations and preparing foods.

Accompanying relevant prescriptions (or consultations) with recommendations to reinforce better-cooking practices and hygiene will be beneficial until validated methods to reduce food contamination are available. Revised food safety guidelines are needed to provide at-risk communities with adequate cooking temperatures, and times (currently not indicated in most guidelines), to prevent inadvertent ingestion of clostridial spores. If \textit{C. difficile} from foodborne sources indeed results in illness, then minimizing cross-contamination during food production and processing could also reduce the incidence of CDI.

Adoption of higher-temperature cooking and enhanced hygiene practices may be limited: Some individuals may be reluctant to adopt recommendations or may not believe food/environmental contamination is an issue, while others may prefer to continue eating
undercooked foods due to personal or cultural preferences. Nevertheless, providing the information to patients and the community ensures that the highest standards of practice are applied. According on the Oxford Centre for Evidence-Based Medicine guidelines (http://www.cebm.net), it is safe to say there is already enough epidemiological and bench research data from diverse sources (Weese et al. 2009; Gould and Limbago 2010; Rodriguez-Palacios et al. 2010; Rodriguez-Palacios and LeJeune 2011), theoretical rationale and mechanistic-based reasoning to harmlessly and systematically advise susceptible communities to cook their foods better (Table 13) and lower their exposure to potential sources of C. difficile (Fig 27 and 28), at least during periods considered of high susceptibility for CDI. With coordination, monitoring the impact of such measures could further assess whether CDI is linked to foods, especially among patients prone to recurrence.

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Table 13. Minutes of heat needed to inhibit 90% of *C. difficile* spores at achievable household cooking temperatures.

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<th>Currently recommended minimum internal temperatures for cooking</th>
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<td></td>
<td>Steaks, fish, and batch pasteurization</td>
<td>Most ground meats</td>
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<td>63°C (145°F)</td>
<td>71°C (160°F)</td>
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<tr>
<td>Gravy, 0% fat; mean ± SD (95% C.I.)</td>
<td>55.9 ± 21.8 (21.3 to 90.6)</td>
<td>56.8 ± 30.5 (18.9 to 94.7)</td>
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<td>Lean ground beef, 3% fat mean ± SD (95% C.I.)</td>
<td>99.4 ± 62.3 (22 to 176)</td>
<td>71.2 ± 38.7 (9.6 to 132)</td>
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<tr>
<td>Ground beef, 30% fat mean ± SD (95% C.I.)</td>
<td>45.8 ± 10.8 (28.7 to 63)</td>
<td>46.8 ± 10.9 (33.1 to 60.4)</td>
</tr>
<tr>
<td>Collectively, mean ± SD (95% C.I.) of the mean</td>
<td>69.6 ± 45.4 (42.1 to 96.9)</td>
<td>57.3 ± 27.8 (41.3 to 73.4)</td>
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*a*Food safety agencies recommend the use of commercially available ‘Food Thermometers’ to ensure proper cooking

[http://www.fsis.usda.gov/is_it_done_yet/brochure_text/index.asp#SMIT].

*b*To achieve food safety standards of 6 log₁₀ units of microbial reduction, cooking temperatures between 3.2 and 11.4 minutes at 96°C (6 x [95% C.I.]; superscripted ‘b’) would be a safe starting point. Adapted from Rodriguez-Palacios & LeJeune (2011)(Rodriguez-Palacios and LeJeune 2011) with permission from the American Society of Microbiology [http://aem.asm.org/cgi/content/full/77/9/3085/DC1].
**Box 1. Risk and exposure factors for acquisition of C. difficile spores and infections**

**Well-known Risk Factors** (these can be host- or environment-dependent):
- Antimicrobials/antacids increases with “sophisticated” drugs, combinations or long treatments.
- Elderly (over 65 years old) are more likely to get sick.
- Debilitating illness such as cancer, or immune-suppressive conditions/medications.
- Colon diseases such as inflammatory bowel disease or colorectal cancer.
- Abdominal surgery or gastrointestinal procedure.
- Having had *C. difficile* infection already.
- Current or past hospitalization or contact with CDI patients.
- Living in a nursing home/long-term care facility.

**Possible exposure risk factors** (environment-dependent; investigation is underway):
- Through contaminated foods (difficult to predict/notice; variable; *C. difficile* has been found in food with good quality – smell and taste).
- Healthy animals shedding *C. difficile* (difficult to predict/notice; animals look normal).
- Diseased animals suffering CDI that shed *C. difficile* in feces (predictable, possible but unclear role in human disease; animal-to-animal has been partly documented).
- Animal waste (occupational risk is possible but unknown; occupational risk has been documented for laboratory personnel working with human fecal specimens).
- Contaminated environment (difficult to predict/notice unless obvious influence of potential source of *C. difficile*; for instance, sewage from human/animal facilities; *C. difficile* has been found in recreational waters).

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Figure 27. Box 1 - Risk and exposure factors for acquisition of *C. difficile* spores and infection
Box 2. Expanded recommendations to reduce the risk of acquiring *C. difficile* spores

- **Hand washing.** Hospital guidelines recommend an alcohol-based hand sanitizer to eliminate most microorganisms but it is largely ineffective in destroying *C. difficile* spores; thorough hand washing with soap and water is effective in removing spores from hands—this is the best. Health care workers in human/animal hospitals, affected patients, and everyone who suspects exposure would benefit from hand washing—this would also benefit others. *Very relevant for household kitchens* and for personnel working in farms, food processing plants or restaurants.

- **Contact precautions.** In [human/animal] hospitals, affected subjects can be isolated [in private rooms/stables/cages], and workers wear disposable gloves and gowns to prevent spread. Apart from voluntary isolation in the community, isolating animals discharged from hospitals with a diagnosis of CID should be encouraged and communicated to related household relatives or farm workers, whichever applies. Contact with hospital visitation pets should be minimized outside hospitals (in hospitals their emotional benefit to patients could be balanced with reinforcing other precautions; controversial). Avoid exposure to animal or animal manure regardless of their apparent health status. Beware that *C. difficile* spores that go down the drain or hill will remain dormant and alive for months or years.

- **Thorough cleaning.** *C. difficile* spores can survive routine household disinfectants. In any setting, all surfaces and equipment should be carefully cleaned with a detergent and a hospital-grade disinfectant approved for *C. difficile* or chlorine bleach diluted with water at a 1:10 ratio (contact exposure should be of at least 5-10 minutes; note that the effect of bleach disappears with evaporation or in presence of dirt or abundant organic matter). Especially important on food preparation areas, avoid cross-contamination because *C. difficile* has been isolated from raw meats and vegetables. Keep pets outside of food preparation areas as they may be carriers of *C. difficile*.

- **Use antimicrobials and antacids only if necessary.** Antibiotics are often prescribed for viral illnesses that aren't helped by these drugs. Consult your doctor if you think an antibiotic is needed. Ask your doctor to prescribe one that has a narrow range and that you can take for the shortest time possible. How antacids predispose to *C. difficile* infections is not fully understood, but it may be in part because they block the killing effect of gastric acidity and thus allow the ingestion of otherwise acid-susceptible microorganisms. Since antibiotics and antacids may disrupt the intestinal flora for several (2-6 weeks) try to follow this precautions for as long as possible. Ideally make them a healthy habit for your benefit and the benefit of your loved ones.

- **Increase cooking time and temperatures.** Studies have shown that *C. difficile* spores survive 2 hours of extended cooking if using internationally recommended minimum internal cooking temperature (71°C, as cited on retail food labels). The currently recommended minimum temperatures for difficult-to-cook meals (whole turkey) is 85°C, although at this temperature *C. difficile* spores can be markedly inhibited, it may require at least 15 minutes, leaving the possibility that surviving spores may re-grow within foods after heating. Cooking at boiling temperatures for at least 3 minutes seems to be a reasonable strategy to inhibit most spores from foods. However, proper chilling and storage of foods soon after heating is absolutely needed to prevent bacterial growth.

- **Adopt complementary food safety measures available for at-risk populations.** Although publicized minimum temperatures are inadequate to destroy *C. difficile*, currently available guidelines have practical measures that may be suitable to enhance food safety against clostridial spores, including chilling and storage practices. (http://www.fsis.usda.gov/is_it_done_yet)

- **Consider risk assessment features against *C. perfringens*.** Food poisoning associated with *C. perfringens* is one of the major foodborne illnesses in the USA. A freely available comprehensive report exists addressing the risk of such pathogen in partly cooked and ready-to-eat meat and poultry products. These data could be useful as *C. difficile* appears to be more susceptible to heat than *C. perfringens*. (http://www.fsis.usda.gov/PDF/CPerfringens_Risk_Assessment_Tagged.pdf)

Figure 28. Box 2 - Expended recommendations to reduce the risk of acquiring *C. difficile* spores
References


Chapter 12 — Conclusions

- *C. difficile* can be shed by food animals at the time of harvest at concentrations equivalent to super-shedding thresholds reported for *E. coli* O157. Despite the isolation of emerging PCR ribotype 078 from finishing cattle 24 hours prior to harvest, fecal shedding of *C. difficile* was transient (no animal tested twice in consecutive relevant samplings which corresponded to 24h and 1 week apart).

- The shedding in feedlot cattle did not increase with increased use of antimicrobials in over a half of all animals in the lot. Antimicrobials were used during the first month for treatment of typical, but serious feedlot cattle respiratory diseases.

- The prevalence of *C. difficile* in food animals during the summer is very low (0-0.6%) compared to horses (7.2%). This indicates that the risk of food contamination at harvest is low.

- High shedding levels from few food animals might be sufficient to contaminate processing facilities. Studies sampling more farms and processing facilities in winter would help to determine factor to explain why the prevalence of food contamination is the highest in winter.
• The potential for carcass and food contamination at the time of animal harvest exist and could be amplified if derived foods are improperly cooked and stored. Thermal studies showed that spores can recover, germinate, and multiply if heating is not lethal.

• High sub-lethal cooking temperatures favor the selection of emerging \textit{C. difficile} strains, especially emerging PCR ribotype 078. Therefore cooking recommendations need to be accompanied with preharvest and postharvest intervention measures to lower the load of contaminating spores.

• Wild birds and possibly wild ruminants may play an important role on dissemination of \textit{C. difficile} in agricultural ecosystems. Starlings may allow for efficient dissemination of \textit{C. difficile} in an agricultural region, while long-range migratory waterfowl could do it a larger distances. Waterfowl can also contaminate environments visited by elderly, like public parks, and therefore serve as a novel factor for \textit{C. difficile} exposure.

• Food safety risk associated with ingestion of toxigenic \textit{C. difficile} from foods is therefore real, especially in times of growing trends for cooking and eating minimally processed foods.

• Further studies are needed to identify critical control points to reduce the risk of food and environmental contamination with \textit{C. difficile}. If foods and animals are sources for zoonotic transmission, interventions to minimize exposure should result in a reduction of \textit{C difficile} infections in the community.
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