Published in final edited form as:

Anaerobe. 2013 August ; 22: 1–5. doi:10.1016/j.anaerobe.2013.05.005.

A Clinical and Epidemiological Review of Non-toxigenic *Clostridium difficile*

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Abstract

Clostridium difficile is a significant nosocomial threat to human health and is the most commonly identified cause of antibiotic associated diarrhea. The development of *C. difficile* colitis requires production of toxins A and/or B, but some strains do not express these proteins. These non-toxigenic *C. difficile* (NTCD) have garnered attention for their capacity to colonize humans and potentially reduce the risk for symptomatic colitis caused by toxigenic strains. Isolates of NTCD have been obtained from the environment as well as from animal and human sources. Studies in a hamster CDI model have demonstrated a protective effect of NTCD against toxigenic infection. The extent to which this protective effect of NTCD occurs in humans remains to be defined. Evidence for a therapeutic or preventive role for NTCD is limited but clinical prophylaxis studies are ongoing. NTCD potentially represents an exciting new tool in preventing CDI and its recurrences.

Keywords

Clostridium difficile; non-toxigenic *Clostridium difficile*; probiotic; hamsters; humans

1. Introduction and Background

In the late 1970s it was demonstrated that the antibiotic-associated disease known as pseudomembranous colitis was associated with a clostridial toxin and that infection by *Clostridium difficile* was the likely etiology [1–5]. Soon after, strains of *C. difficile* that lacked toxin production were isolated from patients and were termed “non-toxigenic” [6]. In the years that followed, many studies were published regarding non-toxigenic *C. difficile* (NTCD) including data regarding their epidemiology and the protective effect against infection by toxigenic *C. difficile*. The purpose of this review is to summarize available clinical and epidemiological data on NTCD with the goal of updating clinicians and investigators in the field, identifying knowledge gaps regarding NTCD, and aiding in the design of future studies.

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Human *C. difficile* infection (CDI) is caused by isolates that produce at least one of two toxins, A and B. Initial studies with mutant *C. difficile* isolates that lack either toxin A or B showed that production of either toxin is sufficient for disease in hamsters [7, 8]. However, some studies suggest that toxin B is the more potent toxin in CDI pathogenesis [9]. Clearly, CDI in humans can be caused by strains of *C. difficile* that only produce toxin B, while to date, strains that produce only toxin A have not been isolated from symptomatic patients [10]. The genes that produce toxins A and B (*tcdA* and *tcdB*, respectively) are in close proximity to genes that may be responsible for regulating their expression (*tcdC* and *tcdR*) and/or the release of the bioactive forms of the toxin (*tcdE*). This 19.6kb region of the *C. difficile* genome is referred to as the pathogenicity locus (PaLoc). Although the role of *tcdA* and *tcdB* in *C. difficile* pathogenesis is well accepted, the *in vitro* vs. *in vivo* roles of TcdC, TcdR, and TcdE are still not clearly defined. However, it is clear is that NTCD isolates do not contain an intact PaLoc, do not produce toxin A or toxin B, and are not typically implicated in symptomatic infection [11–14]. In addition to strains that lack an intact PaLoc, other NTCD strains exhibit dysregulation of the *tcdA* and *tcdB* genes and do not express enough bioactive toxin to cause disease. These strains are “phenotypically” non-toxigenic. One such example is the M90 strain that contains the PaLoc, but does not produce detectable levels of toxin, possibly due to poor gene transcription [15]. Another group of strains, classified as toxinotype XI or ribotype 033, contain a fragment of the *tcdA* gene, but do not produce detectable toxin A or B [16].

2. Methods

Articles for this review were identified by searching PubMed (<http://www.ncbi.nlm.nih.gov/pubmed/>) and Google Scholar (<http://scholar.google.com/>) with the following terms: “non-toxigenic *Clostridium difficile*” and “nontoxigenic *Clostridium difficile*.” Additional manuscripts were identified by reviewing the bibliographies of published works.

3. Natural History of Non-toxigenic Isolates

The evolutionary history of NTCD has not been definitively established. Whether non-toxigenic lineages once produced toxin or whether toxin-producing lineages evolved from non-toxigenic ancestors is not entirely clear. Corthier and Muller reported that toxin production was diminished or lost completely in mutant clones that evolved during infection of germ-free mice that were pre-colonized with the yeast, *Saccaromyces boulardii*, or when the mice were fed a semi-synthetic diet [17]. Incidentally, these non-toxigenic mutants were able to protect mice against subsequent toxigenic challenge, suggesting a protective role (see below in section 6). Regardless, a phylogenetic reconstruction of non-toxigenic lineages and closely related toxigenic lineages would certainly help clarify the evolution of NTCD; however, only one genome sequence of a non-toxigenic isolate is available to date [11].

4. Epidemiology

Among healthy volunteers without symptoms, the prevalence of *C. difficile* carriage has been reported as between 4% and 7.6% [18, 19]. Of the positive isolates in these studies, non-toxigenic strains accounted for 42% to 50% of the total. This percentage of NTCD is similar to what has been observed in asymptomatic hospitalized patients (Table 1) [20]. Another way to estimate the prevalence of NTCD is through the characterization of stool samples sent for testing for the presence of toxigenic *C. difficile*. Although this method does not take into account the entire patient population, it focuses on individuals who are likely at risk of developing CDI since they had symptoms that prompted stool studies. The proportion of *C. difficile* isolates that are non-toxigenic has varied considerably depending on the patient population and clinical setting (Table 1). The variability in these data likely results

from the different populations studied as well as differences in how isolates were determined to be toxigenic. For example, in the presence of a mixed population (non-toxigenic and toxigenic), NTCD strains are likely to be missed if not enough strains are selected to be tested from a culture. Conversely, if the method used to detect toxin is insensitive, one would expect to overestimate the prevalence of NCTD. However if data are compared from a single laboratory using patient samples from an entire healthcare system, these differences are less likely to be secondary to laboratory variation and more likely to be from patient characteristics (Table 1) [21].

The proportion of positive isolates that are non-toxigenic might also vary by age; one study found isolates from patients greater than 60 years of age were 6.9% non-toxigenic while those from patients younger than 20 years of age were 22.8% non-toxigenic (Table 1) [21]. These data support other data showing NTCD isolates are more prevalent in younger patients, especially infants less than one year old (Table 1). However, one must be careful when comparing rates of NTCD between symptomatic and asymptomatic patients as these are two fundamentally different populations (Table 1). For the hospitalized infants, this high percentage of NTCD carriage may reflect nosocomial spread of a single non-toxigenic strain [22]. Alternatively, there may be transient colonization of NTCD that may be replaced later by a different strain [23]. In addition, there may also be differences in neonatal gastrointestinal microbiota and immune status that make these individuals more prone to colonization by non-toxigenic rather than toxigenic isolates. For example, Rousseau and colleagues demonstrated changes in the intestinal microbiota composition of infants colonized by *C. difficile* compared to those who were not colonized [24]. However, this study did not find a major difference in microbiota composition between toxigenic and non-toxigenic colonization, highlighting that there are still major gaps in our understanding of the epidemiology and biology of NTCD among infants.

Humans are not the only source of non-toxigenic isolates. Dogs and cats in a veterinary setting have been reported to carry non-toxigenic isolates as have pigs, horses, and poultry in an agricultural setting [25–28]. NTCD isolates have also been isolated from the health care environment as well as from rivers and soil samples, where they reached 30.8% of the total isolates examined [28–30]. Thus, NTCD isolates exist outside the human host and may be ubiquitous in the environment.

5. Can Non-toxigenic *C. difficile* Cause Disease?

Although NTCD isolates are thought of as benign and non-pathogenic, there are several reports of association with infection in susceptible patients. A case report from France describes a 16-year-old boy with osteosarcoma who developed a non-toxigenic *C. difficile* chronic septic arthritis and osteomyelitis in a prosthetic knee joint [31]. In another case, a 48-year-old man who had surgery to drain a subdural hematoma later developed a brain abscess that was infected with both non-toxigenic and toxigenic *C. difficile* strains [32]. Lastly, a study observing patients with diarrhea on a hematology/oncology ward found that those with non-toxigenic strains of *C. difficile* had a longer duration of diarrhea compared to those with a toxigenic strain [33]. However, those patients were also exposed to antimicrobials and chemotherapeutics, making it unclear whether NTCD was the true cause of the diarrhea. In addition, our group and others have cultured NTCD isolates from toxin positive patients [18, 34]. This observation suggests that NTCD isolates may be involved in mixed (toxigenic and non-toxigenic) infections and such cases could be misidentified as solely associated with the non-toxigenic strain.

6. A Protective Effect?

In 1983, Wilson and Sheagren reported that NTCD could protect against toxigenic infection in a hamster model of the disease [35]. They found that animals given a NTCD isolate prior to a toxigenic *C. difficile* challenge had a higher survival (93%) compared to animals given non-toxicogenic and toxigenic strains together (32%) and to animals only given a toxigenic strain (21%).

Borriello and Barclay later investigated this effect in more detail using the hamster model [36]. They showed that the NTCD isolate had to be alive and present at the time of toxigenic *C. difficile* exposure for the protective effect to be evident. If the NTCD isolate was heat-treated prior to administration to hamsters or if it was eliminated pharmacologically by vancomycin, the protective effect was lost. In addition, NTCD did not protect against infection from other clostridial pathogens. However, over time the investigators found that even the hamsters which were successfully colonized by NTCD eventually became infected by toxigenic *C. difficile* and died. These important findings were limited by a lack of mechanistic evidence to explain how NTCD provides specific protection against CDI.

To better simulate natural *C. difficile* exposure, Sambol and colleagues used spores of NTCD in their hamster inoculation experiments, as opposed to the suspensions of vegetative cells used in the aforementioned studies [37]. A protective effect in hamsters was observed using combinations of three different non-toxicogenic (REA types M3, M23, and T7) and toxigenic (REA types B1, J9, and K14) strains that were originally isolated from human patients. In this study, the protective effect appeared to be more durable compared to prior studies as protected hamsters survived up to 106 days.

As patients who develop CDI have often received antibiotics over a contiguous period of time, Merrigan, *et al.* administered clindamycin to hamsters continuously over several days to more accurately model how patients develop CDI [38]. These hamsters were either given a clindamycin-resistant or a clindamycin-susceptible NTCD strain. The hamsters given clindamycin-resistant NTCD were protected when later challenged with toxigenic *C. difficile*. In contrast, the hamsters given the clindamycin-susceptible non-toxicogenic strain did not become colonized with NTCD and were not protected from a toxigenic *C. difficile* challenge while receiving clindamycin. However, if NTCD was given at least 2 days after the clindamycin was stopped, there was NTCD colonization and protection from toxigenic *C. difficile*. Similar results were also shown using ceftriaxone and NTCD strains resistant to ceftriaxone [39]. These data show how the protective effect of NTCD would be limited in the setting of patient antibiotic use as antibiotic-susceptible NTCD would not be able to colonize these patients. However, using antibiotic-resistant NTCD strains as was done in these studies could potentially risk the transfer of resistance to other bacteria in the GI tract.

Despite an incomplete understanding of how NTCD isolates provided protection against CDI, these early results soon informed practice in veterinary applications. For example, the protective effect of NTCD was employed in an agricultural setting in an attempt to prevent infection in pigs in which *C. difficile* has emerged as a problem, especially in piglets [40]. Piglets given a NTCD isolate orally had a lower rate of *C. difficile* associated disease compared to untreated control piglets.

Evidence supporting a protective effect of NTCD has also been found in humans. A meta-analysis of four studies in which hospitalized patients were monitored with weekly rectal swab cultures demonstrated that asymptomatic colonization by *C. difficile* was associated with a lower rate of subsequent CDI (3.6% vs. 1.0%) [20]. Notably, 46% of the asymptotically colonized patients carried NTCD, suggesting some of the benefit of colonization may be related to NTCD. It was based on the prior studies showing a beneficial

effect in hamsters that prompted Seal and colleagues to administer a NTCD isolate in a therapeutic fashion to human patients [41]. Two patients who had relapsing CDI despite multiple courses of traditional antimicrobial treatments were given three doses of the M1 strain, a non-toxigenic *C. difficile*, as a live culture. Although the bacteria were administered after symptoms had stopped, these patients did not relapse further and did not have any side effects from the bacteriotherapy, suggesting a therapeutic benefit. This study was reported in 1987 and appears to have been the first documented use of NTCD in humans.

The next study of NTCD in humans was not published for another 25 years. Villano, *et al.* recently reported a phase 1 study showing administration of NTCD spores to healthy subjects was generally well tolerated [42]. The NTCD spores used in this study were REA type M3 and were one of the same strains that was shown to be protective in hamsters [37, 38]. In one group, 100 million spores were given twice a day for 5 days (the highest daily dose) and NTCD was detected in the stool two days after the last dose. However, follow-up cultures one to two weeks later failed to show colonization, indicating that colonization with NTCD is unlikely to occur in the presence of an unperturbed microbiota. In another group the subjects were pretreated with vancomycin prior to administration of the spores which were given over a 14-day period. In 44% of these subjects, NTCD was detected in the stool 1–2 weeks after the last dose of spores, but no colonization was detected beyond an additional month. Overall, these data demonstrated that oral administration of a NTCD isolate did not have major side effects and could be used to colonize healthy subjects. A follow up phase 2 study is being conducted in patients with CDI with the goal to prevent disease recurrence [42, 43]. The use of an oral NTCD preparation as a “probiotic” would be a major milestone in the treatment of recurrent CDI and would be an easier and more palatable option compared to fecal bacteriotherapy.

7. Conclusions

Non-toxigenic *C. difficile* do not produce toxin A or toxin B and are usually not associated with symptomatic infection. There is an established body of work demonstrating a protective effect of NTCD colonization against toxigenic infection in the hamster model. However, with human patients the evidence is still growing and there are several yet unanswered questions. For example, the mechanisms whereby NTCD provide protection against CDI remain unclear. Whether the benefit is simply due to competition for a niche in the gastrointestinal tract or results from more complex effects on mucosal immunity or nutrient acquisition remains to be solved. In addition, little is known regarding the natural history of NTCD colonization in humans and it is unclear what factors allow some patients to become colonized when others do not. Lastly, NTCD colonization appears to be associated with a lower risk of subsequent infection in humans [20], but it is not clear whether colonization is in itself protective or whether those patients who are prone to be colonized have some other, unidentified protective host factor(s).

With respect to using NTCD as a therapeutic, it has been recently shown that intentional colonization of healthy subjects with NTCD is safe [42]. The results of the ongoing phase 2 trial should help assess the safety and efficacy of NTCD in preventing disease recurrence in patients with recurrent CDI [43]. An additional indication of NTCD that warrants further study is its use as primary prophylaxis against CDI in susceptible patients such as those receiving antimicrobials.

Acknowledgments

This work was supported by National Institutes of Health grants 1U19AI090871-01 (V.B.Y., and D.M.A) and 1K01AI09728101A1 (S.T.W.).

Abbreviations

NTCD non-toxigenic *Clostridium difficile*

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Table 1

Reported proportion of *C. difficile* isolates that were non-toxicogenic, by clinical setting, age (when available), specimen source (symptomatic or asymptomatic patients), and method used to identify NTCD. Patients were considered symptomatic if the specimen was ordered to evaluate for CDI (noted symptoms in parenthesis).

Clinical setting and age (when available)	Specimen source	Method used to identify NTCD	Total no. of <i>C. difficile</i> isolates	% Non-toxicogenic	Reference
Inpatient wards-Chicago, IL, USA	S (N/A)	Culture, CCCA, PCR	153	8.5	[44]
Inpatient wards-Sacramento, CA, USA	S (diarrhea)	Culture, EIA, PCR	97	26	[45]
Inpatient oncology ward-Sacramento, CA, USA	S (diarrhea)	Culture, PCR	21	14	[29]
Hospitalized patients-Slovenia	S (N/A)	Culture, PCR	690	6.5	[28]
Nursing homes-Virginia, USA	S (N/A)	Culture, CCCA, PCR	489	4.3	[21]
Inpatient wards-Virginia, USA	S (N/A)	Culture, CCCA, PCR	1058	11	[21]
Outpatient clinics-Virginia, USA	S (N/A)	Culture, CCCA, PCR	260	17	[21]
Inpatients and outpatients-Michigan, USA	S (N/A)	Culture, CCCA, PCR	791	3.0	[46]
Mixed clinical setting, age > 60 years-Virginia, USA	S (N/A)	Culture, CCCA, PCR	1479	6.9	[21]
Mixed clinical setting, age 20 – 59 years-Virginia, USA	S (N/A)	Culture, CCCA, PCR	977	16	[21]
Mixed clinical setting, age < 20 years-Virginia, USA	S (N/A)	Culture, CCCA, PCR	342	23	[21]
Hospitalized children, age 0–5 years-Brazil	S (diarrhea)	Culture, CCCA, PCR	10	10	[47]
Inpatients-Minnesota/Massachusetts, USA	AS	Culture, CCCA	192	46	[20]
Neonatal unit-Belgium	AS	Culture, CCCA	223	53	[30]
Hospitalized infants, age 0–12 months-France	AS	Culture, EIA, cytotoxin cell culture assay	24	63	[48]
Neonatal intensive care unit-Japan	AS	Culture, PCR	55	96	[22]
Healthy infants in daycare, age 0–3 years-France	AS	Culture, EIA, PCR	38	71	[23]
Outpatient and inpatient infants, age 0–1 year-France	AS	Culture, PCR	87	82	[49]
Outpatient and inpatient infants, age 1–2 years-France	AS	Culture, PCR	12	58	[49]

Abbreviations: AS = asymptomatic; CCCA = cell culture cytotoxicity assay; EIA = enzyme immunoassay; N/A = not available; PCR = polymerase chain reaction; S = symptomatic;