Laboratory identification of anaerobic bacteria isolated on *Clostridium difficile* selective medium

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Running title:

Bacteria growing on C. difficile medium

Abstract

Despite increasing interest in the bacterium, the methodology for *Clostridium difficile* recovery has not yet been standardised. Cycloserine cefoxitin fructose taurocholate (CCFT) has historically been the most used medium for *C. difficile* isolation from human, animal, environmental and food samples, and presumptive identification is usually based on colony morphologies. However, CCFT is not totally selective. This study describes the recovery of 24 bacteria species belonging to 10 different genera other than *C. difficile,* present in the environment and foods of a retirement establishment that were not inhibited in the *C. difficile* selective medium. These findings provide insight for further environmental and food studies as well as for isolation of *C. difficile* on supplemented CCFT.

Keywords

Cycloserine cefoxitin fructose taurocholate medium; cefotaxime; bacteria identification; 16S ribosomal DNA sequence analysis.

Introduction

Many studies have reported changes in the epidemiology of *Clostridium difficile* and its presence in foods, animals and the environment. Interest in these types of *C. difficile* samples continues to expand and the possibility of zoonotic and food transmission of the bacterium is still the main focus of several research reports. However, an isolation procedure for research purposes has not yet been standardised. In recent years, a large number of studies have focused on the improvement of differential media and culture methods [1-3], including ethanol shock, sample enrichment in a selective broth, or the use of chromogenic and other pre-made agars. However, pre-made agars are expensive and thus unaffordable for many research groups. Furthermore, they are used for the clinical recovery of *C. difficile* from faecal samples and not for the semi-quantification of viable spores [4]. Since it was first proposed by George et al. [5], cycloserine-cefoxitin fructose (CCF) has been the most commonly used medium for *C. difficile* isolation from human, animal, environmental and food samples. The addition of taurocholate, desoxycholate or cholate has also been shown to induce germination of *C. difficile* spores when they are incorporated in CCF [3,6]. Other modifications to improve this media have been proposed; Delmée et al. [7] included cefotaxime instead of cefoxitin, which increases the sensitivity and specificity of the medium. This selective agent is also more soluble than cefoxitin, facilitating its homogeneous distribution in the agar.

Few studies have focused on the identification of other bacterial species growing in CCF. George et al. [5] reported the growing of *Lactobacillus spp*., unidentified yeast and unidentified anaerobic Gram-negative rods on CCF. Only one further study [8] described other *Clostridium* colonies growing in cycloserine-cefoxitin fructose taurocholate (CCFT), including *Clostridium sporogenes*, *Clostridium cadaveris*, *Clostridium perfringens*, *Clostridium bifermentans* and *Clostridium septicum*.

The objective of this study was to identify by comparative 16S ribosomal DNA sequence analysis the spectrum of bacteria cultured on CCFT, using surface and food samples. Growth of isolates was also tested in modified CCFT medium (with cefotaxime) and strains were further characterized for susceptibility to two selective agents, cefotaxime and cycloserine.

Materials and methods

The study was conducted over four months, from March to June 2013, and included 188 food samples and 246 surface samples [9]. The meals sampled were composed of raw and/or cooked ingredients, according to the daily menu. Every Friday morning, samples from the week were

transported to the laboratory for immediate analysis. The food preparation date, analysis date, quantity, and ingredients for each sample were recorded. Samples from surfaces were taken on two different occasions with a 65-day interval between them. A variety of areas (total area of approximately 100 cm^2) were swabbed before or after routine cleaning, including residents' rooms and other common areas [9].

Culture was performed on CCFT as described previously [9] in an anaerobic workstation (LedTechno, Heusden-Zolder, Belgium) at 37 °C. Colonies other than those with the characteristic morphology of *C. difficile* were then subcultured on Columbia agar plates with 5% horse blood (Biomérieux, Marcy-l'Étoile, France). Total DNA was harvested from a single colony and extracted as previously described [10]. Molecular identification of bacteria by 16S ribosomal DNA sequence analysis was performed using the primers and conditions described by Simpson et al. [11]. Sequencing and product purification were performed as described previously [12]. Following sequencing, consensus sequences were created using the Geneious program (http://www.geneios.com). The genus and species of each consensus sequence were deduced from a comparison with the non-redundant nucleotide database (http://blast.ncbi.nlm.nih.gov) using the basis local alignment search tool (BLAST). A 99% identity was used as a threshold for species identification [13].

All of the isolated strains were subcultured on modified-CCFT agar to include the selective agents cycloserine (400 µg/mL) and cefotaxime (3.6 µg/mL). After incubation for 48 h in an anaerobic atmosphere at 37 °C, the plates were examined to verify bacterial growth in the modified medium. Additionally, all isolates were tested for susceptibility to cycloserine and cefotaxime antimicrobials. The test was performed by paper disc diffusion according to the French Society of Microbiology (FSM) (www.sfm-microbiologie.org) guidelines. For cefotaxime, the test was performed with a 30 µg standard disc (Becton-Dickinson, Erembodegem, Belgium). For cycloserine, as commercial standard discs are not available, the test was adapted to the protocol previously described by Mith et al. [14] with a final concentration of 120 µg of cycloserine in the disc. The plates were incubated for 48 h in an anaerobic workstation. The antibacterial activity was evaluated by measuring the diameter of inhibitory zones in millimetres using Top Craft digital callipers (Globaltronics GmbH & Co. KG, Germany). Means were then calculated from the results of three determinations. The entire tests were performed in duplicate. *Bacteroides fragilis* ATCL 25285 was tested as a quality control.

Results and discussion

Ethanol shock was not used in the course of this study, nor was alcohol selection of microorganisms conducted; we can therefore describe a wider range of species capable of growing in this medium. On the other hand, for both food and surface samples, no colony growing was observed in more than half of the plates analysed. Furthermore, cultured colonies were observed in low numbers, which facilitated the identification of different morphologies despite not having used the ethanol shock step.

From food samples, a total of 59 strains were isolated and identified by 16S rDNA sequencing analysis. Results revealed a total of 7 bacterial genera comprising 20 different species. The bacteria most frequently isolated belonged to the genera *Lactobacillus, Clostridium* and *Enterococcus.* Within these, the dominant species were identified as *Lactobacillus rhamnosus* (n=6), *Enterococcus faecium* (n=5) and *Enterococcus faecalis* (n=5) (Table 1). *C. sporogenes* (n=12) was the most common clostridia isolated. In agreement with the results of the present study, Limbago et al. (2012) [8] reported a total of 13 isolates identified as *C. sporogenes* obtained from ground beef and ground turkey after culture on *C. difficile* selective medium.

For environmental surfaces, a total of 8 different bacterial species were identified. Most of these species have been previously observed as able survive for months on surfaces [15]. *E. faecalis* (n=26) and *Eggerthella lenta* (n=14) were the bacteria most frequently isolated from the areas sampled. Regarding the genus *Clostridium,* only one isolate (*Clostridium tertium*) was obtained. Other species identified were *E. faecium* (n=2), *Staphylococcus haemolyticus* (n=2), *Staphylococcus capitis* (n=1), *Pediococcus pentosaceus* (n=2) and *Finegoldia magna* (n=2) (Table 2).

In the present study, all the described strains isolated from food and surface samples were able to grow in CCFT in the same culture conditions established for *C. difficile* recovery. The estimated concentration in the researcher-prepared agar of D-cycloserine was 400 µg/mL and 3.6 µg/mL for cefoxitin (with an average 20 mL of CCFT per plate). In the modified-CCFAT, which included the selective agents cefotaxime and cycloserine in the same concentrations, all of the isolated strains were also able to grow, except the only strain identified as *Weisella viridescens.*

Previously reported data describe a *C. difficile* minimal inhibitory concentration ≥1,024 µg/ml for D-cycloserine in 16 different strains of *C. difficile* [5]. However, in the available antibiotic management guidelines there are no disk breakpoints or critical concentrations for this drug. In relation to cefotaxime, according to the FSM, the sensitivity and resistant zone diameters proposed

are ≥21 mm and <15 mm, and the critical concentrations for susceptibility and resistance are ≤4 mg/L and >32 mg/L for strict anaerobes. However, it must be taken into account that these values refer only to therapeutic breakpoints.

For most of the isolated strains, the observed zone of inhibition was lower or equal to the size of the *C. difficile* inhibition zone. Results obtained from a D-cycloserine disc diffusion test (120 µg/disc) showed that for all of the isolates belonging to the genus *Clostridium, Paenibacillus, Pediococcus, Propionibacterium, Staphylococcus* and *Paenibacillus,* no inhibition zone was present in the plate. For the genus *Lactobacillus*, no inhibition zone was observed for any of the isolates except *Lactobacillus graminis* and *Lactobacillus salivarius,* for which zones of 22.7 mm and 28.3 mm in diameter were respectively detected. Regarding the genus *Enterococcus,* all of the species studied displayed an inhibition diameter between 13 mm and 16 mm except *Enterococcus gallinarum,* which had a maximum diameter of 22 mm. *E. lenta* showed a diameter of 29.6 mm while *F. magna* had a diameter of 26 mm. For cefotaxime (30 µg/disc), the results were more heterogeneous. Isolates belonging to the genus *Lactobacillus,* including *L. rhamnosus* and *L. graminis* showed full resistance to cefotaxime (no inhibition zone), while two other species of this genus, *Lactobacillus sakei* and *Lactobacillus casei,* had diameters of 19 mm and 20.5 mm respectively. Regarding the genus *Clostridium*, most of the species showed an inhibition zone ≥ 10 mm and ≤ 32 mm (*Clostridium orbiscidens* 31.3 mm; *C. sporogenes* 20.6 mm; *Clostrodium baratii* 15.6 mm; *Clostridium butyricum* 11.8 mm). Only three species, *C. tertium, Clostridium subterminale* and *C. difficile* presented full resistance to the drug. Most of the isolates belonging to the genus *Enterococcus* showed resistance (no inhibition zone) with only the *E. gallinarum* strain presenting an inhibition zone, 16.6 mm in diameter. *S. capitis* and *S. haemolitycus* also showed full resistance to cefotaxime (no inhibition zone). Other strains like *Paenibacillus lautus, Propionobacterium acnes, F. magna* and *E. lenta* had diameters of 18.6 mm, 23.2 mm, 22.9 mm and 22.4 mm respectively. While *P. pentosaceus* had a diameter of 16.6 mm*, Pediococcus acidilactici* showed no inhibition zone in the plate, indicating full resistance.

A total of 70 out of the 188 samples analysed (70.7%) were composed entirely of cooked ingredients while 55 (29.3%) contained one or more raw ingredients, such as lettuce, tomato, mushroom or raw meat. These percentages may explain why only 20 strains were isolated from raw food (mostly from fresh vegetables) while 40 strains were isolated from cooked food samples (all of them composed of meat or fish as the main ingredient). Samples were frozen before analysis, which may affect survival of some of the bacterial groups [16]. Regarding fresh vegetables, they can harbour large and diverse populations of bacteria. A previous study [17] demonstrated significant

differences in bacterial community structure dependant upon the type of vegetables involved, and also treatments undertaken in the course of production. In this context, several factors could play a role in the lower recovery of strains from raw food samples. Methods of cleaning and sanitizing vegetables can cause a significant reduction in the total plate count [18]. Dominant taxa in vegetables belong to aerobic groups, like *Pseudomonas, Xantomonas* or other non-Enterobacteriaceae species; therefore they are not detectable under the anaerobic culture conditions of this study [17, 19]. Regarding cooked foods, most of the bacteria found were classified in genus *Clostridium*, *Enterococcus* and *Lactobacillus*. Several studies have addressed the survival of *Clostridium* spores in extreme conditions in the environment. While freezing temperatures seem to have little impact on the viability of most of the spores, their viability at different temperatures varies by species. For example, viable spores of *C. sporogenes* and *C. butyricum* can survive temperatures of 100 °C for hours [20, 21]. Enterococci have shown an important heat resistance and, depending on the isolates and species, they can survive pasteurization temperatures [22]. Some species of *Lactobacillus* have also been shown to have the potential to survive pasteurisation. However, their resistance depends on genetic variations among strains, the physiological status of the cells and other environmental factors [23, 24]. Therefore, it is not surprising that these groups of bacteria (specially *Clostridium* and *Enterococcus*) were isolated more frequently from samples comprising fish or meat (even if they were cooked) as contamination with this faecal species would have occurred more frequently in slaughterhouse conditions compared to contamination in the environment.

On the other hand, the use of antimicrobial agents in animal production has caused an increase in the resistance of Enterobaceriaceae and other bacterial families, with higher production of β-lactamases, which hydrolyse the beta-lactam ring and inactivate the beta-lactams. The results are a high prevalence of extended-spectrum β-lactamase (ESBL)-producing Enterobacteriaceae in meat products. While the connection between ESBL-producing bacteria in food animals, retail meats and humans has been previously suggested [25], few publications describe ESBL-resistance in bacteria from vegetables, or identify which species were detected in which vegetable types [19]. In the present study we selected a final cefoxitin (CCFT) and cefotaxime (modified CCFT) concentration of 3.6 µg/mL. The epidemiological cut-off value (ECOFF) available for cefoxitin ranges between 4 µg/mL (*Staphylococcus aureus*) and 8 µg/mL (*Escherichia Coli, Klebsiella spp., Salmonella spp., Staphylococcus spp.*). The epidemiological cut-off value available for cefotaxime varies between ≤ 0.25 µg/mL (*Escherichia coli, Klebsiella spp., Streptococcus spp.*), 0.5 µg/mL (*Citrobacter spp., Enterobacter spp., Streptococcus spp.*), 1 µg/mL (*Yersinia enterocolitica, Serratia spp*.), 2-4 µg/mL (*Staphylococcus spp*.) and 32 µg/mL (*Pseudomonas aeuroginosa*). Most of the strains selected in

this study have probably already acquired resistance (at least to cefotaxime), therefore it is not surprising that twice as many isolates were obtained from cooked foods, including in most of the cases meats.

As in the case of meats and Enterobacteriaceae, several fermented foods have recently been suggested as potential vehicles for the exchange of antibiotic resistance genes between acid lactic bacteria and other pathogens in the gastrointestinal tract [26]. As most of the *Lactobacillus* species isolated in this study presented resistance to both of the drugs, it will be interesting to determine in the course of future studies whether the resistance of these strains results from an intrinsic mechanism or are due to genes encoding possible transferable resistance determinants.

In relation to the surface samples, the species belonging to genus *Enterococcus*, including *E. faecalis* and *E. faecium*, were frequently isolated from different swabs (kitchen, residents' rooms, private bathrooms and common areas). These species have been commonly found in clinical samples [15, 27] and observed to persist between 5 days and 4 months in hospital environments and on other inanimate surfaces [28, 29]. In this nursing home, residents' rooms are cleaned and disinfected daily using bleach-based disinfectants (sodium hypochlorite 10%). Automated gaseous decontamination of residents' rooms (stabilised hydrogen peroxide 6%) is also performed weekly; isolates from bathroom walls and bathroom floors were only obtained when sampling was performed before cleaning routine. Doorknobs, bedsides, cistern buttons, toilet seats and chamber pots were found contaminated after cleaning in only one resident's room (D), which may indicate less effort and time spent on cleaning this room. Beds were also found to be contaminated after being cleaned in three different rooms, but in each case isolates were obtained from the beds of dependant residents. The dependant classification was used for residents who were confined to bed; this means that at the moment of cleaning and at the moment of sampling the residents were present in the bed, which hinders cleaning procedures and also favours rapid recontamination of the sample surface. *E. faecalis* was isolated from the armrest of one invalid chair. This chair was in the resident's room, however it was not treated as part of the cleaning routine. Room walls and room floors were most frequently contaminated before cleaning was performed. The only floor (room D) that was contaminated after cleaning was also from a dependent resident receiving nursing assistance with the continuous circulation of the nursing staff a likely source of the floor contamination. It should be noted that this room (D) was inhabited by a patient diagnosed with *C. difficile* infection (CDI) nine days before the study began and positive for the bacterium at the moment of sampling. For residents suffering from CDI, the protocol implementing by the healthcare facility prescribes the automated gaseous decontamination of the room every day.

However, in this specific case, the critical health status of the patient required continuous monitoring by the nurses and medical assistants, resulting in the constant movement of medical personnel around the room. Therefore, although special measures were taken by the staff (double gloving if manipulating faeces, constant disinfection of hands), automated gaseous decontamination was not possible, at least before surface sampling was performed. The flow of personnel in this room could also have contributed to the fact that this was the room most contaminated after cleaning. There was one other resident positive for *C. difficile* at the moment of sampling (room E), however while the bacterium was detected in their faeces, CDI was not diagnosed and therefore special protocols of disinfection were not applied.

Besides *Enterococcus*, *E. lenta* was the most commonly bacterium isolated. *E. lenta* is an anaerobic Gram-positive non-sporulating bacteria poorly studied due to difficulties with phenotypic identification. It is recognised as a part of the normal human intestinal microbiome but it has been also associated with gastrointestinal infections. A recent study identified *E. lenta* in 33 patients suffering intra-abdominal pathology with median age of 68 years [30]. In relation to elderly people and gut microbiota, decreased microbial diversity is correlated with increased age. Furthermore, individuals living in short or long-term residential facilities have been shown to have less diversity in microbiota than those living in the community [31]. It seems that long-term residential subjects have a higher proportion of Bacteroidetes in their gut, whereas elderly people in the community have a higher proportion of Firmicutes [32]. Reductions in some clostridia or bifidobacteria species and proliferation of opportunistic bacteria such as *E. faecalis* were also reported in hospitalized elderly patients [33]. In this study, only one isolate obtained from the internal doorknobs of the kitchen staff bathroom was identified as *C. tertium*. These findings could suggest that *Clostridium* species were sub-dominant in faecal microbiota of these elderly residents, and explains why other species present in higher proportions and resistant to the selective agents used in the medium were more commonly isolated.

In conclusion, this study focuses on the identification of bacteria growing on selective media (CCFT and modified CCFT). These *C. difficile* home-made culture media have a relatively low cost but offer high sensitivity for research purposes. Data reported provide the identification of the spectrum of bacteria growing on CCFT, which could also help further environmental screening studies in nursing homes and other healthcare environments.

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Table 1. 16S rDNA sequencing identification of bacteria growing on the CCFAT medium isolated from food samples after CCFT enrichment

Isolated bacterium	Total number of isolates	Sample Weeks[1]	N° of isolates/ Week[2]	Samples composed of one or more raw ingredients	Sample composed of cooked ingredients only
Genus *Clostridium*					
Clostridium baratii	1	29/03	1	0	1
Clostridium butyricum	2	10/05	2 [A]	1	1
Clostridium orbiscindens	1	24/05	1	0	1
Clostridium sporogenes	12	22/03 [C]	4 [A]	1	3
		26/04	2	0	2
		03/05	2	1	1
		17/05	1	0	1
		24/05	1	1	0
		07/06	1	0	1
		28/06	1	0	1
Clostridium subterminale	4	22/03	1	0	1
		05/04	2 [A]	0	2
		12/04 [D]	1	0	1
Genus *Enterococcus*					
Enterococcus casseliflavus	3	07/06	1	1	0
		14/06	1	0	1
		28/06	1	1	0
Enterococcus durans	3	29/03 [B]	2	1	1
		31/05	1	0	1
Enterococcus faecalis	5	29/03	1	0	1
		10/05 [E]	2[A]	1	1
		20/06 [F]	1	0	1
		28/06	1	1	0
Enterococcus faecium	5	24/05	1	0	1
		14/06	1	0	1
		20/06 [F]	1	1	0
		28/06	2	0	2
Enterococcus gallinarum	1	14/06	1	0	1
Genus *Lactobacillus*					
Lactobacillus sakei	3	29/03	1	0	1
		12/04	1	1	0
		28/06	1	0	1
Lactobacillus salivarius	1	28/03	1	1	0
Lactobacillus rhamnosus	6	29/03 [B]	1	0	1
		05/04	1	0	1
		12/04 [D]	1	0	1
		19/04	1	0	1
		10/05 [E]	1	0	1
		24/05	1	0	1
Lactobacillus casei	2	19/04	1	1	0
		31/05	1	1	0
Lactobacillus graminis	1	17/05	1	1	0
Genus *Paenibacillus*					
Paenibacillus lautus	1	24/05	1	0	1
Genus *Pediococcus*					
Pediococcus pentosaceaus	5	03/05	1	0	1
		10/05	1	1	0
		17/05	2	2	0
		28/06	1	0	1
Pediococcus acidilactici	1	10/05	1	1	0
Genus *Propionobacterium*					
Propionobacterium acnes	1	22/03 [C]	1	0	1
Genus *Weisella*					
Weisellla viridescens	1	14/06	1	0	1

[1] The date refers to the Friday on which samples from the proceeding week were collected and transported to the laboratory for immediate analysis.

[2] Number of the different bacterial species obtained from food samples in each week of sampling

[A] Two isolates were from food prepared on the same day but in a different services

[B,C,D,E,F] Two different colonies were subcultured from the same sample

402

403 **Table 2.** Different bacteria from the nursing home environment isolated on CCFT

Sampling area	N° of samples	Isolated bacterium	N° of isolates	Specific information regarding the isolate area
Kitchen		-	-	
External kitchen doorknobs	4	-	-	
Internal Kitchen doorknobs	4	-	-	
Refrigerators handles	2	-	-	
Cover of the food warmer (bain marie)	2	*Eggerthella lenta*	1	
Kitchen cutting board for meat	2	-	-	
Kitchen cutting board for vegetables	2	*Eggerthella lenta*	1	
Slicer machine	2	*Pediococcus pentosaceus*	1	
Oven handle	2	-	-	
Touch control kitchen faucet	4	*Eggerthella lenta*	1	
Meal deliverey carts (for rooms and canteen)	14	*Enterococcus faecalis*	1	Carts for canteen
Trays (for rooms and canteen)	8	*Enterococcus faecalis*	1	Tray for canteen
Kitchen wall	2			
Kitchen floor	2			
Kitchen staff bathroom and locker room				
External doorknobs	9	*Eggerthella lenta*	1	
Internal doorknobs	9	*Clostridium tertium*	1	Toilet internal doorknob
Toilet seat	4	*Eggerthella lenta*	2[1]	
Cistern flush button	2			
Paper towel dispenser	2	*Eggerthella lenta*	1	
Shower controls	2			
Sink faucet	2			
Soap dispenser	2			
Towel bar	2			
Control knob (radiator)	2			
Bathroom wall	2			
Bathroom floor	2	*Eggerthella lenta*	1	
Light switch	2	*Eggerthella lenta*	1	
Residents' rooms				
External doorknobs	8	*Eggerthella lenta*	1	Room E
Internal doorknobs	8	*Enterococcus faecium*	1	Room F
		Enterococcus faecalis	1	Room D
Bedside	8	*Finegoldia magna*	1	Room D
		Enterococcus faecalis	1	Room F
		Eggerthella lenta	1	Room E
Bed	8	*Finegoldia magna*	1	Room D
		Enterococcus faecalis	2	Room G/B
Invalid chair	1	*Enterococcus faecalis*	1	Room D
Room wall	8	*Enterococcus faecalis*	1	Room F
Room floor	8	*Enterococcus faecalis*	4	Room D/E/F/B
Private bathrooms				
External doorknobs	8	*Enterococcus faecalis*	2	Room D/0
		Staphylococcus haemolyticus	1	Room E
Internal doorknobs	8	*Staphylococcus haemolyticus*	1	Room D
Sink faucet	8	*Enterococcus faecalis*	1	Room A
		Staphylococcus capitis	1	Room E
		Eggerthella lenta	1	Room E
Cistern flush button	8	*Enterococcus faecalis*	2	Room D/C
Toilet brush handle	8			
Toilet seats	8	*Enterococcus faecalis*	1	Room D
Toilet support bar	6	*Eggerthella lenta*	1	Room F
Towel	8	*Enterococcus faecalis*	4	Room D/G/0/A
Chamber pot	1	*Enterococcus faecalis*	1	Room D
Bathroom wall	8	*Eggerthella lenta*	1	Room E
Bathroom floor	8	*Enterococcus faecalis*	1	Room E
Common areas				
Couch	2			
Coffee table	2			
Elevator control panels	12	*Enterococcus faecalis*	2[1]	
		Pediococcus pentosaceus	1	
Staircase railings	4	*Enterococcus faecium*	1	
Hall wall	2			
Hall floor	2	*Enterococcus faecalis*	1	

404 [1] One isolate from each sampling day.
405 Sampling before cleaning: rooms 0, C, E, F
406 Sampling after cleaning: rooms A, B, D, G
407 Rooms with residents tested positive for *C. difficile* at the time of sampling: D, E