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Credit author statement

Rodriguez C: conceptualization, methodology, formal analysis, writing-original draft

Mith H: conceptualization, sampling collection, data collection and analysis

Taminiau B; Garcia-Fuentes E; Korsak N; Delmée M; Daube G: conceptualization, supervision, review and editing.

Bouchafa L, Van Broeck J, Soumillon K, Ngyuvula E: methodology, resources, data curation and analysis

FIRST ISOLATION OF CLOSTRIDIOIDES DIFFICILE FROM SMOKED AND DRIED FRESHWATER FISH IN CAMBODIA

Rodriguez C[1,2#*], Mith H[3#], Taminiau B[4], Bouchafa L[4], Van Broeck J[5], Soumillon K[5], Ngyuvula E[5], García-Fuentes E[1,2**], Korsak N[4], Delmée M[5], Daube G[4].

[1]UGC Aparato Digestivo, Hospital Universitario Virgen de la Victoria, Málaga Spain.
[2]Instituto de Investigación Biomédica de Málaga (IBIMA), Málaga, Spain.
[3]Faculty of Chemical and Food Engineering, Institute of Technology of Cambodia. Russian Federation Blvd., PO Box 86, Phnom Penh, Cambodia.
[4]Laboratory of Food Microbiology, Fundamental and Applied Research for Animals and Health Center (FARAH), Department of Food Sciences, Faculty of Veterinary Medicine, University of Liege, Belgium.
[5]National Reference Center *Clostridium difficile*, Microbiology Unit, Catholic University of Louvain, Avenue Hippocrate 54, Bte B1. 5405, 1200 Brussels, Belgium.

[#]equal contribution

Correspondence:
*corresponding author: cris.rdrz@gmail.com
**corresponding author: edugf1@gmail.com
Hospital Civil,
Laboratorio de Investigación,
Plaza del Hospital Civil s/n,
Málaga 29009, Spain.

Abstract

In Cambodia, freshwater aquaculture is the most important source of food production. Fresh fish meat is considered a highly perishable food that requires the use of different manipulations and preservation techniques to inhibit the proliferation of undesirable bacteria. These bacteria are naturally present in the raw product or could be acquired during manipulation by cross-contamination. Many studies worldwide have investigated the epidemiology of *Clostridioides difficile* (*C. difficile*) in food, but to date, there are no publications about the bacterium in ready-to-eat fish or descriptions in Cambodia. The objective of this study was to assess the presence of *C. difficile* in one of the main food supplies of this country, smoked freshwater fish, originating from different provinces. A total of 25 samples were collected directly from local markets, yielding 4 *C. difficile* isolates and an overall recovery rate of 16%. Most of the isolates were toxigenic and classified as rare PCR profiles, and they were resistant to clindamycin. These findings indicate contamination during handling and/or contamination of the raw fish, followed by insufficient heat treatment to kill the spores. The presence of *C. difficile* in smoked and dried fish implies a potential risk of human exposure, contamination and infection.

Keywords

Clostridioides difficile, smoked dried freshwater fish, cross-contamination, ready-to-eat food, antibiotic resistance, food contamination

1. Introduction

Clostridioides difficile (historically classified as *Clostridium difficile*) is a spore-forming anaerobic bacterium ubiquitous in the natural environment and is considered the leading cause of nosocomial antibiotic-associated diarrhea in developed countries. Although the infection is classically related to healthcare settings, several cases in the community have been detected in recent years. Some recent regional studies reported a prevalence between 40% and 50% of community *C. difficile* infection (CDI) cases, with a proportion that increases yearly but decreases during winter months (Crobach et al., 2019; Suarez-Bode et al., 2019). These findings have led to the further study of bacterial routes of transmission.

Different types of soils, farmlands and rivers are often contaminated with bacterial spores and constitute a potential source of transmission for both animals and humans. Some evidence of this direct or indirect exposure is the contamination of rivers, puddle water, animal manure, composts, farm environments and food products (Rodriguez et al., 2018). In this context, the presence of *C. difficile* in some fresh food products has been repeatedly reported in Europe and America in the last decade. Cross-contamination of carcasses at slaughterhouse has been suggested (Hampikyan et al., 2018; Rodriguez et al., 2014; Rodriguez et al., 2013), and the bacterium has been observed in uncooked meat with a prevalence of approximately 10% and a maximum of 20% (Lee et al., 2018; Rodriguez et al., 2014; Rodriguez-Palacios et al., 2007). Vegetables such as potatoes, beetroots, onions and carrots have been found to be contaminated and therefore involved in potential foodborne transmission of the bacterium (Tkalec et al., 2019; Lim et al., 2018, Eckert et al., 2013). Spores of *C. difficile* have also been isolated from seafood, with a prevalence of 11.6% and 23.2% in mussels and clams, respectively (Agnoletti et al., 2019). Further studies also detected the bacterium in fresh salmon, shrimp and scallops (Norman et al., 2014; Metcalf et al., 2011). The presence of *C. difficile* has been previously demonstrated in saltwater samples from the sea coastline, rivers and lakes (Al Saif et al., 1996); therefore, contamination of shellfish probably occurs directly in the marine environment. However, for other fish products, especially those that undergo postmortem manipulation, cross-contamination during their transformation cannot be excluded (Arvanitoyannis et al., 2008). In the context of processed food, only a few reports found *C. difficile* in ready-to-eat foods, such as salads (Romano et al., 2018), or other cooked meals, including a ready-to-eat sample composed of pork sausage, mustard sauce and carrot salad (Rodriguez et al., 2015a).

In Cambodia, freshwater aquaculture is one of the most important sources of food production. Fresh fish meat is considered a highly perishable food that requires the use of different manipulations and preservation techniques not only to reduce water activity but also to inhibit the development of several undesirable bacteria (Hubackova et al., 2014). These bacteria are naturally present in the raw product or could be acquired during manipulation by cross-contamination

Many studies worldwide have investigated the epidemiology of *C. difficile* in humans, animals and food, but to date, there are no publications about the bacterium in ready-to-eat fish or descriptions in Cambodia. The objective of this study was to investigate the presence of *C. difficile* in one of the main food supplies of this country, smoked and dried freshwater fish, originating from five different provinces. The aim was to determine how healthy individuals in the community may be exposed to *C. difficile* by food ingestion. *C. difficile* isolates obtained for the first time in Cambodia were characterized by PCR ribotyping, toxin gene profiling and antibiotic resistance.

2. Materials and methods

2.1 Sample collection

Twenty-five samples of smoked and dried freshwater fish were collected from local markets and producing sites from five provinces, Battambang, Kampong Chhnang, Kampong Cham, Kampong Thom and Siem Reap, in Cambodia (Supplementary file 1). Each sample was collected from one individual sampling point to exclude the detection of positive samples in a single stall and to obtain a better representation of the extent of bacterial distribution. These samples, often found available at local markets and sold as ready-to eat products, corresponded to nine species of smoked and dried freshwater fish (Supplementary file 2). All collected samples were wrapped in aseptic plastic bags, frozen and transported to the laboratory. The samples were aseptically ground, lyophilized and stored at -80°C prior to further analysis.

2.2 *C. difficile* isolation and identification

Culture of fish samples was performed following the protocol and with the same selective medium (cycloserine cefotaxime fructose taurocholate) used in Rodriguez et al. (2019). In this study, two parallel plates were used for each single sample. For the detection of spores present in low numbers, enrichment cultures were also performed. Suspected colonies were identified by morphological criteria, subcultured onto blood agar and checked using a *C. difficile* latex agglutination rapid test kit DR 1107A (Oxoid, FR). Multiple colonies were taken when morphologies suggested more than one type of PCR ribotype or when the presumptive colonies were too small to ensure isolation on blood agar. Confirmation of *C. difficile* was performed by detection of a species-specific internal fragment of the *tpi* gene and detection of genes for toxin A (*tcdA*), toxin B (*tcdB*) and binary toxin (*cdtA*) by classical PCR (Rodriguez et al., 2013). PCR ribotyping based on capillary gel electrophoresis was performed using the primers and the method proposed by Bidet et al. (1999) and Fawley et al. (2015), respectively. International nomenclature was used for *C. difficile* strains that presented a PCR ribotype profile matching the Cardiff ribotypes (Anaerobic Reference Unit (ARU), UK) from the strain collection available in our laboratory. Otherwise, strains were identified with an internal nomenclature system beginning with UCL (database at the Catholic University of Louvain, National Reference Laboratory for *C. difficile* in Belgium) or as rare profiles if the strains presented new PCR ribotype profiles never detected in our laboratory before. The results were further analyzed using the web-based database WEBRIBO (Indra et al., 2008).

2.3 Antimicrobial susceptibility testing

Resistance to erythromycin (15 µg), vancomycin (5 µg), clindamycin (2 µg), tetracycline (30 µg), metronidazole (5 µg) and moxifloxacin (5 µg) (Oxoid) was tested through a disc diffusion assay on Brucella Blood Agar with hemin and vitamin K1 (Oxoid) according to the French Society of Microbiology protocols (SFM, 2017). Zone diameters were measured after 24 h of anaerobic incubation at 37°C and interpreted as previously described (Rodriguez et al., 2014). *Bacteroides fragilis* ATCL 25285 was included as a quality control.

3. Results and discussion

3.1 Prevalence of *C. difficile*

A total of 25 samples were collected directly from local markets, yielding four *C. difficile* isolates and an overall recovery rate of 16%. Only a few previous studies have

investigated the presence of the bacterium in sea products, and most of them focused on bivalve mollusks (Figure 1). In fish, Al Saif and Brazier (1996) reported negative results when investigating the bacterium in 107 samples obtained from fishery stores in Cardiff. Norman et al. (2014) detected the presence of the bacterium in frozen whole wild-caught pink salmon (whose origin was Alaska) collected from a local grocery in Texas. A third study (Metcalf et al. 2011) reported the isolation of *C. difficile* in fresh perch and in fresh salmon purchased on different days and from different grocery stores. Both studies with positive results identified the strains as toxigenic and belonging to PCR ribotype 078. This PCR ribotype has been largely isolated from animals and food (Krutova et al., 2018; Rodriguez et al., 2018) and is associated with interspecies clonal transmission between animals and humans (Knight and Riley, 2019). To the best of our knowledge, this is the first study reporting the presence of *C. difficile* in ready-to-eat smoked and dried freshwater fish. Furthermore, to date, no study has previously investigated this bacterium and its epidemiology in Cambodia.

Positive samples were collected from 3 different provinces (Battambang, Kampong Chhanang and Kampong Cham) separated within a radius of approximately 500 km and from 4 different sellers. Samples with *C. difficile* spores belonged to 3 fish species, *Ompok bimaculatus, Paralaubuca typus* and *Clarias macrocephallus* (supplementary file 2). All of them are found in quiet slow-flowing rivers, often muddy or stagnant water, sandy streams or inundated fields. Aquatic pollution caused by human activities is directly associated with important health problems for both animals and humans. Due to recreational as well as other pollution activities in coastal and river city waters, such as sewage and drain discharges, this environment is increasingly contaminated by microorganisms, especially those with fecal origins (Kacar et al., 2017). Discharges of fecal matter-contaminated water could be associated with an increase in *C. difficile* presence in the aquatic medium, and therefore, it represents a risk for public health. A previous study investigating the rivers and the coastline of the Bristol Channel found the presence of *C. difficile* with spore counts ranging from 3 to 6 cfu/100 ml and a prevalence of 43.7%. Furthermore, most of the isolates obtained were identified as toxigenic. These findings demonstrated that the population could be exposed to *C. difficile* directly via sea water and indirectly via seafood.

In Cambodia, traditional smoking is the most common procedure to preserve food due to the general low level of industrialization. This process includes salting, evisceration and wood smoking, with a final temperature in the product of approximately 80°C. The total smoking time can vary between 1 and 5 days based on the fish species, the type of firewood and the kiln. Therefore, all of these variable factors will finally determine the quality of the final product (Slamova et al., 2017). As with other clostridial species, *C. difficile* is able to survive in unfavorable environments due to its sporulation capacity. In processed foods, it has been shown that food preservatives, such as nitrites, cannot kill *C. difficile*, but they can inhibit the growth of the bacterium (Lim et al., 2016). The thermal resistance of *C. difficile* spores depends on the matrix. In meat, previous studies reported minimal destruction of spores at 70°C for 3 hours and the recovery of viable spores even after incubation at 85°C- 90°C for 10 minutes (Redondo-Solano et al., 2016; Rodriguez-Palacios et al., 2011; Lawley et al., 2009). This resistance to thermal treatments and food preservatives could explain the presence of the bacterium in chorizo and other ready-to-eat foods, such as summer sausage, Braunschweiger sausage and a meal sample composed of pork sausage, mustard sauce and salad. The isolates belonged mainly to PCR ribotypes 078 and 027 (Rodriguez et al., 2015a; Songer et al., 2009; Harvey et al., 2011).

In our study, 3 out of 4 isolates were positive for the presence of genes encoding toxins A and B, but the *cdtA* gene fraction of the binary toxin CDT was not detected in any of the samples. Only one isolate was negative for all toxin genes. Following our nomenclature system, none of the PCR ribotypes identified had profiles that corresponded to the ARU Cardiff collection. Furthermore, only 1 out of 4 isolates (PCR ribotype UCL36) had a ribotype profile matching our available strain collection (database at the Catholic University of Louvain, National Reference Center for *C. difficile* in Belgium). PCR ribotype UCL36 is a nontoxigenic type that has been previously isolated from human feces, pig carcasses and soils in recreative walking areas (Rodriguez et al., 2019; Rodriguez et al., 2015b). In our previous study, we showed that in a neighbor-joining phylogenetic tree constructed with multilocus sequence typing results, the *C. difficile* type with the closest genetic proximity to PCR ribotype UCL36 was the toxigenic PCR ribotype 081. The same results were observed when the strains were analyzed using multilocus variable tandem-repeat analysis (Rodriguez et al., 2015b). The remaining 3 isolates were classified as rare profiles, as they had never been detected in our laboratory before. Using the Webribo database, all of the isolates were identified as a new ribotype.

In Asia, *C. difficile* epidemiology and infection are not well understood. In Cambodia, there are no previous studies about the presence of the bacterium in humans, animals or food. However, in neighboring countries, including Malaysia, Indonesia, Thailand and Singapore, the reported prevalence of infection in humans is high, ranging between 9% and 11%, and the most common PCR ribotypes described are 017 and 369 (toxin profile A-B+CDT- (Collins and Riley, 2019)). Some surveys in local hospitals also described the presence of PCR ribotypes 078 and 027 in human feces and in the environment, both of which are binary toxin-positive strains (Jia et al., 2016; Jin et al., 2016). Antibiotic resistance of these *C. difficile* Asian isolates to clindamycin, metronidazole, ciprofloxacin and moxifloxacin has been reported (Wang et al., 2018), even among nontoxigenic isolates (Moura et al., 2013). In our study, all of the isolates were resistant to clindamycin, and one isolate with a rare profile presented resistance to moxifloxacin and tetracycline. Resistances to clindamycin have been previously described in strains identified as PCR ribotype 36 from animal origin, as well as in other toxinogenic ribotypes isolated from humans, cattle and pigs (Rodriguez et al., 2015b). Treatments with clindamycin have been associated with diarrheic outbreaks of CDI (Johnson et al., 1999; Thibault et al., 1991; Tedesco et al., 1974), and clinical practice guidelines recommend restrictions in its use as a function of local epidemiology (McDonald et al., 2018). Moxifloxacin use has been associated with increased cases of CDI (Wenisch et al., 2014), and resistance to this drug was described in different PCR ribotypes isolated from humans, cattle and pigs (Rodriguez et al., 2015b). Furthermore, corresistance of newly emergent *C. difficile* PCR ribotypes towards moxifloxacin, clindamycin, tetracycline and erythromycin has been recently described in Asia (Chow et al., 2017).

One positive sample (PCR ribotype classified as rare and toxigenic) was detected by direct culture and was also positive after enrichment. The total count of spore levels in this sample was 100 cfu/g. With our method, the limit of detection is 50 cfu/g for the direct culture and 10 cfu/g for the enrichment culture (Rodriguez et al., 2018). The remaining three positive samples were detected only after an enrichment step, indicating that the spore load of the samples was low. Previous studies in foods also reported low levels of *C. difficile* spores, with a mandatory enrichment method to isolate the bacterium. The fish contamination could have originated after thermic treatment, from the food handlers, or by contact with contaminated surfaces. However, it is also possible that the bacterium was

present in the intestine of the fish and that contamination occurred during gutting/handling. At the treatment temperature (approximately 80°C), there is a sublethal effect, but the recovery of viable spores is still possible. This may explain why, in 3 samples, the bacterium was not detected by direct culture but isolated after making conditions of growth very favorable for this pathogen. In animal models with antibiotic treatment, bacterial colonization and diarrhea occur at less than 10 cfu (Larson and Borriello, 1990; Lawley et al., 2010), but in humans, the dose of infection has not yet been established, and it is likely that it varies between strongly susceptible and healthy individuals. Furthermore, it is possible that continuous exposure to the bacterium by foods or contact with a contaminated environment could finally lead to the development of infection.

4. Conclusions

Our results describe for the first time the presence of the pathogen *C. difficile* in ready-to-eat smoked and dried freshwater fish. Furthermore, we isolated for the first time the bacterium in Cambodia, underlining the need for additional epidemiological studies in this country. *C. difficile* was detected before and after enrichment, which indicates contamination during handling and/or contamination of the raw fish, followed by insufficient heat treatment to kill the spores. Most of the PCR ribotypes isolated were toxigenic and belonged to rare PCR ribotype profiles never detected in our laboratory before. In Cambodia, there are no previous studies about the presence of the bacterium in humans, animals or food; therefore, at this moment, it is not possible to establish further relationships between food and human isolates. As aquaculture is one of the main sources of food production in this country, the population may be continuously exposed to the bacterium. Further studies on the proportion of spore-forming bacteria in the gut microbiota of humans and animals in the Cambodian population are needed to determine if there is bacterial adaptation as a function of the levels of exposure.

Disclosure of Potential Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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Ethical Statement

An ethical statement is not applicable.

Figure 1. Presence and characterization of *C. difficile* isolates in different types of seafood: bivalve mollusk, fish and decapod crustaceans. Data from Refs. Norman et al., 2012; Metcalf et al., 2011; Pasquale et al., 2011; Pasquale et al., 2012; Agnoletti et al., 2019; Candel-Pérez et al., 2019

Supplementary files

Supplementary file 1. Smoked and dried freshwater fish originated from provinces in Cambodia

Supplementary file 2. Smoked and dried fish samples originating from nine freshwater fish species in Cambodia. (a) *Clarias macrocephallus*, (b) *Paralaubuca typus*, (c) *Cirrhinus siamensis*, (d) *Micronema bleekeri*, (e) *Rasbora tornieri*, (f) *Ompok bimaculatus*, (g) *Thynnichthys thynnoides*, (h) *Belodontichthys truncatus*, (i) *Clupeoides borneensis.*

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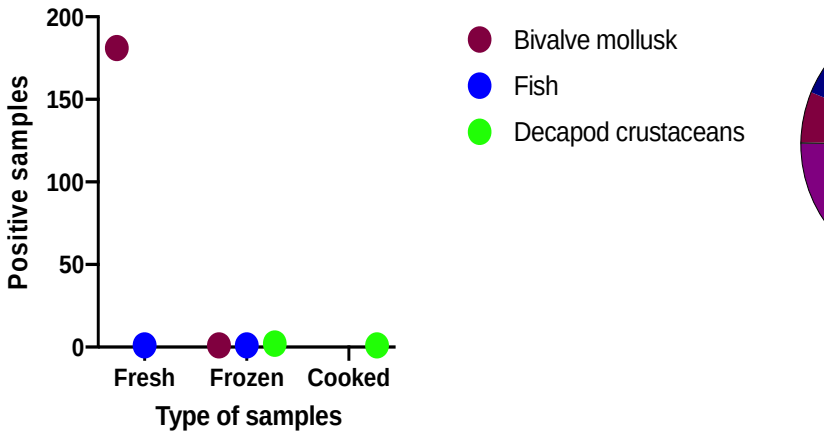
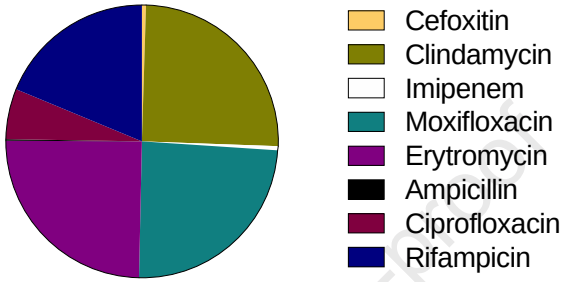
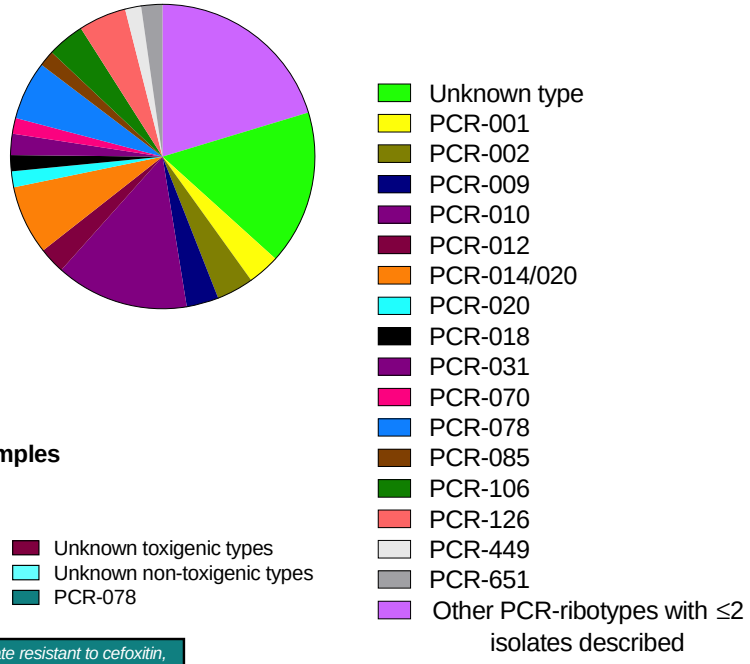
C. difficile in seafood, 177 isolates



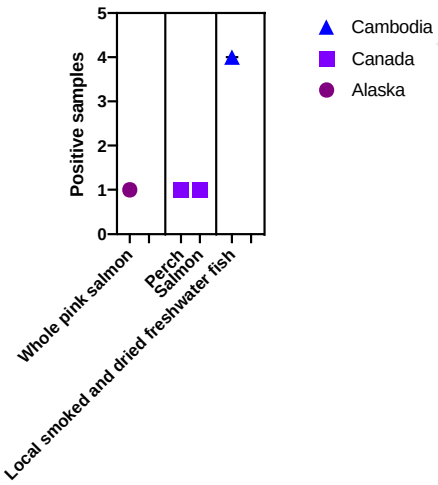
Antibiotic resistances detected in seafood, 177 isolates



C. difficile PCR-Ribotypes in seafood, 177 isolates

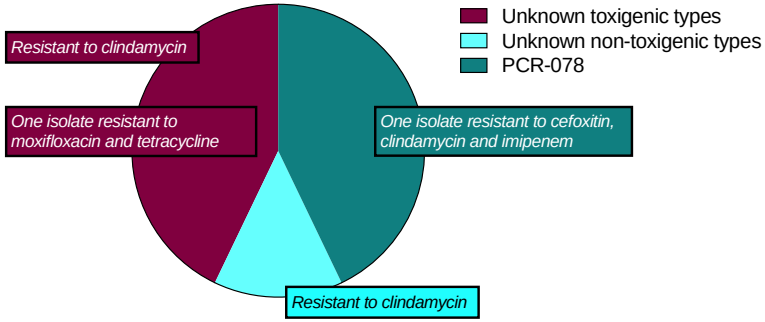


Presence of _C. difficile_ in fish samples



*Graph includes available data in the literature and the findings of the present work

C. difficile PCR-Ribotypes in fish samples



*Graph includes available data in the literature (n=3; PCR-078) and the findings of the present work (n=4; unknown PCR-ribotypes)

Disclosure of Potential Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.