Toxigenic *Clostridium difficile* PCR ribotypes from wastewater treatment plants in Southern Switzerland

Running title: *Clostridium difficile* from wastewater treatment plants

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The occurrence of *Clostridium difficile* in 9 wastewater treatment plants in the Ticino Canton (Southern Switzerland), was investigated. The samples were collected from raw sewage influents and from treated effluents. Forty seven out of 55 characterized *C. difficile* strains belonged to 13 different reference PCR ribotypes (009, 010, 014, 015, 039, 052, 053, 066, 070, 078, 101, 106, and 117), whereas 8 strains did not match any of those available in our libraries. The most frequently isolated ribotype (40%) was 078, isolated from 6 wastewater treatment plants, whereas ribotype 066, a toxigenic emerging ribotype isolated from patient admitted to hospitals in Europe and Switzerland, was isolated from the outgoing effluent of 1 plant. The majority of the isolates (85%) was toxigenic. Forty nine per cent of them showed the toxigenic profile A’B’CDT^+^, whereas 51% showed the profile A’B’CDT^−^.

Interestingly, 8 ribotypes (010, 014, 015, 039, 066, 078, 101, and 106) were among the riboprofiles isolated from symptomatic patients admitted to the hospitals of the Ticino Canton in 2010. Despite the limitation of sampling, this study highlight that toxigenic ribotypes of *C. difficile*, involved in human infections, may occur in both incoming and outgoing biological wastewater treatment plants. Such finding arises concern about the possible contamination of water bodies receiving wastewater treatment plant effluents and for the safe re-use of treated wastewater.

**INTRODUCTION**

*Clostridium difficile* is a Gram positive, anaerobic, endospore forming bacterium isolated for the first time by Hall and O’Toole (16) as a commensal microorganism of the intestinal microbiota of healthy newborn infants. *C. difficile* is commonly considered a nosocomial pathogen causing antibiotic associated diarrhea and pseudomembranous colitis (5). Toxins are considered the main virulence factors of this microorganism (38). Toxigenic strains of *C. difficile* produce different toxins: toxin A (tcdA, an enterotoxin), toxin B (tcdB, a cytotoxin) that directly mediate diarrhea and
colitis (35, 12) and sometimes an additional toxin, the binary toxin (CDT). A correlation between binary toxin production and severity of C. difficile infection has been reported by Barbut et al. (4), although a clear role in pathogenesis has yet to be demonstrated. C. difficile associated diarrhea is one of the most common nosocomial infection worldwide and a significant cause of health care-associated morbidity and mortality, particularly among the elderly people (17, 23, 28). Outbreaks of C. difficile infections (CDI) with increased gravity and significant mortality have been related to the emergence of highly virulent strains B1/NAP1/027 (toxinotype III) and Ribotype 078 (toxinotype V) in North America, Europe and Asia (15, 23, 24, 27) sharing similar virulence markers. The CDI caused by 078 ribotype are increasing, particularly in young people, with no previous contact with hospitals, and in community acquired infections (6, 13, 17).

Considering that the community acquired CDI are on the increase in Western countries (10, 32), a possible role of contaminated food and environment in the diffusion of this pathogen has been hypothesized (19, 34). Recently, some authors described the occurrence of C. difficile in vegetable potentially subjected to be irrigated with contaminated water. In 1996, Al Saif and Brazier (1) reported C. difficile contamination in 7 out of 300 unwashed raw vegetable samples (carrot, cucumber, mushroom, onion, potato, and radish) on sale in retail outlets; 5 isolates were toxin A positive. Bakri et al. (3) analyzed 40 ready-to-eat salads and found 3 samples contaminated with C. difficile: 2 isolates belonged to ribotype 017 (A‘B‘) and 1 to ribotype 001 (A+B+). Metcalf et al. (30) reported the occurrence of C. difficile in 5 of 111 vegetable samples (ginger, carrot, and eddoes): 3 of them were ribotype 078/NAP 7/toxinotype V, genetically indistinguishable from the hypervirulent ribotype 078 associated with severe CDI in humans.

According to Dubberke et al. (11), environment and animals may, thus, be an important reservoir and source of exposition of pathogenic strains of C. difficile.

Only few studies, however, have reported the isolation of C. difficile from water ecosystems (1, 33, 40, 45). Laine et al. (25) described an extensive waterborne gastroenteritis outbreak occurred in
autumn 2007 in Finland, as a consequence of the accidental contamination of the drinking-water network with sewage effluents from a municipal wastewater treatment plant (WWTP). *C. difficile* was recovered from drinking water samples and faecal specimens of symptomatic people, together with 6 other pathogens. Viau and Peccia (2009) found *C. difficile* in biosolids issued from a WWTP and Norman et al. (2011) in sewage of an closed and integrated human and swine population in the USA.

This study investigates the occurrence, the genotypic features and toxigenic profiles of *C. difficile* isolated from untreated and treated water from different WWTPs in Southern Switzerland, as treated wastewater could act as carrier of *C. difficile* environmental contamination.

**MATERIALS AND METHODS**

**Sampling.** Samples were collected, from May 12th to in May 13th 2010, from the inlets and outlets of 9 WWTPs located in the Canton Ticino, Southern Switzerland, processing both urban and industrial wastewater. The capacity of the plants ranges from 18,000 to 186,667 population equivalents, corresponding to 60g BOD₅/d per population equivalent as pollution load in the treatment plant influent (BOD₅ stands for “Biological Oxygen Demand”, a standard procedure assessing the biologically oxidable organic carbon in water over a 5 days incubation) The wastewater treatment included grid separation, primary sedimentation and secondary biological treatment (activated sludge process). No disinfection treatments or tertiary water treatments were carried out during the sampling period. Rivers were the receiving bodies of the treated water. The sampling was carried out taking into account the residence time of the wastewater, in order to sample in the outlet the same water sampled in the wastewater inlet. Two samples of wastewater were collected, by sterile bottles, from each WWTP: 1 sub-surface sample was taken from the inflow pond, after the grid separation of the raw wastewater, and 1 sample was taken from the plant outflow pipe.
**Culture conditions.** Ten ml of treated water and 10 ml of pre-filtered (Wathman filter 40) raw wastewater samples were filtered through a 0.45 µm pore size nitrocellulose membrane filter (Millipore, Billerica, MA). Each filter was then immersed in 40 ml of Brain Heart Infusion Broth (Oxoid, Basingstoke, UK) supplemented with 1.0 g/L taurocholic acid sodium salt hydrate (Sigma, St. Louis, USA) and *C. difficile* Selective Supplement (Oxoid). The cultures were incubated at 37°C for 10 days in anaerobic jars with the AnaeroGen (Oxoid) anaerobic atmosphere generating system (37). Thereafter, an alcohol shock was performed by mixing 2 ml (1:1 v/v) of broth cultures with 96% ethanol, left at room temperature for 50 min. and centrifuged at 3,000 rpm for 10 min. After centrifugation, the supernatant was discarded, and an aliquot of the pellet was streaked onto Cefoxitin Cycloserine Egg Yolk (Oxoid) agar. The plates were incubated under anaerobic conditions at 37°C for 48 h.

**Detection of gluD, tcdA, tcdB, cdtA, cdtB genes.** Yellow and rhizoid colonies of spore-forming Gram-positive bacilli growing on CCYE agar, with horse-barn odor, were considered for further testing. From each plate, at 3 or 4 presumptive colonies were sub-cultured for the detection of gluD, tcdA, tcdB, cdtA and cdtB genes.

For DNA extraction, InstaGene Matrix (Bio-Rad Laboratories, Hercules, CA, USA) was used according to the manufacturer’s protocol. Two duplex PCRs were performed for the identification and determination of the toxigenic profiling of the isolates. One duplex was used to target the species-specific internal fragment of the glutamate dehydrogenase gene (*gluD*) and the internal fragment of the *tcdB*; the primer set used for detection of *gluD* gene was described by Paltasing et al. (32); primers NK104 and NK105 were used for the detection of *tcdB* (22). The other PCR was developed to detect the toxin A gene (*tcdA*): a primer set designed at the Department of Microbiology of Leiden University Medical Center (LUMC) in The Netherlands was used to detect the presence of the gene and primers NKV011 and NK9, described by Kato et al. (21), were used, to detect the deletion on 3’ region of *tcdA*. For both PCR, DNA samples were amplified according to a
previously described touchdown procedure (26). The genes that encode for the enzymatic and binding components of the CDT were detected by PCR according to Stubbs et al. (41).

**PCR ribotyping.** PCR ribotyping was performed as described by Bidet et al. (7). Gels were processed and compared with the reference riboprofiles kept in our library or kindly provided by the Department of Microbiology of Leiden University Medical Center in The Netherlands. GelCompare II software (BioNumerics, Applied Maths, Belgium) allowed normalization of each gel and subsequent comparison of the riboprofiles by UPGMA clustering, using a 1.5 of tolerance.

**RESULTS**

Occurrence of *C. difficile* in untreated and treated water from 9 WWTPs was investigated and genotypic characterization of isolates was carried out (Table 1). *C. difficile* was found in all of the 18 water samples analyzed. Out of the 55 *C. difficile* strains identified, 24 (43.6%) harbored *tcdA* and *tcdB* genes, whereas 23 (41.8%) carried also *cdtA* and *cdtB* genes (Table 1). Forty seven *C. difficile* isolates, out of 55, belonged to 13 different PCR ribotypes, namely 078, 010, 014, 009, 015, 039, 052, 053, 066, 070, 101, 106, and 117. Eight strains did not match any known reference riboprofiles being, nevertheless, all positive for *tcdA* and *tcdB* genes. *C. difficile* of ribotype 078 (toxigenic profile A`B`CDT+) was isolated both from incoming raw sewage and treated water of WWTPs 3, 4, 6, and 8. Ribotype 014 strains (toxigenic profile A`B`CDT-) were isolated from incoming raw sewage of WWTPs 1, 6, and 7, whereas ribotype 066 (toxigenic profile A`B`CDT+) was isolated from the treated water of WWTP 8.

**DISCUSSION**

This study deals with a limited time frame sampling period and a limited number of sampling. Nevertheless, the WWTPs investigated had a capacity of more than 600.000 population equivalents and represent the largest WWTPs of the Ticino canton (2812 km²).
Few studies described the occurrence and characterization of *C. difficile* in water, but none of them dealt with the isolation and characterization of this pathogen in WWTPs. Al Saif and Brazier (1) isolated *C. difficile* from rivers, lakes, drainage channels, seawater, and from treated water samples from swimming pools and tap water from domestic supplies in South Wales. These authors reported that the majority (84.6%) of isolates from water was toxin A positive. Accordingly, 85.4% of the *C. difficile* strains isolated in our study were toxin A positive. Simango (40) analyzed 234 drinking water samples (171 household-stored water, 61 well water, and 2 borehole water) collected in a rural community of Zimbabwe and recovered *C. difficile* from 4.8% of well/borehole water and 6.4% of household-stored water. The same author found toxigenic strains of *C. difficile* in 18.2% of isolates from household-stored water and no toxigenic strains in the well/borehole water samples. In 2010, Laine et al. (25) reported an extensive waterborne outbreak in Finland (about 6,500 cases of gastroenteritis) due to the contamination of the community water supply with purified sewage water. Campylobacter, *Salmonella enteritidis*, norovirus, rotavirus, *Giardia*, and *C. difficile* were isolated from patients and water samples. Recently, Zidaric et al. (45) reported that *C. difficile* is widely distributed in Slovenian rivers, as it was isolated from 68% of rivers investigated, being the more frequently contaminated sampling stations those which were closer to the more anthropized areas. These findings led to hypothesize a contribution of sewage to the enrichment of water bodies with *C. difficile* strains. The authors found similarity among *C. difficile* strains isolated from rivers, humans and animals.

In addition, Wéry et al. (44), Wen et al. (43) and Marcheggiani et al. (29) suggested that the environmental diffusion of *Clostridiaceae* via WWPT effluents is highly significant due to the ability of such bacteria to produce spores withstanding harsh environmental conditions. The high rate of spore detachment from sludge-flocks in the secondary sedimentation tanks contribute to a further contamination of the outflow WWPT water. Being the role of WWPT effluents in the distribution of *Clostridium* spp. in water ecosystems widely recognized (8, 39), the possible
spreading of *C. difficile* in water bodies through such effluents become an issue of particular concern.

To the best of our knowledge, our study is the first report on the occurrence of *C. difficile* ribotypes 066 and 078 in treated and untreated wastewater of WWTPs. These findings have far-reaching consequences for public health, as the spreading of *C. difficile* into water ecosystem may facilitate the contact between this pathogen and susceptible hosts. Also Norman et al. (2011) found *C. difficile* toxinotype V from composite sewage samples of an closed and integrated human and swine population; in this work no information about the *C. difficile* ribotypes was given.

In agreement with Zidaric et al. (45), we found an overlap between *C. difficile* genotypes isolated from WWTPs and those isolated from humans in the same area, being 8 out of 13 PCR ribotypes found in WWTPs (namely: 010, 014, 015, 039, 066, 078, 101, and 106) also isolated from symptomatic patients admitted to 8 hospitals of Ticino Canton in 2010 (A. Demarta, unpublished data); in addition, 5 (namely: 014, 015, 053, 078, and 106) of them are comprised within the most frequent toxigenic PCR-ribotypes isolated from symptomatic patients admitted to 97 European hospitals (6).

PCR ribotype 078 was the most frequent among isolates (40%). The frequency of infection caused by this genotype is increasing in several EU countries (6, 15). As reported by Goorhius et al. (15), the frequency of infection ascribed to ribotype 078 is increasing in young population, causing severe forms of illness. In the Netherlands, the incidence of ribotype 078 has increased since the end of 2006 and it has become the third most frequent *C. difficile* ribotype (18). Similarly, in UK the frequency of this ribotype doubled (from 1.8% to 3.5%) from the year 2007 to 2009 (17).

Recently, an infection due to ribotype 078 was also reported for the first time in the Republic of Ireland (9). In November 2008, an incidence survey, commissioned by the European Centre for Disease Prevention and Control, found that *C. difficile* 078 was the third most prevalent ribotype among patients with CDI in hospitals of 34 European countries, whereas in Switzerland this
ribotype top ranked (6). In 2010, in Ticino Canton, ribotype 078 was the second most prevalent human isolate among patients with CDI (A. Demarta, unpublished data). In addition, Hoffer et al. (20) also isolated *C. difficile* 078 from a healthy calf in Switzerland. A role of livestock as reservoir of toxigenic *C. difficile* strains has been highlighted by Hensgens et al. (19). In addition, it is worth noting that the ribotype 066, isolated from the effluent of the WWTP n. 8, harbored genes encoding for toxin A, toxin B and binary toxin. Even though the clinical data on this ribotype are scant (2) the potential ability to produce of all the *C. difficile* toxins make this strain of particular concern from a public health standpoint.

Interestingly, ribotype 014, which has been found in this study only in the raw influents of WWTPs 1, 6, and 7, was the predominate type from human CDI (19.5% of all isolates) in Ticino Canton in the year 2010 (A. Demarta, unpublished data). Bauer et al. (6) and Hensgens et al. (18) ranked this ribotype among the 3 more frequently isolated ribotypes in Europe.

Considering the overlapping of environmental and human *C. difficile* ribotypes reported by Zidaric (2010), a special emphasis should devoted to the environmental tracking of *C. difficile* strains expressing toxins, since in our study the 41.8% of the isolates possessed whole array (A’B’CDT+) of *C. difficile* toxins.

In summary, *C. difficile* can be isolated from a wide variety of environmental matrices (1, 33), including water, making humans and animals potentially subjected to *C. difficile* exposure from multiple sources. Moreover, even though there is no study assessing that human infections can be acquired from the environment, the number and severity of community-associated cases is increasing (13). In this regard, Riley (36) recently speculated on the possible transmission to humans of *C. difficile* ribotypes commonly found in pig farms in the Netherlands. The results of our study showed that both WWTPs incoming sewage and treated water in Ticino Canton were contaminated with toxigenic *C. difficile* strains belonging to ribotypes also found in cases of human CDI in this region. Particularly, the detection of toxinogenic PCR ribotypes 014, 066 and 078 points
out that further ecological and epidemiological studies are necessary to elucidate the public health significance of *C. difficile* in water environment and the health risk associated to the presence of *C. difficile* in WWTP effluents.

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Unk: unknown.

PE: Population equivalents, corresponding to 60g BOD$_3$/d per population equivalent.

* These strains share the same riboprofile.

**Table 1.** PCR ribotype and toxigenic profile of *Clostridium difficile* strains isolated from wastewater treatment plants in Southern Switzerland.