AEM
Journals.ASM.org

Toxigenic *Clostridium difficile* PCR Ribotypes from Wastewater Treatment Plants in Southern Switzerland

Vincenza Romano,[a] Vincenzo Pasquale,[a] Karel Krovacek,[b] Federica Mauri,[c] Antonella Demarta,[c] and Stefano Dumontet[a]

Department of Environmental Sciences, Parthenope University of Naples, Naples, Italy[a]; Department of Biomedical Sciences and Veterinary Public Health, Swedish University of Agricultural Sciences, Uppsala, Sweden[b]; and Cantonal Institute of Microbiology, Bellinzona, Switzerland[c]

The occurrence of *Clostridium difficile* in nine 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 six wastewater treatment plants, whereas ribotype 066, a toxigenic emerging ribotype isolated from patients admitted to hospitals in Europe and Switzerland, was isolated from the outgoing effluent of one plant. The majority of the isolates (85%) were toxigenic. Forty-nine percent of them produced toxin A, toxin B, and the binary toxin (toxigenic profile A^+ B^+ CDT^+), whereas 51% showed the profile A^+ B^+ CDT^-. Interestingly, eight 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 highlights that toxigenic ribotypes of *C. difficile* involved in human infections may occur in both incoming and outgoing biological wastewater treatment plants. Such a finding raises concern about the possible contamination of water bodies that receive wastewater treatment plant effluents and about the safe reuse of treated wastewater.

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 that causes 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 (an enterotoxin, encoded by *tcdA*), toxin B (a cytotoxin, encoded by *tcdB*) that directly mediates diarrhea and colitis (35, 12), and sometimes an additional toxin, the binary toxin (CDT). A correlation between binary toxin production and the 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 infections worldwide and a significant cause of health care-associated morbidity and mortality, particularly among 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) that share similar virulence markers. The CDI caused by ribotype 078 are increasing, particularly in young people with no previous contact with hospitals and in community-acquired infections (6, 13, 14, 17).

Considering that the community-acquired CDI are on the increase in Western countries (10, 32), a possible role of contaminated food and environments in the dispersion of this pathogen has been hypothesized (19, 34). Recently, some authors described the occurrence of *C. difficile* in vegetables potentially exposed to contaminated water through irrigation. 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; five isolates were toxin A positive (A^+). Bakri et al. (3) analyzed 40 ready-to-eat salads and found three samples contaminated with *C. difficile*: two isolates belonged to ribotype 017 (negative for toxin A and positive for toxin B [A^- B^+]) and one belonged 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); three isolates 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), the environment and animals may thus be important reservoirs and sources of exposure to pathogenic strains of *C. difficile*.

Only a 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 that occurred in the autumn of 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 fecal specimens of symptomatic people, together with six other pathogens. Viau and Peccia (42) found *C. difficile* in biosolids issuing from a WWTP, and Norman et al. (31) detected the bacteria in sewage of a closed and integrated human and swine population in the United States.

This study investigates the occurrence, genotypic features, and toxigenic profiles of *C. difficile* isolated from untreated and treated water from different WWTPs in southern Switzerland as treated

Received 2 May 2012 Accepted 2 July 2012
Published ahead of print 13 July 2012
Address correspondence to Vincenzo Pasquale, vincenzo.pasquale@uniparthenope.it.

doi:10.1128/AEM.01379-12

wastewater could act as a carrier of *C. difficile* and result in environmental contamination.

MATERIALS AND METHODS

Sampling. Samples were collected from 12 to 13 May 2010 from the inlets and outlets of nine WWTPs, located in the Canton Ticino, southern Switzerland, that process both urban and industrial wastewater. The capacity of the plants ranges from 18,000 to 186,667 population equivalents, corresponding to 60 g of five-day biological oxygen demand ([BOD_5] a standard measure of the biologically oxidizable organic carbon in water over a 5-day incubation period) per day per population equivalent. 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 processing 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 in sterile bottles from each WWTP; one subsurface sample was taken from the inflow pond after the grid separation of the raw wastewater, and one sample was taken from the plant outflow pipe.

Culture conditions. Ten milliliters of treated water and 10 ml of prefiltered (Whatman 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, United Kingdom) supplemented with 1.0 g/liter taurocholic acid sodium salt hydrate (Sigma, St. Louis, MO) and *C. difficile* selective supplement (Oxoid). The cultures were incubated at 37°C for 10 days in anaerobic jars with an AnaeroGen (Oxoid) anaerobic atmosphere-generating system (37). Thereafter, an alcohol shock was performed by mixing 2 ml (1:1, vol/vol) of broth cultures with 96% ethanol; the culture was then 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 (CCEY; Oxoid) agar. The plates were incubated under anaerobic conditions at 37°C for 48 h.

Detection of *gluD*, *tcdA*, *tcdB*, *cdtA*, and *cdtB* genes. Yellow and rhizoid colonies of spore-forming Gram-positive bacilli growing on CCEY agar with a distinct horse barn odor were considered for further testing. From each plate, three or four presumptive colonies were subcultured for the detection of the *gluD*, *tcdA*, *tcdB*, *cdtA*, and *cdtB* genes.

For DNA extraction, InstaGene Matrix (Bio-Rad Laboratories, Hercules, CA) was used according to the manufacturer's protocol. Two duplex PCRs were performed for the identification and determination of the toxigenic profiles 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* gene; the primer set used for detection of the *gluD* gene was described by Paltansing 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 of the 3′ region of *tcdA*. For both PCRs, DNA samples were amplified according to a previously described touchdown procedure (26). The genes that encode 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. GelCompar II software (BioNumerics; Applied Maths, Belgium) allowed normalization of each gel and subsequent comparison of the riboprofiles by clustering using the unweighted-pair group method using average linkages (UPGMA) with a tolerance of 1.5.

TABLE 1 PCR ribotype and toxigenic profile of *C. difficile* strains isolated from wastewater treatment plants in southern Switzerland

WWTP no.	Capacity (PE)[a]	Inflow isolate characterization		Outflow isolate characterization	
		Ribotype	Toxigenic profile	Ribotype	Toxigenic profile
1	37,500	014	$A^+ B^+ CDT^-$	078	$A^+ B^+ CDT^+$
		014	$A^+ B^+ CDT^-$	078	$A^+ B^+ CDT^+$
		014	$A^+ B^+ CDT^-$	009	$A^- B^- CDT^-$
2	62,000	039	$A^- B^- CDT^-$	Unknown	$A^+ B^+ CDT^-$
		070	$A^+ B^+ CDT^-$	Unknown	$A^+ B^+ CDT^-$
		070	$A^+ B^+ CDT^-$	Unknown	$A^+ B^+ CDT^-$
3	18,000	078	$A^+ B^+ CDT^+$	078	$A^+ B^+ CDT^+$
		078	$A^+ B^+ CDT^+$	078	$A^+ B^+ CDT^+$
		078	$A^+ B^+ CDT^+$	106	$A^+ B^+ CDT^-$
4	25,000	078	$A^+ B^+ CDT^+$	078	$A^+ B^+ CDT^+$
		078	$A^+ B^+ CDT^+$	078	$A^+ B^+ CDT^+$
		Unknown[b]	$A^+ B^+ CDT^-$	078	$A^+ B^+ CDT^+$
5	186,667	078	$A^+ B^+ CDT^+$	015	$A^+ B^+ CDT^-$
		078	$A^+ B^+ CDT^+$	015	$A^+ B^+ CDT^-$
		078	$A^+ B^+ CDT^+$	015	$A^+ B^+ CDT^-$
6	125,000	078	$A^+ B^+ CDT^+$	053	$A^+ B^+ CDT^-$
		014	$A^+ B^+ CDT^-$	078	$A^+ B^+ CDT^+$
		052	$A^+ B^+ CDT^-$	Unknown[b]	$A^+ B^+ CDT^-$
		117	$A^+ B^+ CDT^-$		
7	43,500	014	$A^+ B^+ CDT^-$	101	$A^+ B^+ CDT^-$
		014	$A^- B^- CDT$	101	$A^+ B^+ CDT^-$
		010	$A^- B^- CDT^-$	010	$A^- B^- CDT^-$
8	78,500	078	$A^+ B^+ CDT^+$	078	$A^+ B^+ CDT^+$
		078	$A^+ B^+ CDT^+$	078	$A^+ B^+ CDT^+$
		078	$A^+ B^+ CDT^+$	066	$A^+ B^+ CDT^+$
9	24,000	010	$A^- B^- CDT^-$	Unknown	$A^+ B^+ CDT^-$
		010	$A^- B^- CDT^-$	Unknown	$A^+ B^+ CDT^-$
		010	$A^- B^- CDT^-$	Unknown	$A^+ B^+ CDT^-$

[a] PE: population equivalents, corresponding to 60 g of BOD_5/day per population equivalent.
[b] These strains share the same riboprofiles.

RESULTS

Occurrence of *C. difficile* in untreated and treated water from nine 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 the *tcdA* and *tcdB* genes, whereas 23 (41.8%) also carried the *cdtA* and *cdtB* genes (Table 1). Of 55 *C. difficile* isolates, 47 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 riboprofile although all of them were positive for the *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 for sampling and a limited number of samplings. Nevertheless, the WWTPs investigated had a capacity of more than 600,000 population equivalents and represent the largest WWTPs of Ticino Canton (2,812 km^2).

A 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, and 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 samples, 61 well water samples, and 2 borehole water samples) 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 samples. 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 by purified sewage water. *Campylobacter*, *Salmonella enterica* serovar 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, with the more frequently contaminated sampling stations being those located closer to more populated areas. These findings led us to hypothesize that sewage contributes to the contamination 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 dispersion of *Clostridiaceae* via WWTP effluents is highly significant due to the ability of such bacteria to produce spores that withstand harsh environmental conditions. The high rate of spore detachment from sludge flocks in secondary sedimentation tanks contributes to a further contamination of the outflow WWTP water. As the role of WWTP effluents in the distribution of *Clostridium* spp. in water ecosystems is widely recognized (8, 39), the possible spreading of *C. difficile* in water bodies through such effluents becomes 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 ecosystems may facilitate contact between this pathogen and susceptible hosts. Also Norman et al. (31) found *C. difficile* toxinotype V from composite sewage samples of a 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, with 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 eight hospitals of Ticino Canton in 2010 (A. Demarta, unpublished data); in addition, five ribotypes (namely, 014, 015, 053, 078, and 106) comprised 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 European Union (EU) countries (6, 15). As reported by Goorhius et al. (14, 15), the frequency of infection ascribed to ribotype 078 is increasing in young populations, 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 the United Kingdom the frequency of this ribotype doubled (from 1.8% to 3.5%) from 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 ranked at the top (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. The role of livestock as a reservoir of toxigenic *C. difficile* strains has been highlighted by Hensgens et al. (19). In addition, it is worth noting that ribotype 066, isolated from the effluent of WWTP 8, harbored genes encoding toxin A, toxin B, and the binary toxin. Even though the clinical data on this ribotype are scant (2), the potential ability to produce of all the *C. difficile* toxins makes 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 predominant 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 three more frequently isolated ribotypes in Europe.

Considering the overlap between environmental and human *C. difficile* ribotypes reported by Zidaric (45), a special emphasis should be placed on the environmental tracking of *C. difficile* strains expressing toxins since in our study 41.8% of the isolates possessed the 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 subject to *C. difficile* exposure from multiple sources. Moreover, even though there is no study assessing whether human infections can be acquired from the environment, the number and severity of community-associated cases are 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 WWTP 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 environments and the health risk associated with the presence of *C. difficile* in WWTP effluents.

ACKNOWLEDGMENTS

We are indebted to O. Petrini (Cantonal Institute of Microbiology, Bellinzona, Switzerland) for his help in planning the study and critically reviewing the manuscript, E. J. Kuijper and C. Harmanus (Department of

Microbiology, Leiden University Medical Center, the Netherlands) for their assistance during the molecular characterization, and R. Koller (Dipartimento del Territorio e dell'Ambiente, Bellinzona, Switzerland) for his technical assistance during sampling.

REFERENCES

1. **Al Saif N, Brazier JS.** 1996. The distribution of *Clostridium difficile* in the environment of South Wales. J. Med. Microbiol. **45**:133–137.
2. **Arvand M, Hauri AM, Zaiss NH, Witte W, Bettge-Weller G.** 2009. *Clostridium difficile* ribotypes 001, 017, and 027 are associated with lethal C. difficile infection in Hesse, Germany. Euro Surveill. **14**:pii19403. http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19403.
3. **Bakri MM, Brown DJ, Butcher JP, Sutherland AD.** 2009. *Clostridium difficile* in ready-to-eat salads, Scotland. Emerg. Infect. Dis. **15**:817–818.
4. **Barbut F, et al.** 2005. Clinical features of *Clostridium difficile*-associated diarrhoea due to binary toxin (actin-specific ADP-ribosyltransferase)-producing strains. J. Med. Microbiol. **54**:181–185.
5. **Barbut F, Petit JC.** 2001. Epidemiology of *Clostridium difficile*-associated infections. Clin. Microbiol. Infect. **7**:405–410.
6. **Bauer MP, et al.** 2011. *Clostridium difficile* infection in Europe: a hospital-based survey. Lancet **377**:63–73.
7. **Bidet P, Barbut F, Lalande V, Burghoffer B, Petit JC.** 1999. Development of a new PCR-ribotyping method for *Clostridium difficile* based on ribosomal RNA gene sequencing. FEMS Microbiol. Lett. **175**:261–266.
8. **Bonadonna L, et al.** 2002. Fate of bacterial indicators, viruses and protozoan parasites in a wastewater multi-component treatment system. New Microbiol. **25**:413–420.
9. **Burns K, et al.** 2010. Infection due to *C. difficile* ribotype 078: first report of cases in the Republic of Ireland. J. Hosp. Infect. **75**:287–291.
10. **Dial SJ, Delaney AC, Barkun AN, Suissa S.** 2005. Use of gastric acid-suppressive agents and the risk of community-acquired *Clostridium difficile*-associated disease. JAMA **294**:2989–2995.
11. **Dubberke ER, et al.** 2011. The ecology and pathobiology of *Clostridium difficile* infections: an interdisciplinary challenge. Zoonoses Public Health **58**:4–20.
12. **Elliott B, Chang BJ, Golledge CL, Riley TV.** 2007. *Clostridium difficile*-associated diarrhea. Intern. Med. J. **37**:561–568.
13. **Freeman J, et al.** 2010. The changing epidemiology of *Clostridium difficile* infections. Clin. Microbiol. Rev. **23**:529–549.
14. **Goorhuis A, et al.** 2008. Emergence of *Clostridium difficile* infection due to a new hypervirulent strain, polymerase chain reaction ribotype 078. Clin. Infect. Dis. **47**:1162–1170.
15. **Goorhuis A, et al.** 2008. *Clostridium difficile* PCR ribotype 078: an emerging strain in humans and in pigs? J. Clin. Microbiol. **46**:1157–1158.
16. **Hall IC, O'Toole E.** 1935. Intestinal flora in newborn infants with a description of a new pathogenic anaerobe, *Bacillus difficilis.* Am. J. Dis. Child. **49**:390–402.
17. **Health Protection Agency Centre for Infections.** 2009. Healthcare-associated infections in England: 2008–2009 report. Health Protection Agency, London, United Kingdom. http://www.hpa.org.uk/webc/HPAwebFile/HPAweb_C/1252326222452.
18. **Hensgens MP, et al.** 2011. *Clostridium difficile* infection in an endemic setting in the Netherlands. Eur. J. Clin. Microbiol. Infect. Dis. **30**:587–593.
19. **Hensgens MP, et al.** 2012. *Clostridium difficile* infection in the community: a zoonotic disease? Clin. Microbiol. Infect. **18**:635–645.
20. **Hoffer E, Haechler H, Frei R, Stephan R.** 2010. Low occurrence of *Clostridium difficile* in fecal samples of healthy calves and pigs at slaughter and in minced meat in Switzerland. J. Food Prot. **73**:973–975.
21. **Kato H, et al.** 1999. Deletions in the repeating sequences of the toxin A gene of toxin A-negative, toxin B-positive *Clostridium difficile* strains. FEMS Microbiol. Lett. **175**:197–203.
22. **Kato H, et al.** 1998. Identification of toxin A-negative, toxin B-positive *Clostridium difficile* by PCR. J. Clin. Microbiol. **36**:2178–2182.
23. **Kuijper EJ, et al.** 2006. Emergence of *Clostridium difficile*-associated disease in North-America and Europe. Clin. Microbiol. Infect. **12**(Suppl 6): 2–18.
24. **Kujiper EJ, et al.** 2007. Update of *Clostridium difficile*-associated disease due to PCR ribotype 027 in Europe. Euro Surveill. **12**:pii714. http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=714.
25. **Laine J, et al.** 2010. An extensive gastroenteritis outbreak after drinking-water contamination by sewage effluent, Finland. Epidemiol. Infect. **139**: 1105–1113.
26. **Lemee L, et al.** 2004. Multiplex PCR targeting *tpi* (triose phosphate isomerase), *tcdA* (toxin A), and *tcdB* (toxin B) genes for toxigenic culture of *Clostridium difficile.* J. Clin. Microbiol. **42**:5710–5714.
27. **Lim PL, et al.** 2008. Increasing incidence of *Clostridium difficile*-associated disease, Singapore. Emerg. Infect. Dis. **14**:1487–1489.
28. **Limbago BM, et al.** 2009. *Clostridium difficile* strains from community-associated infections. J. Clin. Microbiol. **47**:3004–3007.
29. **Marcheggiani S, et al.** 2008. Microbiological and 16S rRNA analysis of sulphite-reducing clostridia from river sediments in central Italy. BMC Microbiol. **8**:171. doi:10.1186/1471-2180-8-171.
30. **Metcalf DS, Costa MC, Dew WMV, Weese JS.** 2010. *Clostridium difficile* in vegetables, Canada. Lett. Appl. Microbiol. **51**:600–602.
31. **Norman KN, et al.** 2011. Prevalence and genotypic characteristics of *Clostridium difficile* in a closed and integrated human and swine population. Appl. Environ. Microbiol. **77**:5755–5760.
32. **Paltansing S, et al.** 2007. The incidence of *Clostridium difficile*-associated disease (CDAD) in the Netherlands and recognition of an outbreak due to the new emerging PCR ribotype 027 strain. Clin. Microbiol. Infect. **13**: 1058–1064.
33. **Pasquale V, et al.** 2011. Isolation and characterization of *Clostridium difficile* from shellfish and marine environments. Folia Microbiol. (Praha) **56**:431–437.
34. **Pasquale V, et al.** 2012. Occurrence of toxigenic *Clostridium difficile* in edible bivalve molluscs. Food Microbiol. **31**:309–312.
35. **Poxton IR, McCoubrey J, Blair G.** 2001. The pathogenicity of *Clostridium difficile.* Clin. Microbiol. Infect. **7**:421–427.
36. **Riley T.** 2009. New development in the laboratory diagnosis and epidemiology of *Clostridium difficile* infection, abstr S335. Abstr. 19th Eur. Congr. Clin. Microbiol. Infect. Dis. European Society of Clinical Microbiology and Infectious Diseases, Basel, Switzerland.
37. **Rodriguez-Palacios A, Staempfli HR, Duffield T, Weese JS.** 2007. *Clostridium difficile* in retail ground meat, Canada. Emerg. Infect. Dis. **13**:485–487.
38. **Rupnik M, Wilcox MH, Gerding DN.** 2009. *Clostridium difficile* infection: new developments in epidemiology and pathogenesis. Nat. Rev. Microbiol. **7**:526–536.
39. **Schijven JF, de Bruin HA, Hassanizadeh SM, de Roda Husman AM.** 2003. Bacteriophages and *Clostridium* spores as indicator organisms for removal of pathogens by passage through saturated dune sand. Water Res. **37**:2186–2894.
40. **Simango C.** 2006. Prevalence of *Clostridium difficile* in the environment in a rural community in Zimbabwe. Trans. R. Soc. Trop. Med. Hyg. **100**: 1146–1150.
41. **Stubbs S, et al.** 2000. Production of actin-specific ADP-ribosyltransferase (binary toxin) by strains of *Clostridium difficile.* FEMS Microbiol. Lett. **186**:307–3012.
42. **Viau E, Peccia J.** 2009. Survey of wastewater indicators and human pathogen genomes in biosolids produced by class A and class B stabilization treatments. Appl. Environ. Microbiol. **75**:164–174.
43. **Wen Q, Tutuka C, Keegan A, Jin B.** 2009. Fate of pathogenic microorganisms and indicators in secondary activated sludge wastewater treatment plants. J. Environ. Manage. **90**:1442–1447.
44. **Wéry N, Monteil C, Pourcher AM, Godon JJ.** 2010. Human-specific fecal bacteria in wastewater treatment plant effluents. Water Res. **44**:1873–1883.
45. **Zidaric V, Beigot S, Lapajne S, Rupnik M.** 2010. The occurrence and high diversity of *Clostridium difficile* genotypes in rivers. Anaerobe **16**: 371–375.