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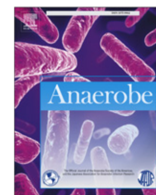

Clinical microbiology

The occurrence and high diversity of *Clostridium difficile* genotypes in rivers

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Clostridium difficile is mainly associated with nosocomial infections but can be present also in other environments. In this study we compared three methods (culturing with and without ethanol shock and real-time PCR) for detection of *C. difficile* in water and have used them on a series of river water samples. *C. difficile* was present in 17 of 25 rivers tested (68.0%) and in 42 of 69 water samples tested (60.9%). Positive sampling sites correlated with increased population densities. Isolates were distributed into 34 PCR ribotypes, of which more than half are present also in humans and animals. PCR ribotype 014 was the predominate type (16.2% of all isolates).

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1. Introduction

Clostridium difficile is considered as one of the important causes of health care-associated infections, however, the number and severity of community-associated or community acquired cases is rising [1,2]. Additionally, *C. difficile* is recognized as emerging pathogen of animals [3–7].

C. difficile infection is traditionally associated with health care settings, thus the hospital is the best-studied environment with respect to diversity of *C. difficile* genotypes, their prevalence and persistence [8–10]. But survival and/or multiplication in other environments can broaden the spectrum of sources of infection and modes of transmission between humans and other reservoirs.

Only a limited number of studies describe isolation of *C. difficile* from soil and water and many of them were looking at the soil associated with animals [11–14]. *C. difficile* was so far reported in different water samples only twice. Al Saif and Brazier found *C. difficile* in 43.7% of sea water samples, 87.5% of river samples, 46.7% of lake samples, 50.0% swimming pools, and in 5.5% of tap water samples [11]. In another study water in small farms was tested during the study on *C. difficile* presence in chickens in Zimbabwe and 6.0% of samples were positive [14].

Typing of *C. difficile* strains and comparison of types present in humans, animals and in the environment is important to understand the possible transmission routes. Currently used molecular typing methods for *C. difficile* are PCR ribotyping, PFGE and to lesser extend REA [15]. Toxigenic strains can be further grouped according to changes in their toxin coding region into toxinotypes [16].

Here we report detection of *C. difficile* in river waters with culturing and molecular methods, the distribution of toxigenic and nontoxigenic strains in water and comparison of genotypes (toxinotypes and PCR ribotypes) isolated from rivers with genotypes isolated from humans and animals.

2. Materials and methods

2.1. Sampling

Sampling was performed between March 2009 and August 2009 in parallel to national surveillance of surface waters. The GPS positions, water temperature, dates and times were taken on standard sampling points and at the time of national water monitoring and are available upon request.

Altogether 25 rivers were tested. Number of sampling sites per river varied from 1 to 11 and total number of sampling sites was 54. Out of 54 sampling sites 44 sites were sampled once, eight were sampled twice, and two different sites were sampled three or four times, respectively. Therefore, the total number of collected samples from 54 sampling sites was 69.

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Water (1 L) was collected in the laminar flow of the river at depth of 30 cm (according to ISO5667-6), in sterile plastic container, transferred to the laboratory and processed within 24 h. Until processing water samples were stored at room temperature.

2.2. Culturing of *C. difficile* from water samples

Two hundred mL were filtered through 0.45 μm cellulose nitrate membrane filter (Whatman) using Millipore filtering system. For each sample two filters were prepared and incubated on selective agar plates CDALT (*C. difficile* medium (Oxoid) supplemented with cefoxitine and cycloserine (*C. difficile* selective supplement, SR0096E; Oxoid), 7% sheep blood, lysozyme (5 mg/L) and sodium cholate (0.1%)). Plates with filters were incubated from 3 to 5 days in anaerobic jars at temperature 37 °C.

After incubation one or more *C. difficile* colonies (according to morphological properties) from first filter were directly transferred to a fresh blood agar plate (COH, bioMerieux). Remaining bacterial growth was swabbed from the filter and used for DNA isolation and tested with real-time PCR as described below.

From second filter entire bacterial growth was swabbed and resuspended in 700 μL of saline solution. Equal amount of absolute ethanol was added, incubated for 30 min at room temperature and centrifuged at 10 000×*g* for 5 min. Entire pellet was inoculated on CDALT plate. One or more recovered *C. difficile* colonies were sub-cultured on blood agar plates.

2.3. Real-time PCR for detection of toxigenic and nontoxigenic *C. difficile*

Total DNA was isolated from bacteria swabbed from the filter with QIAGEN DNA isolation kit (QIAamp DNA Mini Kit).

For the detection of toxigenic strains real-time PCR specific for *tcd*B was used as described before [17]. For the detection of non-toxigenic strains we used primers LokF (5′-GAAATGTG CAAAATCTTTTGAAGA-3′) and LokR (5′-CCTCYAAAGTACTGAGTCAC TTAATTACATC-3′) targeting the 115 bp region that is replacing toxin encoding PaLoc in nontoxigenic strains. A specific probe LokS (5′-ACGAAGAGGAGCTAACAGAGGAAAA-3′) was used as an internal probe.

Real-time PCR was performed in 20 μL volume using LightCycler TaqMan Master kit (Roche) and Light Cycler 2.0 (Roche). The cycling conditions were as follows: activation step of 10 min at 95 °C, 40 cycles of 10 s at 95 °C for denaturation, 30 s at 57 °C for annealing and 13 s at 72 °C for elongation.

2.4. Characterization of isolates

Pure cultures were confirmed as *C. difficile* by amplification of *cdd3*, located downstream from the pathogenicity locus (PaLoc) using primers Tim 6 (5′-TCC AAT ATA ATA AAT TAG CAT TCC A-3′) and Struppi 6 (5′-GGC TAT TAC ACG TAA TCC AGA TA-3′) [7]. For toxinotyping and PCR ribotyping crude DNA was prepared with Chelex-100 from *C. difficile* culture grown on blood agar plate for 48 h. Toxinotyping was performed as described before [16]. Binary toxin genes were detected by PCR, as described by Stubbs et al. [18]. The PaLoc-negative genotype was confirmed by PCR using Lok1/Lok3 primers [19]. PCR ribotyping was done using primers and conditions described by Bidet et al. [20]. PCR ribotypes were designated either with standard Cardiff nomenclature (001,…) if the profile corresponded to one of 25 reference PCR ribotype strains available or with internal nomenclature (SLO 001,…).

3. Results

3.1. Comparison of three methods for detection of *C. difficile* in water samples

We have used three methods for detection of *C. difficile* in water samples: (1) culture on selective medium with spore germination enhancers, (2) culture on selective medium with spore germination enhancers and subsequent alcohol shock, and (3) method 1 combined with real-time PCR on mixed cultures grown on primary selective media.

Real-time PCR for nontoxigenic strains was evaluated before use on eight nontoxigenic *C. difficile* strains from our strain collection. With primers and conditions described here 7 out of 8 toxin A-negative/toxin B-negative strains were detected. Sixteen toxigenic *C. difficile*, *Clostridium perfringens* and *Clostridium sordelii* tested negative.

From 69 water samples included in the study 42 (60.9%) were positive with at least one of the methods (Table 1). Only in 13 samples (18.8%) all three methods were positive for *C. difficile* and in 14 samples (20.3%) *C. difficile* was detected with only one of the methods used (Table 1).

The most sensitive method was culture of *C. difficile* in combination with alcohol shock (35 of 69 samples; method 2, Table 1), followed by culturing combined with real-time PCR detection (31 of 69 samples; method 3, Table 1). Simple growth on selective medium detected *C. difficile* only in 17 of 69 samples (method 1, Table 1). More than one genotype per sample was usually found when cultivation with alcohol shock method was used and less often after cultivation without alcohol shock (data not shown).

3.2. The presence and toxigenic status of *C. difficile* in river waters

In seventeen rivers of 25 (68.0%) *C. difficile* was present in at least one sample. In the case where several sampling sites along a single river were tested there was no clustering of *C. difficile* positive sites; *C. difficile* positive sampling site could be followed by *C. difficile* negative sampling site and also genotypes detected in two subsequent sampling sites were rarely identical (Fig. 1).

Ten sampling sites of a total number of 54 sampling sites were tested more than once. Two of them were negative on both sampling occasions, four were negative at least once, and four sites were positive on all samplings.

Nontoxigenic *C. difficile* was detected in 26 samples representing 61.9% of all positive samples (26/42) and 37.7% of all tested samples (26/69). In nine samples nontoxigenic strains were detected only with real-time PCR, in seven samples nontoxigenic strains were

Table 1
Comparison of two culture methods and real-time PCR for detection of *Clostridium difficile*.

	Method 1	Method 2	Method 3	Number of samples (%)
	Culture method without EtOH	Culture method with EtOH	Real-time PCR from mixed growth on filter culture	
	+	+	+	13 (18.8%)
	−	+	+	12 (17.4%)
	−	−	+	4 (5.8%)
	+	−	+	2 (2.9%)
	+	−	−	1 (1.4%)
	+	+	−	1 (1.4%)
	−	+	−	9 (13.0%)
	−	−	−	27 (39.1%)
Total number of samples	17	35	31	69 (100.0%)

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