MICROBIOLOGY

Clostridioides difficile Isolated from Water; Prevalence, Antibiotic Susceptibility and Toxigenic Activity

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ABSTRACT

Objective: **Evaluate the prevalence, antibiotic susceptibility and toxigenic activity of *Clostridioides difficile* (*C. difficile*) detected in water samples collected from Mansoura city, Egypt.**

Methods: **A total of 200 water samples were collected. Standard microbiological methods including enrichment/selective cultures were used for isolation and identification of *C. difficile*. Epsilon tests were used to detect resistance/susceptibility of the isolates to clindamycin, metronidazole and vancomycin antibiotics. Xpect CD Toxin A/B test was used to detect their toxigenicity.**

Results: **Prevalence of *C. difficile* isolates was 19.0% (38 isolates / 200 collected samples) and the majority (78.9%) of them were toxigenic. Distribution of isolates was as follows; 17 from water supply intake pipe (13 of them were toxigenic), 13 from waste water treatment (12 of them were toxigenic), 5 from house tap water (two of them were toxigenic) and three from public swimming pool (all of them were toxigenic). Variable degrees of resistance to clindamycin and metronidazole were detected. All isolates were sensitive to vancomycin.**

Conclusion: The study has illustrated that *C. difficile* is widespread in water sources including the municipal water supply from domestic housing and swimming pool water samples. Water can be a source for antibiotic resistant toxin producing *C. difficile* transmission.

KEY WORDS

clostridium, colitis, resistance, toxins, water

INTRODUCTION

Clostridioides difficile (formerly *Clostridium difficile; C. difficile*), an anaerobic bacterium, can live in the small intestine as a component of the normal microbiota of approximately 3% of adults and 66% of young children and infants[1)]. The number of cases and deaths associating community acquired and nosocomial *C. difficile* infection (CDI) is increasing[2)]. In clinical practice, nosocomial CDI prolongs the stay of hospitalized patients, so various methods of prevention, such as probiotics, are being investigated[3)].

C. difficile can form spores outside the host that can persist over extended periods because of their moderate resistance to heat and chemicals including most sanitizers[4)]. Moreover, the spores can survive temperature of 71℃ for up to 2 h[5)] and can pass easily through the stomach because they have a high level of resistance to acids[6)]. Toxin A (that causes primary cellular damage and increases fluids in the colon), toxin B (that increases cellular damage) and the binary toxin, CDT (that enhances the activity of toxins A and B) are the main and most virulence factors of *C. difficile*[7)].

Vancomycin (VA) and metronidazole (MZ) antibiotics were recommended for treatment of severe and non-severe *C. difficile* infections, respectively[8)]. Furthermore, clindamycin (CM) is one of the most significant risk antibiotics for developing of CDI[9)].

Actually, *C. difficile* is the most frequent pathogen implicated in nosocomial diarrhea, toxic megacolon and pseudomembranous colitis in hospitals. Additionally, *C. difficile* ribotypes 078 and 027 are the most concerning strains when it comes to human infection and food animals. Nevertheless, the emergence of other strains of *C. difficile* such as ribotype 001, ribotype 014, and ribotype 017 has been reported. It is established that community acquired *C. difficile* infection (CA-CDI) is increasing and that ribotype 078 is frequently implicated which differs from 027 that is commonly linked to healthcare settings[10)].

The sources of CA-CDI remain unclear. *C. difficile* is encountered in humans and animals with the potential of being disseminated to the population through water sources. The conducted study aimed to evaluate water as a potential source for CA-CDI by screening *C. difficile* prevalence of in water samples collected from Mansoura city and nearby areas, Egypt and testing the ability of the isolates to secret toxins and resist the antibiotics; CM, MZ and VA.

METHODS

Totally, 200 water samples were collected from different water sources and sites within Mansoura city and its surroundings in the north Delta of Egypt. All water samples were collected in sterilized bottles. The sampling sites included the municipal water supply intake pipe and effluent outlet from the sewage treatment works located in Mansoura. Sediment was collected from the bottom of the water stream for most of the water samples. A public swimming pool and municipal water from domestic housing were also sampled. The distribution of the 200 samples was as follows; 50 from water supply intake pipe, 50 from waste water treatment, 70 from house tap water and 30 from public swimming pool.

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Table 1: Water supply intake pipe isolates; MIC values of selected antibiotics against *C. difficile* isolates by E-tests

Antibiotics	MIC (µg/mL) breakpoints			Number of *C. difficile* isolates			MIC values (µg/mL) of *C. difficile* isolates and control																
	S	I	R	S	I	R	Isolate number																ATCC
				(%)	(%)	(%)	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)	(16)	(17)	9689
CM[1]	≤ 2	4	≥ 8	11 (64.7%)	5 (29.4%)	1 (5.9%)	4.0	1.0	2.0	8.0	0.5	0.25	4.0	0.25	0.5	4.0	0.5	4.0	0.5	4.0	0.25	2.0	0.5	1.0
MZ[1]	≤ 8	16	≥ 32	16 (94.1%)	1 (5.9%)	0 (0%)	8.0	0.06	0.25	16.0	0.03	1.0	8.0	1.0	0.06	8.0	0.5	4.0	0.5	8.0	0.5	0.5	0.5	2.0
VA[2]	≤ 2	-	≥ 2	17 (100%)	- (0%)	0	1.0	0.5	0.25	2	0.5	0.25	1.0	0.5	0.25	0.5	0.25	1.0	0.5	1.0	0.5	0.25	1.0	0.5

CM, clindamycin; **I**, intermediate; **MIC**, minimum inhibitory concentration; **MZ**, metronidazole; **R**, resistant; **S**, sensitive; **VA**, vancomycin.

1) The breakpoints defined by Clinical and Laboratory Standards Institute (CLSI) [14].

2) The breakpoints defined by European Committee for Antimicrobial Susceptibility Testing (EUCAST) [15].

Table 2: Waste water treatment isolates; MIC values of selected antibiotics against *C. difficile isolates* by E-tests

Antibiotics	MIC (µg/mL) breakpoints			Number of *C. difficile* isolates			MIC values (µg/mL) of *C. difficile* isolates and control													
	S	I	R	S	I	R	Isolate number													ATCC
				(%)	(%)	(%)	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	9689
CM[1]	≤ 2	4	≥ 8	7 (53.8%)	5 (38.5%)	1 (7.7%)	0.25	4.0	0.5	0.25	4.0	0.25	1.0	4.0	16.0	0.5	4.0	0.5	4.0	1.0
MZ[1]	≤ 8	16	≥ 32	12 (92.3%)	1 (7.7%)	0 (0%)	0.06	1.0	0.25	0.5	0.25	0.06	0.25	8.0	0.25	0.5	1.0	0.5	16.0	2.0
VA[2]	≤ 2	-	≥ 2	13 (100%)	-	0 (0%)	0.25	1.0	0.5	0.5	0.25	0.5	0.25	2	0.5	0.5	1.0	0.25	0.5	0.5

CM, clindamycin; **I**, intermediate; **MIC**, minimum inhibitory concentration; **MZ**, metronidazole; **R**, resistant; **S**, sensitive; **VA**, vancomycin.

[1] The breakpoints defined by Clinical and Laboratory Standards Institute (CLSI) [14].

[2] The breakpoints defined by European Committee for Antimicrobial Susceptibility Testing (EUCAST) [15].

Table 3: House tap water isolates; MIC values of selected antibiotics against *C. difficile* isolates by E-tests

Antibiotics	MIC (µg/mL) breakpoints			Number of *C. difficile* isolates			MIC values (µg/mL) of *C. difficile* isolates and control					
	S	I	R	S	I	R	Isolate number					ATCC
				(%)	(%)	(%)	(1)	(2)	(3)	(4)	(5)	9689
CM[1]	≤ 2	4	≥ 8	3 (60.0%)	2 (40.0%)	0 (0%)	0.5	4.0	0.25	4.0	1.0	1.0
MZ[1]	≤ 8	16	≥ 32	5 (100%)	0 (0%)	0 (0%)	0.25	4.0	0.06	8.0	1.0	2.0
VA[2]	≤ 2	-	> 2	5 (100%)	-	0 (0%)	0.5	1.0	0.5	1.0	0.5	0.5

CM, clindamycin; **I**, intermediate; **MIC**, minimum inhibitory concentration; **MZ**, metronidazole; **R**, resistant; **S**, sensitive; **VA**, vancomycin.

1) The breakpoints defined by Clinical and Laboratory Standards Institute (CLSI) [14].

2) The breakpoints defined by European Committee for Antimicrobial Susceptibility Testing (EUCAST) [15].

Enrichment/selective cultures were used for isolation and identification of *C. difficile*. Approximately 2g of the sediment from the water samples was enriched in 9 ml of *C. difficile* Moxalactam Norfloxacin (CDMN) broth (Oxoid, Hampshire, UK) with 0.1% sodium taurocholate and incubated for 7 days at 37°C in anaerobic jar. For the water samples that had little amount of sediment or mixed sediment that cannot be separated from the water, approximately 2 ml of the mixture, water and sediment, was enriched in 9 ml of CDMN broth and incubated at the same conditions. Also, 200 ml of water samples was filtered for water samples that did not have any amount of sediment including swimming pool water samples, and tap water samples by using a water filtering system that had a 0.45 mm filter. The filters then were enriched in about 30ml of CDMN broth and incubated as described above[11].

For all samples, after incubation, 2 ml of the bacterial broth was added to an equal amount of absolute ethanol, incubated in room temperature for 1h and then centrifuged at 1,792 G-force for 10 min. The pellet was streaked on CDMN agar (Oxoid) and incubated for 48 h in anaerobic jar at 37°C[11]. After incubation, the plates were checked for *C. difficile* colonies by morphology and odor, which is usually white and round with a distinctive smell similar to horse manure. Confirmation

Table 4: Public swimming pool isolates; MIC values of selected antibiotics against C. difficile isolates by E-tests

Antibiotics	MIC (µg/mL) breakpoints			Number of *C. difficile* isolates			MIC values (µg/mL) of *C. difficile* isolates and control			
	S	I	R	S	I	R	Isolate number			ATCC
				(%)	(%)	(%)	(1)	(2)	(3)	9689
CM1)	**≤ 2**	**4**	**≥ 8**	1 (33.3%)	1 (33.3%)	1 (33.3%)	4.0	8.0	1.0	1.0
MZ1)	**≤ 8**	**16**	**≥ 32**	2 (66.7%)	1 (33.3%)	0 (0%)	16.0	4.0	8.0	2.0
VA2)	**≤ 2**	**-**	**> 2**	3 (100%)	-	0 (0%)	0.5	0.5	0.5	0.5

CM, clindamycin; **I**, intermediate; **MIC**, minimum inhibitory concentration; **MZ**, metronidazole; **R**, resistant; **S**, sensitive; **VA**, vancomycin.

1) The breakpoints defined by Clinical and Laboratory Standards Institute (CLSI) [14].

2) The breakpoints defined by European Committee for Antimicrobial Susceptibility Testing (EUCAST) [15].

was done by API20A test (BioMérieux, Marcy l'Etoile, France) anaerobically according to the manufacturer's instructions[12]. Reference strain (*C. difficile* ATCC 9689) was used as a positive control in all steps (Oxoid).

The susceptibility/resistance of *C. difficile* isolates to CM, MZ and VA antibiotics was tested using Epsilon test (E-test; BioMérieux) as described before[13]. The plates were incubated at 37□ for 48 h. The minimum inhibition concentration (MIC) values for CM and MZ were compared with the breakpoints established by Clinical and Laboratory Standards Institute[14], while that for VA with those defined by European Committee for Antimicrobial Susceptibility Testing[15]. *C. difficile* ATCC 9689 was used as a positive control in all steps (Oxoid).

The toxin A/B production by *C. difficile* isolates was tested by the Xpect CD Toxin A/B test (Oxoid). Briefly, under anaerobic conditions, the thioglycolate broth of the isolates was incubated for 24 h at 37□, appropriate amount of the broth was mixed with equal amount of Brain Heart Infusion (BHI) broth and incubated for 72 h at 37□ then the BHI broth culture was used according to the manufacturer's instructions to detect the toxin A/B[12]. The toxigenic (A+/B+/CDT-) *C. difficile* ATCC 9689 (Oxoid) was used as a positive control. All experiments were performed in triplicate to ensure accuracy of the results.

RESULTS

The total prevalence of *C. difficile* isolates in the collected water samples was 19.0 % (38 isolates / 200 collected samples). The distribution of the isolates was as follows; 34.0% from water supply intake pipe (17 isolates / 50 samples), 26.0% from waste water treatment (13 / 50), 7.1% from house tap water (5 / 70) and 1.0% from public swimming pool (3 / 30).

All antibiotic susceptibility/resistance data of the research samples was shown in tables 1-4. Variable degrees of resistance to CM and MZ were detected. Totally, 34.2% and 7.9% of the isolates were intermediately resistant (13 / 38) or resistant (3 / 38) to CM, respectively. Although, full resistance to MZ was not detected, three of the 38 isolates were intermediately resistant (7.9%) to MZ. All isolates were sensitive to VA.

The majority (78.9%) of C. difficile isolates were toxigenic (30 toxin producing isolates / 38 isolates). The distribution of the toxigenic isolates was as follows; 76.5% of the isolates from water supply intake pipe (13 / 17), 92.3% of the isolates from waste water treatment (12 / 13), 40.0% of the isolates from house tap water (2 / 5) and 100.0% of the isolates from public swimming pool (3 / 3).

DISCUSSION

Reports describing *C. difficile* detection in water are not numerous. Thirty-eight samples were positive for *C. difficile* isolates from the two hundred water samples that were collected during the current study (38 / 200 =19.0%) as follows; 17 isolates from water supply intake pipe, 13 isolates from waste water treatment, 5 isolates from house tap water and 3 isolates from public swimming pool. Similarly, in the United Kingdom, *C. difficile* was reported in 5.6% of the tested tap water samples[16]. The recovery of *C. difficile* in municipal water in the current study maybe expected given the high prevalence encountered in the water intake station. Although water processing by a combination of filtration and chlorination steps, it is evident that a proportion of the *C. difficile* passes through the process and enter the municipal system. Yet, acquiring CDI from municipal water supplies is likely low, probably in part because the very low levels of contamination that is could be present.

In agreement with our results, it was reported that 50% of the collected swimming pool water samples were contaminated with *C. difficile* and the researchers theorized that this high prevalence was due to contamination of the swimming pool water by young babies who are known to have a high carriage of the pathogen[16]. It should also be noted that spores of *C. difficile* can readily be removed by filtration which would suggest that there was a defect in the filtration process of the swimming pool in the conducted study.

High rate of water contamination (77%) was reported from Canada and the authors attributed their result to water contamination from adjacent pig farm[17]. Moreover, higher rates of *C. difficile* detection in waste water treatment plant effluent were reported (100%) by other two different studies[18,19].

In the conducted study, although all isolates were sensitive to VA, variable degrees of resistance to CM and MZ were detected. as shown in tables 1-4. Sensitivity of all *C. difficile* isolates to VA was reported by many studies[9,20-22]. Similarly, sensitivity of 99.13% of *C. difficile* isolates to VA was reported[23].

Aspevall and his colleagues[20] reported that 16% of the isolates were sensitive to CM, while 11% of *C. difficile* isolates from patients were resistant. Drummond and his research team[24] reported that 67% 8% of *C. difficile* strains recovered from clinical CDI cases were resistant to CM with 25% intermediate and 8% were sensitive. *C. difficile* resistance rate to MZ was reported as 0.00, 0.11% and 1.00%, respectively[9,23,25]. Resistance to MZ is very rare, however decreased sensitivity is emerging[26].

Reduced susceptibilities of *C. difficile* isolates to multiple antibiotics, including imipenem, erythromycin, CM, tetracycline, rifampicin and daptomycin, was reported and three of the isolates (PCR ribotypes 251, 244 and SLO 002,) were associated with human and animal hosts[27].

The explanation of acquiring variable degrees of resistance to CM and MZ in the current study is that these two antibiotics are commonly used to treat infections such as MRSA (CM) or many anaerobes and parasites (MZ). Coming along with the side effect of getting CDI, we could conclude that the relatively high resistance of *C. difficile* isolates from water to CM and MZ could be evidence that the isolates would be related to clinical sources. This may suggest that the *C. difficile* encountered in water is derived from both animal and human sources. Regardless of the source of *C. difficile*, there is a need to determine the extent to which waterborne strains of this pathogen contribute to the CA-CDI.

In the conducted study, majority (78.9%) of *C. difficile* isolates were toxigenic. Similar rate of toxigenic *C. difficile* isolates (79.2%)

was reported[11]. The results would confirm that toxigenic *Cl. difficile* is widely distributed and could contribute to the high occurrence of CA-CDI. On the other hand, Lower rate of toxigenic *C. difficile* isolates (38.1%) was reported and the majority of them were toxinotype 0 that is commonly encountered in clinical settings and toxinotype XII that is not commonly found in clinical cases, but could be found in animals[28].

About 6,500 cases of gastroenteritis was reported from Finland due community water supply contamination by sewage water. *C. difficile*, *Salmonella enterica* serovar Enteritidis, Campylobacter, rotavirus, norovirus and Giardia were detected in water samples and isolated from the patients[29]. A recent study reported that 19.2% of *C. difficile* human strains shared a genomic relationship with one or more water strains and this study supported the growing hypothesis regarding the role of *C. difficile* water contamination in CDI transmission[30].

It is known that *C. difficile* peaks in late Fall-early Winter although it is unknown if this can be related to greater prevalence of the pathogen or greater susceptibility of the host. Further studies should focus on determining the prevalence of *C. difficile* across a wider geographical area and if seasonal effects exist. In addition, it is important to compare the ribotypes and toxinotypes isolated from water with those animals and human to trace the sources of water contamination.

CONCLUSION

This research aimed to evaluate the prevalence of *C. difficile* in water, its toxigenic activity and its resistance to CM, MZ and VA antibiotics. The study has illustrated that *C. difficile* is widespread in water sources including the municipal water supply from domestic housing and swimming pool water samples. The resistance of *C. difficile* to MZ and VA is very rare, but decreased sensitivity is emerging. The majority of *C. difficile* isolates were toxigenic. Water can be a source for antibiotic resistant toxin producing *C. difficile* transmission and should be considered as a potential risk for CA-CDI.

ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by the author.

CONFLICT OF INTEREST

Author declares that there is no conflict of interest associated with this study.

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