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Survey, characterization and antimicrobial susceptibility of *Clostridium difficile* from marine bivalve shellfish of North Adriatic Sea

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ABSTRACT

Clostridium difficile is a major cause of infectious diarrhea associated to healthcare settings. Community-acquired infections are increasingly reported in the last decade and exposure other than to symptomatic patients rather to contaminated foods or animals is feasible. Occurrence of *C. difficile* in shellfish raises concern because spores can survive the cooking temperatures given that shellfish is often consumed poorly cooked or raw. Aim of our study was to investigate whether shellfish represents a reservoir of *C. difficile* human PCR-ribotypes (RTs). 702 shellfish samples of farmed and wild bivalve mollusc species were collected over the 2015–2017 period in North Adriatic Italian Sea to investigate contamination with *C. difficile* and characterize the isolates in terms of genotypic variability and antimicrobial resistance profile. *C. difficile* was detected in 16.9% (CI: 14.1%–19.8%) samples: 11.6% mussels and 23.2% clams. Compared to mussels, clams were significantly associated with detection of *C. difficile* (OR = 2.4, $P < 0.01$). Overall 113 *C. difficile* isolates were genotyped and 75 (66.4%) were toxigenic. Fifty-three different RTs were identified. 40.7% *C. difficile* isolates were among the RTs most commonly involved in human infection in Europe. The profile of antimicrobial susceptibility was determined by E-test; microbiological resistance was frequent against clindamycin (17%), erythromycin (23%), rifampicin (8.8%) and moxifloxacin (10.6%). All isolates were susceptible to metronidazole and one showed MIC > ECOFF for vancomycin. *C. difficile* strains showed high variety in RTs, most of them already detected in other animals or known as highly virulent and epidemic in humans. These results prompt towards investigating on specific risk mitigation measures against *C. difficile* and are preliminary for any source attribution and risk assessment study.

1. Introduction

Clostridium difficile is recognized as the leading cause of life-threatening infectious diarrhea associated to healthcare settings. *C. difficile* infection (CDI) severity ranges from mild diarrhea to severe, in some instances fatal, diseases such as pseudomembranous colitis and fulminant colitis (Heinlen and Ballard, 2010). In the last two decades, epidemiology of CDI has dramatically changed concurrently with an increase in incidence, severity, recurrence and mortality rates of infection (Freeman et al., 2010; Khanna et al., 2012; Lessa, 2013; Vindigni and Surawicz, 2015). This change has been attributed to the emergence of *C. difficile* strains identified as PCR-ribotype (RT) 027 with increased virulence and resistance to several antibiotics (Clements et al., 2010; Goorhuis et al., 2007; He et al., 2013).

CDI is currently considered as one of the most important antimicrobial-resistant threats in the United States (CDC, 2013) and cause of infection in European hospitals, with a significant economic burden to healthcare system (Heimann et al., 2018; Lessa et al., 2015). In addition, CDI is increasingly reported in the community (CA-CDI), often in young people or people without a previous hospitalization or antibiotic treatment (Bauer et al., 2011; Kuntz et al., 2011; Eyre et al., 2013; Wiegand et al., 2012).

Recent studies suggest that, besides symptomatic patients, other sources as pet, food producing animals, meat and vegetables at retail, may have an important role in *C. difficile* transmission (Eckert et al., 2013; Eyre et al., 2013; Jöbstl et al., 2010; Keessen et al., 2011; Rupnik and Songer, 2010; Gupta and Khanna, 2014). Although the epidemiological linkage between CDI and consumption of foods of animal origin remains unclear (Hover and Rodriguez-Palacios, 2013; Søes et al., 2014; Warriner et al., 2017; Weese, 2009), several studies have shown

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an overlap of *C. difficile* RTs between humans and animals (Al Saif and Brazier, 1996; Álvarez-Pérez et al., 2017; Arroyo et al., 2005; Hensgens et al., 2012). In particular, a study carried out in Dutch farms has demonstrated the occurrence of clonal strains in pigs and farmers, suggesting a possible interspecies transmission of *C. difficile* (Knetsch et al., 2015). Notably, these strains were RT 078, a type frequently isolated in CA-CDI (Hensgens et al., 2012; Rupnik et al., 2008).

Given the uncertainty on whether *C. difficile* is to be considered a foodborne pathogen (Warriner et al., 2017) the occurrence of *C. difficile* in shellfish raises concerns because *C. difficile* spores can survive cooking temperatures (Lund and Peck, 2015; Rodriguez-Palacios et al., 2010) and edible bivalve molluscs (EBMs) are often consumed poorly cooked or raw. So far, few studies have been performed. Metcalf et al. (2011) found *C. difficile* in 5/199 (4.8%) seafood and fish sampled in Canadian retails and 4/5 toxigenic strains were RT078, toxinotype V. In Italy *C. difficile* was detected in 49% (26/53) of EBMs collected at retail in Naples, some samples were harvested in the Gulf of Naples, and 58% (15/26) of these isolates resulted toxigenic (Pasquale et al., 2012). Later on a study on EBMs harvested or retailed in the same area detected *C. difficile* in 3.9% (36/925) of the analyzed samples and 52% (19/36) of the isolated strains were toxigenic (Troiano et al., 2015). The high variability in the occurrence of *C. difficile* in EBMs, according to studies available so far, may be due to differences in terms of samples collected as well as different sampling points along the food chain.

Aim of our research was to collect prevalence data about *C. difficile* in farmed or wild EBMs in the North Adriatic Italian Sea: *Mytilus galloprovincialis* (*M. galloprovincialis*), *Chamelea gallina* (*C. gallina*) and *Ruditapes philippinarum* (*R. philippinarum*) at post-harvest level, and to characterize *C. difficile* isolates according to toxinotype, ribotype and antimicrobial resistance.

2. Materials and methods

2.1. Study design and sample collection

A cross-sectional study was carried out from December 2015 to August 2017 (21 months) by selecting at least 300 mussel (*M. galloprovincialis*) and 300 clam (*R. philippinarum* and *C. gallina*) samples breaded or captured in the north-east Italian fishing area, belonging to the Veneto and Friuli Venezia Giulia Regions, and not undergone to any decontamination treatment. This sample size supposes 50% prevalence of *C. difficile* in this area (i.e. the higher uncertainty on real prevalence) and provides an estimation of *C. difficile* prevalence with 95% confidence and 6% precision. Given the cost of logistics to randomly select EBMs from the wild shellfish population, the study used samples that were collected as sentinel indicators for the surveillance of the classification of production areas for EBMs according to EU regulations (Reg. EC N° 854/2004) as well as regional legislation. This surveillance is regulated by plans that, despite not being fully harmonized between Regions, are based on censed, mapped and differently sized fishing areas that are sampled regularly over time with a risk-based sampling strategy aimed to detect the worst scenario of water bacterial contamination. Sampling is performed by official authorities, and microbiologic testing included detection of *Salmonella* and quantification of *E. coli* (Most Probable Number). These samples were reused for the *C. difficile* cross-sectional study using a selection strategy based on a grid approach to the geographic area to investigate: each fishing area was sampled with a frequency that was proportional to the geographical surface, i.e. the larger the area the higher the sampling frequency. Seasonality, if any, was avoided by distributing the sampling over a broad period.

Three different Laboratories from the Istituto Zooprofilattico Sperimentale delle Venezie were involved in the study. To guarantee uniformity of study enrolment we set up a sample enrollment tool (SET) using the business intelligence QlikView software (Qlik View 11, ver. 11 11.20.12354.0 SR6.0.11440.0 SR2, Qlik Tech International) capable to monitor continuously our laboratory informative system (LIMS) for newly submitted samples that, according to qualitative sample features, sample size, time, and geographic origin were eligible for the *C. difficile* study. SET alerted, supported and monitored the laboratory personnel in the sample enrollment process. The enrollment of clams was uniformly monthly distributed over the study period, whereas all eligible mussels were enrolled because they undergo a seasonal fishing variation which made difficult to fix a month limit.

To investigate the genetic and phenotypic variance all *C. difficile* isolates from the cross-sectional study were characterized.

2.2. Clostridium difficile isolation and identification

Samples were transported at 4 ± 2 °C and processed within few hours after collection. To isolate *C. difficile*, 20 g of sample (flesh and intravalvular liquid) were homogenized by Stomacher (Stomacher 400, Seward Limited, Worthing, UK). Ten grams of homogenate were then inoculated into 40 mL of the *C. difficile* selective medium Taurocholate Cefoxitin Cycloserine Fructose Broth (TCCFB, Oxoid Limited, Basingstoke, UK) and incubated at 37 °C for 10 days in anaerobic atmosphere. After incubation samples were screened by real-time PCR targeting a specific *C. difficile* 16S rDNA region as described by Bandelj et al. (2013). Positive broth-cultures were alcohol shocked. Two milliliters of broth-culture were mixed with 2 mL of 96% ethanol (Sigma-Aldrich, St. Louis, MO, USA), left for 60′ at room temperature and then centrifuged at 3800 × g for 10 min. After centrifugation the supernatant was discarded and the pellet was streaked onto *Clostridium difficile* Agar Base (Oxoid Limited, Basingstoke, UK) supplemented with 5% horse defibrinated red blood cells (Biolife Italiana, Milan, Italy), 1 g/L esculin (Sigma-Aldrich, St. Louis, MO, USA), *Clostridium difficile* Moxalactam and Norfloxacin selective supplement (CDSA, Oxoid Limited, Basingstoke, UK) and incubated at 37 °C for 48 h in anaerobic conditions. Suspected colonies were isolated on Blood agar (Biolife Italiana, Milan, Italy) supplemented with 1 g/L esculin (Sigma-Aldrich, St. Louis, MO, USA) and 5% horse defibrinated red blood cells (Biolife Italiana, Milan, Italy) and incubated at 37 °C for 24–48 h in anaerobic atmosphere. Subsequently one colony per each sample was randomly selected and confirmed as *C. difficile* using MALDI-TOF MS (Microflex Biotyper LT, Bruker Daltonics GmbH, Bremen, Germany) and latex agglutination test (*C. difficile* test kit, Oxoid Limited, Basingstoke, UK). Isolates were stored in Cryobank vials (Nalge Nunc International, Rochester, UK) at −80 °C until molecular and antimicrobial susceptibility testing. The combined molecular and bacteriological detection methods used were previously proved not affecting the overall *C. difficile* isolation rate (Drigo et al., 2015).

2.3. Other microbiological methods

Samples were analyzed for *Salmonella* spp. detection according to the ISO 6579-1:2017 and for quantification of *E. coli*, according to the five-tube, three dilution Most Probable Number (MPN) (ISO 16649-3:2015).

2.4. Molecular typing

C. difficile DNA extraction was performed on 48 h blood agar culture using MagMAX™ Total Nucleic Acid Isolation Kit (Applied Biosystems, Foster City, CA, USA) and the MICROLAB STARLET automatic extractor (Hamilton Robotics, Bonaduz, Switzerland). All isolates included in this study were screened by PCRs for the species-specific gene *tpi*, for the toxin genes *tcdA*, *tcdB*, the binary toxin genes *cdtA* and *cdtB* and the *tcdC* regulatory gene deletions as previously described (Antikainen et al., 2009; Lemee et al., 2004; Stubbs et al., 2000). Toxinotyping and PCR-ribotyping were performed as described by Rupnik et al. (1998) and Bidet et al. (1999) respectively. Strains belonging to the predominant PCR-ribotypes circulating in Europe (kindly provided by the European

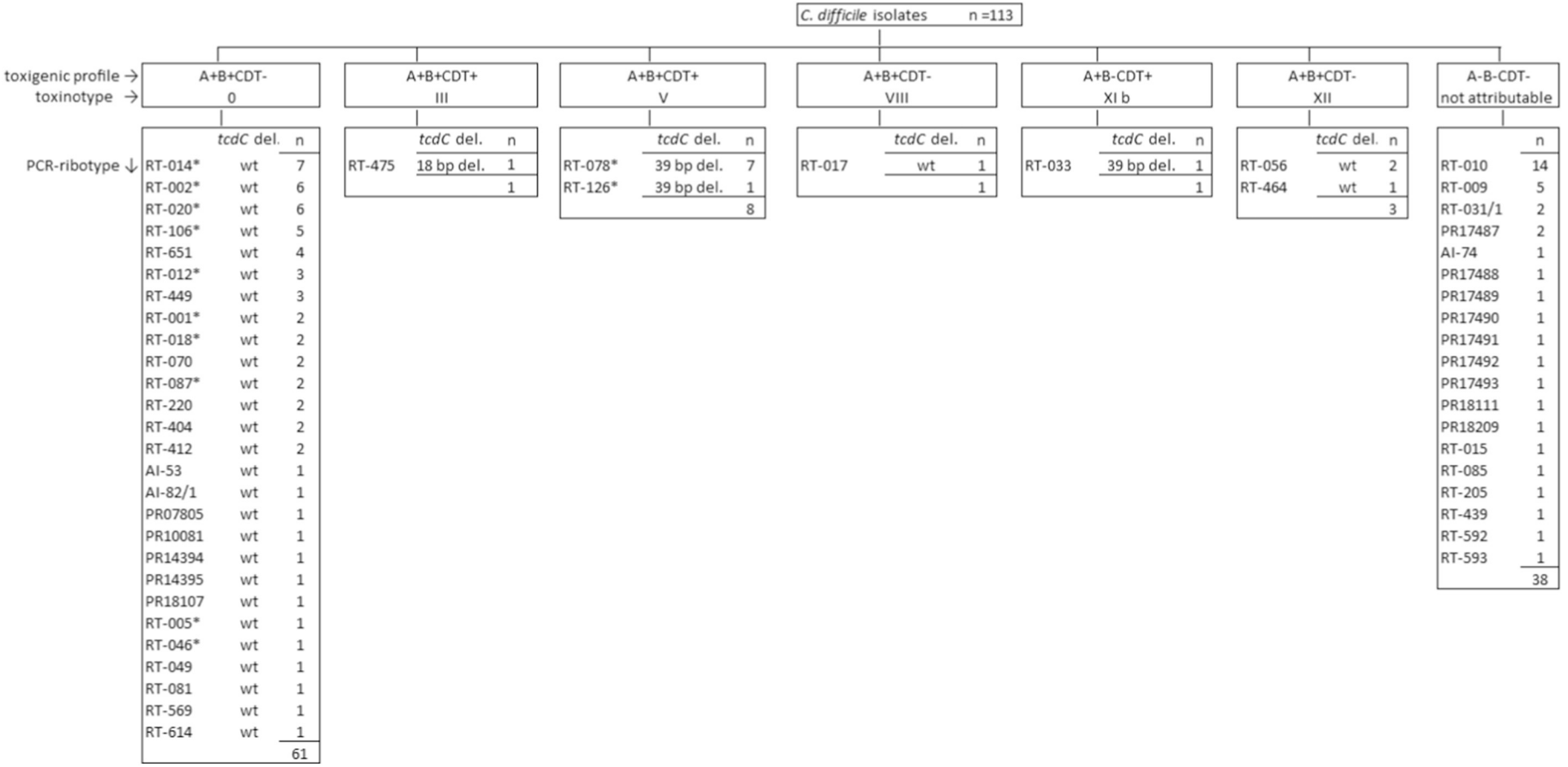

Fig. 1. The schema illustrates 113 *C. difficile* isolates from molluscs aggregated according to the PCR-ribotype and toxinotype. The number of isolates (n) is shown for each RT. The most prevalent RTs in European hospitals are highlighted by an asterisk. The *tcdC* column reports the occurrence of 18 or 39 bp deletion of this gene; absence of deletions is reported as wild type (wt).

Centre for Disease Prevention and Control-ECDC) were used as reference collection. Isolates showing a RT pattern different from those of reference strain were further analyzed by capillary gel electrophoresis–based PCR-ribotyping and were classified according to the WEBRIBO-database (http://webribo.ages.at).

2.5. Antimicrobial susceptibility testing

The susceptibility to clindamycin, moxifloxacin, rifampicin, erythromycin, metronidazole and vancomycin was assessed by E-test (bioMérieux, Marcy l'Etoile, France) onto pre-reduced Brucella blood agar plates supplemented with 5 mg/L haemin, 1 mg/L vitamin K1 and 5% defibrinated sheep red blood cells (bioMérieux, Marcy l'Etoile, France). MIC values were recorded after 48 h of incubation in anaerobic chamber and analyzed according to the epidemiological cut-off (ECOFF) (EUCAST, http://www.eucast.org). Isolates with MIC > 2 mg/L for erythromycin, vancomycin and metronidazole, with MIC > 4 mg/L for moxifloxacin, with MIC > 16 mg/L for clindamycin and with MIC > 0.004 mg/L for rifampicin were classified with reduced antimicrobial susceptibility. *C. difficile* ATCC 700057, *Bacteroides fragilis* ATCC 25285, *Bacteroides thetaiotaomicron* ATCC 29741 and *Staphylococcus aureus* ATCC 29213 were used as quality controls. In order to detect heteroresistance to metronidazole, agar plates were inspected after further five days of incubation in the same test conditions, according to Peláez et al. (2008) and Álvarez-Pérez et al. (2017). Multidrug-resistance (MDR) was defined as reduced susceptibility against at least three classes of antibiotics.

2.6. Data analysis

Data were stored in the LIMS. Key performance indicators for the project management were produced using Qlik reports (Qlik View 11, ver. 11 11.20.12354.0 SR6.0.11440.0 SR2, Qlik Tech International). The toxigenic isolate profile was defined after detection of toxin genes and not detection of toxins themselves. Descriptive statistics were provided. The association between variables was explored by chi-square test and assessed by logistic regression, a 95% significance level for hypothesis testing was used. Data handling and mining was performed in R statistical software (R Core Team, 2017).

3. Results

3.1. Sampling results, C. difficile detection and strains isolation

From December 2015 to August 2017 (21 months) 702 shellfish samples were analyzed: 387 (55.1%) mussels (*M. galloprovincialis*) and 315 (44.9%) clams (220 *R. philippinarum* and 95 *C. gallina*). Overall 16.9% (118/702; CI: 14.1%–19.8%) shellfish samples were contaminated with *C. difficile*: 11.6% (45/387; CI: 8.6%–15.2%) in mussels and 23.2% (73/315; CI: 18.6%–28.2%) in clams.

Clams were significantly associated with detection of *C. difficile* (OR = 2.4; CI: 1.6–3.7; $P < 0.01$) in a logistic regression model that accounts for the sampling month and year.

Among the 118 samples contaminated with *C. difficile* none was also contaminated with *Salmonella* spp. Eighty-three (69.5%) samples contaminated with *C. difficile* complied with the microbiologic requisite for immediate consumption of live bivalve molluscs (< 230 *E. coli* in 100 g of mollusc flesh and absence of *Salmonella* spp.), indicating, according to current legislation for such samples, a marginal risk of faecal contamination.

3.2. Molecular characterization of isolates

During storage five isolates (four from *M. galloprovincialis* and one from *R. philippinarum*) extinguished, the remaining 113 strains, 41 from *M. galloprovincialis*, 55 from *R. philippinarum* and 17 from *C. gallina*, were genotyped after *tpi* detection; results are summarized in Fig. 1 and Table 1 supplementary.

Among 113 isolates 75 (66.4%) resulted toxigenic (Table 1 supplementary). In particular, 29/55 (52.7%) isolates from *R. philippinarum* showed the $A^+B^+CDT^-$ toxigenic molecular profile, 5/55 (9.1%) $A^+B^+CDT^+$, 1/55 (1.8%) $A^+B^-CDT^+$ and the remaining 20 isolates (36.4%) were non-toxigenic.

Among isolates from *M. galloprovincialis* 23/41 (56.1%) had the $A^+B^+CDT^-$ toxigenic molecular profile, 4/41 (9.7%) $A^+B^+CDT^+$ and 14 isolates (34.1%) were non-toxigenic, whereas 13/17 (76.5%) isolates from *C. gallina* were $A^+B^+CDT^-$ and four (23.5%) were non-toxigenic.

Between the isolates with toxigenic profile $A^+B^+CDT^+$, eight displayed a 39 bp deletion of the *tcdC* regulatory gene (seven RT078, toxinotype V, one RT126, toxinotype V) and one, belonging to RT475, toxinotype III, displayed a 18 bp deletion of the *tcdC*. Between the isolates with toxigenic profile $A^+B^-CDT^+$ only one (RT033, toxinotype XI b) displayed a 39 bp deletion of the *tcdC* (Table 1 supplementary and Fig. 1).

Toxigenic profiles were evenly distributed in 65.9% and 66.7%, mussels (*M. galloprovincialis*) and clams (*C. gallina* and *R. philippinarum*), respectively. Given the high number of RTs and toxigenic profiles this sample size had not enough power for testing the association with the shellfish species.

Overall 53 different RTs were identified (Fig. 1 and Table 1 supplementary). Among toxigenic strains the most common RTs detected were 014 (8 isolates), 078 (seven isolates), 002 and 020 (both 6 isolates), 106 (5 isolates), 651 (4 isolates), 012 and 449 (both 3 isolates), whereas among non-toxigenic strains the most frequently RTs detected were 010 (14 isolates), 009 (5 isolates), 031/1 and PR17487 (both 2 isolates). The epidemic RT027 strain was never detected in our study collection.

A considerable number of *C. difficile* isolates collected during the study (46/113, 40.7%) belonged to RTs recognized as frequent cause of CDI in European hospitals: RT001, RT002, RT005, RT012, RT014, RT015, RT017, RT018, RT020, RT046, RT078, RT087, RT106, RT126 (Freeman et al., 2015).

3.3. Antimicrobial susceptibility testing of isolates

Results of antimicrobial susceptibility testing are reported in Table 1. In general, 20/113 (17.7%) and 25/113 (22.1%) strains had MIC > 256 mg/L for clindamycin and erythromycin, respectively. All strains highly resistant to clindamycin (MIC > 256 mg/L) were also highly resistant to erythromycin (MIC > 256 mg/L). MICs above ECOFF for rifampicin and moxifloxacin were observed in 10/113 (8.8%) and 12/113 (10.6%) of the analyzed isolates, respectively. The higher moxifloxacin MICs were observed in *C. difficile* strains belonging to RT014, RT018, RT046, RT078, RT106, RT126 and PR10081.

Six strains showed a MDR pattern, five of them were toxigenic. One of the MDR toxigenic strains, an isolate RT018, was resistant to erythromycin (MIC > 256 mg/L), moxifloxacin (MIC > 32 mg/L), and rifampicin (MIC > 32 mg/L). Three other strains (RT078, RT106 and RT046) showed a MIC > 256 mg/L for clindamycin and erythromycin and a MIC > 32 mg/L for moxifloxacin, while one isolate PR10081 was resistant to clindamycin (MIC > 256 mg/L), erythromycin

Table 1
Distribution of MIC values for clindamycin (CLI), erythromycin (ERI), metronidazole (MTZ), vancomycin (VAN), rifampicin (RIF) and moxifloxacin (MXF) of the 113 *C. difficile* analyzed in the study.

	Antibiotic concentration (mg/L)																										
	0.002	0.003	0.004	0.006	0.008	0.012	0.016 - 0.094 [a]	0.125	0.19	0.25	0.38	0.50	0.75	1.0	1.5	2	3	4	6	8	12	16	24	32[b]	48 – 192 [c]	256 [d]
CLI	nt[e]	nt	nt	nt	nt	nt										1	8	21	38	21	4					20
ERY	nt	nt	nt	nt	nt	nt						1	9	25	34	19										25
MTZ	nt	nt	nt	nt	nt	nt		5	35	28	24	8	12		1											
VAN	nt	nt	nt	nt	nt	nt					25	43	41	3			1									
RIF	23[f]	65	15	6															1					3	nt	nt
MXF												2	24	46	28	2								11	nt	nt

Vertical bars indicate the epidemiological cut-off (ECOFF) of the antibiotic
[a] concentrations tested in this range : 0.016, 0.023, 0.032, 0.047, 0.064, 0.094 mg/L
[b] MIC >32 mg/L
[c] concentrations tested in this range: 48, 64, 96, 128, 192 mg/L
[d] MIC >256 mg/L
[e] not tested
[f] MIC ≤0.002 mg/L

(MIC > 256 mg/L), moxifloxacin (MIC > 32 mg/L) and rifampicin (MIC > 32 mg/L).

All analyzed strains were susceptible to metronidazole. One isolate (PR07805) showed MICs higher than ECOFF for vancomycin. Heteroresistance to metronidazole was investigated in 80/113 randomly selected isolates and observed in five strains, three of them toxigenic (data not shown). In particular, two MDR strains (RT046 and RT106, respectively) resulted heteroresistant to this antibiotic.

4. Discussion

The role of animals and food of animal origin in *C. difficile* transmission to humans and thereafter an involvement in CDI, especially community-acquired CDI, is still controversial mostly because of so many sources of possible exposure to humans, the absence of an infectious dose for CDI onset and no strong evidence of CDI linked to food consumption (Warriner et al., 2017). For these reasons, Warriner et al. (2017) suggested a one health prevention approach to reduce the overall environmental and animal *C. difficile* exposure to humans. Within this vision, knowledge of *C. difficile* prevalence in EBMs and characterization of strains isolated may contribute to the understanding the possible role of raw or scarcely processed EBMs consumption in *C. difficile* transmission.

C. difficile was detected in 16.9% post-harvest mussel and clam samples selected with a risk-based sampling strategy in the North Adriatic Sea. Pasquale et al. (2012) detected 49% *C. difficile* contaminated samples out of 53 investigated and Troiano et al. (2015) 3.9% out of 925 in the Gulf of Naples area, using convenience sampling of EBMs collected at different points of the food chain, from farms and natural banks to fishmongers. Differences in proportion of *C. difficile* detection may be attributed to different sampling strategies and different seafood species investigated. Indeed we found that clams have 2.4 more odds to be contaminated by *C. difficile* compared to mussels, suggesting that distance from sea bottom is a risk factor for *C. difficile* carriage. The former live buried in sand, whereas the latter grow on ropes suspended far from sea bottom. Shellfish capacity of siphoning and their ability to accumulate pathogenic microorganisms mirrors water contamination with bacteria from humans, animals and the environment (Al Saif and Brazier, 1996) and can be influenced by several environmental factors, included water temperature and salinity (Oliveira et al., 2011). We observed that samples from November 2016 to April 2017 were more frequently *C. difficile* contaminated compared to other months of the study period yet this finding was not related to a specific sampling area nor was the study a time series suitable to evaluate seasonality.

It has to be stressed here that samples we analyzed are EBMs animals and not seafood because they were collected at post-harvest and pre-retail stage, but further information on whether isolates are toxinogenic, ribotypes been detected in CDI cases and the antimicrobial resistance profile adds valuable information for risk assessment studies. To the scope it also contributes the information that while none of the samples with *C. difficile* was also contaminated with *Salmonella* spp., 69.5% of them were compliant with the microbiologic requisite for the classification of production areas from which EBMs can be destined to direct human consumption (Reg. EC N° 854/2004).

Overall 66.4% of the *C. difficile* isolates collected in this study were toxigenic, which is higher yet consistent with 52.7% previously reported in samples from the Gulf of Naples (Troiano et al., 2015).

We found a high variety in RTs among *C. difficile* isolates (53 RTs/113 isolates), 40.7% of them belonging to RTs commonly isolated in European hospitals (Freeman et al., 2015). Among these, the hypervirulent RT078 (toxinotype V) and RT126 (toxinotype V) frequently isolated from humans, but also from pigs and cattle (Goorhuis et al., 2008), represented 11% (8 isolates) of the toxigenic *C. difficile* strains analyzed during the study (Fig. 1). Both RT078 and RT126, as other highly virulent types, show alterations in the *tcdC* regulatory gene which probably play a role in increased toxin production (Warriner et al., 2017). Our results are consistent with Pasquale et al. (2012) and Troiano et al. (2015), reporting 11% RT078 and 22% RT126 among *C. difficile* from the Gulf of Naples. It has to be noted that in Italy RT078 is commonly detected in swine and rarely in humans (Spigaglia et al., 2015; Spigaglia et al., 2016), whereas RT018 is the main cause of CDI in Italian hospitals (Spigaglia et al., 2010). Only two RT018 isolates were detected in our sampling area. The highly virulent RT027 was not detected at all in our study, in accordance with previous studies reporting that infections with RT027 in humans are more frequently hospital-acquired than community-acquired and there is little evidence that this type has zoonotic source (Hensgens et al., 2012).

The emergence of *C. difficile* strains resistant to several antibiotics represents an increasing problem for the prevention and treatment of CDI (Baines et al., 2008) and has a central role in driving CDI epidemiological changes. In this study, 23% (26/113) isolates were resistant to at least one antibiotic class. Resistance to both erythromycin and clindamycin was observed in 17% of *C. difficile* strains examined, while a limited number of strains were MDR. One RT018 out of two isolates detected in this study was resistant to erythromycin, rifampicin and moxifloxacin, while the other one was fully susceptible to tested antimicrobials.

One MDR PR07805 ribotype showed a slight reduced susceptibility to vancomycin, the first-line antibiotic for CDI treatment together with metronidazole.

Although all strains were fully susceptible (MIC < ECOFF) to metronidazole five isolates (one RT046, one RT010, one RT009 and two RT106) showed heteroresistance to this antibiotic. Strains with reduced susceptibility to metronidazole have increased in the last years together with the number of treatment failures after metronidazole therapy (Freeman et al., 2015). Reduced susceptibility to this antibiotic is reported to be unstable, for these reasons Peláez et al. (2008) suggests to evaluate MIC with fresh not previously frozen *C. difficile* cultures. Moreover, lower metronidazole MICs were reported when measuring MIC by E-test compared to agar dilution method (Poilane et al., 2000). In our study *C. difficile* isolates were stored at −80 °C before MIC testing by E-test, both conditions may have contributed to an overestimation of *C. difficile* susceptibility to metronidazole.

We did not evaluate the detection thresholds of the combined molecular-culture method we used in this study nor did we count *C. difficile* spores in shellfish samples, however Eckert et al. (2013) using a similar procedure was able to detect from one to three vegetative cells and from six to 15 spores in 20 g salad samples. Knowledge of the detection limit is of importance for the evaluation of the sensitivity of our *C. difficile* detection method, whereas knowledge of the amounts of viable cells and spores are to be used in risk assessment to understand whether a microbiologic hazard may represent a risk for consumers. The former limitation may result in an underestimation of prevalence whereas the latter is of no use because there is no defined infectious dose of *C. difficile* for humans as CDI onset requires concurrent specific risk factors, mostly an important alteration of the person's gut microbiota.

5. Conclusions

The linkage between food and CDI is still controversial. In a recent review Warriner et al. (2017) remarked that to date there is no evidence of a direct linkage between consumption of food and CDI onset, whereas human exposure may be zoonotic, water-borne, environmental and person to person. Given that, post-harvested molluscs harboring toxigenic RTs commonly cause of CDI together with the human habit to consume shellfish entirely or partially raw, may favor the transmission of toxigenic *C. difficile* via shellfish to susceptible individuals. Results from this study indicate that EBMs harvested in the North Adriatic Italian Sea are often contaminated by *C. difficile*. More than half isolates are toxigenic and almost half of them belong to RTs involved in CDI, some recognized highly virulent and epidemic in humans. This study may contribute to assess the burden of *C. difficile* in shellfish and estimate its transmission from seafood to humans, which is part of the complex ongoing evaluation on whether there is any linkage between animals and food from animals with CDI.

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References

Al Saif, N., Brazier, J.S., 1996. The distribution of *Clostridium difficile* in the environment of South Wales. J. Med. Microbiol. 45, 133–137.
Álvarez-Pérez, S., Blanco, J.L., Harmanus, C., Kuijper, E., García, M.E., 2017. Subtyping and antimicrobial susceptibility of *Clostridium difficile* PCR ribotype 078/126 isolates of human and animal origin. Vet. Microbiol. 199, 15–22.
Antikainen, J., Pasanen, T., Mero, S., Tarkka, E., Kirveskari, J., Kotila, S., Mentula, S., Kononen, E., Virolainen-Julkunen, A.R., Vaara, M., Tissari, P., 2009. Detection of virulence genes of *Clostridium difficile* by multiplex PCR. APMIS 117, 607–613.
Arroyo, L.G., Kruth, S.A., Willey, B.M., Staempfli, H.R., Low, D.E., Weese, J.S., 2005. PCR ribotyping of *Clostridium difficile* isolates originating from human and animal sources. J. Med. Microbiol. 54 (pd 2), 163–166.
Baines, S.D., O'Connor, R., Freeman, J., Fawley, W.N., Harmanus, C., Mastrantonio, P., Kuijper, E.J., Wilcox, M.H., 2008. Emergence of reduced susceptibility to metronidazole in *Clostridium difficile*. J. Antimicrob. Chemother. 62, 1046–1052.
Bandelj, P., Logar, K., Usenik, A.M., Vengust, M., Ocepek, M., 2013. An improved qPCR protocol for rapid detection and quantification of *Clostridium difficile* in cattle feces. FEMS Microbiol. Lett. 341, 115–121.
Bauer, M.P., Notermans, D.W., van Benthem, B.H., Brazier, J.S., Wilcox, M.H., Rupnik, M., Monnet, D.L., van Dissel, J.T., Kuijper, E.J., 2011. *Clostridium difficile* infection in Europe: a hospital-based survey. Lancet 377, 63–73.
Bidet, P., Barbut, F., Lalande, V., Burghoffer, B., Petit, J.C., 1999. Development of a new PCR-ribotyping method for *Clostridium difficile* based on ribosomal RNA gene sequencing. FEMS Microbiol. Lett. 175, 261–266.
CDC, Centers for Disease Control and Prevention, 2013. Antibiotic Resistance Threats in the United States. https://www.cdc.gov/drugresistance/pdf/ar-threats-2013-508.pdf, Accessed date: 21 February 2019.
Clements, A.C., Magalhães, R.J., Tatem, A.J., Paterson, D.L., Riley, T.V., 2010. *Clostridium difficile* PCR ribotype 027: assessing the risks of further worldwide spread. Lancet Infect. Dis. 10, 395–404.
Drigo, I., Mazzolini, E., Bacchin, C., Tonon, E., Puiatti, C., Bano, L., Spigaglia, P., Barbanti, F., Agnoletti, F., 2015. Molecular characterization and antimicrobial susceptibility of *Clostridium difficile* isolated from rabbits raised for meat production. Vet. Microbiol. 181, 303–307.
Eckert, C., Burghoffer, B., Barbut, F., 2013. Contamination of ready-to-eat raw vegetables with *Clostridium difficile* in France. J. Med. Microbiol. 61, 1435–1438.
EUCAST, European Committee on Antimicrobial Susceptibility testing MIC Distributions and ECOFFs. http://www.eucast.org, Accessed date: 21 February 2019.
Eyre, D.W., Cule, M.L., Wilson, D.J., Griffiths, D., Vaughan, A., O'Connor, L., Ip, C.L.C., Golubchik, T., Batty, E.M., Finney, J.M., Wyllie, D.H., Didelot, X., Piazza, P., Bowden, R., Dingle, K.E., Harding, R.M., Crook, D.W., Wilcox, M.H., Peto, T.E.A., Walker, A.S., 2013. Diverse sources of *C. difficile* infection identified on whole-genome sequencing. N. Engl. J. Med. 369, 1195–1205.
Freeman, J., Bauer, M.P., Baines, S.D., et al., 2010. The changing epidemiology of *Clostridium difficile* infections. Clin. Microbiol. Rev. 23 (3), 529–549.
Freeman, J., Vernon, J., Morris, K., Nicholson, S., Todhunter, S., Longshaw, C., Wilcox, M.H., 2015. Pan-European longitudinal surveillance of antibiotic resistance among prevalent *Clostridium difficile* ribotypes. Clin. Microbiol. Infect. 21, 248.e9–248.e16.
Goorhuis, A., Van der Kooi, T., Vaessen, N., Dekker, F.W., Van den Berg, R., Harmanus, C., et al., 2007. Spread and epidemiology of *Clostridium difficile* polymerase chain reaction ribotype 027/toxinotype III in The Netherlands. Clin. Infect. Dis. 45, 695–703.
Goorhuis, A., Bakker, D., Corver, J., Debast, S.B., Harmanus, C., Notermans, D.W., Bergwerff, A.A., Dekker, F.W., Kuijper, E.J., 2008. Emergence of *Clostridium difficile* infection due to a new hypervirulent strain, polymerase chain reaction ribotype 078. Clin. Infect. Dis. 47, 1162–1170.
Gupta, A., Khanna, S., 2014. Community-acquired *Clostridium difficile* infection: an increasing public health threat. Infect. Drug Resist. 7, 63–72.
He, M., Miyajima, F., Roberts, P., Ellison, L., Pickard, D.J., Martin, M.J., et al., 2013. Emergence and global spread of epidemic healthcare-associated *Clostridium difficile*. Nat. Genet. 45 (1), 109–113.
Heimann, S.M., Cruz Aguilar, M.R., Mellinghof, S., Vehreschild, M.J.G.T., 2018. Economic burden and cost-effective management of *Clostridium difficile* infections. Med. Mal. Infect. 48, 23–29.
Heinlen, L., Ballard, J.D., 2010. *Clostridium difficile* Infection. Am J Med Sci 340, 247–252.
Hensgens, M.P.M., Keessen, E.C., Squire, M.M., Riley, T.V., Koene, M.G.J., de Boer, E., Lipman, L.J.A., Kuijper, E.J., 2012. *Clostridium difficile* infection in the community: a zoonotic disease? Clin. Microbiol. Infect. 1–11.
Hover, D.G., Rodriguez-Palacios, A., 2013. Transmission of *Clostridium difficile* in foods. Infect. Dis. Clin. N. Am. 27, 675–685.
Jöbstl, M., Heuberger, S., Indra, A., Nepf, R., Köfer, J., Wagner, M., 2010. *Clostridium difficile* in raw products of animal origin. Int. J. Food Microbiol. 138, 172–175.
Keessen, E.C., Gaastra, W., Lipman, L.J.A., 2011. *Clostridium difficile* infections in humans and animals, differences and similarities. Vet. Microbiol. 153, 205–2017.
Khanna, S., Pardi, D.S., Aronson, S.L., Kammer, P.P., Orenstein, R., Sauver, J.L.S.,

Harmsen, W.S., Zinsmeister, A.R., 2012. The epidemiology of community-acquired *Clostridium difficile* infection: a population-based study. Am. J. Gastroenterol. 107 (1), 89–95.
Knetsch, C.W., Connor, T.R., Mutreja, A., van Dorp, S.M., Sanders, I.M., Browne, H.P., Harris, D., Lipman, L., Keessen, E.C., Corver, J., Kuijper, E.J., Lawley, T.D., 2015. Whole genome sequencing reveals potential spread of *Clostridium difficile* between humans and farm animals in the Netherlands, 2002 to 2011. Euro Surveill. 19 (45), 20954.
Kuntz, J.L., Chrischilles, E.A., Pendergast, J.F., Herwaldt, L.A., Polgreen, P.M., 2011. Incidence of and risk factors for community-associated *Clostridium difficile* infection: a nested case-control study. BMC Infect. Dis. 11, 194.
Lemee, L., Dhalluin, A., Testelin, S., Mattrat, M.A., Maillard, K., Lemeland, J.F., Pons, J.L., 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.
Lessa, F.C., 2013. Community-associated *Clostridium difficile* infection: how real is it? Anaerobe 24, 121–123.
Lessa, F.C., Mu, Y., Bamberg, W.M., Beldavs, Z.G., Dumyati, G.K., Dunn, J.R., et al., 2015. Burden of *Clostridium difficile* infection in the United States. N. Engl. J. Med. 372, 825–834.
Lund, B.M., Peck, M.W., 2015. A possible route for foodborne transmission of *Clostridium difficile*? Foodborne Pathog. Dis. 12 (3), 177–179.
Metcalf, D., Avery, B.P., Janecko, N., Matic, N., Reid-Smith, R., Weese, J.S., 2011. *Clostridium difficile* in seafood and fish. Anaerobe 17, 85–86.
Oliveira, J., Cunha, A., Castilho, F., Romalde, J.L., Pereira, M.J., 2011. Microbial contamination and purification of bivalve shellfish: crucial aspects and future perspective – a mini-review. Food Control 22, 805–816.
Pasquale, V., Romano, V., Rupnik, M., Capuano, F., Bove, D., Aliberti, F., Krovacek, K., Dumontet, S., 2012. Occurrence of toxigenic *Clostridium difficile* in edible bivalve molluscs. Food Microbiol. 31, 309–312.
Peláez, T., Cercenado, E., Alcalá, L., Marín, M., Martín-López, A., Martínez-Alarcón, J., Catalán, P., Sánchez-Somolinos, M., Bouza, E., 2008. Metronidazole resistance in *Clostridium difficile* is heterogeneous. J. Clin. Microbiol. 3028–3032.
Poilane, I., Cruaud, P., Torlotin, J.C., Collignon, A., 2000. Comparison of the E test to the reference agar dilution method for antibiotic susceptibility testing of *Clostridium difficile*. Clin. Microbiol. Infect. 6, 155–156.
R Core Team, 2017. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria URL. https://www.R-project.org/.
Rodriguez-Palacios, A., Reid-Smith, R.J., Staempfli, H.R., Weese, S.J., 2010. *Clostridium difficile* survives minimal temperature recommended for cooking ground meats. Anaerobe 16, 540–542.
Rupnik, M., Songer, J.G., 2010. *Clostridium difficile*: its potential as a source of foodborne disease. Adv. Food Nutr. Res. 60, 53–66.
Rupnik, M., Avesani, V., Janc, M., von Eichel-Streiber, C., Delmee, M., 1998. A novel toxinotyping scheme and correlation of toxinotypes with serogroups of *Clostridium difficile* isolates. J. Clin. Microbiol. 36, 2240–2247.
Rupnik, M., Widmer, A., Zimmermann, O., Eckert, C., Barbut, F., 2008. *Clostridium difficile* toxinotype V, ribotype 078, in animals and humans. J. Clin. Microbiol. 46, 2146.
Søes, L.M., Holt, H.M., Böttiger, B., Nielsen, H.V., Andreasen, V., Kemp, M., Olsen, K.E.P., Ethelberg, S., Mølbak, K., 2014. Risk factors for *Clostridium difficile* in the community: a case-control study in patients in general practice, Denmark, 2009-2011. Epidemiol. Infect. 142, 1437–1448.
Spigaglia, P., Barbanti, F., Dionisi, A.M., Mastrantonio, P., 2010. *Clostridium difficile* isolates resistant to fluoroquinolones in Italy: emergence of PCR ribotype 018. J. Clin. Microbiol. 2892–2896.
Spigaglia, P., Drigo, I., Barbanti, F., Mastrantonio, P., Bano, L., Bacchin, C., Puiatti, C., Tonon, E., Berto, G., Agnoletti, F., 2015. Antibiotic resistance pattern and PCR-ribotyping of *Clostridium difficile* strains isolated from swine and dogs in Italy. Anaerobe 31, 42–46.
Spigaglia, P., Barbanti, F., Morandi, M., Moro, M.L., Mastrantonio, P., 2016. Diagnostic testing for *Clostridium difficile* in Italian microbiological laboratories. Anaerobe 37, 29–33.
Stubbs, S., Rupnik, M., Gibert, M., Brazier, J., Duerden, B., Popoff, M., 2000. Production of actin-specific ADP-ribosyltransferase (binary toxin) by strains of *Clostridium difficile*. FEMS Microbiol. Lett. 186, 307–312.
Troiano, T., Harmanus, C., Sanders, I.M.J.G., Pasquale, V., Dumontet, S., Capuano, F., Romano, V., Kuijper, E.J., 2015. Toxigenic *Clostridium difficile* PCR ribotypes in edible marine bivalve molluscs in Italy. Int. J. Food Microbiol. 208, 30–34.
Vindigni, S.M., Surawicz, C.M., 2015. *C. difficile* infection: changing epidemiology and management paradigms. Clin. Transl. Gastroenterol. 6 (7). https://doi.org/10.1038/ctg.2015.24. e99-.
Warriner, K., Xu, C., Habash, M., Sultan, S., Weese, S.J., 2017. Dissemination of *Clostridium difficile* in food and the environment: significant sources of *C. difficile* community-acquired infection? J. Appl. Microbiol. 122, 542–553.
Weese, J.S., 2009. *Clostridium difficile* in food – innocent bystander or serious threat? Clin. Microbiol. Infect. 16, 3–10.
Wiegand, P.N., Nathwani, D., Wilcox, M.H., Stephens, J., Shelbaya, A., Haider, S., 2012. Clinical and economic burden of *Clostridium difficile* infection in Europe: a systematic review of healthcare-facility-acquired infection. J. Hosp. Infect. 81, 1–14.