

A three-year study entailing molecular characterization and epidemiology of *Clostridium difficile* in an Italian tertiary care hospital

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SUMMARY

In Italy, there are limited studies on the molecular epidemiology of *Clostridium difficile*, possibly due to insufficient laboratory diagnostic capacity, low awareness and lack of high-quality surveillance systems. The aim of this study was to evaluate the diffusion of *C. difficile* in a tertiary care hospital and to genotype all the collected strains in order for hospital staff to take corrective action. All specimens were subjected to a CDI diagnostic algorithm. This included highly specific toxin PCRs and multilocus sequence typing (MLST) to obtain clear, unequivocal genotypization. During a three-year study period, as part of routine *C. difficile* testing, 711 stool samples were collected from 522 patients to detect the presence of toxigenic genes. After testing, 106 different samples were identified as toxigenic. The proportions of non-toxigenic and toxigenic isolates were

respectively 8.7% (62/711) and 14.9% (106/711). The most infection findings in wards for toxigenic strains were in Internal Medicine (56), followed by Neurology (11) and Gastroenterology (11). Three novel sequence types (STs) were found. The two most prevalent STs in wards were clade 1 ST-378 (40) and clade 1 ST-379 (33). Other healthcare-acquired strains were clade 4 ST-37 (11) and clade 5 ST-11 (7). Two STs, namely clade 3 ST-5 (10) and clade 1 ST-380 (5), were isolated among external patients. To prevent an increase in outbreak probability, an active surveillance programme combined with proper hand hygiene, environmental cleaning and contact precautions should be implemented.

Keywords: *C. difficile*, MLST, prevalence, sequence type, nosocomial infections.

INTRODUCTION

Clostridium difficile is the leading cause of infectious diarrhoea in the industrialized world. This spore-forming anaerobe bacteria may cause a spectrum of diseases, ranging from pseudomembranous colitis to uncomplicated mild diarrhoea

[1]. It predominantly affects elderly and frail hospital and nursing home patients [2].

C. difficile is known to express up to three toxins: toxin A (TcdA), toxin B (TcdB) and less commonly a third toxin called binary toxin (CDT) [1]. Symptoms are caused by toxins A and B encoded by the *tcdA* and *tcdB* genes located within the pathogenicity locus (PaLoc) [3]. These toxins cause extensive colonic inflammation, epithelial tissue damage, and cell death [1]. By contrast it is not clear if the binary toxin genes increase the virulence of *C. difficile* or if they are simply epidemiological

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logic markers of strains with increased virulence. The development of genotypic methods since the late 1990s has facilitated the understanding of *C. difficile* epidemiology. Genotypic methods are divided into band-based and sequence-based approaches. The most commonly used band-based approach is the PCR ribotyping [4].

The most frequently used sequence-based method is the multilocus sequence typing (MLST). MLST is a microbial genotyping method with a high discrimination potential, using nucleotide sequences of housekeeping gene fragments [5]. Each unique combination of alleles is assigned to a Sequence Type (ST) number that can be used to group MLST results by evolutionary relationships into clades. An Internet-accessible MLST database (<http://pubmlst.org/>) allows the comparison of MLST results from different laboratories maintaining the ownership of the data.

The knowledge of *C. difficile* distribution and genotyping is the first step in monitoring and understanding the epidemiology inside hospitals. In Italy there are limited studies on the *C. difficile* molecular epidemiology, possibly due to insufficient laboratory diagnostic capacity, low awareness, and lack of high-quality surveillance systems [6, 7].

C. difficile infection represents a significant burden on the health care system. Although this infection is sporadic, nosocomial transmission is still important and outbreaks are a constant threat in hospitals [8]. The first objective of this study is to characterize *C. difficile* populations inside a tertiary care hospital in Central Italy, to estimate strain diversity in a variety of wards. The second objective is the genotyping of all toxinogenic strains and the comparison of our results with other previously reported.

■ PATIENTS AND METHODS

This study was carried in a hospital located in Central Italy with 288 beds divided in wards and a mean of 31,000 inpatient days per semester. A ward is a spatial unit provided with rooms where a unique staff of health-care and co-workers are active. All patients admitted from January 2015 to January 2018 were included in the study. In order to optimize the diagnosis of CDI we combined different tests as suggested by Crobach et al. [9] (Fig-

ure 1). The advantage of an algorithm is that tests can be combined in such a way that the percentage of false-positive and false-negative results can be decreased. CDI cases were defined as subject with at least one toxin-positive stool and we considered the total number of patients and analyses, including the analyses repeated for single subjects and changes of result concerning single patients during time. All specimens were obtained from a source cohort of patients aged >18 years who experienced clinically significant diarrhoea, abdominal pain and cramps, lower quadrant tenderness, fever, leucocytosis and hypoalbuminemia according to the current guidelines [10]. In step 2 of the algorithm, all the stool samples were tested for the presence of the glutamate dehydrogenase enzyme and the toxins. *ImmunoCard*[®] GDH Enzyme Assay (Meridian Bioscience inc., United States) detect glutamate dehydrogenase, an enzyme that is produced by both toxigenic and nontoxigenic strains of *C. difficile*. All the strains were also tested with the *ImmunoCard*[®] toxins A/B System (Meridian Bioscience inc., United States). This assay is able to detect both toxin A and B produced by the pathogenicity locus (*PaLoc*) of toxinogenic *C. difficile* which encodes both the toxin A gene (*tcdA*) and the toxin B gene (*tcdB*). Toxin A/B EIAs tend to be the most specific assays, while GDH EIAs is the more sensitive test [9]. The GDH positive strains but negative for toxins were discarded as suggested by the European Centre for Disease Prevention and Control (ECDC) guidelines and according with the Hospital standards.

In step 3, only positive *C. difficile* stool samples to GDH and one of the toxin (A or B) or both, were subjected to alcohol shock procedure to isolate the pathogenic strains. Of the first stool dilution (1:2), 0.5 mL was added and mixed by vortexing to an equal volume of absolute sterile ethanol. After incubation at room temperature for 1h, serial tenfold dilutions were prepared and samples of 1 mL of the serial dilutions were plated on to BHIA medium. Plates were incubated at 37° C under anaerobiosis conditions using a "gas generating kit" system (Oxoid, UK). The plates were placed in an incubator for at least 48 h in 10% CO₂ atmosphere. Isolated bacteria were stored in Bacterial Freezing Kit tubes (Ops Diagnostics, USA) for further analysis.

In step 4, all the isolates were subjected to a highly specific toxin-PCRs. These PCRs can con-

firm the production of TcdA and/or TcdB and, in addition to these toxins, several strains isolated from outbreaks and severe infections have been shown to harbour the genes encoding the binary toxin CDT. For these reasons *tcdA*, *tcdB*, *cdtA* and *cdtB* primers were used, as shown by Persson et al. [11].

Eventually, in step 5, MultiLocus Sequence Typing were performed to have a clear and unique genotypization. All samples positive in the step 2 (GDH+ and Toxin A or B positive or both) and confirmed in step 3 with the molecular assay were subjected to MLST and seven house-keeping gene were sequenced as previously described [5]. ST and clades of *C. difficile* isolates were determined by querying an official website (<http://pubmlst.org/>). The allele sequences of every strain was concatenated into a super-gene alignment and compared each other to construct the phylogenetic

tree [12]. MEGA 6 software (<http://www.megasoftware.net/>) was used to build the tree with a maximum likelihood method.

RESULTS

During the 3-year study period, 711 stool samples were collected from 522 patients. The gender distribution was 367 (51.7%) males and 344 (48.3%) females. The mean age was 74.3 ± 20.5 years. Only following clinical suspicion, 168 samples out of 711 (23.6%) were positive to *Meridian ImmunoCard*[®] GDH Enzyme Assay and, 82.1% (n.584) of these were adults over the age of 65.

After GDH screening, a patient was considered to suffer from infection only if the sample was positive to *Meridian ImmunoCard*[®] toxins A/B System (step 2) and eventually positive at least



Figure 1 - CDI testing algorithm and genotyping.

Table 1 - Total toxinogenic *C.difficile* isolations in wards. All positive strains (GDH+ and toxin A/B or both positive) were subjected to MLST.

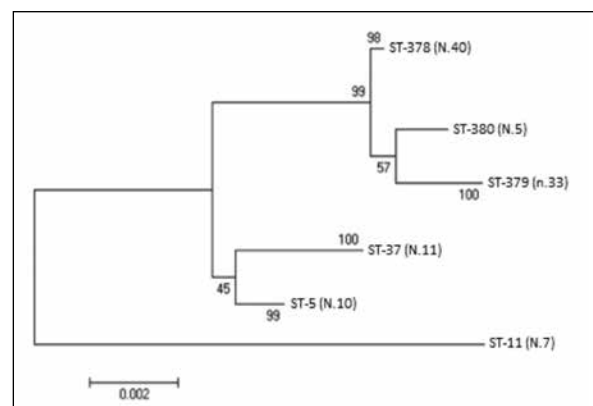
Sequence Type	GDH test	PaLoc	Toxin A	Toxin B	CDT	MLST clade	Cardiology	Dyalisys	Internal Medicine	Neurology	Gastro-enterology	Outpatients	Total CDIs
ST-378	+	+	+	+	-	1	0	6	23	0	11	0	40
ST-379	+	+	+	+	-	1	0	0	33	0	0	0	33
ST-380	+	+	+	+	-	1	0	0	0	0	0	5	5
ST-11	+	+	+	+	+	5	7	0	0	0	0	0	7
ST-5	+	+	+	+	+	3	0	0	0	0	0	10	10
ST-37	+	+	-	+	-	4	0	0	0	11	0	0	11

for one toxin (A or B) after toxin-PCRs (step 4). With this approach, a total of 106 (14.9%) patients were diagnosed with *C. difficile* infection (CDI) (Table 1). The results from the step 2 toxins assay and those confirmed through molecular techniques (step 4) were identical, according to the high specificity of EIAs methods as denoted in literature. The 77.3% of the patients (n = 82) were males and the 22.7% (n = 24) were female with a mean age of 83.3±17.2 years. The highest number of toxinogenic strains in wards was found in Internal Medicine Unit (n = 56), followed by Neurology (n = 11) and Gastroenterology (n = 11). Among all the CDI, the 85.9% were inpatients, while outpatients counted for 15 toxinogenic-positive strains representing the 14.1% of all toxinogenic isolations.

After the toxin-PCRs, 78 strains were toxin A/B positive and CDT negative. Seventeen strains were toxin A/B positive and CDT positive and 11 strains toxin A negative, toxin B positive and CDT negative. In order to obtain a sequence-based genotypization of these six unique banding profiles, we performed MLST by sequencing *adk1*, *atpA1*, *dxr3*, *glyA1*, *recA2*, *sodA5* and *tpi2* genes. After matching the six housekeeping genes with MLST public database (<http://pubmlst.org/>), six different STs were detected. We found three known and three unknown sequence types. All the new loci combinations were sent to the database curator and three novel ST were assigned, ST-378, ST-379 and ST-380. These three new STs, according the classification provided by the software database, belonged to clade 1. The three sequence types, already present in the public database, were clade 4 ST-37, clade 3 ST-5 and clade 5 ST-11 frequently associated with animals. All the CDIs-causative toxinogenic strains and

the matching ward of origin are listed in Table 1. Some strains were isolated from hospital wards like Gastroenterology, Internal Medicine, Dialysis, Cardiology, Neurology, while others from outpatients.

In order to understand the evolutionary relationship of these strains, we constructed a maximum likelihood phylogenetic tree (Figure 2), after the concatenation of alleles sequences from every ST. The phylogenetic tree demonstrated a heterogeneous genetic characteristics of *C. difficile* isolates in this collection. The STs clustered into two groups with one outlier (ST-11). According to the classification provided by the software database (<http://pubmlst.org/>), the closest related strains were the ST-378, ST-379 and ST-380 corresponding to the clade 1. ST-5 and ST-37 apparently grouped together but they were less related, because they belonged to clade 3 and clade 4 respectively. A single clade 5 ST-11 outlier was found.

**Figure 2** - Phylogenetic analysis (Maximum likelihood) based on the alignment of seven housekeeping genes. The number of isolations is reported in round brackets.

■ DISCUSSION

C. difficile continues to be the most common cause of healthcare-associated infection in the developed world. A previous European *C. difficile* hospital-based survey has shown that the incidence of CDI and the distribution of causative types differs greatly from hospital to hospital [13]. This may represent a possible limitation, making difficult the comparison of our data with other publications.

The most common STs (ST-378, ST-379) were found in Internal Medicine and in Gastroenterology wards. Both these types carried toxin A and B but were negative to the binary toxin. The high *C. difficile* rate in these wards was probably due to systemic antibiotics exposure, advanced age, followed by gastric acid-suppressive medications which are the three most notable risk factors for developing CDI [14].

Our results show the presence of ST-378 also in Dialysis, due to frequent transfer of patients from Internal Medicine and other wards. These transfer patients could be a reservoir for *C. difficile* spores and it helps to explain the reason why there was a presence of the two most common ST in just three wards.

ST-380, one of the community-acquired strains, has resulted phylogenetically close to the most common strains isolated in wards. As ST-378 and ST-379 belong to MLST Clade 1 (>100 STs) and carried the same toxins.

Another healthcare-acquired strain was ST-11 (toxin A⁺B⁺, CDT⁺) found in Cardiology ward. This strain, from clade 5, was most distant from the other lineages. Although clade 5 strains are currently present at low frequency, prospective surveillance demonstrates the continued expansion found it in Australia, USA and Europe [15]. The recent trends in epidemiological data show that it is an important type found in the Dutch healthcare system, Belgian provinces and among patients in Holland [16, 17].

In Neurology ward, we found eleven ST-37 strains belonging to Clade 4, also known as the toxin A⁺B⁺ clade and this is in line with our results [5]. Some studies showed that all ST-37 isolates exhibited multi-drug resistance and also indicated that the recurrence rate has increased in recent years following the use of metronidazole and/or vancomycin [18]. This ST has caused widespread disease

in East Asia and recently has been reported as the predominant type in China [19]. Finally, from 12 outpatients, we isolated twelve ST-5 carrying the two main toxins A⁺B⁺ and the binary toxin CDT⁺. This community-acquired strain is a binary-toxin-positive and it is among the 15 most prevalent STs in Europe. This is the most common type in clade 3/HA2 and in contrast with our results, most of literature reports that it is much more prevalent inside hospitals than in community. In a Czech study, samples were collected from 32 healthcare facilities (7 tertiary care hospitals, 24 secondary care hospitals and 1 specialized care hospital) but ST-5 was found in only 1 hospital [20].

Nowadays, ST-17 has been reported as one of the most prevalent genotypes circulating in hospital settings in Italy and it has been accounted for >20% of all Italian isolates [7]. This sequence type is highly transmissible and generally shows a multidrug-resistant phenotype that seem to contribute in conferring an adaptive advantage to these strains, allowing their successful spread in our country [21]. In our study, there is no sign of infection caused by this sequence type. Maybe to start a longer period surveillance could help us to better investigate its presence in our hospital and to understand its spread.

To avoid the increase of outbreak probability, an active surveillance program combined with proper hand hygiene, environmental cleaning and contact precautions, should be improved. Contact precautions are crucial while patients transfer between wards. Healthcare workers, particularly when understaffed, may unintentionally contribute to transmission of infectious diseases through poor infection control practices. Daily cleaning of all rooms with bleach wipes may reduce the incidence of hospital-acquired *C. difficile* infection and routine use of gloves may also be an effective mean to reduce nosocomial transmission of *C. difficile* spores. In addition to reducing the burden of spores in the environment, a key aspect of preventing *C. difficile* infection in older adults is to minimize their vulnerability by avoiding unnecessary antibiotic exposure through antimicrobial stewardship [22].

Conflict of interest

The authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria;

educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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