**Clostridium difficile** PCR Ribotype 018, a Successful Epidemic Genotype

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**Clostridium difficile** infection (CDI) became a public health problem for the global spreading of the so-called hypervirulent **PCR** ribotypes (RTs) 027 and 078, associated with increases in the transmission and severity of the disease. However, especially in Europe, several RTs are prevalent, and the concept of hypervirulence is currently debated. We investigated the toxin and resistance profiles and the genetic relatedness of 312 **C. difficile** strains isolated in a large Italian teaching hospital during a 5-year period. We evaluated the role of **CDI**-related antibiotic consumption and infection control practices on the RT predominance in association with their molecular features and transmission capacity. Excluding secondary cases due to nosocomial transmission, RT018 was the predominant genotype (42.4%) followed by RT078 (13.6%), while RT027 accounted for 0.8% of the strains. RT078 was most frequently isolated from patients in intensive care units. Its prevalence significantly increased over time, but its transmission capacity was very low. In contrast, RT018 was highly transmissible and accounted for 95.7% of the secondary cases. Patients with the RT018 genotype were significantly older than those with RT078 and other RTs, indicating an association between epidemic RT and age. We provide here the first epidemiological evidence to consider RT018 as a successful epidemic genotype that deserves more attention in clinical practice.

**Clostridium difficile** infection (CDI) is one of the most common hospital-acquired infections and the main known cause of antibiotic-associated diarrhea in hospitals in industrialized countries (1). Previous antibiotic treatment, hospitalization, old age, and underlying diseases represent major risk factors for CDI. The disease spectrum ranges from mild diarrhea to severe pseudomembranous enterocolitis, sepsis, and death (2, 3).

In the past decade, many countries in North America and Europe have registered an increase in CDI rates and related severity and mortality (4, 5). These epidemiological changes coincided with the emergence and epidemic spread of **C. difficile** strains belonging to the **PCR** ribotype (RT) 027/North American pulsed-field gel electrophoresis type 1 (NAP1) (6). Indeed, RT027 became the predominant strain in many geographical regions, as documented in different epidemiological studies, and, since 2005, several countries have conducted epidemiological surveillance to monitor its spread (7, 8). It has been reported to occur in at least 38 U.S. states and in 16 European countries, being also responsible for outbreaks (7, 9). Moreover, an increase in community-acquired CDI has been reported; in Europe, this phenomenon correlates with the diffusion of RT078/NAP7 or NAP8 which, like RT027, has been associated with higher disease severity and attributable mortality (1, 10).

RT027 and RT078 are often referred to as hypervirulent genotypes of **C. difficile**, but this definition is not without controversy. Though this term denotes an increase in virulence, it has often been misused to identify epidemic strains that cause outbreaks (7, 11). The success of an epidemic strain relies not only on its ability to cause disease (virulence) but also on its transmissibility. Indeed, depending on the country and the time period, different RTs, such as RT001, RT017, and RT014/020, have been reported as prevalent and associated with severe infections and large outbreaks in hospital settings (12–15).

In Italy, we reported in 2010 for the first time the presence of RT027 and RT078; later, these RTs were detected as minor genotypes (16–19). RT018 has been reported as the predominant genotype circulating in Italian hospitals (20). Longitudinal surveillance data on **C. difficile** epidemiology are lacking, as systematic isolation and molecular typing of **C. difficile** strains are not routinely performed. Between 2009 and 2013, we characterized for infection control purposes 312 **C. difficile** strains, representing 66% of all the CDI cases identified at the San Raffaele hospital. The aims of this study were to analyze the epidemiology of CDI in our hospital during a 5-year period and to investigate the influence of antibiotic consumption and infection control practice on the predominance of different RTs in association with their molecular characteristics and transmission capacity.

**MATERIALS AND METHODS**

**Definitions.** We included in this study the first strain isolated from the first stool sample collected from symptomatic patients; subsequent strains or samples from the same patient, defined as duplicates, were excluded.
CDI was defined as the acute onset of diarrhea with a positive toxin A and B assay (Vidas C. difficile toxin A&B; bioMérieux) and no other documented cause of diarrhea (21, 22). Health care–associated CDI (HA-CDI) was defined as the development of CDI ≥48 h after hospital admission or within 4 weeks after discharge. Community-associated CDI (CA-CDI) was defined as the development of CDI within 48 h of hospital admission in patients with no documented prior hospitalization or long-term care facility stay in the preceding 3 months. Indeterminate CDI was defined as the development of CDI between 4 to 12 weeks after hospital discharge (23). For the epidemiological investigation, the patient case histories were analyzed. An outbreak was defined as ≥2 related CDI cases presenting the same RT and a link confirmed by classical epidemiological investigation. The incidence of CDI was defined as the number of cases per 1,000 admissions or 10,000 patient-days. These data were compared with the CDI incidence registered in other hospitals of the region with similar characteristics. The transmission index for each RT was calculated as the total number of secondary cases divided by the total number of index cases (24).

Bacterial strains, study setting, and data collection. Between January 2009 and December 2013 at the San Raffaele hospital (OSR), a total of 312 nonduplicate toxigenic C. difficile strains were isolated from unformed Vidas toxin A&B–positive stool samples. Clostridium difficile selective agar (CDSA) plates (Becton Dickinson) were used for strain recovery. The OSR is a large (1,400-bed) private university hospital with close to 45,000 (CDSA) plates (Becton Dickinson) were used for strain recovery. The collected as epidemiological data.

Patient genders, ages, dates of admission, wards, and dates of CDI diagnosis were considered statistically significant.

PCR ribotyping. The clonal relatedness of the C. difficile strains was analyzed using PCR ribotyping (16) and by comparing the identified RT to the following reference strains: strains N1 (RT001, NCTC11204) and R20291 (RT027, NCTC13366) from the National Collection of Type Cultures (Health Protection Agency, United Kingdom); strain 630 (RT012, BAA-1382) from LGC Standards (United Kingdom); strains 1470 (RT017), R7605/78 (RT078), and B15 (RT126), kindly provided by M. Rupnik (Maribor University, Slovenia); strains RT002, RT014/020, and RT018, kindly provided by F. Agnolotti (Istituto Zooprofilattico Sperimentale delle Venezie, Italy); and strains RT056, RT106, and RT137, kindly provided by V. Pasquale (Parthenope University of Naples, Italy).

RESULTS Incidence of CDI, antibiotic consumption and infection control measures. Of 8,649 stool samples tested during the study period, 642 (7.4%) were positive for C. difficile. Of these, 473 were from individual patients with CDI, while 169 were duplicate samples. Overall, there was a 22% reduction of samples tested through the study period, from 1,964 samples in 2009 to 1,532 samples in 2013. In total, 312 nonduplicate C. difficile strains were available, representing 66% of all CDI cases identified at the OSR between 2009 and 2013. Figure 1 shows the yearly incidence of CDI (A) and the consumption data of antibiotics that have been considered risk
factors for the occurrence and increasing rate of CDI (B). A mean incidence of 2.13 CDI episodes per 1,000 admissions or 3.05 per 10,000 patient-days was determined in our hospital. The CDI rate decreased between 2009 and 2011 and then due to the occurrence of several epidemic events, peaked in 2012, and fell to a preepidemic level in 2013. Overall, through the whole study period, there were no significant changes in either the CDI incidence or antibiotic use (Fig. 1). We did not find any association between CDI rate and antibiotic consumption. According to data provided for the Lombardy region, the CDI incidence in OSR and in other hospitals with similar characteristics was comparable (data not shown).

C. difficile epidemiological and interpatient transmission data. PCR ribotyping and epidemiological investigation distinguished 243 C. difficile strains isolated from index case patients. Table 1 shows the distribution of the 243 C. difficile strains from index cases among the different RTs. We identified two major RTs accounting for 56% of the C. difficile strains: RT018 (42.4%; 103 of 243 strains) and RT078 (13.6%; 33 of 243 strains). The remaining 44% of the strains belonged to 8 identified RTs (12.3%; 30 of 243 strains) and 46 unidentified RTs (other RTs, 31.7%; 77 of 243 strains); only 2 strains belonged to RT027 (0.8%) (Table 1). Figure 2A shows the isolation trend of C. difficile strains (index cases) belonging to RT018, RT078, and all other RTs (non018 and non078) between 2009 and 2013; while RT018 displayed a fluctuating trend, RT078 presented a significantly increasing trend ($P = 0.02$).

The majority of CDI index cases (65%; 158 of 243 cases) were detected in medical wards, followed by surgical (16%; 39 of 243 cases) and rehabilitation (12.8%; 31 of 243 cases) wards and intensive care units (6.2%; 15 of 243 cases). In particular, as shown in Fig. 2B, the distribution of C. difficile strains belonging to RT018, RT078, and all other RTs was similar in medical and rehabilitation wards. In contrast, in intensive care units, RT078 was detected more frequently than RT018 (15.2% versus 6.8%) and all other RTs (15.2% versus 2.8%; $P = 0.02$).

Ten of 243 C. difficile strains (4.1%) originated from patients with CA-CDI. Of these, 3 were RT018 (3 of 103; 2.9%), 1 was RT078 (1 of 33; 3%), and 6 belonged to other RTs (6 of 107; 5.6%).

Nosocomial transmission events were caused by three RTs, RT018, RT078, and RT014/020, which accounted for 95.7% (66 of 69), 2.9% (2 of 69), and 1.4% (1 of 69) of the secondary cases, respectively. The transmission index of RT018 (0.640) was significantly higher than that of RT078 (0.0606; $P < 0.0001$) or RT014/020 (0.0093; $P = 0.0328$).

Patients infected with RT018 were significantly older than those infected with RT078 or all other RTs. The median ages of patients with RT018, RT078, and all other RTs were 78 years, 71 years, and 69 years, respectively (RT018 versus RT078, $P = 0.0074$; RT018 versus all other RTs, $P < 0.0001$). Only 12.4% of patients with RT018 were younger than 65 years compared to 28.6% of

### Table 1: PCR ribotypes accounting for C. difficile strains isolated from index case patients$^a$

<table>
<thead>
<tr>
<th>PCR ribotype</th>
<th>No. (%) of strains</th>
</tr>
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<tbody>
<tr>
<td>018</td>
<td>103 (42.4)</td>
</tr>
<tr>
<td>078</td>
<td>33 (13.6)</td>
</tr>
<tr>
<td>014/020</td>
<td>14 (5.8)</td>
</tr>
<tr>
<td>002</td>
<td>4 (1.7)</td>
</tr>
<tr>
<td>001</td>
<td>3 (1.2)</td>
</tr>
<tr>
<td>056</td>
<td>3 (1.2)</td>
</tr>
<tr>
<td>012</td>
<td>2 (0.8)</td>
</tr>
<tr>
<td>027</td>
<td>2 (0.8)</td>
</tr>
<tr>
<td>137</td>
<td>1 (0.4)</td>
</tr>
<tr>
<td>106</td>
<td>1 (0.4)</td>
</tr>
<tr>
<td>Other</td>
<td>77 (31.7)</td>
</tr>
</tbody>
</table>

$^a n = 243.$

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**FIG 2** Isolation and distribution of predominant PCR ribotypes. Percentage of C. difficile strains belonging to RT018, RT078, and all other (non-018 and non-078) RTs isolated from index case patients ($n = 243$) between 2009 and 2013 (A) and distribution in surgical, medical, and rehabilitation wards and intensive care units (B).
patients with RT078 ($P = 0.0255$) and 38% of those with all other RTs ($P < 0.0001$).

**Molecular characteristics of C. difficile strains.** Table 2 shows the molecular characteristics of C. difficile strains isolated from index cases. All strains tested positive for toxins A and B. The prevalence of strains with binary toxin was 18.5%; binary toxin was present in all RT078 strains and in 11.2% of other RT strains. The gyrA mutation C245T, conferring a high level of resistance to fluoroquinolones, was present in 56.8% of the strains. In particular, the presence of this mutation was associated with the two major RTs, 100% of the RT018 and 66.6% of the RT078 C. difficile strains, while only 12.1% of the strains belonging to all other RTs carried this mutation (RT018 and RT078 versus other RTs; $P < 0.001$). The presence of the ermB gene was observed in 23% of the strains. It was mainly detected in RT078 (42.4%) and in other RT strains (29%); only 10.7% of the RT018 strains were ermB positive (RT078 and other RTs versus RT018; $P < 0.001$). We did not observe significant changes in the frequency of detection of the gyrA mutation C245T or the gene ermB during the study period.

All RT078 strains presented the 39-bp deletion and the nonsense mutation C184T in the tcdC gene. The two RT027 strains carried the binary toxin genes, the 18-bp deletion, the nucleotide deletion at position 117 in the tcdC gene, and the gyrA mutation C245T, and they were ermB negative.

**DISCUSSION**

Currently, longitudinal data on the burden of CDI in Italy are largely missing and uncertain. A very recent study, conducted in five hospitals in Rome over a 6-year period (2006 to 2011), reported an increase in the CDI incidence from 0.84 to 2.3 per 10,000 patient-days (29). In our study, we observed higher CDI rates, with an average value of 3.05 episodes per 10,000 patient-days, perfectly overlapping with incidence data of other similar hospitals of the Lombardy region. In addition, in our hospital between 2009 and 2013, we noticed a reduction in the CDI incidence that did not reach statistical significance due to the occurrence of an increasing peak in 2012. This peak was associated with the detection of a major number of nosocomial transmission events compared to the other years, partly due to changes in the routine workflow of the microbiology laboratory, which shortened the time to culture the positive toxin A&B stool samples, thus increasing the rate of positive C. difficile culture and improving the detection of outbreaks and epidemiological monitoring. In 2006, the infection control committee of the OSR finalized the first 5-year plan (2007 to 2011) for the management and prevention of health care-associated infections. This plan allowed implementing several important strategies: designation of infection control nurses and doctors in each ward; revision of the guidelines on hand hygiene, environment cleaning, and antiseptic and disinfectant use; introduction of guidelines on antibiotic prophylaxis; and education and training activities of health care workers. Moreover, molecular typing of the major multidrug-resistant organisms (MDROs), including C. difficile, was introduced to rapidly identify and limit epidemic clusters. The implementation of the guidelines on isolation and contact precautions was strengthened to control the spreading of MDROs and reduce the risk of transmission. Although none of these measures specifically targeted C. difficile, they could have contributed, at least in part, to the lowering of the number of CDI episodes between 2009 and 2011.

The use of broad-spectrum β-lactams, cephalosporins, fluoroquinolones, clindamycin, and macrolides has been associated with increasing CDI incidence (30–32). Through the study period, there were no significant changes in the consumption of these antibiotics, and, although infectious disease consultants were regularly involved in antibiotic therapy prescribing for infected patients in all wards, no antimicrobial stewardship programs were implemented at that time. Overall, we did not find any correlation between either the CDI trend or RT prevalence and the consumption of the different classes of antibiotics over time. The high number of interpatient transmission episodes and, consequently, the increase in the CDI incidence observed in 2012 did not seem to be linked to any specific antibiotic class consumption trend.

Although RT027 has been associated with the current CDI epidemic, there has been evidence recently that, in some European countries, the prevalence of this RT is starting to wane (18, 33). Our group reported the presence of RT027 in Italy in 2010; since then, it has been only sporadically detected (16, 17, 19). Here, we confirmed that the prevalence of RT027 is low, accounting for only 0.8% of all the C. difficile strains analyzed. As demonstrated by several studies, the molecular features of C. difficile are related to country and may change over time; thus, it is likely that other epidemic strains of C. difficile could emerge (9, 34–37). In agreement with the few Italian reports available, in our setting the predominant genotype during the whole study period was RT018, which accounted for 42.4% of the strains, followed by RT078, which was responsible for 13.6% of the CDI index cases (18, 20).

As previously reported, the fact that the prevalence of the gyrA mutation C245T was significantly higher in RT018 and RT078 strains than in other RT strains suggested that the use of fluoroquinolones could be a driving force for the selection and spread of successful C. difficile genotypes (2, 20). The ermB gene was observed with a significantly higher frequency in RT078. We also noticed that RT078 was most frequently detected in intensive care
units compared to both RT018 and other RTs. Unfortunately, we
were not able to collect patient clinical data to evaluate the severity
of the C. difficile-associated disease. In Europe, RT078 has been
reported to be the predominant RT identified in CA-CDI cases
(11, 18), but we did not find in our population study any associa-
tion between RT078 and a source of CDI in the community. This
could be partially explained because the majority of our CDI cases
were health care associated; however, the proportion of RT078
and RT018 strains responsible for CA-CDI was similar. Overall,
the prevalence of RT078 significantly increased over time, but the
interpatient transmissibility was very low, as highlighted by the
transmission index (0.0606). In contrast, RT018 accounted for
95.7% of the secondary cases and was highly transmissible, pre-
senting a transmission index 10-fold higher than that of RT078
(0.640). We also found that patients infected with an RT018 strain
were significantly older than those infected with RT078 or other
RT strains, confirming that old age represents an important risk
factor for CDI and suggesting an association between the epi-
demic success of an RT and the elderly (3).

The hypervirulence of RT027 and RT078 has recently become
a matter of debate. Indeed, there has been growing evidence that
RT027 was not a significant predictor of severe CDI and poor
outcome (43–45). For pathogenic bacte-
ria, lower virulence could favor host survival and, consequently,
transmission to new susceptible hosts, and the success of an epi-
demic strain relies on its transmission capacity. In this study, we
provided for the first time epidemiological evidence for inclusion
of RT018 among successful epidemic genotypes. Our findings
were further corroborated by recent works showing that RT018
was not only predominant and responsible for outbreaks in Asia,
particularly in Korea and Japan, but also associated with CDI re-
lapse (43–45).

This study has some limitations. Our data originate from a
single, but very representative, health care setting. Also, only 70%
of the isolates from the CDI cases diagnosed during the study time
were available for the study.

Despite these limitations, the presented results indicate that C.
difficile genotypes other than RT027 (e.g., RT018) deserve more
attention in clinical practice for their epidemic potential and un-
derline the importance of local surveillance program to identify
and monitor areas of endemicity and epidemic C. difficile strains.
Additionally, care should be taken when defining RT027 or RT078
as hypervirulent and using this term as a synonym for epidemic.
More studies are needed to understand the epidemiological dy-
namics of C. difficile genotypes worldwide and to identify the fea-
tures correlated to the epidemic behavior of a C. difficile strain.
Until then, all genotypes should be monitored.

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difficile strains.

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