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FULL PAPER

Prevalence of *Clostridium difficile* and ribotype 027 infection in patients with nosocomial diarrhoea in Southern Italy

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SUMMARY

Clostridium difficile is an emerging cause of healthcare-associated infections. The increasing frequency and severity is attributed to highly virulent ribotypes such as 027.
The aim of this study was to retrospectively analyze the prevalence of CDI and ribotype 027 in 481 clinical samples collected from hospitalized patients and sent to the laboratory of molecular biology, UOC Microbiology and Virology, Azienda Ospedaliera-Universitaria, Policlinico of Bari, Italy. Toxins A+B and DNA *C. difficile* detections were performed using immunochromatographic test and a multiplex real-time PCR assay, respectively.
Overall, 37/366 (10.11%) patients were positive at the immunochromatographic assay. This result was confirmed in 31 (8.47%) samples from 31 different patients by molecular assay. Logist regression confirmed age >50 years (adjusted odds ratio [aOR]: 4.29, 95%CI:1.44-18.50) and hospitalization in the Infectious Diseases (aOR: 3.77, 95%CI: 1.34-9.85) ward were risk factors for CDI. The associated 027 ribotype deletion D117tcd was detected in seven (22.58%) of 31 positive patients. Exploratory analysis of monthly prevalence of 027 ribotype suggested a slight increase after August 2015.
Our results show that a monitoring program is needed to either better assess the diffusion of CDI and ribotype 027 or also to establish the risk factors associated with the transmission in our healthcare facilities.

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INTRODUCTION

Clostridium difficile is a Gram-positive anaerobic bacillus responsible for a range of clinical conditions from mild diarrhea to psudomembranous colitis, toxic megacolon and bowel perforation, commonly known as *C. difficile* infections (CDI) (Bartlett, 2002; Wilcox, 2003; Lyras *et al.*, 2009; Sunenshine and McDonald, 2006; Bartlett, 2006; Monaghan *et al.*, 2008; Rupnik *et al.*, 2009; Surawicz *et al.*, 2013). CDI is an intestinal disease mediated by powerful cytotoxic enzymes that damage the intestinal mucosa (Rupnik *et al.*, 2009; Chen *et al.*, 2015). These cytotoxic enzymes, named toxin A (TcdA) and toxin B (TcdB) alter cytoskeletal actin, causing a reduction of transepithelial resistance, fluid accumulation, and destruction of the intestinal epithelium (Rupnik *et al.*, 2009; Carter *et al.*, 2015; Kuehne *et al.*, 2010; Voth and Ballard, 2005).
Worldwide, CDI continues to be an important public health problem (McCollum and Rodriguez, 2012; Centers for Disease Control and Prevention, 2012). European and American studies have reported an increase of CDI in terms of incidence, morbidity and mortality (Loo *et al.*, 2005; Pepin *et al.*, 2004; Kuijper *et al.*, 2007a; McDonald *et al.*, 2005; Smith, 2005). In Italy it is difficult obtain an overview of CDI epidemiology because a national monitoring program is still lacking (Roncarati *et al.*, 2017; Cioni *et al.*, 2016, Sansone *et al.*, 2009; Davies *et al.*, 2014). In fact, the few Italian data on CDI rates come from a single or a small group of hospitals and report a highly variable incidence.
An important consideration in the increasing incidence and severity of CDI is due to the emergence of a hypervirulent strain, typed as ribotype 027 (Khanna and Pardi, 2012a). This strain is associated with a hyperproduction of toxins A and B, production of a third toxin called binary toxin encoded by tcdC gene and also a delection at nucleotide 117 in the same gene (Bartlett, 2006; Kato *et al.*, 2007; Kuijper *et al.*, 2006; Kuijper *et al.*, 2007b; Labbè *et al.*, 2008; Loo *et al.*, 2005; Kelly and LaMont, 2008; Loo *et al.*, 2005; Pepin *et al.*, 2005; Warny *et al.*, 2005, O'Connor *et al.*, 2009; Warny *et al.*, 2005). In the last ten years, the incidence of diseases associated with 027 strain has increased in Europe, the USA, Canada and Asia (Kuijper *et al.*, 2006; Vonberg *et al.*, 2007; Pituch *et al.*, 2006; McDonald *et al.*, 2006).
Clinical suspicion and timely laboratory diagnosis are crucial for the treatment of CDI. Although a wide range of laboratory tests are available, recent advances in CDI diagnosis include the introduction of molecular tests which have revolutionized the diagnostic approach to CDI (Oldfield *et al.*, 2014; Planche *et al.*, 2013).
The aim of this study was to evaluate the prevalence of CDI in clinical samples collected from hospitalized patients in

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a small area of Apulia, a region of Southern Italy. Moreover, the time series of monthly prevalence values were also evaluated to detect presence of an increasing trend.

MATERIALS AND METHODS

From February 2015 to March 2016, 481 liquid or unformed stool samples collected from 366 hospitalized patients were analyzed in the Laboratory of molecular biology, U.O.C. Microbiology and Virology, Azienda Ospedaliero-Universitaria, Policlinico of Bari. In particular, more than one sample (range: 2-6) was collected on 78 patients. Specimens without added transport medium were transported to the laboratory and immediately tested by an immunochromatographic test (Mascia Brunelli, Milano). Following the recent guidelines (Oldfield *et al.*, 2014), samples resulting positive were then confirmed by a molecular method (Xpert *C. difficile* multiplex real-time PCR assay, Cepheid, Sunnyvale, CA) within 24 hours. A patient was considered infected by *C. difficile* if at least one sample was positive at both tests.
Sample informations (date of sampling, ward, type of specimen, final testing results) together with the data of patients for whom molecular testing was performed (i.e. age and sex) were recorded in an anonymous database by changing sensitive data into alphanumeric codes. No clinical data associated with these specimens was available. As a retrospective study, formal consent is not required.

C. difficile toxin A+B Card

C. difficile toxin A+B Card is a immunochromatographic rapid test for the qualitative detection of *C. difficile* toxin A and toxin B in stool samples. Briefly, a small portion of stool sample was transferred to the extraction tube contained in the kit, shaken to obtain a homogeneous solution and then 100 µl were dispensed into the card's well. The reaction mixture flowed through the absorbent device.

Xpert C. difficile multiplex real-time PCR assay

The Xpert *C. difficile* (Cepheid, Sunnyvale, CA) is a multiplex real-time PCR assay that detects the toxin B gene (*tcd* B), the binary toxin gene (*cdt*) and the tcdC gene deletion at nt 117. DNA extraction and amplification are completely automated and the time is around 45 minutes. Briefly, a swab was dipped into the stool specimen container, then shifted to the sample Xpert *C. difficile* reagent provided in the kit and capped. The specimen was vortexed for ten seconds and then the solution was pipetted into the related cartridge. Next, the cartridge barcode was scanned and placed in the Gene Xpert Instrument (Cepheid) and the test was performed by the Gene Xpert *C. difficile* assay program.

Statistical analysis

The independence of categorical and continuous variables was assessed by two-tailed Fisher's exact Test and two-tailed Kruskal-Wallis Test, respectively, as appropriate. Strength of associations was assessed by odds ratio and 95% confidence interval. Logistic regression analysis was performed combining statistically significant variables (age >50, Infectious Diseases ward) and gender and Internal Medicine ward as adjusting factors. Calculations of all statistical tests were performed by the open source environment R (R Core Team, 2016).
Exploratory analysis of time trends of overall and 027 *C. difficile* infections was performed by Lowess smoothing (Locally weighted scatterplot smoothing) on one-month period prevalences. The smoother span value selected for the analysis was 0.8.

RESULTS

From March 2015 to March 2016, 481 stool samples from 366 patients (193 males and 173 females, M/F=1.12) were collected and analyzed.
Median age of men was 60.35 years (Inter Quantile Range: 41.39-43.10) while the median age of women was 63.76 years (42.76-78.79). The age difference was not statistically significant (*p value*=0.076).
37/366 (10.11%) patients were positive at the immunochromatographic assay. However, only 31 (8.47%) samples from 31 different patients were confirmed positive at the molecular assay.
The median age of 31 patients with CDI was 76.43 years (72.20-80.26), whereas the median age of patients without CDI was 60.30 years (41.00-74.21) and such difference was statistically significant (*p value*<0.001). In particular, age >50 was a risk factor for CDI (7.65% *vs* 0.82%, Odds Ratio [OR]: 4.74, 95% Confidence Interval [95%CI]: 1.42-24.86, *p value*=0.004). Moreover, the prevalence of CDI in male and female patients was not statistically significant (3.83% *vs* 4.65, *p value*=0.453).
The majority of the 366 patients were hospitalized in the Internal Medicine (91) and Infectious Diseases (48) wards. At univariate analysis, the prevalence of CDI among the 366 patients was not statistically significant for Internal Medicine ward (3.28% *vs* 5.19%, OR: 2.04, 95%CI: 0.86-4.66, p value=0.080) while for Infectious Diseases it was

Table 1 - *Evaluation of the logistic regression model for the presence of Clostridium difficile infection among the 366 analyzed patients.*

Variable	*Odds Ratio (95%CI)*	*Adjusted Odds Ratio (95%CI)*	*P value*
Age>50	4.74 (1.42-24.86)	4.29 (1.44-18.50)	0.020
Sex (M Vs F)	2.56 (0.97-6.54)	0.75 (0.35-1.60)	0.459
Internal Medicine (ward)	2.04 (0.86-4.66	2.25 (0.95-5.32)	0.062
Infectious Diseases (ward)	2.56 (0.92-6.43)	3.77 (1.34-9.85)	0.007

Table 2 - *Results obtained by GeneXpert C. difficile on 31 samples positive for Clostridium difficile infection.*

GeneXpert® results	*Number of samples*	*Percentage% (95%CI)*
Toxin B DNA (only)	19	61.29 (42.29-77.58)
Toxin B + Binary Toxin DNA	5	16.13 (6.09-34.47)
Deletion D117tcd	7	22.58 (10.28-41.54)

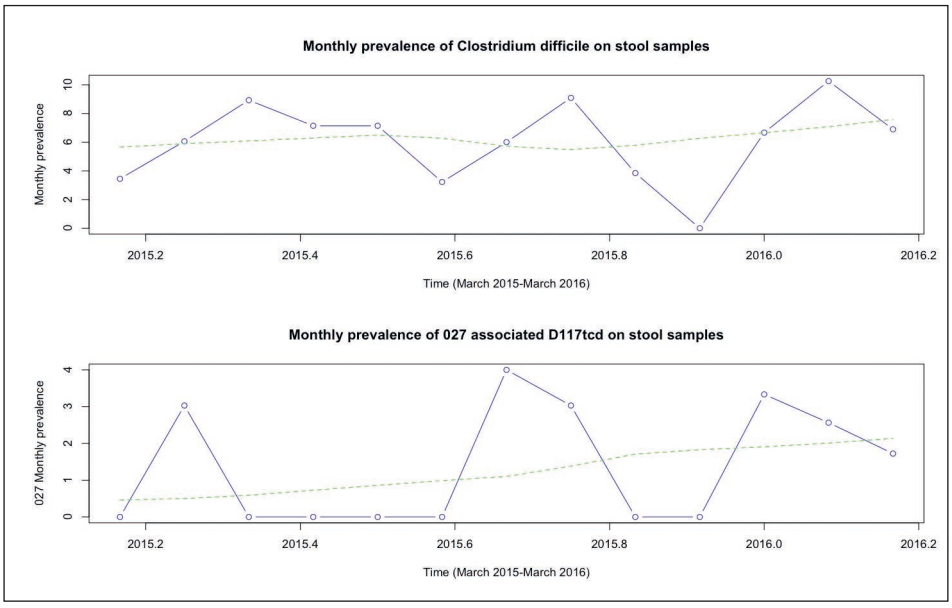

Figure 1 - *Evaluation of trend of monthly prevalences of C. difficile and D117tcd detection on 481 stool samples by Lowess smoothing with smoothing span value 0.8.*

statistically significant (2.18% *vs* 6.28%, OR: 2.56, 95%CI: 0.92-6.43, p value=0.045). Logistic regression model confirmed that age >50 years and the presence of patients in the Infectious Diseases ward were risk factors for CDI (*Table 1*).

Among the 31 patients with CDI, 61.29% had only toxin B DNA and 16.13% both toxin B and binary toxin DNA. Deletion D117tcd which is associated with *C. difficile* ribotype 027 was detected in at least 22.58% (*Table 2*).

Exploratory analysis of the monthly prevalence of *C. difficile* among the 481 stool samples analyzed revealed a quite constant trend of *C. difficile* detection with a minimum value in December 2015 and a maximum in February 2016. On the contrary, the trend of 027-associated D117tcd began to increase slightly after August 2015 with a peak value in September 2015 (*Figure 1*).

DISCUSSION

The present study describes the prevalence of CDI from hospitalized patients and an attempt to evaluate the distribution of ribotype 027 isolated from stool samples from March 2015 to March 2016 in Bari. To our knowledge, this is the first study to evaluate the prevalence of CDI in a important area of South Italy. The prevalence rate of CDI among patients undergoing *C. difficile* testing was 8.47%. More recent studies showed similar rates with only 5%-10% of samples testing positive (American Society for Microbiology, 2010). This prevalence is also similar to those reported by Roncarati *et al.* and Tan *et al.* (Roncarati *et al.*, 2017; Tan *et al.*, 2014). The prevalence rate of CDI in a five-year study of Aschbacher *et al.* in Bolzano was 9.41% with incidence rates ranging from 4.3/10,000 in 2009 to 1.8/10,000 patients/days in 2013 (Aschbacher *et al.*, 2017). On the contrary, a study by Cioni et al. in Internal Medicine wards reported a prevalence rate of 0.96% with an estimated incidence of 5.3/10,000 patients-days (Cioni *et al.*, 2016).

An Italian survey revealed that the average percentage of positive tests for *C. difficile* toxins was 12.2% but only 34% of laboratories used a algorithm for CDI (Spigaglia *et al.*, 2016). Immunochromatographic assay detected toxin A/B in 37 patients (10.11%) according to the result of Spigaglia *et al.* (Spigaglia *et al.*, 2016) but only in 31 was the result confirmed by the molecular assay.

The increasing incidence and severity of CDI has been attributed to the emergence of the hypervirulent strain 027 (McDonald *et al.*, 2005). An update on the spread of *C. difficile* ribotype 027 in Europe was published in 2007 (Kuijper *et al.*, 2007b). We detected seven patients with ribotype 027-linked deletion D117tcd accounting for 22.58% of *C. difficile* positive patients. Such result is in line with that reported in a northern Italian study (Baldan *et al.*, 2010). Moreover, *C. difficile* ribotype 027 and ribotype 078 were firstly detected in Italy in eight and 26 cases of CDI in generally younger patients (Baldan *et al.*, 2010). Ribotype 027 was also detected in 10/24 stool samples collected from patients with severe diarrhoea and clinical suspicion of hypervirulent strain from seven hospitals in Rome and another multicentre study in six tertiary care hospitals in Rome detected ribotype 027 in 270 of 563 patients with CDI (Falcone *et al.*, 2016, Di Bella *et al.*, 2012). In the survey of Spigaglia et al. ribotype 027 accounted for 8% of the clinical isolates (Spigaglia *et al.*, 2016), whereas ribotype 027 accounted for 19% of the 1,196 *C. difficile* clinical isolates from 19 European Countries (Davies *et al.*, 2016).

The hypervirulent fluoroquinolone-resistant *C. difficile* ribotype 027 is considered a cause of severe outbreaks as well as higher death rates, longer hospital stays, and frequent relapses (Bacci *et al.*, 2011). On the contrary, the pathological significance of binary toxin is not yet clear. In fact, Bacci et al. reported a 30-day fatality rate (27.8%) in 72 *C. difficile* non-027 binary toxin positive patients similar to 193 *C. difficile* 027 patients (28.0%). The cumulative risk of death after 60 days at Kaplan Meier analysis was 24.5% in 212 *C. difficile* toxin A and B positive patients, 37.1% in *C. difficile* 027 patients and 30.5% in *C. difficile* non-027 binary toxin positive patients. Barbut et al. reported an association between binary toxin positive *C. difficile* strains (11% of 131 strains) and more severe diarrhea (RR 3.38) and also an increased fatality rate (RR 2.55) (Barbut *et al.*, 2007). However, other studies failed to detect an increase in mortality or relapse rates. In particular, Walk *et al.* reported that *C. difficile* ribotype was not a

predictor of 34 severe CDI cases (Walk *et al.*, 2012). Kim et al. reported that 11 binary toxin positive strains compared to 105 toxin A and B positive strains were associated with a significant increase of leukocytosis and mucoid stool but not with different clinical outcome (Kim *et al.*, 2013). Pilate et al. also failed to detect an increase in mortality of the 33 binary toxin positive patients compared to 66 patients who were carriers of non-toxigenic *C. difficile* despite a higher peripheral leukocytosis (Pilate *et al.*, 2016). Moreover, Reigadas et al. reported no association between 54 non-027 binary toxin-positive patients and poor clinical outcome (OR: 0.793, 95%CI: 0.243-2.591) compared to 265 *C. difficil*e-positive binary toxin-negative patients (Reidagas *et al.*, 2016).

Internal Medicine and Infectious Diseases wards were the most affected by episodes of CDI but the logistic model confirmed only Infectious Diseases ward and older age as risk factors for CDI. Several Italian studies showed that the area of medicine (general medicine and long-term care wards) was the most affected by CDI (Roncarati *et al.*, 2017). Moreover, older age, the use of antimicrobials and proton pump inhibitors, hospital stay before CDI, and previous CDI are well known risk factors for CDI (Roncarati *et al.*, 2017, Cioni *et al.*, 2016). According to some studies, female gender was associated with CDI but this result was not confirmed in our and other studies (Cioni *et al.*, 2016, Boone *et al.*, 2012, Khanna *et al.*, 2012b).

Exploratory analysis of the monthly prevalence of *C. difficile* detection in stools samples revealed a quite constant trend with a peak number of cases in February 2016. Other Italian studies also showed a constant trend of CDI cases despite major fluctuations (Aschbacher *et al.*, 2017, Mellace *et al.*, 2013). The time series of monthly number of cases of CDI analyzed by Polgreeen *et al*. revealed a clear pattern of yearly seasonal variation with peaks mostly in March and an increasing trend in accordance with other studies (Polgreeen *et al.*, 2010). On the contrary, the monthly prevalence of ribotype 027 seems to show an increasing trend in the last months of the observation period but other studies will be needed to confirm such results.

This study shows some weaknesses that should be considered. First, immunochromatographic tests for toxin A/B detections may lack sensitivity (Alcalà *et al.*, 2008). For this reason, the prevalence of CDI was possibly underestimated. Secondly, the observation period is quite short and the presence of a seasonal and cyclical pattern may not be revealed. Thirdly, due to the absence of data regarding length of hospitalization and clinical outcomes, it was not possible to estimate the incidence of CDI and mortality rates, especially for hypervirulent ribotype 027. Moreover, we were not able to ribotype the Xpert positive clinical strains due to the retrospective nature of the study. In fact, *C. difficile* Xpert offers only a very limited discrimination of potential ribotypes. It is also possible that *C. difficile* Xpert may erroneously classify some ribotypes as presumptive 027. In fact, several cases of ribotype misclassification of *C. difficile* Xpert as presumptive 027 were reported. Moreover, *C. difficile* Xpert has been reported to be unable to diagnose multiple infections from different *C. difficile* strains (McMillen *et al.*, 2015, Mentula *et al.*, 2015, Tenover *et al.*, 2011). However, some studies showed that ribotypes other than 027 are increasing in Italy (Barbanti *et al.*, 2016).

Despite this limitations, this is the first study to have estimated the prevalence and time trend of CDI and ribotype 027 in South Italy. Moreover, surveillance data are needed to better assess the diffusion of *C. difficile* 027 and other ribotypes in South Italy and to establish risk factors associated with their transmission in our healthcare facilities. Such data will be also useful to improve specific antibiotic stewardship guidelines to reduce risk of CDI in most interested wards.

Conflict of interest

The authors declare that they have no conflict of interest.

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Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and / or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. For this type of study formal consent is not required.

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