New Microbiologica, **35**, 307-316, 2012

# Laboratory diagnosis of *Clostridium difficile* associated diarrhoea and molecular characterization of clinical isolates

**Giuseppe Russello[1], Antonio Russo[2], Francesca Sisto[3], Maria Maddalena Scaltrito[3], Claudio Farina[1]**

*[1]U.O.C. Microbiologia e Virologia, A.O. Ospedale San Carlo Borromeo, Milano, Italy;*
*[2]U.O.C. Epidemiologia e Statistica, A.O. Ospedale San Carlo Borromeo, Milano, Italy;*
*[3]Dipartimento di Sanità Pubblica-Microbiologia-Virologia, Sezione di Microbiologia, Università degli Studi di Milano, Milano, Italy*

**SUMMARY**

We evaluated a three-step algorithm for laboratory diagnosis of *Clostridium difficile*-associated diarrhoea (CDAD). First, stool specimens were screened using an EIA test for glutamate dehydrogenase detection. Screen-positive specimens were tested by a rapid cytotoxintoxin A/B assay and subjected to stool culture. All cultures positive for *C. difficile* underwent toxigenic culture.

The results showed that toxigenic culture allowed us to recover 37/156 (24.4%) stool samples harbouring toxigenic *C. difficile* that would have been missed by using faecal cytotoxin assay alone. This determined an increase in infection prevalence of 4.2% (from 11.4% to 15.6 %).

Furthermore, to characterize the clinical *Clostridium difficile* isolates and the distribution of PCR ribotypes circulating in the San Carlo Borromeo hospital, molecular typing using semi-automated repetitive-sequence-based PCR (rep-PCR) and PCR ribotyping, and an evaluation of the antibiotic resistance were also performed.

Among them, 71 indistinguishable strains were detected by rep-PCR and 83 by PCR-ribotyping revealing *C. difficile* outbreaks in our hospital. A total of 6 different ribotypes were obtained by PCR ribotyping. The most frequent ribotype was 018 (88.2%) that also showed resistance to moxifloxacin. In one case, uncommon PCR ribotype 186 was also identified.

Key words: *Clostridium difficile*, Toxigenic culture, PCR-ribotyping, rep-PCR, Nosocomial infection.

*Received* October 20, 2011 *Accepted* March 08, 2012

## INTRODUCTION

*Clostridium difficile* is responsible for 15-25% of cases of antibiotic-associated diarrhoea and for more than 95% of cases of pseudomembranous colitis. This anaerobic bacterium is a major cause of nosocomial diarrhoea in hospitalized patients and can be responsible for large outbreaks (Barbut *et al.*, 2000).

To decrease the prevalence of this bacterium and the consequences of its infection it is necessary to perform an accurate and rapid diagnosis, to follow the good hygiene practices, to disinfect the environment daily and to apply a restrictive antibiotic policy.

There are many different approaches that can be used in the laboratory diagnosis of *Clostridium difficile*-associated diarrhoea (CDAD). The use of rapids enzyme immunoassays (EIA) performed directly on stool samples has proved to be poorly sensitive (Alcalà *et al.*, 2008; Sloan *et al.*, 2008). However, the poor performance of toxin enzyme immunoassays was reassessed after the introduction of *C. difficile* glutamate dehydrogenase (GDH) screening as part of two- or three-step algorithms (Reller *et al.*, 2007; Ticehurst *et al.*, 2006). GDH screening is characterized by a high level of sensitivity and a low level of specificity

*Corresponding author*
Claudio Farina
U.O.C. Microbiologia e Virologia
A.O. Ospedale San Carlo Borromeo
Via Pio II, 3 - 20153 Milano, Italia
E-mail: farina.claudio@sancarlo.mi.it

(Sharp *et al.*, 2010). Therefore a GDH positive result must be corroborated by confirmation assays, i.e. enzyme immunoassays (EIA).
However several authors have shown a poor sensitivity of different EIAs test as confirmation assays (Gilligan P., 2010) and recently, it has been shown that the sensitivity of the GDH screening in a two-step algorithm can vary depending on the ribotypes involved in the infection. The lowest sensitivity of the GDH test was observed with ribotypes 002, 027 and 106 (Tenover *et al.*, 2010).
Recently, new molecular methods for the detection of genes encoding *C. difficile* toxin B (*tcdB*) or toxin regulatory genes (*tcdC*) directly in stool samples has been developed. These procedures have shown good sensitivity and can also be used as part of two- or three-step algorithms for confirming of GDH-positive specimens (Larson *et al.*, 2010); Sloan *et al.*, 2008; Thomson *et al.*, 2007). A combination of tests, therefore, should be used to help the physician to establish a diagnosis of CDI.
The aim of this study was to evaluate the two-step algorithm for detection of *Clostridium difficile* and the contribution of toxigenic culture as confirming *test* in GDH-positive specimens in a three-step algorithm.
In order to analyze the spread of strains in different hospital wards and to know which ribotypes are present locally in comparison with the epidemiological Italian data, rep-PCR and PCR ribotyping respectively were performed.
Furthermore, we examined the antimicrobial susceptibility of *C. difficile* isolates to five different antibiotics to evaluate the possible circulation of strains with low susceptibility to metronidazole, vancomycin or quinolones.

## MATERIAL AND METHODS

### Case selection procedure and specimens collection

A total of 1052 consecutive stool specimens belonging to Bristol stool chart types 5-7 (Bauer *et al.*, 2009) were collected from 796 symptomatic patients over a period of 8 months (March 2009-October 2009). These patients were consecutively admitted in the "San Carlo Borromeo" Hospital (Milano, Italy). All stool samples were tested for glutamate dehydrogenase (GDH) antigen and positive specimens were further tested for A and B toxins. Stool samples that resulted positive to the GDH test were also used for culturing.
Ninety-two unique culture positive samples (of 92 consecutive symptomatic patients) were then randomly selected for additional investigations: toxigenic culture, toxins and binary toxin detection via PCR, and rep-PCR and PCR rybotyping for genotyping.
Twenty-one *C. difficile* strains representative of each rep-PCR pattern were further tested for the sensitivity to metronidazole, moxifloxacin, vancomycin, daptomycin or tigecycline using E test strips.

### Clinical characteristics of patients resulted positive to the screening GDH

The clinical characteristics of 92 patients are shown in table 1. The mean age of patients was 72.7 years (range, 23 to 95 years), and the ratio of males to females was 1:1.7. The mean hospital stay of these patients was 34.1 days (range, 9 to 98 days). Most patients presented as preexisting pathologies arterial hypertension (42.3%), neurological disorders (30.4%) and chronic obstructive pulmonary disease (COPD) (28.3%), and the previous antibiotic treatment were administration of third-generation cephalosporins (34.8%), quinolones (29.3%) and carbapenems (28.3%) were the most used.

### *Clostridium difficile* culture

Stool samples were subjected to thermal shock-according to Lahn *et al.* (Lahn *et al.*, 1993). Ten µL of stool sample were suspended in 500 mL of sterile water (B. Braun Melsungen AG, Germany) and heated in thermostatic bath at 65ºC for 20 minutes. Three drops of the faecal layer were immediately spread on Columbia blood agar without any supplement (bioMérieux, Marcy-l'Etoile, France) and incubated in anaerobic atmosphere for 48 hours at 35°C. Suspected colonies (based on morphological criteria, odour and Gram-stain) were re-isolated to obtain pure culture on Columbia blood agar without any supplement. Species identity was confirmed using a commercial biochemical identification system (API 20A, BioMérieux). All stool samples were tested within 2 hours of collection and, in case the tests could not be performed rapidly, they were kept at 4°C until processing.

### GDH assay, toxin A/B detection, and toxigenic culture

Stool samples were tested for GDH antigen, and for toxins A and B, using C. DIFF CHEK™-60 (Wampole Laboratories, Princeton, NJ, USA) and Meridian Premier Toxin A and B (PTAB) EIA (Meridian Bioscience Inc., Cincinnati, OH, USA), respectively. Toxigenic culture was also performed using Meridian Premier Toxin A and B (PTAB) EIA. Each test was performed according to the manufacturer's instructions.

TABLE 1 - *Clinical characteristics of 92 patients selected.*

| *Variables* | *No. of patients (%)* |
|---|---|
| Age (yr)a | 72,7 (23-95) |
| Sex | |
| Male | 43,5 (40) |
| Female | 56,5 (52) |
| Hospital stay (day) | 34,1 (9-98) |
| Preexisting pathology | |
| Myocardial infarction | 7,5 (6) |
| Heart rhythm disturbances | 20,7 (19) |
| Heart valves diseases | 7,5 (6) |
| Pulmonary circulation diseases | 25,0 (23) |
| Peripheral vessels diseases | 18,5 (17) |
| Hypertension | 42,3 (39) |
| Neurological disorders | 30,4 (28) |
| COPD* | 28,3 (26) |
| Diabetes | 18,5 (17) |
| Chronic liver disease | 17,4 (16) |
| Peptic ulcer | 2,1 (2) |
| Malignant neoplasms | 13,0 (12) |
| Rheumatoid arthritis | 2,1 (2) |
| Coagulopathies | 2,1 (2) |
| Anemia | 25,0 (23) |
| Psychiatric disorders | 10,7 (10) |
| Previous drugs treatment | |
| Antiulcers | 23,9 (22) |
| Laxatives | 10,7 (10) |
| Antineoplastics | 1,1 (1) |
| First-generation cephalosporins | 4,3 (4) |
| Third-generation cephalosporins | 34,8 (32) |
| Carbapenems | 28,3 (26) |
| Quinolones | 29,3 (27) |
| Glycopeptides | 6,5 (6) |
| Penicillins | 13,0 (12) |
| Macrolides | 4,3 (4) |
| Folate inhibitors | 1,1 (1) |
| Clinical symptoms | |
| Diarrhea at the admission to hospital | 23 |
| Diarrhea after the admission to hospital | 69 |

*Chronic obstructive pulmonary disease

### Detection of toxin A and B genes and binary toxin gene

DNA was extracted from a 10 mL loop using the UltraClean™ microbial DNA isolation kit (Mo Bio Laboratories, Solona Beach, CA, USA) according to the manufacturer's recommendation. Toxin genes were determined by PCR (Kato *et al.*, 1998) by using 5 µl of DNA extract to evaluate the sensitivity of Meridian Premier Toxin A and B (PTAB) EIA assay.

For detection of binary toxin gene, we followed the method reported on the toxinotypes home page (http://www.mf.uni-mb.si/mikro/tox) (Rupnik *et al.*, 1998) by amplification of 510 bp fragment of *cdtB* gene, which codes for the binding toxin component. PCR conditions and primers were previously described by Stubbs *et al.* (Stubbs *et al.*, 2000).

### rep-PCR

DNA extracted was amplified using the DiversiLab *Clostridium difficile* Kit (Bacterial Barcodes, Inc.) according to the manufacturer's instructions. Thermal cycling parameters were as follows; initial denaturation at 94°C for 2 min; 35 cycles of denaturation at 94°C for 30°s, annealing at 45°C for 30 s, and extension at 70°C for 90 s; and a final extension at 70°C for 3 min. Analysis of rep-PCR products was implemented using a DiversiLab system in which the amplified fragments of various sizes and intensities were separated and detected using a microfluidics LabChip with an 98 Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, California, USA). The isolates relatedness was analyzed by the DiversiLab software, version 3.4, which uses the KL (Kullback-Leibler distance) correlation coefficient to determine distance matrices and the unweighted-pair group method with arithmetic mean (UPGMA) to create dendrograms, electropherograms, virtual gel images and scatter plots. Isolates were considered to be "indistinguishable" when showed >97% similarity and none banding differences, similar when had >95% similarity and 1 band differences and different with <95% similarity and ≥2 band differences.

**PCR-ribotyping**

PCR ribotyping is based on a comparison of patterns of PCR products from the 16S-23S rRNA intergenic spacer region. In this study, PCR-ribotyping analyses were performed using the method of Bidet *et al.* (Bidet *et al.*, 1999). The genomic DNA fingerprinting patterns produced were compared and analyzed with GelCompar II ver. 6.0 (Applied Maths, Sint-Martens-Latem, Belgium), using international reference strains representative of the most widespread PCR-ribotypes, kindly supplied by Dr. Ed Kuijper, European Centre for Disease Prevention and Control-ECDC.

**MIC determinations**

Twenty-one strains were tested against metronidazole, moxifloxacin, vancomycin, daptomycin or tigecyclineusing E test strips (bioMérieux). A suspension of *C. difficile* equivalent to 0,5 McFarland turbidity standard was spread on the agar surface and the plates were incubated in an anaerobic atmosphere.

MICs were read at the point where the elliptical zone of inhibition intersected the MIC scale after incubation for 24 h. Resistance was defined according to the following breakpoints: metronidazole >32 µg/mL, moxifloxacin >4 µg/mL, vancomycin >32 µg/mL, daptomycin >8 µg/mL, and tigecycline >4 µg/mL. Quality controls used for susceptibility testing included *Clostridium difficile* ATCC 9689.

## RESULTS

Among the 1052 fecal samples tested, 780 (74.1%) resulted negative for the GDH screening test, and 272 (25.9%) were positive. Among these, 116 (42.6%) were positive for faecal A and B toxin test (Meridian), 152 (55.9%) were negative and 4 (1.5%) had non-specific results. Out of 272 GDH positive sample 212 were also positive by culture (Table 2).

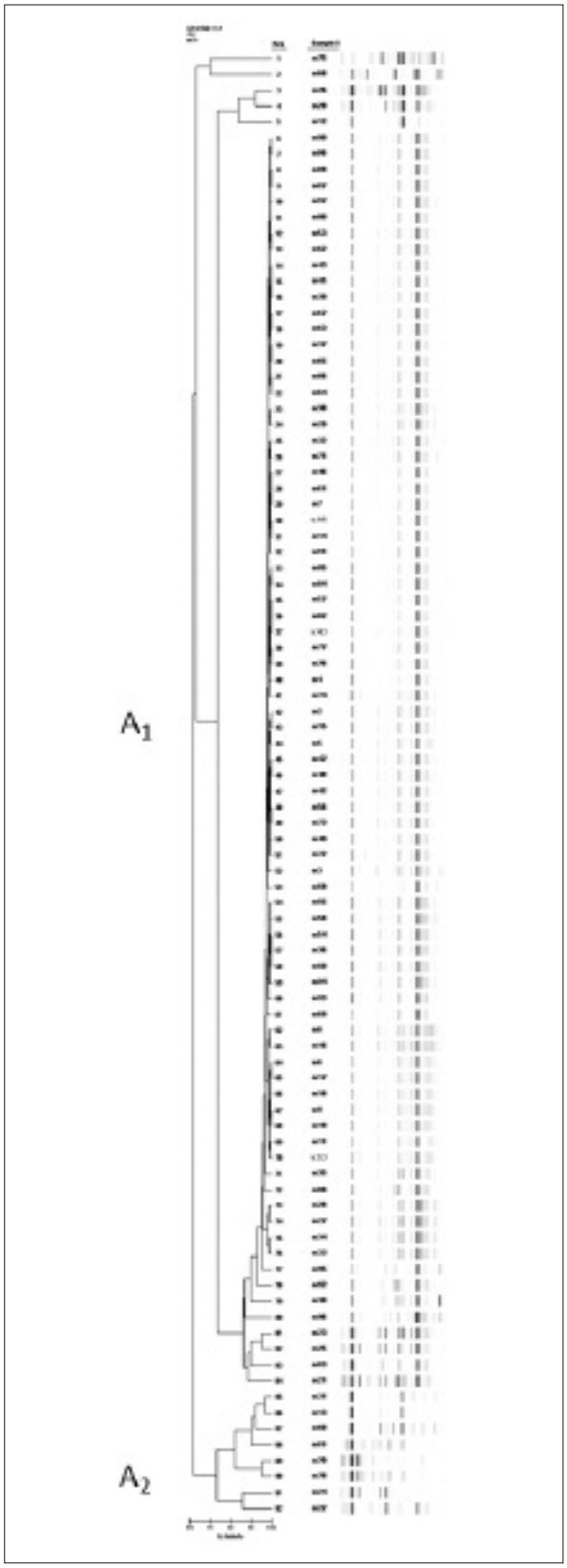
Ninety-two culture positive samples were randomly selected for additional investigations (Table 3). They showed the following results when tested for A and B toxins: 53 (57.6%) were positive, 35 (46.7%) were negative and 4 (4.3%) had non-specific results. These 92 samples were further tested by toxigenic culture. A total of 90 samples (97.8%) were positive, 1 (1.1%) was negative and 1 (1.1%) had non-specific results. The results of this test were confirmed by PCR detection of toxin A&B genes; the strain with an undefined result for toxins by EIA method resulted positive for both toxin genes. Before performing toxigenic

TABLE 2 - *Results of sensitive C. difficile diagnosis (March 2009 till October 2009).*

| *GDH Assay* | *N°(%)* | *Tot. samples* |
|---|---|---|
| + | 272 (25,9%) | 1052 |
| - | 780 (74,1 %) | |
| *Fecal cytotoxin assay* | *N°(%)* | *Tot. samples* |
| + | 116 (42,6%) | 272 |
| NSP | 4 (1,5%) | |
| - | 152 (55,9%) | |

TABLE 3 - *Results of culture, fecal toxin assay (A), toxigenic culture (B), and PCR (C).*

| *Culture* | *Fecal cytotoxin assay* | *N° (%)* |
|---|---|---|
| + | + | 53/92 (57,6%) |
| + | - | 35/92 (46,7%) |
| + | NSP | 4/92 (4,3%) |
| *Fecal cytotoxin assay* | *Toxigenic culture* | *N° (%)* |
| + | + | 53/92 (97,8%) |
| - | + | 34/92 (1,1%) |
| - | - | 1/92 (1,1%) |
| NSP | + | 3/92 (3,3%) |
| NSP | NSP | 1/92 (1,1%) |
| *Toxigenic culture* | *PCR detection* | *N° (%)* |
| + | + | 90/92 (97,8%) |
| - | - | 1/92 (1,1%) |
| NSP | + | 1/92 (1,1%) |




culture, the overall prevalence of *C. difficile* infection was 11.4% (91/796). The use of toxigenic culture allowed us to recover thirty-eight (24.4%) samples that would have been missed by performing only GDH screening and faecal-toxin detection. Consequently, the effective prevalence of *C. difficile* infection was of 15.6% (129/796) after the including the toxigenic culture in the diagnostic algorithm.

Figure 1 and Table 4 show the results of rep-PCR analysis by comparing virtual gel images and superimposing pairs of electropherograms. A total of 21 different patterns were detected among the 92 strains tested (Figure 1). The dendrogram analysis showed two main clusters ($A_1$ e $A_2$), characterized by an average similarity ratio of 62.7%. The first cluster included 14 different patterns and, in particular it shows 71 (78%) indistinguishable strains with an average similarity ratio of 97.5% and no banding differences. These strains were isolated from different patients admitted to all the departments excluding General Surgery Ward. The cluster $A_2$ included 7 different patterns with 2 indistinguishable strains of *C. difficile* (sc13 and sc31) isolated from different patients admitted to the San Carlo Borromeo Hospital at different times in different wards (Rehabilitation Unit and, after two months, Neurology Institute).

The results of PCR-rybotyping performed on *C. difficile* strains, are shown in Table 5. The 92 toxigenic isolates could be classificated into 6 distinct ribotypes. Of these, eighty three (90.1% of the strains and 100% of the strain belonged to subcluster $A_{1B}$) belonged to the PCR ribotype 018, two (2.2%) to PCR ribotype 002 (subcluster $A_{1A}$) and ribotype 106, one (1.1%) to the PCR ribotype 014/020 and ribotype 186 and the remaining three strains (3.3%) belonged to PCR ribotype 078. The presence of binary-toxin genes was also investigated. Eighty eight of 91 (96.7%) strains were negative but three (3.3%) belonging to the PCR ribotype 078 harboured the binary-toxin gene (data not shown). The ribotype 078 strains were recovered from two different

FIGURE 1 - *Dendrogram and virtual gel image fingerprints from the analysis of the 92C. difficile strainsselected in this study. Pearson's correlation created a pairwise percentage similarity matrix and the tree was generated using UPGMA.*

TABLE 4 - *rep-PCR results of 92 clinical isolates of C. difficile.*

| *Pattern* | *Hospital departements* | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *ICU* | *Card* | *Surgery 9 A* | *Surgery 9 B* | *Vascular surgery* | *Gastr* | *Med 3 A* | *Med 3 B* | *Med 3 urg* | *Med 6 A* | *Med 6 B* | *Nef* | *Neu* | *Pne* | *FA* | *Reh* |
| 1 | | | | | | 1 | | | | | | | 1 | | | |
| 2 | | | | | | | | | | | 1 | | | | | |
| 3 | | | | | | | | | | 1 | | | | | | |
| 4 | | | | | | | 1 | | | | | | | | | |
| 5 | | | | | | | | | | 1 | | | | | | |
| 6 | | | | | | | | 1 | | | | | | | | |
| 7 | | | | | | | 1 | | | | | | | | | |
| 8 | | | | | | 1 | | | | | | | | | | |
| 9 | | | | | | | | | | | | | | | | 1 |
| 10 | | | | | | | | | | | 1 | | | | | |
| 11 | 3 | 1 | | 1 | 1 | 9 | 7 | 5 | 6 | 3 | 14 | 5 | 1 | 1 | 1 | 13 |
| 12 | | | | | | | | | | | | 1 | | | | |
| 13 | | | | | | | | | 1 | | | | | | | |
| 14 | | | | | | | | | 1 | | | | | | | |
| 15 | | | 1 | | | | | | | | | | | | | |
| 16 | | | | | | | 1 | | | | | | | | | |
| 17 | | | | | | | | 1 | | | | | | | | |
| 18 | | | | | | | | | | | 1 | | | | | |
| 19 | | | | | | | | 1 | | | | | | | | |
| 20 | | | | | | | | | | | | | | | | 1 |
| 21 | | | | | | | 1 | | | | | | | | | |

[c]ICU, Intensive Care Unit; Card, Cardiology; Urg, Urgency; Gastr, Gastroenterology; Med, Medicine; Nef, Nephrology; Neu, Neurology; Pne, Pneumology; PS, First Aid; Reh, Rehabilitation

patients admitted two months later to the medical ward.

The results of antimicrobial susceptibility test are shown in Table 6. All *C. difficile* strains were susceptible to tigecycline, ($MIC_{50}$ = 0.047 µg/mL and $MIC_{90}$ = 0.047 µg/mL), daptomycin, ($MIC_{50}$ = 0.125 µg/mL and $MIC_{90}$ =1), vancomycin ($MIC_{50}$ = 0.75 µg/mL and $MIC_{90}$ = 1.5 µg/mL) and metronidazole ($MIC_{50}$ = 0.12 µg/mL and $MIC_{90}$ = 0.38 µg/mL).

Eight out 21 (38.1%) strains resulted susceptible to moxifloxacin ($MIC_{50}$>32 µg/mL and $MIC_{90}$ ≥32 µg/mL).

A total of 31 strains (33.7%) were isolated from

TABLE 5 - *PCR-ribotyping results of 92 clinical isolates of C. difficile.*

| *Pattern* | *Hospital departements* | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *ICU* | *Card* | *Surgery 9 A* | *Surgery 9 B* | *Vascular surgery* | *Gastr* | *Med 3 A* | *Med 3 B* | *Med 3 urg* | *Med 6 A* | *Med 6 B* | *Nef* | *Neu* | *Pne* | *FA* | *Reh* |
| 186 | | | | | | | | | | 1 | | | | | | |
| 002 | | | | | | 1 | | | | | | | 1 | | | |
| 078 | | | | | | | 2 | | | | | | | | | 1 |
| 14/020 | | | | | | | | 1 | | | | | | | | |
| 106 | | | | | | | | | | 2 | | | | | | |
| 018 | 3 | 1 | 1 | 1 | 1 | 10 | 9 | 7 | 8 | 2 | 17 | 6 | 1 | 1 | 1 | 14 |

[c]ICU, Intensive Care Unit; Card, Cardiology; Urg, Urgency; Gastr, Gastroenterology; Med, Medicine; Nef, Nephrology; Neu, Neurology; Pne, Pneumology; PS, First Aid; Reh, Rehabilitation

TABLE 6 - *In vitro antimicrobial susceptibility for C. difficile strains representative of each pattern obtained by rep-PCR.*

| *Antimicrobial agents* | *Range(µg/mL)* | *MIC50 (µg/mL)* | *MIC90 (µg/mL)* | *% resistance* |
|---|---|---|---|---|
| Moxifloxacin | 0,032->32 | >32 | >32 | 61,9 (13/21) |
| Metronidazole | <0,016-0,5 | 0,38 | 0,125 | 0 |
| Vancomycin | 0,016-1,5 | 0,75 | 1,5 | 0 |
| Tygecycline | 0,016-0,094 | 0,047 | 0,047 | 0 |
| Daptomycin | 0,094-1 | 0,125 | 1 | 0 |

*Breakpoints: Metronidazole >32 µg/mL, Moxifloxacin >4 µg/mL, Vancomycin >32 µg/mL., Daptomycin >8 µg/mL, and Tigecycline >4 µg/mL

hospitalized geriatric patients of which 6 (19.3%) had different molecular pattern by rep-PCR (four strains belonged to ribotype 018, one strain to ribotype 014/020 and one strain to ribotype 106) and 25 (80.6%) belonged to prevalent cluster $A_{1B}$ and to ribotype 018.

## DISCUSSION

The introduction of the three steps algorithm (direct faecal GDH screening *plus* faecal A and B toxins detection, and toxigenic culture) determined a major improvement in the *Clostridium difficile* infection diagnosis (Moro *et al.*, 2009). However, the sensitivity and specificity, positive and negative predictive values of Premier toxin A and B assay, were 78%, 99.1%, 91.7% and 97.1% respectively (Wilcox *et al.*, 2009). If toxigenic cultures were added to the two-step diagnostic algorithm, it could be possible to recognize also symptomatic patients with negativestools for toxin A and B by EIA test. Delmee *et al.* (Delmee *et al.*, 2005), reported that when negative stools for toxin A and B were tested by toxigenic culture, there was a 3.4% increase in the infection detection rate. The results showed that toxigenic cultures allowed us to recover 38/156 (24.4%) stool samples with toxigenic *C. difficile*, that would have been missed by performing only EIA screening and leading an increased prevalence of *C. difficile* infection of 4.2%.

The false negative results of faecal-toxin detection can be caused by an inhomogeneous distribution of bacteria in the stools, and consequently the amount of toxins changes in different parts of the specimen. This has some implications not only in terms of patient misdiagnosis but also in environmental contamination (Mulligan *et al.*, 1979). To improve the diagnosis and the outbreak prevention it could be better to perform toxigenic culture systematically. Our experience confirms the validity of the three-step testing algorithm even if the toxigenic culture procedure needs to be standardized. Unfortunately, culture is not always recommended in some guidelines probably because of cost and technical difficulties.

Nosocomial *C. difficile* infections lead to extended hospital stays and thus increase the attributable costs. The three-step algorithm is a much more efficient way to detect *C. difficile* infections and will diagnose more cases that could be missed with other algorithms. The possibility to detect more cases reduces the number of nosocomial transmission cases, thereby decreasing the annual costs deriving from prolonged hospitalization.

Culture is the only way to perform antimicrobial susceptibility testing and molecular typing. Both are of crucial interest in the clinical management of individual cases and hospital outbreaks. Typing will trace clonal strains and identify specific virulent groups (Brazier *et al.*, 2001), whereas susceptibility testing might become mandatory in the future since the observation of the emergence of strains with a decreased susceptibility to vancomycin or metronidazole. Although moxifloxacin was reported to have a good activity against Gram positive bacilli including *C. difficile,* reduced susceptibility to this antibiotic could have facilitated the spread of *C. difficile* strains, potentially providing them with a survival advantage in the hospital environment where fluoroquinolone is usually widespread (Kelly and Lamont, 2008). In our study, all the strains were susceptible to metronidazole, vancomycin, daptomycin and tigecycline whereas only strains belonging to ribotype 018 were resistant to moxifloxacin. The presence and resistance of ribotype 018 to fluoroquinolones had already been described in Italy by Spigaglia *et al.* (Spigaglia *et al.*, 2010). Therefore, our results confirm the increase in the number of *C. difficile* strains resistant to fluoroquinolones in Italy and the changes in the prevalence of the type of *C. difficile* isolates.

The manual rep-PCR technique is useful for typing many prokaryotic and eukaryotic organisms, including those of epidemiological significance (Carretto *et al.*, 2008; Hulton *et al.*, 1991; Ligozzi *et al.*, 2010; Versalovic *et al.*, 1993) and the semi-automated rep-PCR system has been recently introduced to type Gram negative bacilli (*Neisseria meningitidis*, *Acinetobacterbaumannii),* fungi *(Aspergillus* spp. and *Candida albicans)* and also Gram positive bacteria (*Staphylococcus aureus*, enterococci) (Li *et al.*, 2004; Tenover *et al.*, 2009). The rep-PCR system (Diversilab) identified 21 *C. difficile* different patterns. Among them, one presented two indistinguishable strains (samples isolates from stool of patients hospitalized in different departments, like Neurology and Gastroenterology) and the other had seventy-one indistinguishable strains (77.2% of isolates) isolated from different patients admitted to all the departments excluding the General Surgery Ward. The subsequent analysis of clones by PCR-ribotyping confirmed the presence of a ribotype 078 *C. difficile* cluster, which is very uncommon in Italy (Baldanti *et al.*, 2010). Ribotype 018 was the most frequent ribotype (90.1%), and all the isolates of this PCR-ribotype proved resistant to fluoroquinolones, suggesting that the increased use of these antibiotics has played a determinant role in selection and spread of these strains (Spigaglia *et al.*, 2010).

The rep-PCR results pointed out a higher level of genetic variability and both dendrogram and similarity matrix generated by the DiversiLab software indicated that not all the samples identified by PCR-ribotyping showed the same level of similarity. In fact, our results show that not all the isolates were clustered by PCR-ribotyping exactly as by rep-PCR and only 28.6% (6/21) of the pattern isolated by rep-PCR were grouped similarly by PCR-ribotyping. In addition, strains belonging to a single PCR-ribotype were classified as belonging to different patterns when analyzed by rep-PCR.

In conclusion, rep-PCR can be proposed as a good and reliable method to type *C. difficile* strains and could be utilized in case of *C. difficile*as a useful option to clinical laboratories to perform molecular typing to improve infection control procedures.

*ACKNOWLEDGEMENTS*

*We are indebted to P. Mastrantonio (Dept. Infectious Diseases, Istituto Superiore di Sanità, Rome) for PCR-ribotyping of the C. difficile isolates, and to J. Brazier (Anaerobe Reference Lab, University Hospital of Wales, UK) for typing the uncommon PCR ribotype 186.*

## REFERENCES

Alcalà L., Sanchez-Cambronero L., Catalan M.P., Sanchez-Somolinos M., Pelaez M.T., Marin M., Bouza E. (2008). Comparison of three commercial methods for rapid detection of Clostridium difficile toxins A and B from fecal specimens. *J. Clin. Microbiol.* **46**, 3833-3835.

Baldan R., Cavallerio P., Tuscano A., Parlato C., Fossati L., Moro M., Serra R., Cirillo D.M. (2010). First report of hypervirulent strains polymerase chain reaction ribotypes 027 and 078 causing severe Clostridium difficile Infection in Italy. *Clin. Infect. Dis.* **50**, 126-127.

Barbut F., Lalande V., Daprey G., Cohen P., Marle N., Burghoffer B., Petit J.C. (2000). Usefulness of simultaneous detection of toxin A and glutamate dehydrogenase for the diagnosis of Clostridium difficile-associated diseases. *Eur. J. Clin. Microbiol. Infect. Dis.* **19**, 481-484.

Bauer M.P., Kuijper E.J., van Dissell J.T. (2009). European Society of Clinical Microbiology and Infectious Diseases (ESCMID): treatment guidance document for Clostridium difficile infection (CDI). *Clin. Microbiol. Infect.* **15**, 1067-1079.

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.

Brazier J.S., Fawley W., Freeman J., Wilcox M.H. (2001). Reduced susceptibility of Clostridium difficile to metronidazole. *J. Antimicrob. Chemother.* **48**, 741-742.

Carretto E., Barbarini D., Farina C., Grosini A., Nicoletti P., Manso E. (2008). Use of DiversiLab semiautomated repetitive sequence-based polymerase chain reaction for epidemiologic analysis on Acinetobacter baumannii isolates in different Italian hospitals. *Diagn. Microbiol. Infect. Dis.* **60**, 1-7.

Delmee M., Broeck J.V., Simon A., le Janssens M., Avesani V. (2005). Laboratory diagnosis of Clostridium difficile associated diarrhoea: a plea for culture. *J. Med. Microbiol.* **54**, 187-191.

Gilligan P.H. (2008). Is a two-step glutamate dehyrogenase antigen-cytotoxicity neutralization assay algorithm superior to the Premier toxin A and B enzyme immunoassay for laboratory detection of Clostridium difficile? *J. Clin. Microbiol.* **46**, 1523-1525.

Hulton C.S., Higgins C.F., Sharp P.M. (1991). ERIC sequences: a novel family of repetitive elements in the genomes of Escherichia coli, Salmonella typhimurium and other enterobacteria. *Mol. Microbiol.* **5**, 825-834.

Kato H., Kato N., Watanabe K., Iwai N., Nakamura H., Yamamoto T., Suzuki K., Shin-moo K., Yunsop C., Wasito E.B. (1998). Identification of toxin A-negative, toxin B-positive Clostridium difficile by PCR. *J. Clin. Microbiol.* **36**, 2178-2182.

Kelly C.P., LaMont J.T. (2008). Clostridium difficile. More difficult than ever. *N. Engl. J. Med.* **359**, 1932-1940.

Lahn M., Tyler G., Däubener W., Hadding U. (1993). Improvement of Clostridium difficile isolation by heat-shock and typing of the isolated strains by SDS-PAGE. *Eur. J. Epidemiol.* **9**, 327-334.

Larson A.M., Fung A.M. Fang F.C. (2010). Evaluation of tcdB real-time PCR in a three-step diagnostic algorithm for detection of toxigenic Clostridium difficile. *J. Clin. Microbiol.* **48**: 124-130.

Li X., Brown N., Chau A.S., Lopez-Ribot J.L., Ruesga M.T., Quindos G., Mendrick C.A., Hare R.S., Loebenberg D., Di Domenico B., McNicholas P.M. (2004). Changes in susceptibility to posaconazole in clinical isolates of Candida albicans. *J. Antimicrob. Chemother.* **53**, 74-80.

Ligozzi M., Fontana R., Aldegheri M., Scalet G., Lo Cascio G. (2010). Comparative evaluation of an automated repetitive-sequence-based polymerase chain reaction instrument versus pulsed-field gel electrophoresis in the setting of a Serratia marcescens nosocomial outbreak. *J. Clin. Microbiol.* **48**, 1690-1695.

Moro M.L., Mongardi M., et al. (2009). Prevenzione e controllo delle infezioni da Clostridium difficile. Documento di indirizzo SIMPIOS (Società Italiana Multidisciplinare per la Prevenzione delle Infezioni nelle Organizzazioni Sanitarie). Giornale Italiano delle Infezioni Ospedaliere, 16: 2-40. http://asr.regione.emilia-romagna.it/wcm/asr/aree_di_programma/fit/fit_rishioinfettivo/pubblicazioni/clos_2009.htm

Mulligan M.E., Rolfe R.D., Finegold S.M., George W.L. (1979). Contamination of a hospital environment by Clostridium difficile. *Curr. Microbiol.* **3**, 173-175.

Reller M.E., Lema C.A., Perl T.M., Cai M., Ross T.L., Speck K.A., Carroll K.C. (2007). Yield of stool culture with isolate toxin testing versus a two-step algorithm including stool toxin testing for detection of toxigenic Clostridium difficile. *J. Clin. Microbiol.* **45**, 3601-3605.

Rupnik M., Avesani V., Janc M., von Eichel-Streiber C., Delmée M. (1998). A novel toxinotyping scheme

and correlation of toxinotypes with serogroups of Clostridium difficile isolates. *J. Clin. Microbiol.* **36**, 2240-2247.

Sharp S., Ruden L.O., Pohl J.C., Hatcher P.A., Jayne L.M., Ivie W.M. (2010). Evaluation of the C. Diff Quik Chek Complete assay, a new glutamate dehydrogenase and A/B toxin combination lateral flow assay for use in rapid, simple diagnosis of Clostridium difficile disease. *J. Clin. Microbiol.* **48**, 2082-2086.

Sloan L.M., Duresko B.J., Gustafson D.R., Rosenblatt J.E. (2008). Comparison of real-time PCR for detection of the tcdC gene with four toxin immunoassays and culture in diagnosis of Clostridium difficile infection. *J. Clin. Microbiol.* **46**, 1996-2001.

Sloan L.M., Duresko B.J., Gustafson D.R., Rosenblatt J.E. (2008). Comparison of real-time PCR for detection of the tcdC gene with four toxin immunoassays and culture in diagnosis of Clostridium difficile infection. *J. Clin. Microbiol.* **46**, 1996-2001.

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.* **48**, 2892-2896.

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.

Tenover F.C., Gay E.A., Frye S., Eells S.J., Healy M., Jr. McGowan J.E. (2009). Comparison of typing results obtained for methicillin-resistant Staphylococcus aureus isolates with the DiversiLab system and Pulse-Field gel electrophoresis *J. Clin. Microbiol.* **47**, 2452-2457.

Tenover FC, Novak-Weekley S., Woods C.W., Peterson L.R., Davis T., Schreckenberger P., Fang F.C., Dascal A., Gerding D.N., Nomura J.H., Goering R.V., Akerlund T., Weissfeld A.S., Jo Baron E., Wong E., Marlowe E.M., Whitmore J., Persing D.H. (2010). Impact of strain type on detection of toxigenic clostridium difficile: comparison of molecular diagnostic and enzyme immunoassay approaches. *J. Clin. Microbiol.* **48** (10), 3719.

Thomson Jr., K.L. Kaul. (2007). Detection of toxigenic Clostridium difficile in stool samples by real-time polymerase chain reaction for the diagnosis of C. difficile-associated diarrhea. *Clin. Infect. Dis.* **45**, 1152-1160.

Ticehurst J.R., Aird D.Z., Dam L.M., Borek A.P., Hargrove J.T., Carroll K.C. (2006). Effective detection of toxigenic Clostridium difficile by a two-step algorithm including tests for antigen and cytotoxin. *J. Clin. Microbiol.* **44**, 1145-1149.

Versalovic J., Kapur V., Mason E.O., Shah U., Jr. Koeuth T., Lupski J.R., Musser J.M. (1993). Penicillin-resistant Streptococcus pneumoniae strains recovered in Houston: identification and molecular characterization of multiple clones. *J. Infect. Dis.* **167**, 850-856.

Wilcox M.H., Eastwood K.A. (2009). Evaluation report: Clostridium difficile toxin detection assays. Centre for Evidence-based Purchasing publication no. CEP08054. Centre for Evidence-based Purchasing, NHS Purchasing and Supplies Agency, National Health Service, London, United Kingdom. http://www.pasa.nhs.uk/pasa/Doc.aspx?Path_%5bMN%5d%5bSP%5d/NHSprocurement /CEP/CEP08054.pdf, (2009).