**Prevalent PCR Ribotypes and Antibiotic Sensitivity of Clinical Isolates of Clostridium difficile.**

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*Clostridium difficile* is an important cause of nosocomial diarrhoea and pseudomembranous colitis (PMC), has a mortality rate that ranges from 15 to 30%. Therefore, in this study we investigated the incidence rate, toxigenicity and susceptibility pattern of *C. difficile* isolates to anti-anaerobic agents. We have also investigated the genotypes of clinical isolates by PCR ribotyping. The study was conducted on 80 patients [40 diarrhoeic and 40 non diarrhoeic] who had history of antibiotic exposure in the previous four weeks as well as on 20 healthy control subjects. The overall incidence rate of *C. difficile* was 35%. None of the healthy controls had positive culture. Analysis of the cytotoxin producing *C. difficile* strains showed that 40% of symptomatic and 20% of asymptomatic patients were infected by the cytotoxigenic strains (p<0.05). A total of 35 *C. difficile* isolates were investigated for their susceptibility to 15 antibiotics using the E test. Amoxycillin/clavulanic acid, ampicillin, meropenem, metronidazole, penicillin, piperacillin, piperacillin/tazobactam, teicoplanin and vancomycin had excellent activity against all isolates of *C. difficile*. Multiple resistance to two or more antibiotics was observed in toxigenic, more than the non-toxigenic strains (ratio, 2.75:1) and in symptomatic than the asymptomatic patients (ratio, 2.5 : 1). Interestingly, the 35 *C. difficile* culture positive patients harboured 10 different, highly diverse PCR ribotypes. Ribotypes 097 (23%) and 078 (17%) were the most prevalent toxigenic ribotypes responsible for over one-third of the cases of *C. difficile* associated diarrhoea (CDAD) seen. In conclusion, we believe that the extent of *C. difficile* involvement in diarrhoeal diseases would be better judged by direct comparison with the isolation rates of other enteric pathogens in antibiotic-associated diarrhoea. In addition, our finding suggest that metronidazole should remain the drug of choice for the therapy of CDAD. It is also concluded that the prevalent PCR ribotypes of *C. difficile* strains isolated in our study are different from those found in Europe.

**INTRODUCTION**

*Clostridium difficile* is an important agent of antibiotic-associated diarrhoea (AAD) and pseudomembranous colitis (PMC) and its causative role in these diseases is well established. It is well known that these diseases complicate antibiotic usage. However, there is evidence which suggests that *C. difficile* infection may not always be preceded by prior antibiotic exposure. Of interest are other studies that showed the presence of *C. difficile*, or its toxin, in adult stool specimens without signs of diarrhoea or colitis.

This organism is now recognized as a major nosocomial pathogen all over the world. It appears that the most important sources of *C. difficile* in a hospital setting are symptomatic patients and asymptomatic carriers who are the main reservoirs of *C. difficile* in the hospital.

Part of the explanation for the pathogenesis of *C. difficile* infection appears to be its ability to competitively overgrow the normal flora of the colon because of changes in the gastrointestinal tract caused by antibiotic therapy. It is now well established that the major virulence factors of *C. difficile* are the (entero) toxin A and (cyto) toxin B. *C. difficile* toxins may alter intestinal epithelial permeability and facilitate bacterial penetration of the intestinal epithelial barrier. The toxins act directly by disassembly of actin microfilaments leading to impairment of tight junctions in human colonocytes. Since the discovery of the causative role of *C. difficile* in AAD and PMC, a variety of laboratory methods have been developed to detect the presence of the organism or its related toxins.

The role of antibiotic susceptibility testing of anaerobes in general and of *C. difficile* specifically, has been questioned over the past decades. Mainly because of the predictable sensitivity pattern of anaerobes to most antibiotics with anti-anaerobic activity as well as lack of simple method for testing, delay in getting pure culture and little correlation between the clinical outcome and
the antibiotic susceptibility testing results. However, resistance of anaerobes to anti-anaerobic antibiotics is on the increase, even to drugs with excellent anti-anaerobic activity like metronidazole, imipenem and B-lactam-B-lactamase inhibitors. Therefore, it is prudent to be abreast of the susceptibility pattern of local isolates to the antibiotics that are available for the treatment of the disease caused by the organism.

Epidemiological studies of C. difficile strains isolated from different patients involve detailed comparison of the different isolates. Several typing schemes have been developed to determine the relatedness of strains of C. difficile associated with infections.

This study was undertaken to investigate the incidence, toxigenicity and antibiotic susceptibility of C. difficile isolates to anti-anaerobic agents. We have also aimed to study the prevalent ribotypes and the relatedness of clinical isolates by PCR ribotyping.

MATERIAL AND METHODS
- Subjects:
  Over 3 years period (January 2003 to December, 2005) 100 subjects selected from internal medicine department, Kasr Al-aini hospital, Cairo university, were divided into the following groups:

i. Symptomatic Patients: 40 patients with symptoms of mild to profuse diarrhoea and gave history of antibiotic usage in the preceding four weeks prior to developing the disease. These patients were presumed to be suffering from antibiotic-associated diarrhoea because of the antecedent antibiotic usage.

ii. Asymptomatic Patients: 40 patients who gave history of antibiotic exposure in the last four weeks without symptoms of diarrhoea.

iii. Healthy Controls: 20 healthy subjects, who had no diarrhoea and gave no history of antibiotic exposure in the last four weeks.

The commonest antibiotics prescribed were cephalosporins, clindamycin, metronidazole, meropenem, amikacin, vancomycin, teicoplanin and imipenem. Most of the patients had received multiple antibiotic therapy.

- All patients and controls were subjected to the following:
  I. Samples collection:
  Single fresh stool specimens (rectal swabs, in Amies transport medium, if collection of stool was not feasible) were obtained from the patients as well as from the healthy controls.

II. Microbiological Methods.
  1. Stool Cultures.
  The stool samples were inoculated directly into Robertson cooked meat (RCM) medium containing 25ml fastidious anaerobe broth (FAB; Lab M) and incubated anaerobically for 48 h at 37°C. Five-hundred microlitres of the FAB from the RCM was heated for 10 min at 80°C. Next, cycloserine-cefoxitin egg yolk agar (CCEYA; oxoid) and cycloserine-cefoxitin fructose agar (CCFA; oxoid) plates were inoculated with one loop of the heated broth and incubated anaerobically for 48 h.

  2. Isolation and Identification of C. difficile Strains.
  Isolates that were Gram-positive bacilli with characteristic horse-dung smell and fluoresced yellowish-green under long-wave UV light (365 nm) were selected and their identity was confirmed as C. difficile by API 20 A (bioMerieux, SA, France). Two reference quality control strains, C. difficile ATCC 9689 and ATCC 17857, and C. perfringens ATCC 13124, were included in each run.

  3. Toxin detection.
  Single colonies were subcultured on pre-reduced Columbia agar base (Oxoid) supplemented with 5% horse blood, vitamin K and haemin and incubated at 37°C under anaerobic conditions for toxin A detection. Toxin A was detected by ELISA (TOX-A) kits (Tech Lab); the procedure was carried out according to the manufacturer’s instructions. Toxin B was detected by cytotoxicity assay on Vero cells. Production of cytopathic effects by filtered supernate of C. difficile RCM broth culture on Vero cells indicated toxin B production. All the isolates were tested for toxin A and B production using commercial C. difficile TOX-A/B TEST kit (Tech Lab, VPI Research Park, Blacksburg,
VA) according to the manufacturer’s package insert.


The susceptibilities of the *C. difficile* isolates to 15 antimicrobial agents (amoxycillin /clavulanic acid, ampicillin, cefotaxime, cefoxitin, cefuroxime, clindamycin, imipenem, meropenem, metronidazole, penicillin, piperacillin, piperacillin/tazobactam, teicoplanin, trovafloxacin, vancomycin) were determined by estimating the minimum inhibitory concentrations (MIC) by using the following methods:

a. **Agar Dilution Method.**

The activity of trovafloxacin (Pfizer, Inc., Groton, CT) was determined by the agar dilution method recommended by the National Committee for Clinical Laboratory Standards (NCCLS,1997) 23. Briefly, serial two-fold dilutions of trovafloxacin were incorporated into Fastidious Anaerobe agar (FAA; Lab M, Bury, UK) with final antibiotic concentrations of 0.03-256 mg/l. An inoculum was prepared in Anaerobe broth (Difco, USA) from a 48 h FAA culture. The suspension was adjusted to 0.5 McFarland turbidity standard. Then the inoculum was applied onto the surface of pre-reduced antibiotic-containing agar using a 35-prong Steers applicator that delivered 10 µl (10^5 CFU) per spot. The plates were incubated in anaerobic jars for 48 h at 37 °C. Anaerobiosis was checked by inclusion of a nutrient agar plate inoculated with *Pseudomonas aeruginosa* and a chemical indicator. After incubation, the MIC was recorded as the lowest concentration of each antibiotic that inhibited visible growth of the organisms.

b. **Epsilometer Test (E-test).**

For the MIC determination by E-test (AB Biodisk, Solna, Sweden), a 48 h bacterial growth suspended in anaerobe broth and adjusted to no. 1.0 McFarland turbidity standard was inoculated onto reduced brucella agar plates (Unipath, Basing-stoke, UK) supplemented with 5% horse blood, haemin 5 µg/ ml and menadione 1 µg/ml. The E-test strips of the antibiotics, other than trovafloxacin, were applied onto the agar surface, after drying for 15 min, and then incubated anaerobically for 48 h in an anaerobic chamber ( H₂ 10%, CO₂ 10%, N₂ 80%). The MIC was read as the interception of the elliptical zone of inhibition with the strip. *C. difficile* and *C. perfringens* control strains were included in each run as control of the media and the susceptibility test.

5. **PCR ribotyping.**

All isolates were typed by the PCR ribotyping method described by O’Neill et al.21. Briefly, after obtaining a pure culture, a single colony was subcultured on fastidious anaerobe agar (FAA; Lab M) supplemented with 6% horse blood and incubated for 24 h at 37 °C. DNA was extracted from a suspension of 10 colonies in 100 µl 5% Chelex 100 (Bio-Rad) by heating at 100 °C for 10 min. Cell debris was removed from the suspension by centrifugation for 10 min at 17000 g. The supernate obtained was then used as the DNA template. Primers P3 (5’-CTGGGAGGTCAAGTCAAAGG) and P4 (5’-GGGCCCTTGTQAAGTGTGACC) were used for the PCR amplification. The reaction mixture (final volume, 100 µl) contained 1.5 mM MgCl₂, 10 mM Tris/ HCl (PH 9), 50 mM KCl, 0.1% Triton X-100, 2.5 U Taq polymerase, 200 mM of each dNTP, 50 pmol of each primer and 10 µl DNA template. The PCR programme was 35 cycles of denaturation at 95 °C for 1 min, annealing at 56 °C for 1 min and extension at 72 °C for 2 min. A negative control was included in each run. Amplification products were concentrated to a final volume of 25 µl by heating at 75 °C for 90 min before electrophoresis at 200V, 100 A in Metaphore agarose gel (FMC) for 3 h at room temperature. DNA fragments were then visualized by staining the gel for 20 min in 0.5% ethidium bromide. Gel images were analysed with GelCompar image analysis software (version 4.0; Applied Maths). Our results then compared with the library of PCR ribotypes already established at the PHLS Anaerobe Reference Unit, Cardiff, UK.

III. Statistical Analysis.

- Results were analysed by the X² Mann-Whitney tests.
- Categorical variables were compared by Fisher’s exact test. A P value of < 0.05 was considered significant.
- All statistical analysis were carried out by using Statistical Package for the Social Sciences software (version 10.0;
RESULTS

I. Bacterial Isolates from patients.

Table 1 shows the distribution of the *C. difficile* isolates from patients and healthy control subjects screened over three years period. Twenty three of the 40 patients with diarrhoea, representing 57.5%, were culture-positive for *C. difficile* while 30% (12/40) of the non-diarrhoeic patients yielded the organism in their stool. However, none of the healthy control subjects had positive culture. The overall incidence of *C. difficile* positive cultures was 35%.

II. Cytotoxin Producing Isolates.

A total of 35 *C. difficile* clinical isolates were studied for toxigenicity. Twenty four (68.6%) of them were toxigenic. Analysis of the cytotoxin producing *C. difficile* strains showed that 16 (69.6%) of the 23 isolates from the diarrhoeic patients and 8 (66.7%) of the 12 isolates from the asymptomatic patients were toxigenic strains i.e. 40% of symptomatic patients and 20% of the asymptomatic patients were infected by the cytotoxigenic strains. This was significant at the 95% confidence limit (p<0.05).

Table 1. The Isolation Rate of *C. difficile* from Stool Samples

<table>
<thead>
<tr>
<th>Source of specimen</th>
<th>No of specimens</th>
<th><em>C. difficile</em> isolates</th>
<th>Toxigenic strains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No (%)</td>
<td>No (%)</td>
</tr>
<tr>
<td>Symptomatic patients</td>
<td>40</td>
<td>23 (57.5%)</td>
<td>16/23 (69.6%)</td>
</tr>
<tr>
<td>Asymptomatic patients</td>
<td>40</td>
<td>12 (30%)</td>
<td>8/12 (66.7%)</td>
</tr>
<tr>
<td>Healthy controls</td>
<td>20</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>35 (35%)</td>
<td>24/35 (68.6%)</td>
</tr>
</tbody>
</table>

III. Antimicrobial Susceptibilities.

The MICs of the antibiotics tested, shown in Table 2, are presented as the concentrations that killed 50% (MIC\(_{50}\)) and 90% (MIC\(_{90}\)) of the isolates. Amoxycillin/clavulanic acid, ampicillin, meropenem, metronidazole, penicillin, piperacillin, piperacillin/tazobactam, teicoplanin and vancomycin had excellent activity against all isolates of *C. difficile* with MIC\(_{90}\)s of 0.38, 0.5, 1.0, 0.19, 1.5, 2.0, 3.0, 0.25 and 0.75 mg/l, respectively. One isolate had decreased susceptibility to vancomycin and teicoplanin with MICs of 3 and 2 mg/l, respectively.

As was expected, 34 (97%) of our 35 strains were resistant to cefoxitin and all of them were resistant to cefotaxime and cefuroxime. Almost half of the *C. difficile* isolates were resistant to clindamycin (17/35; 49%). Of these 17 resistant isolates, 9 (53%) exhibited high-level clindamycin resistance with MIC > 256 mg/l. Of interest, 30 (86%) and 34 (97%) were resistant to imipenem and trovafloxacin with MICs of > 32 and 64 mg/l, respectively.

Table 3 shows the antibiotic resistance profile of 30 multiply resistant *C. difficile* isolates in relation to toxigenicity. A total of 22 toxigenic (73%) and 8 non toxigenic (27%) strains were multiply resistant. Of these, 13 isolates were resistant to 4 antibiotics (cefoxitin, clindamycin, imipenem and trovafloxacin) and 12 were resistant to a combination of cefoxitin, imipenem and trovafloxacin.

Table 4 shows the distribution of the 22 toxigenic strains that were multiply resistant among the symptomatic and asymptomatic patients. More isolates exhibiting resistance to 4 antibiotics came from the symptomatic patients than from the asymptomatic patients by a ratio of 2.5 : 1, whereas those resistant to 3 or fewer...
antibiotics were isolated predominantly from the asymptomatic patients.

A comparison was also made between antibiotic usage and multiple resistance. The average number of antibiotics prescribed per patient was 4.0 (range 0-11) for an average duration of 8.4 days (range 1-22) per prescription. The commonest antibiotics prescribed were cephalosporins (21%), metronidazole (17%), meropenem (14%), amikacin, vancomycin and teicoplanin (7% each) and imipenem (3%). Trovafloxacin has never been prescribed for any patient. Over 50% of the 30 multiply resistant isolates were from patients treated with the cephalosporins, meropenem and metronidazole. Most of the patients had received multiple antibiotic therapy and thus incrimination of specific agent was not possible.

Table 2. Susceptibility of the 35 clinical isolates of C. difficile to 15 antibiotics

<table>
<thead>
<tr>
<th>Antibiotics (breakpoint,mg/l)</th>
<th>MIC (mg/l)</th>
<th>Range</th>
<th>MIC50</th>
<th>MIC90</th>
<th>% Resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxy / clav (8)</td>
<td>0.047 - 0.5</td>
<td>0.125</td>
<td>0.38</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Ampicillin (8)</td>
<td>0.094 - 0.75</td>
<td>0.25</td>
<td>0.5</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Cefotaxime (32)</td>
<td>24 - &gt;256</td>
<td>96</td>
<td>&gt; 256</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Cefoxitin (32)</td>
<td>0.25 - &gt;256</td>
<td>&gt; 256</td>
<td>&gt; 256</td>
<td>97</td>
<td></td>
</tr>
<tr>
<td>Cefuroxime (32)</td>
<td>&gt;256</td>
<td>&gt; 256</td>
<td>&gt; 256</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Clindamycin (4)</td>
<td>0.16 - &gt;256</td>
<td>4</td>
<td>&gt; 256</td>
<td>49</td>
<td></td>
</tr>
<tr>
<td>Imipenem (8)</td>
<td>0.064 - &gt; 32</td>
<td>32</td>
<td>&gt; 32</td>
<td>86</td>
<td></td>
</tr>
<tr>
<td>Meropenem (8)</td>
<td>0.032 – 1.0</td>
<td>0.75</td>
<td>1.0</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Metronidazole (8)</td>
<td>0.023 – 0.19</td>
<td>0.094</td>
<td>0.19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Penicillin (4)</td>
<td>0.125 – 1.5</td>
<td>0.5</td>
<td>1.5</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Piperacillin (32)</td>
<td>0.094 - 3</td>
<td>1.5</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Pipera / tazo (64)</td>
<td>0.047 - 6</td>
<td>2.0</td>
<td>3</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Teicoplanin (2)</td>
<td>0.032 - 2</td>
<td>0.125</td>
<td>0.25</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Trovafloxacin (4)</td>
<td>0.5 - &gt; 256</td>
<td>32</td>
<td>64</td>
<td>97</td>
<td></td>
</tr>
<tr>
<td>Vancomycin (4)</td>
<td>0.125 - 3</td>
<td>0.5</td>
<td>0.75</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

Amoxy / clav, amoxicillin - clavulanic acid; pipera / tazo, piperacillin / tazobactam.

Table 3. Antibiotic resistance profile of 30 multiply resistant C. difficile isolates

<table>
<thead>
<tr>
<th>Resistance groups</th>
<th>Number of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Toxigenic</td>
</tr>
<tr>
<td>Cefox, Clind, Imip, Trovafl</td>
<td>7</td>
</tr>
<tr>
<td>Cefox, Imip, Trovafl</td>
<td>11</td>
</tr>
<tr>
<td>Cefox, Clind, Trovafl</td>
<td>1</td>
</tr>
<tr>
<td>Cefox, Clind, Imip</td>
<td>1</td>
</tr>
<tr>
<td>Cefox, Trovafl</td>
<td>1</td>
</tr>
<tr>
<td>Imip, Trovafl</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>22</td>
</tr>
</tbody>
</table>

Cefox, Cefoxitin ; Clind, Clindamycin ; Imip, Imipenem ; Trovafl, Trovafloxacin.
Table 4. Distribution of Multiply Resistant Toxigenic Strains Among Symptomatic and Asymptomatic Patients.

<table>
<thead>
<tr>
<th>Resistance groups</th>
<th>No. patients that were</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Symptomatic</td>
</tr>
<tr>
<td>Cefox, Clind, Imip, Trovafl</td>
<td>5</td>
</tr>
<tr>
<td>Cefox, Imip, Trovafl</td>
<td>2</td>
</tr>
<tr>
<td>Cefox, Clind, Trovafl</td>
<td>1</td>
</tr>
<tr>
<td>Cefox, Clind, Imip</td>
<td>0</td>
</tr>
<tr>
<td>Cefox, Trovafl</td>
<td>0</td>
</tr>
<tr>
<td>Imip, Trovafl</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>8</td>
</tr>
</tbody>
</table>

Cefox, Cefoxitin ; Clind, Clindamycin ; Imip, Imipenem ; Trovafl, Trovafloxacin.

The distribution of PCR ribotypes among the clinical isolates is shown in Table 5 and the banding of representative ribotypes is demonstrated in Fig.1. As shown in Table 5, 10 distinct, genotypically different ribotypes were identified among the 35 clinical isolates. Ribotype 039 was the single most prevalent non toxigenic ribotype among our isolates (17%). However, ribotypes 097 (23%) and 078 (17%) were the most prevalent toxigenic ribotypes responsible for over one-third of the cases of CDAD seen.

Table 5. Distribution of PCR ribotypes and toxigenic strains of 35 C. difficile isolates

<table>
<thead>
<tr>
<th>Ribotype</th>
<th>Toxin (A/B)</th>
<th>Number (35)</th>
</tr>
</thead>
<tbody>
<tr>
<td>002</td>
<td>+</td>
<td>1</td>
</tr>
<tr>
<td>010</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>012</td>
<td>+</td>
<td>1</td>
</tr>
<tr>
<td>029</td>
<td>+</td>
<td>3</td>
</tr>
<tr>
<td>039</td>
<td>-</td>
<td>6</td>
</tr>
<tr>
<td>051</td>
<td>+</td>
<td>3</td>
</tr>
<tr>
<td>076</td>
<td>+</td>
<td>2</td>
</tr>
<tr>
<td>078</td>
<td>+</td>
<td>6</td>
</tr>
<tr>
<td>097</td>
<td>+</td>
<td>8</td>
</tr>
<tr>
<td>113</td>
<td>-</td>
<td>3</td>
</tr>
</tbody>
</table>
Fig. 1. Representative PCR ribotype profiles of C. difficile isolated from patients. Lanes: 1, 6, 11 and 14, 100 bp ladder. Lane 2, ribotype 029; lanes 3, 5, 7 and 8: ribotype 039. Lane 4 and 12: ribotype 097. Lane 9 and 10: ribotype 078. Lane 13: ribotype 051.

**DISCUSSION**

In spite of the growing number of studies devoted to CDAD in Western countries \(^1\text{3,3,6}\), studies on CDAD in the Middle East are lacking, where information on the incidence of *C. difficile* carriage and CDAD is almost rare \(^8\text{, 24}\). This is partly as a result of inertia in anaerobic bacteriology prompted, until recently, by lack of expertise, technology and facilities for culturing anaerobic pathogens.

*C. difficile* is an important cause of nosocomial diarrhoea \(^25\) and PMC has a mortality rate that ranges from 15 to 30% \(^26\). Therefore, in this study we investigated the incidence rate, toxigenicity and susceptibility pattern of *C. difficile* isolates to anti-anaerobic agents. We have also investigated the genotypes of clinical isolates by PCR ribotyping.

In most studies conducted in developed countries where administration of antibiotics is under strict regulatory and legislative control, the isolation of *C. difficile* in diarrhoeal diseases is considered to be infectious and has often been linked with the appearance of PMC and antibiotic-associated diarrhoea in about 20-25% of the patients \(^1\text{3,3}\). In the present study, the frequency of isolation of *C. difficile* (57.5%) in diarrhoeic patients is significantly higher than in non-diarrhoeic patients (30%) and healthy control subjects (0%) \((p<0.05)\). This isolation rate is obviously higher than the figures obtained from either diarrhoeic or non-diarrhoeic patients in the developed countries \(^1\text{3,3}\). The indiscriminate use of antibiotics may explain, in part, the unusually high isolation rate. The relevance of this finding in clinical setting can only be assessed in comparison with the isolation rate of other enteric pathogens in
stool samples of diarrhoeic patients in order to establish its causative role.

Bartlett et al. 27 reported a relationship between the presence of cytotoxigenic strains of *C. difficile* in the stool and the severity of the disease. In this report it appears difficult to assume that this organism plays any significant role in diarrhoeal diseases especially as cytotoxigenic strains of *C. difficile* which are usually associated with PMC and antibiotic-associated diarrhoea 3 were isolated from relatively high proportion of both the diarrhoeic (69.6%) and non-diarrhoeic (66.7%) patients. Therefore, it is conceivable that the presence of cytotoxigenic strains in the stool specimen may not be a predictive index of antibiotic associated diarrhoea in our population. Although the clinical histories obtained in many cases were not satisfactory, the apparent trend in this study indicates no relationship between the presence of cytotoxigenic strains of *C. difficile* in the stool specimen and the development of diarrhoea. This appears to be in keeping with the report of Burden et al. 28 who found no relationship between cytotoxin titer and severity of the disease. Furthermore, in the experience of Chang and Gorbach 29 and Carroll et al. 30 detection of *C. difficile* in stool specimen may be of more significance in *C. difficile*-associated diarrhoea without pseudomembranous colitis than the detection of cytotoxin production. Systemic symptoms are caused mainly by toxin-mediated inflammatory mediators released in the colon 31. Thus, it is conceivable that the ability of the host to mount an effective antibody-mediated response to the *C. difficile* toxin plays a major role in this regard.

Metronidazole and vancomycin, given orally for 10 days, are the drugs of first choice for the treatment of CDAD 32. Teicoplanin, another potent glycopeptide, is equally efficacious as vancomycin 33. A recent report from Spain, Pelaez et al. 34 described an increase in the number of clinical isolates of *C. difficile* with decreased susceptibility to metronidazole and the emergence of strains with decreased susceptibility to vancomycin thus emphasizing the importance of antibiotic susceptibility testing of anaerobic bacteria, in particular *C. difficile*, in different geographical areas. This prompted us to investigate the susceptibility of clinical isolates of *C. difficile* to establish a base-line susceptibility pattern to 15 antibiotics and assess the level of resistance of our isolates to established anti-anaerobic agents.

The data from our present study show that 90% of the strains were inhibited by 0.75 mg/l of vancomycin and 0.25 mg/l of teicoplanin; all strains were inhibited by concentrations that did not exceed 3 mg/l and 2 mg/l, respectively., with the MICs distributed over a narrow range. These upper limits are higher than the concentrations reported in other studies 16. However, only one strain with decreased susceptibility to vancomycin (MIC, 3 mg/l) and teicoplanin (2 mg/l) was isolated. This strain was from a symptomatic patient with CDAD and thus calls for vigilance and close monitoring of the susceptibility of the future isolates.

Metronidazole was the most active of all the antibiotics. All isolates were inhibited by a concentration that did not exceed 0.19 mg/l and with a narrow range of MIC distribution. This is in contrast to studies that have reported from 3-20% decreased susceptibility to metronidazole 32, 34. Wong et al. 35 has also recently described a single isolate from a collection of 100 strains studied that had MIC of 64 mg /l. Despite these reports, the incidence of metronidazole resistant strains remains very low. Our finding suggest that metronidazole should remain the drug of choice for the therapy of CDAD. This is without prejudice to the fact that metronidazole and vancomycin are equally effective for treatment of *C. difficile*-associated disease 32. In addition to the excellent in vitro activity, cost and concern regarding the emergence of vancomycin-resistant *Enterococci* 36 support its choice as a preferred drug. Even though teicoplanin is as effective as vancomycin 33 its high cost precludes it as the drug of first choice.

All our isolates were susceptible to ampicillin, penicillin and amoxycillin-clavulanic acid but resistant to the cephalosporins (cefotaxime, cefuroxime and cefoxitin) with MIC90s of more than 256 mg/l, a finding consistent some earlier reports 27. Although, imipenem and meropenem have very good activity against anaerobes in general, there was a big difference in their in vitro activities against our *C. difficile* isolates. While meropenem showed excellent activity against all isolates of *C. difficile*, over three-
quarters of strains had high-level resistance to imipenem. In a previous review of 46 isolates by Jones 18 quite a few had MIC 90 of more than 10 mg/l. The explanation for the discrepancy in susceptibility of our isolates to meropenem and imipenem is a subject of further investigation.

The resistance rate of our isolates to clindamycin is much lower than the rate reported elsewhere 38. Nearly 25% of the isolates showed high-level clindamycin resistance. Susceptibility of C. difficile to the quinolones has always been poor particularly to the first and second-generation agents such as norfloxacin and ciprofloxacin 37, 39. Although in general trovafloxacin has good anti-anaerobic activity against most of the Gram-positive and Gram-negative anaerobes, in our study trovafloxacin had very poor activity against C. difficile with about two-thirds of the strains showing high-level resistance. Our data indicates that this drug can not be considered for use in the treatment of disease caused by this organism.

Further analysis of the strains according to toxin production and multiple resistance revealed that all the toxigenic, more than the non-toxigenic strains by a ratio of 2.75:1, were resistant to two or more antibiotics. Resistance to combinations of [cefoxitin, imipenem and trovafloxacin], [cefoxitin, clindamycin and trovafloxacin], [cefoxitin, clindamycin and imipenem] were exclusively observed in the toxigenic group. These results cast new light into the relationship between toxigenic strains and resistance grouping. While C. difficile isolates are routinely resistant to the cephalosporins and imipenem, resistance to clindamycin is less common. The finding of these multiple resistant strains in our study is therefore an important one, particularly as those strains resistant to four antibiotics were associated more with symptomatic patients than asymptomatic patients by a ratio of 2.5:1. A study of larger number of isolates will be needed to confirm this observation.

The data generated from the present study showed that all 35 C. difficile isolates originating from patients were typable by the PCR ribotyping method. Interestingly, the 35 C. difficile culture positive patients harboured 10 different, highly diverse PCR ribotypes. Ribotype 039 was the single most prevalent non toxigenic ribotype among our isolates (17%). However, ribotypes 097 (23%) and 078 (17%) were the most prevalent toxigenic ribotypes responsible for over one-third of the cases of CDAD seen. From an epidemiological point of view, this is an interesting finding, in that the dominant ribotypes causing diarrhoea in our isolates are completely different from those seen in Europe 40, 41, particularly the UK 22, Hungary 41 and Poland 42. Fifty-five per cent of C. difficile infections seen in UK hospitals are caused by ribotype 001 22, While ribotype 087 accounted for 39% of all isolates in Hungary 41. In the Polish study, all the environmental isolates and 11 of 31 neonatal isolates belonged to ribotype 001 42.

In conclusion, CDAD is almost unknown in the absence of antibiotic use and it has been demonstrated that the risk of this disease among hospitalized patients increases with the use of clindamycin and the presence of clindamycin-resistant strains 43 as well as the use of cephalosporins 44. We believe that the extent of C. difficile involvement in diarrhoeal diseases in our environment would be better judged by direct comparison with the isolation rates of other enteric pathogens in antibiotic-associated diarrhoea. In addition, our finding suggest that metronidazole should remain the drug of choice for the therapy of CDAD. It is also concluded that the prevalent PCR ribotypes of C. difficile strains isolated in our study are different from those found in Europe. Further work is needed to elucidate the factors responsible for the geographical differences and the ability of one strain to cause more systemic symptoms than another.

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**REFERENCES**

Aetiology of pseudomembranous colitis.


طراز الحساسية للمضادات الحيوية والأمراض الجينية المنتشرة للمعزولات الإكلينيكية من
ميكروب المتمثلي العصيرة

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إن ميكروب المتمثلي العصيرة يعتبر مسبب هام للإسهال المستشقفي والتهاب القولون الغضائي الكاذب الذي له معدلات وفيات تراوح بين 15% و 30%، ولذا أجريت هذه الدراسة لإستقصاء معدل حدوث وتوليد الذيفان لهذا الميكروبات وطراز حساسيته للمضادات الحيوية الخاصة بالبكتيريا اللاهوائية. بالإضافة إلى الاستقصاء عن النمط الجيني لمعزولات الميكروبات الإكلينيكية (PCR ribotyping). باستخدام اختبار البلمرة المستحلل خلال فترة ثلاث سنوات أجريت الدراسة على 80 مريضا تم علاجهم بالمضادات الحيوية خلال فترة الأربع أسابيع السابقة (10 مريضاً يعانون من الإسهال وال10 الآخرين لم تظهر عليهم الأعراض) بالإضافة إلى 20 من الأصحاء كمجموعة ضابطة.

وقد أن المعاد الكلي لحدوث ميكروب المتمثلي العصيرة هو 3.3% بينما كانت جميع نتائج المزارع سلبية للأصحاء. كما وجد أن معدل حدوث السلالات المولدبة للذيفان في المرضى الذين يعانون من الإسهال هي 1.9% بينما كانت بنسبة 20% في المرضى الذين لا يعانون من نفس الأعراض. وكانت نتائج اختبارات الحساسية للأموكسيسيلين/كلارايلاتيك أسيدي والأمبيسيللين والميروبيتين والميترودازول والبينيبراسيلين وبيبراسيلين/ناروباباذام والتيموكسيتين والفانكوميسين ممتازة لجميع المعزولات. وكان تعداد المقاومة لعدد إثنين أو أكثر من المعزولات الحيوية سائلاً، بينما أظهرت المعزولات الغير مولدية للذيفان بنسبة 1.6% من الإسهال. وتعتبر معزولاً غير مولدية للذيفان في المرضى الذين يعانون من الإسهال عن المرضى الذين لا يعانون من نفس الأعراض بنسبة 2.5%. كما أنه كان من المدهد وجود 10 أنواع جينية مختلفة بين 35 سلالة من ميكروب المتمثلي العصيرة التي تم علاجها من المرضى في هذه الدراسة. وكانت الألائم الجينية المترابطة في هذه الدراسة 37% و78% في المرضى الذين تم علاجهم بالمضادات الحيوية وتمتلك أكثر من 30% عدم المعاد الذي يعانون من الإسهال.

والخلاصة هي أنه من الأفضل عمل دراسات أخرى على المرضى الذين يعانون من الإسهال بعد العلاج بالمضادات الحيوية لعرفة ما إذا كانت هناك أنواع أخرى من الميكروبات المعوية تعتبر كمسببات للإسهال كما أنه ثبت أن الميترودازول هو المضاد الحيوي الأفضل والأمثل لعلاج مثل هذه الحالات. بالإضافة إلى أن الأنماط الجينية المنتشرة التي تم التعرف عليها في هذه الدراسة مختلفة عن التي هي منتشرة لدى الدول الأوروبية.