Development of Clostridium difficile resistance to Piperacillin/Tazobactam over a period of ten years.

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Abstract:

Background: *Clostridium difficile* has emerged as one of the most problematic and challenging bacterial pathogens of the past decade. It became a human pathogen of note in the 1970s. Antibiotic treatment is the main risk for CDI outbreak. CDI occurs when the intestinal microflora is disrupted, allowing *C. difficile* to colonize the gut and produce toxins. One major cause of the CDI increase is the development of resistance to antibiotics. Piperacillin/Tazobactam for antibiotic treatment has been shown to have inhibitory effect on *C. difficile* sporulation or cytotoxic production. It is widely used as first line antibiotics. Our knowledge and understanding of this organism has grown remarkably in the past few years. Increasing awareness along with rapid and accurate diagnosis are extremely important, thus allowing prompt initiation of appropriate treatment and infection control to prevent serious complications and spread of the disease. Appropriate use of antibiotics only when indicated would further prevent developing of new cases. However, more knowledge about *C. difficile* resistance development to antibiotics is required in order to control and defeat this disease.

Objective: The purpose of this study is to investigate if *C. difficile* has developed resistance to Piperacillin/Tazobactam over a period of ten years. If that shows to be the case, it would indicate that the situation now is more severe for preventing outbreak of CDI, and measures should be taken to avoid further resistance development.

Method: The chosen indicator for antibacterial resistance development is comparing MIC (minimum inhibitory concentration) values. A retrospective cross-sectional study was performed on 200 stool samples, from one group of patients collected 2005 and another group of patients collected 2015. The purpose was to investigate the development of *C. difficile* resistance to Piperacillin/Tazobactam. MIC test (also called E-test) was used to determine the minimum inhibitory concentration level (mg/L). The mean MIC values for Piperacillin/Tazobactam in two groups were compared and analyzed.

Results: The mean MIC value from the 2005 group was 11.20 mg/L, and from the 2015 group it was 13.69 mg/L. This means that it requires a higher concentration of Piperacillin/Tazobactam to kill *C. difficile* strains year 2015 compared to ten years earlier. The statistical significance of this study is p<0.05.

Conclusion: This study indicates that *C. difficile* resistance to Piperacillin/Tazobactam has increased during a period of ten years. This result emphasizes the fact that inappropriate uses of Piperacillin/Tazobactam must be prevented to avoid further resistance development. However, there are some confounding factors in this study, and the recommendation is then to follow up this study with further investigation to confirm the results.
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Introduction

1.1 Clostridium Difficile Infections (CDI)

*C. difficile* has emerged as one of the most problematic and challenging bacterial pathogens of the past decade. It was initially discovered in the 1930s, but it only became a human pathogen of note in the 1970s [1]. *C. difficile* has taken on epidemic proportions in North America and Europe since the early 2000s, leading to devastating outbreaks of *C. difficile* infection (CDI) in hospitals [1]. Unfortunately it has also been a steep rise in mortality. It shows that the attributable mortality of CDI during the period 2005-2011 has increased from 5.7 to 6.9% [2].

CDI is defined with the presence of toxin producing *C. difficile* in stool without reasonable evidence of another cause of diarrhea, or pseudomembranous colitis as diagnosed during endoscopy, after colectomy or on autopsy.

Gastrointestinal problems with nausea, ache or diarrhea associated with antibiotic treatment is common. The symptoms can usually quickly regress at the termination of the treatment, but in some cases the patient may develop a colitis caused by *C. difficile* toxin. In conjunction with antibiotic treatment the normal intestinal flora is affected unfavorably, making it possible for toxigenic *C. difficile* to grow in the intestine with diarrhea as a result. The risk of *C. difficile* problems is greater during treatment with cephalosporin, clindamycin than with other antibiotics [4].

Once CDI is diagnosed in a patient, immediate implementation of appropriate infection control measures is mandatory in order to prevent further spread within the hospital. These include early diagnosis of CDI, surveillance, education of staff, appropriate use of isolation precautions, hand hygiene, protective clothing, environmental cleaning and cleaning of medical equipment, good antibiotic management, and specific measures during outbreaks [3]. Additional treatment include discontinuation of unnecessary antimicrobial therapy, which results in that one third of the patients recover [4].

A specific recommendation for the antibiotic treatment of CDI cannot be made, but a clear guideline on the treatment of CDI is needed for clinical practice. Metronidazole is used to treat mild cases, and vancomycin is recommended for severe cases. Vancomycin or Fidaxomicin should be used to treat recurrences. In cases with several recurrences, a treatment option is fecal microbiome transfer [3, 5].
1.2 Piperacillin/Tazobactam

Piperacillin/Tazobactam is a parenteral preparation with very broad spectrum comprising gram-positive and gram-negative aerobic and anaerobic bacteria [6]. It contains two active ingredients; Piperacillin, which is a penicillin-type antibiotic, and Tazobactam, which is a medicine that prevents bacteria from inactivating Piperacillin. It is used to treat bacterial infections.

Piperacillin is an antibiotic that has the ability to kill a wide variety of bacteria. It works by interfering with the formation of bacterial cell walls, and the effect is depending on the time the free concentration in serum exceed the bacterial MIC values [7].

Resistance can arise due to beta lactamase production, several of which are inhibited by Tazobactam. In addition, resistance may arise from the production of altered penicillin-binding proteins [7].

Tazobactam has no antibacterial effect but makes Piperacillin becomes stable to most beta-lactamase producing bacteria. Tazobactam therefore increases the range of bacteria that Piperacillin can kill [8].

Piperacillin is excreted unchanged in the urine and to some extent also bile. Tazobactam is metabolized in part to an inactive metabolite, but is also excreted in the urine and bile [7].

1.3 Development of C. difficile resistance to antibiotics

Antibiotic treatment is the main risk for CDI outbreak. CDI occurs when the intestinal microflora is altered or disrupted, allowing C. difficile to colonize the gut and produce toxins [4]. One major cause of the CDI increase is the development of resistance to antibiotics. Resistance to a wide range of antibiotics allow C. difficile to colonize and infect in the presence of drugs [9, 10]. New properties of antibiotic resistance in C. difficile clinical isolates are appearing, like resistance to multiple antibiotics, reduced susceptibility to antibiotics used for treatment and rapid spreading and persistence of resistance [11, 12].

Piperacillin/Tazobactam for antibiotic treatment has been shown to have inhibitory effect on C. difficile sporulation or cytotoxic production [13, 14]. It is widely used as first line antibiotics. Unfortunately there are findings that shows that inappropriate use of antibiotics is a key factor contributing to the global problem of bacterial resistance [15]. It is then suspected that some C. difficile strains have developed resistance to Piperacillin/Tazobactam. Oral vancomycin and metronidazole have provided the mainstream of treatment for C. difficile infection over recent years. However there is also a growing concern about the failure of current treatments to prevent and treat CDI [16].
Our knowledge and understanding of this organism has grown remarkably in the past few years. Increasing awareness along with rapid and accurate diagnosis are extremely important, thus allowing prompt initiation of appropriate treatment and infection control to prevent serious complications and spread of the disease [2]. Appropriate use of antibiotics only when indicated would further prevent developing of new cases. However, more knowledge about C. difficile resistance development to antibiotics is required in order to control and defeat this disease.

1.4 Minimum inhibitory concentration

Minimum inhibitory concentrations (MICs) are defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation. The unit measured is mg/L.

MIC scores are important in diagnostic laboratories to confirm resistance of microorganisms to an antimicrobial agent and also to monitor the activity of new antimicrobial agents. MIC score is used to choose which antibiotics to administer to patients with specific infections and to identify an effective dose of the antibiotic. This is important because populations of bacteria exposed to an insufficient concentration of a particular drug or to a broad-spectrum antibiotic can evolve resistance to these drugs. Therefore, MIC values helps in preventing evolution of drug-resistant microbial strains [17].

1.5 Objective

The purpose of this study is to investigate if C. difficile has developed resistance to Piperacillin/Tazobactam over a period of ten years. If that shows to be the case, it would indicate that the situation now is more severe for preventing outbreak of CDI, and measures should be taken to avoid further resistance development.
Materials and methods

2.1 Study population

The stool samples used in this study come from previous collected samples at Örebro University Hospital (USÖ) in Örebro, Sweden. Both primary care and inpatient care. The samples were not selected in any other way than that they were submitted to the clinical microbiology laboratory with the issue of Clostridium difficile infection, e.g. diarrhea etc., during the given period.

This study included frozen bacterial isolates from \textit{C. difficile} positive stool samples collected 2005 and 2015. The first 100 samples from each year were selected from a list with sample number, the patient’s social security number and date.

2.2 Collecting and handling of the stool samples

Collection of stool samples at Örebro University Hospital (USÖ) in Örebro, Sweden, was done according general sampling instructions. The material used were feces tube, spoon and transportation sleeve.

In brief the sample was taken with spoon from stools collected in a clean container, and then put into a feces tube. Only loose stools were used for this analysis. Relevant clinical data and possible antibiotic treatment was specified for each sample, as well as \textit{C. difficile} issue. Samples were then stored in the fridge awaiting transport. The samples were sent to the clinical microbiology laboratory, USÖ, for culture and toxin analysis.

Toxin negative samples were disposed directly. For toxin positive sample a preliminary answer was given, and the final answer was later sent when the supplementary cultivation was completed. The toxin positive isolates were frozen for long term storage in the freezer with a temperature of -70 degrees C, for later studies.

2.3 MIC test

The performance of the MIC test took place at USÖ laboratory during the period August to December 2016. The samples were taken from the freezer in the laboratory. The process was the same for samples from 2005 and 2015.

Clinical \textit{C. difficile} isolates were recovered from -70 °C freezer by inoculation on FAAP non-selective agar plates (Fastidious anaerobe agar; Acumedia, Neogen corp, USA, 5% horse blood; SVA, Sweden), and anaerobic incubation at 37 °C for 48h. 10 samples at a time were processed.
For e-test pre-warmed MUHBP (Muller Hinton Agar: 5% horse blood and b-NAD, E test technical manual Biodisk http://www.abbiordisk.se/pdf/etm_index.htm), were used. All cultured \textit{C. difficile} isolates were inspected for characteristic colony morphology and odour. Colony growth was grouped as follows:

- Proper growth of \textit{C. difficile} colony.
- No growth of the bacterial colony.
- Mixed growth of \textit{C. difficile} and other contaminating bacterial species.
- Only growth of other bacterial species colony.

\textit{C. difficile} identity was further verified using a combined EIA for \textit{C. difficile} GDH and toxin A/B (C.diff QuikChek complete test, Techlab, Blacksburg, VA, USA)

\textit{C. difficile} colonies were vortexed in saline (0,85 % NaCl) to an optical density of 3,5 – 4,5 Mc Farland and spread evenly on MUHBP plates by using a rotating disk, The plates were allowed to dry slightly before application of a Piperacillin/Tazobactam MIC test strip (Liofilchem) and anaerobic incubation at 37 °C for 24-48 h.

Results from the MIC-tests were read by visually detecting where the line were bacterial growth crossed the scale on the E-test strip. The unit measured was mg/L, and measurement results were recorded on a list of all the numbered samples.

2.4 Statistical analysis

The study is a retrospective quantitative observation study of the cross-sectional type. The material shows univariate statistical discrepancy, and groups from the various test sessions are unpaired. Values are distributed as ratio scale and exhibit normal distribution. Since there were two groups compared, 2005 and 2015, t-test statistical analysis method was chosen to be performed to clarify the significance of the result. Means were considered significantly different when \( p < 0.05 \).

2.5 Ethical consideration

No ethical approval was applied for, since this is a retrospective non-intervention study. All data regarding the stool samples, including social security number, were handled confidentially.
Results

3.1 Sample characteristics

*C. difficile* resistance to Piperacillin/Tazobactam was analyzed by measuring and comparing the MIC value on test strip in totally 194 samples, 98 from 2005 and 96 from 2015. Initial number of samples were 100 from 2005 and 100 from 2015. In about 90% of the cultures a correct growth of *C. difficile* was found. In cases where no such growth was found the plate was discarded, and the growth of culture was repeated. The reason for the dropout (2% resp. 4%) was finally failure in *C. difficile* colony growth on agar plates. The dropouts, distribution of gender and age among the patients from where the samples were taken is presented in table 1.

<table>
<thead>
<tr>
<th></th>
<th>2005</th>
<th>2015</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of samples</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Dropouts</td>
<td>2 (2%)</td>
<td>4 (4%)</td>
</tr>
<tr>
<td>Male</td>
<td>37 (38%)</td>
<td>49 (51%)</td>
</tr>
<tr>
<td>Female</td>
<td>61 (62%)</td>
<td>47 (48%)</td>
</tr>
<tr>
<td>Mean age</td>
<td>70</td>
<td>72</td>
</tr>
</tbody>
</table>

3.2 Results 2005 samples.

The 2005 results were obtained by visually detecting values on the MIC test strips, where the line between the living and the killed bacterial colony was detected on the scale. This indicated the level of resistance to Piperacillin/Tazobactam of that particular *C. difficile* isolate. Samples from 98 patients were used when analyzing the MIC test value for 2005. The distribution of readings of MIC values can be seen in table 2.

3.3 Results 2015 samples.

The 2015 results were obtained in the same way as for the 2005 results, by visually detecting values on the Liofilchem MIC test strips. Samples from 96 patients were used when analyzing the MIC test value for 2015. The distribution of readings of MIC values can be seen in table 2.
USÖ Läkarprogrammet

Table 2. E-test results (MIC) of Piperacillin/Tazobactam strip scale, for clinical *C. difficile* isolates from 2005 and 2015. Results are presented in number of samples with corresponding MIC value.

<table>
<thead>
<tr>
<th>MIC (mg/L)</th>
<th>2005</th>
<th>2015</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>6</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>29</td>
<td>20</td>
</tr>
<tr>
<td>12</td>
<td>42</td>
<td>43</td>
</tr>
<tr>
<td>16</td>
<td>7</td>
<td>14</td>
</tr>
<tr>
<td>24</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>32</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>48</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>

3.4 Comparing results 2005 vs. 2015 samples.

From the 98 respective 96 MIC values the mean value and standard deviation could then be calculated. The mean MIC value for the 2005 group was 11.20 mg/L, and for the 2015 group it was 13.69 mg/L. The standard deviations for the same years were 5.25 mg/L resp. 7.87 mg/L, as can be seen in table 3. The difference in mean value between 2005 and 2015 was 2.49. See figure 1.

In order to examine possible statistically significant differences between the mean MIC values of 2005 compared to 2015 the data was analyzed using t-test. The result is t-value of 2.078 and the statistical significance p<0.05. The result gives a statistical significant difference between mean MIC values 2005 and 2015.

Table 3. Results of MIC test strip scale on 2005 and 2015 samples presented in mean MIC value and standard deviation.

<table>
<thead>
<tr>
<th>Mean MIC</th>
<th>2005</th>
<th>2015</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard deviation</td>
<td>5.25 mg/L</td>
<td>7.87 mg/L</td>
</tr>
</tbody>
</table>

Figure 1. Mean MIC value (Piperacillin/Tazobactam) for clinical *C. difficile* isolates from the 2005 group and from the 2015 group, as well as the standard deviations for the same years.
Discussion

The purpose of this study was to identify if any development of *C. difficile* resistance to the antibiotic Piperacillin/Tazobactam can be detected over a period of ten years. It is important to follow this resistance development since Piperacillin/Tazobactam is widely used as first line antibiotics.

According to the results presented in this study, there is a significant difference in Piperacillin/Tazobactam mean MIC values between clinical *C. difficile* strains isolated in 2005 and 2015. The mean MIC value are higher 2015 compared to ten years earlier, which indicates that *C. difficile* resistance to Piperacillin/Tazobactam has increased.

Studies have shown that Piperacillin/Tazobactam rapidly reduce gut bacterial populations below the limits of detection by the end of the Piperacillin/Tazobactam instillation period. Despite such widespread disruption of gut bacterial populations, *C. difficile* populations remained principally as spores, but with no sustained proliferation or high-level cytotoxin production observed [14]. Other studies have shown isolates of *C. difficile* to be susceptible to Piperacillin/Tazobactam [9, 10]. However, any resistance development to Piperacillin/Tazobactam would change this.

Quantifying resistance within and between bacterial and host populations is a complex challenges in terms of laboratory methodology and sampling design [18]. There are various antibiotic sensitivity testing methods to choose from. Some examples are dilution methods, disk diffusion method, E-test, automated antimicrobial susceptibility testing systems, mechanism-specific tests and genotypic methods [19]. The method used in this study was the E-test, in the report called MIC value test. The reason for choosing E-test was that it provides a convenient quantitative test of antibiotic resistance of a clinical isolate.

To our knowledge, no other study with the purpose to retrospectively investigate the development of *C. difficile* resistance to Piperacillin/Tazobactam has been done. However, other investigations to find and follow up the development of resistance to Piperacillin/Tazobactam in general have been undertaken. One conclusion is that inappropriate uses of Piperacillin/Tazobactam must be prevented by antimicrobial handling efforts, as well as infection-control practices, to avoid drug resistance development [20].

Another study showed that resistance to Piperacillin/Tazobactam had increased over a period of nine years in *Escherichia. coli*, *Klebsiella spp.* and in *AmpC-inducible Enterobacteriaceae*, though not in *Pseudomonas mirabilis* or *P. aeruginosa* [21].
With these studies in mind, and the fact that 10 years is a relatively short period, the development of antibiotic resistance to conventional preparations may soon end up in a critical situation. The development of new antibiotic is a quiet time and cost consuming industry. If new developed and launched antibiotics shows to have short life length on the market, there will be no business case in product research and development.

Study limitations

Originally Biomerieux brand MIC test strips were supposed to be used. This was standard equipment at the laboratory. However these strips had been recalled from the market. The reason for recalling the Etest® PIP/TAZO/CON-4 PTC 256 was because the test results from the affected product indicated that antibiotic therapy using PIP/TAZO could stop or slow the growth of certain bacteria when it may not actually be effective in treating those bacteria [22]. The strips were then changed for Liofilchem branded instead, even though the reliability of this MIC test strip was questioned in a report from EUCAST 2015 [23]. This could have an impact and gives an uncertainty to the result. The use of MIC test strip without evidence based results may then be considered as a confounder.

A second confounder could be the relatively high standard deviations in relation to found difference in mean MIC values. The difference in mean MIC values found is 2.49 mg/L, while the standard deviation range from 5.25 mg/L to 7.87 mg/L. This means that the difference in mean MIC values are found within the standard deviations from each group. A bigger difference in mean MIC value compared to the standard deviations would have given the study less uncertainty in the result. The main reason for the relatively high standard deviations is suspected to be the small size of the study. A bigger study, with more isolates included, may have come to better results regarding standard deviations.

A third possible confounder in the study is the uneven gender distribution in the two groups. The fact that 2005 group consists of 62% females while 2015 group has a more even gender distribution with 48% female participants makes the result less reliable. Due to the uncertain effect of the gender influence, this must be kept in mind as a potential error when reading the results.

**Conclusion:**

This study has found a significant difference in Piperacillin/Tazobactam mean MIC values for *C. difficile* between 2005 and 2015. The mean MIC value is more than 20% higher 2015 compared to ten years earlier, which indicates that *C. difficile* resistance to Piperacillin/Tazobactam has increased. This result could be considered clinically relevant, and emphasizes the fact that inappropriate uses of Piperacillin/Tazobactam must be prevented to avoid further resistance development.
References


