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Antimicrobial susceptibility of Brazilian *Clostridium difficile* strains determined by agar dilution and disk diffusion

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ABSTRACT

Clostridium difficile is a leading cause of diarrhea in hospitalized patients worldwide. While metronidazole and vancomycin are the most prescribed antibiotics for the treatment of this infection, teicoplanin, tigecycline and nitazoxanide are alternatives drugs. Knowledge on the antibiotic susceptibility profiles is a basic step to differentiate recurrence from treatment failure due to antimicrobial resistance. Because *C. difficile* antimicrobial susceptibility is largely unknown in Brazil, we aimed to determine the profile of *C. difficile* strains cultivated from stool samples of inpatients with diarrhea and a positive toxin A/B test using both agar dilution and disk diffusion methods. All 50 strains tested were sensitive to metronidazole according to CLSI and EUCAST breakpoints with an MIC_{90} value of 2 μg/mL. Nitazoxanide and tigecycline were highly active *in vitro* against these strains with an MIC_{90} value of 0.125 μg/mL for both antimicrobials. The MIC_{90} were 4 μg/mL and 2 μg/mL for vancomycin and teicoplanin, respectively. A resistance rate of 8% was observed for moxifloxacin. Disk diffusion can be used as an alternative to screen for moxifloxacin resistance, nitazoxanide, tigecycline and metronidazole susceptibility, but it cannot be used for testing glycopeptides. Our results suggest that *C. difficile* strains from São Paulo city, Brazil, are susceptible to metronidazole and have low MIC_{90} values for most of the current therapeutic options available in Brazil.

Introduction

Clostridium difficile is a spore-forming Gram-positive bacillus. This microorganism produces two major toxins, enterotoxin A and cytotoxin B, that can cause diarrhea, pseudomembranous colitis, colon dilation, sepsis, and even death.[1]

The incidence and severity of *C. difficile* infections (CDI) is growing in many countries due in part to the dissemination of

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a hyper virulent strain known as North America Pulse type 1 (NAP1) or ribotype 027.[2]

Until the 1980s, there was little interest in researching new antibiotics for the treatment of CDI because most patients responded well to treatment with metronidazole or oral vancomycin. More recently, infection recurrence and the limitations of the available therapeutic options have become clearer.[3] There are still doubts about the accuracy and correlation of different methods used to evaluate *in vitro* antibiotic sensitivity as well as the sensitivity of *C. difficile* strains to the recommended treatment regimens.

This study assessed the susceptibility profiles of a collection of *C. difficile* strains cultivated from stools of inpatients with diarrhea in six tertiary hospitals in São Paulo, Brazil. We also aimed to evaluate if disk diffusion method could be an alternative for susceptibility testing of the main drugs used in the treatment of CDIs. It was not a purpose of the study to evaluate the epidemiological data of the patients.

Materials and methods

Clostridium difficile strains

Consecutive clinical strains of *Clostridium difficile* ($n = 50$) were cultivated from stool samples of inpatients with diarrhea in six tertiary hospitals in São Paulo from March to December 2013 (one sample per patient). Stool samples were randomly selected for culture based on their positivity when tested with the ProScpect™ *C. difficile* Toxin A/B Microplate Assay (Thermo Scientific). Stool cultures for *C. difficile* were carried out as previously described, with modifications.[4] In summary, approximately 0.5 g of feces were mixed with 0.5 mL 95% ethanol, vortexed and incubated at room temperature (20–25 °C) for 1 h. The suspension was vortxed again and two drops were plated on *Brucella* agar supplemented with 5% horse blood and 0.2% sodium taurocholate. Plates were incubated in a 2.5 L anaerobic jar containg the Atmosphere Generation System AnaeroGen (Oxoid-Thermo Scientific) for 72 h at 36 °C. Identification to the species level was achieved by MALDI-ToF MS using the MALDI Biotyper LT System (Bruker).

The strains were stored in 10% skim milk at −70 °C and subcultured on *Brucella* agar with 5% horse blood twice before utilization in susceptibility tests.

Antimicrobial susceptibility testing

The antimicrobials tested in this study were: metronidazole (Sigma-Aldrich), moxifloxacin (Sigma-Aldrich), nitazoxanide (Farmoquímica), teicoplanin (Sigma-Aldrich), tigecycline (Pfizer), and vancomycin (Sigma-Aldrich).

Disk diffusion was performed as described by Erikstrup et al.[5] Cultured strains were suspended in thioglycollate broth to a density of 1.0 McFarland ($\approx 3.0 \times 10^8$ CFU/mL) with the aid of DensiCheck® (bioMérieux). The suspension was then seeded onto *Brucella* Blood Agar supplemented with 10% sterile defibrinated lysed horse blood, hemin (5 μg/mL) and vitamin K (1 μg/mL). To optimize the growth of *C. difficile*, plates were pre-reduced for 24 h in an anaerobic atmosphere generated by the AnaeroGen system (Oxoid-Thermo Scientific) before use. For inoculum preparation, inoculation and incubation the 15-15-15 rule was followed.[5]

After 24 h of incubation at 36 °C in anaerobic atmosphere, generated with the aid of the AnaeroGen Atmosphere Generation Systems (Oxoid-Thermo Scientific), inhibition zone diameters were measured under reflected light considering 100% inhibition. Duplicate tests were performed for each strain on two separate days. The inhibition zone diameters were correlated with the minimum inhibitory concentrations (MICs) obtained by agar dilution for each strain and drug combination.

For nitazoxanide and metronidazole 6-mm paper disks were prepared by adding 10 μL of a 0.5 mg/mL solution in dimethyl sulfoxide (Sigma), while for vancomycin and teicoplanin 6-mm paper disks with a potency of 5 μg were prepared by adding 10 μL of a 0.5 mg/mL solution in reagent grade water. A 30 μg teicoplanin disk (Oxoid-Thermo Scientific) was also tested. For tigecycline and moxifloxacin, we used commercially available disks (Oxoid-Thermo Scientific) containing 15 and 5 μg, respectively. There are currently no interpretative criteria for disk diffusion when testing *C. difficile*.

For agar dilution bacterial strains were tested according to the Clinical and Laboratory Standards Institute (CLSI)[6] and the European Committee on Antimicrobial Susceptibility Testing (EUCAST)[7] guidelines. Bacterial suspensions were prepared in thioglycollate broth to 0.5 McFarland turbidity ($\approx 1.5 \times 10^8$ CFU/mL) before 1 μL of each suspension was transferred to the agar plates with the aid of a Steers replicator. Plates containing antibiotic were stamped starting from the lowest concentration. The MIC was defined as the lowest antibiotic concentration inhibiting visible growth after 48 h of incubation at 37 °C in anaerobiosis. Tests were performed in duplicates.

The CLSI breakpoints for MICs were used for metronidazole and moxifloxacin,[8] while EUCAST criteria were used for metronidazole and vancomycin.[7] The interpretative criteria are summarized in Table 2. There are currently no interpretative criteria for tigecycline, teicoplanin, and nitazoxanide; consequently, only MIC_{50} and MIC_{90} values were calculated. For teicoplanin and nitazoxanide, the ECOFFinder spreadsheet[9] was used to estimate epidemiological cut-off values (ECOFFs). *C. difficile* ATCC 700057 strain was used for quality control and tested simultaneously with each batch of antimicrobial susceptibility tests.

Statistical analysis

For metronidazole, moxifloxacin, and vancomycin categorical agreement between agar dilution and tentative interpretative criteria for disk diffusion was evaluated using CLSI and EUCAST breakpoints for agar dilution. The errors were classified as previously described.[10]

Results

From March 1st to December 31st 2013 there were 1884 patients for which the detection of *C. difficile* toxins A and B by ELISA was ordered by the attending physicians. A total of 239 (12.7%) patients had a positive test. The mean age of patients