Journal of Global Antimicrobial Resistance 14 (2018) 246–252

Contents lists available at ScienceDirect

Journal of Global Antimicrobial Resistance

journal homepage: www.elsevier.com/locate/jgar

Comparison between disk diffusion and agar dilution methods to determine in vitro susceptibility of *Corynebacterium* spp. clinical isolates and update of their susceptibility

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ARTICLE INFO

Article history:
Received 8 August 2017
Received in revised form 11 May 2018
Accepted 11 May 2018
Available online 18 May 2018

Keywords:
Corynebacterium spp.
Antimicrobial susceptibility
Disk diffusion
Agar dilution
Resistance

ABSTRACT

Objectives: Although *Corynebacterium* spp. are part of the microbiota of the skin and mucous membranes, human infections caused by *Corynebacterium* spp. have been reported and the multidrug resistance pattern of the recovered isolates was emphasised. Due to the usefulness of disk diffusion in daily practice, the purpose of this study was to compare disk diffusion with agar dilution to determine disk diffusion breakpoints and to review the antimicrobial susceptibility of the most frequent *Corynebacterium* spp. isolated in clinical samples.
Methods: Susceptibility to 20 antimicrobial agents of 143 *Corynebacterium* spp. isolates recovered from relevant clinical samples was determined. Comparison between the disk diffusion and agar dilution methods for eight antimicrobial agents was performed to establish new breakpoints using simple linear regression analysis.
Results: All of the isolates tested were susceptible to vancomycin, minocycline and linezolid. A typical susceptibility profile to β-lactam antibiotics among the different species included was not observed. Almost all isolates showed resistance to macrolides and lincosamides. Using a simple linear regression method, it was possible to establish breakpoints for penicillin, erythromycin, clindamycin, gentamicin and ciprofloxacin. However, the low correlation coefficient obtained for vancomycin, minocycline and trimethoprim/sulfamethoxazole did not allow establishment of breakpoints for the disk diffusion method.
Conclusion: The disk diffusion method could only be used to evaluate susceptibility to penicillin, erythromycin, clindamycin, gentamicin and ciprofloxacin. These data show that the presence of a *Corynebacterium* spp. isolate in a clinical sample demands the performance of antimicrobial susceptibility testing since the susceptibility profile is not predictable.

1. Introduction

Corynebacterium spp. are part of the microbiota of the skin and mucous membranes [1,2]. Since they are usually found as commensals, they are often considered as mere contaminants when isolated from clinical samples [1–3]. However, human infections caused by *Corynebacterium* spp. have been reported and the multidrug resistance pattern of the recovered isolates was emphasised [1,4].

The European Committee on Antimicrobial Susceptibility Testing (EUCAST) (http://www.eucast.org/) and Clinical Laboratory and Standard Institute (CLSI) have published minimum inhibitory concentration (MIC) breakpoints for *Corynebacterium* spp. However, the CLSI has not established breakpoints for the disk diffusion method [5]. Due to the usefulness of disk diffusion in daily practice, the purpose of this work was to compare disk diffusion with agar dilution to determine breakpoints for the disk diffusion method. Moreover, the antimicrobial susceptibilities of 143 *Corynebacterium* spp. isolates to 20 antimicrobial agents were analysed.

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https://doi.org/10.1016/j.jgar.2018.05.009

2. Materials and methods

2.1. Bacterial strains

A total of 143 isolates of non-lipophilic *Corynebacterium* spp. strains were collected from clinically relevant samples of patients at Hospital de Clínicas 'José de San Martín' of Universidad de Buenos Aires (Buenos Aires, Argentina). All of the strains were grown on 5% sheep blood agar in 5% CO_2 at 35 °C and were stored at −70 °C in brain–heart infusion broth with 20% glycerol until use. Identification was performed by conventional phenotypic methods as previously described [1,2,4]. Phenotypic identification of colonies included morphology, Gram staining, catalase activity, lipophilicity and biochemical methods using the algorithm previously described by Funke et al. [1,2].

Bacterial isolates were also identified by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF/MS) using the direct colony on-plate extraction method as previously described [6]. The MALDI Biotyper Library v.3.0 and MALDI Biotyper software v.3.1 (Bruker Daltonik GmbH, Bremen, Germany) were used. Lower cut-off scores for identification were used (≥1.5 for genus level and ≥1.7 for species level). A score of <1.5 was not considered a reliable identification, as suggested by Barberis et al. [6]. A minimum difference of 10% between the top and next closest score was required to differentiate a genus or species [6].

Molecular identification was used as the gold-standard method to compare the results obtained by MALDI-TOF/MS and conventional phenotypic methods. 16S rRNA gene sequencing was performed for characterisation of all isolates. When 16S rRNA gene sequencing did not allow a correct identification to species level, a secondary gene target (*rpoB*) was used. PCR was performed as previously described [7]. The sequences of the primers used for 16S rRNA amplification were Rp2 (5′-ACGGCTACCTTGTTACGACTT-3′) and fD2 (5′-AGAGTTTGATCATGGCTCAG-3′) [8], and for the *rpoB* gene were Rp (5′-CGWATGAACATYGGBCAGGT-3′) and fD (5′-TCCATYTCRCCRAARCGCTG-3′) [8]. Sequencing of the PCR products was performed on both DNA strands using an ABI Prism® 3100 Bioanalyzer (Applied Biosystems/Hitachi, Seoul, South Korea) at the Macrogen Inc. sequencing facility (Seoul, South Korea). The sequences were analysed using BLAST v.2.0 software (http://www.ncbi.nlm.nih.gov/BLAST/). A ≥99.0% and ≥95.0% similarity cut-off for the 16S rRNA and *rpoB* genes, respectively, were required for species identification [9].

2.2. Antimicrobial susceptibility testing

Antimicrobial susceptibility was determined by agar dilution following the recommendations of the CLSI as the reference method [10]. Twenty antimicrobial agents representative of the main antimicrobial groups were tested, including penicillin (PEN), cefalotin (CEF), cefotaxime (CTX), imipenem (IPM), meropenem (MEM), gentamicin (GEN), amikacin (AMK), erythromycin (ERY), clindamycin (CLI), vancomycin (VAN), teicoplanin (TEC), tetracycline (TET), minocycline (MNO), tigecycline (TGC), ciprofloxacin (CIP), levofloxacin (LVX), moxifloxacin (MXF), linezolid (LNZ), trimethoprim/sulfamethoxazole (SXT) and rifampicin (RIF). Drug powders for the agar dilution test were obtained commercially or were provided by their respective manufacturers (Sigma-Aldrich, Buenos Aires, Argentina). Following CLSI recommendations for susceptibility testing by the disk diffusion method [11], eight antimicrobial agents were tested three times under the same conditions on the same day: PEN (10 μg); ERY (15 μg); CLI (2 μg); VAN (30 μg); GEN (10 μg); CIP (5 μg); SXT (1.25/23.75 μg); and MNO (30 μg). Antimicrobial disks were provided by Oxoid Ltd. (Basingstoke, UK). The susceptibility test medium was Mueller–Hinton agar (Oxoid Ltd.). Plates were read and the mean of triplicate zone diameters of each drug for each isolate was determined following overnight incubation at 35 °C in ambient air for 24–48 h. All isolates were further screened by the d-test method using ERY (15 μg) and CLI (2 μg) disks to determine macrolide–lincosamide–streptogramin B (MLS_B) resistance [11].

MIC breakpoints were those established by the CLSI for *Corynebacterium* spp. [5]. For CEF, AMK, MNO, TEC, LVX and MXF, the breakpoints used were those for *Staphylococcus* spp. [12], and for TGC those established by the US Food and Drug Administration (FDA) for *Staphylococcus* spp. were used [13]. Control strains were *Staphylococcus aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212 for the agar dilution test and *S. aureus* ATCC 25923 for the disk diffusion test.

Correlation between the disk diffusion and agar dilution methods was applied between *Corynebacterium* spp. isolates that were not clonally related as previously determined by degenerate oligonucleotide-primed PCR [14].

2.3. Statistical analysis

To establish breakpoints for the disk diffusion method, a simple linear regression model was used. Disk diffusion test breakpoints were determined on the basis of the MIC breakpoints near the linear least-squares regression line between the MIC and the diffusion zone data. These breakpoints were then rounded to the nearest whole number, which was subject to error rate-bounding analysis in order to maximise intermethod agreement. Data were entered into Statistica™ 6.0 statistical package (https://www.R-project.org/). Pearson correlation coefficients (r values), linear regression equations and categorical error rates were generated for each antimicrobial agent indexed by susceptibility test method. The criteria used to consider the statistical procedure accuracy was a Pearson correlation value (r) >0.75.

Errors were determined using the methods published by the CLSI as follows: very major (VME), i.e. susceptibility result by disk diffusion method and resistance by MIC; major (ME), i.e. resistance result by disk diffusion method and susceptibility by MIC; and minor (mE), i.e. intermediate result by disk diffusion method and a resistant or susceptible category for the agar dilution MIC. VME <1.5% and ME <3% were considered acceptable values [15].

3. Results and discussion

MIC values (MIC_{50}, MIC_{90} and MIC ranges) for *Corynebacterium* spp. isolates tested by the agar dilution method are shown in Table 1.

The results of the correlation between the disk diffusion and agar dilution methods are shown in Table 2. Only breakpoints for PEN, ERY, CLI, GEN and CIP could be established by simple linear regression and the error rate-bounding method.

In the last decades, *Corynebacterium* spp. isolates have appeared in clinically relevant samples, demanding the performance of antimicrobial susceptibility testing in order to initiate adequate antimicrobial treatment. Knowing the epidemiology and susceptibility patterns could help to decide the best treatment option.

3.1. β-Lactam antibiotics

The mechanism of resistance to β-lactam antibiotics in the genus *Corynebacterium* is still unknown; however, resistance is likely due to decreased membrane permeability or decreased affinity for these antibiotics [16].

In this study, the activity of β-lactam antibiotics was not uniform in all of the *Corynebacterium* spp., and moreover, a variation in the susceptibility profile was observed intraspecies. Lower activity of β-lactam antibiotics was observed in