Development of the EUCAST disk diffusion antimicrobial susceptibility testing method and its implementation in routine microbiology laboratories

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Abstract

With the support of ESCMID and European countries, EUCAST has developed a disk diffusion test with zone diameter breakpoints correlated with the EUCAST clinical MIC breakpoints. The development of the EUCAST disk diffusion method and quality control criteria are described, together with guidance on quality control and implementation of the method in clinical microbiology laboratories. The method includes the use of Mueller–Hinton agar without supplements for non-fastidious organisms and with 5% mechanically defibrinated horse blood and 20 mg/L β-NAD for fastidious organisms, a standardized inoculum resulting in confluent growth, an incubation time of 16–20 h, a reading guide on how to read zone diameters on individual species-agent combinations and zone diameter breakpoints calibrated to the EUCAST clinical MIC breakpoints. EUCAST recommendations are described in detail and updated regularly on the EUCAST website (http://www.eucast.org).

Keywords: Antimicrobial susceptibility testing, disk diffusion, European Committee on Antimicrobial Susceptibility Testing, MIC, Mueller–Hinton agar, zone diameter breakpoints

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Introduction

Disk diffusion is one of the oldest approaches to antimicrobial susceptibility testing (AST) and remains one of the most widely used AST methods in routine clinical microbiology laboratories. The method is versatile in that it is suitable for testing the majority of bacterial pathogens, including the more common fastidious bacteria, almost all antimicrobial agents can be tested and it requires no special equipment. When performed according to recommendations, disk diffusion is a reproducible and accurate method for AST [1,2]. Several of the European national antimicrobial breakpoint committees, including BSAC in the UK [3], CA-SFM in France [4], DIN in Germany [5] and SRGA in Sweden [6], developed their own disk diffusion methods for AST, but there was no common method calibrated to European breakpoints. Following the harmonization of European MIC breakpoints [7] by the European Committee on Antimicrobial Susceptibility Testing (EUCAST), the committee initiated the development of a standardized disk diffusion method calibrated to the harmonized MIC breakpoints. In common with most other disk diffusion techniques, the EUCAST method is based on the principles defined in the report of the International Collaborative Study of Antimicrobial Susceptibility Testing [8] and on the experience of expert groups worldwide.

The need for a standardized disk diffusion method calibrated to EUCAST clinical MIC breakpoints became obvious from responses to a questionnaire sent by EUCAST to EUCAST European national representatives in 2007. The questionnaire responses indicated that the disk diffusion methods used most widely included Mueller–Hinton (MH)

agar with an inoculum corresponding to a McFarland 0.5 turbidity standard, as described by Bauer *et al.* [9]. Many laboratories followed the performance standards published by the United States Clinical and Laboratory Standards Institute (CLSI) [10,11] or local modifications of the CLSI method. The opinions expressed in the questionnaire strongly supported the development of a European disk diffusion method, based on the widely used Kirby–Bauer method [9] and calibrated to EUCAST clinical MIC breakpoints. It was also evident that a common medium for fastidious organisms instead of separate media for *Streptococcus* spp. and *Haemophilus influenzae* would facilitate laboratory work. In response to these demands, EUCAST, in collaboration with and financed by The European Society for Clinical Microbiology and Infectious Diseases (ESCMID), developed a standardized disk diffusion method based on MH agar with an inoculum density equivalent to a McFarland 0.5 standard and with the specific aim to develop a common medium for fastidious organisms. These objectives have been achieved and zone diameter breakpoints calibrated to the EUCAST clinical MIC breakpoints have been established by analysis of MIC-zone diameter correlations, inhibition zone diameter distributions and MIC distributions. This paper describes the development and calibration of the disk diffusion method, how quality control targets and ranges were developed and validated, and presents guidance on how to implement the EUCAST disk diffusion method in the routine laboratory.

Basic Materials and Methodology

The following description of the EUCAST disk diffusion methodology is a summary of the methodology detailed in a manual on the EUCAST website [12]. The first version of the manual was released in December 2009 and it is updated annually. The described technique must be adhered to without modification in order to obtain reliable results. Tables including organisms covered by the EUCAST disk diffusion method, and corresponding methodology recommendations for each of these, are available in the EUCAST disk diffusion test manual and, from 2014, will also be in a table on the first page of the EUCAST breakpoint tables.

Preparation of media

Unsupplemented MH agar is used for non-fastidious organisms and MH agar supplemented with 5% (v/v) mechanically defibrinated horse blood and 20 mg/L β-NAD ('Mueller–Hinton fastidious', MH-F) for fastidious organisms. MH agar is prepared according to the manufacturer's instructions and supplements are added after cooling to 42–45°C. Agar is dispensed in Petri dishes to achieve an even depth of 4.0 mm with a maximum variation of $\pm$0.5 mm.

Preparation of inoculum

The inoculum suspension is prepared by selecting several morphologically similar colonies (when possible) from overnight growth (16–24 h of incubation) on a non-selective medium with a sterile loop or a cotton swab and suspending the colonies in sterile saline (0.85% NaCl w/v in water) to the density of a McFarland 0.5 standard, approximately corresponding to 1–2 $\times$ 10^8 CFU/mL for *Escherichia coli*. The density of the suspension is preferably measured with a photometric device that has been calibrated with a McFarland standard according to the manufacturer's instructions. Alternatively, the density of the suspension can be compared visually to a 0.5 McFarland turbidity standard. The density of the suspension is adjusted to McFarland 0.5 by addition of saline or more organisms. *Streptococcus pneumoniae* is preferably suspended from colonies on a blood agar plate to the density of a McFarland 0.5 standard. When *S. pneumoniae* is suspended from colonies on a chocolate agar plate, the inoculum must be equivalent to a McFarland 1.0 standard in order to contain a sufficient number of viable cells. All inoculum suspensions should optimally be used within 15 min and always within 60 min of preparation.

Inoculation of agar plates

A sterile cotton swab is dipped into the inoculum suspension and the excess fluid removed by turning the swab against the inside of the tube to avoid over-inoculation of plates, particularly for Gram-negative organisms. The inoculum is spread evenly over the entire surface of the agar plate by swabbing in three directions or by using an automatic plate rotator.

Application of antimicrobial disks

Antimicrobial disks should be handled and stored according to the manufacturer's instructions. Disks are applied firmly on the agar surface within 15 min of inoculation of the plates. It is important that zone diameters can be reliably measured and the maximum number of disks on a plate depends on the size of the plate, the organism and the antimicrobial agents tested. The number of disks on a plate should be limited so that unacceptable overlapping of zones is avoided. A maximum of six disks can be accommodated on a 90-mm circular plate and 12 on a 150-mm circular plate.

Incubation of plates

Within 15 min of application of antimicrobial disks, the plates are inverted and incubated at 35 $\pm$ 1°C for 16–20 h, unless