

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 ± 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 $1\text{--}2 \times 10^8$ 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 $35 \pm 1^\circ\text{C}$ for 16–20 h, unless

otherwise stated in the disk diffusion test manual on the EUCAST website [12]. Unsupplemented MH agar plates are incubated in air and MH-F agar plates in $5 \pm 1\%$ CO₂ in air. The efficiency of incubators varies so appropriate numbers of plates in stacks should be determined as part of the laboratory's quality assurance programme. Small stacks of plates with a space between stacks are more likely to ensure uniform and rapid heating of all plates.

Examination of plates after incubation

A correct inoculum and satisfactorily streaked plates should result in an even confluent lawn of growth. If individual colonies can be seen, the inoculum is too light and the test should be repeated. The age of the culture, the nutritional requirements of the strains or failure to comply with recommendations should be considered before retesting. If zone edges are jagged, the evenness of streaking of the plate should be improved.

Measurement of inhibition zone diameters and interpretation of results

After incubation, inhibition zones are read at the point where no obvious growth is detected by the unaided eye when the plate is held about 30 cm from the eye. The inhibition zone diameters are measured to the nearest millimetre with a ruler, calliper or an automated zone reader. Unsupplemented MH agar plates are read from the back of the plate with reflected light against a dark background whereas MH-F agar plates are read from the front with the lid removed and with reflected light. For haemolytic streptococci on MH-F agar plates, inhibition of growth and not inhibition of haemolysis should be read. If double zones are visible, the inner zone should be read, unless otherwise specifically stated. Specific reading instructions are given in the EUCAST disk diffusion test manual [12] and in the EUCAST reading guide [13]. Zone diameters are interpreted and categorized as susceptible, intermediate or resistant according to the EUCAST clinical breakpoint tables [14].

Quality control

Defined control strains (Table 1) are used to monitor test performance. For antimicrobial agents that are part of routine panels, control tests should optimally be set up daily. In addition to the daily routine quality control tests, each new batch of MH agar (i.e. a different batch (new lot) of agar powder or a switch in the manufacturer of agar for in-house-produced plates or each new shipment of commercial pre-poured plates) should be tested to ensure that all zones are within the acceptable ranges defined in the EUCAST routine quality control tables [15].

TABLE 1. Strains recommended by EUCAST for routine quality control

Organism	Strain collection number	Characteristics
<i>Escherichia coli</i>	ATCC 25922	Susceptible, wild type
	NCTC 12241	
	CIP 76.24	
	DSM 1103	
	CCUG 17620	
	CECT 434	
<i>Pseudomonas aeruginosa</i>	ATCC 27853	Susceptible, wild type
	NCTC 12903	
	CIP 76.110	
	DSM 1117	
	CCUG 17619	
	CECT 108	
<i>Staphylococcus aureus</i>	ATCC 29213	Weak β -lactamase producer
	NCTC 12973	
	CIP 103429	
	DSM 2569	
	CCUG 15915	
<i>Enterococcus faecalis</i>	CECT 794	Susceptible, wild type
	ATCC 29212	
	NCTC 12697	
	CIP 103214	
	DSM 2570	
	CCUG 9997	
<i>Streptococcus pneumoniae</i>	CECT 795	Low-level, chromosomally mediated penicillin resistant
	ATCC 49619	
	NCTC 12977	
	CIP 104340	
	DSM 11967	
<i>Haemophilus influenzae</i>	CCUG 33638	Susceptible, wild type
	NCTC 8468	
	CIP 54.94	
	CCUG 23946	

ATCC, American Type Culture Collection, USA; NCTC, National Collection of Type Cultures, UK; CIP, Collection de Institut Pasteur, France; DSM, Deutsche Stammsammlung für Mikroorganismen und Zellkulturen, Germany; CCUG, The Culture Collection University of Gothenburg, Sweden; CECT, Colección Española de Cultivos Tipo, Spain.

Development of Methodology

Medium

Mueller–Hinton medium is the only generic, commercially available medium for AST and it has been recommended by the CLSI and CA-SFM for more than 25 years. Many laboratories and manufacturers of pre-poured plates have long traditions and extensive knowledge of production and use of MH media. MH was therefore the obvious choice for EUCAST when deciding on the medium. However, there is some variation in MH from different manufacturers and between batches from the same manufacturer. Some of this variation (e.g. cation content) affects antimicrobial activity and some affects the growth of the bacteria, which in turn may affect the size of inhibition zones, and each new batch of MH agar must be quality controlled to ensure that inhibition zones are within EUCAST ranges.

Development of MH-F medium

For fastidious organisms EUCAST did not want to recommend different media for different organisms. We aimed to develop a common medium for *H. influenzae*, *S. pneumoniae*, streptococcus groups A, B, C and G, viridans group strepto-

cocci, *Campylobacter* spp., *Pasteurella multocida*, *Listeria monocytogenes*, *Corynebacterium* spp., *Neisseria* spp. and rapidly growing anaerobes. National antimicrobial breakpoint committees have previously developed or adopted different media for fastidious organisms. The CLSI [10] and CA-SFM [4] recommend MH agar supplemented with 5% sheep blood for streptococci and *Haemophilus* Test Medium (HTM) for *H. influenzae*. The BSAC [3] and SRGA [6] have both recommended Iso-Sensitest agar (Thermo Fisher Ltd, Basingstoke, UK) supplemented with 5% mechanically defibrinated horse blood and 20 mg/L β -NAD. We exchanged the Iso-Sensitest agar for MH agar and found that this medium, named 'Mueller–Hinton fastidious' (Mueller–Hinton agar with 5% defibrinated horse blood and 20 mg/L β -NAD, MH-F), supports good growth of most of the fastidious organisms listed above, including *H. influenzae* (21st European Congress of Clinical Microbiology and Infectious Diseases (ECCMID), poster 749; 22nd ECCMID, posters 671, 676 and 682). However, MH-F has been shown to be inadequate for growth of *Neisseria gonorrhoeae* and anaerobes.

MH-F agar was compared with a similar medium where sheep blood was used instead of horse blood. However, with sheep blood the growth of *H. influenzae* was inadequate unless the concentration of β -NAD was increased at least five-fold (to 100 mg/L), which would significantly increase the cost of the medium. At a concentration of 20 mg/L in MH-F agar, β -NAD from seven different manufacturers [Acros (Fair Lawn, NJ, USA), BDH (VWR International, Radnor, PA, USA), Biomol (Hamburg, Germany), Fluka (Sigma-Aldrich, Steinheim, Germany), ICN Biomedicals (Irvine, CA, USA), Merck (Whitehouse station, CA, USA) and Sigma-Aldrich] resulted in good and very similar growth of *H. influenzae* NCTC 8468 and inhibition zone diameters were within ± 1 mm for all investigated antimicrobial agents, including β -lactam agents, tetracyclines and trimethoprim-sulphamethoxazole. Concentrations of β -NAD as low as 10 mg/L supported good growth and resulted in reliable inhibition zone diameters for *H. influenzae* and *S. pneumoniae*, but 20 mg/L β -NAD was selected for MH-F medium to allow for some variation in purity, quality and concentration between manufacturers.

MH-F plates prepared at the EUCAST Laboratory for Antimicrobial Susceptibility Testing (Växjö, Sweden) between 2009 and 2012 using a total of 11 different batches of MH agar from four manufacturers (Oxoid, Bio-Rad, BBL and bioMérieux) and more than 150 batches of defibrinated horse blood have shown reproducible inhibition zones for *S. pneumoniae* ATCC 49619 and *H. influenzae* NCTC 8468 over time, with random variation within the quality control ranges similar to that for other organisms and agents on unsupplemented MH agar (Fig. 1).

Inoculum preparation, inoculation of plates and application of disks

The preparation and handling of inoculum suspensions, inoculated plates and antimicrobial disks affect the size of inhibition zone diameters in disk diffusion tests and therefore require careful standardization. EUCAST recommends that the inoculum suspension should optimally be used within 15 and always within 60 min of preparation to ensure the correct number of viable cells. Numbers of colony forming units in suspensions of overnight cultures of *E. coli* ATCC 25922, *S. pneumoniae* ATCC 49619 and *H. influenzae* NCTC 8468 adjusted to the density of a McFarland 0.5 turbidity standard were shown to be within the recommended range ($1-2 \times 10^8$ CFU/mL) when left at ambient temperature (20–22°C) for 15 and 60 min. However, the effect of leaving the inoculum suspension at ambient temperature for more than 15 min has not been investigated for all organisms and we strongly recommend that the inoculum suspension is used within 15 min. Disks should be placed on inoculated plates within 15 min and then placed in the correct incubation atmosphere within another 15 min. If inoculated plates are left at room temperature for longer periods of time before the disks are applied, the organisms may begin to grow prior to disk application, resulting in erroneous reduction in sizes of inhibition zones. When inoculated plates were left at room temperature for 2 h before application of disks, inhibition zone diameters for *E. coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 (with 9 and 11 antimicrobial agents, respectively, representing different classes of antimicrobial agents) were 1–3 mm smaller compared with when disks were applied immediately. Systematic accumulation of such deviations will significantly affect the results and can contribute to misinterpretation of susceptibility testing results. Furthermore, if the plates are left at room temperature for longer than 15 min after disks have been applied, pre-diffusion may result in erroneously large zones of inhibition.

Incubation of plates

Unless otherwise stated in the EUCAST disk diffusion test manual [12], plates are incubated at $35 \pm 1^\circ\text{C}$ for 16–20 h. MH plates are incubated in air and MH-F plates in 5% CO_2 . Repeated reading of inhibition zones of quality control strains after 16, 18 and 20 h incubation, respectively, yielded inhibition zone diameters that varied randomly within ± 1 mm for each antimicrobial agent tested (with 6–11 antimicrobial agents, depending on the strain, representing different classes of agents). However, prolonging incubation beyond 20 h resulted in growth of colonies within the inhibition zones for some organism-agent combinations. Incubation beyond 20 h is permitted only for species and/or antimicrobial agents for which a longer incubation has been validated (e.g. for

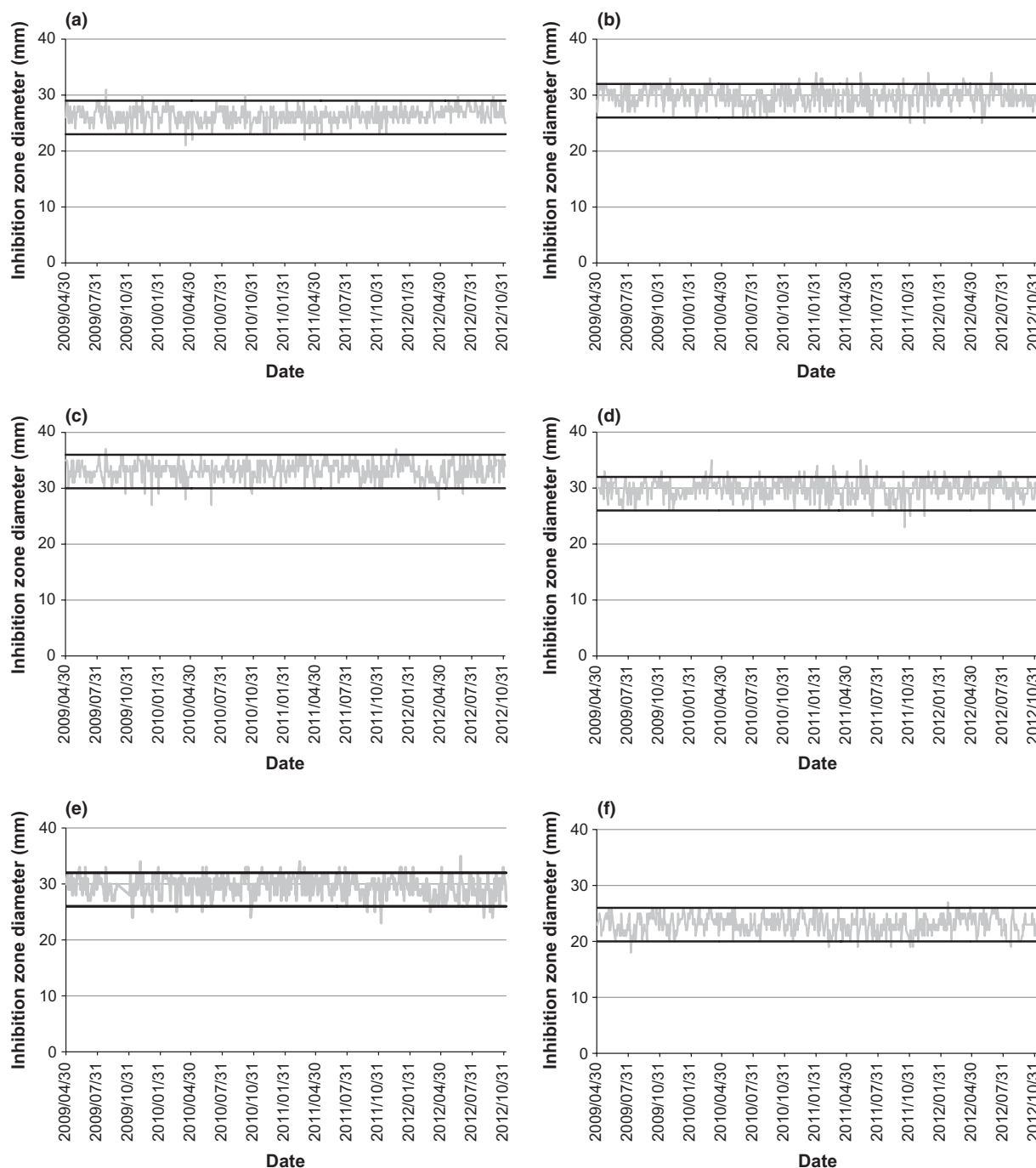


FIG. 1. Examples of reproducibility of inhibition zones over time for quality control strains. Strains were routinely tested on in-house prepared MH and MH-F plates (212 and 125 batches, respectively, including 11 different batches of MH agar from four manufacturers). For each strain-agent combination, the inhibition zone was read once daily. Altogether, 15 laboratory technicians were involved in setting up and reading tests. Thick black lines show EUCAST QC limits. (a) *S. aureus* ATCC 29213 with erythromycin 15 µg disk on MH agar ($n = 697$ with eight zone diameters (1.1%) out of range). (b) *S. pneumoniae* ATCC 49619 with erythromycin 15 µg disk on MH-F agar ($n = 707$ with 16 zone diameters (2.3%) out of range). (c) *S. aureus* ATCC 29213 with rifampicin 5 µg disk on MH agar ($n = 695$ with 12 zone diameters (1.7%) out of range). (d) *S. pneumoniae* ATCC 49619 with rifampicin 5 µg disk on MH-F agar ($n = 704$ with 29 zone diameters (4.1%) out of range). (e) *S. aureus* ATCC 29213 with trimethoprim-sulphamethoxazole 25 µg disk on MH agar ($n = 674$ with 37 zone diameters (5.5%) out of range). (f) *S. pneumoniae* ATCC 49619 with trimethoprim-sulphamethoxazole 25 µg disk on MH-F agar ($n = 705$ with seven zone diameters (1.0%) out of range).

Campylobacter jejuni and *coli* with all agents and for enterococci with glycopeptides).

Reading of zones

In any disk diffusion test, the reading of zones is the most difficult variable to standardize. Reading of zones includes measuring the zone diameter, inspecting the zone edge and the detection of colonies within the inhibition zone. For some organism-agent combinations (e.g. *S. aureus* and benzylpenicillin) a sharp zone edge indicates the presence, and for others (e.g. enterococci and vancomycin) the absence, of a resistance mechanism. Excluding contamination, the presence of growth within a zone may be due to resistance, but for some organism-agent combinations is typical for susceptible strains (e.g. *Stenotrophomonas maltophilia* with trimethoprim-sulphamethoxazole). In order to improve standardization of reading, EUCAST has published reading guidelines in the EUCAST disk diffusion test manual [12] and illustrative pictures in the EUCAST reading guide [13].

Accuracy and Reproducibility

Validation of accuracy using available quality control criteria

The technical aspects of the EUCAST [12] and the CLSI [10] disk diffusion methodologies are almost identical for non-fastidious organisms and EUCAST has checked and then adopted several of the quality control strains and the criteria recommended by CLSI. There is a small difference in the standard incubation time, 16–18 h in the CLSI method and 16–20 h in the EUCAST method, and for a few agents EUCAST recommends lower disk contents than CLSI (Table 2). In order to validate materials and testing conditions used for the EUCAST disk diffusion test, inhibition zones for CLSI quality control strains, *E. coli* ATCC

25922, *Pseudomonas aeruginosa* ATCC 27853 and *S. aureus* ATCC 25923, were generated using CLSI disk contents. Repeated testing was performed with a total of five different batches of MH agar from three manufacturers (two batches from Oxoid, two from BBL and one from bioMérieux) and disks from Oxoid. For each recommended CLSI zone diameter range, a median value was calculated (the 'CLSI target'). The measured mean inhibition zone diameters were compared with the 'CLSI targets'. All mean values were within ± 2 mm of the 'CLSI targets', with the majority being within ± 1 mm (Table 3).

As part of the validation of materials and testing conditions (as described above), tests to establish control ranges were performed for (i) *S. aureus* ATCC 29213 and *E. faecalis* ATCC 29212 on MH, (ii) *S. pneumoniae* ATCC 49619 and *H. influenzae* NCTC 8468 on MH-F, (iii) additional antimicrobial agents and (iv) agents with a different disk content to CLSI (EUCAST disk content). All other test characteristics were identical to those used for the CLSI control strains. From these tests, EUCAST quality control ranges were calculated as described in the section on establishment of EUCAST quality control ranges (see below). Batches of MH agar from additional manufacturers (Bio-Rad, MAST and Liofilchem) were subsequently tested when available. Disks from one or more additional manufacturer (BD, Bio-Rad, I2a, Liofilchem, MAST, Oxoid and Rosco) were tested if there was a difference of >1 mm between CLSI and EUCAST results.

Reproducibility

The reproducibility of the EUCAST disk diffusion test was investigated by analysis of data for quality control strains and data for clinical isolates tested between 2009 and 2012 at the Department of Clinical Microbiology, Växjö Central Hospital, Sweden, which was the first laboratory where the EUCAST methodology was implemented. A total of 212 and 125 in-house prepared batches of MH and MH-F agar plates, respectively, (including a total of 11 different batches of MH agar from four manufacturers) were used in routine antimicrobial susceptibility

TABLE 2. Differences in disk content between EUCAST and CLSI disk diffusion methods

Antimicrobial agent ^a	EUCAST disk content	CLSI disk content
Benzylpenicillin	1 unit	10 units
Ampicillin	2 and 10 μg ^b	10 μg
Amoxicillin-clavulanate	2–1 and 20–10 μg ^c	20–10 μg
Piperacillin	30 μg	100 μg
Piperacillin-tazobactam	30–6 μg	100–10 μg
Cefotaxime	5 μg	30 μg
Ceftaroline	5 μg	30 μg
Ceftazidime	10 μg	30 μg
Gentamicin (test for HLAR)	30 μg	120 μg
Vancomycin	5 μg	30 μg
Linezolid	10 μg	30 μg
Nitrofurantoin	100 μg	300 μg

HLAR, high-level aminoglycoside resistance.

^aCeftriaxone 30 μg and cefepime 30 μg are also under consideration for lower disk contents in the EUCAST disk diffusion test.

^b2 μg for *Haemophilus influenzae*, *Pasteurella multocida*, *Listeria monocytogenes*, *Staphylococcus saprophyticus* and streptococci.

^c2–1 μg for *Haemophilus influenzae*, *Moraxella catarrhalis* and *Pasteurella multocida*.

TABLE 3. Comparison of EUCAST mean zone diameters^a and 'target values'^b for CLSI recommended quality control strains

	Number of antimicrobial agents/total number of agents tested		
	<i>E. coli</i> ATCC 25922	<i>P. aeruginosa</i> ATCC 27853	<i>S. aureus</i> ATCC 25923
≤ 2 mm from 'CLSI target'	29/29	15/15	31/31
≤ 1 mm from 'CLSI target'	24/29	10/15	30/31
=CLSI target	8/29	3/15	9/31

^aEUCAST mean values were each calculated from a total of ≥ 20 separate tests on MH agar from Oxoid (two batches), BBL (two batches) and bioMérieux (one batch).

^bMedian values of published CLSI control zone diameter ranges.

tests during this time period, and inhibition zones were read by a total of 15 different technicians. Several lots of antimicrobial disks (≥ 5 per agent) were used during this time period. Of the investigated 48 combinations of quality control strains and antimicrobial agents, most showed excellent reproducibility with random variation within the control range and very few zone diameters out of range (e. g. Fig. 1). For control ranges established by EUCAST, only four combinations had $>5\%$ readings out of range, *S. aureus* ATCC 29213 with trimethoprim-sulphamethoxazole (5.5%), *S. pneumoniae* ATCC 49619 with tetracycline (5.2%) and *H. influenzae* NCTC 8468 with cefotaxime (6.7%) and trimethoprim-sulphamethoxazole (8.8%). Of these, *H. influenzae* with trimethoprim-sulphamethoxazole has been shown to be particularly sensitive to variation in media (manufacturer and batch), presumably due to varying content of thymidine. However, testing of clinical isolates ($n = 147$) on MH-F agar prepared with MH from Oxoid and BBL resulted in similar susceptibility categorization with only a few minor errors for both media. For clinical isolates, annual zone diameter distributions for 2009–2012 were very similar for all organism-antimicrobial agent combinations tested, with medians within ± 1 mm (e. g. Fig. 2).

Quality Control Criteria

Following testing of control strains as described above, EUCAST has, when possible, adopted the CLSI criteria for quality control strains for disk diffusion tests [11]. In cases where EUCAST recommendations for strains, medium or disk contents are different from those of CLSI, EUCAST has developed separate criteria. EUCAST quality control tables list both acceptable ranges and target values (Table 4). The targets are based on the median value of the CLSI range and have been checked for accuracy during the development of the EUCAST methodology as described above. A few discrepancies between the 'CLSI targets' and the EUCAST results have been identified (e.g. *E. coli* ATCC 25922 with meropenem 10 μg disks and *P. aeruginosa* ATCC 27853 with gentamicin 10 μg and tobramycin 10 μg disks). When discrepancies have been confirmed in tests in multiple laboratories, some of these have been revised in collaboration with the CLSI (e. g. *P. aeruginosa* with gentamicin 10 μg and tobramycin 10 μg disks) [11].

Establishment of EUCAST quality control ranges

When there were no CLSI quality control criteria, targets and ranges were developed by EUCAST. The targets were initially based on the mean values of 20 separate tests for each strain-agent combination, using a total of five different batches of MH agar from three manufacturers (two batches from

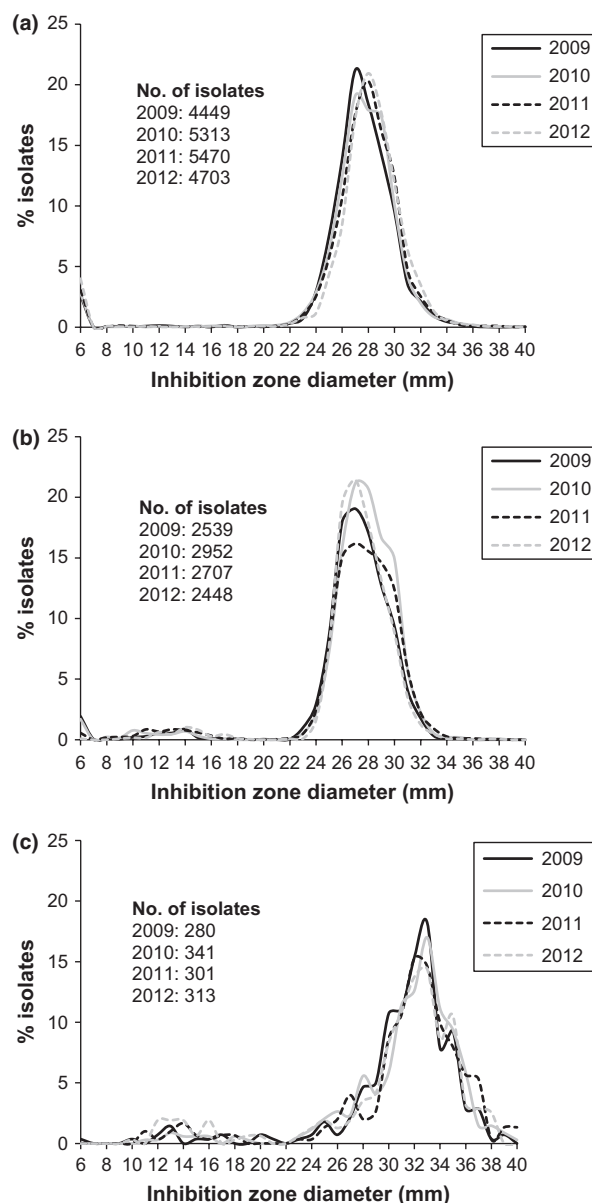


FIG. 2. Reproducibility of the EUCAST disk diffusion method as illustrated by three comparisons of annual zone diameter distributions. For each organism-antimicrobial agent combination, the distributions are highly reproducible with similar medians and ranges. (a) *E. coli* vs. cefotaxime 5 μg . (b) *S. aureus* vs. cefoxitin 30 μg . (c) *S. pneumoniae* vs. tetracycline 30 μg .

Oxoid, two from BBL and one from bioMérieux) and disks from Oxoid. All testing was performed in parallel with testing of the CLSI control strains as described above. EUCAST control ranges were based on mean values ± 2 SD, with a minimum range of ± 3 mm. All targets and ranges were re-evaluated with additional media and disk batches from a range of manufacturers. Control criteria were revised when there were data to support a change, and since version 1.0 of

the EUCAST QC tables, 11 zone diameter ranges have been slightly modified.

Establishment of Zone Diameter Breakpoints Calibrated to the EUCAST Clinical MIC Breakpoints

For most antimicrobial agents, there is good correlation between MIC values and inhibition zone diameters [8,16]. This is especially true when species-specific correlations are calculated. The establishment of EUCAST clinical MIC breakpoints is described elsewhere [17–19] and techniques for establishing zone diameter breakpoint correlates have recently been reviewed by Kronvall *et al.* [20]. We utilize several of these techniques when establishing species-specific (e.g. for *S. pneumoniae* and *H. influenzae*) or group-specific (e.g. for Enterobacteriaceae and staphylococci) zone diameter breakpoints calibrated to the EUCAST clinical MIC breakpoints. MIC-zone diameter correlates and MIC and zone diameter distributions were analysed. Large series of MIC determinations and parallel disk diffusion tests (100–1000 per organism-antimicrobial agent combination) were performed at the EUCAST Laboratory of Antimicrobial Susceptibility Testing in collaboration with several other laboratories. In addition, we have had access to MIC-zone diameter distributions produced with CLSI methodology (courtesy of Dr R.N. Jones at JMI Laboratories, North Liberty, Iowa, USA).

Determination of epidemiological cut-off (ECOFF) values from wild-type MIC and zone diameter distributions

The use of wild-type MIC distributions to distinguish wild-type isolates from those with acquired resistance mechanisms and to establish ECOFFs has been described by Kahlmeter *et al.* [7]. The MIC distributions in the EUCAST database are based on more than 50 000 isolates for some antimicrobial agent-organism combinations. These MIC distributions are composed of collated data from a large number of investigators and laboratories. The zone diameter distributions, all from tests performed with the EUCAST disk method as described in this paper, were originally all from the EUCAST Laboratory for Antimicrobial Susceptibility Testing, but data from other laboratories using the EUCAST disk diffusion test have been added subsequently. By analysis of MIC and zone diameter distributions, we have defined wild-type distributions from which MIC and zone diameter epidemiological cut-off values (ECOFFs) were established (e.g. Fig. 3b) [21].

Calibrating inhibition zone diameters to MIC values and/or resistance mechanisms

The zone diameter breakpoints were established by performing simultaneous MIC determination and disk diffusion tests on 10–1000 isolates per species, particularly isolates with MIC values close to EUCAST breakpoints or inhibition zone diameters close to the low end of the wild-type population. When establishing EUCAST zone diameter breakpoints, MIC determination was performed by both reference methodology (i.e. broth microdilution according to the International Standards Organisation

TABLE 4. Excerpt from the EUCAST routine quality control tables version 3.0

Escherichia coli ATCC 25922

(NCTC 12241, CIP 76.24, DSM 1103, CCUG 17620, CECT 434)

Mueller-Hinton agar, McFarland 0.5, air, 35±1°C, 18±2h. Read zone edges as the point showing no growth viewed from the back of the plate against a dark background illuminated with reflected light.

Antimicrobial agent	MIC (mg/L)		Disk content (µg)	Inhibition zone diameter (mm)	
	Target ^a	Range ^b		Target ^a	Range ^c
Amikacin	1–2	0.5–4	30	23	19–26
Cefadroxil	–	–	30	17	14–20
Cefotaxime	0.06	0.03–0.12	5	28	25–31
Cefoxitin	4	2–8	30	26	23–29
Ceftriaxone	0.06	0.03–0.12	30	32	29–35
Cefuroxime	4	2–8	30	23	20–26
Ciprofloxacin	0.008	0.004–0.015	5	35	30–40
Gentamicin	0.5	0.25–1	10	23	19–26
Meropenem	0.015–0.03	0.008–0.06	10	31	28–34
Piperacillin-tazobactam	2/4	1/4–4/4	30–6	24	21–27
Tobramycin	0.5	0.25–1	10	22	18–26
Trimethoprim	1	0.5–2	5	25	21–28
Trimethoprim-sulphamethoxazole	≤0.5/9.5 ^b	–	1.25–23.75	26	23–29

^aCalculated by EUCAST.

^bFrom the International Standards Organisation, ISO 20776-1: 2006, except ranges in bold/italics established by EUCAST.

^cFrom the Clinical and Laboratory Standards Institute, M100-S22: 32:3, 2012, except ranges in bold/italics established by EUCAST. All ranges have been validated by EUCAST.

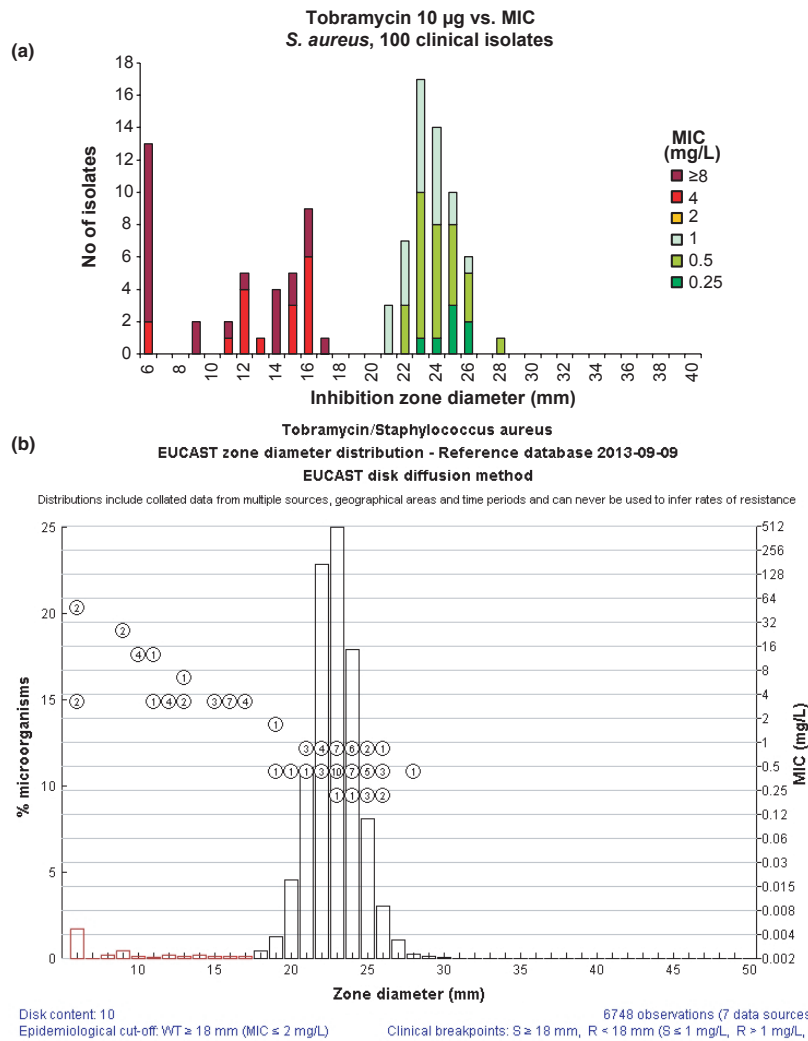


FIG. 3. Examples of MIC-zone diameter correlations for *S. aureus* vs. tobramycin 10 µg as used by EUCAST to determine and validate zone diameter breakpoints. (a) Inhibition zone diameter distribution with corresponding MIC values represented as different coloured bars, as presented on the EUCAST website http://www.eucast.org/eucast_disk_diffusion_test/calibration_and_validation/. (b) Inhibition zone diameter distribution (aggregated clinical data from several test sites) and MIC correlates from Figure 3(a) and additional data from multiple sources, as presented in the EUCAST MIC and zone diameter distribution database <http://mic.eucast.org/Eucast2/>.

(ISO) standard [22]) and other methodologies calibrated to the ISO method (e.g. broth microdilution with media supplemented for fastidious organisms [23,24] and gradient MIC tests). When possible, isolates with known resistance mechanisms (e.g. ESBL-producing Enterobacteriaceae, *Staphylococcus* spp. positive for *mecA* and *Enterococcus* spp. positive for *vanA* and *vanB*) have been used to test the breakpoints. Correlations of MICs and zone diameters for a wide range of organisms and antimicrobial agents are presented on the EUCAST website in two formats. Firstly, as zone diameter bar charts in which bars are subdivided with each MIC value plotted in a colour representing a specific MIC value [16] (e.g. Fig. 3a) and, secondly, as MIC-zone diameter distributions on the zone diameter distribution website [25] (e.g. Fig. 3b). Several MIC-zone diameter distributions are the result

of collaborations with other laboratories, both within and outside Europe. An important principle in the setting of clinical breakpoints by EUCAST is, when possible, to avoid setting breakpoints that divide wild-type distributions of target species [7]. The same principle was applied to the zone diameter breakpoints by checking breakpoints indicated by the MIC-zone diameter correlations against zone diameter distributions for clinical isolates [25].

EUCAST Breakpoint Tables

The EUCAST clinical breakpoint tables v 1.0, with tentative zone diameter breakpoints, were published on the EUCAST website

in December 2009. The breakpoint tables are revised yearly and are published for consultation on the website early in December each year. The finalized revised version is published on 1 January each year and contains revisions of existing breakpoints, breakpoints for added species or new antimicrobial agents. Should there be an urgent need for changing or adding breakpoints during a year, these are published in an addendum. EUCAST clinical breakpoint tables present clinical MIC breakpoints expressed as $S \leq X$ mg/L, $R > X$ mg/L and zone diameter breakpoints expressed as $S \geq X$ mm, $R < X$ mm. The intermediate category is not spelled out and is inferred from the susceptible and resistant breakpoints. Relevant background data on antimicrobial agents and breakpoints, as well as relevant MIC and zone diameter distributions, can be accessed via links in the table on the EUCAST website, where antimicrobial agent names are linked to EUCAST rationale documents, and MIC and zone diameter breakpoints are linked to MIC and zone diameter distributions, respectively.

Implementation of the EUCAST Disk Diffusion Test in Routine Clinical Microbiology Laboratories

Changing from another method to the EUCAST standardized disk diffusion test is not difficult, but needs planning. In order to facilitate this process, EUCAST has published an implementation guide on the EUCAST website [26]. A few important aspects are highlighted and discussed below.

How to select and prepare Mueller–Hinton medium

MH agar may vary between manufacturers and between batches from the same manufacturer and each batch of MH agar used for disk diffusion testing should be tested to ensure that inhibition zone diameters for antimicrobial agents are within EUCAST quality control limits for agents that are used routinely in the laboratory. Laboratories making their own media are encouraged to obtain test samples and to ensure that the new batch meets the quality control criteria prior to buying a large quantity of a batch. By extensively testing a new batch and then purchasing large quantities, the laboratory can ensure long periods of consistent quality. Each new batch of MH agar, disks or supplements should be tested to ensure that inhibition zones for all antimicrobial agents that are part of routine panels are within the acceptable ranges defined in the EUCAST quality control tables.

Agar depth and supplements for fastidious organisms must be consistently as defined for the method. The agar depth should be 4.0 ± 0.5 mm and systematic use of plates that are close to the limits, particularly the lower limit, is more likely to

result in errors due to variation in medium depth. It is crucial that the horse blood is mechanically, not chemically, defibrinated and it must not be lysed. Chemical defibrination will result in inhibition zone diameters out of range for some organism-antimicrobial agent combinations. For β -NAD, a purity of $\geq 98\%$ should be used to ensure adequate growth of *H. influenzae* and to minimize batch-to-batch variation. Several manufacturers provide β -NAD of required purity.

Preparation and incubation of plates

Inoculation of agar plates with the standardized organism suspension can be performed either by hand or by using a plate rotator. Safety regulations prohibit the use of flooding, which may result in splashing and/or production of aerosols of concentrated bacteria. Regardless of whether plates are inoculated by hand or by use of a rotating device, it is important to achieve an even confluent growth for all organisms tested. For Gram-negative organisms, it is particularly important to avoid over-inoculation. This is best achieved by removing excess fluid from the swab before streaking the plates. The growth should be even over the agar surface and jagged zone edges indicate uneven inoculation. Furthermore, it is important not to exceed the incubation period of 16–20 h because prolonged incubation often results in indistinct zone edges or colonies within the inhibition zones, which might result in reporting isolates as falsely resistant. When testing *H. influenzae*, it is also important to remove excess moisture before inoculation of plates. If condensation can be seen on the inside of the lid, fuzzy zone edges and/or hazes within zones are more likely to be seen with some isolates.

Reading of inhibition zones

When implementing the EUCAST disk diffusion test, training in the reading of zones is essential to ensure consistent reading between technologists. This is best achieved by introducing regular exercises where all laboratory staff read inhibition zones from the same plate with EUCAST quality control strains. The mean and variation of all readings can be compared with the target values and the ranges published in the quality control tables [15]. From experiments performed with staff in routine laboratories we know that standardized reading to within ± 1 mm can be achieved among 15–20 technologists. The quality of reading can furthermore be assessed by comparing the ranges and medians of in-house zone diameter distributions ($n \geq 50$) for routine clinical isolates with those published by EUCAST [25]. For wild-type isolates, the median and width of the in-house distribution should match that of the EUCAST distribution. Wild-type distributions with a median that deviates more than 2 mm from the EUCAST target clearly indicate a systematic difference between the in-house and the recom-

mended method. Wild-type distributions that are wider than the reference distribution are normally the result of variation in reading of zones among staff. This is also likely to be the reason for irregular wild-type distributions. The EUCAST reading guide [13] contains specific instructions and illustrative pictures and is recommended for use during implementation of the EUCAST disk diffusion test and also for continued education of staff.

Routine quality control

Several factors affect inhibition zone sizes, including the medium, preparation of agar plates, inoculum density, age of colonies, quality of disks, incubation temperature, incubation time and reading of zone diameters [1,8,9]. Variations in zone diameters can be due to either in-house factors, such as inadequate training, inconsistency in reading results or inadequate control of equipment or reagents, or to external factors such as variation between batches of media or disks. Variation caused by a change in medium or other reagents should have been detected by testing before these were accepted for routine use. Strictly controlling the in-house variation provides confidence when diagnosing and dealing with external reasons for variation. The EUCAST Laboratory for Antimicrobial Susceptibility Testing in Växjö may be contacted for assistance in investigation of the cause of unexplained variation.

Repeat testing of EUCAST control strains as part of consecutive daily or weekly quality control can be expected to yield individual values randomly distributed within the recommended range and with a mean within ± 1 mm of the EUCAST target if the number of tests is ≥ 10 . A systematic deviation of the mean value, above or below the target for several agents, should be investigated. Guidelines for investigation of quality control results out of range or changes in trends over time are available in the EUCAST disk diffusion test manual [12].

The ranges for the quality control strains are set to accommodate some variation between media and disks and repeated testing of quality control strains is sometimes not sufficient to detect specific problems [27], which may be detected by comparison of results with reference distributions. Comparison of the median and range of zone diameter distributions from a laboratory with the reference distributions available on the EUCAST website [25] can be more effective than testing of quality control strains for detection of systematic errors, or problems with disks or media, as well as problems with specific organism-antimicrobial agent combinations. Examples are shown in Fig. 4, where zone diameter distributions from 17 Swedish laboratories are compared with the EUCAST reference distribution. The highlighted distribution is from a laboratory with excellent reproducibility but with a systematic deviation from the reference distribution. Systematic differences in median values compared with the

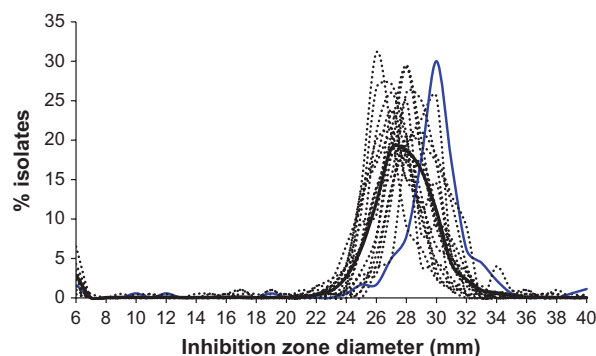


FIG. 4. Inhibition zone diameter distributions for *E. coli* with cefotaxime 5 μg based on consecutive clinical isolates from 17 Swedish laboratories using the EUCAST disk diffusion method. The EUCAST reference zone diameter distribution is shown as a thick black line. Systematic deviations can be detected by comparing the median and range of each graph with the reference distribution. One deviating laboratory is highlighted in blue.

reference distributions will result in erroneous susceptibility categorization, especially for organism-antimicrobial agent combinations where there are resistant isolates with zone diameters close to the susceptible zone diameter breakpoint.

The Future of the EUCAST Disk Diffusion Test

Since 2011, more and more countries, mainly in Europe, have adopted the EUCAST clinical breakpoints and the EUCAST disk diffusion test. EUCAST encourages laboratories with expertise in susceptibility testing to participate in a network of collaborating laboratories interested in contributing to the development and maintenance of the disk diffusion test. With this network, the financial support of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) and the support and interest of National Antimicrobial Susceptibility Testing Committees (NACs), the future of the EUCAST disk diffusion method is secured. Automated susceptibility testing may relieve laboratories of some AST work, but their lack of versatility, the unavailability of some agents and tests for some species, and their long development times, still favour the use of disk diffusion testing for many years to come. All documents and data on the EUCAST website are freely available.

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Transparency Declaration

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References

1. Woods GL. In vitro testing of antimicrobial agents. *Infect Dis Clin North Am* 1995; 9: 463–481.
2. Jones RN. Recent trends in the college of American pathologists proficiency results for antimicrobial susceptibility testing: preparing for CLIA '88. *Clin Microbiol Newsl* 1992; 14: 33–37.
3. Andrews J. BSAC standardized disc susceptibility testing method (version 7). *J Antimicrob Chemother* 2008; 62: 256–278 (updates on <http://www.bsac.org.uk>).
4. Comité de l'Antibiogramme de la Société Française de Microbiologie. Technical recommendations for in vitro susceptibility testing. *Clin Microbiol Infect* 1996; 2 (suppl 1): S11–S25 (updates on <http://www.sfm.asso.fr>).
5. Deutsches Institut für Normung. *Medical microbiology -susceptibility testing of pathogens to antimicrobial agents*. Berlin, Germany: DIN, 2000; 58940–58944.
6. The Swedish Reference Group of Antibiotics. Antimicrobial susceptibility testing in Sweden. *Scand J Infect Dis* 1997; 105: 5–31.
7. Kahlmeter G, Brown DFJ, Goldstein FW et al. European harmonization of MIC breakpoints for antimicrobial susceptibility testing. *J Antimicrob Chemother* 2003; 52: 145–148.
8. Ericsson HM, Sherris JC. Antibiotic sensitivity testing. Report of an international collaborative study. *Acta Pathol Microbiol Scand B Microbiol Immunol* 1971; 217 (suppl): 1.
9. Bauer AW, Kirby WM, Sherris JC, Turck M. Antibiotic susceptibility testing by a standardized single disk method. *Am J Clin Pathol* 1966; 45: 493–496.
10. Clinical and Laboratory Standards Institute. *Performance standards for antimicrobial disk susceptibility tests; approved standard-eleventh edition*. CLSI document: M02-A11. Wayne PA: Clinical and Laboratory Standards Institute, 2012.
11. Clinical and Laboratory Standards Institute. *Performance standards for antimicrobial susceptibility testing; twenty-third information supplement. CLSI document M100-S23*. Wayne PA: Clinical and Laboratory Standards Institute, 2013.
12. The European Committee on Antimicrobial Susceptibility Testing. *EUCAST Disk Diffusion Test Manual*. v 3.0, 2013. Available at: <http://www.eucast.org> (last accessed August 16, 2013).
13. The European Committee on Antimicrobial Susceptibility Testing. *Reading guide. EUCAST disk diffusion method for antimicrobial susceptibility testing*. v 3.0, 2013. Available at: <http://www.eucast.org> (last accessed August 16, 2013).
14. The European Committee on Antimicrobial Susceptibility Testing. *Breakpoint tables for interpretation of MICs and zone diameters*. Version 3.1, 2013. Available at: <http://www.eucast.org> (last accessed August 16, 2013).
15. The European Committee on Antimicrobial Susceptibility Testing. *Routine internal quality control as recommended by EUCAST*. v 3.1, 2013. Available at: <http://www.eucast.org> (last accessed August 16, 2013).
16. The European Committee on Antimicrobial Susceptibility Testing. *Antimicrobial susceptibility testing. calibration and validation*. Available at: <http://www.eucast.org> (last accessed August 16, 2013).
17. The European Committee on Antimicrobial Susceptibility Testing. *Setting breakpoints for new antimicrobial agents*. EUCAST SOP 1.0, 2010. Available at: <http://www.eucast.org> (last accessed August 16, 2013).
18. The European Committee on Antimicrobial Susceptibility Testing. *Setting breakpoints for existing antimicrobial agents*. EUCAST SOP 2.0, 2010. Available at: <http://www.eucast.org> (last accessed August 16, 2013).
19. The European Committee on Antimicrobial Susceptibility Testing. *Review and revision of antimicrobial breakpoints*. EUCAST SOP 3.0, 2013. Available at: <http://www.eucast.org> (last accessed August 16, 2013).
20. Kronvall G, Giske CG, Kahlmeter G. Setting interpretive breakpoints for antimicrobial susceptibility testing using disk diffusion. *Int J Antimicrob Agents* 2011; 38: 281–290.
21. Kronvall G, Kahlmeter G, Myhre E, Galas MF. A new method for normalized interpretation of antimicrobial resistance from disk test results for comparative purposes. *Clin Microbiol Infect* 2003; 9: 120–132.
22. International Standards Organisation. *Reference method for testing the in vitro activity of antimicrobial agents against rapidly growing aerobic bacteria involved in infectious diseases*. Geneva, Switzerland: ISO, 1996; 20776–1.
23. The European Committee on Antimicrobial Susceptibility Testing. *Media preparation for EUCAST disk diffusion testing and for determination of MIC values by the broth microdilution method*. v 3.0, 2013. Available at: <http://www.eucast.org> (last accessed August 16, 2013).
24. Clinical and Laboratory Standards Institute. *Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard -ninth edition*. CLSI document: M07-A9. Wayne, PA: Clinical and Laboratory Standards Institute, 2012.
25. The European Committee on Antimicrobial Susceptibility Testing. *MIC and zone diameter distributions*. Available at: <http://www.eucast.org> (last accessed August 16, 2013).
26. The European Committee on Antimicrobial Susceptibility Testing. *Check list to facilitate implementation of antimicrobial susceptibility testing with EUCAST breakpoints*. Available at: <http://www.eucast.org> (last accessed August 16, 2013).
27. Yechouron A, Dascal A, Stevenson J, Mendelson J. Ability of National Committee for Clinical Laboratory Standards-recommended quality control strains from the American Type Culture Collection to detect errors in disk diffusion susceptibility tests. *J Clin Microbiol* 1991; 29: 2758–2762.