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The critical influence of the intermediate category on interpretation errors in revised EUCAST and CLSI antimicrobial susceptibility testing guidelines

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Abstract

Erroneous assignments of clinical isolates to the interpretative categories susceptible, intermediate and resistant can deprive a patient of successful antimicrobial therapy. The rate of major errors (ME) and very major errors (vME) is dependent on: (i) the precision/standard deviation (σ) of the antibiotic susceptibility testing (AST) method, (ii) the diameter distributions, (iii) clinical breakpoints, and (iv) the width of the intermediate zone. The European Committee on AST (EUCAST) has abandoned or decreased the intermediate zone for several drug/species combinations. This study focused on the effects of discontinuing the intermediate category on the rate of interpretation errors. In total, 10 341 non-duplicate clinical isolates were included in the study. For susceptibility testing the disc diffusion method was used. Error probabilities were calculated separately for diameter values flanking the interpretative category borders. Error probabilities were then applied to the actual numbers of clinical isolates investigated and expected rates of ME and vME were calculated. Applying EUCAST AST guidelines, significant rates of ME/vME were demonstrated for all drug/species combinations without an intermediate range. Virtually all ME/vME expected were eliminated in CLSI guidelines that retained an intermediate zone. If wild-type and resistant isolates are not clearly separated in susceptibility distributions, the retaining of an intermediate zone will decrease the number of ME and vME. An intermediate zone of 2–3 mm avoids almost all ME/vME for most species/drug combinations depending on diameter distributions. Laboratories should know their epidemiology settings to be able to detect problems of individual species/drug/clinical breakpoint combinations and take measures to improve precision of diameter measurements.

Keywords: CLSI, epidemiological cut off, EUCAST, major errors, very major errors
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Introduction

The disc diffusion method was described by Bauer and Kirby in the 1950s/1960s [1]. After more than 50 years, disc diffusion is still a widely used procedure for antimicrobial susceptibility testing (AST) in the clinical microbiological laboratory. National and international guidelines refer to standardized Kirby–Bauer testing, e.g. CLSI or European Committee on AST (EUCAST) guidelines for AST [2,3]. Traditionally, the basis of clinical AST reporting is categorization into susceptible (S), intermediate (I), and resistant (R)—referring to the likelihood of clinical success [4]. The susceptible category is defined as likely therapeutic success for the individual species/drug combination tested if recommended standard dosing is applied [2,5]. The intermediate category is defined as uncertain therapeutic success for the individual species/drug combination tested by EUCAST and is intended for compounds for which dosing can be increased. CLSI defines the intermediate category as a lower response rate than for susceptible isolates, but clinical efficacy if the drug accumulates at the site of infection. The intermediate category represents the 'grey zone' regarding therapeutic success; it also

helps to prevent serious categorization errors resulting from imprecision of inhibition zone readings [4,6]. The resistant category implies a high likelihood of therapeutic failure (EUCAST) or no reliable clinical efficacy (CLSI).

Differences in susceptibility categorization of individual isolates in repeated or in parallel ASTs can be referred to as 'discrepancies'. This statistical category is traditionally split for AST interpretation according to the level of therapeutic implications resulting from false categorization: Discrepancies resulting in erratic assignment of bacterial isolates to adjacent interpretative categories (S to I, I to S, I to R, R to I) are referred to as 'minor errors' (mE) resulting in limited therapeutic consequences [4,6,7]. Erroneous categorization of true-susceptible isolates as resistant are referred to as 'major errors' (ME) leading to unnecessary restriction of therapeutic options that can deprive a patient of a successful therapy. The most serious clinical implications result from 'very major errors' (vME), i.e. categorization of true-resistant isolates as susceptible—as a consequence there is a high likelihood of therapeutic failure. Clinical isolates are often (i) repeatedly tested for antimicrobial susceptibility to monitor clinical course, therapeutic success, and the emergence of resistance and (ii) grown and tested for antimicrobial susceptibility from parallel samples of the same patient, bearing the risk of discrepant and confusing AST reports.

The rates of ME and vME are dependent on: (i) the accuracy of the AST method reflected by the standard deviation (σ) of measurements, and (ii) on the width of the intermediate zone. If the intermediate zone is abandoned, the resistant and susceptible categories become directly adjacent and all minor errors will, by consequence, become ME and vME. EUCAST guidelines have abandoned or decreased the intermediate zone for several drug/species combinations (see Table 1). According to EUCAST the intermediate category has been kept for drugs that can be administered in high dose, but it has been abandoned for compounds with only one approved dosing regimen. However, there are several exceptions, e.g. for ciprofloxacin, ceftazidime, or the aminoglycosides, variable dosing regimens are established but no intermediate category is assigned. For other compounds, e.g. amoxicillin-clavulanic acid, rationale documents for EUCAST AST categories are currently not yet available [8,9]. As a result EUCAST handling of the intermediate zone seems, in part, inconsistent and incomprehensive from the outside. National systems have adopted EUCAST clinical breakpoints (CBPs) and, in part, abandoned or decreased the intermediate zones (British Society for Antimicrobial Chemotherapy) [10]. In contrast, other organizations have kept intermediate zones for all drugs (CLSI) or retained the intermediate zone for more agents than EUCAST (e.g. Comité de l'Antibiogramme de la Société Française de Microbiologie) [2,11].

The aim of this study was to calculate the expected rates of ME and vME with the revised EUCAST and CLSI AST guidelines for disc diffusion testing using a set of zone diameter data generated with isolates of clinically important gram-negative and gram-positive bacteria. The focus of this study was on the effect of either discontinuing or downsizing the intermediate category. Analyses were carried out for (i) a statistically normalized setting (independent of an individual epidemiological situation) and (ii) the actual epidemiological situation of a clinical laboratory.

Materials and Methods

Bacterial strains

In total, 10 341 non-duplicate clinical isolates recovered in our diagnostic laboratory during a period of 24 months from January 2010 to December 2011 were included in the study, comprising 2992 *Escherichia coli*, 317 *Stenotrophomonas maltophilia*, 1610 *Pseudomonas aeruginosa*, 191 *Acinetobacter baumannii*, 3001 *Staphylococcus aureus* and 2230 coagulase-negative staphylococci. For some of the isolates not all zone diameter values were available resulting in lower numbers of data points for certain drug/species combinations (see Table 2).

Susceptibility testing

For susceptibility testing the disc diffusion method according to Kirby–Bauer was used. Antibiotic discs were obtained from Becton Dickinson (Franklin Lakes, NJ, USA). Susceptibility testing was done on Mueller–Hinton agar (Becton Dickinson) using McFarland 0.5 from overnight cultures followed by incubation at 35°C for 16–18 h according to EUCAST recommendations [3]. Inhibition zone diameters were determined using the semi-automated Sirweb/Sirscan system (i2a, Montpellier, France).

Statistical analysis

Error probabilities were calculated separately for all diameter values flanking the interpretative category borders (S vs I, R vs I, S vs R) covering a diameter range that comprised $\geq$95% of all ME/vME expected. An example is shown in Fig. 1.

According to Altman *et al.* [12] normal distribution is a bell-shaped, unimodal and symmetrical probability distribution. It is described by two parameters, the mean (μ) and the standard deviation (σ). The total area under the Gaussian density is equal to 1. As a result of the symmetry, the probability for an observation to be below the mean (μ) is equal to the tail area below the density to the left of the mean of the normal distribution and is equal to 0.5. By similar argument the tail area of the normal density to the right of the mean is equal to

TABLE 1. Clinical breakpoints and normalised error probabilities for European Committee for Antibiotic Susceptibility Testing (EUCAST) and CLSI AST guidelines

	Clinical breakpoints						Width of intermediate range (mm)		Cumulated statistical probability (%) for ME/vME					
	CLSI			EUCAST					σ = 1 mm		σ = 2 mm		σ = 3 mm	
Drug/species/group	S	I	R	S	I	R	CLSI	EUCAST	CLSI	EUCAST	CLSI	EUCAST	CLSI	EUCAST
Enterobacteriaceae														
Ampicillin	≥17	14–16	≤13	≥14		<14	3	0	0.0015	**9.1**	1.5	**13.9**	8.6	**15.7**
Amoxicillin-clavulanic acid	≥18	14–17	≤13	≥17		<17	4	0	<0.0001	**9.1**	2.9	**13.9**	4.2	**15.7**
Piperacillin-tazobactam	≥21	18–20	≤17	≥20	17–19	<17	3	3	0.0015	0.0015	1.5	1.5	8.6	8.6
Cefuroxime	≥18	15–17	≤14	≥18		<18	3	0	0.0015	**9.1**	1.5	**13.9**	8.6	**15.7**
Cefoxitin	≥18	15–17	≤14	≥19		<19	3	0	0.0015	**9.1**	1.5	**13.9**	8.6	**15.7**
Cefpodoxime	≥21	18–20	≤17	≥21		<21	3	0	0.0015	**9.1**	1.5	**13.9**	8.6	**15.7**
Ceftriaxone	≥23	20–22	≤19	≥23	20–22	<20	3	3	0.0015	0.0015	1.5	1.5	8.6	8.6
Ceftazidime	≥21	18–20	≤17	≥22	19–21	<19	3	3	0.0015	0.0015	1.5	1.5	8.6	8.6
Cefotaxime	≥26	23–25	≤22	≥20	17–19	<17	3	3	0.0015	0.0015	1.5	1.5	8.6	8.6
Cefepime	≥18	15–17	≤14	≥24	21–23	<21	3	3	0.0015	0.0015	1.5	1.5	8.6	8.6
Meropenem	≥23	20–22	≤19	≥22	16–21	<16	3	6	0.0015	<0.0001	1.5	0.01	8.6	0.8
Imipenem	≥23	20–22	≤19	≥21	15–20	<15	3	6	0.0015	<0.0001	1.5	0.01	8.6	0.8
Ertapenem	≥23	20–22	≤19	≥25	20–24	<20	3	5	0.0015	<0.0001	1.5	0.1	8.6	1.9
Tobramycin	≥15	13–14	≤12	≥16	14–15	<14	2	2	0.07	0.07	4.9	4.9	16.0	16.0
Amikacin	≥17	15–16	≤14	≥16	14–15	<14	2	2	0.07	0.07	4.9	4.9	16.0	16.0
Gentamicin	≥15	13–14	≤12	≥17	15–16	<15	2	2	0.07	0.07	4.9	4.9	16.0	16.0
Ciprofloxacin	≥21	16–20	≤15	≥22	19–21	<19	5	3	<0.0001	0.0015	0.1	1.5	1.9	8.6
Levofloxacin	≥17	14–16	≤13	≥22	19–21	<19	3	3	0.0015	0.0015	1.5	1.5	8.6	8.6
Norfloxacin	≥17	13–16	≤12	≥22	19–21	<19	4	3	<0.0001	0.0015	2.9	1.5	4.2	8.6
Trimethoprim-Sulfamethoxazole	≥16	11–15	≤10	≥16	13–15	<13	5	3	<0.0001	0.0015	0.1	1.5	1.9	8.6
Stenotrophomonas maltophilia														
Trimethoprim-sulfamethoxazolexazole	≥16	11–15	≤10	≥16		<16	5	0	<0.0001	**9.1**	0.1	**13.9**	1.9	**15.7**
Pseudomonas aeruginosa														
Piperacillin-tazobactam	≥21	15–20	≤14	≥19		<19	6	0	<0.0001	**9.1**		**13.9**		**15.7**
Ceftazidime	≥18	15–17	≤14	≥16		<16	3	0	0.0015	**9.1**	1.5	**13.9**	8.6	**15.7**
Cefepime	≥18	15–17	≤14	≥18		<18	3	0	0.0015	**9.1**	1.5	**13.9**	8.6	**15.7**
Imipenem	≥16	14–15	≤13	≥20	18–19	<18	2	2	0.07	0.07	4.9	4.9	16.0	16.0
Meropenem	≥16	14–15	≤13	≥24	18–23	<18	2	6	0.07	<0.0001	4.9	0.01	16.0	0.8
Tobramycin	≥15	13–14	≤12	≥16		<16	2	0	0.07	**9.1**	4.9	**13.9**	16.0	**15.7**
Amikacin	≥17	15–16	≤14	≥18	15–17	<15	2	3	0.07	0.0015	4.9	1.5	16.0	8.6
Gentamicin	≥15	13–14	≤12	≥15		<15	2	0	0.07	**9.1**	4.9	**13.9**	16.0	**15.7**
Ciprofloxacin	≥21	16–20	≤15	≥25	20–24	<20	5	5	<0.0001	<0.0001	0.1	0.1	1.9	1.9
Levofloxacin	≥17	14–16	≤13	≥20	17–19	<15	3	3	0.0015	0.0015	1.5	1.5	8.6	8.6
Acinetobacter baumannii														
Imipenem	≥16	14–15	≤13	≥23	18–22	<18	2	5	0.07	<0.0001	4.9	0.1	16.0	1.9
Meropenem	≥16	14–15	≤13	≥21	16–20	<16	2	5	0.07	<0.0001	4.9	0.1	16.0	1.9
Tobramycin	≥15	13–14	≤12	≥17		<17	2	0	0.07	**9.1**	4.9	**13.9**	16.0	**15.7**
Amikacin	≥17	15–16	≤14	≥18	16–17	<16	2	2	0.07	0.07	4.9	4.9	16.0	16.0
Gentamicin	≥15	13–14	≤12	≥17		<17	2	0	0.07	**9.1**	4.9	**13.9**	16.0	**15.7**
Ciprofloxacin	≥21	16–20	≤15	≥21		<21	5	0	<0.0001	**9.1**	0.1	**13.9**	1.9	**15.7**
Levofloxacin	≥17	14–16	≤13	≥21	19–20	<19	3	2	0.0015	0.07	1.5	4.9	8.6	16.0
Staphylococcus aureus														
Cefoxitin	≥22		≤21	≥22		<22	0	0	**9.1**	**9.1**	**13.9**	**13.9**	**15.7**	**15.7**
Tobramycin	≥15	13–14	≤12	≥18		<18	2	0	0.07	**9.1**	4.9	**13.9**	16.0	**15.7**
Amikacin	≥17	15–16	≤14	≥18	16–17	<16	2	2	0.07	0.07	4.9	4.9	16.0	16.0
Gentamicin	≥15	13–14	≤12	≥18		<18	2	0	0.07	**9.1**	4.9	**13.9**	16.0	**15.7**
Coagulase-negative staphylococci														
Cefoxitin	≥25		≤24	≥25		<25	0	0	9.1	**9.1**	**13.9**	**13.9**	**15.7**	**15.7**
Tobramycin	≥15	13–14	≤12	≥22		<22	2	0	0.07	**9.1**	4.9	**13.9**	16.0	**15.7**
Amikacin	≥17	15–16	≤14	≥22	19–21	<19	2	3	0.07	0.0015	4.9	1.5	16.0	8.6
Gentamicin	≥15	13–14	≤12	≥22		<22	2	0	0.07	**9.1**	4.9	**13.9**	16.0	**15.7**
Staphylococci (general)														
Penicillin	≥29		≤28	≥26		<26	0	0	9.1	**9.1**	**13.9**	**13.9**	**15.7**	**15.7**
Levofloxacin	≥19	16–18	≤15	≥22	20–21	<19	3	2	0.0015	0.07	1.5	4.9	8.6	16.0
Erythromycin	≥23	14–22	≤13	≥21	19–20	<18	9	2	<0.0001	0.07	<0.0001	4.9	<0.0001	16.0
Clindamycin	≥21	15–20	≤14	≥22	19–21	<19	6	3	<0.0001	0.0015	0.01	1.5	0.8	8.6
Rifampicin	≥20	17–19	≤16	≥26	23–25	<23	3	3	0.0015	0.0015	1.5	1.5	8.6	8.6
Trimethoprim-Sulfamethoxazole	≥16	11–15	≤10	≥17	14–16	<14	5	3	<0.0001	0.0015	0.1	1.5	1.9	8.6

S, susceptible; I, intermediate; R, resistant; σ, standard deviation; ME, major error; vME, very major error.
Statistical probabilities were calculated on the basis of standard deviations of 1 2, and 3 mm in disc diffusion readings assuming equal numbers of isolates for all diameter values, i.e. independent of the epidemiological situation present. The cumulated statistical probability comprises ≥95% of all possible ME/vME.

0.5. Any position along the horizontal *x*-axis can be expressed as a distance of a number of standard deviations from the mean. This distance is known as a standard normal deviate or normal score. It is equivalent to looking at a normal distribution with a mean 0 and a standard deviation 1, which is called the standard normal distribution. The necessary information for the lower and upper tail areas of the standard normal distribution is readily available in statistical tables. See for example table B1: Normal distribution—areas in one tail (z->p) in Altman *et al.* [12]. It can also be computed by

TABLE 2. Error probabilities on the basis of the actual epidemiological situation

Species/drug[a]	Isolates (*n*)	σ	Range of ≥95% ME/vME (mm)	Isolates at risk in the 95% probability range for ME and vME (*N*, %)	EUCAST								CLSI	
					ME (isolates at risk)		vME (isolates at risk)		ME and vME cumulated (isolates at risk)		ME and vME cumulated (% of all isolates)	Ratio ME/vME (%)	ME and vME cumulated	
					n	% (95% CI)	*n*	% (95% CI)	*n*	% (95% CI)			*n*	% (95% CI)
Escherichia coli														
Ampicillin	2991	1.2	12–15	130 (4.4)	8	6 (3–12)	10	8 (4–13)	18	14 (8–20)	0.6	44/56	0	<0.01 (0–0.1)
Amoxicillin-clavulanic acid	2992	1.3	14–19	655 (21.9)	30	5 (3–7)	33	5 (4–7)	63	10 (8–12)	2.1	48/52	0	<0.01 (0–0.1)
Cefuroxime	2987	1.2	16–19	107 (3.6)	8	7 (4–13)	8	7 (4–14)	16	14 (9–22)	0.5	50/50	0	<0.01 (0–0.1)
Cefoxitin	2991	1.5	16–21	171 (5.7)	7	4 (2–8)	14	8 (5–13)	20	12 (8–17)	0.7	32/68	0	<0.01 (0–0.1)
Cefpodoxime	2990	2.4	16–25	350 (11.7)	5	1 (0–2)	23	7 (4–10)	28	8 (5–11)	0.9	18/82	0	<0.01 (0–0.1)
Stenotrophomonas maltophilia														
Trimethoprim-Sulfa-methoxazole	317	2.2	12–21	33 (10.4)	2	5 (1–18)	1	4 (0–16)	3	9 (4–25)	0.9	67/33	0	<0.01 (0–0.1)
Pseudomonas aeruginosa														
Piperacillin-tazobactam CLSI	716	1.1	13–22	76 (10.6)	–	–	–	–	–	–		–	0	<0.0001 (0–0.001)
Piperacillin-tazobactam EUCAST	888	1.1	16–21	148 (16.7)	4	3 (1–7)	6	4 (2–8)	10	7 (4–12)	1.1	40/60	–	
Ceftazidime CLSI	716	0.8	14–18	36 (5.0)	–	–	–	–	–	–		–	0	<0.01 (0–0.1)
Ceftazidime EUCAST	888	0.8	14–17	40 (4.5)	1	3 (1–14)	1	2 (0–11)	2	5 (1–16)	0.2	50/50	–	
Cefepime	1610	0.9	16–19	219 (13.6)	6	3 (1–6)	8	4 (2–7)	14	7 (4–10)	0.9	41/59	0	<0.01 (0–0.1)
Tobramycin	1604	0.6	14–17	100 (6.2)	1	1 (0–5)	2	2 (0–7)	3	3 (1–8)	0.2	33/67	0	<0.01 (0–0.1)
Gentamicin	1599	0.5	13–16	211 (13.2)	2	1 (0–3)	3	1 (0–4)	5	2 (1–6)	0.3	42/58	0	<0.01 (0–0.1)
Acinetobacter baumannii														
Tobramycin	191	1.3	14–19	22 (11.5)	3	12 (2–27)	4	17 (7–37)	6	29 (12–47)	3.1	41/59	0	<0.01 (0–0.1)
Gentamicin	191	1.3	14–19	18 (9.4)	0	2 (0–20)	1	4 (0–23)	1	6 (1–26)	0.5	0/100	0	<0.01 (0–0.1)
Ciprofloxacin	190	1.6	18–23	18 (9.5)	1	6 (3–31)	2	12 (8–43)	3	18 (14–53)	1.6	33/67	0	<0.01 (0–0.1)
Staphylococcus aureus														
Cefoxitin	3001	0.8	20–23	47 (1.6)	1	2 (0–10)	1	2 (0–11)	2	4 (1–13)	0.1	50/50	2	4 (1–13)
Tobramycin	3000	1.0	15–18	30 (1.0)	2	5 (1–19)	2	5 (1–19)	4	10 (3–26)	0.1	50/50	0	<0.01 (0–0.1)
Gentamicin	2998	1.2	15–18	20 (0.7)	2	10 (1–23)	1	5 (1–25)	3	15 (3–30)	0.1	67/33	0	<0.01 (0–0.1)
Coagulase-negative staphylococci														
Cefoxitin	2183	0.8	23–26	154 (7.1)	4	2 (1–6)	6	4 (2–8)	10	6 (3–11)	0.5	40/60	10	6 (3–11)
Tobramycin	2230	1.0	20–23	114 (5.1)	5	5 (2–9)	6	5 (2–10)	11	10 (5–15)	0.5	44/56	0	<0.01 (0–0.1)
Gentamicin	2226	1.2	20–23	83 (3.7)	4	5 (2–13)	6	7 (3–15)	10	12 (7–21)	0.4	44/56	0	<0.01 (0–0.1)
All staphylococci														
Penicillin	1566	0.6	27–30	20 (1.3)	0	0 (0–17)	1	1 (0–18)	1	1 (0–19)	0.1	0/100	nd	nd

CI, confidence interval; σ, standard deviation; ME, major error; vME, very major error.
Statistical error probabilities for diameter values comprising the ≥95% probability range for ME/vME as applied to actual numbers of isolates and their diameter distributions. Resulting specific error probabilities were calculated for individual species/drug combinations.
[a]For ceftazidime, cefotaxime and piperacillin-tazobactam disc loads of CLSI and EUCAST are different: Results are, therefore, listed separately.

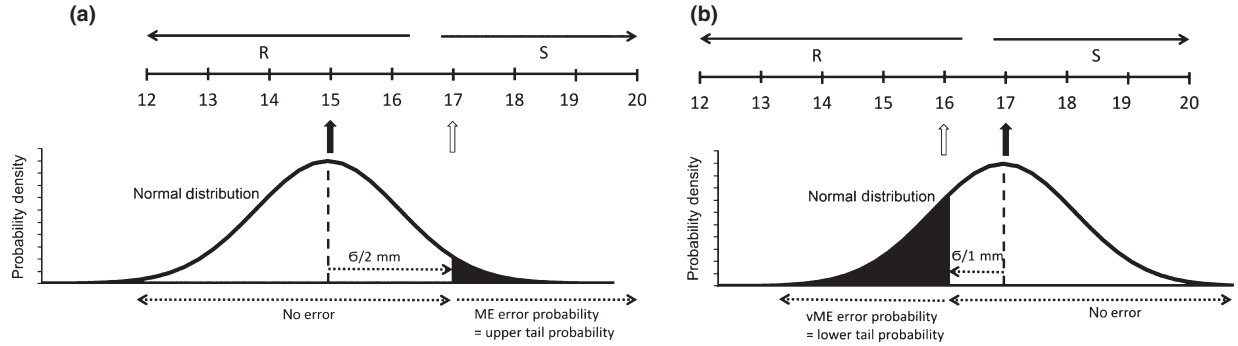

FIG. 1. Principle of the calculation of statistical error probabilities. The diagrams illustrate the calculation principle of error probabilities. European Committee for Antibiotic Susceptibility Testing resistant and susceptible category ranges are indicated above the diameter scale. Example (a) The actually measured diameter value (black arrow) is 15 mm (resistant). The probability for this measurement to represent a major error (ME; false-resistant) equals the probabilities for all diameter values in the susceptible category to be the—per definition unknown—true value. Probabilities can be assumed to be normally distributed, depending on the standard deviation (σ) of measuring. Assuming the true value (white arrow) is 17 mm (susceptible), the ME (false-resistant) probability is equal to the upper tail probability (i.e. upper tail area under the curve for the multiples of σ at 17 mm, black shaded). For example, if the σ is 1 mm, the probability that the true value is 17 mm is equal to 0.0222, i.e. 2.2%. In this example the probability that the actually measured value of 15 mm represents an ME is 2.2%. Example (b) Given an actually measured value of 17 mm (susceptible, black arrow), and a σ of 1 mm, the probability that the—unknown—true value (white arrow) is 16 mm (i.e. the actual measurement of 17 mm would be a false-susceptible result, very major error (vME)) is equal to 0.1587, i.e. 15.87% (lower tail area under the curve for the multiples of σ at 16 mm, black shaded). σ, standard deviation.

statistical packages such as the freely available R (http://www.r-project.org/). The normality assumption is useful in describing real-world data. In particular it is well known that laboratory measurements are not exact. One possible assumption is that the true unknown diameter is normally distributed around the mean diameter obtained by the Kirby–Bauer method with a given σ. When a set of observations has a distribution that is similar to a normal distribution it can be assumed that in the population the distribution of the variable is normal and calculations can be carried out on this basis, as suggested by Altman *et al.* [12]. The assumed probability distribution can be used to calculate the theoretical upper and lower tail areas with respect to different cut-offs (see Fig. 1). In such a case the normal distribution is a theoretical equivalent of the empirical relative frequency distribution.

In the first step of this study the theoretical tail areas exceeding the clinical breakpoints for different μ and σ scenarios were calculated by setting standard deviation (σ) to σ = 1 mm, σ = 2 mm, or σ = 3 mm (Fig. 1). In a second step, to estimate the relevance of the erroneous classifications for laboratory practice, the expected absolute frequencies of erroneously classified measurements were computed for the given observed populations. To calculate 95% CI for error probabilities, Wilson's method was used [13].

Standard deviations of disc diffusion inhibition zones for individual drug/species combinations were calculated from 19 independent readings by 19 experienced persons under standardized ambient conditions (EUCAST recommended) using *E. coli* ATCC 25922, *S. aureus* ATCC 29213, *P. aeruginosa* ATCC 27853, and two clinical strains of *A. baumannii* and *Stenotrophomonas maltophilia* with a resistance pattern consistent with that of the wild type [3].

Software

All calculations were done using the IBM SPSS statistics software version 20 (IBM Corporation, Armonk, NY, USA), the Microsoft Excel 2010 software (Microsoft Corporation, Redmond, WA, USA), and the software R (freely available under http://www.r-project.org/).

Results

Calculation of expected error rates independent of diameter distributions

In a first step, cumulated statistical probabilities for ME and vME were calculated for σ = 1 mm, σ = 2 mm and σ = 3 mm, normalized to equal numbers of isolates for each diameter value, i.e. error rates for the $\geq$95% probability range of ME/vME were calculated independently of actual isolate numbers in diameter distributions. Cumulated statistical probabilities for ME and vME significantly increased for drugs without an intermediate range: depending on the value of σ, cumulated probabilities of ME/vME ranged from 9.1% (σ = 1 mm) to 15.7% (σ = 3 mm) when no intermediate zone was defined (see Table 1). An intermediate zone of 2 mm resulted in

cumulated ME/vME probabilities of 0.07–15.7% depending on σ. Most intermediate zones as defined by EUCAST and CLSI comprise a range of 3 mm, resulting in cumulated ME/vME probabilities of 0.0015–8.6% depending on σ (Table 1).

Calculation of expected error rates applying individual diameter distributions

Further calculations on error rates applying the diameter distributions as determined in the clinical laboratory were performed for those drug/species combinations with the highest statistical probabilities for ME and vME, i.e. for those combinations lacking an intermediate range in EUCAST and CLSI CBP tables (Table 1, bold numbers). Results for individual drug/species combinations are summarized below.

Escherichia coli

The cumulated ME/vME rate for ampicillin, amoxicillin-clavulanic acid, cefuroxime and cefoxitin according to EUCAST CBPs ranged from 8% to 14% for isolates included in the $\geq$95% probability range for ME/vME (Table 2). The highest absolute number of expected errors was calculated for amoxicillin-clavulanic acid (n = 63 errors in 655 isolates at risk). The high number of isolates at risk was caused by a unimodal diameter distribution that showed a shoulder towards smaller diameter values (Fig. 2). Values of ME and vME were almost equally distributed except for cefpodoxime for which 18% ME and 82% vME were expected. The majority of isolates in the $\geq$95% probability range for ME/vME was situated in the lower third of the wild-type distribution curve (Fig. 2). CLSI has kept intermediate zones for all drugs resulting in error rates <0.01% with no ME/vME to be expected in the present population of isolates at risk (95% probability level of ME/vME).

Stenotrophomonas maltophilia

The cumulated rate of expected ME/vME for trimethoprim-sulfamethoxazole and EUCAST CBPs was 9%. The absolute number of isolates with errors was comparably low (n = 3). The cumulated ME/vME rate for trimethoprim-sulfamethoxazole applying CLSI CBPs was <0.01% (n = 0).

Pseudomonas aeruginosa

Applying EUCAST CBPs resulted in cumulated ME/vME rates for piperacillin-tazobactam, ceftazidime, cefepime, tobramycin and gentamicin of 2–7% for isolates in the 95% probability range for ME/vME. The relation of ME and vME was almost equal with a slight tendency to more vME for cefepime, tobramycin and piperacillin-tazobactam (ME/vME relations of 41/59%, 33/67% and 40/60%, see Table 2). If applying the CLSI CBPs, no ME/vMEs were expected (Table 2). The $\geq$95% probability range for ME/vME flanking EUCAST CBPs comprised significant parts of the diameter distribution curves for piperacillin-tazobactam, cefepime, tobramycin and gentamicin (Fig. 2). Notably, EUCAST CBPs for piperacillin-tazobactam, cefepime and gentamicin divided the wild-type population (Fig. 2).

Acinetobacter baumannii

For tobramycin, gentamicin and ciprofloxacin cumulated ME/vME rates in the EUCAST system were 29%, 6% and 18%, respectively. For ciprofloxacin the expected ME/vME relation was shifted towards vME (ratio ME/vME 33/67%, Table 2). If applying the CLSI CBPs no ME/vME were expected (Table 2).

Staphylococcus aureus

Applying EUCAST CBPs cumulated ME/vME rates ranged from 2% to 4% for isolates in the 95% ME/vME probability range for cefoxitin, tobramycin and gentamicin. In the case of gentamicin, more expected ME than vME were calculated (67% vs 33%, Table 2). Regarding cefoxitin, expected ME/vME rates using CLSI guidelines equalled those applying EUCAST guidelines. Tobramycin and gentamicin calculations showed no expected ME/vME applying CLSI CBPs; the $\geq$95% probability range for ME/vME adjacent to EUCAST CBPs comprised low numbers of isolates (see Table 2, Fig. 2).

Coagulase-negative staphylococci

When applying EUCAST CBPs, cumulated ME/vME rates for cefoxitin, tobramycin and gentamicin ranged from 6% to 12% for isolates in the 95% ME/vME probability range. For cefoxitin slightly more expected vME than ME were calculated (60% vs 40%, Table 2). Regarding cefoxitin, the expected ME/vME rates using CLSI guidelines equalled those for EUCAST guidelines. With tobramycin and gentamicin no ME/vME were expected applying CLSI CBPs. Distribution curves showed that wild-type and tobramycin and gentamicin resistant populations were not clearly separated because of the formation of a 'transition population' (Fig. 2).

All staphylococci (*Staphylococcus aureus* and coagulase-negative staphylococci)

The rate of expected ME/vME applying EUCAST CBPs for penicillin G was 1% for isolates at risk (95% probability level of ME/vME, Table 2). However, the absolute number of errors was low (n = 1) because few clinical isolates showed diameter values in the 95% probability range of ME/vME (see Fig. 2). For penicillin G, wild-type and resistant populations were clearly separated (Fig. 2). Error rates in the CLSI system could not be determined for penicillin G because data were only available

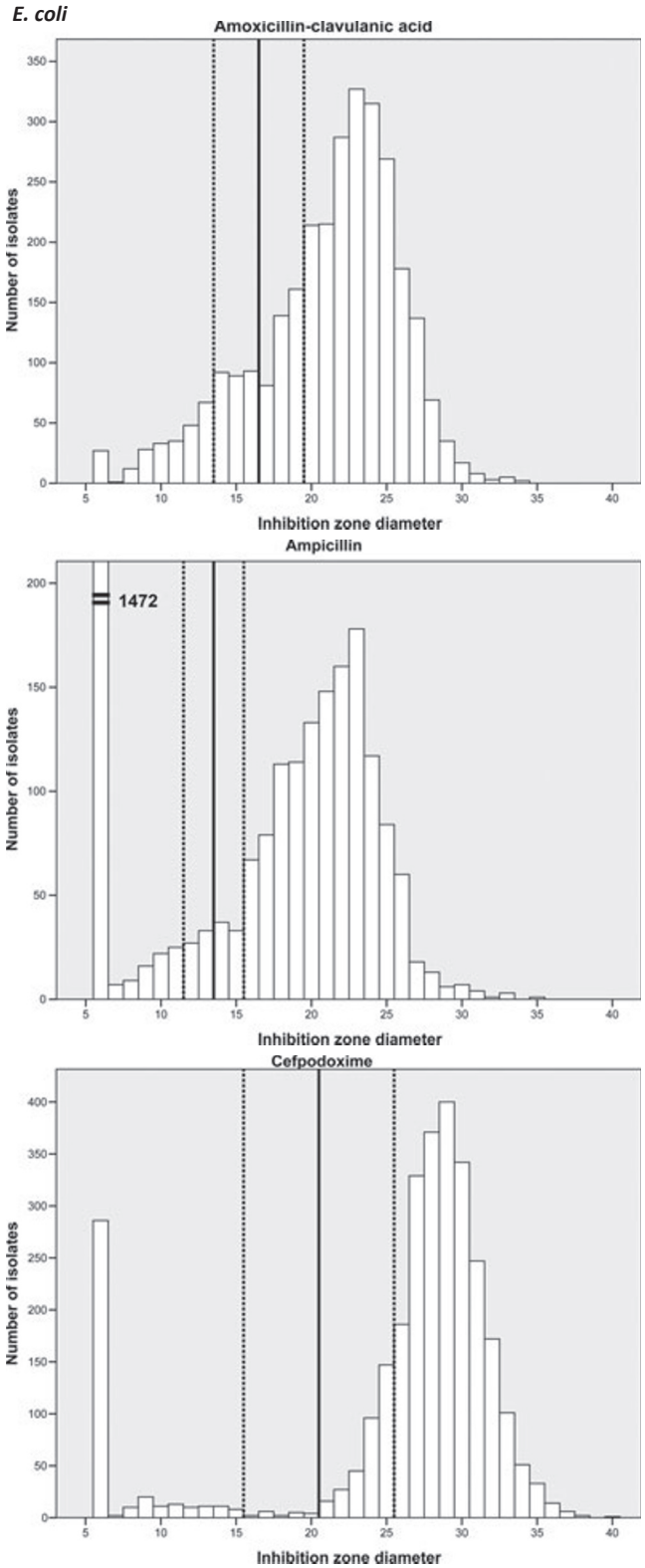

FIG. 2. Diameter distributions, European Committee for Antibiotic Susceptibility Testing (EUCAST) clinical breakpoints, and probability ranges of $\geq$95% for major error/very major error (ME/vME). Diameter distributions are displayed for species/drug combinations with high probabilities of ME/vME (see Table 1, bold numbers). EUCAST clinical breakpoints are indicated by black lines, borders of the $\geq$95% probability range for ME/vME are indicated by dotted lines.

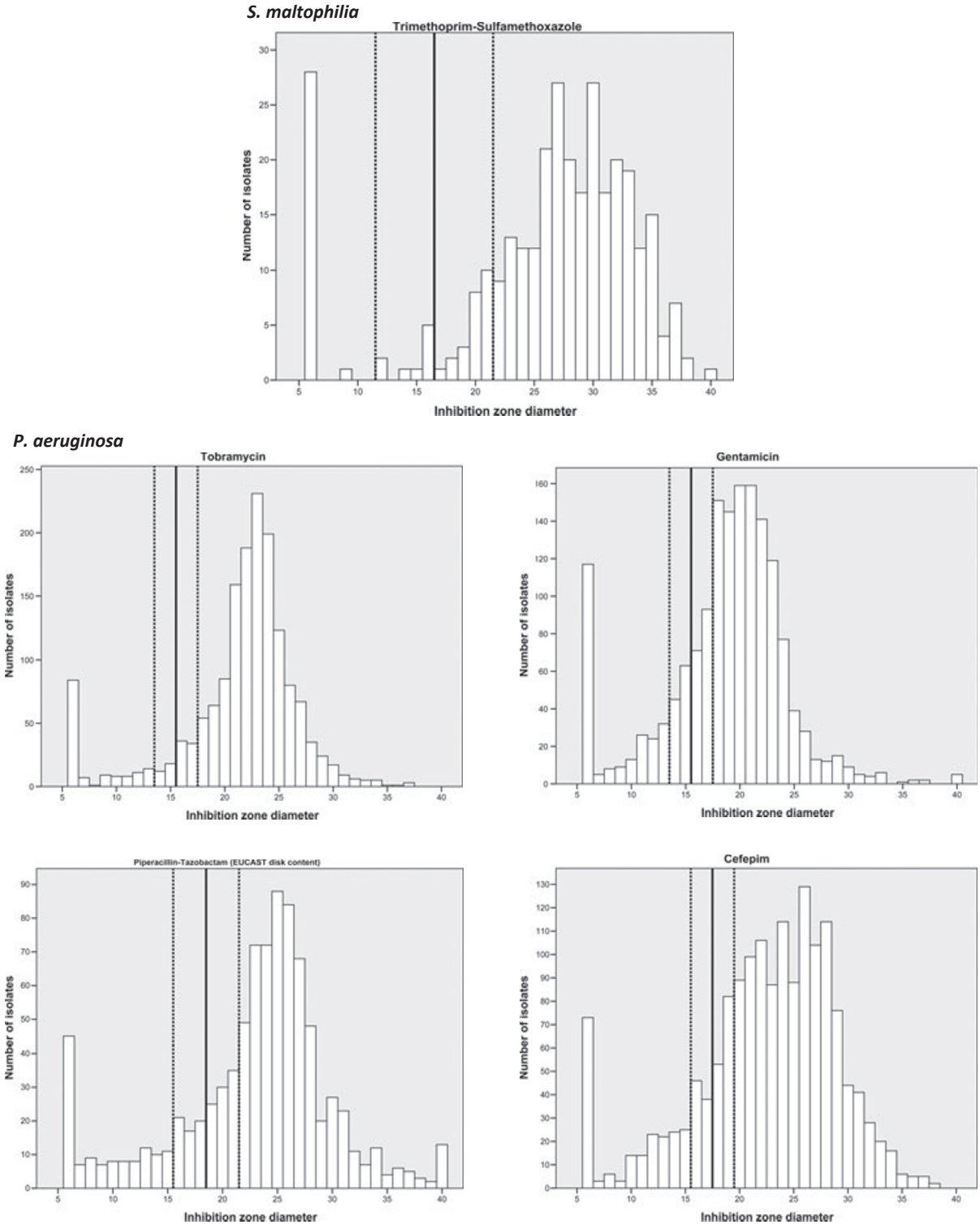

FIG. 2. Continued.

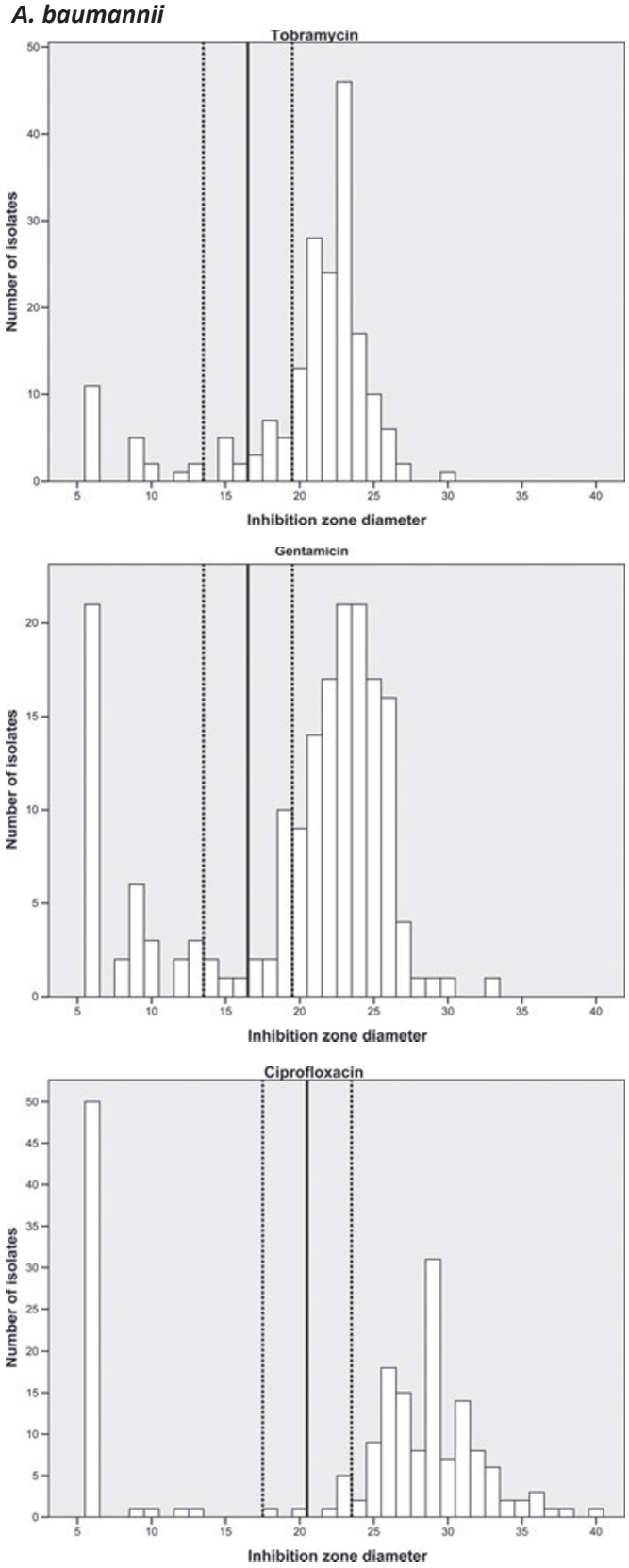

FIG. 2. Continued.

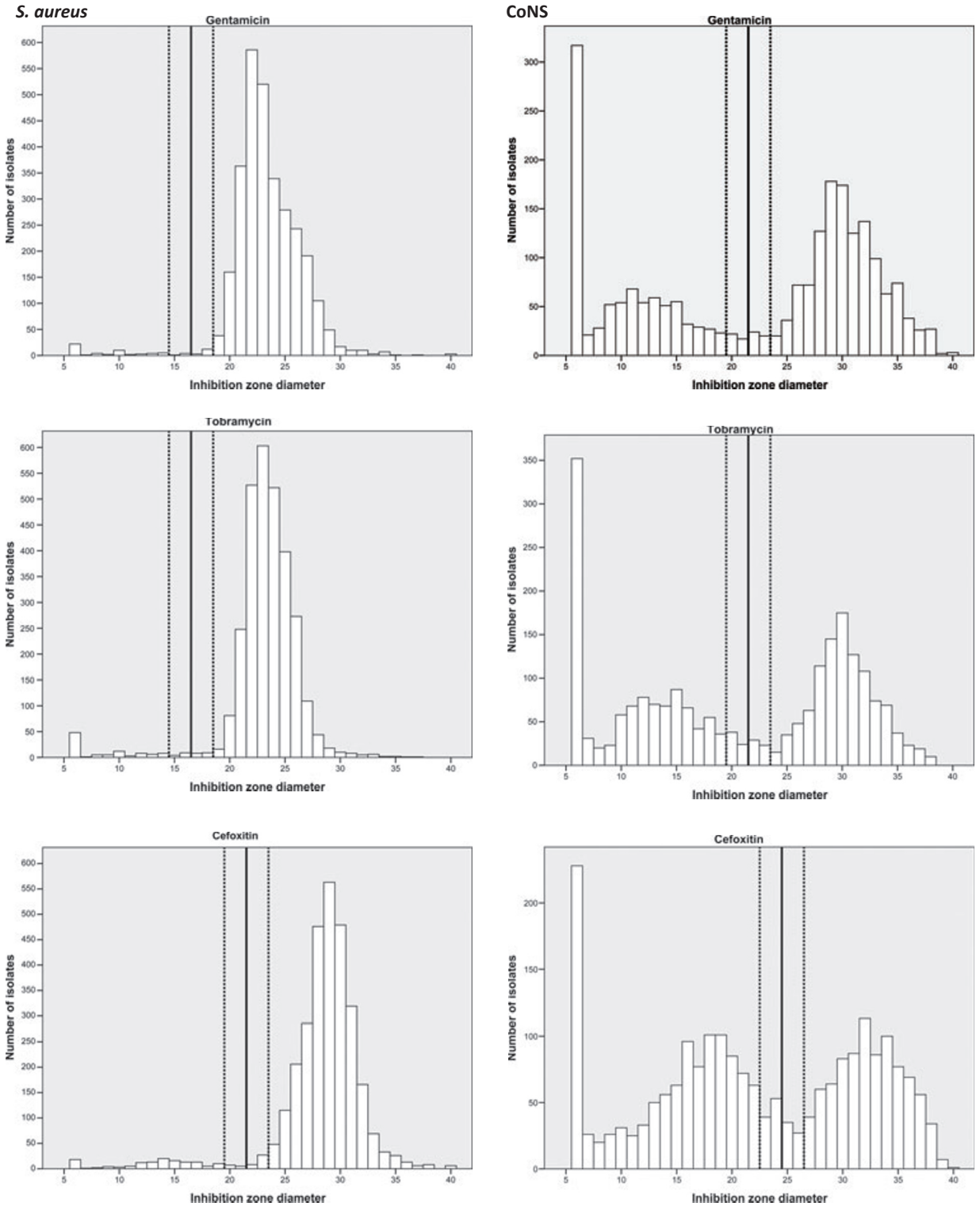

FIG. 2. Continued.

for the EUCAST recommended disc content (1 μg/disc EUCAST vs 10 μg/disc CLSI).

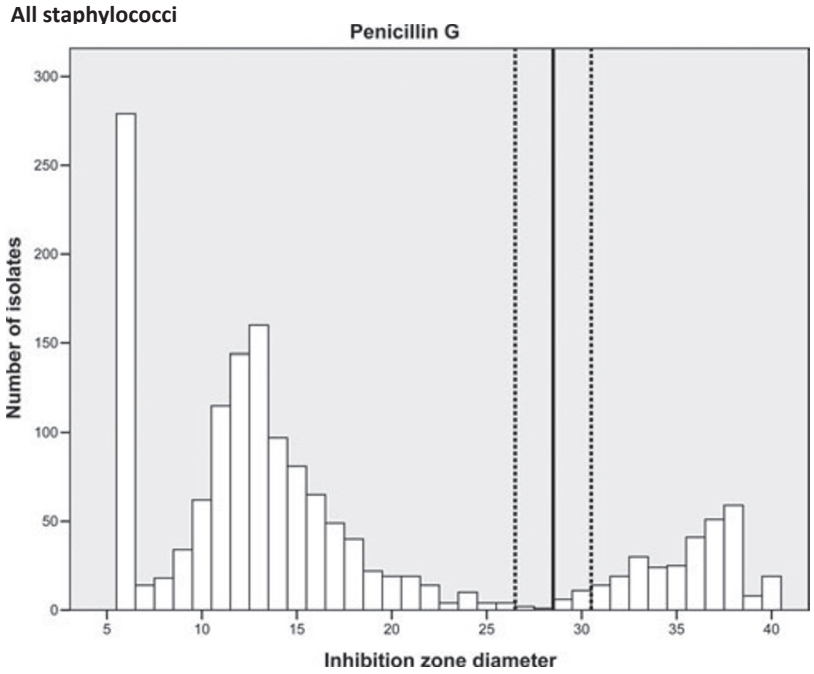

FIG. 2. Continued.

Discussion

The definition of CBPs is a complex process integrating epidemiological cut-off (ECOFF) values from MIC distributions, correlating these ECOFFs to zone diameters in a scattergram, and subsequently relating the putative CBPs to pharmacokinetic (PK)/pharmacodynamic (PD) data. The final step is validation of proposed CBPs from ECOFF and PK/PD data by means of clinical outcome studies [4].

Several methods can be used to define CBPs on the basis of disc diffusion, e.g. normalized resistance interpretation, the classical error-rate-bounded method, modifications of the error-rate-bounded method, or detailed modelling of the spread of errors [4,6,7,14–17]. CLSI uses a variant of the error-rate-bounded method, incorporating an intermediate zone that influences the rate of mE, ME and vME [14]. EUCAST defines harmonized MIC CBPs on the basis of ECOFFs and PK/PD parameters and correlates MIC CBPs to zone diameter values using the 'MIC-coloured zone diameter histogram technique' [16,18]. EUCAST has, in part, abandoned the intermediate category with the view to facilitate AST interpretation and to avoid splitting wild-type populations referring to PK/PD data. Concomitantly, CBPs for drug/species combinations without an intermediate zone relate to high-dose therapy only [8]. Abandoning or decreasing the intermediate zone that had in the past been established to decrease the rate of ME and vME in AST interpretation runs the risk of an increased frequency of interpretative errors and may result in so-called type I errors [6,19]. As a consequence, serious treatment failures may result from false categorization. In the case of extended spectrum β-lactamase producers EUCAST and CLSI proposed clinical categorization based on AST readings alone—independent of whether the mechanism itself is present [2,9]. This strategy poses a paradigm change as interpretative reading is abandoned and diameter (or MIC) testing alone becomes the single parameter to predict clinical outcome [20]. Adopting such a strategy must be accompanied by ensuring the most accurate and reproducible S/I/R categorization because otherwise serious treatment errors may result from measurement inaccuracy [21].

The number of ME and vME depends on several variables: (i) the presence of an intermediate zone; (ii) the shape of diameter distribution curves; and (iii) the precision of measurements.

The presence and width of an intermediate zone are critical for the rate of ME/vME [4,6]. Examples for the influence of an intermediate zone with several drugs are the error probabilities shown in Tables 1 and 2.

The absolute number of isolates with diameter measurements adjacent to R/S CBPs, and so the absolute number of errors, is dependent on the shape of the diameter distribution curves. For clearly separated wild-type and resistant popula-

tions the setting of an S/R CBP at the ECOFF produces low numbers of ME and vME as few isolates cluster around the CBP (e.g. penicillin G and staphylococci, Table 2, Fig. 2). If, however, the wild-type is not clearly separated from the resistant population the number of errors increases as more isolates cluster around the CBP (e.g. diameter distribution of amoxicillin-clavulanic acid in *E. coli*, Table 2, Fig. 2). To address this problem, the separation of wild-type and resistant populations by determination of the optimal disc content for a specific species/drug combination has been suggested [22,23].

Setting the CBP equal to the ECOFF encloses part of the wild-type population in the $\geq$95% probability range for ME/vME, inevitably leading to ME. In contrast, setting the CBP close to a resistant population would avoid ME, but increase the probability for vME. Increasing the S/R zone diameter breakpoint above the ECOFF would avoid vME, but split the wild-type population between different categories [16,18,24].

The relation of ME to vME in a clinical laboratory depends on the individual diameter distributions (see Fig. 2). Three basic scenarios are possible: (i) diameter measurements of isolates included in the $\geq$95% ME/vME range are evenly distributed resulting in equal numbers of expected ME and vME (e.g. tobramycin and *S. aureus*, and amoxicillin-clavulanic acid and *E. coli*, Table 2, Fig. 2); (ii) more measured diameter values are greater than the S/R breakpoint, i.e. categorized susceptible leading to higher numbers of vME (e.g. cefpodoxime and *E. coli*, ciprofloxacin and *A. baumannii*, or tobramycin and *P. aeruginosa*, Table 2, Fig. 2); (iii) more measured diameter values are lower than the S/R breakpoint, i.e. categorized resistant and the probability for ME is relatively increased (e.g. gentamicin and *S. aureus*, see Table 2, Fig. 2).

The critical influence of an individual epidemiological situation (reflected in the shape of a diameter distribution curve) is illustrated by tobramycin and *P. aeruginosa*. The EUCAST diameter distribution shows a low number of isolates with diameters of 14–17 mm (95% ME/vME probability range). The CBP (16 mm) is equal to the ECOFF [25]. In our study population, however, a significant number of isolates showed inhibition zones of 14–17 mm (n = 100 out of 1604, i.e. 6.2% of all *P. aeruginosa* isolates in this study). The resulting ME/vME rate of 3% was comparably low because of a low σ of 0.5 mm. In-depth analysis of these isolates showed that a significant proportion (44.1%, corrected for duplicates) originated from patients in the cystic fibrosis or lung transplantation unit where tobramycin is a mainstay drug. Recording zone diameters will give laboratories information about their local epidemiology, which influences error rates. Local adaptations of general breakpoints will be facilitated, a strategy that was repeatedly recommended to improve correct assignment of strains to interpretative categories [16,24,26–29].

The precision of diameter measurements as reflected by σ significantly influences the number of ME/vME (see Table 1). σ is itself dependent on factors like inoculum, agar composition, disc content or incubation time, in addition to intra-person and inter-person variances: the higher the value of σ, the broader the $\geq$95% ME/vME probability range: e.g. in the present study σ for cefpodoxime and *E. coli* was 2.4 mm resulting in an interval of 10 mm containing $\geq$95% of all ME/vME vs ampicillin with σ = 1.2 mm resulting in an interval width of 4 mm (see Table 2 and Fig. 2). The influence of σ on error rates is best illustrated by σ mean values as determined for *E. coli* and *P. aeruginosa* isolates in this study (σ = 1.5 mm vs σ = 0.8 mm for *E. coli* and *P. aeruginosa*, respectively, as determined from σ values listed in Table 2). These values of σ result in mean ME/vME rates of 12% in *E. coli* vs 5% in *P. aeruginosa*. The error rates used for calculations in this work may even be underestimated as factors like inoculum, agar composition, disc content or incubation time (standardized in the present work) additionally contribute to σ besides inter-person variance. EUCAST accepts quality control ranges of 5–10 mm for individual drug/species combinations. Assuming that this reflects a range of $\pm$ 2*σ, the accepted one-fold σ range is from 1.25 to 2.5 mm. Separation of resistant and wild-type isolates by CBPs can be ensured by taking into account σ and associated error probability ranges. Hence, the width of the intermediate category represents the measurement inaccuracy.

The assumption of normality is one possible approximation to reality but frequently a reasonable one. Our study demonstrates the consequences of removing the intermediate zone and illustrates challenges, difficulties and problems attached to the setting of CBPs. Although the problem of reporting ME and vME is apparently limited to a comparably small part of the population (highest cumulated ME/vME rate of 3.1% for tobramycin in *A. baumannii* relating to the complete study population, see Table 2), the reliable classification of isolates in the $\geq$95% ME/vME probability range (close to CBPs) is of vital importance for clinical decisions and monitoring therapeutic success.

Four conclusions can be drawn from this study. (i) If the wild-type is not clearly separated from the resistant population an intermediate or 'grey' zone should be kept, decreasing ME/vME numbers. Assigning isolates with uncertain classification to an intermediate category avoids producing a feigned impression of precision in AST classification. This will prevent confusion of clinicians receiving discrepant (S/R) results for isolates with true diameter values close to the CBPs originating from parallel samples or from samples that are tested

consecutively for monitoring therapeutic success. (ii) An intermediate zone of at least 2–3 mm avoids almost all ME/vME for most species/drug combinations (Table 1). (iii) Laboratories should know their individual diameter distributions to detect problems with individual species/drug/CBP combinations. (iv) Clinical microbiologists should be aware of the individual drug/σ combinations in their laboratories to monitor the precision of diameter measurements. Measures should be taken to decrease σ of diameter measurements and enhance reproducibility, e.g. by training of personnel or by automation of diameter measurements.

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Transparency Declarations

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