

Relative contribution of biological variation and technical variables to zone diameter variations of disc diffusion susceptibility testing

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Objectives: Disc diffusion is still largely based on manual procedures. Technical variations originate from inoculum preparation, variations in materials, individual operator plate streaking and reading accuracy. Resulting measurement imprecision contributes to categorization errors. Biological variation resembles the natural fluctuation of a measured parameter such as antibiotic susceptibility around a mean value. It is deemed to originate from factors such as genetic background or metabolic state. This study analysed the relative contribution of different technical and biological factors to total disc diffusion variation.

Methods: For calculation of relative error factor contribution to disc diffusion variability, five experiments were designed keeping different combinations of error factors constant. A mathematical model was developed to analyse the individual error factor contribution to disc diffusion variation for each of the tested drug–species combinations.

Results: The contribution of biological variation to total diameter variance ranged from 10.4% to 98.8% for different drug–species combinations. Highest biological variation was found for *Enterococcus faecalis* WT and vancomycin (98.8%) and for penicillinase-producing *Staphylococcus aureus* and penicillin G (96.0%). Average imprecision of automated zone reading revealed that 1.4%–5.3% of total imprecision was due to technical variation, while materials, i.e. antibiotic discs and agar plates, contributed between 2.6% and 3.9%. Inoculum preparation and manual plate streaking contributed 6.8%–24.8% and 6.6%–24.3%, respectively, to total imprecision.

Conclusions: This study illustrates the relative contributions of technical factors that account for a significant part of total variance in disc diffusion. The highest relative contribution originated from the operator, i.e. manual inoculum preparation and plate streaking. Further standardization of inoculum preparation and plate streaking by automation could potentially increase the precision of disc diffusion and improve the correlation of susceptibility reports with clinical outcome.

Introduction

Disc diffusion is one of the mainstay methods for antimicrobial susceptibility testing (AST). However, in contrast to the recent advances in microbial identification by the introduction of nucleic acid amplification techniques and MALDI-TOF MS into the clinical laboratory, the prospects of improved AST are considered less predictable.¹

Disc diffusion is still largely based on manual procedures for preparation and reading of agar plates and zone diameters. Technical variations of disc diffusion AST thus originate from both operator-dependent factors, i.e. inoculum preparation, plate streaking or reading precision, and operator-independent variations in materials, e.g. agar plates and antibiotic discs, or incubation

conditions.^{2–4} Technical variation significantly contributes to categorization errors and, thus, erroneous treatment of patients.^{5–7} Many attempts were made to further standardize disc diffusion testing in the 1960s and 1970s, mainly focusing on standardizing basic parameters such as agar type, agar depth, disc content, time of pre-diffusion, atmospheric conditions, the reading endpoint or the ideal inoculum density.^{2,3} Current EUCAST and CLSI guidelines for AST are essentially based on these early studies and describe a structured and standardized methodology.^{8,9} In addition to technical variations, zone diameter distribution variations also comprise individual strain variations, i.e. the biological diversity of a species and/or genotype, respectively.⁷ This biological variation appears as random fluctuations in zone diameter values around a mean, which usually produce a Gaussian distribution.

Automation of laboratory procedures is of increasing importance for clinical microbiology laboratories as it is regarded as essential for further standardization of microbiological techniques with respect to higher quality, more rapid availability and improved reproducibility of results.^{10–12} However, scientific literature systematically analysing the impact of automation on the quality of microbiological results is rare.¹³ Automation of zone diameter reading has already been shown to increase the precision and reproducibility of disc diffusion AST.¹⁴ Other technical variables may similarly be standardized by automation to improve the reliability and reproducibility of disc diffusion and the resulting clinical susceptibility categorizations and treatment recommendations.

To quantify the relative contribution of different technical factors to the total variation of the disc diffusion method, we analysed the contribution of technical and biological factors to the overall zone diameter variation in disc diffusion AST to provide a basis for targeting the most promising technical aspects for further standardization. Technical variables analysed were: (i) zone diameter reading; (ii) materials (i.e. agar plates, antibiotic discs, swabs); (iii) inoculum preparation; and (iv) inoculation of AST agar plates.

Methods

Relative contribution of variables and experimental designs

For the calculation of the relative contribution of individual factors (Table 2) to the total inaccuracy of disc diffusion AST, the following experiments (A–E) were designed, each of which had a different combination of error factors that were kept constant or left variable. The resulting matrix is shown in Figure 1. Experiments A–E were arranged according to increasing

complexity of variable factors. In detail, Experiments A–E were carried out as follows.

Experiment A

One hundred repeat zone diameter measurements were made of the same inhibition zone on the same Mueller–Hinton agar plate of EUCAST quality control strains using the Sirscan automated zone reader (i2a, Montpellier, France).

Experiment B

One hundred repeat zone diameter measurements were made for EUCAST quality control strains of 100 individual inocula (0.5 McFarland standard) prepared from 100 individual colonies picked from the same agar plate by the same operator using the same lot of antibiotic discs and the same lot of Mueller–Hinton agar plates, eliminating inter-operator variance in plate streaking. All zone diameters were recorded using the Sirscan automated zone reader.

Experiment C

One hundred repeat zone diameter measurements were made for EUCAST quality control strains from 100 individual Mueller–Hinton agar plates prepared from the same inoculum (0.5 McFarland standard) streaked by 10 different operators (10 plates for each operator) using the same lot of antibiotic discs and the same lot of Mueller–Hinton agar plates, using the Sirscan automated zone reader.

Experiment D

We measured *n* zone diameter measurements for EUCAST quality control strains from the internal quality control of the clinical microbiology laboratory of the Institute of Medical Microbiology, University of Zurich over a 2 year period of time carried out weekly using the Sirscan automated

Experiment	Variation factors				
	Technical factors			Biological factors	
	Reading imprecision	Material variations	Operator factors		
			Inoculum preparation		Plate streaking ^a
A	Variable	Constant	Constant	Constant	Constant
B	Variable	Variable	Variable	Constant	Constant
C	Variable	Variable	Constant	Variable	Constant
D	Variable	Variable	Variable	Variable	Constant
E	Variable	Variable	Variable	Variable	Variable

Figure 1. Design matrix of experiments A–E according to variation factors. ^aThe plate-streaking variation factor refers to inter-operator variation. Intra-operator variations cannot be eliminated.

zone reader. Individual drug–strain combinations show different numbers as the composition of the AST panels varied. Exact numbers can be retrieved from Table 1. Production lots of Mueller–Hinton agar plates and antibiotic discs as well as inoculum preparation and operators who performed streaking of AST plates were variable.

Experiment E

We used n zone diameter measurements of n individual clinical strains using the Sirscan automated zone reader as the reference populations that were defined above. Strains, Mueller–Hinton agar plates, antibiotic discs, inoculum preparation and operators were always different.

Bacterial strains

For experiments A, B, C and D we used EUCAST reference strains *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212 and *Pseudomonas aeruginosa* ATCC 27853 (for the specific experimental designs see the ‘Relative contribution of variables and experimental designs’ section). Reference populations of Experiment E were derived from non-duplicate clinical strains isolated over a 4 year period from 2010 to 2014 in the clinical microbiology laboratory of the Institute of Medical Microbiology, University of Zurich. Isolates of the same species were considered duplicate(s) if they: (i) originated from the same patient; and (ii) showed one major AND two minor differences in AST interpretation at a maximum. All duplicates were excluded from the analysis. The numbers of drug–species combinations are available in Table 1.

Phenotype definitions and reference populations

The WT was defined as all isolates of a given species that are devoid of an acquired resistance mechanism to the drug analysed. The biological variation of the WT was calculated for each drug–species combination separately. In the case of *S. aureus* and penicillin G the EUCAST reference strain (ATCC 29213) used for Experiments A, B, C and D is penicillinase positive and PBP2a negative. Therefore, the reference population comprised those non-WT isolates that harboured a penicillinase but were devoid of PBP2a. Thus, the biological variation of *S. aureus* and penicillin G was calculated for the penicillinase-positive non-WT population.

WT populations of Experiment E were defined using EUCAST epidemiological cut-offs (ECOFFs) with the exception of the tetracycline’s WT populations, for which CLSI 2015 clinical breakpoints (CBPs) were used as EUCAST ECOFFs were not available. The *S. aureus* penicillinase-positive reference population comprised isolates that were penicillin G resistant PLUS isolates that were penicillin G susceptible AND showed a sharp penicillin G zone edge AND were cefoxitin susceptible applying EUCAST 2014 CBPs.

Susceptibility testing

For susceptibility testing, the disc (i2a, Montpellier, France) diffusion method was carried out using Mueller–Hinton agar (Becton Dickinson, Franklin Lakes, NJ, USA) and a 0.5 McFarland standard dilution from overnight cultures as an inoculum followed by incubation at 35°C for 16–18 h according to EUCAST recommendations.¹⁵ McFarland standard dilutions were prepared by visual comparison of the test tubes with freshly prepared and vortexed 0.5 McFarland turbidity standards manufactured according to EUCAST guidelines, and double-checked using a calibrated Densicheck instrument (bioMérieux, Marcy-l’Étoile, France) calibrated and used according to the recommendations of the manufacturer.

Statistical model

Measured disc diameters are random; they scatter around a mean with a certain standard deviation. Changing any experimental conditions, i.e.

factors, possibly implies a change in mean and standard deviation, hence the distribution of disc diameters may be shifted and/or stretched. The experimental setup outlined above does not allow deconvolution of the individual effects of all the factors.

For each experiment the result of each measurement was decomposed additively using the different factors. For the fixed components of each experiment their contribution was given by a simple offset, i.e. a fixed effect. For those components that varied between measurements we considered a random contribution, i.e. a random effect.

For example, for Experiment A we had only one random effect for reading imprecision. For Experiment E, five random effects were used (reading imprecision, material variation, inoculum preparation, plate streaking and biological variation) (Figure 1).

Biological variation always referred to a specific phenotype of a given drug–species combination, e.g. the WT population or a specific non-WT population.

For each of the five experiments we estimated the mean and variance of the disc diameter measurements. These five estimated variances determined the variances of the five random effects. We hence had five equations and five unknowns leading to a unique solution. Unfortunately it could contain negative values, which are not admissible for variances. To get at least an approximate solution in each case, we implemented a pragmatic approach. Whenever one element of the solution was negative we set it to zero, i.e. the factor had negligible variability. The procedure was equivalent to minimizing the sum of squared differences between the original and approximate solutions.

Software

Disc diameter data collected by the Sirscan device were electronically archived using SIRweb software v100.32 (i2a, Montpellier, France). Statistical analyses were performed in the ‘R’ programming language version 3.0.2, which is freely available at <http://www.r-project.org/>.

Results

Statistical parameters of variation (variance, standard deviation and the coefficient of variation) increased from Experiment A to Experiment E, i.e. with increasing number of variables (Table 1 and Figure 2). Median diameters and standard deviations of Experiment D closely matched EUCAST quality control target values and accepted quality control diameter ranges (Table 1 and Figure 2). The average coefficient of variation for Experiment A was 1.5% for all species except for *S. aureus* (2.3%). Variances, standard deviations and coefficients of variation for Experiments B, C and D were similar for all species investigated, whereas variance in Experiment E was particularly high in *P. aeruginosa* (12.7 mm²; Table 1).

With respect to individual drug–species combinations, biological variation contributed to a variable extent to total diameter variance, ranging from 10.4% to 98.8% in WT ATCC strains (Table 2). Considering the average of all drug–species combinations, biological variation was highest in *P. aeruginosa* (81.3%) followed by *S. aureus* (64.0%), *E. coli* (53.9%) and *E. faecalis* (47.0%). Antibiotic classes did not behave uniformly and relative biological variation for individual drug classes was species-dependent, e.g. average biological contribution for aminoglycoside measurement variation was 82.4% for *S. aureus* and *P. aeruginosa*, but 23.9% for *E. coli*. For the latter species and aminoglycosides, operator dependence (i.e. plate streaking) was the most significant error factor (40.2%; Table 2). In contrast, average biological contribution for fluoroquinolone measurement variation was 49.3% and

Table 1. Basic summary statistics and EUCAST QC ranges/targets of experiments A–E

Drug	<i>E. coli</i>						<i>S. aureus</i>					<i>E. faecalis</i>					<i>P. aeruginosa</i>							
	experiment	measurements (n)	median diameter (mm)	variance (mm ²)	SD (mm)	coefficient of variation (%)	EUCAST QC range	EUCAST QC target	experiment	measurements (n)	median diameter (mm)	variance (mm ²)	SD (mm)	coefficient of variation (%)	EUCAST QC range	EUCAST QC target	experiment	measurements (n)	median diameter (mm)	variance (mm ²)	SD (mm)	coefficient of variation (%)	EUCAST QC range	EUCAST QC target
Penicillin G									A	100	17	0.1	0.3	1.9										
									B	100	16	1.0	1.0	6.2										
									C	100	17	1.4	1.2	6.8										
									D	201	15	1.2	1.1	7.3	12–18	15								
									E	881	13	33.6	5.8	40.9										
Ampicillin	A	100	20	0.04	0.2	1.2											A	100	17	0	0.1	0.8		
	B	100	20	0.8	0.9	4.3											B	100	18	0.9	0.9	5.2		
	C	100	20	1.4	1.2	5.8											C	100	19	1.7	1.3	7.1		
	D	117	20	2.6	1.6	8	16–22	19									D	108	18	2.1	1.5	8.0	15–21	18
	E	1389	21	7.8	2.8	13.4											E	2779	18	7.5	2.7	15.5		
Amoxicillin/clavulanic acid	A	100	24	0.3	0.5	2.1																		
	B	100	23	0.8	0.9	3.8																		
	C	100	24	1.0	1.0	4.1																		
	D	117	22	2.0	1.4	6.4	18–24	21																
	E	4778	24	5.8	2.4	10.2																		
Piperacillin/tazobactam	A	100	22	0.01	0.1	0.5											A	100	25	0.4	0.6	2.5		
	B	100	24	0.6	0.8	3.4											B	100	27	1.0	1.0	3.8		
	C	100	24	1.0	1.0	4											C	100	27	1.0	1.0	3.7		
	D	117	23	2.6	1.6	6.8	21–27	24									D	134	26	2.3	1.5	6.0	23–29	26
	E	4773	26	7.8	2.8	11.0											E	2694	24	13.6	3.7	14.9		
Cefalotin	A	100	21	0	0	0																		
	B	100	18	0.8	0.9	5																		
	C	100	19	2.0	1.4	7.6																		
	D	119	20	2.9	1.7	8.5	NA	NA																
	E	9052	18	10.2	3.2	17.0																		
Cefuroxime	A	100	22	0.1	0.3	1.5																		
	B	100	24	0.8	0.9	3.8																		
	C	100	24	1.0	1.0	4																		
	D	118	24	2.3	1.5	6.2	20–26	23																
	E	4774	25	6.8	2.6	10.4																		
Cefoxitin	A	100	25	0.3	0.5	2.1			A	100	26	0.8	0.9	3.5										
	B	100	26	0.8	0.9	3.6			B	100	26	1.0	1.0	4.0										
	C	100	27	0.8	0.9	3.6			C	100	27	1.2	1.1	4.2										
	D	119	26	3.6	1.9	7.2	23–29	26	D	200	26	2.3	1.5	5.6	24–30	27								
	E	4773	26	7.8	2.8	10.6			E	5934	27	6.8	2.6	9.7										

Ceftazidime	A	100	24	0	0	0		
	B	100	26	0.8	0.9	3.2		
	C	100	27	1.2	1.1	4.1		
	D	118	26	1.7	1.3	4.9	23-29	26
	E	4772	28	7.8	2.8	10.3		
Cefpodoxime	A	100	23	0.8	0.9	4.2		
	B	100	24	1.0	1.0	4.2		
	C	100	25	1.4	1.2	4.8		
	D	116	25	2.3	1.5	6	23-28	26
	E	4785	27	10.9	3.3	12.1		
Ceftriaxone	A	100	29	0.5	0.7	2.5		
	B	100	30	1.2	1.1	3.8		
	C	100	31	2.3	1.5	4.7		
	D	115	30	1.4	1.2	4	29-35	32
	E	4766	31	7.3	2.7	8.7		
Cefotaxime	A	100	28	0.0	0	0		
	B	100	28	1.2	1.1	3.8		
	C	100	29	1.4	1.2	4.3		
	D	118	28	1.7	1.3	4.8	25-31	28
	E	4769	31	11.6	3.4	11.1		
Cefepime	A	100	31	0.4	0.6	1.8		
	B	100	33	1.2	1.1	3.2		
	C	100	33	2.0	1.4	4.1		
	D	120	32	1.7	1.3	4.1	31-37	34
	E	4726	32	9.6	3.1	9.6		
Ertapenem	A	100	31	0.04	0.2	0.8		
	B	100	31	1.2	1.1	3.4		
	C	100	33	2.3	1.5	4.5		
	D	111	33	5.3	2.3	6.9	29-36	33
	E	4484	33	10.9	3.3	10.1		
Meropenem	A	100	34	0.3	0.5	1.4		
	B	100	35	2.6	1.6	4.6		
	C	100	36	1.7	1.3	3.6		
	D	113	31	3.2	1.8	5.8	28-34	31
	E	4628	33	10.2	3.2	9.7		
Imipenem	A	100	28	0.8	0.9	3.1		
	B	100	32	2.3	1.5	4.6		
	C	100	33	2.0	1.4	4.2		
	D	108	30	2.3	1.5	4.8	26-32	29
	E	4737	30	9.6	3.1	10.2		
Vancomycin	A	100	16	0	0.2	1.1		
	B	100	16	0.5	0.7	4.5		
	C	100	17	0.6	0.8	4.7		
	D	112	15	0.5	0.7	4.7	10-16	13
	E	2775	16	2.5	1.6	9.6		

A	100	24	0.2	0.4	1.8		
B	100	24	1.1	1.0	4.3		
C	100	25	0.7	0.8	3.3		
D	133	23	1.6	1.3	5.5	21-27	24
E	2867	23	12.0	3.5	15.0		

A	100	29	0.3	0.5	1.7		
B	100	29	1.3	1.2	4.0		
C	100	29	1.1	1.0	3.5		
D	134	28	2.1	1.4	5.3	24-30	27
E	2744	25	13.5	3.7	14.8		

A	100	33	0.4	0.6	1.9		
B	100	30	1.9	1.4	4.5		
C	100	32	2.4	1.6	4.9		
D	134	30	2.6	1.6	5.4	27-33	30
E	2395	31	14.3	3.8	12.2		

A	100	27	0.1	0.3	1.1		
B	100	26	4.0	2.0	7.6		
C	100	25	2.6	1.6	6.3		
D	134	24.5	2.3	1.5	6.2	20-28	24
E	2582	27	13.0	3.6	13.1		

Continued

Biological variation and technical variables of disc diffusion

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Table 1. Continued

Drug		<i>E. coli</i>						<i>S. aureus</i>						<i>E. faecalis</i>						<i>P. aeruginosa</i>					
		experiment	measurements (n)	median diameter (mm)	variance (mm ²)	SD (mm)	coefficient of variation (%)	EUCAST QC range	EUCAST QC target	experiment	measurements (n)	median diameter (mm)	variance (mm ²)	SD (mm)	coefficient of variation (%)	EUCAST QC range	EUCAST QC target	experiment	measurements (n)	median diameter (mm)	variance (mm ²)	SD (mm)	coefficient of variation (%)	EUCAST QC range	EUCAST QC target
Nalidixic acid	A	100	28	0.1	0.3	1.1																			
	B	100	27	1.0	1.0	3.6																			
	C	100	28	2.0	1.4	4.9																			
	D	117	26	2.3	1.5	5.9	22–28	25																	
	E	6981	26	5.3	2.3	9.1																			
Norfloxacin	A	100	34	0.1	0.3	1			A	100	23	0.5	0.7	3.2											
	B	100	32	0.8	0.9	2.8			B	100	24	0.8	0.9	3.9											
	C	100	32	2.0	1.4	4.2			C	100	24	0.5	0.7	2.9											
	D	111	31	4.8	2.2	7	28–35	32	D	107	22	1.4	1.2	5.2	18–24	21									
	E	6361	32	12.3	3.5	10.7			E	2630	24	7.8	2.8	11.4											
Ciprofloxacin	A	100	37	0.2	0.4	1.2			A	100	27	0.04	0.2	0.7			A	100	21	0.2	0.4	1.9			
	B	100	34	1.4	1.2	3.5			B	100	25	1.7	1.3	5.1			B	100	22	1.2	1.1	4.9			
	C	100	35	2.9	1.7	4.8			C	100	26	1.2	1.1	4.2			C	100	24	1.1	1	4.4			
	D	115	32	4.8	2.2	7	30–40	35	D	145	25	3.6	1.9	7.5	21–27	24	D	79	23	2.6	1.6	7.0	19–25	22	
	E	6230	33	11.6	3.4	10.1			E	2451	27	6.3	2.5	9.5			E	1523	23	5.2	2.3	10.1			
Levofloxacin	A	100	35	0.4	0.6	1.6			A	100	28	0.5	0.7	2.6			A	100	22	0.1	0.3	1.2			
	B	100	34	1.0	1.0	2.9			B	100	28	1.7	1.3	4.6			B	100	22	0.5	0.7	3.3			
	C	100	33	1.7	1.3	3.8			C	100	29	1.4	1.2	4.2			C	100	24	1.1	1	4.3			
	D	117	32	6.8	2.6	8.3	29–37	33	D	200	27	2.3	1.5	5.5	23–29	26	D	110	22	2.7	1.6	7.6	19–25	22	
	E	6603	32	12.3	3.5	10.5			E	2628	28	6.3	2.5	8.9			E	2320	22	4.8	2.2	9.8			
Gentamicin	A	100	23	0.3	0.5	1.9			A	100	22	0.2	0.4	1.6			A	100	18	0.2	0.5	2.7			
	B	100	24	0.5	0.7	2.9			B	100	22	0.8	0.9	4.2			B	100	17	0.7	0.9	5.0			
	C	100	24	1.2	1.1	4.7			C	100	24	1.0	1.0	4.3			C	100	18	0.5	0.7	4.1			
	D	116	23	3.2	1.8	8	19–26	23	D	200	23	2.0	1.4	6.1	19–25	22	D	112	16	1.8	1.3	8.6	12–18	15	
	E	9436	23	4.4	2.1	9.1			E	7058	23	5.3	2.3	9.9			E	2350	17	7.6	2.8	16.7			
Tobramycin	A	100	21	0.4	0.6	2.8			A	100	21	0.3	0.5	2.1			A	100	20	0.2	0.5	2.2			
	B	100	21	0.8	0.9	4.2			B	100	22	1.2	1.1	5.0			B	100	21	1.0	1.0	4.6			
	C	100	22	1.0	1.0	4.3			C	100	23	0.8	0.9	3.7			C	100	23	0.8	0.9	3.9			
	D	120	23	1.7	1.3	5.7	18–26	22	D	199	22	2.0	1.4	6.2	20–26	23	D	134	23	1.8	1.4	6.0	20–26	23	
	E	9231	21	5.3	2.3	10.8			E	6936	22	5.3	2.3	10.2			E	2990	22	10.3	3.2	14.3			
Amikacin	A	100	24	0.2	0.4	1.9			A	100	23	0.3	0.5	2.0			A	100	24	0.1	0.3	1.2			
	B	100	23	0.6	0.8	3.6			B	100	22	0.8	0.9	4.0			B	100	23	1.0	1.0	4.3			
	C	100	24	1.4	1.2	4.8			C	100	23	0.8	0.9	3.8			C	100	24	1.8	1.3	5.5			
	D	117	23	3.6	1.9	8.2	19–26	23	D	150	22	2.0	1.4	6.6	18–24	21	D	134	24	2.5	1.6	6.7	18–26	22	
	E	7819	23	5.8	2.4	10.6			E	3484	22	5.8	2.4	10.9			E	2602	23	10.4	3.2	13.8			

QC, quality control.

Experiment	Measurements (n)	Inhibition zone diameter measurements (n)																																								Median diameter (mm)	Variance (mm ²)	SD (mm)	Coefficient of variation (%)
		6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40									
A	100	0	0	0	0	0	0	0	0	0	0	0	0	6	94	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	20	0.1	0.2	1.2					
B	100	0	0	0	0	0	0	0	0	0	0	0	4	32	45	16	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	20	0.7	0.9	4.3					
C	100	0	0	0	0	0	0	0	0	0	0	0	10	23	28	32	5	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	20	1.3	1.2	5.8						
D	117	0	0	0	0	0	0	0	0	0	2	4	13	13	35	28	14	5	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	20	2.6	1.6	8.0						
E	1389	0	0	0	0	0	0	0	68	99	134	208	391	512	710	710	716	601	430	287	152	82	46	17	0	0	0	0	0	0	0	0	0	0	0	0	21	8.0	2.8	13.4					

Figure 2. Diameter distributions and related summary statistics for Experiments A–E exemplified by *E. coli* and ampicillin. Colours reflect the relative count of strains within a given inhibition zone category for each experiment (A–E) individually: green, no strains; yellow to red, a low number to a high number of strains.

36.5% for *S. aureus* and *E. coli* versus 82.7% for *P. aeruginosa* and 0% for *E. faecalis*. The highest biological contribution was calculated for WT *E. faecalis*, vancomycin and gentamicin (high-load disc) (98.8% and 91.9%, respectively; Table 2) and for penicillinase-producing *S. aureus* and penicillin G (96%). For fluoroquinolones and *S. aureus*, the contribution of biological factors to total variation decreased with increasing potency/generation of the drug (70.6%, 40.7% and 28.0% for norfloxacin, ciprofloxacin and levofloxacin, respectively; Table 2).

The contribution of measurement imprecision from automated zone diameter readings to total population zone diameter variation was low, on average ranging from 1.5% to 5.3% for all drug–species combinations (Table 2). Exceptions were seen in *S. aureus* and cefoxitin, the fluoroquinolones and erythromycin (relative contribution of 9.5%, 11.1% and 21.2%, respectively).

The average contribution of materials, e.g. lot variations in antibiotic discs and Mueller–Hinton agar plates, to total variation ranged from 2.4% to 3.9% for the tested species. Material-related variations were above average for certain drug–species combinations, e.g. proportions of >10% were found for *E. coli*, imipenem, ceftriaxone and cefepime (10.4%–18.3%). In contrast, no contribution of material-related variations was observed for *E. coli* and penicillins, fluoroquinolones, aminoglycosides and tetracyclines (0%). For *P. aeruginosa*, the impact of material variations was particularly high for the carbapenems (imipenem 28.8%).

Overall, manual inoculum preparation and operator-dependent plate streaking were the most important technical factors contributing to diameter measurement variation, ranging from 6.8% to 24.8% and from 6.6% to 24.3%, respectively (Table 2).

Both factors exceeded the biological contribution and were the main contributors to total variation for fluoroquinolones, aminoglycosides and *E. coli* (average of 27.3% and 34.6%, and of 32.2% and 40.2%, respectively; Table 2) and for fluoroquinolones and *E. faecalis* (average of 50.5% and 47.6%; Table 2).

Discussion

Some authors have generally commented on the contribution of technical parameters and biological variability to total AST

variation. However, systematic data on the contribution of individual method-dependent variables to AST variation are rare to non-existent for both disc diffusion and microdilution methods.¹⁶ In this study, a statistical model was constructed to allow separation of the relative influences of technical parameters and biological variation in disc diffusion AST.

Generally, the relative contribution of biological variation was different for individual drug–species combinations, varying from 10.4% to 98.8% (Table 2). Of note, the biological contribution to total diameter variations in *P. aeruginosa* was higher as compared with the other species investigated (average of 81.3% versus 47.0%, 53.9% and 64.0% for *E. faecalis*, *E. coli* and *S. aureus*, respectively; Table 2). This is reflected in the higher variance of *P. aeruginosa* WT populations (Experiment E; Table 1), but equal technical variation parameters for all species (Experiment D; Table 1). These results underline the particularly high variability of AST data in *P. aeruginosa* populations due to intrinsic biological traits affecting various classes of antimicrobials.^{17,18}

The relative contribution of biological factors to total diameter variation of bacterial populations may also reflect the relative number of determinants contributing to innate drug susceptibility. This is exemplified by the fluoroquinolones and *S. aureus*: less potent drugs such as norfloxacin are presumably affected by a variety of comparably weak, unspecific and regulated mechanisms, such as efflux pumps, that are present in the WT (‘genetic background’).¹⁹ Clinical resistance to the more potent drugs, such as ciprofloxacin and levofloxacin, requires mutations in *gyrA/parC*, which are absent in the WT.²⁰ Since a large number of mechanisms may affect innate drug susceptibility levels for the weaker drugs, the relative biological variance may be higher. This hypothesis is paradigmatically reflected in the decreasing relative contribution of biological factors for norfloxacin, ciprofloxacin and levofloxacin and the *S. aureus* WT (70.6%, 40.7% and 28.0%, respectively; Table 2), reflecting different drug potencies in increasing order.

Three principal settings exist considering the relation of technical and biological variability of zone diameter measurements:

- (i) Lower biological variability, higher technical variability. In this setting, it can be desirable to improve precision and reproducibility of measurements; examples from this study are the

Table 2. Relative contribution (%) of individual variation factors to total species WT zone diameter variance

Drug class	Drug	S. aureus						E. faecalis						E. coli						P. aeruginosa					
		reading	imprecision	material variations	inoculum preparation	operator variation	biological variation	reading	imprecision	material variations	inoculum preparation	operator variation	biological variation	reading	imprecision	material variations	inoculum preparation	operator variation	biological variation	reading	imprecision	material variations	inoculum preparation	operator variation	biological variation
Penicillins	penicillin G ^a	0.3	2.9	0.0	0.8	96.0																			
	ampicillin						0.1	11.6	13.3	20.9	54.1	0.7	0.0	15.3	19.7	64.2									
	amoxicillin/clavulanic acid											4.0	0.0	16.7	20.0	59.3									
	piperacillin/tazobactam											0.1	0.0	18.0	21.1	60.8	2.8	0.0	8.6	8.7	79.8				
Cephalosporins	cefalotin											0.0	0.0	8.4	19.9	71.7									
	cefuroxime											1.5	0.0	16.5	18.3	63.7									
	cefoxitin	9.5	0.0	10.5	13.1	66.9						2.9	0.0	28.8	27.9	40.4									
	ceftazidime											0.0	7.6	2.5	5.6	84.4	1.4	0.0	8.1	4.7	85.7				
	cefepodoxime											0.0	4.3	4.7	10.6	80.3									
	ceftriaxone											6.5	18.3	0.0	1.6	73.5									
	cefotaxime											7.7	0.0	7.9	11.4	73.0									
	cefepime											3.3	10.4	0.0	5.7	80.6	1.8	0.9	7.0	5.0	85.2				
Carbapenems	ertapenem											0.5	0.0	22.6	30.6	46.3									
	meropenem											2.2	8.1	15.5	6.5	67.7	2.7	5.8	4.6	8.4	78.4				
	imipenem											8.3	14.0	1.4	0.0	76.4	0.5	28.8	0.0	0.0	70.6				
	average β-lactams	4.9	1.5	5.3	6.9	81.4						2.7	4.5	11.3	14.2	67.3	1.9	7.1	5.7	5.4	80.0				
Glycopeptides	vancomycin						0.8	0.4	0.0	0.0	98.8														
Fluoroquinolones	nalidixic acid											1.5	0.0	37.1	51.0	10.4									
	norfloxacin	14.6	0.0	10.2	4.6	70.6						1.2	0.0	30.1	39.6	29.1									
	ciprofloxacin	11.4	0.0	26.0	22.0	40.7	1.5	0.0	50.2	48.4	0	1.9	0.0	32.8	36.6	28.6	1.1	0.0	9.1	7.3	82.5				
	levofloxacin	7.6	0.0	33.8	30.6	28.0	2.5	0.0	50.7	46.8	0	1.5	0.0	37.1	51.0	10.4	1.3	0.0	8.4	7.4	82.9				
	average fluoroquinolones	11.1	0.0	22.0	17.6	49.3	2.0	0.0	50.5	47.6	0	1.7	0.0	27.3	34.6	36.5	1.2	0.0	8.7	7.4	82.7				
Aminoglycosides	gentamicin	3.6	0.0	12.5	12.5	71.3	0.4	7.6	0.0	0.1	91.9	3.3	0.0	34.0	47.4	15.3	0.0	0.0	6.0	8.9	85.0				
	tobramycin	0.2	3.9	3.4	5.5	87.0						5.0	0.0	34.1	35.7	25.1	2.0	0.0	10.9	7.9	79.3				
	amikacin	2.9	7.3	1.1	0.0	88.8						2.6	0.0	28.5	37.6	31.4	0.8	3.8	4.9	7.7	82.9				
	average aminoglycosides	2.2	3.7	5.7	6.0	82.4						3.6	0.0	32.2	40.2	23.9	0.9	1.3	7.3	8.2	82.4				
Tetracyclines	tetracycline	0.3	10.0	11.4	4.9	73.4						0.7	0.0	34.7	38.0	26.6									
	minocycline	2.8	0.6	7.2	10.8	78.7						0.8	0.0	17.2	21.4	60.7									
	tigecycline	5.2	0.0	47.9	46.8	0.0																			
	average tetracyclines	2.8	5.0	29.7	25.9	36.7						0.8	0.0	25.9	29.7	43.6									
Macrolides/Lincosamides	erythromycin	21.2	0.0	16.3	12.7	49.8																			
	clindamycin	0.5	0.0	26.2	22.9	50.4																			
Sulfa-drugs	trimethoprim-sulfamethoxazole	0.5	18.8	2.2	0.0	78.4						1.6	0.0	26.3	25.7	46.4									
Rifamycins	rifampicin	2.6	0.0	16.4	10.5	70.5																			
Oxazolidinones	linezolid	1.6	0.0	11.0	13.4	74.0																			
Average all drugs		5.3	2.7	14.8	13.2	64.0	1.5	2.4	24.8	24.3	47.0	2.4	2.6	18.4	22.6	53.9	1.4	3.9	6.8	6.6	81.3				

^aThe EUCAST *S. aureus* quality control strain ATCC 29213 harbours the *blaZ* β -lactamase. Biological variation thus does not reflect the WT as in all other drug-species combinations tested, but the regulated penicillinase resistotype. Inhibition zone variability in the same genotype and the related variation in susceptibility categorization is very high due to regulation of gene expression. Therefore, detection of the presence or absence of the regulated resistance mechanism is of critical importance. In the case of *S. aureus* and penicillin G, EUCAST recommends screening for the presence of penicillinase by inspection of the inhibition zone edge. For other important resistance mechanisms, such as ESBL or plasmid-encoded *ampC*, data on the regulation of the resistance mechanism are largely lacking.

fluoroquinolones and the aminoglycosides in *E. coli* or the fluoroquinolones in *S. aureus* (Table 2). Several drug species combinations in this study illustrate specific technical challenges of the disc diffusion method. The influence of drug stability on AST variability is illustrated e.g. by imipenem, a relatively unstable compound, for which materials, such as antibiotic discs, contributed 14% to total AST variation in the *P. aeruginosa* ATCC WT strain (Table 2).²¹ The influence of individual growth characteristics on AST variation is best illustrated by *S. aureus*, cefoxitin, the fluoroquinolones or erythromycin. Staphylococci produce rather fuzzy zone edges that most likely contribute to the higher general reading variation for *S. aureus* (5.3% versus 1.5%–2.4% for the other species; Table 2). In particular, reading variation for cefoxitin, the fluoroquinolones or erythromycin (all producing fuzzy zone edges) was significantly higher (9.5%, 11.1% and 21.2%, respectively; Table 2) than reading variation for the aminoglycosides (average of 2.2%), for which sharp zone edges are usually observed.

(ii) Higher biological variability, lower technical variability. This setting was observed for many drug–species combinations in this study (Table 2). In such settings, practical consequences differ for WT and non-WT populations:

- (a) WT populations. If the biological contribution to AST variability is significantly higher than the technical contribution, the WT population is assumed to be highly variable, as was observed e.g. for *P. aeruginosa* in this study (Table 2). As a consequence of such high intrinsic variability, the risk of random susceptibility categorization increases significantly if the WT population is split by a CBP.⁶ CBPs equal to or lower than the ECOFF will reliably prevent erratic categorization of the WT in settings with higher biological variability and lower technical variability.
- (b) Non-WT populations. If the biological contribution to diameter variability is significantly higher than the technical contribution in a genetically homogeneous non-WT population, CBPs should not split this population for the same reasons that were outlined above for the WT.⁶

If, however, non-WT and WT populations overlap, CBP setting cannot completely avoid splitting non-WT and/or WT populations.²² In this case, screening for the presence or absence of the corresponding resistance mechanisms is necessary to prevent erroneous clinical categorization resulting from biological variation. For example, the EUCAST *S. aureus* quality control strain ATCC 29213 is non-WT to penicillin G since it harbours the regulated *blaZ* β -lactamase.⁹ The biological contribution to total methodological variation is very high (96.0%) due to high inhibition zone variability (ranging from 6 to 36 mm) for the same genotype (*blaZ*+, MRSA strains were excluded). The EUCAST CBP for *S. aureus* and penicillin is set at 26 mm, splitting this *blaZ*+ population. The detection of the presence or absence of the regulated resistance mechanism (*blaZ*) is, however, of critical importance as an *in vitro* penicillin G-susceptible *blaZ*+ *S. aureus* isolate may become resistant *in vivo* due to up-regulation of gene expression.²³ In the case of *S. aureus* and penicillin G, EUCAST thus recommends screening for the presence of penicillinase by inspection of the inhibition zone edge for all isolates that display inhibition zones in the susceptible range. Unfortunately,

data on the regulation of other important resistance mechanisms, such as ESBL or plasmid-encoded *ampC*, are widely lacking, and most likely biological variation necessitates a requirement for detection of an ESBL, AmpC or carbapenemase resistance mechanism similar to that described above for *S. aureus* and *blaZ*.

- (iii) If the relative contribution of technical and biological variation is comparable, erroneous clinical categorization depends equally on technical and biological factors. Improving technical precision and detection of resistance mechanisms—at least for the regulated and highly variable ones—is necessary to improve the quality of AST reporting.

In summary, the higher the biological contribution to zone diameter variability of a genetically homogeneous population, the more important it is not to split this population into different clinical categories and to determine the presence or absence of resistance mechanisms, as otherwise erratic categorizations will inevitably occur, even if measurements are technically very precise and reproducible. The present work illustrates the species–drug combination-specific biological contribution to zone diameter variability. Thus, these data may facilitate reliable CBP setting.

Furthermore, the present work illustrates the relative contribution of factors, such as inoculum preparation and operator skills, that account for a significant part of technical variance in disc diffusion AST. As technical imprecision contributes significantly to AST classification errors, further improvement of AST precision and reproducibility will affect the reliability of AST reports with respect to the forecast probability of the predicted therapeutic success or failure of individual drug–species combinations as reflected in clinical AST categories (susceptible versus resistant).^{6,14} The highest relative contribution to technical variation originated from operator influence, i.e. mainly inter-operator variations in plate streaking, followed by inoculum preparation and materials. The laboratory cannot influence factors such as the precision of disc charging or agar composition to improve current AST. However, further standardization of operator influence and inoculum preparation by mechanization and automation could potentially increase the precision of disc diffusion AST, as has previously been shown for zone diameter readings.⁵

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None to declare.

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