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Standardization of Operator-Dependent Variables Affecting Precision and Accuracy of the Disk Diffusion Method for Antibiotic Susceptibility Testing

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Parameters like zone reading, inoculum density, and plate streaking influence the precision and accuracy of disk diffusion antibiotic susceptibility testing (AST). While improved reading precision has been demonstrated using automated imaging systems, standardization of the inoculum and of plate streaking have not been systematically investigated yet. This study analyzed whether photometrically controlled inoculum preparation and/or automated inoculation could further improve the standardization of disk diffusion. Suspensions of *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 of 0.5 McFarland standard were prepared by 10 operators using both visual comparison to turbidity standards and a Densichek photometer (bioMérieux), and the resulting CFU counts were determined. Furthermore, eight experienced operators each inoculated 10 Mueller-Hinton agar plates using a single 0.5 McFarland standard bacterial suspension of *E. coli* ATCC 25922 using regular cotton swabs, dry flocked swabs (Copan, Brescia, Italy), or an automated streaking device (BD-Kiestra, Drachten, Netherlands). The mean CFU counts obtained from 0.5 McFarland standard *E. coli* ATCC 25922 suspensions were significantly different for suspensions prepared by eye and by Densichek ($P < 0.001$). Preparation by eye resulted in counts that were closer to the CLSI/EUCAST target of 10^8 CFU/ml than those resulting from Densichek preparation. No significant differences in the standard deviations of the CFU counts were observed. The interoperator differences in standard deviations when dry flocked swabs were used decreased significantly compared to the differences when regular cotton swabs were used, whereas the mean of the standard deviations of all operators together was not significantly altered. In contrast, automated streaking significantly reduced both interoperator differences, i.e., the individual standard deviations, compared to the standard deviations for the manual method, and the mean of the standard deviations of all operators together, i.e., total methodological variation.

The basic parameters of disk diffusion antibiotic susceptibility testing (AST), such as agar type, agar depth, incubation conditions, the reading endpoint, and the ideal inoculum density, have been increasingly standardized in the past (1, 2). Current CLSI and EUCAST recommendations offer detailed protocols for these basic aspects that provide a high level of standardization (3, 4). While improved reproducibility of zone diameter reading has been demonstrated using software-guided imaging systems instead of visual reading by eye, other factors causing AST variation, such as inoculum preparation or agar plate streaking, both performed by human operators, are more difficult to standardize (5).

Systems for standardizing the inoculum for disk diffusion AST have been evaluated previously (6, 7). The results were reported to be comparable but not superior to the standard method, i.e., visual adjustment of the inoculum solution to 0.5 McFarland standard turbidity. Systematic studies evaluating the impact of automated agar plate streaking on the results of AST are, however, rare despite recent articles emphasizing the potential role of full laboratory automation for the standardization of diagnostic microbiology (8–10).

AST methods are used to generate individual treatment recommendations (the AST report) for clinicians. Methodological variation contributes to categorial errors and, thus, erroneous treatment of patients (11, 12). Therefore, further improvement in the reproducibility and precision of disk diffusion AST will lead to more appropriate AST reports and related treatment recommendations.

Having shown recently that operator-dependent factors contribute significantly to the zone diameter variation of disk diffusion AST results, we here evaluated whether photometrically controlled inoculum preparation, inoculation with flocked swabs, and automated inoculation of agar plates could further improve the standardization of disk diffusion testing (13).

MATERIALS AND METHODS

Bacterial strains. *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 were used in this study. These strains represent the EUCAST and CLSI quality control (QC) strains for AST.

Susceptibility testing. Susceptibility testing was done by disk diffusion according to EUCAST recommendations (4). In brief, preparation of a 0.5 McFarland standard inoculum from overnight cultures was followed by incubation of Mueller-Hinton agar plates at 35°C in ambient air. All Mueller-Hinton agar plates were incubated in the fully automated Sirscan system, comprising an automated incubator and automated reading equipment. Incubation time was fixed to 16 h. Antibiotic disks (see Table 2 for the compounds tested) were obtained from i2a, Montpellier, France,

Received 28 August 2015 Returned for modification 26 September 2015
Accepted 7 October 2015

Accepted manuscript posted online 14 October 2015

Citation Hombach M, Maurer FP, Pfiffner T, Böttger EC, Furrer R. 2015. Standardization of operator-dependent variables affecting precision and accuracy of the disk diffusion method for antibiotic susceptibility testing. J Clin Microbiol 53:3864–3869. doi:10.1128/JCM.02351-15.

Editor: N. A. Ledeboer

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and Mueller-Hinton agar was obtained from Becton Dickinson, Franklin Lakes, NJ. Inhibition zone diameters were recorded using the Sirweb/Sirscan system (i2a) to standardize reading precision (5).

Operator influence on inoculum standardization. Suspensions of *E. coli* ATCC 25922 and *S. aureus* ATCC 29213 of 0.5 McFarland standard turbidity were prepared by 10 experienced operators using colonies from overnight growth on Columbia sheep blood agar (bioMérieux, Marcy l'Etoile, France) by visual comparison of the test tubes to freshly prepared and vortexed 0.5 McFarland turbidity standards manufactured according to EUCAST guidelines and by using a calibrated Densichek instrument (bioMérieux) (4). To assist visual inoculum adjustment, bacterial suspensions were compared to McFarland standards against a white background with black lines (i.e., a Wickerham card) (14). All preparations and measurements were done in triplicate. Immediately after preparing the 0.5 McFarland standard bacterial suspensions using both methods, the absorbance at 600 nm was measured using an Eppendorf BioPhotometer plus (Eppendorf AG, Hamburg, Germany). In addition, inoculum concentrations were determined by diluting bacterial suspensions 100,000-fold (100 μl into 900 μl of a 0.85% NaCl solution five times) and subsequent plating of 100 μl of the final dilution onto Columbia sheep blood agar (bioMérieux) within 15 min from the initial preparation. Plates were incubated for 24 h at 35°C, and colonies were counted manually. The number of CFU per milliliter of bacterial suspension was calculated by multiplying colony counts by 10^6. The same lots of media and disks were used for the experiments.

Operator influence on agar plate inoculation. A single 0.5 McFarland standard bacterial suspension was prepared from an overnight culture of *E. coli* ATCC 25922 according to EUCAST recommendations (4). This suspension was split between 8 experienced operators, each of whom inoculated 10 square Mueller-Hinton II agar plates (12 by 12 cm; Becton Dickinson) according to EUCAST recommendations using regular cotton swabs (Paul Hartmann AG, Heidenheim, Germany). Antibiotic disks were obtained from i2a and were applied to the agar within 15 min from streaking, the plates were incubated as recommended by EUCAST, and inhibition zone diameters were recorded as described above. This experiment was repeated twice, but instead of cotton swabs, the same operators either used regular, dry, flocked swabs (product number 519CS01; Copan, Brescia, Italy) or applied 50 μl of the bacterial suspension to the agar plates and used the InoqulA BT automated inoculation device (BD-Kiestra, Drachten, Netherlands) for spreading of the inoculum. The same lots of media and disks were used for the experiments.

Statistical calculations. Student's *t* test was used to test for statistically significant differences in CFU counts obtained from 0.5 McFarland standard bacterial suspensions prepared by eye using visual comparison to a reference solution and by the Densichek instrument. The Levene variance homogeneity test with the Brown-Forsythe modification (Levene/Brown-Forsythe test) for homogeneity was used to test for significant differences in individual operator standard deviations (SDs) on a 95% probability level ($P < 0.05$ was considered significant). The Kolmogorov-Smirnov and Shapiro-Wilk tests were applied to test for normal distribution of measured values on a 95% probability level.

Software. All calculations were done using IBM SPSS statistics software, version 20 (IBM Corporation, Armonk, NY), and Microsoft Excel 2010 software (Microsoft Corporation, Redmond, WA).

RESULTS

No difference was found between the mean CFU counts of *S. aureus* ATCC 29213 prepared by eye and using Densichek ($P = 0.900$) (Table 1). For *E. coli* ATCC 25922, the mean CFU counts ± standard deviations were $(0.94 \pm 0.18) \times 10^8$ CFU/ml when density was adjusted by eye and $(0.62 \pm 0.20) \times 10^8$ CFU/ml with the Densichek instrument, a significant difference ($P < 0.001$). As EUCAST and CLSI recommend a general inoculum of 1×10^8 CFU/ml for disk diffusion AST in their current guidelines, preparation by eye achieved results that were closer to the target value than those of preparation using Densichek (15, 16). To what extent these differences will influence actual diameter values and clinical categorization, however, cannot be derived from the results of this study.

TABLE 1 Comparison of absorbance values and CFU counts obtained from 0.5 McFarland standard bacterial suspensions prepared by visual comparison to a reference solution or using the Densichek instrument[a]

Bacterium, parameter	Value obtained from suspension prepared using:			
	Eye		Densichek	
	A_{600}	CFU/ml	A_{600}	CFU/ml
S. aureus ATCC 29213				
Mean	0.11	0.20×10^8	0.10	0.18×10^8
SD	0.02	0.12×10^8	0.01	0.10×10^8
E. coli ATCC 25922				
Mean	0.15	**0.94×10^8**	0.12	**0.62×10^8**
SD	0.03	0.18×10^8	0.02	0.20×10^8

[a] Levene's test of equality of standard deviations was applied to test for significant differences in standard deviations of absorbance and CFU counts. The Mann-Whitney test was applied to test for differences in mean CFU counts. *P* values equal to or smaller than 0.05 were considered statistically significant. Values that are significantly different are in boldface.

No significant differences were observed between SDs of absorbance values or CFU counts obtained from bacterial suspensions of *E. coli* ATCC 25922 or *S. aureus* ATCC 29213 adjusted to 0.5 McFarland standard by eye or by the Densichek instrument (using Levene's test of equality of standard deviations for eye versus Densichek, $P = 0.371$ and $P = 0.420$ for *E. coli* and *S. aureus*, respectively) (Table 1).

All mean inhibition zone diameters of repetitive measurements for *E. coli* ATCC 25922, with the exception of the results for meropenem and manual streaking, were in the EUCAST quality control (QC) range irrespective of the inoculation method, i.e., cotton swabs, flocked swabs, or automated streaking (Table 2). However, the percentages of individual measurements that were situated in the EUCAST QC range showed differences, increasing from 92.0% of average measurements in the QC range for manual streaking with cotton swabs to 93.3% for manual streaking with flocked swabs and 98.1% for automated streaking (Table 2). The average SD of measurements was lower (0.93 mm) for the automated streaking instrument than for inoculation using cotton swabs (1.19 mm) and flocked swabs (1.11 mm).

Changes in inhibition zone diameter SDs were further analyzed with respect to individual drug classes. The SDs generally decreased for both the flocked swab and automated streaking compared to those for cotton swabs. However, for the flocked swab, this effect was statistically significant only for beta-lactam inhibitor combinations (amoxicillin-clavulanic acid and piperacillin-tazobactam, SD decrease of 21.6%), whereas for the automated streaking instrument, significant decreases of 12.5% to 33.9% were found for 6 of 9 drug classes (penicillins, beta-lactam inhibitor combinations, cephalosporins, aminoglycosides, fluoroquinolones, and colistin) (Table 3). No significant changes in SDs were observed for the carbapenems (ertapenem, meropenem, and imipenem), tetracyclines (tetracycline and minocycline), or trimethoprim-sulfamethoxazole for either flocked swab or auto-

TABLE 2 Drug-specific mean measurements and standard deviations of inhibition zone diameters and percentages that are within EUCAST quality control ranges for *E. coli* ATCC 25922

Antibiotic	EUCAST QC range (mm)	Value (mm unless % is indicated) for measurements of inhibition zone diam obtained using:											
		Cotton swab				Flocked swab				Automated streaking			
		Range	% of measurements in range	Mean	SD	Range	% of measurements in range	Mean	SD	Range	% of measurements in range	Mean	SD
Ampicillin	16–22	18–23	99	20.1	1.16	19–23	98	20.4	0.93	18–23	99	20.6	0.83
Amikacin	19–26	21–27	98	24.3	1.18	23–27	92	25.2	0.93	22–25	100	23.6	0.72
Amoxicillin-clavulanic acid	18–24	21–26	84	23.6	0.97	22–25	96	23.3	0.73	21–24	100	22	0.77
Cephalothin	—[a]			18.8	1.42			18.3	1.33			18.9	1.1
Cefepime	31–37	30–37	99	33.3	1.37	30–37	99	33.9	1.16	31–37	100	33.4	1.23
Cefotaxime	25–31	25–31	100	28.7	1.22	26–31	100	28.6	0.86	26–29	100	27.6	0.75
Cefoxitin	23–29	25–29	100	27.2	0.9	25–29	100	27.2	0.91	25–28	100	26.8	0.84
Cefpodoxime	23–28	22–28	99	25.2	1.2	24–28	100	25.4	0.88	23–26	100	24.2	0.82
Ceftazidime	23–29	24–30	99	26.9	1.1	26–30	98	27.3	1.02	25–30	99	26.7	0.97
Ceftriaxone	29–35	28–35	98	31.1	1.42	28–35	100	31.3	1.33	28–33	100	30.5	1.15
Cefuroxime	20–26	22–26	100	24	0.97	22–26	100	23.9	0.88	21–25	100	22.9	0.67
Ciprofloxacin	30–40	30–38	100	35.1	1.7	34–40	100	38.4	1.7	33–38	100	36.1	1.02
Colistin	—			15.7	0.72			15	0.71			14.9	0.63
Ertapenem	29–36	30–37	99	33	1.47	29–36	100	32.5	1.38	28–34	100	31.4	1.38
Gentamicin	19–26	22–27	99	24	1.14	23–27	98	24.6	0.87	22–26	100	23.9	0.67
Imipenem	26–32	29–37	54	33.3	1.41	28–34	85	30.9	1.47	26–35	84	30.9	1.85
Levofloxacin	29–37	30–36	100	33.3	1.26	32–40	80	36.1	1.54	32–37	100	33.9	1.01
Meropenem	28–34	32–38	41	35.6	1.29	30–36	89	33	1.2	30–34	100	31.9	1.07
Minocycline	—			23.5	1.03			23.5	1.14			23.3	0.78
Nalidixic acid	22–28	25–31	64	28	1.38	25–31	55	28.2	1.41	25–30	79	27.7	0.96
Norfloxacin	28–35	30–36	99	32.4	1.36	31–39	70	34.8	1.72	31–35	100	32.5	0.83
Piperacillin-tazobactam	21–27	22–26	100	24.3	0.97	22–26	100	24.3	0.78	23–26	100	24.2	0.72
Tetracycline	—			25.8	0.9			24.9	0.9			23.7	0.78
Tobramycin	18–26	20–24	100	22	0.95	21–25	100	23.3	0.92	20–24	100	22.3	0.77
Trimethoprim-sulfamethoxazole	23–29	24–29	100	25.6	1.16	24–29	100	26.6	1.14	24–29	100	26.5	1.04
Avg			92	27	1.19		93.3	27.2	1.11		98.1	26.4	0.93

[a] —, not available.

TABLE 3 Drug class-specific changes in standard deviations of inhibition zone diameter measurements[a]

Parameter	SD of inhibition zone diam measurements (mm) or % change in SD for:								
	Penicillins	Inhibitor combinations	Cephalosporins	Carbapenems	Aminoglycosides	Quinolones	Tetracyclines	Trimethoprim-sulfamethoxazole	Colistin
SD using standard manual method[b]	1.16	0.97	1.2	1.39	1.09	1.42	0.96	1.16	0.72
Change in SD using flocked swabs	−0.23	**−0.21**	−0.15	−0.04	−0.18	0.17	0.06	−0.02	−0.01
% change using flocked swabs	−19.8	**−21.6**	−12.5	−2.9	−16.5	12.0	6.3	−1.7	−1.4
Change in SD using automated streaking	**−0.33**	**−0.22**	**−0.26**	0.04	**−0.37**	**−0.46**	−0.18	−0.12	**−0.09**
% change using automated streaking	**−28.4**	**−22.7**	**−21.7**	2.9	**−33.9**	**−32.4**	−18.8	−10.3	**−12.5**

[a] Inhibition zone diameter values for all drug classes showed normal distributions (Kolmogorov-Smirnov and Shapiro-Wilk tests, $P < 0.001$). The Levene/Brown-Forsythe test was applied to test for homogeneity of SDs. *P* values equal to or smaller than 0.05 were considered statistically significant. SDs that are significantly different are in boldface.

[b] The manual method using regular cotton swabs, representing the currently accepted standard, was the comparator method.

mated streaking compared to the SDs for cotton swab inoculation (Table 3).

The range (i.e., heterogeneity) of standard deviations for individual operators decreased significantly with the flocked swab system compared to the range of SDs for cotton swabs (Levene/Brown-Forsythe test for homogeneity, $P = 0.011$). However, the mean standard deviations for all operators were not significantly different when the flocked swab system and cotton swabs were used ($P = 0.780$). In contrast, using automated streaking, both the range of individual operator standard deviations ($P = 0.033$) (Fig. 1) and the mean standard deviation for all operators decreased significantly compared to the individual ranges and overall means of SDs for the manual methods ($P = 0.005$).

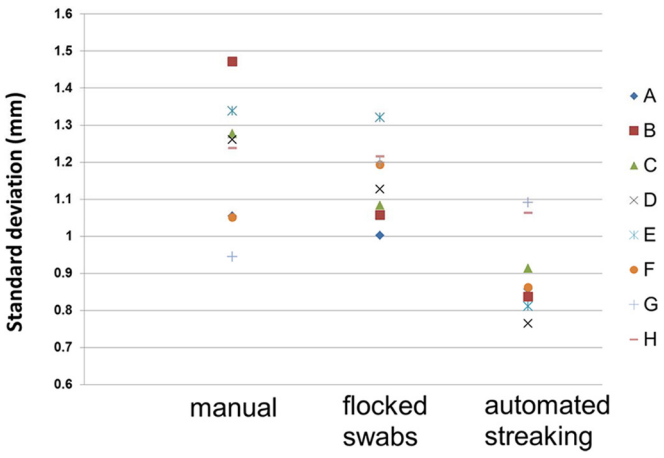

FIG 1 Ranges of standard deviations for inhibition zone diameters from 10 independent replicate measurements by eight operators (A to H) for all drugs on average using the three inoculation methods. The Levene/Brown-Forsythe test for homogeneity was applied to test for significant differences in individual operator standard deviations. *P* values equal to or smaller than 0.05 were considered statistically significant. The range (i.e., heterogeneity) of individual operator SDs significantly decreased with the flocked swab method ($P = 0.011$). The mean SD for all operators obtained using the flocked swab method was not significantly different from that obtained using the manual method ($P = 0.780$). With the use of the automated streaking method, both the range of individual operator SDs ($P = 0.033$) and the mean SD for all operators ($P = 0.005$) significantly decreased compared to those obtained using the manual method.

DISCUSSION

Operator skills, e.g., with respect to inoculum preparation and plate inoculation, account for a significant part of the methodological variation in disk diffusion AST (13). Methodological variation influences the precision and reproducibility of measurements and, thus, affects the reliability of AST reports in terms of the predicted therapeutic success or failure of individual drugs. Even a 1-fold standard deviation of 1 mm to 1.5 mm can cause significant major and very major error rates, in particular for problematic bacterial populations like extended-spectrum β-lactamase (ESBL)- or AmpC-positive isolates (12). In particular, for settings with clinical breakpoints of species-drug combinations for which intermediate zones have been abandoned or with narrow intermediate zones, the cumulated probabilities of major and very major error rates can reach a critical level (11). In consequence, further improvements regarding standardization of the disc diffusion method are desirable. We have shown in a previous work that highly standardized zone diameter readings can be obtained using an automated zone reader (5). Hence, the impact of reading imprecision on the total methodological variation of the disk diffusion method can be reduced to a negligible proportion. The highest relative contributions to methodological variation originate from operator influence (mainly plate inoculation, followed by inoculum preparation) and the quality of consumables, such as agar plates and antibiotic disks (13). Standardization of consumables for disk diffusion testing cannot be influenced by the laboratories but remains a responsibility of the manufacturers.

In the present study, we questioned, however, whether the operator influence on inoculum preparation and plate inoculation could be further standardized. Early studies indicated that bacterial growth that is not confluent but shows densely grouped colonies may be ideal for disk diffusion reproducibility. However, this approach would require different seeding densities for individual species, e.g., 6,400 to 7,500 CFU/cm^2 for *S. aureus* or 1,700 CFU/cm^2 for *E. coli* (2). Therefore, Ericsson and Sherris decided to recommend an inoculum of 1×10^8 CFU/ml, which leads to confluent growth for virtually all species (2). Both EUCAST and CLSI have based their current guidelines on this recommendation (15, 16). To achieve confluent growth, preparation of a 0.5 McFarland standard bacterial suspension, preferably by using a photometric device, is recommended, although visual comparison with 0.5

McFarland standards is accepted and is still widely practiced (15, 16). It has to be noted that a 0.5 McFarland standard suspension will result in 10^8 CFU/ml for *E. coli* but not for other species, such as *S. aureus*. In this study, inoculum preparation using a photometric device (the Densichek instrument) was not more accurate than inoculum preparation by visual comparison of the test tube to a 0.5 McFarland turbidity standard in front of a Wickerham card, i.e., a white background with black lines (Table 1): the nonphotometric preparation resulted in a mean inoculum density of 0.94×10^8 CFU/ml for *E. coli*, closer to the proposed EUCAST and CLSI target value of 1.0×10^8 CFU/ than the mean inoculum density found for the photometrically adjusted suspension (0.62×10^8 CFU/ml) (Table 1). Furthermore, the variability in CFU counts displayed no significant differences between the counts resulting from Densichek and from visual preparation (Levene's test of equality of standard deviations for *E. coli* and *S. aureus* for eye versus Densichek, $P = 0.371$ and $P = 0.420$, respectively) (Table 1). Therefore, the contribution of inoculum variability to total methodological variation of the disk diffusion AST method could not be reduced by photometric measurements using the Densicheck instrument.

Improvement of the plate inoculation procedure was achieved using both flocked swabs and automated streaking instead of regular cotton swabs. Most importantly, following inoculation using either flocked swabs or automated streaking, the mean zone diameters for a broad panel of antibiotics all remained within the EUCAST quality control ranges for *E. coli* ATCC 25922 (Table 2). The use of flocked swabs significantly decreased interoperator heterogeneity with respect to individual standard deviations ($P = 0.011$) but did not improve the mean of the standard deviations of all operators ($P = 0.780$). In contrast, agar plate inoculation using automated streaking led to a significant decrease of both the interoperator heterogeneity with respect to individual standard deviations ($P = 0.005$) and the mean of the standard deviations of all operators ($P = 0.033$) (Fig. 1). Regarding the SDs of individual zone diameter measurements, the flocked swab inoculation method could significantly reduce the SD for only one of nine drugs belonging to different classes (SD reduction of 21.6% for beta-lactam inhibitor combinations; $P < 0.05$) (Table 3). Automated inoculation significantly reduced the SDs of individual zone diameter measurements for six of nine drugs compared to the SDs of measurements obtained using cotton swabs (SD reductions of 12.5% to 33.9%). Furthermore, improved standardization when applying automated streaking was mirrored in higher numbers of individual measurements situated in the EUCAST QC range than were obtained with manual streaking (92.0%, 93.3%, and 98.1% of individual measurements in QC range for manual streaking, manual streaking with flocked swabs, and automated streaking, respectively) (Table 2).

For this study, we used EUCAST QC ranges and methodological recommendations. However, the results may apply similarly to CLSI standards, as the CLSI recommendations concerning inoculum preparation from overnight cultures, incubation conditions, agar plate inoculation, disk application, and incubation times are essentially the same.

A limitation of this study is the use of ATCC strains that depict an idealized model for variation studies. Clinical strains will, most likely, yield more variability. However, the advantage of such highly characterized strains is their comparability both for the existing QC/variation ranges of EUCAST and CLSI and for interlaboratory purposes.

To summarize, photometrically controlled preparation of bacterial suspensions equal to 0.5 McFarland standard turbidity did not decrease variation in CFU counts, and therefore, photometrically controlled preparation using the Densicheck instrument did not decrease the contribution of inoculum preparation to the total methodological variation of the disk diffusion method. The use of flocked swabs for inoculation of disk diffusion agar plates can help to standardize AST results among a heterogeneously skilled group of operators in an individual clinical laboratory by significantly decreasing interoperator variations. However, the use of flocked swabs will not decrease the mean standard deviation of an individual clinical laboratory. The use of an automated agar plate streaking device has the potential to further standardize AST results by decreasing the total methodological variation.

ACKNOWLEDGMENTS

We thank the team of the bacteriology laboratory for technical assistance, and we are much obliged to Forouhar Mouttet for proper statistical analyses.

This work was supported by the University of Zurich.

All authors have no conflicts of interest to declare.

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