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Variation of MIC measurements: the contribution of strain and laboratory variability to measurement precision

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Objectives: Although testing of antimicrobial agents for susceptibility has inherent variability like any assay, it is generally held that there are also real differences in susceptibility between strains. In the routine laboratory, variability of the MIC measurement may be sufficient to mask real strain differences. We determined which factors contributed to the variability, using linezolid against *Staphylococcus aureus* as one example.

Methods: Twenty-five *S. aureus* strains were sent to five different laboratories in quadruplicate in a blinded fashion. Laboratories determined MICs of linezolid using Etest. Results of 22 strains corresponding to 440 observations were available for analysis. Sources of variability were explored and quantified using an ANOVA approach.

Results: The overall geometric mean MIC was 1.8 mg/L, comparable to that of the published WT distribution of 1.7 mg/L (www.eucast.org). The total variation amounted to ~1.3 2-fold dilutions for a one-sided CI of 95% and two 2-fold dilutions for a CI of 99%. Variation between laboratories and variation between strains contributed 10% and 48%, and in a subset analysis averaging 17% and 26%, respectively. Strain-to-strain variation (biological variation) could not be reliably determined, even with four replicates.

Conclusions: This analysis serves as an example of an approach to discerning various sources of MIC variation. Here, at best, a single measurement of an MIC may provide an indication of whether it likely belongs to the WT distribution. Only repeated measurements of MICs for individual strains within one laboratory may provide an indication of differences in susceptibility between strains.

Introduction

When the susceptibilities of a large number of strains of a single species to an antimicrobial agent are determined, the resulting WT MIC distribution has been shown to follow a log-normal distribution[1–3] or a normal distribution for disc diffusion zone diameters.[4–6] Many MIC distributions can be found on the EUCAST web site (www.eucast.org) and are specific for each drug–species combination. Most of the WT distributions extend over four to five 2-fold dilutions. One feature that is used to describe these distributions is the epidemiological cut-off. This is the MIC value that represents the 'upper end' of the WT distribution.

The WT distribution of a specific drug–species combination, as presented on the EUCAST web site, is a compilation of MIC distributions from multiple sources. Each of these distributions also has a log-normally distributed WT population, but locations of these distributions are often slightly different. Differences are observed even when distributions from different laboratories of a single strain (e.g. a quality control strain) are compared. In other words, WT distributions encompass both variation between strains (biological variation)[7] and variation between measurements (assay variation within and between laboratories).

The exact sources of the variation and the relative contributions from the various sources are not well-characterized and few studies have looked into this issue.[8] The most important factors in assay variation include inter-laboratory variation[9] and intra-laboratory variation to which biological variation is added.[10] All of these contribute to overall variability and can be driven by random and systematic error. The error rate will also differ by laboratory (the term error is used here rather than bias or precision because the MIC is the reference for susceptibility testing).[11] It is important

Table 1. Linezolid susceptibility characteristics of the *S. aureus* total dataset and subset 1 (non-GISA) and subset 2 (GISA)

	Strains (*n*)	Isolates (*n*)	Observations (*n*)	Mean $\log_2$ MIC (mg/L)	Geometric mean MIC (mg/L)	SD $\log_2$ MIC (mg/L)	SEM $\log_2$ MIC (mg/L)
Total	22	88	440	0.86	1.82	0.72	0.034
Subset 1	10	40	200	1.29	2.45	0.61	0.043
Subset 2	12	48	240	0.51	1.42	0.60	0.039

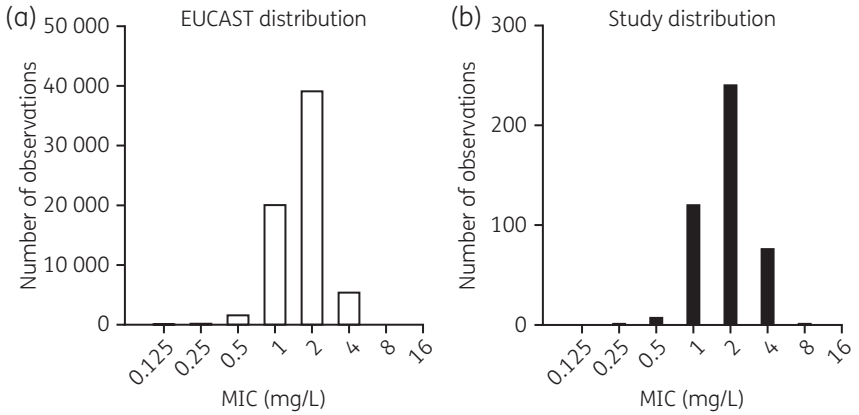

Figure 1. Frequency distribution of MIC values from EUCAST (a) and this study (b).

to understand and distinguish between these factors for interpretation of MICs when measured in the routine laboratory.

Increasingly, individual dosing regimens are designed to reach certain pharmacodynamic targets, especially in the critically ill.[12,13] These targets are unified by the MIC and the value of the MIC therefore has a large impact on the value of the pharmacodynamic determinant of efficacy, such as *f*AUC/MIC or $\%fT_{>MIC}$, especially since the MIC is measured on a logarithmic interval scale.

In the present study, we attempted to separate various sources of variation in MIC measurements using linezolid and *Staphylococcus aureus* as an example. Strains were sent to five different laboratories in a blinded manner in quadruplicate. The MIC distribution was subsequently analysed using a classical ANOVA approach and the sources of variability explored and quantified.

Methods

The study was conducted in five clinical microbiology laboratories in the Netherlands. All study materials were supplied by one central laboratory. Each laboratory received, in a blinded fashion, 100 serially numbered isolates of 25 unique strains comprising 10 MRSA, 6 glycopeptide intermediately susceptible *S. aureus* (GISA) and 9 heterogeneous GISA (hGISA) in quadruplicate. The characteristics of these strains have been described and confirmed in full detail elsewhere.[14] The laboratories were unaware of the glycopeptide and linezolid phenotype and unaware that they had received the same strain in quadruplicate. In retrospect, three strains were excluded from the study because screening results were inconsistent with their original glycopeptide resistance phenotype. A total of 440 observations remained for the present analysis.

Testing of linezolid was performed using Etest (AB BIODISK, Solna, Sweden) on Mueller–Hinton agar (Becton Dickinson, Cockeysville, MD, USA) as recommended by the manufacturer. MIC values were rounded up where necessary to the standard 2-fold dilution series for initial analysis. Analyses were performed using the GLM, UNIVARIATE, TTEST and NPAR1WAY procedures in SAS software.[15] For statistical evaluations, all data were log-transformed (base 2; expressed as $\log_2$ MIC) and arithmetic means of these presented. EUCAST linezolid distributions were taken from the EUCAST web site.[16]

Results

Characteristics of test MIC distribution and similarity with the EUCAST MIC distribution

Table 1 shows the characteristics of the total MIC distribution of the 22 strains. Figure 1(a and b) shows the linezolid MIC distributions as displayed on the EUCAST web site and from the present study, respectively. The shape of the two distributions appears to be very similar. The arithmetic means of the log-transformed MICs and coefficients of variation (CVs) of the distributions were 0.86 and 76.7% for the dataset and 0.72 and 69.2% for the EUCAST distribution. Thus, the difference in means was minor, at 0.14 $\log_2$. However, although the shapes of the distributions were similar and there seemed to be no difference between the two distributions by eye, they did differ (Kolmogorov–Smirnov two-sample test, $P = 0.0015$) and the means were significantly different statistically (*t*-test, $P < 0.001$). This may be attributed in part to the fact that the EUCAST-published pooled distribution includes distributions generated by a range of test methods, rather than just Etest.

Sources of variability

Two main sources of variability (error) were considered: strain-to-strain variation and variation between laboratories. Table 2 provides an overview of the MIC frequency distributions for the main sources of variation and indicates there was significant

Table 2. Distribution of MICs over laboratories (reflecting differences in laboratory assessments) and strains (reflecting intra- and inter-laboratory differences in MIC assessment)

	Laboratory (occurrences)					Strain code (occurrences)																				
MIC (mg/L)	1	2	3	4	5	A	b	c	D	e	f	h	i	j	K	L	M	N	O	P	Q	r	S	t	u	x	z	Total
0.25	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1
0.5	4	2	0	1	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	7
1	36	29	12	26	17	0	5	6	0	12	14	3	14	11	0	3	1	0	3	1	1	11	1	2	8	7	17	120
2	44	41	52	55	44	16	15	13	10	7	1	13	6	9	8	14	15	7	14	13	10	9	12	18	12	13	1	236
4	4	15	23	6	27	4	0	1	10	1	0	4	0	0	11	3	4	13	2	6	9	0	7	0	0	0	0	75
8	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1
Total	88	88	88	88	88	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	440
Mode	2	2	2	2	2	2	2	2	2	1	1	2	1	1	4	2	2	4	2	2	2	1	2	2	2	2	1	2
Geometric mean	1.46	1.70	2.22	1.68	2.16	2.30	1.68	1.68	2.83	1.37	0.87	2.07	1.23	1.37	3.14	2.00	2.22	3.14	1.74	2.38	2.64	1.37	2.46	1.87	1.52	1.57	0.97	1.82
Range	4	5	4	4	3	2	2	3	2	3	3	3	2	2	3	3	3	2	5	3	3	2	3	2	2	2	3	6

Strains indicated with upper case letters belong to subset 1 (non-GISA) and strains indicated with lower case letters belong to subset 2 (GISA).

between-laboratory variation and between-strain variation in the assessments of MICs. A two-way ANOVA was performed initially to quantify the sources of variability. Table 3 provides the main results of this analysis. For the total dataset of 440 observations, more than 58% of the variation could be explained and close to half of the total variation was attributed to strain-to-strain differences. Around 10% of the total variability could be explained by differences between laboratories. Since differences between strains appeared to be a major factor explaining total variation, we first explored whether glycopeptide phenotype was an important factor. This appeared to be the case—of the variation observed due to strain-to-strain variability, 61% could be attributed to differences in glycopeptide phenotype. Therefore, an ANOVA was performed to determine whether there were significant differences in linezolid susceptibility between MRSA, hGISA and GISA strains. This showed a significant difference between the three groups ($F = 91.2$, $df = 2$, $P < 0.001$). Differences between hGISA and GISA were not significant and were therefore pooled for further analysis. A separate analysis for each of the five laboratories confirmed these results (data not shown). In the remaining analyses, two subsets were therefore distinguished; MRSA isolates are referred to as non-GISA (subset 1) and hGISA/GISA isolates as GISA (subset 2).

Table 1 shows the MIC characteristics of the two subsets of isolates. The data indicate that there were significant differences in $\log_2$ means between the two subsets and amounted to slightly less than one 2-fold dilution, but their standard deviations were almost identical. However, there was considerable variation between measurements within each subset. In the next two sections the sources of the variation are explored further.

Sources of error

For each of the two subsets a two-way ANOVA was performed to further explore sources of variation with an emphasis on strain-to-strain variability and variability between laboratories. Table 3 provides the main results of this analysis for both subsets. For subset 1 (non-GISA), slightly more than one-third of the variability could be explained by variation between laboratories and variation between strains. Thus, almost two-thirds (sum of squares of 48.27 out of 75.18 in the table) was assay variation. For subset 2 (GISA), assay variation was around half the variation (42.48 out of 85.98).

Inter-laboratory variability

There was a systematic and significant difference in test results between the laboratories for both subsets. Differences between laboratories explained 18% of the total variation in subset 1 and close to 17% in subset 2. To express the impact of the differences between laboratories in terms of MIC values, Table 4 provides the mean $\log_2$ MIC and mean standard deviation for each of the five laboratories. This provides an indication of the systematic differences between the laboratories. The maximum mean difference found between laboratories is 0.65 $\log_2$ for the non-GISA strains and 0.68 $\log_2$ for the GISA strains. Thus, if an MIC is measured in one laboratory, it could, on average, be more than half a 2-fold dilution higher in another laboratory.

Table 3. Results of two-way ANOVA with laboratory and strain as fixed effects for the total dataset and subset 1 (non-GISA) and subset 2 (GISA)[a]

		Sum of squares (% of total error)					
	Observations (n)	total variation	strain variation	laboratory variation	Explained	R^2	Unexplained assay variation
Total	440	227.82 (100%)	109.22 (47.9%)	23.57 (10.3%)	132.79 (58.3%)	0.58	95.03 (41.7%)
Subset 1	200	75.18 (100%)	13.38 (17.8%)	13.53 (18.0%)	26.91 (35.8%)	0.36	48.27 (64.2%)
Subset 2	240	85.98 (100%)	29.18 (33.9%)	14.31 (16.6%)	43.48 (50.6%)	0.51	42.48 (49.4%)

[a]Strain and laboratory effects: $P < 0.001$.

Table 4. Mean and SD of $\log_2$ MICs per laboratory, the SD of the mean of the strain means and the mean of the SD of the strain means for all four replicates for subset 1 (non-GISA) and subset 2 (GISA)

	Subset 1				Subset 2			
Laboratory	mean $\log_2$ MIC ($n = 40$)	SD mean ($n = 40$)	SD means ($n = 10$)	mean SD ($n = 10$)	mean $\log_2$ MIC ($n = 48$)	SD mean ($n = 48$)	SD means ($n = 12$)	mean SD ($n = 12$)
1	1.05	0.39	0.20	0.30	0.13	0.53	0.36	0.37
2	1.25	0.74	0.51	0.51	0.35	0.60	0.43	0.39
3	1.55	0.55	0.47	0.26	0.81	0.53	0.45	0.22
4	0.98	0.53	0.34	0.42	0.56	0.58	0.47	0.34
5	1.63	0.54	0.29	0.45	0.69	0.51	0.47	0.14
Mean	1.29	0.55	0.36	0.39	0.51	0.55	0.44	0.29

Intra-laboratory variability

The variability within each laboratory was estimated by determining the mean variance and standard deviation in each laboratory for each of the replicate measurements. The average standard deviation of the four replicates in each laboratory gives an indication of the intra-laboratory variability. These are also shown in Table 4. The average standard deviation of the replicates in each laboratory varied from 0.26 to 0.51 $\log_2$ for subset 1 and 0.14 to 0.39 $\log_2$ for subset 2. Overall, this is a quarter to a half of a 2-fold dilution step and the 95% CIs amount to around two-thirds of a 2-fold dilution step to both sides of the $\log_2$ mean.

Strain-to-strain variability (biological variation)

If the findings above are correct, i.e. there are true differences in MIC between strains, apart from the differences already noted above for the two subsets, then the MICs for strains should be significantly different from each other. This was tested using a one-way ANOVA for each of the subsets, based upon 20 observations per strain. Figure 2 shows plots of differences between strains. Both the non-GISA and GISA subsets showed some significant differences between strains after adjustment for multiple comparisons, indicating that not all strains had the same MIC biologically.

Implications for the clinical laboratory

To determine whether differences between strains can be reliably detected by a clinical laboratory, the following analysis was performed. First, strains were sought that appeared to be statistically different strains from the mean of the whole population studied using the 20 replicate measurements from each strain for each of the two subsets. These showed three strains in subset 1 and four strains in subset 2 to be significantly different from the pooled mean (Nelson adjustment in the GLM LSMEANS procedure/option). Using four replicate measurements, in three of the five laboratories for subset 1 (non-GISA) and one laboratory for subset 2 (GISA), no significant differences were detected for any of these strains. One strain was detected as significantly different in two of five laboratories for subset 1 and four of five laboratories for subset 2. Similarly, for the total dataset, 9 of 22 strains appeared to be significantly different from the mean after adjustment. Only 1 of the 22 strains was attributed as significantly different in five of five laboratories.

Discussion

In this analysis, we have attempted to explain the variability observed for MIC measurements, taking linezolid and *S. aureus* as an example. The analysis showed that over half of the variability could be explained by differences between strains and inter-laboratory differences. The remainder of the variability is due to assay variance. Although true differences between strains appeared to be present, these could not be detected reliably even with four replicate measurements. We did, however, detect differences in linezolid susceptibility between non-GISA MRSA strains and GISA strains. The fact that these groups behave differently was unexpected and could possibly be explained by unique growth and kill characteristics.

With regard to the consequences of these findings, when a single MIC determination is undertaken in the routine microbiological laboratory, it is often assumed that the value is 'reasonably

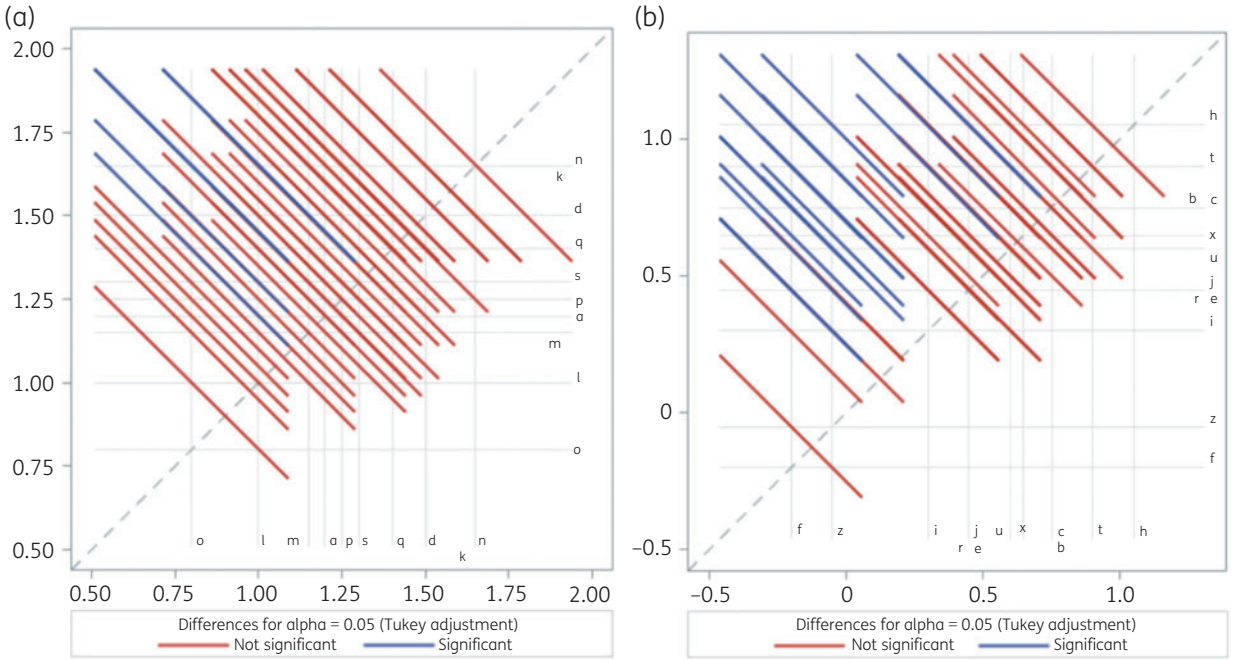

Figure 2. Plots of differences between strains for subset 1 (non-GISA; a) and subset 2 (GISA; b). Axes indicate $\log_2$-transformed MIC values, letters indicate strains and the lengths of the bars indicate the 95% CIs. Each bar indicates a comparison between two strains.

accurate'. The assumption is likely to be based on an extrapolation of experience in the clinical chemistry laboratory, where assays usually have CVs of 10%–15% or less. However, there is an important difference from chemistry assays. The measurement of an MIC involves growing a collection of bacterial cells that are not all identical. They are part of a biological system of cells, each with its own complement of drug receptors and other constituents. These factors are augmented by differences in environmental conditions, such as slight differences in media used, set-up conditions or incubation temperature. Repeated measurements are therefore likely to give different results as the cellular mixture of each culture is slightly different due to natural variation. In this regard, it has been estimated that biological variation explains 10%–90% of the variation in disc diffusion testing, dependent on the species–antimicrobial combination.[10] The total variability, including variability between testing methods, is contained in the WT distributions as presented on the EUCAST web site (www.eucast.org). However, the total variability observed over all strains or measurements—more than 66 000 for linezolid and *S. aureus*—can be broken down into a number of factors that each contribute to overall variability.

Here we attempted to discriminate between each of these contributions except for variation between testing methods. Roughly, half of the total variability can be explained by differences between laboratories and strains; the other half is random variation inherent to this biological assay. The implication is that for an individual MIC that is reported from a laboratory, a large part of the variation of the test is not explained by biological differences between strains. It follows that it is impossible to provide an accurate estimate of the 'true' MIC with a single measurement and multiple replicates—preferably a large number—need to be performed in order to determine the 'true' MIC (its so-called 'expected value') for a strain and its CI (degree of uncertainty). The four replicates in each laboratory in our study were insufficient to estimate the expected value and CI.

If the true distribution of MICs in a single laboratory is not known—and it usually is not—the inter-laboratory variation will add a significant margin of error to that observed in the individual laboratory. If the MIC distribution within an individual laboratory has been established with multiple tests over time, then the overall accuracy of MIC measurements will be slightly higher. Unfortunately, in our study, the total number of strains and replicates was insufficient to define MIC distributions confidently, resulting in reduced capacity to detect outliers. The variability between laboratories here was lower than in an earlier study, where it was estimated to be around 50%.[9]

The inter-laboratory variation observed in this study was the same as that observed in quality control studies and in the construction of the EUCAST MIC database (https://mic.eucast.org/Eucast2/).[16] Inter-laboratory variation is a standard feature of MIC testing and can be attributed to technical variation in the large number of variable components and conditions of the assay.[17] Whether the differences observed here between laboratories are systematic or random would require a confirmation study.

For the clinician, therefore, the report of a single measurement of MIC has limited accuracy if the value is within the WT distribution. The distributions of the various sources of error in that single measurement overlap considerably and, in general, the only conclusion that can be drawn from an individual MIC determination is whether it is likely to be part of the WT distribution or not. Most clinical breakpoints do take the WT distributions into account. It is a general EUCAST rule, for instance, that clinical breakpoints avoid, wherever possible, splitting the WT distribution. For this reason,

reporting using the interpretive categories of susceptible and resistant are adequate for treatment purposes, even if in one specific laboratory differences between strains could be demonstrated.[7] It is only when MICs have been measured outside the WT distribution that the 'exact' value of an MIC may have some value in clinical reporting.

This study has some strengths and limitations. The strength of the study is that the laboratories were unaware that they had been sent four replicates of the same strains and therefore were completely blinded. The sample size was large enough to necessitate several days to complete the measurements in each laboratory; the individual replicates were not numbered consecutively and were sufficiently distant in number to ensure that day-to-day variability would be factored in. Our estimates of variability seem to be a reasonable reflection of daily practice and include technical variation. In addition, because of the unexpected significant differences in linezolid susceptibility between non-GISA and GISA strains, this led to two separate analyses. The results of the two subsets were within the margin of error and the estimates of variability could therefore be regarded as representative. Limitations of the study include the small number of replicates sent to each laboratory and the use of a non-reference method. Because of the relatively large variability between observations of a single strain, the estimates of the 'true' MIC for each strain contain a margin of error that precluded showing 'true' differences between strains. However, given this margin of error, a large number of replicate measurements would have been needed and does not affect the conclusion that a single measurement is not discriminating. Another important limitation is that we only looked at one species–antimicrobial combination. The results of the present analysis may therefore not reflect that of other combinations. However, we provide this analysis as an approach to discerning contributions to variability, which was the primary objective of the study.

In summary, this analysis serves as an example of how an approach to discerning various sources of MIC variation could be taken. We conclude that an accurate measurement of an MIC for an individual strain—at least for the tested isolate and antibiotic combination—in the routine clinical laboratory is not possible due to both inter- and intra-laboratory assay variation. The total variation extends to a 95% CI that covers ∼1.3 2-fold dilutions and a 99% CI of two dilutions from the mean. For an individual laboratory the variation is somewhat lower but in routine practice is insufficiently low to consider a single MIC measurement accurate.

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