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Reproducibility of Control Strains for Antibiotic Susceptibility Testing

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Inter- and intralaboratory reproducibility of susceptibility testing requires stable control strains. The Food and Drug Administration diffusion procedure recommends the Seattle strains of *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (ATCC 25922) for this purpose. It was of interest to determine the present reproducibility of control cultures maintained in various laboratories over several years. Fifteen cultures each of *S. aureus* and *E. coli* were obtained from laboratories in different parts of the country. Their performance was compared with strains directly derived from ATCC. Diffusion susceptibility tests using a modified overlay technique were made with four replicates. Seven of the eight statistically significant differences in responses of the staphylococci were to penicillin, methicillin, or cephalothin. One culture was a penicillinase producer with a zone 15 mm less than the standard strain. Eleven of the 15 cultures showed no significant deviations or differences greater than 2 mm from the results with the strain derived directly from ATCC. All except the penicillinase producer were of identical phage type. Among 150 organism-antibiotic combinations tested with *E. coli*, all but one reading were within 2 mm of the standard. Four of the six statistically significant differences were in a culture from one laboratory. The stability of the cultures appears to have been influenced by the method of storage. Cultures that were kept frozen during extended storage were remarkably stable. Significant differences were found in cultures from four of five laboratories that maintained cultures in refrigerators or at ambient temperature.

Recommended quality control procedures for antimicrobial disk susceptibility tests require daily testing of *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923) (3, 4). These strains, originally isolated and used for quality control purposes in the University of Washington Hospital Clinical Laboratories, have now been maintained in many different laboratories for up to 7 years. This has raised the possibility that contamination or genetic drift may have occurred in some clones similar to that which was shown with the Oxford *Staphylococcus* by Oeding and Östervold (5). A study was therefore designed to determine whether any differences could be detected in control strains used routinely in clinical laboratories.

Laboratories in different parts of the country were requested to send working cultures of their control strains with information on their original source, the date obtained, and maintenance procedures used. These cultures were then compared with strains derived directly from the American Type Culture Collection using a closely standardized agar overlay diffusion procedure.

MATERIALS AND METHODS

Cultures. Cultures of *E. coli* and *S. aureus* submitted by 15 laboratories were tested. All were said to be derived from *E. coli* (ATCC 25922) and *S. aureus* (ATCC 25923) or from the "Seattle" control strains, which were the original source of the ATCC strains. The standard strains from the American Type Culture Collection with which all other cultures were compared had been subcultured only twice prior to the study.

Storage. The cultures from participating laboratories were streaked immediately upon receipt onto a blood agar plate and incubated overnight. One colony was inoculated into 3 ml of Trypticase soy broth supplemented with 1% yeast extract (TSY) and incubated until turbid. Blood agar plates were streaked from the broth and incubated overnight, and the growth was used to prepare a heavy suspension in a mixture of 50% TSY broth and 50% heat-inactivated fetal calf serum. A few drops of the suspension were added to 2-dram (ca. 3.55-g) vials

containing glass beads and shaken to ensure that the beads were coated. Vials were stored at −70°C.

Susceptibility testing. Vials of the organisms to be tested were transferred from the −70°C freezer to a dry ice-ethanol bath, and single glass beads were removed and inoculated into 3 ml of TSY broth for overnight incubation. The resulting broth cultures were streaked onto blood agar plates and incubated overnight. Five colonies were inoculated into 3 ml of TSY and incubated for 2 to 4 h, and the optical density was adjusted with TSY broth to 0.14 ± 0.01 at 580 nm in a Bausch & Lomb Spectronic 20 spectrophotometer. A 0.1-ml amount of the standardized suspension was then added to each of two tubes containing 8 ml of aqueous 1.5% agar at 45°C, and duplicate 150-mm plates of Mueller-Hinton agar (Difco) containing 60 ml of medium were overlaid evenly with the agar suspension. Antibiotic disks were applied within 15 min and the plates were incubated overnight. Zone diameters were measured with calipers on the surface of the plates. All cultures of either *S. aureus* or *E. coli* were tested at the same time on two different occasions and read independently by two readers. Thus, there were four plates for each culture and a total of eight measurements.

Antibiotics. Disks (BBL) of the same lot number for each antibiotic were used throughout. Their content was that recommended by the Food and Drug Administration (FDA) (3) and the National Committee for Clinical Laboratory Standards (NCCLS) (4). The antibiotics tested were those indicated in Tables 2 and 4.

Phage typing of *S. aureus* strains. All cultures of *S. aureus* were typed with the following phages: 3A, 3C, 6, 29, 42D, 42E, 47, 52, 52A, 53, 54, 55, 71, 75, 77, 80, 81, 83A, 84, 85, 94, 187, and D11.

Biotyping of *E. coli* cultures. *E. coli* cultures were tested for the biochemical reactions listed in Table 31 of Edwards and Ewing (2) with the omission of KCN, gelatin, and cellobiose and the addition of nitrate, esculin, and trehalose. Reactions were read daily for 3 days.

Statistical analysis. An analysis of variance was performed on the *S. aureus* and *E. coli* data for each drug under study. The primary purpose was to estimate the variability associated with the mean response to each drug, where response was measured as the diameter of the zone of inhibition in millimeters. Components of variability associated with differences among laboratories, between readers, and between days were separated. Using the standard deviation estimated from the analysis of variance, Dunnett's *t* test for comparing multiple means to a control mean was performed to determine, separately for each drug, which laboratory means differed from the standard ATCC control strain (1).

RESULTS

***S. aureus* cultures.** All cultures were phage type 29/52/80/81+, except culture 3 which was untypable. The susceptibility test results are summarized in Table 1, which lists all mean zone diameters that differed from the ATCC standard by more than 1 mm and identifies all statistically significant differences. The culture from laboratory 3 was not *S. aureus* ATCC 25923, as evidenced by its penicillin resistance and phage type. With the exception of one erythromycin zone, differences of >1 mm were only found with the penicillins and cephalothin. Cultures from laboratories 5, 9, and 20 showed zone sizes that were significantly different from the standard strain. Six of the 15 cultures did not differ from the standard strain by more than 1 mm with any antibiotic tested.

The mean zones of the standard strain and the ranges of the mean zone sizes for all 16 cultures of *S. aureus*, including the standard strain, are shown in Table 2. The mean zone diameters are not comparable to control values recommended by FDA and NCCLS (3, 4) because a different technique was used which involved a heavier inoculum. Penicillin and cephalothin zones had the largest ranges. The right column lists the ranges for the 11 cultures that did not differ significantly (Table 1) from the standard. Reproducibility with these was excellent: only the mean penicillin zone sizes ranged more than 2 mm.

***E. coli* cultures.** Twelve of the *E. coli* cultures showed identical reactions with the 30 biochemical tests. Three cultures (11, 20, and 22) differed in one reaction, and culture 5 differed in three reactions. The results of the susceptibility tests are summarized in Table 3. Five cultures gave mean zone diameters differing from the standard strain by more than 1

TABLE 1. *Differences of >1 mm in mean zone diameters of S. aureus cultures from the standard ATCC 25923*

Laboratory of origin	Differences of >1 mm in mean zone diameter[a]			
	Penicillin	Oxacillin	Cephalothin	Erythromycin
2	−1.5			
3	−15.3[b]		−2.2	
4	−1.9		−1.2	
5	+4.4[b]		+3.9[c]	
8	−1.9		−1.1	
9	+3.6[b]	+2.5[c]	+1.5	+1.9[c]
11	−1.4			
18	+1.4		−1.1	
20	+7.1[b]	+1.9	+6.6[b]	

[a] No difference of >1 mm with tetracycline, chloramphenicol, clindamycin, kanamycin, streptomycin, or cultures from laboratories 1, 12, 15, 19, 21, and 22.

[b] Significance of mean difference from standard strain by Dunnett's test: $P < 0.01$.

[c] Significance of mean difference from standard strain by Dunnett's test: $P < 0.05$.

mm. Three of these, including culture 5, yielded results that were significantly different. Culture 11 showed four significant differences, and the others showed one each. Five other strains with statistically significant differences in ampicillin zones were not included in this table because the differences were 1 mm or less. This reflected a very narrow range of ampicillin readings as well as the fact that the ampicillin zones of the standard strain were larger than those of the other 15 cultures.

Table 4 lists the mean zones of the standard strain and the ranges of the mean zone sizes of all cultures. Results with cultures from laboratories 5 and 11 are excluded from the ranges listed in the right column. The other 13 cultures performed very closely to the standard.

Relationship of method of storage to performance. It appears that the method of storage influenced the stability of the cultures. Table 5 correlates methods and duration of extended storage with performance of the *S. aureus* cultures. None of the eight cultures that had been stored at −70°C showed any significant differences from the standard strain. One purporting to be of commercial origin was that which differed in phage type and penicillin susceptibility from the others. This was probably a case of contamination or error after receipt from the manufacturer, because FDA diffuson tests (3, 4) of four other *S. aureus* cultures from the same manufacturer indicated that they were satisfactory. Three of the five cultures stored in the refrigerator or at room temperature were found to show significant differences from the standard.

Table 6 presents the correlation of methods and duration of storage with the performance of the *E. coli* cultures. Significant differences greater than 1.2 mm from the standard strains were found with two of the four cultures of *E.*

TABLE 2. *Ranges (maximum − minimum) of mean zone diameters with 16 cultures of S. aureus ATCC 25923 from different sources*

Drug	Mean zone[a] of standard ATCC strain (mm)	Range of all 16 cultures (mm)	Range excluding laboratories 3, 5, 9, and 20
Penicillin	27.4	22.5	2.4
Oxacillin	18.9	2.8	1.3
Cephalothin	27.6	8.7	1.3
Tetracycline	22.6	1.5	0.7
Chloramphenicol	19.8	1.8	1.0
Clindamycin	21.6	1.8	1.2
Erythromycin	21.4	2.4	1.2
Kanamycin	19.3	1.0	1.0
Streptomycin	15.2	0.9	0.8

[a] The mean zone diameters are not comparable to control values recommended by FDA (3) and NCCLS (4) because a different technique was used involving a heavier inoculum.

TABLE 4. *Ranges (maximum − minimum) of mean zone diameters with 16 cultures of E. coli ATCC 25922 from different sources*

Drug	Mean zone[a] of standard ATCC strain (mm)	Range of all 16 cultures (mm)	Range excluding laboratories 5 and 11 (mm)
Ampicillin	16.5	1.2	1.8
Carbenicillin	20.2	3.3	1.5
Cephalothin	15.2	2.2	1.2
Tetracycline	21.5	2.2	0.8
Chloramphenicol	21.1	1.9	1.2
Gentamicin	16.6	1.7	1.0
Kanamycin	16.8	2.2	1.1
Streptomycin	13.1	1.7	0.5
Polymyxin	12.9	0.7	0.6
Nitrofurantoin	20.3	1.1	1.1

[a] The mean zone diameters are not comparable to control values recommended by FDA (3) and NCCLS (4) because a different technique was used involving a heavier inoculum.

TABLE 3. *Differences of >1 mm in mean zone diameters of E. coli cultures from the standard ATCC 25922*

Laboratory	Differences of >1 mm in mean zone diameter[a]								
	Ampicillin[b]	Carbenicillin	Cephalothin	Tetracycline	Chloramphenicol	Gentamicin	Kanamycin	Streptomycin	Nitrofurantoin
1							−1.1		−1.1
3	−1.2[c]								
4									−1.1
5		+1.8[d]	−2.0						
11		+2.6[c]		+1.3[c]	+1.3	−1.1	−1.6[c]	−1.1[d]	

[a] No difference of >1 mm with polymyxin B or cultures 2, 8, 9, 12, 15, 18, 19, 20, 21, and 22.
[b] The mean ampicillin zones from five additional cultures differed significantly from the standard, but since these differences were 1 mm or less they are regarded as clinically not of interest.
[c] $P < 0.01$.
[d] $P < 0.05$.

TABLE 5. *Correlation of method of storage with significant differences from standard S. aureus ATCC 25923*

Method of storage	Mean duration of storage (mo)	Total no. of laboratories	No. of cultures with statistically significant differences	Antibiotics
Frozen	33	8	0	
Commercial	7	2	1	Penicillin
Refrigerated	48	1	1	Penicillin, cephalothin
Room temp	31	2	1	Penicillin, cephalothin
Room temp under oil	31	2	1	Penicillin, oxacillin, erythromycin

TABLE 6. *Correlation of method of storage with significant differences of >1.2 mm[a] from standard E. coli ATCC 25922*

Method of storage	Mean duration of storage (mo)	Total no. of laboratories	No. of cultures with statistically significant differences	Antibiotics
Frozen	33	8	0	
Commercial	7	2	0	
Refrigerated	48	1	0	
Room temp	31	2	1	Carbenicillin
Room temp under oil	31	2	1	Carbenicillin, kanamycin, tetracycline

[a] The mean ampicillin zones from six cultures differed significantly from the standard, but since these differences were less than 1.2 mm they are regarded as clinically not of interest.

coli that had been stored at room temperature.

There was no evidence of a relationship between duration of storage and stability of the cultures.

FDA diffusion tests. The 15 *E. coli* cultures and the standard were tested against the antibiotics listed above by the FDA diffusion test procedures (3, 4). Four of 144 tests were outside FDA limits. On repeat tests all were within limits, except culture 5, which was again out of control with chloramphenicol. One result that was in control on the first test was 1 mm below the FDA limit for cephalothin on the repeat test. Results with carbenicillin, for which there are no FDA limits, were compared with the limits recommended by NCCLS. In the two series of tests, 9 of 32 carbenicillin zones were 1 to 2 mm below the recommended control values of 24 to 29 mm. The ATCC strain gave a mean value of 24.5 mm, and the overall mean of results from all strains was 24.2 mm. This suggests that the NCCLS limit may need revision.

The *S. aureus* cultures were also tested by the FDA diffusion test procedure. With the exception of culture 3, which was the penicillinase producer, all cultures were within the FDA recommended control limits. Since no limits have been established for oxacillin, we calculated the limits to be 19 to 23 mm, based on over 200 measurements from the routine quality control procedures in our laboratory. Only culture 9 was out of control, having an oxacillin zone of 24 mm. On a repeat test, this culture was in control.

DISCUSSION

Quality control of antibiotic susceptibility testing presumes the stability of standard organisms. When inhibitory zone diameters are outside the limits of FDA or NCCLS recommendations, the sources of error will first be sought in media production or in technique. The results from this study, which used a highly reproducible diffusion procedure, indicate that there are detectable differences in the performance of standard *E. coli* and *S. aureus* cultures from different laboratories. With the exception of the penicillin-resistant culture, however, none of the *S. aureus* cultures was found to yield a zone diameter outside the FDA ranges when tested by the routine standardized diffusion test. Most of the significant changes in susceptibilities of the *Staphylococcus* were with the penicillins and cephalothin. This was especially evident when the data were expressed as ranges of mean zone diameters. However, cultures from 11 of the 15 laboratories showed no significant deviations from the results with the strain derived directly from ATCC.

In the case of *E. coli* cultures, four of the six significant differences were with a culture from one laboratory. When this culture and culture 5, which had a different biotype, are excluded, the ranges of the zone diameters did not exceed 1.6 mm with any antibiotic tested. Only culture 5 was outside the FDA limits on both the initial and repeat tests using the standardized diffusion procedure. This culture had a light inner

zone around the chloramphenicol disk that was not detected by the overlay procedure.

The method of storage appears to have influenced the stability of the strains. Disregarding the very small differences found with ampicillin and *E. coli*, none of the cultures that were kept frozen during extended storage showed significant differences from the standard strains. In contrast, 5 of the 10 cultures from laboratories that stored their strains in the refrigerator or at ambient temperature showed significantly different zone diameters from the ATCC strains. All of these cultures had been maintained for over 2 years.

These results show that 2 of the 30 cultures received were of different origin to the ATCC strains and unsuitable for control purposes. There was evidence of some genetic shift in cultures received from laboratories that stored them under conditions in which some growth as well as cell death could occur. Fortunately, this did not appear to have taken them outside the recommended FDA control limits, although the possibility of further shift exists. Excellent stability can be anticipated if basic stocks are maintained under nonmetabolizing conditions such as frozen or lyophilized. Replacement of working stock cultures from frozen portions on a weekly or monthly basis appears to be a satisfactory procedure.

LITERATURE CITED

1. **Dunnett, C. W.** 1964. New tables for multiple comparisons with a control. Biometrics 20:482–491.
2. **Edwards, P. R., and W. H. Ewing.** 1972. Identification of *Enterobacteriaceae*, 3rd ed. Burgess Publishing Co., Minneapolis, Minn.
3. **Federal Register.** 1972. Rules and regulations. Antibiotic susceptibility discs. Fed. Regist. 37:20525–20529.
4. **National Committee for Clinical Laboratory Standards.** 1975. Performance standards for antimicrobial disc susceptibility tests. National Committee for Clinical Laboratory Standards, Villanova, Pa.
5. **Oeding, P., and B. Östervold.** 1959. The stability of Oxford staphylococcus H and other penicillin reference strains. Acta Pathol. Microbiol. Scand. 46:149–155.