Quality assurance of antimicrobial susceptibility testing by disc diffusion

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Quality assurance is essential to ensure the quality of antimicrobial susceptibility test by diffusion methods. Routine internal quality control testing with a range of control strains is a major part of the quality assurance process, as it facilitates monitoring of the performance of the test. Most standardized methods include tables of acceptance zone size ranges for control stains and, in addition to checking that control zone diameters are within the published ranges, rules or statistical approaches may be applied to indicate deviations from acceptable performance. If control tests indicate unacceptable performance, the source(s) of the error should be investigated and may include problems with media, antimicrobial discs, inoculum and plate reading. Participation in external quality assessment schemes provides an independent assessment of performance although the number of strains distributed in such schemes is limited. Internal quality assessment in which routine tests are repeated with the identity of the organisms blinded is a useful complementary approach to external quality assessment and may detect problem areas not highlighted by other control methods. Education is an important part of the quality assurance process. Knowledge of atypical results for different organism-agent combinations may provide warning of possibly erroneous results, and an understanding of the limitations and sources of error in disc diffusion methods contributes significantly to the recognition, resolution and avoidance of errors.

Introduction
There are multiple factors that may affect the performance of susceptibility tests and standardized methods are more likely to be reproducible than unstandardized methods. Quality assurance is the overall process by which the quality of the test results can be guaranteed. A major part of this process is the internal quality control testing which is routinely undertaken to monitor the precision and accuracy of the test procedure, the performance of reagents used in the test and the performance of the persons carrying out the tests. However, there are additional aspects that contribute to quality assurance, including participation in external quality assessment schemes, internal quality assessment and the validation process, in which atypical or contradictory results can be detected. Education is an important part of the quality assurance process as an understanding of the techniques, together with their limitations and pitfalls, contributes significantly to the recognition, resolution and avoidance of errors.

Internal quality control
The basis of routine quality control is the inclusion of control strains to detect abnormal performance of the test, and this is recommended for all susceptibility testing methods.

Control organisms
Control organisms should be genetically stable and remain unaltered during long-term storage. Organisms susceptible to antimicrobial agents are commonly used but resistant organisms are necessary when testing for particular mechanisms of resistance. These resistant organisms may be genetically less stable and must be carefully monitored for loss of resistance mechanisms. Every laboratory needs to maintain a collection of the strains used in quality control procedures.

The recommended control organisms for susceptibility testing[1] can be obtained lyophilised from the National Collection of Type Cultures (NCTC; London, UK), the American Type Culture Collection (ATCC; Manassas, VA, USA) or in various formats from commercial sources (e.g. Culti-Loops, Oxoid, Basingstoke, UK; QC Sticks, Mast Laboratories, Bootle, UK; Qualiswabs, Becton-Dickinson, Oxford, UK). Cultures should be reconstituted as recommended by the manufacturer, and stored frozen at -70°C in small aliquots in 7-10% glycerol broth on glass beads. The control strains can also be dried on gelatin discs, each of which provides material for one subculture.[2]

Working cultures should be stored on nutrient agar slopes (non-fastidious strains) or chocolate agar slopes (fastidious strains) at 4-8°C. Before testing, the strains should be subcultured to agar plates to obtain isolated colonies and check for purity. Fresh subcultures from stored aliquots or gelatin discs must be made weekly. When subculturing from cultures stored at -70°C do not allow the entire contents of the frozen vial to thaw, but remove a bead or scrape a small quantity from the top of the broth and return the remainder to the freezer without delay.

Unexplained alteration in the mean zone diameters with a control strain may indicate contamination of the culture or mutational changes. If this is suspected a fresh culture should be obtained either by subculture from stored aliquots or by purchasing new cultures.

Performance limits for control organisms
Acceptable ranges for control zone diameters have been published for some disc diffusion methods.[1,3-5] If performance limits for a particular antimicrobial agent tested in the laboratory have not yet been published, in-house limits should be established fro temporary use until limits are published for the agent. Strict adherence to the method is essential and on at least 30 days zone diameters should be accurately measured to the nearest millimetre. Temporary performance limits are then calculated as the mean $\pm$ two standard deviations (S.D.).

Routine testing of control organisms
Appropriate control organisms should be tested daily for all antimicrobial agents routinely tested in the laboratory. Zone diameters should be measured accurately with callipers or ruler and recorded. The zone diameters should be towards the middle of the acceptable range. However, most methods do not specify what should be done if zones are outside the acceptable range. A single control organism-antimicrobial combination out of range will not necessarily require action unless the result is far out of range or there is other indication of a problem (e.g. small control zone together with a higher frequency of resistant isolates than normal). Two successive results either above or below the control limits indicate a likely systematic problem resulting in error in the test. More than two results outside the limits (in any direction) in a series of 20 tests may also indicate error. If zone diameters are consistently either above or below the mean of the range, a systematic error may be indicated, particularly if this is true for several agents. In this case the methodology should be checked, including the depth of agar, time

between inoculation and application of discs, time between application of discs and incubation, inoculum and incubation conditions.[1]

Control charts are a simple graphical means of monitoring performance. The simplest approach is to plot the daily readings on a chart (a Shewhart diagram) with upper and lower control limits marked (Figure 1). Readings outside the control limits and trends in control one sizes can be assessed visually from examination of the diagram. A more formal mathematical approach would be to use rules of the type described by Westgard & Klee[6] for interpreting clinical chemistry control data. In clinical chemistry these rules relate to the S.D. of the control analyte concentration, so applying this approach to control zone limits set for a standardized disc diffusion method is not entirely appropriate. However, if the control zone ranges are treated as mean ±2 S.D., some of the Westgard rules can be applied (Figure 1), for example as follows.

(i) One control result is outside the limits (Westgard rule 1_{2s}). This may be a warning of an emerging problem. Routine test results for that day may be accepted if there is not other evidence of problems in the current tests.

(ii) Two consecutive control results are outside the limits in the same direction (Westgard rule 2_{2s}). This indicates an error in the routine method which must be acted on.

(iii) Ten consecutive results are within the limits but on the same side of the mean of the range (Westgard rule 10). Results may be accepted but this indicates a possible problem that should be investigated.

A simple statistical method to detect shifts in performance or trends in performance of controls is the cumulative sum (CUSUM).[6] The difference between the mean of the acceptable range and the observed reading (either + or -) is cumulatively added to previous differences. If the control results are within range and equally distributed above and below the man the CUSUM will remain relatively constant. A shift in zone diameters, repeated results on one side of the mean or a gradual drift in zone diameters will be seen as an increasing or decreasing CUSUM. This calculation may be plotted to give a visual indication of changes (Figure 2).

Frequency of testing

The time taken to set up and record the performance of control strains is significant and some methods suggest that the frequency of control testing can be reduced once the method has been shown to be performing adequately.[3,4] What constitutes adequate performance is arguable, but the NCCLS[4] suggest that performance is acceptable if in daily testing no more than two in 20 results are out of range. The frequency of testing may be reduced to once weekly if no more than three of 30 results on daily testing are outside the control limits. If any weekly control test is out of range corrective action is required.

Control with reference histograms

In Sweden, reference histograms of species-specific zone diameter distributions are available.[3] Laboratories using the Swedish system can compare the zone diameter distributions for their won isolates with the reference histograms. If the distributions of zone diameters for the susceptible and, if available, resistant populations are similar to those in the reference histograms it is a good indication that the laboratory is adhering closely to the standardized method and is producing acceptable results.

Control of media

New batches of medium received from the manufacturer should be tested to ensure that the batch is comparable with the previous batch. This can be established by performing tests in parallel with old and new batches, including tests with medium supplemented for fastidious organisms. The routine control strains should be used but as these are mostly susceptible strains, additional strains with known resistances should also be tested.

Each new batch of medium, including that with supplements for fastidious organisms, should be tested for satisfactory growth of organisms before use. Quality control tests with all agents routinely tested should be included. If zone diameters are outside the performance limits there may be problems with the medium. Larger or smaller zones may indicate that the plates are, respectively, too think or too thick. If the pH of the medium is too high, smaller zones may be seen with tetracycline and larger zones with aminoglycosides; and the opposite may be seen if the pH is too low.

Control of antimicrobial discs

Discs for diffusion methods are manufactured within strict control limits and should perform adequately provided they are handled correctly within the laboratory. Stocks of discs should be stored below 8°C and preferably at –20°C. Working stocks should be refrigerated with an indicating desiccant and protected from light. To prevent condensation and possible loss of potency, discs must be allowed to

reach room temperature before opening for use. Discs must not be used after the expiry date shown on the packaging. Discs containing ß-lactamase inhibitors or imipenem are particularly vulnerable to moisture and loss of potency. If routine control tests give zone diameters around discs for a single antibiotic smaller than the lower performance limit, or if there is a gradual reduction in control zone diameter with a particular agent, there may be a problem with deterioration of the agent. In this case the discs in use should be discarded and replaced with fresh discs from stock.

With discs containing a ß-lactam agent and a ß-lactamase inhibitor the susceptible control strains will detect deterioration of the ß-lactam agent but not the ß-lactamase inhibitor. Deterioration of the inhibitor may be indicated by reduction in zone diameter with the ß-lactamase-producing strain *Escherichia coli* NCTC 11954 (ATCC 35218) or *E. coli* NCTC 11560.

Control of inoculum

The standard inoculum required in the BSAC[1] and several other disc diffusion methods (but not the NCCLS) yields semi-confluent growth. This can be achieved by a variety of methods but must be monitored. It is important that all members of staff are familiar with the chosen method and that the inoculum is correct for all species. If there are difficulties achieving the required inoculum with particular species, the chosen method of inoculum preparation must be reassessed. The use of a standardized method of preparing the inoculum is much more reliable than 'experience' in direct suspension of colonies, and success in achieving the correct inoculum is dependent on the reliability of the chosen method. The use of spectrophotometer to determine the absorbance of a suspension followed by appropriate dilution is the most accurate method. Visual comparison with a turbidity standard is an approach recommended in most standard methods. Both these methods should produce the correct inoculum size for the majority of isolates. If the growth is not semi-confluent the test must be regarded as unsatisfactory and repeated.

Use of resistant control strains

Acceptable results obtained from testing susceptible control strains do not guarantee accurate results with all patient isolates. *Streptococcus pneumoniae* 12977 (ATCC 49619) is a strain with intermediate penicillin resistance and is used to check the method for detection of reduced susceptibility to penicillin. *Staphylococcus aureus* NCTC 12493 is a methicillin heteroresistant strain which can be used to check that test conditions are acceptable for the detection of methicillin/oxacillin resistance. Other control strains with reduced susceptibility, particularly low-level resistance, to antimicrobial agents would be useful but no strains have been specifically recommended.

Recognition of atypical results

Some susceptibilities are predictable because species are inherently resistant to particular agents, resistant strains have not been isolated or there is cross-resistance. Other resistances may be very rare locally. However, it should be remembered that resistance may change over time and there may be differences between centres. A set of such rules[7] can contribute significantly to checking the quality of reported results and may be incorporated in computerized validation and reporting systems. Atypical results should be investigated by repeating the test and verifying the purity and identification of the organism.

External quality assessment

In external quality assessment (proficiency testing) schemes, organisms with susceptibility defined by reference methods are distributed as unknown strains from a central laboratory to participating laboratories.[8] Results of routine tests are returned from participating laboratories to the central laboratory, which provides feedback on the performance of the individual laboratory and on the overall performance of all laboratories in the scheme. Analysis of results in relation to methods used may also be provided. The benefits of such schemes are that they allow laboratories to compare their performance with that of other laboratories, they provide some evidence of performance that is recognized by accreditation authorities, they provide some insight into national and international performance, and they have educational value in highlighting problem areas. However, if errors are rare they may not be detected by external quality assessment, and the number of strains distributed is relatively small.

Internal quality assessment

In internal quality assessment specimens or organisms are reprocessed on the same day as the original, with the identity of the specimens blinded.[9] After reports are produced, the results with the original and reprocessed specimen are compared and discrepancies noted. As the number of

specimens reprocessed can be much larger than in external quality assessment, multiple discrepancies with the same species-antimicrobial combination may indicate areas where errors are likely and remedial action is required. Particular combinations can also be targeted if there are specific technical concerns.

Troubleshooting

If the control procedures indicate that there may be an error in the test this must be investigated. Some common problems[10] are detailed in the Table, together with appropriate measures for correcting them.

References

1. Andrews, J.M. for the BSAC Working Party Report on Susceptibility Testing. (2001). BSAC standardized disc susceptibility testing method. *Journal of Antimicrobial Chemotherapy* **48**, *Suppl. 1,* 43-57.

2. Snell, J.J.S. (2000). Preservation of control strains. In *Quality Assurance: Principles and Practice in the Microbiology Laboratory,* (Snell, J.J.S., Brown, D.F.J. & Roberts, C., Eds), pp. 69-76. Public Health Laboratory Service, London.

3. Kahlmeter, G., Olsson-Liljequist B. & Ringertz, S. (1997). Antimicrobial susceptibility testing in Sweden. IV. Quality assurance. *Scandinavian Journal of Infectious Diseases* **105**, *Suppl.,* 24-31.

4. National Committee for Clinical Laboratory Standards. (2000). *Performance Standards for Antimicrobial Disk Susceptibility Tests – Seventh Edition: Approved Standard M2-A7.* NCCLS, Wayne, PA.

5. Comite de l'Antibiogramme de la Societe Francaise de Microbiologie. (1996). Technical recommendations for in vitro susceptibility testing. *Clinical Microbiology and Infection* **2**, *Suppl. 1*, S11-25.

6. Westgard, J.O. & Klee, G.G. (1994). Quality maagemtn. In *Tietz Textbook of Clinical Chemistr*y, 2nd edn, (Burtis, C.A. and Ashwood, E., Eds), pp. 564-75. W.B. Saunders Co., Philadelphia, PA.

7. Livermore, D.M., Winstanley, T. & Shannon, K. (2001). Interpretative reading: recognizing the unusual and inferring resistance mechanisms from resistance phenotypes. *Journal of Antimicrobial Chemotherapy* **48**, *Suppl. 1,* 59-64.

8. Snell, J.J.S. (2000). External quality assessment. In *Quality Assurance: Principles and Practice in the Microbiology Laboratory,* (Snell, J.J.S., Brown, D.F.J. & Roberts, C., Eds), pp. 77-90. Public Health Laboratory Service, London.

9. Farrington, M. (2000). Internal quality assessment at the specimen level. In *Quality Assurance: Principles and Practice in the Microbiology Laboratory,* (Snell, J.J.S., Brown, D.F.J. & Roberts, C., Eds), pp. 91-104. Public Health Laboratory Service, London.

10. Brown, D.F.J. (2000). Quality assurance of antimicrobial susceptibility testing. In *Quality Assurance: Principles and Practice in the Microbiology Laboratory,* (Snell, J.J.S., Brown, D.F.J. & Roberts, C., Eds), pp. 151-68. Public Health Laboratory Service, London.

Table. Sources of error in disc diffusion testing and appropriate remedial action.

Problem	Appropriate Action
Errors in measurement of zone sizes of control organisms	Ensure that observers define zone edges according to established laboratory practice
Defining the edge of the zone is liable to variation and illumination may be variable	Standardize illumination
Contamination or mutational changes in the control cultures	Take a fresh culture from stock or obtain a fresh culture from an appropriate culture collection
Problems with the medium	Ensure the medium is prepared as recommended by the manufacturer. A medium other than the susceptibility medium may have been inadvertently used. If a new batch of medium is unacceptable obtain a fresh batch from the manufacturer. Where possible purchase a large quantity of a satisfactory batch of medium.
Problems with antimicrobial discs	Occasionally discs with excessively high or low content may be encountered and should be replaced. Labile agents in discs may deteriorate as a result of mishandling in the laboratory. Ensure that discs are stored correctly and that they are allowed to reach room temperature before opening. Low disc usage may be a problem in some smaller laboratories. Ensure that discs have not passed the expiry date. Some antimicrobials such as metronidazole and some quinolones are sensitive to light and must be stored in the dark.
Inoculum too heavy or too light	Check the method of preparing the inoculum and if necessary adjust the method. If a turbidity standard is used check the preparation and storage. If a spectrophotometer is used ensure it is working correctly.
Zone diameters too large or too small with all antimicrobials	Check the depth of the agar and, if correct, adjust the amount of agar dispensed. Check the inoculum size. Ensure that discs are applied to inoculated plates within 15 min and that plates are incubated within15 min of application of discs.