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1. Introduction

Most of the clinically important bacteria causing infections in humans are capable of exhibiting resistance to antimicrobial agents commonly used for the treatment. Therefore, upon isolation of the organism in the clinical microbiology laboratory, characterization frequently also employs tests to detect its antimicrobial susceptibility. Thus, the report produced by clinical microbiology laboratory for the physician, also includes organism’s susceptibility profile to different antimicrobials along with its identification [1]. Antimicrobial susceptibility testing (AST) is performed on bacteria that are isolated from clinical specimens to determine if the bacterial etiology of concern can be killed or inhibited by antimicrobial drugs that are potential choices for therapy, at the concentrations of the drugs that are attainable at the site of infection using the dosing regimen indicated in the drug product’s labeling. The results of AST are generally reported with interpretive categories. The category “susceptible” indicates that the bacteria are inhibited by the usually achievable concentrations of antimicrobial agent when the dosage recommended to treat the site of infection is used. The “intermediate” category defines the bacteria for which the response rates to usually attainable blood and tissue levels of antimicrobial agent are lower compared to susceptible isolates. The intermediate category plays the role of a buffer zone between the susceptible and resistant categories, but also indicates a number of other possibilities; the antimicrobials which are concentrated at the site of infection may be regarded as options for treatment (e.g., nitrofurantoin for the urinary tract infections). The “resistant” category, however, defines the bacteria which are not inhibited by the usually achievable concentrations of the agent with normal dosage regimens and that the clinical efficacy of the agent against the isolate may not be sufficient [2]. Clinicians consider these interpretations to determine which antimicrobial agent might be effective in treating the particular patient. The primary role of routine microbiology laboratories is to provide accu-
rate and timely antimicrobial susceptibility test results for guiding the treatment of infectious diseases. In order to achieve that, the microbiologist should inform the clinician about whether an infectious agent is present in the patient’s specimen and which antimicrobial agent should provide the optimum therapy. Although the importance of antimicrobial susceptibility testing is well established, the procedure itself is very sensitive to changes in the environment and test conditions. Therefore, it is crucial that each variable in the procedure should be standardized and carefully controlled. With more reliable susceptibility results, infectious disease specialists and public health leaders can be able to recognize emerging resistance and novel resistance patterns. Additionally, the results of AST can be applied to define the agent of choice for empirical therapy, establish institutional or nationwide policies for prescribing of antibiotics, conduct epidemiological studies or resistance surveillance, and to evaluate the efficacy of newly developed agents. Owing to numerous variables that may affect the results, rigorous quality control is of utmost importance for susceptibility testing. Properly performed quality control would aid in providing accurate, reproducible and timely results. In this chapter the components of a quality assurance program for antimicrobial susceptibility testing will be highlighted.

2. Overview of the antimicrobial susceptibility testing methodologies

Fleming was first to report the inhibitory effect of penicillin on agar by observing a zone of growth inhibition of staphylococcal colonies grown next to a *Penicillium* contaminant on an agar plate. Fleming also made two significant contributions to the field of AST in the 1920s. In 1924, he introduced the use of the ditch plate technique for evaluating antimicrobial qualities of antiseptic solutions [3]. Fleming’s second contribution to modern AST was the development of broth dilution technique using turbidity as an end-point determination [4]. Filter paper disks incorporating penicillin were utilized by Vincent & Vincent for assaying this newly discovered compound in 1940s [5]. Agar dilution AST method was also described in the 1940s [6]. At an early stage, it was realized that there were many variables affecting AST methods [7]. In 1961, World Health Organization (WHO) published a report on standardization of AST methodology [8]. The broad application of AST was introduced to clinical laboratories by the efforts of Bauer, Kirby and co-workers, with the method known as Kirby-Bauer disk diffusion method which is still the most widely used AST technique in the world [9]. Bergeron & Ouellette highlighted the shortcomings of the phenotypic approach to AST and concluded that different bacterial species have different susceptibilities to the same antibiotic, and that there is no international agreement on breakpoints for interpretation of antimicrobial susceptibility tests [10]. The need for developing standardized AST methods became a necessity soon after antibiotics became commercially available. During World War II, following penicillin, other antibiotics were discovered and used. Although these new antibiotics were regarded as “wonder drugs” at the time of their introduction, emergence of resistant strains followed. With the emergence of bacterial resistance to antimicrobials and the changing properties of different bacteria to different classes of antimicrobials, the need for the performance of AST on pathogens became a practical necessity.
Nationwide attempts were made to standardize AST methodologies; Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) (USA) [11], Werkgroep Richtlijnen Gevoeligheidsbepalingen (Netherlands) [12], Comité de l’Antibiogramme de la Société Française de Microbiologie (France) [13], the Swedish Reference Group for Antibiotics (Sweden) [14], Deutsches Institut für Normung (Germany) [15], the British Society for Antimicrobial Chemotherapy (UK) [16], they all published guidelines to improve the methodology and interpretation of AST. Recently, the European Committee on Antimicrobial Susceptibility Testing (EUCAST), a non-profit organization under the auspices of European Society of Clinical Microbiology and Infectious Diseases (ESCMID), developed and published AST guidelines. Breakpoint and QC tables for disk diffusion and minimum inhibitory concentration (MIC) testing can freely be accessed on organization’s website [17].

In clinical laboratories, widely adopted AST methods are disk diffusion and broth dilution methods. In disk diffusion method, disks impregnated with antimicrobial agents are used. The disks are placed onto agar plates which are preinoculated with the suspension of the microorganism being tested. The basic principle of the disk diffusion method is the diffusion of the antimicrobial agent into the medium which occurs when the disks come into contact with the moist surface of the plate. The concentration of the agent reduces logarithmically as the distance from the disk is increased. After the incubation period the plates are observed for the circular inhibition zone created around the disk which is due to the inhibitory effect of the antimicrobial agent on the microorganism. Within the zone the concentration of the agent is sufficient to inhibit growth, whereas at the point where the concentration of the agent is no longer enough to inhibit growth, the organism is able to grow and forms a lawn of bacteria around the disk. To interpret the test results, the radius of the inhibition zone is measured and compared against the predefined values provided by the guidelines [18]. The most widely used guidelines are the CLSI and EUCAST guidelines [2, 17]. CLSI divides the results into three categories for most of the organism-agent combinations; susceptible, intermediate and resistant, whereas EUCAST uses only two categories, susceptible and resistant.

In the dilution methods, however, the susceptibility of the microorganisms to antimicrobial agents is determined whether in tubes (macrobroth dilution method) or in microtube wells molded into a plastic plate (microbroth dilution method). Both broth dilution methods use the same principle; first serial two-fold dilutions of the antimicrobial agent to be tested are made in the tubes/wells containing broth, and then same amount of bacterial suspension is distributed on each tube/well. At the end of the incubation period, the tubes/wells are examined for turbidity which is the indicator of bacterial growth in broth. The tubes/wells remain clear where the concentration of the agent is high enough to inhibit the bacterial growth, whereas at lower concentrations of the agent, the bacteria may grow which causes the tube/well become turbid. The lowest concentration of antimicrobial agent that prevents the in vitro growth of bacteria is defined as the minimal inhibitory concentration (MIC) [18]. As in the disk diffusion method, the MIC values are compared against the predefined values provided by the guidelines and their interpretive category is determined and reported.
3. Quality assurance program for antimicrobial susceptibility testing

Clinical microbiology laboratories are an integral part of the total healthcare delivery system. Quality assurance (QA) is the overall process by which a laboratory can verify that a laboratory does its job well. While QA and quality control (QC) share the similar purposes, their meanings and functions are different [19]. QA can be defined as the overall program by which the quality of the test results can be guaranteed [20]. It evaluates and ensures that procedures provide relevant and timely data in the delivery of healthcare services. QA is primarily concerned with broader measures and monitors the performance of laboratory in total and covers all three phases of testing; pre-analytical, analytical and post-analytical. QC, in the other hand, is responsible for monitoring of the analytical phase of testing only and ensures that the daily tests are working properly [21]. QC and QA, only together provide measures for controlling how correct the tests are being performed because QC by itself often does not detect problems in time to prevent harmful results. For example, if >5% of Enterobacter, Serratia, or Citrobacter isolates are susceptible to ampicillin, it likely indicates a problem with insufficient inoculum [22]. Although daily or weekly QC test results are in acceptable limits, such an error can be overlooked until enough data have been accumulated and evaluated which can sometimes take weeks.

Standard processes are required to establish quality measures to be monitored. Standardization of AST has been achieved by CLSI, and in part by EUCAST. The processes defined in CLSI guidelines help clinical laboratories to perform QC tests, measure their results and provide corrective action recommendations covering a broad spectrum of error types. Each laboratory should establish its own quality requirements for testing processes. Only with established quality goals, laboratories can determine whether acceptable quality is being achieved, identify processes that are not performing satisfactorily and are in need of improvement, or to plan new processes to reach a specified level of quality [21]. And to ensure that all the established quality goals are achieved, a comprehensive QA program should be functional in a clinical laboratory.

The major components of a comprehensive QA program for AST, with the relative amount of effort required to be spent on each component given in parantheses, can be listed as follows [23]:

- Clinically relevant testing strategies (15%)
- Testing of reference QC strains (15%)
- Technical competency (15%)
- Organism antibiogram verification (15%)
- Supervisor review of results (15%)
- Procedure manual (10%)
- Cumulative antibiogram (5%)
The goals of the QC program as set by the CLSI [24, 25] includes to monitor the following:

- the precision (repeatability) and accuracy of AST procedures
- the performance of reagents used in the tests
- the performance of persons who carry out the tests and read the results

The continuous monitoring of the performance is best achieved, but not limited to, by the testing of QC strains.

3.1. Developing relevant antimicrobial susceptibility testing strategies

Only organisms likely to be the cause of an infection should be tested for antimicrobial susceptibility which necessitates the differentiation should be done between the normal flora that resides at the site of the infection and the actual organism causing the infection. Some important factors are to be considered to decide which bacterium or bacteria from a clinical specimen must be included in the AST; such as the body site from which the organism was isolated, the presence of other bacteria and the quality of the specimen from which the organism was grown, the host’s status, the ability of the bacterial species to cause infection at the body site from which the specimen was obtained, etc. [1, 26].

3.2. Selecting antimicrobials to test and to report

Each laboratory is unique in its capability, resources, level of experience or institutional needs. Therefore, the decision of which antimicrobials to test depends on each laboratory’s specifications and cannot be generalized. The decision involves the opinions of infectious diseases specialist and the pharmacist and should also be in concordance with the hospital formulary. Generally, a laboratory defines 10 to 15 antimicrobial agents for routine testing against various organisms or organism groups, which is called antimicrobial panel or battery. In CLSI’s M100 documents Table 1A (Suggested Groupings of Antimicrobial Agents With FDA Clinical Indications That Should Be Considered for Routine Testing and Reporting on Nonfastidious Organisms by Clinical Microbiology Laboratories in the United States) is a valuable source of information to refer to when such tables are to be created at the local level [2]. Because the identity of the bacterial isolate is often not known at the time the AST is performed, some drugs, which are inappropriate to report for that particular isolate, may be tested. These results, however, should be suppressed in the final report.

The goal of the clinical microbiology laboratory is to create a report which will direct the clinician to use the least toxic, most cost-effective and most clinically effective agent that is available. This is accomplished by using the selective-reporting protocol provided by the CLSI. CLSI categorizes antimicrobial agents generally into four groups, Group A, B, C and U. Group A includes the primary agents whose results to be reported first. The results of
Group B drugs should be selectively reported because these are generally broader spectrum agents. However, if the isolate is resistant to the primary agents, the patient cannot tolerate drugs in Group A, the infection has not responded to the therapy with the primary agents, a secondary agent would be a better clinical choice for the particular infection or that the patient has organisms isolated from another site also, and a secondary agent might be more appropriate for treating both organisms, then the results of Group B drugs can be reported [26]. Group C includes alternative or supplemental agents for special cases; such as resistant strains, for patients allergic to primary drugs, for treatment of unusual isolates or for epidemiological purposes. And finally, Group U, includes the agents that are used only or primarily in the treatment of urinary tract infections (e.g., nitrofurantoin, norfloxacin).

Selective-reporting, also called cascade-reporting, improves the clinical relevance of the reports produced and minimizes the selection of multiresistant strains by avoiding the use of broad spectrum agents when narrow spectrum option is susceptible.

3.3. Standardization of the antimicrobial susceptibility testing methodology

The procedural steps of each method must be followed strictly in order to obtain reproducible results. Standardization of AST methodology helps to optimize bacterial growth conditions so that the inhibition of growth can be attributed to the antimicrobial agent and the effects of nutrient limitations, temperature differences or other environmental conditions can be eliminated. And it also optimizes conditions for maintaining antimicrobial integrity and activity so that the failure to inhibit bacterial growth can be attributed to the organism’s resistance mechanisms [1].

The standardized components of AST include:

**Bacterial inoculum size:** Preparation of the inoculum is one of the most critical steps in any susceptibility test method. Inoculum suspensions are prepared using either a log-phase or direct-colony suspension. When direct-colony suspension method is used, 4 to 5, fresh (16- to 24-hour old) colonies, rather than a single colony, should be selected to minimize the possibility of testing a susceptible colony only and missing the resistant mutants dispersed in other colonies. McFarland turbidity standards are used to standardize the number of bacteria in the inoculum. McFarland standards can be prepared by adding specific volumes of 1% sulfuric acid and 1.175% barium chloride to obtain a barium sulfate solution with a specific optical density. The most commonly used is the McFarland 0.5 standard, which provides turbidity comparable with that of a bacterial suspension containing approximately 1.5 \(10^8\) CFU/mL (CFU: colony-forming unit). Once standardized, the inoculum suspensions should be used within 15 minutes of preparation. False-susceptible results may occur if too few bacteria are tested, and false-resistant results may be the outcome of testing too many bacteria [26].

**Growth medium:** The most frequently used growth media are Mueller-Hinton broth and Mueller-Hinton agar. The standardized variables regarding these media should include: its formulation, pH, cation concentration and thymidine content, thickness of agar (disk diffusion test), and supplements such as blood and serum.
Incubation conditions (atmosphere, temperature, duration): Different organisms require different incubation conditions. Moreover, some antimicrobial agents require different incubation length or temperature than the other disks used for the same organism (e.g., oxacillin with *Staphylococcus* spp.). The user should refer to CLSI M100 tables which give detailed testing conditions for each organism or organism group [2].

Antimicrobials concentrations to be tested: The contents of antimicrobial disks in disk diffusion test and concentrations of antibiotic solutions to be tested in dilution tests are also included in CLSI documents [2].

3.4. Quality control testing with reference quality control strains

Routine QC testing with a range of QC strains is the backbone of the internal QC testing. QC strains are well characterized organisms with defined susceptibility or resistance mechanisms to the antimicrobial agent(s) tested. Testing of QC strains helps to concurrently monitor the performance of the test and ensures that the test is being performed properly. The results obtained with the QC strains should be in predefined, acceptable ranges; for disk diffusion test, between the predefined inhibition zone diameters, and for MIC tests in predefined MIC ranges. If deviations from the acceptable limits are observed, it indicates unacceptable performance and the source(s) of the error should be investigated. CLSI recommends to use various QC strains for different aspects of AST. The list of QC strains can be found in the M100 tables which are updated on a yearly basis. Because of the introduction of new drugs, the changes effecting the existing drugs, or the emergence of new resistance mechanisms which should be investigated by the laboratory, the users are always referred to the latest update available. The QC strains recommended by CLSI are divided in two as being regular „QC strains“ and „supplemental QC strains“. Each laboratory performing AST with CLSI’s reference methods should include QC strains in regular QC tests, however, the supplemental strains are only required if they are used to assess a new test, for training new personnel, investigation of special susceptibility or resistance characteristics, etc., and are not required to be included in the routine QC of AST [2].

CLSI’s European counterpart, EUCAST, also publishes guidelines for the use of QC strains for AST, however, compared with the comprehensive battery of QC strains suggested by the CLSI, EUCAST is limited to six QC strains at the moment [27]. The guidelines of EUCAST are continuously evolving and on areas where EUCAST’s experience is not able to cover yet, EUCAST does not refrain from making referrals to relevant CLSI documents. However, one big difference between the QC strains recommended by CLSI and EUCAST is that, EUCAST’s recommendation for *Haemophilus influenzae* NCTC 8468 in contrast to CLSI’s *H. influenzae* ATCC® 49247. The strain EUCAST chose as a QC strain is susceptible to β-lactam antibiotics whose inhibition zones are easier to read than the ATCC® strain which is a β-lactamase negative, ampicillin resistant (BLNAR) strain. The suggested QC strains by CLSI with their specifications are listed in Table 1 [2].
| QC Strain | Test(s), for which strain is primarily used |
|-----------|--------------------------------------------|
| **Escherichia coli ATCC® 25922** | Disk diffusion and MIC of Enterobacteriaceae, Pseudomonas aeruginosa, Acinetobacter spp., Burkholderia cepacia, Stenotrophomonas maltophilia |
| | MIC of other non-Enterobacteriaceae |
| | Screening and confirmatory tests for ESBLs (negative) |
| | Disk diffusion and MIC of Neisseria meningitidis (for ciprofloxacin, nalidixic acid, minocycline, and sulfisoxazole) |
| **Escherichia coli ATCC® 35218** | Disk diffusion and MIC for β-lactam/β-lactamase inhibitor combination drugs of Enterobacteriaceae, Pseudomonas aeruginosa, Acinetobacter spp., Burkholderia cepacia, Stenotrophomonas maltophilia, Staphylococcus spp. |
| | MIC for β-lactam/β-lactamase inhibitor combination drugs of other non-Enterobacteriaceae |
| | Testing of amoxicillin-clavulanic acid for Haemophilus spp. |
| **Klebsiella pneumoniae ATCC® 700603** | Screening and confirmatory tests for ESBLs (positive) |
| **Klebsiella pneumoniae ATCC® BAA-1705** | Confirmatory test for suspected carbapenemase production in Enterobacteriaceae (MHT positive) |
| **Klebsiella pneumoniae ATCC® BAA-1706** | Confirmatory test for suspected carbapenemase production in Enterobacteriaceae (MHT negative) |
| **Pseudomonas aeruginosa ATCC® 27853** | Disk diffusion and MIC of Pseudomonas aeruginosa, Acinetobacter spp., Burkholderia cepacia, Stenotrophomonas maltophilia |
| | MIC of other non-Enterobacteriaceae |
| **Staphylococcus aureus ATCC® 25923** | Disk diffusion of Staphylococcus spp. and Enterococcus spp. |
| | Screening test for β-lactamase production of Staphylococcus aureus group and coagulase negative Staphylococci (negative) |
| | Screening test for mecaA-mediated oxacillin resistance using cefoxitin in Staphylococcus aureus group and coagulase negative Staphylococci (mecaA negative; disk diffusion susceptible) |
| | Screening test for inducible clindamycin resistance in Staphylococcus aureus group and coagulase negative Staphylococci with disk diffusion (D-zone test) (negative) |
| | Screening test for high-level mupirocin resistance in Staphylococcus aureus group (mupA negative; disk diffusion susceptible) |
| **Staphylococcus aureus ATCC® 29213** | MIC of Staphylococcus spp. |
| QC Strain | Test(s), for which strain is primarily used |
|-----------|-------------------------------------------|
| QC Strain Test(s), for which strain is primarily used | Screening test for β-lactamase production in *Staphylococcus aureus* group and coagulase negative *Staphylococci* (positive) |
| | Screening test for oxacillin resistance in *Staphylococcus aureus* group (susceptible) |
| | Screening test for *mecA*-mediated oxacillin resistance using cefoxitin in *Staphylococcus aureus* group (*mecA* negative; MIC susceptible) |
| | Screening test for inducible clindamycin resistance in *Staphylococcus aureus* group, coagulase negative *Staphylococci* and *Streptococcus* spp. β-hemolytic group with broth microdilution (no growth) |
| | Screening test for high-level mupirocin resistance in *Staphylococcus aureus* group (*mupA* negative; MIC susceptible) |
| Staphylococcus aureus ATCC® 43300 | Screening test for oxacillin resistance in *Staphylococcus aureus* group (resistant) |
| | Screening test for *mecA*-mediated oxacillin resistance using cefoxitin in *Staphylococcus aureus* group (disk diffusion and MIC) and coagulase negative *Staphylococci* (disk diffusion) (*mecA* positive) |
| Staphylococcus aureus ATCC® BAA-976 | Screening test for inducible clindamycin resistance in *Staphylococcus aureus* group, coagulase negative *Staphylococci* and *Streptococcus* spp. β-hemolytic group with broth microdilution (no growth) |
| Staphylococcus aureus ATCC® BAA-977 | Screening test for inducible clindamycin resistance in *Staphylococcus aureus* group, coagulase negative *Staphylococci* and *Streptococcus* spp. β-hemolytic group with broth microdilution (growth) |
| Staphylococcus aureus ATCC® BAA-1708 | Screening test for high-level mupirocin resistance in *Staphylococcus aureus* group (*mupA* positive; disk diffusion and MIC resistant) |
| Enterococcus faecalis ATCC® 29212 | MIC of *Enterococcus* spp. |
| | Screening test for vancomycin MIC ≥8 µg/mL in *Staphylococcus aureus* group (susceptible) |
| | Screening test for high-level aminoglycoside resistance in *Enterococcus* spp. (disk diffusion, broth microdilution, agar dilution: susceptible) |
| QC Strain                              | Test(s), for which strain is primarily used                                                                 |
|---------------------------------------|------------------------------------------------------------------------------------------------------------|
| **Enterococcus faecalis ATCC® 51299** | Screening test for vancomycin resistance in *Enterococcus* spp. (agar dilution: susceptible) checking that medium is acceptable for testing sulfonamides, trimethoprim, and trimethoprim/sulfamethoxazole |
| **Enterococcus faecalis ATCC® 51299** | Screening test for high-level aminoglycoside resistance in *Enterococcus* spp. (broth microdilution, agar dilution: resistant) |
| **Haemophilus influenzae ATCC® 49247** | Disk diffusion and MIC of *Haemophilus* spp. (BLNAR; β-lactamase negative, ampicillin resistant) |
| **Haemophilus influenzae ATCC® 49766** | Disk diffusion and MIC of *Haemophilus* spp. with selected cephalosporins (β-lactamase positive) |
| **Haemophilus influenzae ATCC® 10211** | Checking growth capabilities of medium used for disk diffusion and MIC tests for *Haemophilus* spp. |
| **Neisseria gonorrhoeae ATCC® 49226** | Disk diffusion and MIC of *Neisseria gonorrhoeae* (CMRNG; chromosomally mediated (penicillin) resistant *N. gonorrhoeae*) |
| **Streptococcus pneumoniae ATCC® 49619** | Screening test for inducible clindamycin resistance in *Streptococcus* spp. β-hemolytic group with disk diffusion (D-zone test) and broth microdilution (negative) |
| **Bacteroides fragilis ATCC® 25285** | MIC of anaerobes |
| **Bacteroides thetaiotaomicron ATCC® 29741** | MIC of anaerobes |
| **Clostridium difficile ATCC® 700057** | MIC of anaerobes |
| **Eubacterium lentum ATCC® 43055** | MIC of aeriobes |

**Table 1.** Quality Control Strains Suggested for Antimicrobial Susceptibility Testing by CLSI

### 3.5. Selection, obtaining and maintenance of reference QC strains

When selecting QC strains for routine internal QC testing; the strains that most closely resemble the patient’s isolate should be tested [23]. This will provide that the drugs planned to be tested for the patient can be concomitantly tested with the QC strain. Additionally, same materials and testing conditions used for the clinical isolates can be evaluated. Before obtaining the QC strains, laboratories should decide which strains do fit best to the laborato-
ry’s procedures. For example, if a laboratory does not perform Modified Hodge Test (MHT) to confirm suspected carbapenemase production in Enterobacteriaceae, the Klebsiella pneumoniae ATCC® BAA-1705 (MHT-positive) and Klebsiella pneumoniae ATCC® BAA-1706 (MHT-negative) strains are not necessary for that particular laboratory. QC organisms susceptible to the tested antimicrobials are generally used but resistant QC strains are also necessary when testing for special resistance mechanisms.

The QC strains can be obtained from various suppliers and in many formats. What important is, no matter in what format the strain has been received, the initial reconstitution should be performed according to supplier’s recommendations. For long term storage, stock cultures can be stored in a suitable stabilizer (e.g., trypticase soy broth with 10 to 15% glycerol, 50% fetal calf serum in broth, defibrinated sheep blood or skim milk) at -20°C or below (preferably at -60°C or below). To obtain working control cultures, subcultures from the permanent stock culture are made onto agar plates. Isolated colonies (4 to 5) are selected and subcultured to an agar slant (trypticase soy agar slants for non-fastidious organisms and chocolate agar slants for fastidious organisms) and incubated overnight. These working cultures on agar slants are stored at 2 – 8°C, for no more than three successive weeks. New working control cultures should be prepared at least monthly from permanent stock cultures. Prior to QC testing, growth from an agar slant is subcultured to agar plates and incubated overnight. To use for QC testing, 4 to 5 isolated colonies from the plate are selected. A new working culture should be prepared each day the QC test is being performed [2, 23].

Working control cultures can be used to monitor precision (repeatability) and accuracy of the AST as long as no significant change in the mean zone diameter or MIC value, not attributable to faulty methodology, is observed. Laboratories usually do not have problems with the maintenance of susceptible QC strains owing to the stability of these strains, however, QC strains with particular resistance mechanisms are harder to maintain since they may be less genetically stable. Repeated subcultures can cause the loss of resistance mechanisms and unsatisfactory performances can be experienced. Documented problems have arisen with the QC strains which carry their specific resistance mechanism on a plasmid (e.g., E. coli ATCC® 35218 and K. pneumoniae ATCC® 700603) [2]. Suboptimal storage conditions and repeated cultures may cause the spontaneous loss of the plasmid encoding the β-lactamase and off-the-limit results may be encountered.

3.6. Frequency of QC testing

Appropriate QC organisms should be tested daily for all antimicrobial agents routinely included in the antimicrobial battery until a laboratory achieves “satisfactory performance”. CLSI makes the definition of “satisfactory performance” as obtaining unacceptable results in no more than 1 out of 20 or 3 out of 30 results obtained in consecutive test days for each antimicrobial agent/organism combination. Once this satisfactory performance is obtained, a laboratory can convert from daily QC testing to weekly QC testing. As long as all QC test results are within the acceptable limits, the laboratory can continue weekly testing, however on occasions when a modification in the test is made, consecutive QC testing is required (Table 2, adapted from reference 2).
| Day(s)* | Modification in the Test |
|---------|-------------------------|
| 1       | Start to use new shipment or lot number of disks/MIC panels or prepared agar plates |
|         | Start to use disks from a new manufacturer |
|         | Expand or reduce the dilution range in MIC testing |
|         | Repair of instrument that affects the AST results |
| 5       | Start to use prepared agar plates (disk diffusion), broth or agar (MIC) from a new manufacturer |
|         | Convert inoculum preparation/standardization method from visual adjustment of turbidity to use a photometric device which has its own QC protocol |
|         | Update of the software which affects the AST results |
| 20 or 30| Use new method for MIC test (e.g., convert from visual reading to instrument reading of panel, convert from overnight to rapid MIC test) |
|         | Use new manufacturer of MIC test |
|         | Change method of measuring zones in disk diffusion test (e.g., start using an automated zone reader) |
|         | Convert inoculum preparation/standardization method to a method that is dependent on user technique |

* Number of days of consecutive QC testing required

Table 2. Required Quality Control Frequency after Modifications in the Test

For both, disk diffusion and MIC testing, addition of any new antimicrobial agent to the existing panel requires 20 or 30 consecutive days of satisfactory testing before it can be tested on a weekly schedule.

3.7. Corrective action

Corrective action is defined as the “action to eliminate the cause of a detected nonconformity or other undesirable situation” [28] and in regard to AST, is needed whenever any of the weekly QC results are not within the acceptable limits. The factors causing for the deviation in the results are various but can be divided in two as being results due to identifiable errors and results with no error identified [24, 25]. Identifiable errors, also named obvious errors, are easy to detect and also easy to correct. Most usual reasons causing for identifiable errors include; use of the wrong disk, use of the wrong QC strain, contamination of the strain or media, use of the wrong incubation temperature or conditions. If the reason causing the out-of-range results is one of the identifiable errors, the test must be carried out again the day the error is observed. If results of the repeat test are in acceptable limits, no further correc-
tive action is necessary. On the other hand, if the reason causing for the error cannot be identified, the test must be carried out again the day the error is observed, preferably with a new working culture or subculture, but should also be monitored for a total of five consecutive test days. During five consecutive days, if all results are within the acceptable limits no additional corrective action is required. However, if any of the results are outside the acceptable limits, additional corrective action is required. At this point, a systematic error, rather than a random should be suspected and the components of AST should be thoroughly investigated. The reasons include; wrong measurement, clerical errors, problems in the adjustment of turbidity, past expiration date materials, failure in providing proper growth conditions (temperature, atmosphere), improper storage of disks, contamination of QC strain, loss of characteristics, inoculum prepared from an old plate (> 24 hours), etc. In order to start to routine QC testing, satisfactory performance for another 20 or 30 consecutive days is required once the reason causing the error is detected and corrected.

When an out-of-range QC results necessitates a corrective action, the factors listed in Table 3 should be considered for troubleshooting (Table 3, adapted from references 24 and 25).

| QC Strain | Use of the wrong QC strain |
|-----------|-----------------------------|
|           | Improper storage            |
|           | Inadequate maintenance (e.g., use of the same working culture for >1 month) |
|           | Contamination               |
|           | Nonviability                |
|           | Changes in the organisms (e.g., mutation, loss of plasmid) |
| Testing supplies | Improper storage or shipping conditions |
|           | Contamination               |
|           | Use of a defective agar plate (too thick or too thin) |
|           | Inadequate volume of broth in tubes or wells |
|           | Use of damaged plates, panels, cards, tubes (e.g., cracked, leaking) |
|           | Use of expired materials    |
| Testing process | Use of the wrong incubation temperature or conditions |
|           | Inoculum suspensions were incorrectly prepared or adjusted |
|           | Inoculum prepared from a plate incubated for the incorrect length of time |
|           | Inoculum prepared from differential or selective media containing anti-infective agents or other growth-inhibiting compounds |
|           | Use of wrong disk/reagents, ancillary supplies |
|           | Improper disk placement (e.g., inadequate contact with the agar) |
|           | Incorrect reading or interpretation of test results |
|           | Transcription error         |
| Equipment | Not functioning properly or out of calibration (e.g., pipettes) |

Table 3. Factors Frequently Causing Out-of-range Results
3.8. Documentation of the quality control test results

Results from all QC tests should be documented on a QC log sheet [23]. On this log sheet information regarding the following are required: the date, the technician who performed the test, antimicrobial agents used (potency, lot, expiration date, etc.), media used (lot, expiration date, etc.). Once the log sheet has been filled by the technician who performed and read the test, a second technician, or the supervisor, should check the results. Also, corrective actions taken, if any, and their outcomes should be noted.

A useful and simple way of monitoring QC results is to use the Shewhart diagram, in which the daily readings are plotted on a chart with upper and lower control limits marked [29]. It provides the visual assessment of the results but can also provide in depth information if a more formal mathematical approach is followed [20]. An example of presenting daily QC results on a Shewhart diagram is given in Figure 1. The famous rules of Westgard and Klee [30] can be easily adopted to the QC of disk diffusion test in which the control diameters are treated as mean ±2 SD [20].

One QC result lies outside the limits (Westgard rule 1): It is a warning, whether it’s a random error or the beginning of an emerging problem. Routine test results for that day may be reported if there is no other evidence of problems in the current tests. It does not require corrective action by itself, unless the result is far out of range or there are other indications of a problem.

Two consecutive QC results are outside the limits in the same side of the mean of the range (Westgard rule 2): Indicates an error in the test methodology (a systematic error), corrective action is required.

Ten consecutive QC results falling on one side of the mean (Westgard rule 10): Results may be accepted but this likely indicates a systematic problem which should be acted on.

![Figure 1. Example for daily disk diffusion QC results for Escherichia coli ATCC® 25922 vs. ampicillin plotted on a Shewhart diagram (acceptable zone limits: 16 – 22 mm).](image-url)
3.9. Organism - Antimicrobial susceptibility test result verification

One of the most widely used supplemental QC measure is the use of susceptibility test results to verify results generated on patient results. Species with “typical” antibiograms are useful in verification of the identification as well as the susceptibility results. CLSI suggests some results to be confirmed before they are reported, these mostly include rare resistance phenotypes. The rare resistance phenotypes are divided in three categories; Category I: not reported or only rarely reported to date, Category II: uncommon in most institutions, and Category III; may be common, but is generally considered of epidemiological concern. Since category I includes the least encountered and most significant results, it is highly important to detect these results before being reported unnoticed and to follow the necessary steps for the verification. Unusual resistance phenotypes which require confirmation are given in Table 3 (adapted from reference 2).

| Category | Observed susceptibility result |
|----------|--------------------------------|
| I        | NS to carbapenems, extended-spectrum cephalosporins or fluoroquinolones in *H. influenzae* |
|          | NS to extended-spectrum cephalosporins, meropenem or minocycline, R to ampicillin or penicillin in *N. meningitidis* |
|          | NS to linezolid or vancomycin in *S. pneumoniae* |
|          | NS to ampicillin, penicillin, extended-spectrum cephalosporins, daptomycin, ertapenem, meropenem, linezolid or vancomycin in β-hemolytic group *Streptococcus* |
|          | NS to daptomycin, ertapenem, meropenem, linezolid, or vancomycin, R to quinupristin-dalfopristin in viridans group *Streptococcus* |
| II       | I or R to carbapenems in *Enterobacteriaceae* |
|          | I or R to 3rd generation cephalosporins or fluoroquinolones in *Salmonella* and *Shigella* spp. |
|          | R to colistin/polymyxin in *A. baumannii* |
|          | I or R to colistin/polymyxin in *P. aeruginosa* |
|          | I or R to trimethoprim-sulfamethoxazole in *S. maltophilia* |
|          | R to amoxicillin-clavulanic acid, R to ampicillin without accompanying β-lactamase production in *H. influenzae* |
|          | NS to extended spectrum cephalosporins in *N. gonorrhoeae* |
| Category | Observed susceptibility result |
|----------|--------------------------------|
| I to ampicillin, penicillin, I or R to rifampin, NS to azithromycin in *N. meningitidis* | |
| R to linezolid, NS to daptomycin for *Enterococcus* spp. | |
| NS to daptomycin, R to linezolid, I or R to quinupristin-dalfopristin, vancomycin MIC = 4 µg/mL or vancomycin MIC ≥ 8 µg/mL for *S. aureus* | |
| NS to daptomycin, I or R to quinupristin-dalfopristin or vancomycin, R to daptomycin in coagulase-negative *Staphylococcus* spp. | |
| I or R to fluoroquinolone, imipenem, meropenem, quinupristin-dalfopristin, rifampin in *S. pneumoniae* | |
| I or R to quinupristin-dalfopristin in β-hemolytic group *Streptococcus* | |
| III | R to amikacin, gentamicin, and tobramycin in *Enterobacteriaceae* |
| I or R to extended spectrum cephalosporins in *E. coli*, *Klebsiella* spp. or *P. mirabilis* | |
| I or R to carbapenem in *A. baumannii* | |
| R to amikacin, gentamicin, and tobramycin, or carbapenem in *P. aeruginosa* | |
| I or R to fluoroquinolone in *N. gonorrhoeae* | |
| I or R to chloramphenicol or fluoroquinolone in *N. meningitidis* | |
| R to vancomycin or high-level aminoglycoside in *Enterococcus* spp. | |
| R to oxacillin in *S. aureus* | |
| R to amoxicillin, penicillin or extended spectrum cephalosporins in *S. pneumoniae* using nonmeningitis breakpoints | |

NS: nonsusceptible, I: intermediate, R: resistant

Table 4. Unusual Resistance Phenotypes Which Require Confirmation

The general approach to be followed is, for all three categories, to confirm the identification of the organism and the AST. If the results are confirmed, the infection control should be informed about the case.
3.10. Real-time review of results

Accuracy of the susceptibility test results should be continuously monitored. This is mostly accomplished by daily reviewing of the data that is being produced. Profiles which are likely, somewhat likely, somewhat unlikely and nearly impossible should be identified, whether manually or with the help of a software programmed to recognize different patterns of susceptibility data [1]. Prompt recognition of unusual resistance or inconsistent susceptibility helps the laboratory to timely confirm the susceptibility results. In order to confirm the results, first step is to exclude the transcriptional and reading errors and make sure of the purity of the inoculum which has been tested. If no errors are found in the previous steps, the identification of the organism should be confirmed and the susceptibility test be repeated, preferably with another method. In cases where no errors are detected and the unusual resistance is confirmed, the clinician may be warned and measures can be taken to limit the spread of this unusual resistance.

3.11. Education

Education is an important component of the QA process. Having knowledge about the methods also provides the understanding of their limitations and pitfalls. A well-educated technician may timely recognize atypical results and is aware of the approach to follow for the resolution and avoidance of errors [20]. A very efficient way of training in-service personnel is the end-point interpretation control [24, 25]. Laboratory workers, who perform AST, are provided with a set of selected disk diffusion plates and are asked to read the results. The recorded results are then compared by an experienced reader, e.g., the laboratory director, and the individual performances of each technician is evaluated and if necessary, corrected. It significantly helps to minimize variation in the interpretation of zone sizes among laboratory workers.

3.12. External quality assessment

In external quality assessment (EQA) programs, a central laboratory distributes test strains with known susceptibility profiles to all participant laboratories. Each participating laboratory tests and reports the results to the central laboratory. Once all the results are returned from participants, the central laboratory evaluates the results and prepares a feedback report. The benefit of participating in such program is that each individual laboratory can assess its own performance compared with other laboratories, at national and international levels, it functions as an educational tool, and also provides the evidence of performance required by the accrediting bodies. On the other hand, the number of strains distributed in a year is relatively small, which brings the disadvantage of the rare errors going unnoticed [20]. Also, in contrast to internal QC, which is capable of acting on problems encountered on daily basis, it takes quite a while for the EQA feedback reports to be sent to the participating laboratories, thus corrective action is delayed.

3.13. Internal quality assessment

Internal quality assessment (IQA) is a complementary activity to EQA in which routine tests are repeated on the same day as the original, but this time, with the identity of the specimen
blinded. After the reports are produced, the results are compared and discrepancies noted. This activity helps to monitor the precision and accuracy of the test procedure and may highlight problem areas not detected by other QC methods. It monitors not only the performance of the test and reagents, but also the performance of the persons carrying out the tests [20]. The EQA and the IQA are complementary activities, while IQA focuses on monitoring a single laboratory on a daily basis, EQA compares the performance of different laboratories and is important for maintaining long-term accuracy of the AST methods employed [21].

3.14. Proficiency testing programs

They are a type of EQA in which simulated patient specimens are sent to participating laboratories. Again, the reports are produced by each laboratory, and returned to the central laboratory for evaluation. In the United States, government mandates that clinical laboratories be accredited and licensed. The government and licensing agencies are using proficiency testing as an objective method for the accreditation of laboratories [21]. In 1988, the U.S. Congress passed the Clinical Laboratory Improvement Amendment (CLIA ’88) which mandated proficiency testing (PT) as a major part of the laboratory accreditation process [31]. The initial CLIA ’88 proposal called for two PT specimens per year but final legislative rule, published in 2003, expanded this to study five samples three times per year. The definition of failure is defined as two of five incorrect results on two of the three consecutive PT surveys [32].

4. Quality control of automated antimicrobial susceptibility test systems

According to the work load and the resources a laboratory has, a laboratory can choose to use one of many types of commercial automated antimicrobial susceptibility test systems. Most of these systems use the principle of turbidimetric detection of bacterial growth in a broth medium by use of a photometer which periodically examines the test wells [26]. The most widely used systems in the world are VITEK 2 System (bioMérieux Vitek, Hazelwood, MO), BD Phoenix System (BD Diagnostic Systems, Sparks, MD), MicroScan WalkAway SI (Siemens Healthcare Diagnostics, Sacramento, CA) and TREK Sensititre (ARIS 2X, Trek Diagnostis Systems, Cleveland, OH). Each device has its own QC procedure and commercial susceptibility testing devices are not addressed in CLSI standards. CLSI only describes methods regarding generic reference procedures, however these reference methods are used by the US Food and Drug Administration before clearance is given to a commercial system for marketing in the US to evaluate its performance.

5. Conclusion

Although great improvement has been done in AST methodology and automated susceptibility systems have been introduced which provide same-day results, it should be considered that there are still many variables not covered by the standard methods. First of all, the
laboratory test conditions are far different from in vivo conditions where the organism and the antimicrobial agent do actually interact. Factors, such as bacterial inoculum size, pH, cation concentration and oxygen tension differ greatly depending on the site of infection [1]. In spite of all these limitations, the clinical microbiology laboratory should follow the most up-to-date guidelines to serve the patients in the best possible way. With a well constructed QA program in operation, a laboratory should aim to ensure that the right test is carried out on the right specimen, and that the right result and right interpretation is delivered to the right person at the right time.

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