



REVIEW ARTICLE

# THE GUIDING PRINCIPLES ON ANTIMICROBIAL SUSCEPTIBILITY TESTING

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**Historically, medical practitioners and veterinarians selected antimicrobials to treat bacterial infectious diseases based primarily on past clinical experiences. However, with the increase in bacterial resistance to traditionally used antimicrobials, it has become more difficult for clinicians to empirically select an appropriate antimicrobial agent. As a result, *in vitro* antimicrobial susceptibility testing (AST) of the relevant bacterial pathogens, from properly collected specimens, should use validated methods. The goal of *in vitro* antimicrobial susceptibility testing is to provide a reliable predictor of how an organism is likely to respond to antimicrobial therapy in the infected host. This type of information aids the clinician in selecting the appropriate antimicrobial agent, aids in developing antimicrobial use policy, and provides data for epidemiological surveillance. Such epidemiological surveillance data provide a base to choose the appropriate empirical treatment (first-line therapy) and to detect the emergence and/or the dissemination of resistant bacterial strains or resistance determinants in different bacterial species.**

**Key words:** AST, Disk diffusion, Broth dilution, Agar dilution, Minimum inhibitory concentration.

## INTRODUCTION

The spread of multiple antimicrobial-resistant pathogenic bacteria has been recognised by the World Organisation for Animal Health (OIE), the Food and Agriculture Organisation (FAO) and the World Health Organization (WHO) as a serious global human and animal health problem. The development of bacterial antimicrobial resistance is neither an unexpected nor a new phenomenon. It is, however, an increasingly troublesome situation due to the frequency with which new emerging resistance phenotypes are occurring among many bacterial pathogens and even commensal organisms.

Historically, many infections could be treated successfully based on the clinician's past clinical experience (*i.e.* empirical therapy). Resistance has been observed to essentially all of the antimicrobial agents currently approved for use in human and veterinary clinical medicine. This,

combined with the variety of antimicrobial agents currently available, makes the selection of an appropriate agent an increasingly more challenging task. This situation has made clinicians more dependent on data from *in vitro* antimicrobial susceptibility testing, and highlights the importance of the diagnostic laboratory in clinical practice. There is a number of antimicrobial susceptibility testing (AST) methods available to determine bacterial susceptibility to antimicrobials. The most widely used testing methods include broth microdilution or rapid automated instrument methods that use commercially marketed materials and devices. The selection of a method is based on many factors such as practicality, flexibility, automation, cost, reproducibility, accuracy, and individual preference. Standardisation and harmonisation of AST methodologies, used in epidemiological surveillance of antimicrobial drug resistance, are

critical if data are to be compared among national or international surveillance/monitoring programmes of OIE Member Countries. It is essential that AST methods provide reproducible results in day-to-day laboratory use and that the data be comparable with those results obtained by an acknowledged 'gold standard' reference method. In the absence of standardised methods or reference procedures, susceptibility results from different laboratories cannot be reliably compared. The method used to select samples for inclusion in antimicrobial resistance surveillance programmes, as well as the methods use for primary bacterial isolation, are also important factors that should be standardised or harmonised to allow direct comparison of data between different regions; consideration of these issues is addressed in an OIE document (OIE, 2003). As the science of AST has progressed, a greater understanding of the multiple factors that could affect the overall outcome of susceptibility testing has become clearer. This review provides guidelines and standardisation for AST methodologies, and interpretation of antimicrobial susceptibility test results.

#### **Antimicrobial susceptibility testing methodologies**

The following requirements should be respected: i) bacteria subjected to AST must be isolated in pure culture from the submitted sample ii) standard reference methods should be used for identification so that the subject bacteria are consistently and correctly identified to the genus and/or species level iii) bacterial isolates considered to be the most important and a sampling of other isolates, should be stored for future analysis (either lyophilisation or cryogenic preservation at -70°C to -80°C).

The following factors influencing AST methods should be determined, optimised, and documented in a detailed standard operating procedure: i) once the bacterium has been isolated in pure culture, the optimum concentration of the inocula must be determined to obtain accurate susceptibility results. Bacteria or other organisms used in AST testing should be from a fresh culture ii) the composition and preparation of the agar and broth media used (e.g. pH, cations, thymidine or thymine, use of supplemented media). Performance and sterility testing of media lots should also be determined and documented as well as employed

procedures iii) the content of antimicrobial in the carrier (antibiotics used in microtitre plates, disk, strip, tablet) iv) composition of solvents and diluents for preparation of antimicrobial stock solutions v) growth and incubation conditions (time, temperature, atmosphere e.g. CO<sub>2</sub>) vi) agar depth vii) number of concentrations tested per broth and agar dilution viii) the test controls to be used, including the reference organisms used ix) the subsequent interpretive criteria.

For these reasons, special emphasis has to be placed on the use of documented procedures and validated, well documented methods, as sufficient reproducibility can be attained only through the use of such methodology.

#### **Selection of antimicrobial susceptibility testing methodology**

The selection of an AST methodology may be based on the following factors: i) ease of performance ii) flexibility iii) adaptability to automated or semi-automated systems iv) cost v) reproducibility vi) reliability vii) accuracy viii) the organisms and the antimicrobials of interest in that particular OIE Member Country ix) availability of suitable validation data for the range of organisms to be susceptibility tested.

#### **Antimicrobial susceptibility testing methods**

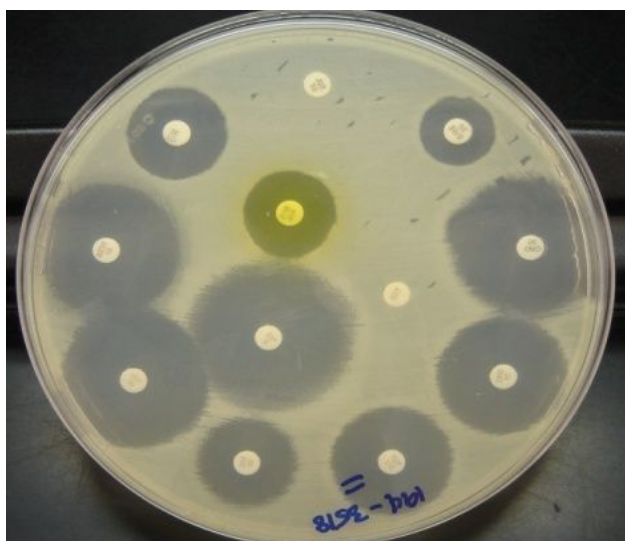
The following three methods have been shown to consistently provide reproducible and repeatable results when followed correctly (Threlfall *et al* 1999; Walker, 2000): i) disk diffusion ii) broth dilution iii) agar dilution.

#### **Disk diffusion method**

The disk diffusion susceptibility method (Jorgensen and Turnidge, 2007; Bauer *et al* 1966; Clinical and Laboratory Standards Institute, M2-A10, 2009) is simple and practical and has been well standardized. Disk diffusion refers to the diffusion of an antimicrobial agent of a specified concentration from disks, tablets or strips, into the solid culture medium that has been seeded with the selected inoculum isolated in a pure culture. Disk diffusion is based on the determination of an inhibition zone proportional to the bacterial susceptibility to the antimicrobial present in the disk.

The test is performed by applying a bacterial inoculum of approximately  $1-2 \times 10^8$  CFU/ml to the surface of a large (150 mm diameter) Mueller-Hinton agar plate. Up to 12 commercially-prepared, fixed concentrations,

paper antibiotic disks are placed on the inoculated agar surface (**Figure 1**). Plates are incubated for 16-24 h at 35°C prior to determination of results. The diffusion of the antimicrobial agent into the seeded culture media results in a gradient of the antimicrobial. When the concentration of the antimicrobial becomes so diluted that it can no longer inhibit the growth of the test bacterium, the zone of inhibition is demarcated. The diameter of this zone of inhibition around the antimicrobial disk is related to minimum inhibitory concentration (MIC) for that particular bacterium/antimicrobial combination; the zone of inhibition correlates inversely with the MIC of the test bacterium. Generally, the larger the zone of inhibition, the lower the concentration of antimicrobial required to inhibit the growth of the organisms. However, this depends on the concentration of antibiotic in the disk and its diffusibility.



**Fig. 1.** A disk diffusion test with an isolate of *Escherichia coli* from a urine culture. The diameters of all zones of inhibition are measured and those values translated to categories of susceptible, intermediate, or resistant using the latest tables published by the CLSI

#### **Considerations for the use of the disk diffusion methodology**

Disk diffusion is straightforward to perform, reproducible, and does not require expensive equipment. Its main advantages are: i) low cost ii) ease in modifying test antimicrobial disks when required iii) can be used as a screening test against large numbers of isolates iv) can identify a subset of isolates for further testing by other methods, such as determination of MICs.

Manual measurement of zones of inhibition may be time-consuming. Automated zone-reading devices are available that can be integrated with laboratory reporting and data-handling systems. The disks should be distributed evenly so that the zones of inhibition around antimicrobial discs in the disc diffusion test do not overlap to such a degree that the zone of inhibition cannot be determined. Generally this can be accomplished if the discs are no closer than 24 mm from centre to centre, though this is dependent on disk concentration and the ability of the antimicrobial to diffuse in agar.

#### **Broth and agar dilution methods**

The aim of the broth and agar dilution methods is to determine the lowest concentration of the assayed antimicrobial that inhibits the growth of the bacterium being tested (MIC, usually expressed in mg/ml or mg/litre). However, the MIC does not always represent an absolute value. The 'true' MIC is a point between the lowest test concentration that inhibits the growth of the bacterium and the next lower test concentration. Therefore, MIC determinations performed using a dilution series may be considered to have an inherent variation of one dilution. Antimicrobial ranges should encompass both the interpretive criteria (susceptible, intermediate and resistant) for a specific bacterium/antibiotic combination and appropriate quality control reference organisms. Antimicrobial susceptibility dilution methods appear to be more reproducible and quantitative than agar disk diffusion. However, antibiotics are usually tested in doubling dilutions, which can produce inexact MIC data.

Any laboratory that intends to use a dilution method and set up its own reagents and antibiotic dilutions should have the ability to obtain, prepare and maintain appropriate stock solutions of reagent-grade antimicrobials and to generate working dilutions on a regular basis. It is then essential that such laboratories use quality control organisms (see below) to assure accuracy and standardisation of their procedures.

#### **Broth dilution**

One of the earliest antimicrobial susceptibility testing methods was the macrobroth or tube-dilution method (Ericsson and Sherris, 1971). This procedure involved preparing two-fold dilutions of antibiotics (*e.g.* 1, 2, 4, 8, and 16 mg/ml) in a liquid growth medium dispensed in

test tubes (Jorgensen and Turnidge, 2007; Ericsson and Sherris, 1971).

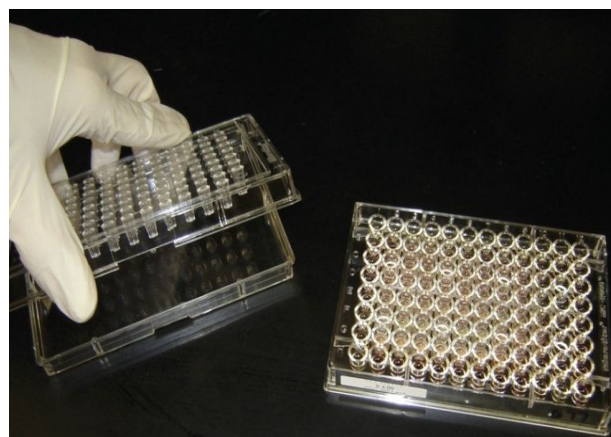
Broth dilution is a technique in which a suspension of bacterium of a predetermined optimal or appropriate concentration is tested against varying concentrations of an antimicrobial agent (usually serial two fold dilutions) in a liquid medium of predetermined, documented formulation. The broth dilution method can be performed either in tubes containing a minimum volume of 2 ml (macrodilution) or in smaller volumes using microtitration plates (microdilution). Numerous microtitre plates containing prediluted antibiotics within the wells are commercially available (**Figure 2**).

The use of identical lots in microdilution plates may assist in the minimisation of variation that may arise due to the preparation and dilution of the antimicrobials from different laboratories. The use of these plates, with a documented test protocol, including specification of appropriate reference organisms, will facilitate the comparability of results among laboratories.

The antibiotic containing tubes were inoculated with a standardized bacterial suspension of  $1-5 \times 10^5$  CFU/ml. Following overnight incubation at 35°C, the tubes were examined for visible bacterial growth as evidenced by turbidity. The lowest concentration of antibiotic that prevented growth represented the minimal inhibitory concentration (MIC). The precision of this method was considered to be plus or minus 1 two-fold concentration, due in large part to the practice of manually preparing serial dilutions of the antibiotics (Balows, 1972).

The advantage of this technique was the generation of a quantitative result (*i.e.* the MIC). The principal disadvantages of the macrodilution method were the tedious, manual task of preparing the antibiotic solutions for each test, the possibility of errors in preparation of the antibiotic solutions, and the relatively large amount of reagents and space required for each test.

Due to the fact that most broth microdilution antimicrobial test panels are prepared commercially, this method is less flexible than agar dilution or disk diffusion in adjusting to changing needs of surveillance/monitoring programme. Because the purchase of the antimicrobial plates/equipment may be costly, this methodology may not be feasible for some laboratories.



**Fig. 2.** A broth microdilution susceptibility panel containing 98 reagent wells and a disposable tray inoculators

### **Agar dilution**

Agar dilution involves the incorporation of varying concentrations of antimicrobial agent into an agar medium, usually using serial twofold dilutions, followed by the application of a defined bacterial inoculum to the agar surface of the plate. These results are often considered as the most reliable for the determination of an MIC for the test bacterium/antimicrobial combination. The advantages of agar dilution methods include: i) the ability to test multiple bacteria, except bacteria that swarm, on the same set of agar plates at the same time ii) the potential to improve the identification of MIC endpoints and extend the antibiotic concentration range iii) the possibility to semi-automate the method using an inoculum-replicating apparatus. Commercially produced inoculum replicators are available and these can transfer between 32 and 36 different bacterial inocula to each agar plate.

Agar dilution methods also have certain disadvantages, for example: i) if not automated, they are very laborious and require substantial economic and technical resources ii) once the plates have been prepared, they normally should be used within a week iii) the endpoints are not always easy to read nor is the purity of the inoculum easy to verify. Agar dilution is often recommended as a standardised AST method for fastidious organisms (NCCLS, document M31-A2, 2002), such as anaerobes, *Campylobacter* and *Helicobacter* species.

### **Antimicrobial gradient method**

The antimicrobial gradient diffusion method uses the principle of establishment of an

antimicrobial concentration gradient in an agar medium as a means of determining susceptibility. The E test (bioMérieux AB BIODISK) (**Figure 3**) is a commercial version available in the United States. In E test, thin plastic test strips are impregnated on the underside with a dried antibiotic concentration gradient and are marked on the upper surface with a concentration scale. As many as 5 or 6 strips may be placed in a radial fashion on the surface of an appropriate 150-mm agar plate that has been inoculated with a standardized organism suspension like that used for a disk diffusion test. After overnight incubation, the tests are read by viewing the strips from the top of the plate. The MIC is determined by the intersection of the lower part of the ellipse shaped growth inhibition area with the test strip. The gradient diffusion method has intrinsic flexibility by being able to test the drugs the laboratory chooses. E-test strips cost approximately \$2–\$3 each and can represent an expensive approach if more than a few drugs are tested. This method is best suited to situations in which an MIC for only 1 or 2 drugs is needed or when a fastidious organism requiring enriched medium or special incubation atmosphere is to be tested. *e.g.* penicillin and ceftriaxone with pneumococci (Huang *et al* 1992; Jorgensen *et al* 1994; Citron *et al* 1991). Generally, E-test results have correlated well with MICs generated by broth or agar dilution methods (Huang *et al* 1992; Jorgensen *et al* 1994; Citron *et al* 1991; Baker *et al* 1991; Rennie *et al* 2008). However, there are some systematic biases toward higher or lower MICs determined by the E-test when testing certain organism-antimicrobial agent combinations (Jorgensen *et al* 1994; Prakash *et al* 2008). This can represent a potential shortcoming when standard MIC interpretive criteria derived from broth dilution testing (Prakash *et al* 2008) are applied to E-test MICs that may not be identical.

#### **Interpretation of susceptibility test results**

The results of a susceptibility test must be interpreted by the laboratory prior to communicating a report to a patient's physician. Optimal interpretation of MICs requires knowledge of the pharmacokinetics of the drug in humans and information on the likely success of a particular drug in eradicating bacteria at various body sites (CLSI, document M23-A3, 2008). This is best accomplished by referring to an expert source such as the CLSI, which

publishes interpretive criteria for MICs of all relevant antibiotics for most bacterial genera (CLSI, document M100-S19, 2009). Indeed, both MIC values and disk diffusion zone diameters must be interpreted using a table of values that relate to proven clinical efficacy of each antibiotic and for various bacterial species (CLSI, M2-A10, 2009). The CLSI zone size and MIC interpretive criteria are established by analysis of 3 kinds of data: (1) microbiologic data, including a comparison of MICs and zone sizes on a large number of bacterial strains, including those with known mechanisms of resistance that have been defined either phenotypically or genotypically; (2) pharmacokinetic and pharmacodynamic data; and (3) clinical studies results (including comparisons of MIC and zone diameter with microbiological eradication and clinical efficacy) obtained during studies prior to FDA approval and marketing of an antibiotic (CLSI, document M23-A3, 2008).



**Fig. 3.** A *Staphylococcus aureus* isolate tested by the E-test gradient diffusion method with vancomycin (VA), daptomycin (DM), and linezolid (LZ) on Mueller-Hinton agar. The minimum inhibitory concentration of each agent is determined by the intersection of the organism growth with the strip as measured using the scale inscribed on the strip

A “susceptible” result indicates that the patient’s organism should respond to therapy with that antibiotic using the dosage recommended normally for that type of infection and species (CLSI, document M23-A3, 2008 and M100-S19, 2009). Conversely, an organism with a MIC or zone size interpreted as “resistant” should not be inhibited by the concentrations of the antibiotic

achieved with the dosages normally used with that drug (CLSI, document M23-A3, 2008 and M100-S19, 2009). An intermediate result indicates that a microorganism falls into a range of susceptibility in which the MIC approaches or exceeds the level of antibiotic that can ordinarily be achieved and for which clinical response is likely to be less than with a susceptible strain. Exceptions can occur if the antibiotic is highly concentrated in a body fluid such as urine, or if higher than normal dosages of the antibiotic can be safely administered (*e.g.* some penicillins and cephalosporins). At times, the “intermediate” result can also mean that certain variables in the susceptibility test may not have been properly controlled, and that the values have fallen into a “buffer zone” separating susceptible from resistant strains (CLSI, document M23-A3, 2008 and M100-S19, 2009). Generally, reporting of a category result of susceptible, intermediate, or resistant provides the clinician with the information necessary to select appropriate therapy. Reporting of MICs could aid a physician in selecting from among a group of similar drugs for therapy of infective endocarditis or osteomyelitis, in which therapy is likely to be protracted. It is important that the tables used for susceptibility test interpretations represent the most current criteria. Indeed, the CLSI documents are reviewed and updated frequently, usually once per year. Use of old or outdated information from the original editions of FDA-approved drug labels or older CLSI tables could represent a serious shortcoming in the reporting of patients’ results.

#### **Antimicrobial susceptibility testing guidelines**

A number of standards and guidelines are currently available for antimicrobial susceptibility testing and subsequent interpretive criteria throughout the world (Kahlmeter *et al* 2003). Amongst others, these include standards and guidelines published by: Clinical Laboratory and Standards Institute (CLSI/NCCLS, USA), British Society for Antimicrobial Chemotherapy (BSAC, UK), Comité de l’Antibiogramme de la Société française de Microbiologie (CASFM, France), Swedish Reference Group for Antibiotics (SIR, Sweden), Deutsches Institut für Normung (DIN, Germany), Japanese Society for Chemotherapy (JSC, Japan), Commissie richtlijnen gevoeligheidsbepalingen (CRG, the Netherlands). At this time, only the CLSI/NCCLS has developed protocols for susceptibility testing of bacteria of animal origin

and determination of interpretive criteria (NCCLS, document M31-A2). However, protocols and guidelines are available from a number of standards organisations and professional societies (*i.e.* Clinical and Laboratory Standards Institute, British Society for Antimicrobial Chemotherapy, Japan Society for Chemotherapy (JSC), Swedish Reference Group for Antibiotics (SIR), Deutsches Institut für Normung, Comité de L’Antibiogramme de la Société Française de Microbiologie, Werkgroep richtlijnen gevoeligheidsbepalingen, and others) for susceptibility testing for similar bacterial species that cause infections in humans. It is possible that such guidelines can be adopted for susceptibility testing for bacteria of animal origin, but each country must evaluate its own AST standards and guidelines. Additionally, efforts focusing on both standardisation and harmonisation of susceptibility/resistance breakpoints on an international scale are progressing. These efforts have primarily focused on the adoption of the standards and guidelines of the CLSI, which provide laboratories with methods and quality control values enabling comparisons of AST methods and generated data (NCCLS Document M31-A2, White *et al* 2001). For those OIE Member Countries that do not have standardised AST methods in place, the adoption of CLSI standards would be an appropriate initial step towards acceptable methods and harmonisation.

As a first step towards comparability of monitoring and surveillance data, Member Countries should be encouraged to strive for harmonised and standardised programme design (Threlfall *et al* 1999). Data from countries using different methods and programme design may otherwise not be directly comparable (Threlfall *et al* 1999; Leegard *et al* 2000). Notwithstanding this, data collected over time in a given country may at least allow the detection of emergence of antimicrobial resistance or trends in prevalence of susceptibility/resistance in that particular country (Petersen *et al* 2003). However, if results achieved with different AST methods are to be presented side by side, then comparability of results must be demonstrated and consensus on interpretation achieved.

#### **Current test methods and future directions**

The antimicrobial susceptibility testing methods discussed in this article provide reliable results when used according to the procedures defined by the CLSI or by the manufacturers of the

commercial products. However, there is considerable opportunity for improvement in the area of rapid and accurate recognition of bacterial resistance to antibiotics. There is a need for development of new automated instruments that could provide faster results and also save money by virtue of lower reagent costs and reduced labor requirements. To accomplish this, it will likely be necessary to explore different methodologic approaches for detection of bacterial growth. The direct detection of resistance genes by polymerase chain reaction or similar techniques has limited utility, because only a few resistance genes are firmly associated with phenotypic resistance (e.g. *mecA*, *vanA*, and *vanB*) (Tenover, 1989). There are hundreds of  $\beta$ -lactamases, and numerous mutations, acquisitions, and expression mechanisms that result in fluoroquinolone, aminoglycoside, and macrolide resistance (Rice and Bonomo, 2007); too many to be easily detected by current molecular techniques. Thus, it seems likely that phenotypic measures of the level of susceptibility of bacterial isolates to antimicrobial agents will continue to be clinically relevant for years to come.

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## CONCLUSION

Although a variety of methods exist, the goal of *in-vitro* antimicrobial susceptibility testing is the same: to provide a reliable predictor of how a microorganism is likely to respond to antimicrobial therapy in the infected host. This type of information aids the clinician in selecting the appropriate antimicrobial agent, provides data for surveillance, and aids in developing antimicrobial use policies.

*In vitro* antimicrobial susceptibility testing can be performed using a variety of formats, the most common being disk diffusion, agar dilution, broth macro dilution, broth micro dilution, and a concentration gradient test (e.g. E test®).

Each of these protocols requires the use of specific testing conditions, including media, incubation conditions and times, and the identification of appropriate quality control organisms along with their specific QC ranges. The AST methods provide reproducible results in day-to-day laboratory use and the data of these methods are comparable with those results obtained by an acknowledged 'gold standard' reference method.

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