

SECTION 2.1.

LABORATORY DIAGNOSTICS

CHAPTER 2.1.1.

LABORATORY METHODOLOGIES FOR BACTERIAL ANTIMICROBIAL SUSCEPTIBILITY TESTING

SUMMARY

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 be performed using validated methods. Thus, AST is an important component of prudent antimicrobial use guidelines in animal husbandry worldwide and veterinarians in all countries should have these data available for informed decision-making.

Although a variety of methods exist, the goals of in-vitro antimicrobial susceptibility testing are either to provide a reliable predictor of how an organism is likely to respond to antimicrobial therapy in the infected host or to assess for surveillance purposes whether there has been development of resistance. 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. The selection of a particular AST method is based on many factors such as validation data, practicality, flexibility, automation, cost, reproducibility, accuracy, standardisation and harmonisation.

The use of genotypic approaches for detection of antimicrobial resistance genes has also been promoted as a way to increase the speed and accuracy of susceptibility testing. Numerous DNA-based assays are being developed to detect bacterial antimicrobial resistance at the genetic level. These methods, when used in conjunction with phenotypic analysis, offer the promise of increased sensitivity, specificity, and speed in the detection of specific known resistance genes and can be used in tandem with traditional laboratory AST methods.

INTRODUCTION

The spread of multiple antimicrobial-resistant pathogenic bacteria has been recognised by the World Organisation for Animal Health (WOAH, founded as OIE), the Food and Agriculture Organization (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, of increasing concern due to the frequency with which new emerging resistance phenotypes are occurring among many bacterial pathogens and commensal organisms, such as resistance to carbapenems, colistin, linezolid, macrolids, etc.

Historically, many infections could be treated successfully according to the clinician's past clinical experience or because susceptibility could be reliably predicted (i.e. empirical therapy); however, this is becoming more the exception than the rule (Walker, 2007). 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 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.

A number of antimicrobial susceptibility testing (AST) methods are available to determine bacterial susceptibility to antimicrobials. The selection of a method is based on many factors such as practicality, flexibility, automation, cost, reproducibility, accuracy, accessibility 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 WOAHA Members. It is essential that AST methods provide reproducible results in day-to-day routine laboratory use and that the data be comparable with those results obtained by an acknowledged 'gold standard' reference method. Currently the reference AST method is the broth micro-dilution method that determines minimum inhibitory concentration (MIC) as described by the ISO (International Organization for Standardization, 2006). 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 used 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 a WOAHA document (Dehaumont, 2004).

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 (WHO, 2017). This document provides guidelines and standardisation for AST methodologies, and interpretation of antimicrobial susceptibility test results.

1. Test requirements

The following requirements should be applied to achieve standardisation of AST methods and comparability of AST results:

- i) the use of standardised AST methods is essential, including the harmonisation of AST test parameters such as media, inoculum, incubation time, quality controls, choice of antimicrobial agents and subsequent interpretive criteria,
- ii) standardised AST methods, including all critical specifications and interpretive criteria, should be clearly defined, documented in detail and used by all participating laboratories,
- iii) all AST methods should generate accurate and reproducible data,
- iv) quantitative susceptibility data (MIC) should be reported,
- v) establishment of national or regional reference laboratories is essential for the coordination of AST methodologies, interpretations and appropriate operational techniques used to ensure accuracy and reproducibility (e.g. quality controls),
- vi) microbiological laboratories should implement and maintain a formal quality management programme (see Chapter 1.1.5 *Quality management in veterinary testing laboratories*),
- vii) laboratories should have acquired a third party accreditation that includes the AST methodologies to be used within the scope of that accreditation. The accreditation body should meet accepted international Laboratory Accreditation Cooperation [ILAC]) standards and guidelines regarding the standards used for the accreditation process. The accreditation standards used should include the requirement for participation in proficiency testing programmes,
- viii) specific bacterial reference/quality control strains are essential for determining intra- and inter-laboratory quality control, quality assurance and proficiency testing.

2. Selection of antimicrobials for testing and reporting

Selecting the appropriate antimicrobials for susceptibility testing can be difficult given the vast numbers of agents available. The following guidelines should be noted:

- i) the FAO/WOAH/WHO expert workshop on non-human antimicrobial usage and antimicrobial resistance recommends creating a list of veterinary and human critically important antimicrobials for susceptibility testing and reporting,

- ii) selection of the most appropriate antimicrobials is a decision best made by each WOH Member in consultation with the appropriate bodies and organisations,
- iii) antimicrobials in the same class may have similar *in-vitro* activities against select bacterial pathogens. In these cases, a representative antimicrobial should be selected that predicts susceptibility to other members of the same class,
- iv) certain microorganisms can be intrinsically resistant to particular antimicrobial classes; therefore it is unnecessary and misleading to test certain agents for activity *in vitro*. The type of intrinsic resistance has to be determined for these organisms from either the scientific literature or through testing,
- v) the number of antimicrobials to be tested should comply with the guideline used (CLSI/EUCAST/ISO) and at least contain class representatives to ensure the relevance and practicality of AST (see also WHO, 2017).

Periodic review of microorganisms that are currently predictably susceptible to certain antimicrobial agents is recommended to ensure that emergent, unexpected resistance is detected. Emerging resistance may also be suspected following poor response or treatment failure to a standard antimicrobial treatment regime.

3. 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 other selected 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, a standardised concentration of the inoculum must be prepared using a nephelometer or spectrophotometer to ensure a defined number of colony forming units to obtain accurate and repeatable susceptibility results. Bacteria or other organisms used in AST testing should be from a fresh 24-hour culture,
- ii) the composition and preparation of the agar and broth media used (e.g. pH, cations, thymidine or thymine, use of supplemented media) should comply with guidelines (CLSI/EUCAST/ISO). Performance and sterility testing of media lots should also be determined and documented as well as the procedures used,
- iii) the content, range/interval and concentration of the antimicrobials used (microtitre plates, disk, strip, tablet) should follow guidelines (CLSI/EUCAST/ISO) and be relevant to the species tested,
- iv) composition of solvents and diluents for preparation of antimicrobial stock solutions,
- v) growth and incubation conditions (time, temperature, atmosphere e.g. CO_2),
- vi) agar depth,
- vii) the test controls to be used, including the reference organisms used,
- viii) the subsequent interpretive criteria (clinical breakpoints, epidemiological cut-off values – ECOFFs).

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.

4. Selection of antimicrobial susceptibility testing methodology

The selection of an AST methodology may be influenced by 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 WOH Member,
- ix) availability of suitable validation data for the range of organisms to be susceptibility tested.

5. Antimicrobial susceptibility testing methods

The following three methods have been shown to consistently provide reproducible and repeatable results when followed correctly (Clinical and Laboratory Standards Institute [CLSI], 2008; Walker, 2007):

- i) disk diffusion,
- ii) broth dilution,
- iii) agar dilution,

5.1. Disk diffusion method

Disk diffusion refers to the diffusion of an antimicrobial agent from a disk or tablet containing a specified concentration of the agent tablets into a solid culture medium (normally Müller–Hinton agar) that has been inoculated with a pure culture (see Section 3). The disk diffusion result is determined by measurement of the diameter of the inhibition zone around the disk, the diameter being proportional to the bacterial susceptibility to the antimicrobial present in the disk.

The diffusion of the antimicrobial agent into the 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 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 antimicrobial agent in the disk and its diffusibility. Antimicrobial agents that are very large molecules diffuse poorly in agar making disk diffusion methods unreliable for these compounds. For this reason disk diffusion methods are not recommended for example for the susceptibility testing of colistin/polymyxin (Matuschek *et al.*, 2018).

Note: Disk diffusion tests based solely on the presence or absence of a zone of inhibition without regard to the size of the zone of inhibition are not acceptable AST methodology.

5.1.1. Considerations for the use of the disk diffusion methodology

Disk diffusion is easy to perform, reproducible if standardised, and does not require expensive equipment. Its main advantages are:

- i) low cost,
- ii) ease in modifying test by changing 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.
- v) the procedure is controlled by inclusion of appropriate control organisms for which a target zone size range is available (or has been derived) for each of the relevant antimicrobial agents being tested in the disk diffusion test procedure.

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 on the agar surface 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. Contamination of culture plates may be harder to detect using automated readers.

The diameter of the zone of inhibition obtained in disk diffusion tests is strongly influenced by the density of the bacterial inoculum applied, underlining the requirement to standardise the inoculum in accordance with guidelines (CLSI, EUCAST, ISO). A denser inoculum than intended will result in reduced zones of inhibition and a sparse inoculum will result in increased zones of inhibition (BSAC [British Society for Antimicrobial Chemotherapy], 2015).

5.2. Broth and agar dilution methods

The aim of the broth and agar dilution methods is to determine the lowest concentration of the antimicrobial that inhibits the visible growth of the bacterium being tested in either broth or on agar (MIC, usually expressed in µg/ml or mg/litre). The range of concentrations tested in broth and agar dilution methods generally includes the breakpoint (clinical or microbiological) with doubling dilutions either side of that value as considered appropriate. However, the MIC does not always represent exactly the concentration which was tested. 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 ±1 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. Target MIC ranges should be available for each antimicrobial agent being tested.

Antimicrobial susceptibility dilution methods are more reproducible than agar disk diffusion which is why broth microdilution is the current reference test method. However, antibiotics are usually tested in doubling dilutions, which can produce inexact MIC data. The continuous range of zone diameter values obtained with disk diffusion can therefore be advantageous in certain circumstances, such as screening large numbers of susceptible isolates.

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 appropriately maintain stock solutions of reagent-grade antimicrobials, to account for the potency of the antimicrobial (supplied by the manufacturer) and to generate complex working dilutions on a regular basis. Published methods should be consulted. It is then essential that such laboratories use quality control organisms (see below) to assure accuracy and standardisation of their procedures.

5.2.1. Broth dilution

Broth dilution is a technique in which a suspension of a bacterium of a predetermined optimal concentration is tested against varying concentrations of an antimicrobial agent (usually serial twofold 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 lyophilised or dried prediluted antibiotics within the wells are commercially available. The use of the same batches of microdilution plates may assist in the minimisation of variation that may arise due to the preparation and dilution of the antimicrobials at 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.

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 the changing needs of the surveillance/monitoring programme.

Because the purchase of antimicrobial plates and associated equipment may be costly, this methodology may not be feasible for some laboratories.

5.2.2. 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. This method may be considered the most reliable for MIC determination for some antimicrobials (fosfomycin, mecillinam) and for certain bacteria where broth dilution methods are not well established.

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 60 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 1–3 weeks depending in quality control (or less, depending on the stability of the antimicrobials tested),
- iii) the endpoints are not always easy to read.

Agar dilution is often recommended as a standardised AST method for fastidious organisms (CLSI, 2015), such as anaerobes and *Helicobacter* species.

5.3. Other bacterial AST and specific antimicrobial resistance tests

Bacterial antimicrobial MICs can also be obtained using commercially available gradient strips that diffuse a predetermined antibiotic concentration. However, the use of gradient strips can be expensive and MIC discrepancies can be found when testing certain bacteria/antimicrobial combinations compared with results of other methods (Ge *et al.*, 2002; Rathe *et al.*, 2009). Gradient strip methods are not recommended for testing the susceptibility of the antimicrobial agent colistin because of the large size of this molecule and its poor diffusion in agar (Matuschek *et al.*, 2018).

Regardless of the AST method used, the procedures should be documented in detail to ensure accurate and reproducible results, and appropriate reference and control organisms should always be tested every time AST is performed in order to ensure accuracy and validity of the data.

The appropriate AST choice can be dependent on the growth characteristics of the bacterium in question, as well as the objective of testing. In special circumstances, novel test methods and assays may be more appropriate for detection of particular resistance phenotypes. For example, chromogenic cephalosporin-based tests (CLSI, 2018) (e.g. nitrocefin) may provide more reliable and rapid results for beta-lactamase determination in certain bacteria, whereas inducible clindamycin resistance in *Staphylococcus* spp. may be detected using a disk diffusion method employing standard erythromycin and clindamycin disks in adjacent positions and measuring the resultant zones of inhibition (e.g. D-zone or D-test) (Zelazny *et al.*, 2005).

Similarly, extended-spectrum beta-lactamase (ESBL) (CLSI, 2018) activity in certain bacteria can also be detected by using standard disk diffusion susceptibility test methods incorporating specific cephalosporins (cefotaxime and ceftazidime) separately and in combination with a beta-lactamase inhibitor (clavulanic acid) and measuring the resulting zones of inhibition. Penicillin-binding protein 2a (PBP 2a) can also be detected in methicillin resistant staphylococci with a latex agglutination test (Stepanovic *et al.*, 2006). It is essential that testing of known positive and negative control strains occurs alongside clinical isolates to ensure accurate results.

Susceptibility testing may also be performed using breakpoint values specifically intended to detect particular mechanisms of bacterial resistance of clinical or public health importance, for example resistance to the carbapenems, which are used prudently to treat highly-resistant bacterial in humans (EUCAST [European Committee on Antimicrobial Susceptibility Testing], 2017).

5.4. Future directions in antimicrobial susceptibility/resistance detection

The use of genotypic approaches for detection of antimicrobial resistance genes has been promoted as a way to increase the rapidity and accuracy of susceptibility testing (Cai *et al.*, 2003; Chen *et al.*, 2005). Numerous DNA-based assays are being developed to detect bacterial antimicrobial resistance at the genetic level. The newest and perhaps most state-of-the-art approach is to use genome sequencing to predict antimicrobial resistance phenotypes via identification and characterisation of the known genes that encode specific resistance mechanisms.

Methods that employ the use of comparative genomics, genetic probes, microarrays, nucleic acid amplification techniques (e.g. polymerase chain reaction [PCR]), and DNA sequencing offer the promise of increased sensitivity, specificity, and speed in the detection of specific known resistance genes (Cai *et al.*, 2003; Chen *et al.*, 2005; Perreten *et al.*, 2005). Genotypic methods have been successfully applied to supplement traditional AST phenotypic methods for other organisms including methicillin-resistant staphylococci, vancomycin-resistant enterococci, and detection of fluoroquinolone resistance mutations (Cai *et al.*, 2003; Chen *et al.*, 2005; Perreten *et al.*, 2005). PCR methods have also been described for beta-lactamases, aminoglycoside inactivating enzymes, and tetracycline efflux genes (Cai *et al.*, 2003; Chen *et al.*, 2005; Frye *et al.*, 2010; Perreten *et al.*, 2005).

Technological innovations in DNA-based diagnostics should allow for the detection of multiple resistance genes and/or variants during the same test. The development of rapid diagnostic identification methods and genotypic resistance testing should help reduce the emergence of antimicrobial resistance, by enabling the use of the most appropriate antimicrobial when therapy is initiated. However, DNA techniques have to be demonstrated to be complementary to AST methods and results.

Additionally, new technological advances may facilitate the ability to probe bacterial species for large numbers of antimicrobial resistance genes quickly and cheaply, thereby providing additional relevant data for surveillance and monitoring programmes (Frye *et al.*, 2010). However, despite the new influx of genotypic tests, documented and agreed upon phenotypic AST methods will still be required in the near future to detect emerging resistance mechanisms among bacterial pathogens and to detect and characterise newly discovered mechanisms of resistance for the development and validation of genetic testing. A literature review (Ellington *et al.*, 2017) considered the role of whole genome sequencing (WGS) in antimicrobial susceptibility testing of bacteria and concluded there was insufficient published evidence to support the use of AST via WGS to replace phenotypic AST in clinical settings for all bacterial species, although certain bacteria (e.g. *Salmonella*, *Staphylococcus aureus*) had been well characterised for that purpose. Subsequently several publications have added support to the use of genetic AST (e.g. McDermott *et al.*, 2016; Zhao *et al.*, 2016). The future of genetic testing in the detection of antimicrobial resistance is promising, but phenotypic testing will remain an important mainstay.

6. Antimicrobial susceptibility breakpoints and zone of inhibition criteria

The primary objective of *in-vitro* AST is to predict how a bacterial pathogen may respond to an antimicrobial agent *in vivo*. The results generated by bacterial *in-vitro* antimicrobial susceptibility tests, regardless of whether disk diffusion or dilution methods are used, are generally interpreted and reported as resistant, susceptible or intermediate to the action of a particular antimicrobial by applying clinical breakpoints. No single formula for selection of optimal breakpoints has been established. The process involves a review of existing data and is influenced by the methods used to select appropriate breakpoints.

Generally, antimicrobial susceptibility breakpoints are established by national standards organisations, professional societies or regulatory agencies. The relevant documents should be consulted. However, there can be notable differences in breakpoints for the same antimicrobial agent within and among countries due to differences between standards setting organisations and regulatory agencies and because of regional or national differences in dosing regimens (Brown & MacGowan, 2010; de Jong *et al.*, 2009; Kahlmeter *et al.*, 2006).

As mentioned previously, antimicrobial susceptibility testing results should be recorded quantitatively:

- i) as distribution of MICs in mg/litre or µg/ml,
- ii) or as inhibition zone diameters in millimetres.

The following two primary factors enable a bacterial isolate to be interpreted as susceptible or resistant to an antimicrobial agent:

- i) The development and establishment of quality control ranges (CLSI, 2015), for disk diffusion or dilution testing, for quality control reference microorganisms.

Establishment of quality control ranges for control organisms is essential for validating test results obtained using a specific AST method. The allowable interpretive category ranges for reference control organisms should be established in addition to determining breakpoints for susceptibility or resistance. The use of reference organisms is a quality control and quality assurance activity.

- ii) The determination of the appropriate interpretive criteria regarding establishment of breakpoints (CLSI, 2015).

This involves the generation of three distinct types of data:

- a) MIC population distributions of the relevant microorganisms,
- b) pharmacokinetic parameters and pharmacodynamic indices of the antimicrobial agent,
- c) results of clinical trials and the outcome of treatment of clinical cases of disease.

The development of a concept known as ‘microbiological breakpoints’, or ‘epidemiological cut-off values’ (the highest MIC value for the bacterium and antimicrobial agent under consideration, where the bacterium is devoid of any phenotypically expressed resistance to that antimicrobial agent), may be more appropriate for some antimicrobial surveillance programmes. Epidemiological cut-off values are derived by examining MIC population distributions for specific bacterial species and antimicrobials performed at several laboratories according to a standardised broth microdilution method. Bacterial isolates that possess any acquired phenotypic resistance (that is, have an MIC above the epidemiological cut-off value) and therefore deviate from the normal wild-type fully-susceptible population are designated as non-wild type (also termed microbiologically resistant) and shifts in susceptibility to the specific antimicrobial/bacterium combination can thus be monitored (Kahlmeter, 2015; Kahlmeter *et al.*, 2006; Turnidge *et al.*, 2006). There is a great advantage in the recording of quantitative susceptibility data in that data may be analysed according to clinical breakpoints as well as by using epidemiological cut-off values.

The development of breakpoint criteria for disk diffusion tests usually involves comparing disk diffusion data against dilution data by creating a scattergram of the bacterial population distribution (representative bacterial isolates), by plotting the zone of inhibition against the logarithm to the base 2 of the MIC for each bacterial isolate for an individual bacterial species. The selection of breakpoints is then based on multiple factors, including regression line analysis that correlates MICs and zone diameters of inhibition, bacterial population distributions, error rate bounding, pharmacokinetics, and ultimately, clinical verification.

7. Antimicrobial susceptibility testing guidelines

A number of national standards and guidelines are currently available. International standards and guidelines for antimicrobial susceptibility testing and subsequent interpretive criteria throughout the world are:

Clinical Laboratory and Standards Institute (CLSI, 2018),
European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2017).

At this time, only the CLSI has developed protocols for susceptibility testing of bacteria of animal origin and determination of interpretive criteria (CLSI, 2018). A veterinary sub-committee (VETCAST) has also been set up under the umbrella of EUCAST. However, protocols and guidelines are available from a number of standards organisations and professional societies, including those listed above 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 CLSI and EUCAST, which provide laboratories with methods and quality control values enabling comparisons of AST methods and generated data (CLSI, 2018; Kahlmeter *et al.*, 2006). For those WOHAM Members that do not have standardised AST methods in place, the adoption of either set of standards would be an appropriate initial step towards acceptable methods and harmonisation.

Many bacteria that cause disease in aquatic animals require growth conditions (e.g. lower temperatures, supplemented or semisolid media) that may vary considerably as compared to terrestrial bacterial pathogens. This necessitated the need for the development of antimicrobial testing methods for bacteria isolated from aquatic species. Further information with regards to methods for disk diffusion or broth dilution antimicrobial susceptibility testing for bacteria isolated from aquatic animals can be referenced in two CLSI documents (CLSI, 2006; 2014b). Further information with regards to methods for disk diffusion or broth dilution antimicrobial susceptibility testing for infrequently isolated or certain fastidious bacteria (e.g. *Campylobacter*, *Pasteurella*) can also be referenced in the CLSI M45-A document (CLSI, 2015).

As a first step towards comparability of monitoring and surveillance data, Members should be encouraged to strive for harmonised and standardised programme design (Brown & MacGowan, 2010; Kahlmeter *et al.*, 2006; White *et al.*, 2001). Data from countries using different methods and programme design may otherwise not be directly comparable (Brown & MacGowan, 2010). 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 compared, then comparability of results must be demonstrated and consensus on interpretation achieved. This will be best accomplished by the use of accurate and reliable documented AST methods used in conjunction with monitoring of AST performance while using well characterised reference microorganisms among participating laboratories.

Table 1. Phenotypic susceptibility testing methods available and their features

Susceptibility testing method	International standard available	Published methods available	Use in national surveillance programmes	Use in susceptibility testing for therapeutic purposes	Breakpoints that may be applied	Test output	Comparability of outputs	Features
Broth (micro) dilution MIC determination	Yes (ISO 20776-1), CLSI, EUCAST	Yes (CLSI, EUCAST)	Yes, broth microdilution MIC determination is preferred	Yes	Clinical breakpoints or epidemiological cut-off values (ECOFFs)	MIC	High	Current reference method. Recording MIC values allows interpretation of the test outputs using different breakpoints (e.g. clinical breakpoint or ECOFF), as well as re-evaluation of historical data if changes occur to breakpoints and evaluation of shifts in MIC. Numerous national surveillance programmes adopt this method. The MIC value can sometimes indicate the likely mechanism of resistance (e.g. high-level amikacin resistance and rRNA methylases) or provide an epidemiological marker. Currently, this is the only method suitable for determining susceptibility to colistin.
Agar dilution MIC determination	No	Yes (CLSI, EUCAST)	Not widely used	Yes	Clinical breakpoints or ECOFFs	MIC	Dependent on congruity of methods used	Reference method. The breakpoints appropriate for broth dilution may not be directly applicable to agar dilution. Currently used in particular for testing certain fastidious organisms.
Breakpoint method	No	Yes (scientific literature)	Not widely used	Yes	The test is performed at a set breakpoint	Resistant or susceptible at selected breakpoint	Dependent on congruity of methods used	Changes to breakpoints in this method result in the inability to interpret historical data. Shifts in susceptibility within the S or R categories cannot be detected. The breakpoint method relies on the growth or absence of growth of bacteria in broth or on agar containing an antimicrobial at a single (breakpoint) dilution.
Gradient strip method	No	Yes (manufacturer)	Not widely used	Yes	Clinical breakpoints or ECOFFs	MIC	High	Provide a convenient alternative method of determining MIC with minimal additional equipment required.
Disc diffusion test	No	Yes (CLSI, EUCAST) A number of different methods are available. These are not in general equivalent.	May be used, but broth microdilution MIC determination is preferred	Yes	Clinical breakpoints (ECOFFs are also available for the EUCAST disc diffusion method).	Diameter of zone of inhibition, interpreted as resistant or susceptible according to test guidelines	Dependent on congruity of methods used	Frequently used to provide an indication of susceptibility for therapeutic purposes. Versatile in that different discs can be used, according to the antimicrobials authorised for treatment. Different methods are not usually equivalent (zone sizes obtained using one method cannot be interpreted using criteria from another, different method). The collection of zone size data can allow shifts in susceptibility to be detected. Disc diffusion methods may be harmonised to a degree with other methods, by using the same breakpoint.

The susceptibility testing method selected should provide details of the method, appropriate controls and quality control ranges and breakpoints. The comparability of outputs obtained in surveillance programmes is not only dependent on the laboratory methodology used but is also dependent on the target population of livestock included in the study and method of sampling.

8. Comparability of results

To determine the comparability of results originating from different surveillance systems, results should be reported quantitatively including information on the performance of the methods, the reference organisms and breakpoints used and the antimicrobial.

AST data, consisting of cumulative and ongoing summary of susceptibility patterns (antibiograms) among clinically important and surveillance microorganisms should be created, recorded and analysed periodically at regular intervals (CLSI, 2014a). Data must also be presented in a clear and consistent manner so that both new patterns of resistance can be identified and atypical findings confirmed or refuted. This data should be available on a central data bank and published yearly.

Cumulative AST data will be useful in monitoring susceptibility/resistance trends in a region over time and assessing the effects of interventions to reduce antimicrobial resistance.

9. Quality control (QC) and quality assurance (QA)

Quality control/quality assurance systems should be established in accordance with chapter 1.1.5 in laboratories performing AST:

- i) quality control refers to the operational techniques that are used to ensure accuracy and reproducibility of AST,
- ii) quality assurance includes, but is not limited to, monitoring, record keeping, evaluating, taking potential corrective actions if necessary, calibration, and maintenance of equipment, proficiency testing, training and QC. A QA programme helps ensure that testing materials and processes provide consistent quality results.

The following components should be determined and monitored:

- i) precision of the AST procedure,
- ii) accuracy of the AST procedure,
- iii) qualifications, competence, and proficiency of the laboratory personnel, as well as the personnel that interpret the results and those that are involved in monitoring of antimicrobial resistance,
- iv) performance of the appropriate reagents.

The following requirements should be respected:

- i) Strict adherence to specified and documented techniques in conjunction with quality control (i.e. assurance of performance and other critical criteria) of media and reagents.
- ii) Record keeping of:
 - a) lot numbers of all appropriate materials and reagents,
 - b) expiration dates of all appropriate materials and reagents,
 - c) equipment calibration and monitoring,
 - d) critical specifications for AST performance (reference results, time, temperature etc.).
- iii) The appropriate reference microorganism(s) should always be used regardless of the AST method employed.
- iv) Reference microorganisms are to be obtained from a reliable source for example, from the American Type Culture Collection (ATCC®), reliable commercial sources, or institutions with demonstrated reliability to store and use the organisms correctly.
- v) Reference microorganisms should be catalogued and well characterised, including stable defined antimicrobial susceptibility phenotypes. Records regarding these reference organisms should include the

established resistant and susceptible ranges of the antimicrobials to be assayed, and the reference to the method(s) by which these were determined.

- vi) Laboratories involved in AST should use the appropriate reference microorganisms in all AST testing.
- vii) Reference strains should be kept as stock cultures from which working cultures are derived and should be obtained from national or international culture collections. Reference bacterial strains should be stored at designated centralised or regional laboratories. Working cultures should not be subcultured from day to day as this introduces contamination and the method of producing working cultures should ensure that stock cultures are rarely used. This may be accomplished with the production of an intermediate stock of cultures derived from the original cultures that are used to create day-to-day working cultures.
- viii) The preferred method for analysing the overall performance of each laboratory should test the working stock of the appropriate reference microorganisms on each day that susceptibility tests are performed.

Because this may not always be practical or economical, the frequency of such tests may be reduced if the laboratory can demonstrate that the results of testing reference microorganisms using the selected method are reproducible. If a laboratory can document the reproducibility of the susceptibility testing methods used, testing may be performed on a weekly basis. If concerns regarding accuracy, reproducibility, or method validity emerge, the laboratory has a responsibility to determine the cause(s) and repeat the tests using the reference materials. Depending on the cause(s), daily reference material use and any other corrective action may be re-initiated.

- ix) Reference microorganisms should be tested each time a new batch of medium or plate lot or batch of disks is used and on a regular basis in parallel with the microorganisms to be assayed.
- x) Appropriate biosecurity issues should be addressed in obtaining and dispersing microorganisms to participating laboratories.

10. External proficiency testing

Laboratories should participate in external quality assurance and/or proficiency testing programmes in accordance with chapter 1.1.5. Laboratories are also encouraged to participate in international inter-laboratory comparisons (e.g. WHO External Quality Assurance System) (Hendriksen *et al.*, 2009). All bacterial species subjected to AST should be included.

National reference laboratories should be designated with responsibility for:

- i) monitoring the quality assurance programmes of laboratories participating in surveillance and monitoring of antimicrobial resistance,
- ii) characterising and supplying to those laboratories a set of reference microorganisms,
- iii) creating, managing, and distributing samples to be used in external proficiency testing,
- iv) creating a central database available on the internet (e.g. European Antimicrobial Resistance Surveillance System [EARSS]) that contains the different susceptibility/resistance profiles for each bacterial species under surveillance.

11. Conclusion

Although a variety of methods exist, the goal of *in-vitro* antimicrobial susceptibility testing for clinical veterinary purposes, surveillance and monitoring 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 judicious use policies (WOAH, 2018).

In-vitro antimicrobial susceptibility testing can be performed using a variety of formats, the most common being disk diffusion, agar dilution, broth macrodilution, broth microdilution, and a concentration gradient test. Each of these procedures requires the use of specific testing conditions and methods, including media, incubation conditions and times, and the identification of appropriate quality control organisms along with their specific QC ranges. 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, antimicrobial susceptibility/resistance results from different laboratories cannot be reliably compared.

The use of genotypic approaches for detection of antimicrobial resistance genes has also been promoted as a way to increase the rapidity and accuracy of susceptibility testing. New technological advances in molecular techniques (e.g. microarray) may facilitate the ability to probe bacterial species for large numbers of antimicrobial resistance genes quickly and cheaply, thereby providing additional relevant data into surveillance and monitoring programs (Ojha & Kostrzynska, 2008; Poxton, 2005). Standardised phenotypic AST methods will still be required to detect novel and emerging resistance mechanisms among bacterial pathogens and to validate their detection via genetic techniques (Ellington *et al.*, 2017).

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NB: There is a WOAH Reference Laboratory for antimicrobial resistance (please consult the WOAH Web site: <https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3>).
Please contact the WOAH Reference Laboratory for any further information on antimicrobial resistance

NB: FIRST ADOPTED IN 2004. MOST RECENT UPDATES ADOPTED IN 2019.