LABORATORY METHODOLOGIES FOR BACTERIAL ANTIMICROBIAL SUSCEPTIBILITY TESTING

SUMMARY

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. 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 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. The selection of a particular AST method is based on many factors such as validation data, practicality, flexibility, automation, cost, reproducibility, accuracy, and individual preference.*

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 antibiotic 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 (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, an increasingly troublesome situation because of 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 according to the clinician's past clinical experience (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 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.

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, 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 Members. 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 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 an OIE 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. This document provides guidelines and standardisation for AST methodologies, and interpretation of antimicrobial susceptibility test results.

1. Test requirements

In order to achieve standardisation of AST methods and comparability of AST results, the following requirements apply:

i)   the use of standardised AST methods and the harmonisation of AST test parameters (including choice of antimicrobial agents and subsequent interpretive criteria) are essential,

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)   all data should be reported quantitatively,

v)   establishment of national or regional designated 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 1.1.4 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/OIE/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 OIE 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 via either the scientific literature or through testing,

v)   the number of antimicrobials to be tested should be limited in order to ensure the relevance and practicality of AST.

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 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 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_2),

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 (clinical breakpoints, epidemiological cut-off values).

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 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,

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.

a)   Disk diffusion method

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 (see section 3.i). Disk diffusion is based on the determination of an inhibition zone proportional to the bacterial susceptibility to the antimicrobial present in the disk.

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.

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.

•   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.

b)   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 visible growth of the bacterium being tested (MIC, usually expressed in µg/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

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 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 prediluted antibiotics within the wells are commercially available. 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.

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.

- **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 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 a week (or less, depending on the antimicrobials tested),

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 (CLSI, 2006c), such as anaerobes and *Helicobacter* species.

c)   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 very expensive and MIC discrepancies can be found when testing certain bacteria/antimicrobial combinations compared with agar dilution results (Ge *et al.*, 2002; Rathe *et al.*, 2009).

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

The appropriate AST choice will ultimately depend on the growth characteristics of the bacterium in question. 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, 2008) (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, 2008) activity in certain bacteria can also be detected by using standard disk diffusion susceptibility test methods incorporating specific cephalosporins (cefotaxime and ceftazidime) in combination with a beta-lactamase inhibitor (clavulanic acid) and measuring

the resulting zones of inhibition. Also penicillin-binding protein 2a (PBP 2a) can 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.

d) 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 antibiotic resistance at the genetic level. The newest and perhaps most state-of-the-art approach is 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.

6. Antimicrobial susceptibility breakpoints and zone of inhibition criteria

The objective of *in-vitro* AST is to predict how a bacterial pathogen may respond to the 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. No single formula for selection of optimal breakpoints has been established. The process involves a review of existing data and is influenced by the subjectivity of individuals tasked with selecting the 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 decisions on 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 milligrams per litre or µg/ml,

ii) or as inhibition zone diameters in millimetres.

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

i) The development and establishment of quality control ranges (CLSI, 2006c), using diffusion when possible and dilution testing, for quality control reference microorganisms.

Establishment of quality control ranges is essential for validating test results obtained using a specific AST method. The allowable Iinterpretive category ranges for the reference organisms should be established prior to determining breakpoints for susceptibility or resistance. The use of reference organisms is a quality control and quality assurance activity. However, it is only necessary to require the use of reference organisms.

ii) The determination of the appropriate interpretive criteria regarding establishment of breakpoints (CLSI, 2006c).

This involves the generation of three distinct types of data:

- MIC population distributions of the relevant microorganisms,
- pharmacokinetic parameters and pharmacodynamic indices of the antimicrobial agent,
- results of clinical trials and experience.

The interpretation of the data involves creating a scattergram from the bacterial population distribution (representative bacterial species), by plotting the zone of inhibition against the logarithm to the base 2 of the MIC for each bacterial pathogen. 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.

The development of a concept known as 'microbiological breakpoints', or 'epidemiological cut-off values', which is based on the population distributions of the specific bacterial species tested, may be more appropriate for some antimicrobial surveillance programmes. In this case, bacterial isolates that deviate from the normal wild-type susceptible population would be designated as resistant, and shifts in susceptibility to the specific antimicrobial/bacterium combination could be monitored (Kahlmeter *et al.*, 2006; Turnidge *et al.*, 2006). There is a great advantage in the recording of quantitative susceptibility data in that such data may be analysed according to clinical breakpoints as well as by using epidemiological cut-off values.

7. Antimicrobial susceptibility testing guidelines

A number of standards and guidelines are currently available for antimicrobial susceptibility testing and subsequent interpretive criteria throughout the world (CLSI, 2008; Kahlmeter *et al.*, 2006). Amongst others, these include standards and guidelines published by:

British Society for Antimicrobial Chemotherapy (BSAC, UK),
Clinical Laboratory and Standards Institute (CLSI, USA),
Comité de l'Antibiogramme de la Société française de Microbiologie (CASFM, France),
Commissie richtlijnen gevoeligheidsbepalingen (CRG, the Netherlands),
Deutsches Institut für Normung (DIN, Germany),
European Committee on Antimicrobial Susceptibility Testing (EUCAST),
Japanese Society for Chemotherapy (JSC, Japan),
Swedish Reference Group for Antibiotics (SRGA, Sweden).

At this time, only the CLSI (formerly NCCLS) has developed protocols for susceptibility testing of bacteria of animal origin and determination of interpretive criteria (CLSI, 2008). 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, 2008; Kahlmeter *et al.*, 2006). For those OIE 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, 2006a; 2006b). 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, 2006c).

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 presented side by side, 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.

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 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, 2009). 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) (see also Chapter 1.1.4)

Quality control/quality assurance systems should be established in accordance with Chapter 1.1.4 Quality management in veterinary testing laboratories, 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:

• lot numbers of all appropriate materials and reagents,

• expiration dates of all appropriate materials and reagents,

• equipment calibration and monitoring,

• 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 crate 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 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 1.1.4 Quality management in veterinary testing laboratories. 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 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 (World Organisation for Animal Health, 2010).

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. Additionally, 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). Despite the new influx of genotypic tests

however, standardised phenotypic AST methods will still be required in the near future to detect emerging resistance mechanisms among bacterial pathogens.

REFERENCES

BROWN D. & MACGOWAN A. (2010). Harmonization of antimicrobial susceptibility testing breakpoints in Europe: implications for reporting intermediate susceptibility. *J. Antimicrob. Chemother.*, **65**, 183–185.

CAI H.Y., ARCHAMBAULT M., GYLES C.L. & PRESCOTT J.F. (2003). Molecular genetic methods in the veterinary clinical bacteriology laboratory: current usage and future applications. *Anim. Health Rev.*, **4**, 73–93.

CHEN S., ZHAO S., MCDERMOTT P.F., SCHROEDER C.M., WHITE D.G. & MENG J. (2005). A DNA microarray for identification of virulence and antimicrobial resistance genes in *Salmonella* serovars and *Escherichia coli. Mol. Cell. Probes*, **19**, 195–201.

CLINICAL AND LABORATORY STANDARDS INSTITUTE (CLSI) (2008). Document M31-A3. Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals, Approved Standard, Third Edition. CLSI, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898, USA.

CLINICAL AND LABORATORY STANDARDS INSTITUTE (CLSI) (2009). Document M39-A3. Analysis and Presentation of Cumulative Antimicrobial Susceptibility Test Data; Approved Guideline, Third Edition. CLSI, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898, USA,

CLINICAL AND LABORATORY STANDARDS INSTITUTE (CLSI) (2006a). Document M42-A, Methods for Antimicrobial Disk Susceptibility Testing of Bacteria Isolated from Aquatic Animals; Approved Guideline CLSI, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898, USA,

CLINICAL AND LABORATORY STANDARDS INSTITUTE (CLSI) (2006b). Document M49-A, Methods for Broth Dilution Susceptibility Testing of Bacteria Isolated from Aquatic Animals; Approved Guideline. CLSI, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898, USA,

CLINICAL AND LABORATORY STANDARDS INSTITUTE (CLSI) (2006c). Document M45-A. Methods for Antimicrobial Dilution and Disk Susceptibility of Infrequently Isolated or Fastidious Bacteria; Approved Guideline. CLSI, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898, USA,

DEHAUMONT P. (2004). OIE International Standards on Antimicrobial Resistance. *J. Vet. Med.* [Series B], **51**, 411–414.

DE JONG A., BYWATER R., BUTTY P., DEROOVER E., GODINHO, K., KLEIN U., MARION H., SIMJEE, S., SMETS, K., THOMAS, V., VALLE, M., & WHEADON A. (2009). A pan-European survey of antimicrobial susceptibility towards human-use antimicrobial drugs among zoonotic and commensal enteric bacteria isolated from healthy food producing animals. *J. Antimicrob. Chemother.*, **63**, 733–744.

FRYE J.G., LINDSEY R.L., RONDEAU G., PORWOLLIK S., LONG G., MCCLELLAND M., JACKSON C.R., ENGLEN M.D., MEINERSMANN R.J., BERRANG M.E., DAVIS J.A., BARRETT J.B., TURPIN J.B., THITARAM S.N. & FEDORKA-CRAY P.J. (2010). Development of a DNA microarray to detect antimicrobial resistance genes identified in the National Center for Biotechnology Information Database. *Microb. Drug Resist.*, **16**, 9–19.

GE B., BODEIS S., WALKER R.D., WHITE D.G., ZHAO S., MCDERMOTT P.F. & MENG J. (2002). Comparison of Etest and agar dilution for in vitro antimicrobial susceptibility testing of *Campylobacter. J. Antimicrob. Chemother.*, **50**, 487–494.

HENDRIKSEN R.S., SEYFARTH A.M., JENSEN A.B., WHICHARD J., KARLSMOSE S., JOYCE K., MIKOLEIT M., DELONG S.M., WEILL F.X., AIDARA-KANE A., LO FO WONG D.M., ANGULO F.J., WEGENER H.C., & AARESTRUP F.M. (2009). Results of use of WHO Global Salm-Surv external quality assurance system for antimicrobial susceptibility testing of *Salmonella* isolates from 2000 to 2007. *J. Clin. Microbiol.*, **47**, 79–85.

KAHLMETER G., BROWN D.F., GOLDSTEIN F.W., MACGOWAN A.P., MOUTON J.W., ODENHOLT I., RODLOFF, A., SOUSSY C.J., STEINBAKK M., SORIANO F. & STETSIOUK. (2006). European committee on antimicrobial susceptibility testing (EUCAST) technical notes on antimicrobial susceptibility testing. *Clin. Microbiol. Infect.*, **12**, 501–503.

OJHA S. & KOSTRZYNSKA M. (2008). Examination of animal and zoonotic pathogens using microarrays. *Vet. Res.*, **39**, 4–26.

Perreten V., Vorlet-Fawer L., Slickers P., Ehricht R., Kuhnert P. & Frey J. (2005). Microarray-based detection of 90 antibiotic resistance genes of gram-positive bacteria. *J. Clin. Microbiol.*, **43**, 2291–2302.

Petersen A., Aarestrup F.M., Hofshagen M., Sipila H., Franklin A. & Gunnarsson E. (2003). Harmonization of antimicrobial susceptibility testing among veterinary diagnostic laboratories in five Nordic countries. *Microb. Drug Resist.*, **9**, 381–388.

Poxton I.R. (2005). Molecular techniques in the diagnosis and management of infectious diseases: do they have a role in bacteriology? *Med. Princ. Pract.*, **14**, 20–26.

Rathe M., Kristensen L., Ellermann-Eriksen S., Thomsen M.K. & Schumacher H. (2009). Vancomycin-resistant *Enterococcus* spp.: validation of susceptibility testing and in vitro activity of vancomycin, linezolid, tigecycline and daptomycin. *APMIS.*, **118**, 66–73.

Stepanovic S., Hauschild T., Dakic I., Al-Doori Z., Svabic-Vlahovic M., Ranin L. & Morrison D. (2006). Evaluation of phenotypic and molecular methods for detection of oxacillin resistance in members of the *Staphylococcus sciuri* group. *J. Clin. Microbiol.*, **44**, 934–937.

Turnidge J., Kahlmeter G., & Kronvall G. (2006). Statistical characterisation of bacterial wild-type MIC value distributions and the determination of epidemiological cut-off values. *Clin. Microbiol. Infect.* **12**:418-425.

Walker R.D. (2007). Antimicrobial susceptibility testing and interpretation of results. *In:* Antimicrobial Therapy in Veterinary Medicine, Giguere S., Prescott J.F., Baggot J.D., Walker R.D., Dowling P.M. eds. Ames, IA, Blackwell Publishing.

White D.G., Acar J., Anthony F., Franklin A., Gupta R., Nicholls T., Tamura Y., Thompson S., Threlfall J.E., Vose D., van Vuuren M., Wegener H., & Costarrica L. (2001). Standardisation and harmonisation of laboratory methodologies for the detection and quantification of antimicrobial resistance. *Rev. Sci. Tech. Off. int. Epiz.,* **20**, 849–858.

World Organization for Animal Health (OIE) (2010). Chapter 6.9. Responsible and prudent use of antimicrobial agents in veterinary medicine. OIE *Terrestrial Animal Health Code,* Volume 1. OIE, Paris, France.

Zelazny A.M., Ferraro M.J., Glennen A., Hindler J.F., Mann L.M., Munro S., Murray P.R., Reller L.B., Tenover F.C. & Jorgensen J.H. (2005). Selection of strains for quality assessment of the disk induction method for detection of inducible clindamycin resistance in staphylococci: a CLSI collaborative study. *J. Clin. Microbiol.*, **43**, 2613–2615.

*
* *

NB: There is an OIE Reference Laboratory for Antimicrobial resistance
(see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list:
http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/).
Please contact the OIE Reference Laboratory for any further information on
Antimicrobial resistance