

Antimicrobial Susceptibility Testing

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Summary

Antimicrobial susceptibility testing (AST) is an in vitro procedure for determining the susceptibility of a bacterium to an antimicrobial agent. A number of methods are available and used in Australia and New Zealand including the Clinical and Laboratory Standards Institute (CLSI) methods, the calibrated dichotomous sensitivity test (CDS), and the commercially available antimicrobial susceptibility cards for veterinary laboratories for use on the Vitek 2 (Biomerieux). The rise in antimicrobial resistance (AMR) in bacteria from humans and animals has led to the publication of international guidelines for the use of antimicrobial agents in food-producing animals and the creation of a number of international surveillance programs to monitor the susceptibility profiles of antimicrobial agents. In Australia an AMR Prevention and Containment Steering Group has been established to develop and implement a national approach to AMR. Of importance is the surveillance and monitoring of AMR and this necessitates laboratories accurately test and report using standardised methodology and interpretive criteria. Recommendations on antimicrobial usage may change; therefore, laboratories must continually check the relevant local Regulatory Authorities. The different standards, methods and the impact of increased antimicrobial resistance on veterinary testing are discussed in this ANZSDP.

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Part 1. Introduction

Antimicrobial susceptibility testing (AST) refers to *in vitro* methods used to determine the susceptibility of a bacterium to an antimicrobial agent.¹ The results assist veterinarians to determine the most appropriate antimicrobial agents to treat infections. AST also is an important tool to monitor the emergence and spread of antimicrobial resistance (AMR). Antimicrobial resistance genes are transferred between bacteria by horizontal transfer involving the mechanisms of conjugation, transduction and transformation. Transfer also can occur from commensal bacteria with inherent resistance. Spread of bacteria containing antimicrobial-resistance genes occurs via direct contact between and within human and animal populations or via zoonotic bacteria along the food chain. Antimicrobial over-use is a major selector mechanism for the development of AMR in bacteria.^{2,3,4} The increase in AMR has led to a global approach for monitoring and managing the risk of the spread of AMR, with proposals for restricted use of some antimicrobial agents in animals so as to preserve these for human use. To enable data from AMR surveillance to be compared and interpreted reliably, it is important that laboratories use standardized procedures for AST.

This ANZSDP provides information on the principles and practices of AST, an overview of some of the methods available (with an emphasis on the preferred methods to be used in

Australia and New Zealand), and notes on antimicrobial susceptibility or resistance profiles of selected bacteria. The information aims to give veterinary laboratories an understanding and increased awareness of the issues created by the rise of AMR in human and veterinary medicine and the impact on veterinary testing.

Increasing AMR and the Impact on Veterinary Testing

The increase in AMR and decreased effectiveness of antimicrobial agents used in human medicine has led to a global focus on AMR in zoonotic bacteria, prompting recommendations for risk management from the World Organisation for Animal Health (Office International des Epizooties, OIE) and the World Health Organisation (WHO).^{5,6} The OIE publishes and constantly updates a list of ‘Critically Important Antimicrobials for Veterinary Use’,⁷ sets standards for the responsible use of antimicrobial agents in animals (Chapter 6.9 of the Terrestrial Animal Health Code http://www.oie.int/index.php?id=169&L=0&htmfile=chapitre_1.6.9.htm), and Chapter 6.3 of the Aquatic Animal Health Code http://www.oie.int/index.php?id=171&L=0&htmfile=chapitre_1.6.3.htm), and encourages harmonisation and coordination of national and international AMR surveillance and monitoring programs. An AMR website (<http://www.oie.int/en/for-the-media/amr/>) provides links to documentation detailing recommendations for controlling resistance, harmonising surveillance and monitoring programs, prudent use of antimicrobial agents in veterinary medicine (including both terrestrial and aquatic species), conducting risk assessments, and providing laboratory methodologies. The Scientific and Technical Review 31(1), Antimicrobial Resistance in Animal and Public Health,⁸ reviews a number of topics including prudent use and existing veterinary guidelines, and the responsibilities of all levels of the supply chain including regulatory bodies, veterinarians and farmers; the evidence for the spread of AMR genes via the food chain; and the harmonisation of technical requirements for the registration of veterinary medicinal products.

Similar to the OIE list of critically important antimicrobials for veterinary use, WHO has published a list of ‘Critically Important Antimicrobials for Human Medicine’.⁹ The list is to be used when developing policy to manage the risk of the spread of AMR bacteria through the food chain, with the aim of preserving the effectiveness of these critical antimicrobial agents for human use.^{5,9}

Zoonotic bacteria that are the focus of AMR surveillance and monitoring programs in a number of countries include *Salmonella*, *Campylobacter*, *E. coli*, *Staphylococcus aureus* and commensal bacteria *Enterococcus* species, in particular *E. faecium*, from food-producing animals.^{9,10,11,12,13,14} Database information is more easily shared if the one standardized AST method is used by all laboratories.⁶

In Australia, the registration and permitted usage of veterinary medicines is controlled by the Australian Pesticides and Veterinary Medicine Authority (APVMA)¹⁵ (<http://www.apvma.gov.au/>), which receives advice from The National Health and Medical Research Council. In 1998, a joint expert technical advisory committee on AMR (JETACAR) was established to provide expert scientific advice on the threat posed by antibiotic resistant bacteria to human health by the selective effect of agricultural use, and medical overuse, of antibiotics. A report, known as the ‘JETACAR Report’, made recommendations for the management of AMR based on regulatory controls, monitoring and surveillance, infection prevention strategies, education, and research.¹⁶ To implement these recommendations and to provide governance and leadership on dealing with AMR, the Department of Health together with the Department of Agriculture (previously Department of Agriculture, Fisheries and Forestry) established the Australian AMR Prevention and

Containment Steering Group (AAMRPC) in February 2013. The Department of Agriculture will provide AMR- related information on their website.

In New Zealand, registration of veterinary medicines is controlled by the Ministry for Primary Industries, Food Safety Group (<http://www.foodsafety.govt.nz/industry/acvm/index.htm>).¹⁷ In 2005, an expert panel was convened by the New Zealand Food Safety Authority (NZFSA) to review the impact of antimicrobial agents used in animals and plants.¹⁸

Part 2. Antimicrobial Susceptibility Testing Methods

A number of methods and corresponding guidelines exist, worldwide, for bacteria of human importance, but there are a reduced number of methods and guidelines established for bacteria isolated from terrestrial animals, and even fewer for aquatic species. The aim of establishing guidelines is to predict how the infecting bacterium will respond to antimicrobial therapy using interpretive criteria based on a number of factors including pharmacokinetics and efficacy studies. Establishing guidelines for animals is complicated by the fact there are many terrestrial and aquatic host species and not all respond in the same way; therefore, it can take many years before sufficient data are generated to establish guidelines. Particular bacteria from different hosts may have nutritional and growth requirements beyond those for which the interpretive guidelines have been established; consequently, meaningful results cannot be obtained using the media and conditions required by AST. This issue is exacerbated when establishing antimicrobial guidelines for testing bacteria from aquatic animals.

AST methods involve culturing a sample to obtain a pure isolate and testing to determine which antimicrobial agents inhibit the growth of, or kill the pathogen.¹⁹ The methods may use broth dilution, agar dilution or disk diffusion methods.

A number of antimicrobial susceptibility methods and standards are available and their use varies within and between countries. The OIE Terrestrial Manual 2012 Guideline 2.1 'Laboratory Methodologies for Bacterial Antimicrobial Susceptibility Testing,'²⁰ recommends standardization of AST methods and lists the requirements for this to be achieved: that they produce accurate and reproducible data able to be reported quantitatively, that designated national or regional laboratories be accredited, that quality management programs are in place in those laboratories, and that designated quality control strains are used. OIE recommends the use of established guidelines when selecting appropriate antimicrobial agents for testing and for this to be based on the lists of veterinary and human antimicrobials designated as critically important. AST testing methodology should follow established and validated methods; the OIE recommends the disk diffusion method, the broth dilution method and the agar dilution method, and preferably the CLSI standards¹⁹ for these methods.

In the disk diffusion method,²¹ a paper disk impregnated with a standard concentration of an antimicrobial agent is placed onto the surface of an agar medium onto which a bacterium has been lawn-inoculated at a standardized concentration of cells per mL. The antimicrobial agent diffuses through the agar resulting in a concentration gradient. Diffusion through the agar is based on the molecular size of the antimicrobial agent, factors that may be present in the agar, and agar concentration. Interpretive criteria are based on the relationship between minimum inhibitory concentration (MIC) and zone diameter size, which is analysed against the pharmacokinetics of the antimicrobial agent in normal dosing regimes. The final *in vitro* criteria are obtained following studies of clinical efficacy and response outcomes.²² The disk

diffusion method is cost-effective, easy to perform on small or large numbers of isolates, and does not require expensive equipment.

The broth dilution method is based on serial dilutions of an antimicrobial agent in tubes, or microtitre plates, to which a standard concentration of the test bacterium is added.¹⁹ Results are read as a MIC in mg/mL, that is, the lowest concentration of antibiotic that completely inhibits the growth of the bacterium; this is the first tube showing no discernible growth. If the contents of the tubes are cultured onto agar plates then the plate with no growth represents the tube with the minimal bactericidal concentration (MBC) of antimicrobial and may be different to the tube indicating the MIC. MBC is not normally done for the broth dilution test. Some antimicrobial agents are commercially available, prediluted in microtitre trays.

The agar dilution method is similar to the broth dilution method in that the antimicrobial agent is serially diluted and the dilutions added to the agar medium. The test bacterium is spot-inoculated at a standardised concentration on to the agar surface. Agar plates are prepared with antimicrobial concentrations that encompass the quality control range and the breakpoints. Agar plates containing antimicrobial concentrations must be used within a week of preparation, and therefore, the method requires more media preparation than the disk diffusion method and is more likely to be used by laboratories with a high sample throughput.¹⁹ Inoculum replicating instruments assist in semi-automating the method. Disadvantages of the method include difficulty in verifying the purity of the applied bacterium, and the endpoints being not always easy to read.

Both the broth dilution and the agar dilution method may be labour intensive if automated equipment is not used.²⁰

Other agar-based AST methods include gradient strips, the E-test²³ (Biomerieux), which consists of commercially produced paper strips containing a concentration gradient of antimicrobial agent. Some automated AST methods are available and these include the Vitek system from Biomerieux, and the Microscan Walkaway System from Microscan.

All these methods are based on phenotype. In medical laboratories there is increasing use of genotypic methods for detecting the presence of antimicrobial resistance genes in bacteria, and for erythromycin and methicillin resistance there is good correlation between genotype and phenotype.²⁴

A number of antimicrobial susceptibility test guidelines have been produced by standards organisations.²⁰ In Australian and New Zealand medical and veterinary laboratories, the most commonly used method is the CLSI method,^{19,25} which is also the predominant method used in the USA. In Australia some medical and veterinary laboratories also use the CDS method²⁶ (<http://web.med.unsw.edu.au/cdstest/>). For countries that have not established standardized AST methods and guidelines, the OIE, and in Australia the JETACAR report,¹⁶ recommends the adoption of the CLSI methods.²⁰

Methods of the Clinical and Laboratory Standards Institute (CLSI)

CLSI, originally called The National Committee for Clinical Laboratory Standards, is an organisation in the United States of America that, through a subcommittee of volunteers who are experts in the field, writes and maintains methods for AST.^{19,25} Information and methods can be purchased from CLSI.^{19,25} Some of the manuals produced by CLSI are listed in Part 3.

Each of the CLSI documents is given a specific number, for example M2-A7, where M denotes a Microbiology document; 2 is the number assigned to the specific document for disk diffusion; A means an approved document and 7 indicates, in this instance, the 7th edition of

the M2 document. Most antimicrobial susceptibility documents are revised every three years. Documents relevant to veterinary microbiology laboratories are now denoted by the prefix VET.

The CLSI manual VET01-A3, 'Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals; approved standard',¹⁹ provides recommendations for antimicrobial agents that should be tested for different diseases in different animal species, and gives interpretive guidelines for these antimicrobial agents and the animal species. Pharmacokinetic and pharmacodynamic parameters are available for some antimicrobial agents in certain animals, but many interpretive criteria are based on data from humans. Be aware that this document might contain some antimicrobials that are prohibited or restricted for use in food-producing animals in Australia and New Zealand. Antimicrobial agents prohibited or restricted for use in food-producing animals in Australia and New Zealand are listed in Part 3.

Terminology may differ between countries and between methodologies, and in the CLSI methods a glossary defines the terms used.

Critical factors in the CLSI method for disk diffusion

The disk diffusion method is based on the original work of Bauer *et al.*, (1966).²¹ The diffusion of an antimicrobial agent through the agar establishes a concentration gradient. The growth of the test bacterium inoculated at a set concentration (colony forming units, CFU/mL) establishes a zone where the concentration of the antimicrobial agent is sufficient to inhibit the growth of the bacterium. A zone size that indicates susceptibility or resistance is established after considerable validation of MIC and correlation of zone size, pharmacokinetic parameters and pharmacodynamic indices of the antimicrobial agent, and results of clinical trials.²¹

The following factors are critical to the accuracy and repeatability of the disk diffusion method and are based on the CLSI guidelines.^{19,22} This list is not exhaustive and the CLSI manuals should be consulted.

Inoculum

Critical factors are the method of preparation (from colony or log phases of growth in a broth), and turbidity.

The inoculum must be prepared using a colony from log phase of growth (18-24 hours growth on plate media) suspended in sterile normal saline, Mueller-Hinton broth, tryptic soy broth, or lab lemco broth. It must be prepared to a turbidity of a 0.5 McFarland standard, which corresponds to 1.5×10^8 CFU/mL. The inoculum must be used within 15 minutes of preparation. The McFarland standard can be purchased commercially or prepared in house (see Part 4).

An inoculum can also be prepared from a broth culture grown for 2-8 hours to reach log phase of growth; this method is used for rapidly growing bacteria only. The suspension is again adjusted to the correct turbidity. Overnight cultures must not be used.

Inoculating the plate

Plates can be inoculated by flooding with the cell suspension and removing the excess with a sterile Pasteur pipette. Alternatively, a sterile cotton-tipped swab is used to inoculate the plate using the lawn inoculation technique (streak back and forth from top of the plate to the bottom of the plate, turn the plate 60 degrees and repeat, then and turn another 60 degrees and

repeat). Disks are placed onto the surface of the plate either individually with sterile forceps or with a disk dispenser, available commercially (Oxoid, Becton Dickinson).

Disks

Disks are stored in the freezer or refrigerator. They must be allowed to equilibrate to room temperature for a minimum of 15 minutes and a maximum of two hours to minimise condensation and reduce the possibility of diluting the concentration of the antimicrobial agent in the disk. The expiry date must be checked. A frost-free freezer must not be used to store disks or media. Disks must be used at the concentration stated in the CLSI document. A variation in zone size may be observed for disks from different commercial companies. Results must adhere to the quality control guidelines stated in the CLSI manual. A disk must not be relocated on a plate once it has touched the agar as many antimicrobial agents diffuse instantaneously through the agar. On a 100 mm Petri dish only five disks should be tested at any one time.

Agar medium

Mueller-Hinton agar must be used as it has a defined set of components and contains low quantities of substances that inhibit certain antimicrobial agents. Agar containing thymine and thymidine inhibit the antibacterial activity of trimethoprim, and the presence of para-aminobenzoic acid (PABA) and the structural analogues antagonise the activity of sulphonamides. Calcium and magnesium ions influence the susceptibility of *Pseudomonas* strains to aminoglycosides and these ions must be within defined limits. These cations also adversely affect the susceptibility of tetracycline against a number of bacterial species.

The pH must be between 7.2 and 7.4 once the agar has set, as a pH of less than 7.2 causes aminoglycosides, quinolones and macrolides to lose potency, or in the case of tetracyclines, enhance potency. The reverse can happen with a pH of greater than 7.4.

Plates must be dried to remove excess moisture. This can be done in a 37°C incubator for 10-15 minutes with the plate inverted and the lids ajar. Plates must be poured to a depth of 4 mm. This corresponds to 60-70 mL of medium for a 150 mm Petri dish, and 25-30 mL for 100 mm Petri dishes.

Nutritional requirements

Organisms such as *Streptococcus* spp, *Pasteurella multocida* and *Mannheimia haemolytica* may require the addition of 5% defibrinated sheep blood for optimal growth.

Incubation temperature, atmosphere and time

Disks must be firmly attached to the agar, and plates incubated inverted (agar base uppermost) to avoid moisture dripping from the lid onto the disks and interfering with the concentration of the antimicrobial agent in the disk.

For most bacteria, incubation is conducted in ambient air. Incubation in CO₂ compared to air can result in an increase or decrease in zone size for different antimicrobials because CO₂ is absorbed into the agar to become carbonic acid, which leads to an increase in pH. Depending upon the antimicrobial agent, an increase in pH will either increase or decrease the zone size.

Non-fastidious bacteria are incubated at 35°C for 16-18 hours. Fastidious bacteria are incubated according to CLSI guidelines (e.g. *Haemophilus* species using Haemophilus test medium for 16-18 hours in 5% CO₂; *Streptococcus* species using Mueller-Hinton agar with 5% sheep blood, for 20-24 hours and in 5% CO₂. *Staphylococcus aureus* should be incubated in ambient air for the oxacillin-salt agar screening test. For *Streptococcus* spp, *Haemophilus* spp and *Actinobacillus pleuropneumoniae*, incubation in 5% CO₂ is recommended).

There is a CLSI method for *Actinobacillus pleuropneumoniae* using both disk diffusion and broth dilution. As yet there is no CLSI methodology for *Avibacterium paragallinarum* or *Haemophilus parasuis*.

Inoculum growth

After incubation, the growth must be checked to ensure it is even and confluent, and that it is not too thick or too thin (indicating an incorrect inoculum concentration) as this will affect the accuracy of the zone sizes. An ideal cell density is when colonies touch each other; isolated colonies indicate the inoculum density is too light.

Measuring zones of growth inhibition

The diameter (in mm) of the zone of 'no growth' around a disk is measured using either a ruler or calipers. If measuring from the back of the plate, the plate is held over a black surface and examined using reflected light from a desk light. Recording zones for *Streptococcus* species may be more easily read from the top of the plate with the lid off. If two zones are seen, ensure the growth is pure and not caused by a contaminated inoculum. The innermost zone is measured.

Where a zone contains individual colonies, ensure the growth is pure; if pure this indicates a resistant subpopulation within the test organism. An individual colony within the zone is subcultured and the AST is repeated. If individual colonies are again seen within the zone, check the purity. If pure, measure the colony-free zone within the zone.

Proteus mirabilis may show as a thin swarm, which covers the zone. The swarming growth is ignored and the obvious zone is measured.

A zone with a feathered edge is measured at the point where there is obvious demarcation.

Zones for trimethoprim-sulfamethoxazole (and for trimethoprim and sulfamethoxazole individually) may be difficult to read due to an unclear demarcation of the rim of the zone. This occurs because the antimicrobial may not inhibit the bacterium until it has undergone several generations of growth. The zone is measured at the point where there is an 80% reduction in growth.

CLSI Guidelines for Aquatic Animals

The CLSI methods²⁵ are available to order from the CLSI website. The methods are: VET03/VET04-S1 Performance Standards for Antimicrobial Susceptibility Testing of Bacteria Isolated from Aquatic Animals; First Informational Supplement, June 2010; VET03-A Methods for Antimicrobial Disk Susceptibility Testing of Bacteria Isolated from Aquatic Animals; Approved Guideline June 2006; and VET04-A Methods for Broth Dilution Susceptibility Testing of Bacteria Isolated from Aquatic Animals; Approved Guideline. VET03/VET04-S1 contains guidelines for disk diffusion for oxytetracycline, oxolinic acid, gentamycin, erythromycin, florfenicol, ormetoprim-sulfadimethoxine and trimethoprim-sulfamethoxazole for bacteria that grow on Mueller-Hinton agar at 22°C ±2°C in ambient air after 44-48 hours. No guidelines are given for specific bacteria or specific aquatic animals. These guidelines have created a number of problems for the CLSI Subcommittee on Veterinary Antimicrobial Susceptibility Testing Aquaculture Working Group, because insufficient pharmacokinetic and pharmacodynamic data exist for different aquatic hosts. Also, bacteria from aquatic animals have particular growth requirements for temperature and may or may not have a growth requirement for NaCl, or require minimal nutrient media (e.g. for the growth of many *Flavobacterium* and *Tenacibaculum* species). The current guideline is for Group 1 aquatic organisms, that is, those bacteria that grow on standard Mueller-Hinton

agar, and are readily cultured at 20-24°C or 26-30°C. The specified quality control (QC) organisms are *E. coli* and *Aeromonas salmonicida* subspecies *salmonicida*. However, the latter is exotic to Australia and New Zealand, and therefore only *E. coli* can be tested for QC. Further work needs to be done for bacteria from aquatic sources, particularly those that have particular growth requirements, gliding bacteria, halophiles, and slow-growing bacteria. Further information needs to be obtained, such as pharmacokinetic and pharmacodynamic data, to enable interpretive criteria to be developed for the many species of aquatic animals including freshwater and saltwater fish, cold water species, tropical species, crustaceans, and molluscs.

Calibrated Dichotomous Sensitivity Test (CDS) Method

The CDS method was developed in 1975²⁶ and is used by some medical and veterinary laboratories in Australia. It is maintained by the microbiology department at the Prince of Wales Hospital in New South Wales (NSW). Similar to the CLSI disk diffusion method, the CDS method is a disk diffusion method based on a correlation between zone sizes of inhibition and quantitative MIC, using the agar dilution method as a gold standard. An antibiotic is calibrated by plotting the zone sizes recorded from a large number of strains of a bacterial species against the log MIC of each antibiotic. It is referred to as a dichotomous test because it divides susceptibilities into two categories, sensitive and resistant, and does not recognise an intermediate category. The CDS method is conservative, selecting the lower end of the range of break point MICs. In the interpretation of results, a uniform zone size with an annular radius (measured from the edge of the disk to the edge of the zone of no growth) of 6 mm (18 mm diameter) indicates a susceptible organism. It is the point of diffusion on the sigmoid curve that enables the greatest discrimination. A zone size of less than 6 mm indicates a resistant organism. There are exceptions to this standard interpretation and these are listed in the CDS manual. Thus, an annular zone size of 6 mm will correspond to a set MIC, in mg/L, for a particular antibiotic. The CDS method is said to increase the specificity of the test by the using the dichotomous cut-off values but in some cases a few marginally sensitive strains may be called resistant and therefore the CDS method may be less sensitive for some bacterial strains compared to other methods.

Factors critical to the accuracy of the CDS method include inoculum concentration and preparation, media, disk potency, incubation temperature and incubation atmosphere. The inoculum is made by stabbing a colony with a straight wire and emulsifying in sterile normal saline to provide a concentration of 10^7 CFU/mL to result in a confluent and uniform growth. This facilitates the visualisation, on the plates, of the production of enzymes that inactivate antimicrobial agents.

The CDS method uses Sensitest agar (Oxoid). The disk potency is designed to promote the uniform cut-off zone between resistant and susceptible results. The CDS manual provides details of testing for a number of bacteria. In the application to veterinary medicine, the CDS method has been calibrated for apramycin, marbofloxacin, neomycin, and spectinomycin. Tables are provided from which results for other antimicrobial agents are extrapolated and these include ceftiofur, cefovexin, enrofloxacin, lincospectin, ofloxacin, orbifloxacin and tylosin. For example, ceftiofur is tested using benzylpenicillin as a surrogate antibiotic, or erythromycin is used as a surrogate antibiotic disk for tylosin. The method is available online (<http://web.med.unsw.edu.au/cdstest/>).

Concentration Gradient (E-Test) Method

The E-Test (Epsilonometer) method²³ is available commercially (Biomérieux) and can be used for aerobes and anaerobes. It consists of a strip of filter paper impregnated with a defined gradient of 20, two-fold dilutions of the antimicrobial agent being tested. The strips are applied to an agar plate on which a test bacterium has been lawn-inoculated according to the manufacturer's instructions. After incubation the result as an MIC is read where the edge of the zone of growth inhibition intersects the strip.

Automated Systems

Vitek antimicrobial susceptibility cards (Biomérieux)

The automated bacterial identification system, the Vitek, available from Biomérieux, has a veterinary card for AST of Gram-negative bacilli and Gram-positive cocci. It is based on photometry where the bacterium utilises a substrate, which results in a colour and density change that is detected by phototransistor detectors.

The Vitek 2 AST-GN38 card for Gram-negative bacilli is based on the USA Food and Drug Authority (FDA) indications for use, and may contain some antimicrobials that are not approved for use in Australia or New Zealand. The card contains wells that test for antimicrobial agents at different concentrations and result in an MIC value. The following antimicrobials are on the AST-GN38 card: amikacin, amoxicillin/clavulanic acid, ampicillin, cephalexin, cefpirome, cefpodoxime, ceftiofur, chloramphenicol, enrofloxacin, gentamicin, imipenem, marbofloxacin, nitrofurantoin, piperacillin, polymyxin B, rifampicin, tetracycline, tobramycin and trimethoprim/sulfamethoxazole. The card also tests for extended-spectrum beta-lactamases (ESBLs).

The Vitek 2 AST-GP69 card is used for susceptibility testing of *Staphylococcus* species, *Enterococcus* species and *Streptococcus agalactiae*. Antimicrobial agents include ampicillin, ampicillin/sulbactam, benzylpenicillin, ceftiofur screen, chloramphenicol, clindamycin, enrofloxacin, erythromycin, fusidic acid, gentamicin, imipenem, kanamycin, marbofloxacin, mupirocin, nitrofurantoin, oxacillin, tetracycline, trimethoprim/sulfamethoxazole, and vancomycin (prohibited for use in animals). It also tests for inducible clindamycin resistance.

MicroScan Automated Microbiology System

This system is based on photometry and fluorometry. Antimicrobial susceptibility testing is available.

Antimicrobial susceptibility testing for *Mycoplasma* and *Ureaplasma*

Generally, diagnostic laboratories are unable to perform susceptibility testing on *Mycoplasma* and *Ureaplasma* species (Mollicutes) because of the specialised nature of working with *Mycoplasma*, as well as the absence of guidelines in the CLSI document for veterinary laboratories. It is difficult to establish a standard medium for the growth of all *Mycoplasma* and *Ureaplasma* due to the nutritional diversity of these organisms. However, some guidelines have been established based on broth dilutions and minimum inhibitory concentrations.²⁷

Molecular methods and new phenotypic methods for detecting antimicrobial resistance

Polymerase chain reaction (PCR) methods are available for the detection of resistance genes and are used by medical laboratories for the rapid detection of multi-resistant bacteria.

Currently, veterinary laboratories aren't required to test for resistance genes in veterinary isolates but a requirement may exist in the future, especially with increasing evidence of methicillin-resistant, coagulase-negative staphylococci in food-producing animals.²⁸

Resistance genes that can be detected by PCR include the *mecA* gene for the detection of methicillin/oxacillin resistance in *Staphylococcus aureus* and coagulase-negative Staphylococci, and *vanA* and *vanB* genes for the detection of vancomycin resistance in *Enterococcus* species. In addition, fluoroquinolone resistance mutations, betalactamases, aminoglycoside inactivating enzymes and tetracycline efflux genes can be detected by PCR.^{20,29}

Matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF MS) has potential to identify antimicrobial resistant bacteria and results have been published for the identification of vancomycin resistant enterococci (VRE) and methicillin resistant *Staphylococcus aureus* (MRSA); however, at this stage, detection of AMR by this method is not a routine diagnostic test.^{30,31}

Factors Influencing Antimicrobial Susceptibility Methods

Limitations of antimicrobial susceptibility testing

One AST method cannot overcome all the growth and nutritional variations encountered when attempting to culture microorganisms. The disk diffusion, broth and agar dilution methods rely on rapidly growing bacteria with normal nutritional and growth requirements. Uncertainty in test outcomes arises for bacteria that have particular nutritional requirements, growth/atmosphere requirements, or are slow growing.¹⁹

Methods for the Detection of Antimicrobial Resistance Mechanisms^{19,32}

Background

With the rise in AMR there is an increasing need for laboratories to screen bacteria for antimicrobial resistance mechanisms. Resistance mechanisms important for medical treatment and/or epidemiological information include β -lactamases (in staphylococci and Gram-negative bacteria), extended spectrum β -lactamase-producing and carbapenemase-producing *Enterobacteriaceae*, acquired AmpC β -lactamase-producing *Enterobacteriaceae*, methicillin-resistant *S. aureus* and *S. pseudointermedius*, glycopeptide non-susceptible *S. aureus*, and vancomycin resistant *Enterococcus faecalis* and *E. faecium*, various mechanisms of fluoroquinolone resistance in campylobacter, *E. coli* and salmonellae.³² Penems and monobactams are not approved for use in veterinary medicine,¹⁹ but resistance has been found in pigs and dogs overseas. The location of resistance genes on plasmids and other mobile genetic elements facilitates their spread between microorganisms. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) has published guidelines for detection methods (http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Resistance_mechanisms/EUCAST_detection_of_resistance_mechanisms_v1.0_20131211.pdf)³² and CLSI details testing for resistance mechanisms in VET01-A4 and VET01-S2.

Laboratories should consult the CLSI documents VET01-A4 and VET 01-S2 and EUCAST Resistance Mechanisms documents for comprehensive information on resistance mechanisms background and methodology. Note that the EUCAST document is for medical laboratories. A very useful summary of antimicrobial classes, the subclass designation, antimicrobial

agents and antimicrobial resistance mechanism is contained in the table (glossary 1) in VET01-S2.

Veterinary isolates of significance

Veterinary laboratories should screen bacteria resistant to a number of antimicrobials for resistance mechanisms. Any third or fourth generation cephalosporin (ceftiofur, cefovecin, ceftriaxone, cefquinome) resistant *E. coli* and *Salmonella* species should be tested for ESBLs and AmpC.

Any *E. coli*, *Klebsiella* species and *Salmonella* species showing resistance to multiple antimicrobial agents should be tested for ESBLs and AmpC. This approach could also be applied to environmental species such as *Acinetobacter* species.

Coagulase-positive *Staphylococcus* should be tested for resistance to methicillin or oxacillin; *S. aureus* (using ceftiofur) and *S. pseudointermedius* (using oxacillin only).

It is not necessary to routinely test all *Enterobacteriaceae* for resistance mechanisms, only those showing resistance to key antimicrobials in susceptibility tests as resistance tests are not predictive of susceptibility to β -lactams.^{19,32}

Methods

The following methods are recommended by CLSI (VET01-A4, VET01-S2 tables 9A & 9B) and EUCAST (Resistance Mechanisms) and the relevant documents should be consulted.

AmpC β -lactamases

AmpC β -lactamases are types of β -lactamases that hydrolyse both broad and extended spectrum cephalosporins (cephalothin, cefazolin, ceftiofur), many penicillins, and β -lactamase inhibitor- β -lactam combinations. They are either encoded on the chromosome or carried on plasmids. They are not inhibited by β -lactamase inhibitors such as clavulanic acid.

As AmpC β -lactamases are not inhibited by clavulanic acid, they won't be detected in the ESBL disk methods described under '*ESBLs in Enterobacteriaceae*'. This resistance mechanism may occur in *Enterobacteriaceae*, particularly *Enterobacter*, *Citrobacter*, *Serratia*, *E. coli* and *Salmonella*. The major producers of acquired AmpCs (encoded by plasmid-mediated genes) in the medical field are *E. coli*, *K. pneumoniae*, *K. oxytoca*, *Salmonella enterica* and *P. mirabilis*. *Acinetobacter baumannii* and many other *Enterobacteriaceae* may produce natural AmpCs. These are capable of conferring high-level resistance to cephalosporins and to penicillin- β -lactamase inhibitor combinations.³²

There are a number of methods to test for AmpC β -lactamases. EUCAST recommends using cefepime as the indicator cephalosporin as this agent is not hydrolysed by AmpC β -lactamases.³² Alternatively, cloxacillin can be used in the combination disk test using two cephalosporin indicators (cefotaxime and ceftazidime) with clavulanic acid and cloxacillin together, or on agar plates supplemented with 200-250 mg/L cloxacillin.³² A commercial test, the Mast D68C disk test (Mast Group), has good sensitivity and specificity.³³ The test consists of four disks containing (A) cefpodoxime, (B) cefpodoxime and an ESBL inhibitor, (C) cefpodoxime and an AmpC inhibitor, and (D) cefpodoxime and both ESBL inhibitors. A suspension (0.5 MacFarland) of the test isolate is lawn-inoculated onto Mueller Hinton agar and the disks placed on the agar surface and incubated in air for 18-24 hours. The results are interpreted according to the manufacturer's instructions. In comparison, a chromogenic medium, the Cica-Beta test, had low sensitivity.

It is suggested that veterinary laboratories test any ceftiofur resistant *E. coli* or *Salmonella* for ESBLs including AmpC.

ESBLs in Enterobacteriaceae

CLSI and EUCAST recommend screening for ESBLs in *Klebsiella pneumoniae*, *K. oxytoca*, *Klebsiella* species, *E. coli* and *Proteus mirabilis*. EUCAST divides these into group 1 *Enterobacteriaceae* (*E. coli*, *Klebsiella* species, *P. mirabilis*, *Salmonella* species and *Shigella* species), and group 2 *Enterobacteriaceae*. Group 2 are *Enterobacteriaceae* with inducible chromosomal AmpC (*Enterobacter* species, *Citrobacter freundii*, *Morganella morganii*, *Providencia stuartii*, *Serratia* species and *Hafnia alvei*). The EUCAST detection of resistance mechanisms document is directed at medical laboratories and so not all isolates recommended for screening will be relevant to veterinary laboratories as some of these won't be pathogens requiring antimicrobial treatment; however, screening of relevant veterinary pathogens or highly resistant bacteria is recommended.

Both CLSI and EUCAST give disk diffusion zone guidelines for cefotaxime, ceftriaxone, ceftazidime, cefpodoxime, that suggest when *Enterobacteriaceae* should be tested for ESBLs. For example, if a disk diffusion zone is obtained of <21 mm for cefotaxime (5 µg) according to EUCAST, or ≤27 mm according to CLSI, then an ESBL test should be conducted. Note: breakpoints may differ between CLSI and EUCAST methods. When testing for ESBLs either cefotaxime or ceftriaxone and ceftazidime must be tested, or cefpodoxime can be tested alone. CLSI recommends screening strains that show MICs above the normal susceptible population, but below standard breakpoints for certain extended-spectrum cephalosporins. Screening for ESBLs is done using the breakpoints as outlined in tables 9A and 9B. ESBL-producing strains will show a decrease in the MIC in the presence of clavulanic acid. ESBL-producers should be reported as resistant to all penicillins and cephalosporins. Check the CLSI document for reporting guidelines.

Several ESBL detection methods are available and may be based on inhibition of ESBL activity by clavulanic acid, seen as enhanced activity of a cephalosporin in the presence of clavulanic acid. EUCAST recommends testing for inhibition of ESBL activity by clavulanic acid using either the combination disk test, the double-disk synergy test, the ESBL gradient test or the broth microdilution test and gives interpretation guidelines for these four tests. The double disk method relies on optimal spacing of the disks on the plate. CLSI gives guidelines and interpretation for the disk method and provides information for preparing the double disks. Cefpodoxime combination disks (code DD0029) for the detection of ESBLs in *Enterobacteriaceae* (*E. coli*, *Klebsiella* species) that don't produce inducible AmpC are available from Oxoid (Thermo Scientific). Interpretation of zone sizes is given for these disks. A combination disk range (code CD01/02/03/04) containing cefpodoxime, ceftazidime, cefotaxime and ceftipime each in combination with clavulanic acid is available. An ESBL is present if there is an increase in zone size of ≥5 mm between the combination disk compared to cephalosporin alone. Various disk combinations for ESBL detection including a flowchart (ZT146 Mastdisc_Flowchart v1.0) for detection of ESBLs is available from the Mast Group, (http://www.mastgrp.com/catalogue_products_in_sublist.asp?cat=1&SubProduct_Type=1206). Chromogenic agars are available as rapid screening tools for ESBLs, but do not detect all ESBLs. To date the use of chromogenic agars have not been included in the CLSI veterinary or EUCAST methods.

The Etest ESBL strip may have a high rate of inconclusive results.³² ESBL testing is available on the automated system, Vitek 2 (Biomerieux) AST GN card.

The nitrocefin β-lactamase test (also referred to as the rapid test) is not suitable for the detection of ESBLs in *Enterobacteriaceae*.^{19,32}

Molecular tests are also available. These include PCRs for the detection of ESBL genes and ESBL gene sequencing.³²

ESBLs in Staphylococcus, Streptococcus and anaerobes

β -lactamase production in penicillin-resistant *Staphylococcus* can be detected using the β -lactamase identification sticks (code BR0066) available from Oxoid (Thermo Scientific). The sticks are impregnated with a solution of nitrocefin, a chromogenic cephalosporin, which is hydrolysed in the presence of an ESBL, indicated by a colour change from yellow to red. Nitrocefin sticks are recommended for testing *Staphylococcus* and *Bacteroides* but not for detecting ESBLs in *Enterobacteriaceae*.¹⁹

Some bacteria will not produce β -lactamase unless the enzyme is induced by exposure to a β -lactam antimicrobial agent. This can be achieved by incubation of the nitrocefin test strip for up to an hour and then testing the growth from the zone margin around an oxacillin disk.¹⁹

Pigmented *Staphylococcus* species may give rise to a false-positive result and in this case Oxoid recommends testing using nitrocefin solution, code SR0112.

MRSA and MRSP

Methicillin-resistant *Staphylococcus aureus* (MRSA), oxacillin-resistant *S. aureus* (ORSA) and *Staphylococcus pseudointermedius* (MRSP) are resistant to β -lactam antimicrobial agents that include the penicillins and cephalosporins due to the acquisition of the *mecA* gene. The *mecA* gene encodes the variant penicillin-binding protein 2a (PBP2a).

CLSI method VET01-A4 details the different detection disks recommended to detect methicillin/oxacillin resistance depending upon the species of *Staphylococcus*. *S. aureus* and *S. lugdunensis* should be tested for resistance to oxacillin using a ceftiofur disk.¹⁹ *S. lugdunensis* is an emerging invasive skin pathogen in humans, but has been implicated in respiratory tract and deep tissue infections in companion animals.³⁴ *S. pseudointermedius* should be tested with oxacillin, not ceftiofur. The plate is observed under a light and the zone of growth inhibition is examined for any discernable growth, which indicates oxacillin resistance. Coagulase-negative *Staphylococcus* species (CoNS), including *S. epidermidis*, should be tested using the ceftiofur disk, which has higher specificity and equal sensitivity to the oxacillin disk for CoNS. Tests for MRSA/ORSA must be incubated for a full 24 hours and at 35°C \pm 2°C. At temperatures above 37°C, MRSA/ORSA may not be detected. Results are reported as oxacillin susceptible or resistant, even when ceftiofur is used as a surrogate for oxacillin.¹⁹ Molecular tests are also available to detect the *mecA* gene.

It is recommended that veterinary laboratories test any penicillin-resistant *S. aureus* and *S. pseudointermedius* for MRSA/ORSA. All *Staphylococcus* species and *S. pseudointermedius* with a disk diffusion <17 mm or MIC > 0.5 μ g/mL are reported as resistant to oxacillin. These breakpoints also indicate *mecA*-mediated resistance in *S. pseudointermedius*.¹⁹ CLSI document VET01-A4¹⁹ should be consulted for comprehensive guidelines on testing and reporting.

Definitions and notes on susceptibility and resistance patterns for some bacteria

AmpC β -lactamases

AmpC β -lactamases are types of β -lactamases that hydrolyse both broad and extended spectrum cephalosporins (cephalothin, cefazolin, ceftiofur), many penicillins, and β -lactamase inhibitor- β -lactam combinations. They are either encoded on the chromosome or carried on plasmids. They are not inhibited by β -lactamase inhibitors such as clavulanic acid.

AmpC β -lactamases may be produced by many Gram-negative bacteria including *Enterobacter*, *Citrobacter*, *Serratia* and may occur in *E. coli*.^{19,32} It is suggested that veterinary laboratories test any ceftiofur resistant *E. coli* or *Salmonella* for ESBLs including AmpC.

Anaerobes and microaerophilic bacteria

Disk diffusion is not recommended for anaerobes and testing should be performed according to CLSI M11.¹⁹ For microaerophilic organisms such as *Campylobacter* species, the European Committee in Antimicrobial Susceptibility Testing (EUCAST) has recommended MIC scores based on epidemiological cut-off values (http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Consultation/Campylobacter_wide_consultation_August_2012.pdf).

Beta-lactamase

This is a bacterial enzyme capable of destroying the activity of beta-lactam agents by hydrolysing the beta-lactam ring portion of the molecule. There are many different types of beta-lactamases with specific activity against different beta-lactam agents. β -lactam antimicrobials include penicillin, cephem (cephalosporin), penem (carbapenem) or monobactam according to additional ring structures or substituent groups added to the β -lactam ring.¹⁹

Breakpoints

The breakpoints, or interpretive criteria, are the MIC and disk diffusion values (zone of growth inhibition measured in mm) recorded for a bacterium that are interpreted as susceptible, intermediate or resistant to an antimicrobial agent.¹⁹

Campylobacter species

CLSI states that disk diffusion is not reliable for *Campylobacter* species;¹⁹ however, EUCAST has published recommended guidelines (see under *anaerobes and microaerophilic bacteria*).

Carbapenemases

Carbapenems are broad-spectrum β -lactam antimicrobial agents used against *Enterobacteriaceae* and are a last line of defence for treatment of serious infections in humans. They are not approved for use in veterinary medicine. Carbapenemases are β -lactamases that hydrolyse penicillins, mostly cephalosporins and to some extent carbapenems, and are produced by some *Enterobacteriaceae*, including *E. coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. Detection of carbapenemases are required for infection control in hospitals, but not for categorisation of antimicrobial susceptibility.³²

Enterococci

Enterococci generally do not cause infections in animals. They are common inhabitants of the gastrointestinal tract of animals and humans. Some notes are mentioned here because of their importance in antimicrobial resistance in medical microbiology. *Enterococcus* species are inherently resistant to many antimicrobial agents such as clindamycin, oxacillin, and cephalosporins and are increasingly developing resistance to ampicillin, vancomycin (vancomycin resistant enterococcus, VRE), streptogramins (virginiamycin) and aminoglycosides. *E. faecalis* is susceptible to ampicillin and penicillin whereas *E. faecium* is often resistant.^{19,35} Enterococci resistant to penicillin and ampicillin, because of production of low-affinity penicillin-binding proteins (PBPs), will be detected by the disk diffusion method.

Strains that are resistant due to the production of β -lactamase are not reliably detected by the disk diffusion method. The direct nitrocefin-based β -lactamase test is used.¹⁹

Enterobacteriaceae

Enterobacteriaceae may produce many different types of β -lactamases such as plasmid-mediated TEM-1, TEM-2 and SHV-1 enzymes. Mutations in the genes encoding TEM-1, TEM-2 and SHV-1 β -lactamases are termed extended-spectrum β -lactamases (ESBLs). ESBLs are resistant to penicillin and cephalosporins (but not cephamycins, ceftiofur and cefotetan) and monobactams. ESBLs are inhibited by clavulanic acid. It is recommended that laboratories test for ESBLs in some of the *Enterobacteriaceae* – see under ‘*methods for detection of resistance mechanisms*’.¹⁹

Aminoglycosides (amikacin, gentamicin and tobramycin) are usually active against *Enterobacteriaceae*. Resistance to fluoroquinolones (ciprofloxacin, levofloxacin) is variable between countries.¹⁹ Fluoroquinolones are prohibited in food-producing animals in Australia.¹⁵

Extended-spectrum β -lactamase (ESBLs)

Gene mutations in plasmid-regulated β -lactamases result in enzymes termed extended-spectrum β -lactamases (ESBLs) that have the ability to inactivate penicillins, and expanded-spectrum cephalosporins including oxyimino- β -lactam compounds (cefuroxime, third- and fourth-generation cephalosporins and aztreonam) but not cephamycins or carbapenems. There are different types of ESBLs but most belong to the Ambler class A of β -lactamases and are inhibited by β -lactamase inhibitors such as clavulanic acid, sulbactam and tazobactam. ESBLs are produced by some *Enterobacteriaceae*, especially *E. coli*, *Klebsiella pneumoniae*, *K. oxytoca*, *Proteus mirabilis*, and environmental Gram-negative bacteria such as *Acinetobacter* species.¹⁹ It is recommended that veterinary laboratories screen any third or fourth generation cephalosporins (ceftiofur, ceftriaxone, ceftiofur) resistant isolates for potential ESBL production. See under ‘*methods for detection of resistance mechanisms*’.

Food-producing animals

Food producing animals are defined as animals reared for the production of meat and other food products, such as milk and eggs, and include cattle, sheep, goats, pigs, poultry (including game species), buffalo, ratites, camelids, finfish, crustaceans and molluscs; however, some jurisdictions may have different definitions.^{19,36}

Growth promotants

Growth promotants (which may include antibiotics) are defined as substances used to increase the rate of weight gain, and/or the food conversion efficiency, in animals.³⁶

Listeria

Due to the slow growth of *Listeria* species, this organism should be tested using a microbroth dilution method.¹⁹

Off-label use

This term describes the use of a veterinary medicine to treat an animal in a way that is not described on the registered label. This may include use in a different species; at a different dose rate, frequency or duration of use; or with a different withholding period. It also includes use of antimicrobials that are registered for use in humans or have limited registration for veterinary use. There are a number of legal restrictions for off-label use in food-producing animals.^{19,36}

Pharmacodynamics

This term describes the study of the action of drugs, including biochemical and physiological effects, mechanisms of action and chemical structure.³⁷

Pharmacokinetics

The term describes the study of drugs in the body. It takes into account the mechanisms of absorption, distribution in the body, the rate of action of the antimicrobial agent, the duration of the effect, and the elimination from the body.³⁷

Pseudomonas aeruginosa

P. aeruginosa is intrinsically resistant to narrow-spectrum penicillins, first and second-generation cephalosporins, trimethoprim and sulphonamides. Antimicrobial agents used for *P. aeruginosa* include extended-spectrum penicillins, such as ticarcillin and piperacillin; extended-spectrum cephalosporins, such as ceftazimide and cefepime; carbapenems; aminoglycosides; and fluoroquinolones; however, resistance is increasing. Ciprofloxacin remains the most active of the fluoroquinolones. Beta-lactamases may be produced by *P. aeruginosa*. Resistance to only amikacin but not gentamycin or tobramycin, is unusual.¹⁹

Staphylococcus aureus

Resistance to commonly used antimicrobials is increasing. Over 90% of *S. aureus* from human sources are resistant to penicillin. Resistance to penicillin is due to a penicillinase enzyme, a type of β -lactamase that hydrolyses the beta-lactam ring of penicillin. For this reason penicillin should be used to test *S. aureus* susceptibility against all penicillinase-labile penicillins. All penicillin-resistant isolates from veterinary sources should be tested against cefoxitin as a surrogate test disk for oxacillin— see under ‘*methods for detection of resistance mechanisms*’.¹⁹ Oxacillin and methicillin are semisynthetic penicillins that are resistant to β -lactamase. Some strains of *S. aureus* have developed resistance to these antimicrobial agents. They are termed oxacillin-resistant *S. aureus* (ORSA) and methicillin-resistant *S. aureus* (MRSA) due to the production of the *mecA* gene.^{28,38} Methicillin is no longer used for testing (or treatment) and oxacillin is the antimicrobial generally tested for in the laboratory. Previously, detection of MRSA was undertaken on test medium containing 5% NaCl and a methicillin disk of 5 or 10 μ g. More recently, testing has been replaced with either oxacillin or cefoxitin disks. ORSA are resistant to all penicillinase-stable penicillins including oxacillin, methicillin, and cloxacillin. ORSAs are usually resistant to macrolides, lincosamides and tetracyclines and may also be resistant to fluoroquinolones and aminoglycosides. CLSI recommends using a 30 μ g cefoxitin disk to predict *mecA*-mediated oxacillin resistance in staphylococci. A zone size of ≤ 20 mm is resistant and ≥ 19 mm is susceptible. A slight haze around an oxacillin disk indicates heteroresistance, i.e. a subpopulation resistant to oxacillin. The CLSI manual should be consulted for a more comprehensive description of AST for *Staphylococcus* species.¹⁹ Detecting the *mecA* gene by PCR is the most accurate method for detecting MRSA and ORSA.^{38,39}

Coagulase-negative staphylococci (CoNS) are generally more resistant to antimicrobial agents than *S. aureus*. CoNS are usually not significant in most food-producing animals and therefore AST is not required.¹⁹ Some laboratories testing samples from zoo animals or laboratory animals may be required to test for these species. Prevalence studies indicate CoNS from livestock sources especially food-producing animal are an increasing source of methicillin resistance.²⁸

Some bacteria may be resistant to macrolides such as erythromycin (which is restricted for use in food-producing animals) and lincosamides such as clindamycin. This is due to the

production of the *erm* gene which produces an RNA methylase enzyme that modifies the ribosomal binding site of macrolides, lincosamides and streptogramins B.

Salmonella species and *Shigella* species

A note for veterinary laboratories recommends that aminoglycosides, and first and second generation cephalosporins, may appear active in AST but are not clinically effective and should not be reported.¹⁹

Trueperella (Arcanobacterium) pyogenes

This organism is inherently sensitive to penicillin and usually antimicrobial susceptibility testing is not required.¹⁹

Quality Control¹⁹

QC is the process a laboratory uses to monitor the test procedure to make sure the test is working correctly. The laboratory should have a written QC method for assessment of the antimicrobial disks and the media. QC should be conducted on each new batch of medium and disks. Zones must be within the guidelines stated in the CLSI document. QC testing initially begins daily and then proceeds to weekly testing once 30 consecutive tests have been recorded and when, for each microbial agent, there are no more than three of 30 zone diameters out of range. Any zone that is out of range on two successive days must be investigated and the test repeated using a new batch of disks. QC is performed each test day for AST performed less than once a week.

If a new antimicrobial is introduced into the laboratory, a new test system is undertaken, or a major change to the method is adopted, then the zone sizes must be tested for 30 consecutive days before proceeding to weekly testing. For example, a change from manual to automated reading.

If conducting a corrective action, the following may need to be checked: zone diameters, turbidity standard, inoculum suspension, storage of media and disks, expiry dates of media and disks, incubator temperature, incubating atmosphere, correct Type strain, purity of growth, competency of the person conducting the test, pH of the medium and depth of the agar.

The QC results can be used for measurement of uncertainty as required by National Association of Testing Authorities, Australia (NATA).

Maintenance of QC strains¹⁹

A collection of reference strains must be maintained and stored in a culture system. The correct Type strains, as stated in the CLSI manual, must be used. Stock cultures are stored long-term in either liquid nitrogen (-196°C), in a -80°C freezer, or freeze-dried using appropriate cryoprotectants or freeze-drying medium. Working stock cultures are prepared monthly from a permanent stock culture that has been subcultured two or three times after reconstitution from storage. Working stocks are maintained on trypticase soy agar slants, or on chocolate agar slants for fastidious organisms. QC testing is performed on a working stock that has been subcultured one to two times. All procedures and testing must be documented. A troubleshooting guide is provided in CLSI VET 01-A4 (previously M31-A3).

Quality Assurance

Quality assurance is a program, usually offered by an external laboratory, to monitor the overall performance of a test within a laboratory.¹⁹

In Australian and New Zealand veterinary laboratories, quality assurance is performed within the IFM Quality Services proficiency program.

Guidance on Safety and Containment Requirements

Adhere to normal procedures when working with bacteria in a PC2 laboratory. For organisms that require a higher biosecurity level, AST must be performed within the specified containment level.

Part 3. Guidelines, Prohibited Antimicrobials and Reporting

Antimicrobial use in veterinary medicine: controls, guidelines and reporting

This section provides information on antimicrobials that are prohibited or have restricted use in food-producing animals so that AST results are reported appropriately, as recommended by the Sub-Committee on Animal Health Laboratory Standards (SCAHLs).⁴⁰

Due to the world-wide concern for human and animal health regarding AMR,⁸ the World Health Organisation (OIE) has ranked antimicrobials according to their importance in human medicine with some being reserved for human use only as part of a worldwide strategy for managing the risk of the spread of antimicrobial resistant bacteria from animals to humans through the food chain. The focus is on fluoroquinolones, vancomycin, macrolides and third and fourth generation cephalosporins.⁵ The bacteria of concern are *Salmonella*, *Campylobacter*, *Staphylococcus aureus*, *Escherichia coli*, and *Enterococcus* species, particularly *Enterococcus faecium* (normal flora in animals). The website should be checked for any changes, (http://www.who.int/foodborne_disease/resistance/cia/en/).

The OIE publishes a list of antimicrobials of veterinary importance⁷ to be used by authorities to develop policy for responsible and prudent use of antimicrobials for treatment of food-producing animals within the OIE and WHO recommendations, and how they can be used; for example, as single animal therapy, herd treatment, or prohibiting off-label use.

In line with the worldwide focus on managing the risk of AMR, SCAHLs has recommended that laboratories do not routinely report antimicrobial susceptibility results for bacterial isolates from food-producing animals where the antimicrobial is prohibited, and/or of critical importance in human medicine.⁴⁰ Alternatively, the report should indicate the restricted use. In Australia, ceftiofur, chlortetracycline, enrofloxacin, erythromycin and gentamicin are prohibited or limited for use in food-producing animals.⁴⁰

The CLSI document VET01-A4 has guidelines for routine testing and reporting of antimicrobial agents for use in food-producing and companion animals; however, to ensure relevance to Australian and New Zealand requirements, laboratories should regularly check the relevant websites. Laboratories in Australia should check the information on antimicrobial use in food-producing animals on the APVMA website (<http://www.apvma.gov.au/>). The APVMA controls the registration of veterinary medicines and the Veterinary Manual of Requirements and Guidelines (Vet MORAG,¹⁵ at http://www.apvma.gov.au/morag_vet) provides information on data requirements and guidelines for application to register, or approve, agricultural chemical products. This site has the Public Chemical Registration Information System (PUBCRIS), which allows a search for agricultural and veterinary chemical products (includes antimicrobial agents) registered for use in Australia and provides label information detailing approved use.

In New Zealand, use of antimicrobials in the agricultural sector is overseen by the Agricultural Compounds and Veterinary Medicines (ACVM) Group of the NZFSA and registration of veterinary medicines is controlled by the Ministry for Primary Industries, Food Safety.¹⁷ A review of the antimicrobial resistance in animals and plants and recommendations has been undertaken (http://www.foodsafety.govt.nz/elibrary/industry/steering-group2004/Review_Impact-Prepared_Appointed.pdf).

The respective websites should be consulted regularly for updated information.

Antibiotics prohibited or restricted for use in food-producing animals in Australia

Many antibiotics used for companion animals are prohibited for use in food-producing animals and restrictions for their use must be adhered to unless allowed for single animals.

The following is a list of antimicrobials for which 'off-label' use is restricted. These can be found in the Australian Veterinary Association (Prescribing and Dispensing Guidelines).⁴¹ These may vary between states, so relevant state and territory legislation should be consulted; a list of relevant legislation and websites is contained in the AVA Prescribing and Dispensing guidelines at www.ava.com.au.

For example, restrictions in WA are contained in the Veterinary Chemical Control and Animal Feeding Stuffs Regulations, 2006, which state the following antimicrobial agents are prohibited in animal feed: chloramphenicol, sulfathiazole, sulfaguanidine, sulfanilamide, sulfamonomethoxine, sulfachloropyridazine, sulfapyridine, sulfafurazole, sulfamethoxydiazine, sulfacetamide sodium, sulfanitran, phthalylsulfacetamide and sulfacetamide.

Table 1. Antibiotics prohibited for use or with off-label restrictions in food-producing animals in Australia*

Antimicrobial	Details
Aminoglycosides	Prohibited use includes gentamicin and streptomycin. Restricted off-label use for neomycin
Avoparcin	A glycopeptide antibiotic. It was withdrawn from the market worldwide in 2001 due to its association with vancomycin resistance in <i>E. faecium</i>
Cephalosporins	Have never been approved for use in Australian poultry; ceftiofur is registered for use in cattle for treatment of respiratory and foot infections only
Chloramphenicol	Not to be used to treat any food-producing animals or horses
Fluoroquinolones	Use is prohibited in all food-producing animals
Metronidazole and Nitrofurantoin	Including the derivatives furaltadone, furazolidone, nifursol, nitrofurazone. Not to be reported for food-producing animals
Nitroimidazoles	With the exception of dimetridazole for use in birds
Sulphonamides	Restricted off-label use
Virginiamycin	Proposed as prohibited for use as a growth promotant and that therapeutic or prophylactic use in food-producing animals be limited to 21 days in chicken broiler stock, and 28 days in cattle and sheep in any 12-month period

*Australian Pesticides and Veterinary Medicines Authority, http://www.apvma.gov.au/registration/not_permitted.php, and guidelines for prescribing, authorising and dispensing veterinary medicines (available online).³³

In Australia, the use of penicillin, tetracycline, oxytetracycline and chlortetracycline were removed from use as growth promotants in livestock over thirty years ago. The tetracyclines can be used as prescription-only veterinary medicines for addition to feed (see Vet MORAG website).

In human health, vancomycin resistant enterococci (VRE) are of great concern and increasing resistance has been blamed on use in food-producing animals. The following is provided as background information for veterinary laboratories. Avoparcin is a glycopeptide class of antimicrobial agent, the same class as vancomycin, and is associated with increased resistance to vancomycin. It was used in Australia for 19 years as a growth promotant in pigs and poultry and to a small extent as a calf weaning mixture (AVPMA website). Its use was banned world-wide in 2001. Enterococci are naturally resistant to some antibiotics such as cephalosporins, aminoglycosides, and clindamycin and are often resistant to tetracyclines and erythromycin. Enterococci are part of the intestinal flora of man and animals. In humans they can be pathogenic in some situations such as post-surgery and may cause septicaemia, endocarditis and minor urinary tract infections. Treatment of enterococcus infections is limited to penicillin, ampicillin, amoxicillin, a glycopeptide (such as vancomycin or teicoplanin), or a streptogramin such as quinupristin/dalfopristin. Increasing resistance to vancomycin in enterococci is of concern in the medical field as vancomycin resistant enterococci (VRE) are usually multi-drug resistant, which has led to restrictions on vancomycin use. Although vancomycin was never used in veterinary preparations in the USA, there is a high rate of VRE in humans in that country indicating other factors may be responsible for the development of VRE.⁴¹ *VanA* and *VanB* are usually found in *E. faecium* and *E. faecalis*. *VanC* is found in other *Enterococcus* species.^{42,43} Resistance to virginiamycin is also a public health concern due to the need to protect the use of quinupristin/dalfopristin for treatment of VRE infections.^{44,45}

No antimicrobials are registered for use in aquaculture but the APVMA does from time to time issue permits for use of tetracycline- and florfenicol-containing products. Tetracycline- and sulphonamide-containing products (sulfadimidine, sulfamerazone, sulfadiazine) are registered for use in ornamental fish. The APVMA website and PUBCRIS should be referred to for current information.

Other antimicrobial classes and their resistance mechanisms are presented in the CLSI document VET01-A4, in the text and in the glossary.¹⁹

The following table is a summary of antibiotic classes registered for use in animals in Australia, and has been revised from Barton (2004).⁴⁶ It can be used as a guide to ensure appropriate results are reported for AST testing.

Table 2. Antibiotic classes registered for use in animals** in Australia

Antibiotic class	Pigs	Birds*	Sheep	Cattle	Dogs & cats	horses
Aminoglycosides						
Apramycin	+	+ ⁷	-	+	-	-
Framycetin	-	-	+ ¹¹	+ ¹¹	+ ^{9,11}	+ ¹¹
Gentamicin#	#	#	#	#	+	+
Neomycin	+	+	+	+	+	+
Spectinomycin ¹⁷	+	+	-	-	-	-
Streptomycin	-	-	-	+ ¹³	+	-
Amphenicols						
Chloramphenicol#	#	#	#	#	+	+
Florfenicol	+	-	-	+ ¹⁸	-	-
Orthosomycins						
Avilamycin	-	+ ¹⁹	-	-	-	-
Carbapenems & monobactams						
	-	-	-	-	-	-
Cephalosporins						
Ceftiofur	-	-	-	+ ¹	+ ¹⁴	+ ¹
Cefuroxime	-	-	-	+ ²	-	-
Cephalonium	-	-	-	+ ^{2, 10}	+ ^{10, 14}	-
Cephapirin	-	-	-	+ ³	-	-
Cephalexin	-	-	-	-	+	-
Bambermycins						
Flavophospholipol ✕	✕	✕	-	✕	-	-
Fluoroquinolones#						
Enrofloxacin#	#	+ ¹⁵	#	#	+	-
Difloxacin# ⁵	#	#	#	#	-	-
Marbofloxacin#	#	#	#	#	+	-
Orbifloxacin#	#	#	#	#	+	-
Ibafloxacin#	#	#	#	#	+	-
Fusidic acid	-	-	-	-	+ ^{9, 10}	-
Glycopeptides⁵	-	-	-	-	-	-
Lincosamides						
Clindamycin	-	-	-	-	+	-
Lincomycin	+	+	-	+ ²	+	-
Macrolides						
Erythromycin	+	+ ⁸	+	+	-	+ ¹⁶
Kitasamycin ◆	◆	-	-	-	-	-
Oleandomycin	-	-	-	+ ²	-	-
Spiramycin	-	-	-	-	+ ²¹	-
Tilmicosin	+	-	-	+	-	-
Tylosin	+	+ ⁸	-	+	-	-
Tulathromycin	+	-	-	+	-	-

Antibiotic class	Pigs	Birds*	Sheep	Cattle	Dogs and cats	horses
Arsenicals roxarsone	-	+ ⁸	-	-	-	-
Nitrofurans Nitrofurazone #	#	#	#	#	+ ⁹	+
Nitroimidazoles Dimetridazole Metronidazole#	+ #	+ ²² + ²³	- #	- #	- +	- + ²³
Novobiocin	-	-	-	+ ²	+	-
Penicillins Ampicillin + cloxacillin Amoxycillin Benzylpenicillin ⁵ Cloxacillin Procaine penicillin	- + - - +	- + ⁸ - - -	- + - + ¹⁰ +	+ ² + ⁴ - + ^{2,10} +	- + ⁴ - + ¹⁰ +	- + - + ¹⁰ +
Pleuromutilins Tiamulin	+	+ ⁸	-	-	-	-
Polyethers (Ionophores)	✦	✦	-	✦	-	-
Polypeptides Polymixin Zn bacitracin	- -	- +	+ ¹¹ + ¹¹	+ ¹¹ + ¹¹	+ ^{9, 11} + ^{9, 11,}	+ ^{9, 11} + ^{9, 11}
Quinoxalines Olaquinox	✧	-	-	-	-	-
Rifamycins Rifampicin	-	-	-	-	-	+ ¹⁶
Streptogramins virginiamycin	-	+ ¹²	+	+	-	+ ²³
Sulphonamides ²⁰ various sulphonamides often in combination with trimethoprim or diaveridine	+	+ ⁸	+	+	+	+
Tetracyclines Chlortetracycline ⁵ Doxycycline Oxytetracycline ⁹	+ - +	+ + ⁶ + ⁸	+ ⁹ - +	- - +	+ + +	- - +

Key

** some tetracycline and sulphonamide containing products are registered for use in ornamental fish but no antimicrobials are registered for use in aquaculture; APVMA from time to time issues permits for use of tetracycline and florfenicol products for use in aquaculture.

* - includes commercial poultry & cage & aviary birds

- use of these products is specifically prohibited in food producing animals

✧ - avoparcin has not been registered since mid-2000 and no other glycopeptides are registered for use in animals

✦ - registered for use as a growth promotant - no related human antibiotics

◆ registered as a growth promotant in pigs and for control of *Mycoplasma pneumonia*

✧ registered as a growth promotant in calves and also included in an intramammary preparation (treatment of mastitis)

- ✦ - registered as coccidiostats in broiler and layer chickens and cattle and goats; for control of bloat and subclinical ketosis in cattle; as growth promotants in cattle and pigs - no related human antibiotics
 ✨ - registered as a growth promotant and for control of some chronic intestinal diseases in pigs

- 1 – registered for respiratory infections only; individual animal treatment only – not mass medication; not for topical, oral or intramammary route in food-producing animals; not for treatment of mastitis; not for bobby calves
 2- registered only as an intramammary preparation
 3 – registered only as an intra-uterine preparation
 4 – includes preparations containing clavulanate
 5 – no products registered in Australia
 6 – registered for use in pigeons & cage & aviary birds only
 7 – registered for use in non-laying chickens
 8 – cannot be used in poultry which are producing or may in the future produce eggs for human consumption
 9 – registered as a topical preparation
 10 – ophthalmic preparation
 11 – ophthalmic and aural preparations
 12 – for use in meat chickens
 13 – in combination with other antimicrobials - oral preparation in calves; intramammary preparation
 14 – registered for use in dogs only, not cats
 15 – registered for use in exotic species and birds (non-food-producing)
 16 – erythromycin plus rifampicin – combined use under permit for treatment of foals with *Rhodococcus equi* infection
 17 – in combination with lincomycin
 18 – not for use in cattle that are producing or may in the future produce milk or milk products for human consumption; not for use in veal calves; injectable formulations not for use in pigs or cattle intended for breeding
 19 – for use only in meat chickens as a growth promotant and for prevention of necrotic enteritis
 20 – triple sulpha formulation registered for use in ornamental fish
 21 – in combination with metronidazole – oral preparation only
 22 – for use in breeder pigeons, breeder game birds, breeder caged birds – not to be used in egg layers, meat chickens, turkeys – restricted to indications only
 23 – not to be used in any food producing species of animal

Antibiotics prohibited for use in food-producing animals in New Zealand

In New Zealand, the registration of veterinary medicines is controlled by the Ministry for Primary Industries. The following are taken from the list of prohibited substances that must not be used during the life of an animal from which any product is used for human consumption.¹⁷

Table 3. Antibiotics prohibited for use in food-producing animals in New Zealand*

Antimicrobial	Details
Chloramphenicol	Not to be used to treat any food-producing animals
Nitrofurans class	Including but not limited to furaltadone, furazolidone, nitrofurazone, ninhydrone
Nitroimidazoles	Including but not limited to metronidazole or ronidazole

*Industry food safety within the Ministry for Primary Industries. Prohibited substances. Restricted veterinary medicines (<http://www.foodsafety.govt.nz/elibrary/industry/prohibited.htm>)

CLSI Documents

CLSI is one of the main standards organisations throughout the world that publish and maintain documents detailing AST methods and guidelines. CLSI publishes and maintains

the only protocols recommended by the OIE for the susceptibility testing of bacteria from animals including aquatic animals. The main protocol for testing bacteria from animals is VET01-A4 and for aquatic animals is VET03/VET04-S1. Table 4 lists some of the documents available for purchase from CLSI (<http://www.clsi.org/>). M45 details methods for fastidious bacteria such as *Pasteurella*, *Corynebacterium*, *Erysipelothrix rhusiopathiae*, *Listeria monocytogenes*, and *Vibrio* species. This protocol is based on interpretive guidelines obtained from medical pharmacokinetic and pharmacodynamic data and for the time being is all that is available for the interpretation of results from these bacteria isolated from animals, and as such, results must be interpreted with caution.

Table 4 Documents published by CLSI

Former document code	New document code	Title
M2	M02-A11	Performance standards for antimicrobial disk susceptibility tests; approved standard—eleventh edition. Vol. 32 No. 1. Jan 2012
M6	M06-A2	Protocols for evaluating dehydrated Mueller-Hinton agar; approved standard—second edition. Vol. 26 No. 6. Jan 2006
M7	M07-A9	Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard—ninth edition. Vol. 32 No. 2. Jan 2012
M11	M11-A8	Methods for antimicrobial susceptibility testing of anaerobic bacteria; approved standard—eight edition. Vol. 32 No. 5. Feb 2012
M23	M23-A3	Development of <i>in vitro</i> susceptibility testing criteria and quality control parameters; approved guideline—third edition. Vol. 28 No. 27. Oct 2008. This document describes the details of the data required to establish interpretive criteria, QC parameters and how the data are to be presented for evaluation.
M31	See VET01-A4 and VET01-S2	
M39	M39-A4	Analysis and presentation of cumulative antimicrobial susceptibility test data; approved guideline—fourth edition. Vol. 34 No. 2. Jan 2014
M100	M100S24	Performance standards for antimicrobial susceptibility testing; twenty-fourth informational supplement. Vol. 34 No. 1. Jan 2014. This is the main document used by medical laboratories
M24	M24-A2	Susceptibility testing of <i>Mycobacteria</i> , <i>Nocardia</i> , and other aerobic <i>Actinomycetes</i> ;

		approved standard—second edition. Vol. 31 No. 5. March 2011
M26	M26-A	Methods for determining bactericidal activity of antimicrobial agents; approved guideline. Vol. 19 No. 18. Sept 1999
M27	M27-S4	Reference method for broth dilution antifungal susceptibility testing of yeasts; fourth informational supplement. Dec 2012
M31-A3	VET01-A4	Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals; approved standard—fourth edition. Vol. 33 No. 7. July 2013
	VET01-S2	Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals; second informational supplement. July 2013
M37-A3	VET02-A3	Development of <i>in vitro</i> susceptibility testing criteria and quality control parameters for veterinary antimicrobial agents; approved guideline—third edition. Vol. 28 No. 7. Feb 2008
M38	M38-A2	Reference method for broth dilution antifungal susceptibility testing of filamentous fungi; approved standard—second edition. Vol. 28 No. 16. April 2008
M42/M49-S1	VET03/VET04-S1	Performance standards for antimicrobial susceptibility testing of bacteria isolated from aquatic animals; first informational supplement. June 2010
M42-A	VET03-A	Methods for antimicrobial disk susceptibility testing of bacteria isolated from aquatic animals; approved guideline. Vol. 26 No. 23. June 2006
M49A	VET04-A	Methods for broth dilution susceptibility testing of bacteria isolated from aquatic animals; approved guideline. Vol. 26 No. 24. June 2006
M45	M45-A2	Methods for antimicrobial dilution and disk susceptibility testing of infrequently isolated or fastidious bacteria; approved guideline—second edition. Vol. 30 No. 18. Aug 2010. Established for medical laboratories but contains information on <i>Pasteurella</i> , <i>Corynebacterium</i> , <i>Erysipelothrix rhusiopathiae</i> , <i>Listeria monocytogenes</i> , <i>Vibrio</i> species, however the interpretive criteria have not been applied to animals
	VET05-R	Generation, presentation, and application of

		antimicrobial susceptibility test data for bacteria of animal origin; a report. September 2011
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Documents can be ordered as print or electronic documents at the CLSI website under documents and under microbiology at the following link.
<http://shopping.netsuite.com/s.nl/c.1253739/sc.7/category.2396/f>

Part 4. Reagents

Preparation of a 0.5 McFarland Standard¹⁹

A 0.5 McFarland turbidity standard is used to make the inoculum for disk susceptibility testing.

Reagents:

- Anhydrous barium chloride BaCl₂ 1% w/v
- Cold pure sulphuric acid H₂SO₄ 1% v/v

Add 0.5 mL of 1% BaCl₂ to 99.5 mL of 1% H₂SO₄. Stir to mix the suspension evenly. Distribute 5 mL into clear glass tubes with the same diameter, or the same tubes in which the inoculum will be prepared. Store tubes at room temperature in the dark. The turbidity is equivalent to a density of 1.5×10^8 cells. CLIS document M02-A11 has details of this method.

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