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Editorial: Assessing the antimicrobial susceptibility of bacteria obtained from animals†

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The accurate performance of antimicrobial susceptibility testing of bacteria from animal sources and the correct presentation of the results is a complex matter. A review of the published literature revealed a number of recurring errors with regard to methodology, quality control, appropriate interpretive criteria, and calculation of MIC_{50} and MIC_{90} values. Although more subjective, there is also no consensus regarding the definition of multiresistance. This Editorial is intended to provide guidance to authors on how to avoid these frequently detected shortcomings.

Keywords: antimicrobial susceptibility testing, interpretive criteria, MIC_{50}, MIC_{90}, multiresistance

Introduction

In recent years, antimicrobial resistance in bacteria of animal origin, including food-producing animals, pet and companion animals, fish and other aquatic animals as well as wild animals, has gained particular attention. Consequently, an increasing number of studies that include antimicrobial susceptibility testing have been published. However, an analysis of recently published articles revealed a number of frequently occurring shortcomings, which may have an impact either directly on the quality of the results obtained or on the conclusions drawn. This editorial is intended to highlight the major pitfalls and provide guidance for authors and reviewers on the correct performance of antimicrobial susceptibility testing, as well as the presentation of the obtained results and the proper comparison of data from different studies.

Methodology

Several methods, like disc diffusion, Etest, agar dilution, broth microdilution and broth macrodilution, are suitable for *in vitro* antimicrobial susceptibility testing (AST). Whichever method is used, the tests have to be performed in accordance with an internationally accepted procedure, such as those published by the Clinical and Laboratory Standards Institute (CLSI),[1] the British Society for Antimicrobial Chemotherapy (BSAC),[2] the Deutsches Institut für Normung e.V. (DIN)[3] and the Comité de l'Antibiogramme de la Société Française de Microbiologie (CA-SFM),[4] among others. The documents issued by these bodies are regularly updated and, since the methodologies and interpretative criteria change over time, it is important to follow the latest edition. Among these bodies, the CLSI is unique in that it produces separate documents for use in human and veterinary microbiology. The CLSI also differs from the other bodies in that its documents are not freely available, but must be purchased.

The status of the various types of documents is clarified below. For example, the CLSI differentiates between 'standards' and 'guidelines'. A 'standard' is a document that clearly identifies specific and essential requirements for materials, methods and practices to be used in an unmodified form. A standard may, in addition, contain discretionary elements, which are clearly identified. In contrast, a 'guideline' is a document describing criteria for a general operating practice, procedure or material for voluntary use. A guideline can be used as written or modified by the user to fit specific needs.

The current CLSI document for testing antimicrobial susceptibilities of bacteria isolated from animals, M31-A3,[1] is an approved standard and cannot be used in a modified form. Clear and precise instructions on how to perform AST *in vitro* are given. They include, for example, the medium to be used (including any supplements required to support the growth of specific bacteria), the inoculum density, the incubation time, the temperature and the test conditions. These instructions are

not optional, but are strict rules that must be adhered to for good laboratory practice. Thus, statements such as 'Susceptibility testing mainly followed the recommendations given in the CLSI document M31-A3' are not acceptable. Any deviation from the approved test conditions, such as the use of a different medium or extended incubation times for slow-growing bacteria, have to be specified and justified by the authors.

Most AST documents cover the testing of numerous different bacterial species. However, for several bacterial pathogens relevant to the veterinary field, such as *Haemophilus parasuis* or *Riemerella anatipestifer*, no approved methodology exists. If authors adopt a method approved for a phylogenetically closely related organism, it must be stated clearly that the method used has not been approved for the species tested, but for another member of the same genus (e.g. if the method for testing *Haemophilus influenzae* is used to test *H. parasuis*). Whenever susceptibility testing is undertaken on bacteria for which there is no approved standard available, the methodology chosen has to be validated first, as detailed in CLSI document M37-A3.[5]

Quality controls (QCs)

It is essential to test approved AST reference strains in parallel with the test strains for QC purposes. Lists of approved reference strains are included in the documents mentioned. They also contain acceptable MIC and zone diameter ranges for these reference strains, and clearly state the methodology (e.g. broth microdilution) and the medium (e.g. Mueller–Hinton agar) that the values relate to.

The reference strains must be relevant to the bacterial species tested, e.g. *Escherichia coli* ATCC® 25922 may be used when testing Enterobacteriaceae. Furthermore, authors must ensure (i) that reference strains are suitable for QC of the antimicrobial agents tested, (ii) that the range of concentrations (in broth microdilution) tested spans the entire approved QC ranges and (iii) that discs (in disc diffusion tests) contain the quantity of antimicrobial for which the QC ranges are approved.

Interpretation of the results

AST studies seek to categorize bacterial isolates as susceptible, intermediate or resistant to each antimicrobial tested, on the basis of the MICs or the zone diameters obtained. Such classification requires approved interpretive criteria. Currently, two different types of interpretive criteria are available: clinical breakpoints and epidemiological cut-off values.[6] The precise emphasis of a particular study will dictate which criteria must be applied. If data are intended to guide a therapeutic approach (i.e. the aim of the study is to determine which antimicrobial agents are most likely to lead to therapeutic success), clinical breakpoints must be applied. Epidemiological cut-off values should be used to describe MIC distributions of bacteria without clinical context. Clinical breakpoints and epidemiological cut-off values may be very similar or even identical for some bacteria/drug combinations; however, authors need to understand that epidemiological cut-off values are determined by a different approach than clinical breakpoints and do not necessarily take into account the results of clinical efficacy studies, dosing and route of administration of the antimicrobial agents, nor the drug's pharmacokinetic and pharmacodynamic parameters in the respective animal species. The term 'breakpoint' should be used exclusively for clinical breakpoints and 'susceptible', 'intermediate' and 'resistant' categories should also be reserved for classifications made in relation to the therapeutic application of antimicrobial agents. When reporting data using epidemiological cut-off values, the term 'resistant' is inappropriate; instead, bacteria should be reported as 'wild-type' if the MIC or zone diameter falls within the wild-type range, or 'non-wild-type' if the MIC is higher or the zone diameter smaller than the wild-type range.

The CLSI document M31-A3[1] lists exclusively clinical breakpoints and includes the largest collection of approved clinical breakpoints for bacteria of animal origin currently available, a considerable number of which represent veterinary-specific breakpoints. Many of the latter have been approved for specific disease conditions often caused by particular bacterial species in defined animal host species. For example, approved clinical breakpoints for enrofloxacin in cattle apply exclusively for bovine respiratory diseases due to *Pasteurella multocida*, *Mannheimia haemolytica* and *Histophilus somni*. The use of these breakpoints for other bovine bacteria and different disease conditions, e.g. *Staphylococcus aureus* from bovine mastitis, is unacceptable. Thus, the scope of application of the veterinary-specific breakpoints is clearly defined and cannot be altered.

All standards for performance of AST contain interpretive criteria that refer specifically to that particular methodology. Thus, a certain methodology and its associated interpretive criteria are an entity, and as such belong together. It is not good practice to 'mix and match' testing methodologies and interpretive criteria issued by different organizations. Authors who perform Etests must refer to the interpretive criteria given by the manufacturer of the Etest strips. Since these interpretive criteria are not veterinary-specific, but are adopted from human medicine, their true value for veterinary pathogens is unknown. The same holds for CLSI-approved breakpoints adopted from human medicine and listed in CLSI document M31-A3.[1]

Authors who describe AST of animal isolates often use incorrect or outdated interpretive criteria derived from their own or others' previous publications. This is also bad practice and often results in cumulative errors. Authors must ensure that correct (at the time of submission) interpretive criteria are used. In addition, there is an onus on reviewers to verify whether the correct interpretive criteria were used.

When comparing rates (percentages) of resistance between published studies, authors must make sure that the same methodologies and the same interpretive criteria have been used. Interpretive criteria often change over time and lowering the breakpoint(s) for a specific antimicrobial agent will result in a higher percentage of isolates being classified as resistant, even if the MIC/zone size distribution of the population has not changed. As a consequence, an artefactual increase in the percentage of resistant strains may be noted. Publication of the full MIC distributions for each species/drug combination reduces the potential for this error, since the data can be reanalysed by others if interpretive criteria change. Such tables or histograms are often large and, due to limitations on journal space, may need to be provided as supplemental material.

Before performing disc diffusion, authors need to make sure that the discs contain the correct quantity of antibiotic

for which interpretive criteria are available. It is unfortunate that although a range of discs with varying amounts of the antimicrobial agent are commercially available for many antimicrobial agents, zone diameter interpretive criteria are commonly available only for a single specific disc strength. For example, discs charged with 10, 15 or 30 μg erythromycin are available, but CLSI interpretive criteria and QC ranges for reference strains refer only to zone diameters around a 15 μg disc.[1] Since it is not possible to adjust the values measured with a 10 μg or a 30 μg disc to the approved values for a 15 μg disc, zone sizes obtained with a 10 μg or a 30 μg disc can neither be interpreted nor validated.

A standard dilution series for AST consists of doubling antibiotic concentrations and includes the reference concentration 1 mg/L (e.g. 0.125, 0.25, 0.5, 1, 2, 4 and 8 mg/L etc.). Etest strips indicate half-log values and, so, MICs determined by Etest should be 'rounded up' to the next highest value on the standard series. For example, if an Etest indicates that growth is inhibited at 0.38 mg/L (which is not a concentration in the standard series), the MIC should be rounded up and reported as 0.5 mg/L.

MIC_{50} and MIC_{90} values

MIC_{50} and MIC_{90} values as well as the range of values obtained are important parameters for reporting results of susceptibility testing when multiple isolates of a given species are tested. The MIC_{50} represents the MIC value at which ≥50% of the isolates in a test population are inhibited; it is equivalent to the median MIC value. Given n test strains and the values y_1, $y_2 \ldots y_n$ representing a graded series of MICs starting with the lowest value, the MIC_{50} is the value at position $n \times 0.5$, as long as n is an even number of test strains. If n is an odd number of test strains, the value at position $(n+1) \times 0.5$ represents the MIC_{50} value. The MIC_{90} represents the MIC value at which ≥90% of the strains within a test population are inhibited; the 90th percentile. The MIC_{90} is calculated accordingly, using $n \times 0.9$. If the resulting number is an integer, this number represents the MIC_{90}; if the resulting number is not an integer, the next integer following the respective value represents the MIC_{90}. MIC_{50} and MIC_{90} values should always be presented as concentrations on the standard AST dilution series. If a statistical package is used to calculate the values, intermediate values should never be used. It should be noted that MIC_{50} and MIC_{90} values are not necessarily suitable parameters to describe bimodal or trimodal MIC distributions, although a discrepancy of several dilution steps between the MIC_{50} and MIC_{90} values, e.g. MIC_{50} at 0.25 mg/L and the MIC_{90} at 16 mg/L, might point towards the presence of at least two subpopulations that differ distinctly in their MICs to a given antimicrobial agent. As an example, in a test population of 70 strains, the MIC_{50} is the value at position 35 and the MIC_{90} is the one at position 63 in a graded series of MICs starting with the lowest MIC value at position 1. In a test population of 71 strains, the MIC_{50} is the value at position 36 and the MIC_{90} is the one at position 64 in the aforementioned graded series of the MICs.

Although MIC_{50} and MIC_{90} values can also be calculated for small test populations of e.g. 10–30 strains, under such conditions few strains with high MICs will have a disproportionately high influence on the MIC_{50} and MIC_{90} values. Thus, researchers are encouraged not to overemphasize MIC_{50} and MIC_{90} data obtained from small test populations. Since the significance of MIC_{50} and MIC_{90} increases with the number of strains tested, sufficiently large test populations should be used for most meaningful statements on MIC_{50} and MIC_{90} values.

Multiresistance

The term 'multiresistance' exclusively refers to acquired resistance properties. Bacteria may exhibit intrinsic (primary) resistance to certain antimicrobial agents. Intrinsic resistance may be based on either the lack or the inaccessibility of the antimicrobial target site among the bacteria in question. In other cases, intrinsically resistant bacteria produce inactivating enzymes, such as species-specific β-lactamases, contain multidrug transporters and/or exhibit permeability barriers.[7,8] Such intrinsic resistances must be excluded when describing multiresistance patterns.

There is no universally accepted definition of 'multiresistance'. As a consequence, this term is used inconsistently in the literature. The following suggestions are intended to provide guidance for the most accurate presentation of multiresistance patterns.

(i) If only phenotypic susceptibility testing is performed, resistance to three or more classes of antimicrobial agents can be referred to as multiresistance. For example, resistance to enrofloxacin, marbofloxacin, difloxacin and orbifloxacin represents resistance to one antimicrobial class, since all agents are fluoroquinolones and resistance is most likely mediated by the same mechanism(s). In the case of fluoroquinolones (and some other antimicrobial classes), resistance to a single representative of this class of antibiotic agent can reasonably be extrapolated to resistance (or reduced susceptibility) to other members of that class. However, single class representatives cannot always be validly defined, e.g. for β-lactams and aminoglycosides. In these cases, resistance is not a class effect and multiple, diverse resistance mechanisms exist, each of which confers resistance to subgroups of the respective antimicrobial class. Resistance to subgroups should be counted separately, e.g. resistance to streptomycin and spectinomycin is distinct from resistance to gentamicin, kanamycin and/or tobramycin.

(ii) If phenotypic susceptibility testing is supplemented with molecular analysis for the resistance genes present, multiresistance should be assessed at the molecular level. Bacterial isolates exhibiting the presence of three or more resistance genes or mutations, all of which are associated with a different resistance phenotype (i.e. affecting different antimicrobial classes or subgroups), may be referred to as multiresistant. Exceptions to this rule would include those cases where a single resistance gene or a gene complex is associated with resistance to structurally and/or functionally different antimicrobial agents, e.g. the gene *cfr* for resistance to phenicols, lincosamides, oxazolidinones, pleuromutilins and streptogramin A antibiotics,[9] or the *erm* genes for combined resistance to macrolides, lincosamides and streptogramin B antibiotics.[10]

Conclusions

As indicated above, conducting AST and subsequent data interpretation is a complex matter. A number of competent

authorities provide instructions for performing AST and data interpretation. Each should be followed precisely. Importantly, protocols for AST and data interpretation from different authorities cannot be interchanged. AST data intended for the recommendation of therapy should be interpreted and reported using clinical breakpoints, whereas AST data intended for surveillance purposes may be reported using epidemiological cut-off values. Moreover, the comparison of data generated in different studies requires not only a common methodology, but also the preferential presentation of the data as MIC distribution, which allows for fast and easy re-evaluation of the original data even if the interpretive criteria change over time.

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Transparency declarations

S. Schwarz and P. S. are members of the Veterinary Antimicrobial Susceptibility Testing (VAST) subcommittee of the CLSI, P. S. acts as a consultant to the pharmaceutical industry. S. Simjee is advisor of the VAST subcommittee of the CLSI and a full-time employee of Elanco Animal Health. N. W. has none to declare. E. V. D. is a member of the SAGAM of EMEA. A. P. J. is Editor-in-Chief of the *Journal of Antimicrobial Chemotherapy* and W. G. is Editor-in-Chief of *Veterinary Microbiology*.

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