Defining Resistance and Susceptibility: What S, I, and R Mean to You

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“Susceptible” and “Resistant” are thrown around in the fields of microbiology, medicine, public health, and epidemiology with great frequency. Unfortunately, these classifications are often used in a manner inconsistent with their correct application. The veterinary literature and textbooks are loaded with inappropriate uses of antibiotic susceptibility testing (AST) results.

Here are a few key concepts to get us started. These key concepts relate to testing in broth. Broth dilution testing will be related to zone diffusion testing later in the document.

- **AST** is an *in vitro* test process where bacterial pathogens isolated from clinical cases are subjected to a series of selected antibiotic concentrations in culture. The most basic concept of AST is that the concentration of bacteria in each culture is constant. It is the concentration of the antimicrobial which changes across the multiple cultures. For example, a series of cultures for E. coli to test susceptibility to oxytetracycline might start at 0.5 µg/ml and proceed through increasing concentrations of 1.0, 2.0, 4.0, and 8.0 µg/ml. These are often called “dilutions” because in testing with broth tubes the initial most concentrated broth solution was created first and then diluted to create the lower concentrations.
  - The term “doubling dilutions” is often used. This may be a confusing term because the concentration cuts in half with each dilution starting with the most concentrated broth, but doubles when you look at the concentrations starting from the lowest to the highest. An appropriate term might be “halving dilutions”, but doubling dilutions is used by convention.

- The result of the susceptibility testing is the lowest (minimal) concentration from the tested series of dilutions that inhibits growth of the organism over a period of 18 – 24 hours. This is termed the **minimal inhibitory concentration (MIC)**.

- The MIC is interpreted based on a series of **breakpoints** which, when determined according to Clinical and Laboratory Standards Institute (CLSI) methods, are correlated to the potential for the drug to have a clinical effect on this *pathogen* for a specific *disease*, in a specific animal species, and for a specific antibiotic regimen. The breakpoints are also termed “interpretive criteria”, which includes both breakpoints and zone diameter ranges.
  - The **susceptible breakpoint** is the highest MIC at which we think there will be a clinical effect of the antibiotic in this specific situation. For example, if the susceptible breakpoint in the example above was 2.0 µg/ml, MIC values of 0.5, 1.0, and 2.0 would all be considered susceptible.
  - The **resistant breakpoint** is the MIC which at or above we think there is very little likelihood the antibiotic will have an effect on the clinical outcome of the infection. In the example above it might be 8.0 µg/ml; if it takes 8 µg/ml or more to inhibit
growth of the organism we would consider the bacteria being tested to be resistant to the antibiotic.

Common abuses of AST

- Where you will see abuse of AST in practice, in text books, and in the literature.
  - Using non-CLSI interpretive criteria and methods, or establishing resistance trends comparing susceptibility testing results from current CLSI interpretive criteria and methods to older data developed from various non-CLSI methods.
  - Applying CLSI interpretive criteria to testing results which were not determined using CLSI methods.
  - Applying CLSI interpretive criteria to animal species, diseases, pathogens, and antimicrobial regimens for which they have not been validated through the development process. (It can be done, but requires a lot of additional interpretation, and it is still a SWAG)
    - Closely related is using veterinary and human CLSI approved breakpoints in veterinary medicine as if they were equivalent in predictive value.
  - Attempting to link zone diameters back to the MIC for a specific pathogen without recognition of the variability of this relationship.
  - Statements such as “pick the antimicrobial with an MIC the most dilutions below the susceptible breakpoint”. This is wrong because…
    - There is typically a wide range of validation across interpretive criteria for the different drugs being considered (e.g., enrofloxacin vs. trimethoprim sulfa for a canine soft tissue infection with Staphylococcus spp.)
    - The pharmacodynamics for the different antimicrobials will vary substantially, making interpretation of effects across different drug concentrations and MICs quite different.
  - Using breakpoints established for injectable formulations for interpretation related to in-feed formulations (e.g., tetracyclines)
  - Comparison of the most easily measured fluid or tissue antimicrobial concentration to the MIC of a target pathogen without consideration of how the concentration is measured (i.e., tissue homogenate, gut contents, or interstitial fluid concentration), local environment characteristics, and binding of the antimicrobial.

To understand the application of veterinary susceptibility testing interpretive criteria, it is necessary to study their origin.

This document seeks to clarify the definition of a breakpoint, the relationship of “S, I, and R” to the clinical outcome of antimicrobial therapy, the relationship between serial dilution and disk diffusion breakpoints, and the inputs for CLSI-approved interpretive criteria.

These notes draw heavily from two CLSI publications with recognition of the efforts and contributions of the CLSI VAST Subcommittee, members of other CLSI committees that have provided valuable guidance and input, and the CLSI staff.
A standard consists of specific, essential requirements for materials, methods, or practices for voluntary use in unmodified form. A guideline provides criteria for a general operating practice, procedure, or material which may be used as written or modified by the user to fit specific needs.

Information from these documents are presented for purposes of general information. Only the official documents should be relied upon for guidance.

Copies of the current editions may be obtained from CLSI, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898, USA. The CLSI office may be reached at 610-688-0100, (fax) 610-699-0700, or on the web at Exoffice@clsi.org. In this paper, quotations from CLSI are presented in boxes or in bullet point format.

Veterinary breakpoints have been developed by the Veterinary Antimicrobial Susceptibility Testing (VAST) Subcommittee of the Clinical and Laboratory Standards Institute (CLSI), formerly the National Committee for Clinical and Laboratory Standards (NCCLS). The VAST first met in 1993.

The CLSI process consists of tripartite participation by academia, government, and industry (pharma, manufacturers, and private labs). In the consensus process, all parties have an opportunity to review and comment on the documents. The CLSI Area Committee on Microbiology consists of the Antimicrobial Susceptibility Testing (AST) Subcommittee (human pathogens) and the VAST Subcommittee (veterinary pathogens).

What is the difference between an MIC (Minimum Inhibitory Concentration), an MBC (Minimum Bactericidal Concentration) and a breakpoint?

**MIC (from VET01-A4)** – “the lowest concentration of an antimicrobial agent that prevents visible growth of a microorganism in an agar or broth dilution susceptibility test.”
An MBC is the lowest dilution where the culture has been completely sterilized. It is not routinely determined. Treatment decisions are made related to MICs, and more specifically, the breakpoint MICs.

**Interpretive criteria/breakpoint (from Vet01-A4)** – “Minimal inhibitory concentration (MIC) or zone diameter value used to indicate susceptible, intermediate, and resistant;

**Susceptible** – a category that implies that an infection due to the strain may be appropriately treated with the dosage regimen of an antimicrobial agent recommended for that type of infection and infection species, unless otherwise indicated;

**Intermediate** – a category that implies that an infection due to the isolate may be appropriately treated in body sites where the drugs are physiologically concentrated or when a high dosage of drug can be used; also indicates a “buffer zone” that should prevent small, uncontrolled, technical factors from causing major discrepancies in interpretations;

**Resistant** – resistant strains are not inhibited by the usually achievable concentration of the agent with normal dosage schedules and/or fall in the range where specific resistance mechanisms are likely (e.g., β-lactamase), and clinical outcome has not been predictable in effectiveness studies.

**Nonsusceptible** – a category used for isolates for which only a susceptible interpretive criterion has been designated because of the absence or rare occurrence of resistant strains. Isolates that have minimal inhibitory concentrations (MICs) above or zone diameters below the value indicated for the susceptible breakpoint should be reported as nonsusceptible; **Note 1:** An isolate that is interpreted as nonsusceptible does not necessarily mean that the organism has a resistance mechanism. It is possible that isolates with MICs above the susceptible breakpoint that lack resistance mechanisms may be encountered within the wildtype distribution subsequent to the time the susceptible-only breakpoint is set; **Note 2:** For strains yielding results in the “nonsusceptible” category, organism identification and antimicrobial susceptibility test results should be confirmed.”

**How is antimicrobial susceptibility testing conducted (properly)?**

**Methods for bacterial susceptibility testing - Microwell dilution method:**

This system uses a plate with wells that contain different concentrations of the selected antimicrobials (or a series of tubes). Ideally we would have a well for each antimicrobial at 1:2 dilution intervals to accurately evaluate the minimal inhibitory concentration (MIC) of the compound for each pathogen. However, practical consideration of cost often mandates focusing on the susceptible and intermediate breakpoint dilutions.
Microwell dilution testing example: The figure below illustrates serial 1:2 dilutions of an antimicrobial being tested against a bacterial isolate. The isolate was first cultured by streaking a swab from the tissue sample on an agar plate. Then, 3-5 colonies of the isolate were collected with a loop and inoculated in a broth culture. The next day, the culture must be within a standardized turbidity range prior to inoculating a standard volume into each well of the plate (or in each tube).

**It is important to realize that the amount of bacteria per well is the same across all drug concentrations. Only the drug concentration changes.**

The numbers below indicate the concentration of antimicrobial in each tube (µg/ml). The greater the number, the higher the concentration of drug in the well or tube. As the MIC and MBC values (defined below) move to the right, this means that a greater concentration of the drug is necessary to inhibit (MIC) or sterilize the culture (MBC). The higher the concentration required for the MIC, the less susceptible the isolate is to the antimicrobial being tested (it takes more drug to inhibit growth).

**The MBC is shown for conceptual purposes only. Only the MIC is used in susceptibility testing.**

As growth moves to the right it means that a higher concentration of the antimicrobial is necessary to inhibit growth of the organism. Once it gets to a certain point (the resistant breakpoint) we predict that the drug will not be able to inhibit the pathogen in the animal.

In the above example, the dilution of 2 µg/ml is the lowest concentration that inhibited visible growth for the 24 hour testing period. Therefore, it is reported as the MIC for this organism. However, the culture is not sterilized at the MIC. In this example, the lowest concentration that sterilized the culture was 8.0. This is called the minimum bactericidal concentration (MBC).

Cost typically prohibits this full-range testing technique for routine use, although more diagnostic laboratories are using an extended-range microwell plate. In many labs testing
focuses on “breakpoints” (defined above) that are selected based on reported serum/plasma pharmacokinetic properties, pharmacodynamic characteristics of the drug, clinical data, and microbiological data for antimicrobials in the species of interest. For example, the CLSI/VAST approved breakpoints for florfenicol and bovine respiratory disease are 2, 4, and 8 µg/ml (for S, I, and R, respectively). Breakpoint testing would only test against the 2 and 4 µg/ml concentrations and are interpreted in this manner.

- A pathogen growing in neither of the wells would be considered susceptible because growth is inhibited by a concentration of ≤ 2 µg/ml
- A pathogen growing only in the 2 µg/ml well would be considered as intermediate because it is not inhibited by the susceptible breakpoint concentration of 2 µg/ml, but is inhibited by the intermediate concentration of 4 µg/ml
- A pathogen growing in both wells would be considered resistant because it is not inhibited by the intermediate breakpoint concentration of 4 µg/ml, and the next concentration that would be tested in a serial dilution is 8 µg/ml, which is the resistant breakpoint. Even if the pathogen growth would be inhibited in the 8 µg/ml well, the resistant breakpoint is ≥ 8 µg/ml, so the finding would be resistant. If growth were not inhibited in the 8 µg/ml well, the finding is still resistant because the concentration required to inhibit growth is > 8 µg/ml.

Here is what a Trek Diagnostics AST plate looks like. The tradename for this system is “Sensititre®). A fixed volume of broth is dropped in each well, containing a standardized number of bacteria. The antibiotic is already in the well in a dried condition, and then becomes suspended or solubilized in the broth to create the appropriate concentration (see plate layout below).

Below is the layout of the bovine/porcine plate

**Methods for bacterial susceptibility testing - Kirby-Bauer (“disk diffusion”):**

A paper disk containing the antimicrobial is placed on an agar plate that has been inoculated with the pathogen. The plate is incubated and the zones of inhibition (absence of any visible bacterial growth) are measured surrounding the disks. The diameter of the zone is correlated back to serial-dilution concentrations used to set “susceptible”, “intermediate susceptibility”, and “resistant” classifications for pathogens. This technique is obviously heavily dependent on quality control. Depth and contents of the agar, inoculum concentration, growth conditions, and antimicrobial contents of the disks must be closely controlled.
The size of the zone of inhibition is dependent on the MIC of the organism, the rate of diffusion of the antimicrobial in the agar (related to depth and composition), duration of incubation, inoculum concentration, and temperature of incubation. To assure that a laboratory is producing valid results, they must periodically test QC (quality control) organisms with known zone diameters to confirm they are adhering to standardized methods.

Practitioners commonly receive susceptibility information from both standard dilution tests and disk diffusion tests. How do MICs and zone diameters relate according to VET01-A4?

Equivalent Minimal Inhibitory Concentration Breakpoints (from VET01-A4)

“Disk diffusion zone diameters correlate inversely with MICs from standard dilution tests, usually broth microdilution. VET01-S2 Tables 2A and 2B list the zone diameters and MIC breakpoints used for the interpretive guidelines. Zone diameters and MIC breakpoints are correlated based upon zone-diameter versus MIC regression, population distributions, pharmacokinetics, and clinical efficacy studies (also see CLSI document M23). However, the zone diameters may not correspond precisely to the listed MIC breakpoints due to differences in the methodologies and the original databases. Regression line analysis should not be used to extrapolate MIC values from measurements of zones of inhibition because, in many cases, the relationship, while mathematically correct, cannot be considered comparable to an MIC derived by actual dilution testing for a given isolate (see CLSI document VET02). Thus, the information provided in VET01-S@ Tables 2A and 2B cannot be used to convert zone diameters to absolute MIC values.”

Approved CLSI/VAST interpretive criteria for food animals

Status as a CLSI/VAST approved veterinary breakpoint means that the breakpoint has been evaluated in light of the data mentioned above and designed to give a reasonable projection of clinical outcome. Remember, that an approved breakpoint is specific for the following factors.

- Animal species
- Disease
When any of these factors are changed, the approved breakpoint may no longer be valid for placing the above combination of factors in a population of animal/disease/pathogen/drug where clinical success is likely or unlikely. For example, none of the breakpoints are approved for predicting clinical efficacy of therapy of enteric diseases. It is very important to know when you are, and when you are not using approved interpretive criteria for susceptibility testing results interpretation.

The Clinical and Laboratory Standards Institute (CLSI), formerly the National Committee for Clinical Laboratory Standards (NCCLS), Veterinary Antimicrobial Susceptibility Testing (VAST) Subcommittee has approved the following veterinary specific breakpoints. The CLSI/VAST Subcommittee periodically updates guidance publications for veterinary susceptibility testing. These documents are produced through a consensus process. These breakpoints are detailed in CLSI VET01-S3, including the specific pathogens associated with these breakpoints. The most recent format lists the interpretive criteria by pathogen rather than by drug. This makes it much easier for a diagnostician or clinician to look up the interpretive breakpoints for the pathogen in question.

To access a publicly available Vet01-S3 go to http://vet01s.edaptivedocs.info/Login.aspx  Click on “Click here to use guest access” in the upper right hand corner. Then click on the document title on the next page “CLSI VET01S ED3:2015” You can navigate pages by using the drag bar at the top of the page. The contents are listed on page 9, and we will focus on tables 2A through 2J. Use the drag bar at the top to navigate to the page of the appropriate table. In the version accessed for this document, the tables are two pages later than listed in the table of contents.

**β-Lactams**
- Ceftiofur
  - Bovine: *Mannheimia haemolytica, Pasteurella multocida, Histophilus somni* respiratory disease
  - *E. coli, Streptococcus agalactiae, Streptococcus dysgalactiae, Streptococcus uberis* mastitis
  - Swine: *Streptococcus suis, Salmonella Cholerasuis, Pasteurella multocida, Actinobacillus pleuropneumoniae* respiratory disease
- Penicillin/Novobiocin
  - Bovine: *Staphylococcus aureus, Streptococcus agalactiae, Streptococcus dysgalactiae, Streptococcus uberis* mastitis

**Macrolides**
- Gamithromycin
  - Bovine: *Mannheimia haemolytica, Pasteurella multocida, Histophilus somni* respiratory disease
Tilmicosin
- Bovine: *Mannheimia haemolytica* respiratory disease
- Swine: *Pasteurella multocida, Actinobacillus pleuropneumoniae, Pasteurella multocida* respiratory disease

Tildipirosin
- Bovine: *Mannheimia haemolytica, Pasteurella multocida, Histophilus somni* respiratory disease
- Swine: *Bordetella bronchiseptica, Pasteurella multocida, Actinobacillus pleuropneumoniae* respiratory disease

Tulathromycin
- Bovine: *Mannheimia haemolytica, Pasteurella multocida, Histophilus somni* respiratory disease
- Swine: *Bordetella bronchiseptica, Pasteurella multocida, Actinobacillus pleuropneumoniae* respiratory disease

Phenicols
- Florfenicol
  - Bovine: *Mannheimia haemolytica, Pasteurella multocida, Histophilus somni* respiratory disease
  - Swine: *Salmonella Cholerasuis, Streptococcus suis, Bordetella bronchiseptica, Pasteurella multocida, Actinobacillus pleuropneumoniae* respiratory disease

Lincosamides
- Pirlimycin
  - Bovine: *Staphylococcus aureus, Streptococcus agalactiae, Streptococcus dysgalactiae, Streptococcus uberis* mastitis
- Clindamycin (often used as class representative)
  - Canine: *Staphylococcus spp., Streptococci*: β-hemolytic group, skin, soft tissue

Pleuromutilins
- Tiamulin
  - Swine: *Actinobacillus pleuropneumoniae* respiratory disease

Fluoroquinolones
- Danofloxacin
  - Bovine: *Mannheimia haemolytica, Pasteurella multocida* respiratory disease
- Enrofloxacin
  - Bovine: *Mannheimia haemolytica, Pasteurella multocida, Histophilus somni* respiratory disease
  - Feline: *E. coli, Streptococcus canis* skin, respiratory

The following breakpoints are included in CLSI VET01-A3 as “generic” breakpoints, where the breakpoint was determined on the basis of published pharmacokinetic parameters in the designated species, pharmacodynamic targets for the drug class, and available target pathogen
susceptibility data. You can tell these apart in the tables because they will have a regimen listed in the comments box. For sponsor supported interpretive criteria, no regimen is necessary since the regimen related to the interpretive criteria is the label regimen.

- **Ampicillin**
  a. **Swine** *Streptococcus suis, Bordetella bronchiseptica, Pasteurella multocida, Actinobacillus pleuropneumoniae* respiratory disease

- **Penicillin G**
  a. **Cattle** *Mannheimia haemolytica, Pasteurella multocida, Histophilus somni* respiratory disease
  b. **Swine** *Streptococcus suis, Pasteurella multocida* respiratory disease
  c. **Swine** *Streptococcus suis, Pasteurella multocida, Actinobacillus pleuropneumoniae* respiratory disease (class representative, injectable only)
  d. **Cattle** *Mannheimia haemolytica, Pasteurella multocida* Respiratory disease (class representative, injectable only)

For some antimicrobials used in veterinary medicine, the CLSI/VAST Subcommittee has found it necessary to use human-derived breakpoints since no sponsor has brought the information to the subcommittee to develop approved breakpoints. The VAST Subcommittee is working on developing “generic” breakpoints for veterinary labels without approved breakpoints and for extralabel uses. The following antimicrobials have human-derived breakpoint criteria adapted by the VAST Subcommittee.

- **Aminoglycosides**
  - amikacin, gentamicin, kanamycin

- **β-lactams**
  - amoxicillin-clavulanic acid, ticarcillin-clavulanic acid
  - ampicillin, oxacillin, penicillin, ticarcillin, imipenem, cefazolin

- **Others**
  - erythromycin, chloramphenicol, trimethoprim-sulfamethoxazole
  - rifampin, sulfisoxazole, tetracyclines, vancomycin

For these antimicrobials, and for extralabel use of antimicrobials with approved veterinary breakpoints, it is necessary to evaluate the susceptibility testing results in light of the MIC breakpoint used and the pharmacokinetics/pharmacodynamics of the animal and pathogen being treated.

**Interpreting susceptibility testing for extralabel applications (for sponsored interpretive criteria related to label regimens) and for human breakpoints.**

When the disk diffusion method is used for extralabel applications, not only are the dilution MICs suspect as to clinical application, but there is also the question of if the zone diameter criteria still correlate to the MICs. The take-home message is to know what susceptibility testing situations have veterinary approved breakpoints. For unapproved breakpoints, “susceptible” is
probably better than “resistant”, as this may place the “S” pathogen in a defined population of zone diameters or MICs, but the “S” result does not necessarily mean that there is an increased chance for clinical success. What about susceptibility testing where CLSI veterinary-specific interpretive criteria are not available? A distinction between veterinary-specific interpretive criteria and criteria adapted from human medicine is made in VET01-A4.

**“Minimal Inhibitory Concentration and Zone-Size Interpretive Criteria**

VET01-S2 Table 2B lists the interpretive criteria for which there are no animal species-specific breakpoints, but for which criteria for infections in humans are available. For those agents for which veterinary-specific interpretive criteria are not available, use caution when using these values in relation to veterinary bacterial isolates for three reasons. First, the value listed in the gray shaded areas listed in VET01-S2 Table 2B were developed in human medicine by comparing zone diameters to MICs in broth or agar dilution tests and from population distributions of zones and/or MICs of known susceptible and resistant strains. Second, the MICs and correlated zone-size distributions were analyzed in relation to the clinical pharmacokinetics of the drug from normal dose-ranges and regimens in humans. Third, the in vitro and pharmacologic data have been analyzed in relation to studies of clinical outcome of treatment of specific human pathogens.

Additionally, caution should be exercised in using the interpretive criteria listed in VET01-S2 Table 2A. These criteria apply to particular uses of the antimicrobial drugs in specific animal species. Extension of these data to other disease indications or other animal species may lead to an incorrect prediction of clinical outcome. Antimicrobial concentrations differ across regions of the body depending on the specific drug, route of administration, drug formulation, and the animal's metabolism. These differences can profoundly affect clinical performance of the drug. Therefore, the subcommittee has listed only approved animal species and pathogens in VET01-S2 Table 2A to define those conditions where interpretive criteria are known to be applicable.”

**How do susceptibility testing results apply to clinical outcomes in the field?**

A susceptibility testing result does not guarantee the treatment outcome of the specific animal from which the isolate was collected. The result indicates that the animal is in a population of an animal-drug regimen-pathogen relationship with a characteristic relationship between the probability of the different possible clinical outcomes. There may be failures with “S” isolates and there may be successes with “R” isolates. When these susceptibility testing criteria are applied to situations where clinical and/or pharmacokinetic data have not been correlated to clinical outcome, then this relationship of “S”, “I”, and “R” to clinical outcome may or may not exist.