Antimicrobial susceptibility testing - Beyond S/I/R

Amanda J. Kreuder, DVM, PhD, DACVIM (LAIM)
Iowa State University, College of Veterinary Medicine, Veterinary Microbiology and Preventive Medicine, Ames, IA 50011; akreuder@iastate.edu

Abstract

Antimicrobial susceptibility testing and interpretation is a critical component of antimicrobial stewardship in bovine practice, yet it remains 1 of the most poorly understood diagnostic tests in veterinary medicine. Improved practitioner understanding of the process of antimicrobial susceptibility testing, clinical breakpoint determination, and the limitations of this critical diagnostic tool are important in ensuring appropriate results interpretation and improving clinical decision making in the context of antimicrobial stewardship.

Key words: antimicrobial susceptibility testing, bovine, antimicrobial stewardship

Introduction

Bovine practitioners are not immune from the rise in antimicrobial resistance that currently threatens both human and animal health, and antimicrobial stewardship is critical to preserving the efficacy of antimicrobials for treatment of bacterial diseases in both humans and animals. One of the key tenets of antimicrobial stewardship is the use of culture and antimicrobial susceptibility testing (AST) to help inform clinical decision making regarding antimicrobial use. Just like any other diagnostic test, however, it is critical that the bovine practitioner understand the appropriate application and limitations associated with the use of AST to be able to utilize the information gained to improve antimicrobial decision-making in clinical practice and to "think beyond S/I/R".

Rethinking the Definition of "S" and "R"

As busy practitioners, we tend to see an "R" on the diagnostic lab report and automatically assume that the bacteria in question is resistant to the antibiotic, and move on to the next antibiotic on the list that says "S" without giving it more thought. To understand what "S" and "R" might really mean, however, we have to understand how the lab arrived at that interpretation. Veterinary diagnostic laboratories in the United States utilize clinical breakpoints that have been established by the Clinical Laboratory Standards Institute (CLSI) Subcommittee on Veterinary Antimicrobial Susceptibility Testing (VAST). A true clinical breakpoint is specific to the bacterial species of interest, location in the body of the infection, the animal species being treated, and the drug dosage regimen being utilized[3,6]. From a clinician's perspective, clinical breakpoints are important because they consider the clinical picture including the dosage, route, and frequency of administration of drug utilized to treat the patient. In regards to clinical breakpoints, "susceptibility" refers to the clinical condition whereby the infection is expected to be susceptible to the antimicrobial administered at a specific dosage and route, while "resistance" indicates that at the dose and route given, the infection is not expected to respond as favorably as it is less susceptible or non-susceptible to the antimicrobial treatment. As clinicians, this is the reason we perform AST, to be able to more appropriately select an antimicrobial for treatment of a disease either in an individual animal or in a group of animals.

Clinical breakpoints can be derived via multiple approaches including microbiological characteristics, pharmacokinetic and pharmacodynamics (PK/PD) parameters, and/or clinical outcome data[5]. It is the application of the clinical breakpoints to the AST result that allows reporting of results as susceptible, intermediate or resistant (S/I/R) to aid in clinical decision-making. There are 2 primary methodologies currently employed to perform AST: disc diffusion and microbroth dilution. Disc diffusion (often referred to as the Kirby-Bauer [KB] test), is a dynamic test involving the diffusion of antibiotics from disc on a plate and yields a qualitative result in the form of a zone diameter. If veterinary clinics choose to perform their own AST, it is most frequently via this method; however, there are several drawbacks to this approach. There are fewer CLSI-approved breakpoints for this approach, and the qualitative result cannot be used for dosage calculations or monitoring for increased resistance development. The most commonly employed method of AST in both human and veterinary diagnostic laboratories is broth microdilution, which is performed using standardized testing panels provided in 96-well plates which contain varying dilutions of antibiotic concentrations. Broth microdilution testing allows for generation of a quantitative result in the form of an MIC (minimum inhibitory concentration) which represents the minimum concentration of an antimicrobial agent that prevents visible growth of a microorganism (typically 90% of the organisms, or MIC90). The MIC or zone diameter datapoint generated from AST are then interpreted using the clinical breakpoints established by CLSI to provide the clinician with the S/I/R designation[3].

In an ideal world, true clinical breakpoints would exist for all pathogens of cattle isolated from all locations in the body for all possible antimicrobial agents; however, due to a number of limiting factors, this is not the case. Table 1 demonstrates the currently available clinical breakpoints established by CLSI VAST that are used by veterinary diagnostic

Table 1. List of CLSI-established bovine-specific clinical breakpoints for use of parenteral antibiotics in cattle along with the dosage regimen used for breakpoint determination.*

		Bacterial species				
Antibiotics routinely tested via AST for bovine isolates	**Dosage regimen used for breakpoint determination****	***M. haemolytica***	***P. multocida***	***H. somni***	***E. coli***	**All other**
Ampicillin	5 mg/lb (11 mg/kg) IM q 24 hrs	Yes	Yes	Yes	Yes	
Ceftiofur	1 mg/lb (2.2 mg/kg) IM (sodium or hydrochloride) 3 mg/lb (6.6 mg/kg) SQ ear base (CCFA)	Yes	Yes	Yes		
Clindamycin (test for lincomycin)						
Danofloxacin	2.7 mg/lb (6 mg/kg) SQ twice 48 hrs apart	Yes	Yes			
Enrofloxacin	3.4 mg/lb (7.5 mg/kg) SQ once	Yes	Yes	Yes		
Florfenicol	9.1 mg/lb (20 mg/kg) IM twice 48 hrs apart [also applies to 18.2 mg/lb (40 mg/kg) SQ once]	Yes	Yes	Yes		
Gamithromycin	2.7 mg/lb (6 mg/kg) SQ once	Yes	Yes	Yes		
Gentamicin						
Neomycin						
Penicillin	10,000 IU/lb (22,000 IU/kg) IM q 24 hrs	Yes	Yes	Yes		
Spectinomycin	4.5 mg/lb (10 mg/kg) SQ q 24 hrs	Yes	Yes	Yes		
Sulfadimethoxine						
Tetracycline (test for oxytetracycline)	9.1 mg/lb (20 mg/kg) IM once for oxytetracycline (may be cautiously applied to SQ dosing)	Yes	Yes	Yes		
Tiamulin						
Tildipirosin	1.8 mg/lb (4 mg/kg) SQ once	Yes	Yes	Yes		
Tilmicosin	4.5 mg/lb (10mg/kg) SQ once	Yes				
Trimethoprim / sulfamethoxazole						
Tulathromycin	1.1 mg/lb (2.5 mg/kg) SQ once	Yes	Yes	Yes		
Tylosin tartrate						

* information summarized from CLSI documents VET08[3] and VET09[5]
** SQ =subcutaneous; IM = intramuscular; q = every

laboratories for interpretation of non-mastitis cattle specimens[3]. As demonstrated in Table 1, all but 1 of the clinical breakpoints available for parenteral antibiotics in cattle are specific only to bovine respiratory disease pathogens such as *M. haemolytica, P. multocida*, and *H. somni*. Why is this important? Because clinical breakpoints have been established for many antibiotics for treatment of bovine respiratory disease pathogens, there is a higher level of confidence in performing AST on these organisms for this condition[5]. In contrast, however, for other bacteria isolated from other disease processes, the confidence in the interpretation of the results of the AST decreases as the clinical breakpoint utilized to determine susceptible vs resistant must be extrapolated from other locations, animal species, and bacterial species. While this information can still be valuable to the practitioner, it is important to recognize this limitation when interpreting these results. As clinical breakpoints are reliant on drug dosing and pharmacokinetic parameters, a breakpoint established in humans or dogs may not necessarily correlate well to use of the same drug at a different dosage or route in cattle. In some cases, no reasonable breakpoint exists in other species from which to extrapolate, and the results may be reported as "no interpretation." This is not meant to frustrate the practitioner, although it frequently does, but is meant to highlight the fact that not enough information is available to make a reasonable recommendation in that case.

Table 2 demonstrates the most common bovine specimens that received culture followed by AST testing at the Iowa State University Veterinary Diagnostic Laboratory (ISU VDL) from 2003-2018. While the majority of specimens were unfortunately classified as "assorted" due to collection and submission of multiple tissue types at necropsy, the most common specified specimen location was respiratory tract followed by gastrointestinal, with all other reported locations making up less than 8% of the total isolates (this includes specified locations such as eye, milk, urinary, CNS, etc). As demonstrated in Table 2, the 5 most common isolates overall representing 86% of the dataset are (from most common to least): *Escherichia coli, Mannheimia haemolytica, Salmonella enterica, Pasteurella multocida*, and *Histophilus somni*. Of these isolates, only *M. haemolytica, P. multocida* and *H. somni* have clinical breakpoints established in cattle, while the first

Table 2. Most common bovine specimens that received culture followed by AST testing from the Iowa State University Veterinary Diagnostic Laboratory (ISU VDL) 2003-2018.

Location from which culture was taken	Number of ASTs performed	Most common isolates (number in parenthesis)
Assorted (i.e., multiple tissues taken at necropsy)	7163	--
Respiratory tract	4588	*M. haemolytica* (1729) *P. multocida* (1477) *H. somni* (943) *Salmonella* sp (309) *B. trehalosi* (74)
Gastrointestinal tract/ fecal	2621	*E. coli* (2090) *Salmonella* sp (478)
All other (eye, CNS, joint, etc.)	1205	--
TOTAL	15577	*E. coli* (5259) *M. haemolytica* (2847) *Salmonella* sp (2447) *P. multocida* (2500) *H. somni* (1453) All others (2101)

and third most common isolates on which AST was performed do not have established clinical breakpoints (the single exception being *E. coli* and ampicillin, which will be further discussed below). This makes interpretation of AST results challenging for both the diagnostic laboratory as well as the practitioner, as all interpretations for these isolates must be extrapolated from data in other species. As both *E. coli* and *Salmonella* are most frequently isolated from the gastrointestinal tract, this also presents several additional issues.

As introduced above, a true clinical breakpoint is specific not only to the bacterial species of interest and animal species being treated, but it is also specific to the location in the body of the infection. This is because the ability of an antibiotic to access the site of infection is critical in determining its clinical effectiveness at that location. There are unfortunately no clinical breakpoints that have been established for cattle for enteric infections. So, while bovine practitioners frequently request AST for enteric isolates, there are no established breakpoints to assist the diagnostic lab and practitioner in selecting appropriate antimicrobial therapy for treatment of infections in the gastrointestinal tract. In fact, no clinical breakpoints have been established in ANY veterinary species for ANY gastrointestinal Enterobacteriaceae infections.[1,15] Therefore, the use of AST to determine appropriate antimicrobial treatment of enteric infections with pathogens such as *E. coli* and *Salmonella* is not recommended, as the concentrations of antimicrobials achievable and needed for disease resolution in the gastrointestinal tract are generally considered to be unknown. This poses significant challenges to veterinary clinicians who are regularly faced with patients suffering from bacterial gastroenteritis and choose to perform AST, yet have little appropriate bovine-specific guidance with which to direct therapy.

Because clinical breakpoints take into consideration patient and drug factors, it is critical to understand that just because an organism tests "resistant" to a particular antibiotic does not necessarily mean that it actually has acquired resistance (i.e., not inherently present or intrinsic) to that antibiotic. Up to this point, we have exclusively looked at resistance from the clinical standpoint and how likely the infection is to respond to the proposed treatment, which is heavily dependent on drug dosing and pharmacokinetic factors. However, when we look at resistance from a bacteriologic standpoint, the terms "susceptible" and "resistant" are instead used to differentiate between 2 populations of bacteria: 1 population that does not typically harbor acquired resistance to an antimicrobial and is thus "susceptible" (i.e., wild type population), and another that does typically harbor acquired resistance mechanisms and is thus considered "resistant" to the antimicrobial (i.e., non-wild type population). The bacteriologic difference between wild type (i.e., do not typically harbor resistance genes) and non-wild type (i.e., typically harbor acquired resistance genes) populations of bacteria is termed an epidemiologic cut-off value (ECOFF or ECV).[6] While clinical breakpoints are used to assist in clinical decision-making, the ECV is recommended to be used to measure resistance development in bacterial species over time, as well as monitor the success of interventions in preventing resistance development. This is because the ECV is determined exclusively from the distribution of observed MIC values in a population of bacteria and is independent of host species or pharmacologic information.

On the surface, it seems that the clinical breakpoint and ECV should be the same value, and in many cases they are. However, the newly adopted (2018) CLSI clinical breakpoint for susceptibility of *E. coli* to ampicillin in cattle provides an excellent example of where this is not the case. The current *E. coli* ampicillin bovine breakpoint is <0.25 µg/mL,[3] while the current human *E. coli* ampicillin breakpoint is <8 µg/mL[4]. The bacteria does not differ between humans and cattle, so why is there such a large difference in clinical breakpoints? In cattle, this breakpoint was derived from PK/PD data and the labeled dose of ampicillin trihydrate given IM once daily; in humans, the breakpoint is for ampicillin sodium given IV 4 times daily. From a bacteriologic standpoint, the human breakpoint also matches the ECV value for *E. coli*, while using the cattle breakpoint means that almost all wild-type bacteria with no acquired resistance will still be classified as "R". If these results were only reported and compared using the "S/I/R" designation, it would appear that there was extensive resistance in *E. coli* in cattle when compared to humans, which is incorrect. This risks the false interpretation that antimicrobial use in livestock species leads to higher rates of antimicrobial resistance in bacteria from animals, which

then may be interpreted as the cause for resistance seen in human infections. Therefore, it is critical that clinical breakpoint interpretation data alone not be utilized to assess for acquired resistance development in bacteria across species. In this context, clinical resistance is better thought of as non-susceptibility to the antibiotic due to the drug formulation, dosage, and frequency, and not always as true bacterial resistance to the antibiotic. In this example, from a clinical standpoint, the use of increased frequency, higher dosages, and different formulations of ampicillin in cattle are likely to move the clinical breakpoint closer the ECV, however, additional research is necessary to support the extent to which this occurs. Thus, it is critically important to know what drug dosage was used to determine a clinical breakpoint when evaluating its utility for interpretation of treatment options for a particular condition.

A Practical Approach to Utilizing AST Results in Bovine Practice

With the knowledge provided above, we can now discuss a practical approach to utilizing AST results in clinical practice. First, it is critical that AST be focused on only potential pathogens. When a bacterial organism is isolated from a diseased animal, we first must ask if it is reasonable to expect that this bacteria is playing a role in the disease process prior to asking what antimicrobial drugs could be used to treat the infection. For many body sites commonly cultured by bovine practitioners (including the upper respiratory tract and gastrointestinal tract), normal flora exist and are routinely able to be cultured. The ability to culture bacteria from these sites, however, does not ensure that they are playing a role in the disease process. In addition, contamination is common when culturing clinical samples and must always be taken into consideration when mixed growth is present instead of pure growth of a bacterial organism. Thus, the bacteriology lab and/or practitioner must frequently determine which of the isolated bacteria are most likely to play a role in the disease process to determine which isolates warrant AST. Requesting or performing AST on isolates that are likely to be normal flora or contaminants has several issues, 1 of which is a lack of clinical breakpoints for these organisms, as discussed above. This, combined with the common existence of both intrinsic and acquired resistance in many of these organisms, may falsely lead the clinician to interpret an infection to be more difficult to treat than necessary, and hinder rather than assist antimicrobial stewardship efforts. When presented with AST results of several organisms isolated from the same location, focusing on the results from the organism(s) most likely to be able to cause clinical disease is likely more beneficial than attempting to find an antibiotic that is listed as "susceptible" for all organisms present. It is also important to keep in mind that MIC values should not be compared across antibiotics (i.e., selection of the lowest numerical value of all of the susceptible results), as the MIC is drug-specific due to the pharmacokinetics of that antibiotic and not an indicator of success across drug classes.

As presented in Table 1, the disease condition for which bovine practitioners can have the most confidence in AST testing interpretation is bovine respiratory disease (BRD) caused by either *M. haemolytica, P. multocida* or *H. somni.* Antimicrobial resistance in these pathogens is of serious concern, as available evidence does suggest that resistance to several antibiotic classes has increased over the past several decades.[14-16] However, it is important to keep in mind that most of the published literature focuses on results from diagnostic laboratory submissions obtained from dead cattle that have been treated multiple times with multiple different antimicrobials.[11,14] It is unclear at this time the significance of isolation of highly resistant pathogens from these chronic cases. Were these resistant bacteria present in high numbers at the start of the infection, and thus responsible for the treatment failure? Or, do they simply represent the only bacteria that are able to be cultured after multiple rounds of antimicrobial therapy failed, due to other factors such as decreased immune function? The universally accepted gold standard for performing culture and AST is to utilize it on untreated, newly diagnosed animals, yet this is not how AST in BRD is typically approached. To truly understand the impact of antimicrobial resistance on treatment outcome, we as a profession likely need to move closer to this ideal through more targeted collection of nasal swabs, deep nasopharyngeal swabs, transtracheal washes or bronchoalveolar lavage from clinical animals prior to treatment.[7,8] While minimal published work evaluating the impact of antimicrobial resistance on clinical outcome in cattle with BRD is available, what has been published does suggest that the presence of resistance at the time of initial treatment affects outcome.[1,12] One additional caveat in regard to the application of clinical breakpoints for BRD pathogens is that all of the current clinical breakpoints were developed using dosages of the antibiotics administered parenterally; these breakpoints should not be extrapolated to in-feed antibiotic administration, and there are currently no clinical breakpoints available in cattle for in-feed administration of antimicrobials[5].

From a practical standpoint, AST should only be performed on clinical specimens and bacterial isolates when it has a high likelihood of providing useful results to instruct either antimicrobial use, or for monitoring trends in populations of pathogenic bacteria. As discussed above, AST testing on enteric isolates presents significant challenges for interpretation due to a lack of clinical breakpoints for enteric infections. When CLSI bovine-specific breakpoints are not available, there is no standard for which breakpoints should be used for a given organism and antibiotic combination, therefore, isolates sent to different labs can receive different interpretations for the same MIC. Beyond interpretation challenges, *E. coli* also represents normal flora of the bovine fecal environment. When we perform AST, the results are generated from a single colony with the assumption that clinical

disease is caused exclusively by this single isolate. As *E. coli* is normal flora, many different biotypes naturally exist in bovine feces, and often the normal non-pathogenic, non-hemolytic *E. coli* are observed to harbor significantly more antimicrobial resistance genes than those that are considered to be enteropathogenic. Because of this, unless enteropathogenic isolates are present, performing AST on enteric *E. coli* isolates is more likely to confuse antimicrobial stewardship efforts rather than assist in appropriate clinical decision-making. Routine treatment of enteric disease should remain focused on appropriate supportive care in the form of fluid therapy, with the addition of antimicrobial therapy as needed for the treatment of bacterial sepsis. There are currently no data to support the use of AST from a single *E. coli* isolate from the feces to inform the clinician as to the most likely cause and best treatment of bacteremia. Isolation of *E. coli* from other body locations, however, does indicate either sepsis or localized infection and can warrant AST testing in those circumstances with the understanding that interpretations will primarily be extrapolated from clinical breakpoints in other species, and the confidence in the results will thus be lower.

For other clinical sites that lack established clinical breakpoints in cattle, valuable information can still be gained from performing AST in many circumstances as long as the limitations of interpretation are considered and actual MIC results are reported by the laboratory. Isolation of a pure culture of bacteria from a "sterile" site such as a joint, the urinary tract, or CNS provides valuable information as to the cause of disease, and use of extrapolated breakpoints in this context, combined with a knowledge of intrinsic resistance and the ECV of the bacteria in question and the pharmacokinetics of the antibiotic(s) available for use, can greatly assist the practitioner in antibiotic selection. The MIC, in combination with knowledge of the pharmacokinetic and pharmacodynamics parameters of the drug in cattle, can be utilized in some cases to customize antibiotic therapy for antimicrobials where extra-label dosing is allowable. However, extra-label limitations on the use of aminoglycosides, fluoroquinolones, and 3rd generation cephalosporins in bovine medicine, along with the requirement for extended withdrawal times with extra-label use, make this approach more challenging than in companion animal species with less restrictions. In these cases, clinical microbiologists at veterinary diagnostic laboratories can often provide the clinician with additional information regarding the extrapolated breakpoints utilized for the provided interpretations to assist in clinical decision making as well.

There are several references available to practitioners to assist in clinical decision making related to AST interpretation. CLSI provides free access to the VET08 "Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animals" document[3] which details all of the clinical breakpoints that have been established in veterinary species; the M100[4] which details human clinical breakpoints is also freely available via the same site: (https://clsi.org/standards/products/free-resources/access-our-free-resources/). For those who wish to further their understanding on the topic, the newly developed CLSI VET09 document "Understanding Susceptibility Test Data as a Component of Antimicrobial Stewardship in Veterinary Settings"[5] provides an excellent resource that includes extensive information about clinical breakpoint development and appropriate interpretations for all veterinary species.

Future Directions

While current veterinary AST procedures primarily rely on the phenotypic testing described above (disc diffusion or broth microdilution), other technologies for both phenotypic and genotypic assessment of antimicrobial resistance do exist and are becoming more commonplace, particularly in human microbiology laboratories. These range from specific phenotypic tests for certain mutations such as the PBP2A latex agglutination test for methicillin resistance in *Staphylococcus* spp,[2] to genotypic tests such as PCR panels or whole genome sequencing to identify the presence of specific resistance genes.[9] While currently only utilized in the research setting, there is significant potential for development of genotypic testing for common resistance genes in bovine pathogens such as *M. haemolytica* to allow for rapid identification of resistance gene acquisition to assist in the direction of therapy.[10,13] However, the continued use of standard phenotypic AST will likely remain critical to clinical medicine, as the presence of resistance genes does not always correlate with phenotypic resistance due to various reasons such as a lack of a functional promoter or additional mutations which have rendered the gene non-functional.

Conclusions

Antimicrobial susceptibility testing is a valuable tool for antimicrobial stewardship, but only when used with the understanding of its benefits and limitations in regards to bovine medicine. Improved understanding of key concepts of AST should assist the practitioner in making improved decisions regarding antimicrobial therapy in bovine patients.

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