Use of Susceptibility Testing in Veterinary Medicine

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Abstract

There has been increased interest in optimizing treatment protocols for antimicrobial agents, with substantial reliance on susceptibility testing of bacterial pathogens isolated from diseased cattle. Antimicrobial susceptibility testing of bovine bacterial pathogens has traditionally used the agar diffusion (Kirby-Bauer) method, which was designed to reflect the antibiotic concentration in serum and interstitial fluid of human patients. The validity of agar diffusion susceptibility breakpoints derived from humans to the treatment of mastitis, diarrhea and respiratory disease in cattle has not been established. The use of susceptibility testing to guide treatment decisions for individual cattle is not recommended until the breakpoints have been validated as being predictive of treatment outcome.

Introduction

A number of methods have been used to determine the susceptibility of bovine pathogens to antimicrobial agents: broth dilution, milk dilution (for mastitis pathogens), agar dilution, determination of mean bactericidal concentration, determination of killing kinetics and the agar diffusion method (Kirby-Bauer test). The first five methods are quantitative, whereas the agar diffusion method is qualitative.[15] Because of issues related to cost and complexity, the broth microdilution method is the recommended gold standard method for *in vitro* susceptibility testing, whereas agar diffusion provides a crude, inexpensive and clinically practical method for determining *in vitro* susceptibility.

Two important concepts (minimum inhibitory concentration [MIC], and breakpoints for MIC) need to be understood when interpreting the results of susceptibility testing. The minimum inhibitory concentration is the lowest antibiotic concentration (expressed in μg/ml) that, under defined *in vitro* conditions, prevents the growth of bacteria within a defined period of time.[5] It is generally accepted that MIC values are very repeatable.[6] Statistics such as MIC_{50} (the median MIC for all isolates) and MIC_{90} (the MIC value which exceeds or equals the MIC for 90% of the isolates) are frequently used to summarize population data. Breakpoints for MIC are specific MIC values used to assign bacteria to one of three categories, susceptible (sensitive), intermediate and resistant, using recommendations from the National Committee for Clinical Laboratory Standards (NCCLS) for testing Veterinary Pathogens.[4] The intermediate term indicates an MIC value that is close to the breakpoint.[5]

The broth dilution method provides a direct measurement of MIC, and determines the ability of the pathogen to grow in the presence of a known antibiotic concentration. The broth dilution test is usually performed as a commercially available microdilution test (Sensititre, Westlake, OH) in a 96 well microtiter plate that permits the testing of 12 antibiotics in a range of eight 2-fold dilutions.[5] The microdilution method starts by using a sterile loop to remove 3-5 representative colonies from a 24-hour bacterial culture plate (use of multiple colonies avoids selection of an atypical variant). The bacterial colonies are then suspended in 5 ml of 0.9% NaCl and the bacterial suspension standardized by adjusting the turbidity to a 0.5 McFarland standard (an index of bacterial concentration approximating 10^7 colony forming units [CFU]/ml). A fixed volume aliquot is then transferred to Mueller-Hinton broth, and an automated inoculation device used to deposit a standardized inoculum into each well of the microtitration tray containing a geometric progression of dehydrated antimicrobial agent concentrations. Growth is recorded by monitoring the turbidity of each well, and the first dilution with non-visible growth considered to be the MIC for that isolate.[41]

The agar diffusion method is also called the Kirby-Bauer method, and the test procedure has changed little since Bauer, Kirby and others standardized the method in 1966.[4,7] Three to 10 representative bacterial colonies are selected from a blood agar plate and suspended in a fixed volume of sterile 0.9% NaCl to achieve the turbidity of a 0.5 McFarlane standard; the suspension is then spread evenly across the surface of an agar plate using a sterile cotton swab. Small circular disks of filter paper or tablets impregnated with antimicrobial agents are placed on the agar plate using flamed forceps or a special applicator and gently pressed down to ensure contact.[7] The agar plate is selected based on the bacterial species being tested and incubated at 37°C overnight. During incubation, antibiotics dissolve from the filter paper or tablets into the surrounding agar and

thereby inhibit bacterial growth. The diameter of the zone of inhibition (Figure 1) is measured in mm and is correlated in some manner with the MIC for the bacteria.[4] Interpretative zone diameters differ for each antibiotic because of differences in MIC and the diffusion and solubility of the antibiotic in agar. Because agar diffusion is qualitative, the method is inferior to the quantitative broth dilution method.

Determination and validation of susceptibility breakpoints

A standardized testing procedure for determining antimicrobial susceptibility has been developed in the United States by the NCCLS. A veterinary subcommittee of NCCLS, called the Veterinary Antimicrobial Susceptibility Testing (VAST) subcommittee, was formed in 1992. The VAST subcommittee published a proposed standard in 1994 and approved standards in 1999 and 2002.[4] The VAST committee recommends an official interpretative MIC breakpoint against specific bacteria at a stated dosage protocol for a specific disease in a species. The MIC breakpoint is determined by considering available in vitro susceptibility data, pharmacokinetic/pharmacodynamic data, and clinical efficacy data; however, this author is unaware of a single refereed publication documenting the relationship between the MIC of bacteria isolated from the site of infection and clinical outcome in individual cattle administered an antimicrobial agent. Accordingly, the recommended MIC breakpoints appear to be based on *in vitro* MIC values, pharmacokinetic/pharmacodynamic data and the results of clinical trials indicating efficacy using a stated dosage protocol. In other words, we are still using the approach described by Hjerpe in his seminal 1976 paper on the treatment of bacterial pneumonia in cattle.[20]

Many problems exist with the currently used susceptibility breakpoints for bacteria isolated from cattle. Accurate antimicrobial susceptibility test breakpoints should be derived using MIC values for 300 to 600 isolates from representative clinical cases from a large geographic area,[4,6] published pharmacokinetic/pharmacodynamic data for cattle, and clinical and bacteriologic cure rates.[6] The results of field studies that measure the rate of clinical cure, using clinically relevant end points such as mortality, weight gain, treatment duration and relapse rate should be reported as a bare minimum. The rate of bacteriologic cure within a specified time interval, using biologically relevant endpoints such as failure to isolate the same pathogen from the affected quarter in cows with mastitis, the feces in calf diarrhea, or a transtracheal wash in pneumonia,

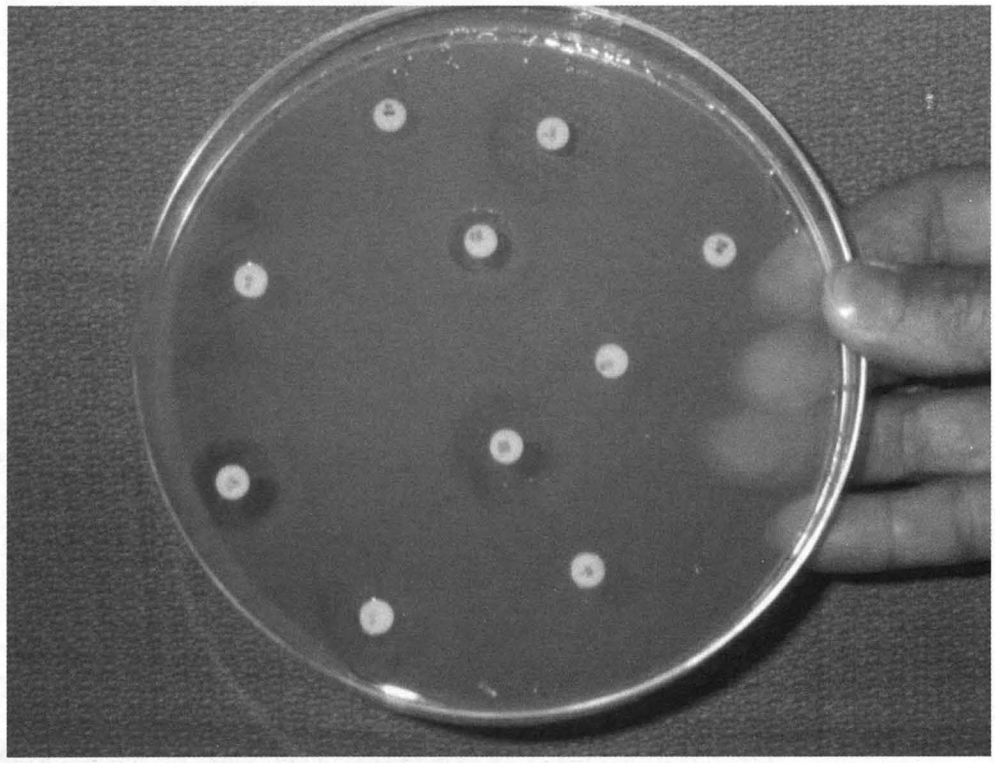

Figure 1. Antimicrobial susceptibility testing using the agar diffusion (Kirby-Bauer) test. The diameter of the zone of inhibition around each antibiotic disk is associated with the MIC value for each antibiotic. Interpretative zone diameters differ for each antibiotic because of differences in the MIC value, diffusion rate and solubility of the antibiotic in agar. Photograph courtesy of Dr. DE Morin.

also provides useful data. Clinical and bacteriologic cure rates may provide a clear breakpoint, or in other situations, this data can be used in conjunction with pharmacokinetic/pharmacodynamic data to suggest the most appropriate breakpoint.[6] Unfortunately, the ideal approach to determine accurate susceptibility breakpoints in cattle is hampered by three main difficulties: 1) limited availability of contemporaneous MIC values from heterogeneous geographic locations, 2) incomplete phamacokinetic/pharmacodynamic data, and 3) complete absence of published field studies validating the suggested susceptibility breakpoints. The effect of disease on the pharmacokinetics of antimicrobial agents has usually been ignored, but differences in the plasma concentration-time profile for oxytetracycline[3,12] and erythromycin[9] exist between healthy and pneumonic calves, and experimental induction of endotoxemia and pyrexia in suckling Holstein calves changes the plasma concentration-time profile for orally or intramuscularly administered amoxycillin trihydrate.[19] Finally, antimicrobial agents may produce beneficial effects separate to their activity against bacteria. A recent example of this is the observation that tilmicosin decreases pulmonary inflammation in bovine pneumonia; this anti-inflammatory effect appears to be mediated, in part, by inducing neutrophil apoptosis[11] and by decreasing macrophage phospholipase A_2 activity and macrophage production of PGE_2.[21]

Specific antibiotics that are considered representative of their class are routinely used in susceptibility testing. For instance, cephapirin is a first generation (narrow spectrum) cephalosporin used to treat mastitis in dairy cows. However, susceptibility testing does not use cephapirin as the test antibiotic, instead, cephalothin is used because it is the recommended representative of first generation compounds.[1] This recommendation ignores the results of an *in vitro* study indicating one dilution difference in MIC_{50} values between cephapirin and cephalothin for *Staphylococcus aureus*, *Proteus mirabilis* and *Citrobacter* spp,[40] and ignores general recommendations that the antibiotic to be used clinically should be tested.[5] Another example is desfuroylceftiofur, the active metabolite of ceftiofur in cattle. Desfuroylceftiofur and ceftiofur have similar MICs for gram negative pathogens, whereas desfuroylceftiofur has 2-3 serial dilutions less antimicrobial activity than ceftiofur against *Staphylococcus aureus*, coagulase negative *Staphylococcus* spp, *Streptococcus uberis* and *Streptococcus dysgalactae*.[31] The VAST subcommittee of the NCCLS recommended that tetracycline be used as the class representative for oxytetracycline, ampicillin be used to test for susceptibility to amoxicillin and hetacillin, oxacillin be used to test for susceptibility to cloxacillin and trimethoprim/sulfamethoxazole be used to test for susceptibility to trimethoprim/sulfadiazine.[4] Susceptibility testing therefore usually does not employ the active antibiotic agents present in commercially available antibiotic treatments for cattle. It is likely that susceptibility test results based on class representatives rather than the active antibiotic agent will lead to erroneous results.

Susceptibility testing in mastitis, calf diarrhea and pneumonia

Important considerations for treating bacterial diseases in cattle are: 1) administering an antibiotic as directed on the label whenever possible, 2) using an antimicrobial agent with an appropriate spectrum of activity, 3) selecting an antimicrobial agent that attains and maintains an effective therapeutic concentration at the site of infection, 4) treating for an appropriate duration, and 5) avoiding adverse local or systemic effects and violative residues. Exisiting limitations in susceptibility testing will be illustrated by examining three economically important and common bacterial diseases of cattle, namely mastitis, calf diarrhea and pneumonia.

Mastitis

Antimicrobial agents are often selected based on availability of labeled drugs, clinical signs in the cow, milk culture results for previous mastitis episodes in the herd, experience of treatment outcome in the herd, treatment cost and withdrawal times for milk and slaughter.[14,15,24] The validity of agar diffusion susceptibility breakpoints derived from humans to the treatment of bovine mastitis has not been established and is extremely questionable because bovine milk pH, electrolyte, fat, protein, and leukocyte concentrations, growth factor composition, and pharmacokinetic profiles are different than those for human plasma, and because human bacterial pathogens are often different from bovine mastitis pathogens. Also, antibiotics are distributed unevenly in an inflamed gland, and high antibiotic concentrations can alter neutrophil morphology or function *in vitro* and thereby inhibit bacterial clearance *in vivo*.[15]

We do not currently have adequate databases of *in vitro* MIC values for clinical mastitis pathogens, although adequate databases are available for subclinical mastitis isolates. Although we have good knowledge regarding the pharmacokinetics of many parenteral antibiotics used to treat clinical mastitis, most pharmacokinetic data has been obtained in healthy cattle and it has not been determined whether pharmacokinetic values in healthy cows are the same as those in cows with clinical mastitis. In addition, pharmacokinetic values for many of the intramammary antibiotics used to treat clinical mastitis are unknown, and we have a lim-

ited understanding of the pharmacodynamics of antibiotics in treating mastitis. More importantly, the breakpoints currently recommended for all parenterally and almost all intramammary administered antibiotics are based on achievable serum and interstitial fluid concentrations in humans after oral or intravenous antibiotic administration. The relevance of these breakpoints to achievable milk concentrations in lactating dairy cows after intramammary, subcutaneous, intramuscular, or intravenous administration is dubious at best. We recently demonstrated that the recommended breakpoints for cephapirin and oxytetracycline were not predictive of treatment outcome in cows with clinical mastitis.[14]

In vitro antimicrobial susceptibility test results have been suspected to be poorly correlated with treatment outcome for clinical mastitis and the value of the agar diffusion method to guide mastitis treatment decisions has been widely questioned.[15,26,30] Susceptibility testing in artificial media (agar or broth) cannot simulate what happens in the mammary gland milk phase of cows with mastitis and therefore cannot predict the outcome of therapy.[26] With the exception of pirlimycin[39] and penicillin-novobiocin,[38] zone diameters in the agar diffusion test have not been related to antibiotic concentrations achieved in the bovine mammary gland with dosage regimens used by veterinarians and dairy producers. It must be emphasized that the preliminary breakpoints for pirlimycin and penicillin-novobiocin combination have not been confirmed because of a lack of appropriate field efficacy data with adequate numbers of susceptible and resistant isolates.[28,41] Moreover, the breakpoints were calculated from phamacokinetic data obtained in healthy mammary glands; it is likely that pharmacokinetic values differ in glands with clinical mastitis.

Results from field studies are available to evaluate the validity of susceptibility breakpoints in guiding treatment of cows with clinical or subclinical mastitis. The results from these field studies suggest that the following antibiotics may have valid (but not necessarily optimal) breakpoints for treating clinical or subclinical mastitis caused by specific bacteria; parenteral penicillin G for subclinical *Staphylococcus aureus* infections, intramammary cephapirin for clinical *Streptococcus* spp infections, and parenteral trimethoprim-sulfadiazine for clinical *Escherichia coli* infections. Of these three antibiotics, the breakpoints for penicillin G and cephapirin have only been validated for bacteriologic cure, whereas the breakpoint for trimethoprim-sulfadiazine is validated for clinical cure.[15] Because duration of infection before treatment, antibiotic dosage, dosage interval and duration of treatment influence treatment outcome, many more field studies must be completed to validate the currently assigned antibiotic break points for pathogens causing clinical mastitis.

Calf diarrhea

The most important determinant of antimicrobial efficacy in treating calf diarrhea is obtaining an effective antimicrobial concentration against bacteria at the two sites of infection (small intestine and blood). The results of fecal antimicrobial susceptibility testing have traditionally been used to guide treatment decisions; however, susceptibility testing in calf diarrhea probably has clinical relevance only when applied to fecal isolates of enterotoxigenic strains of *E. coli* or pathogenic strains of *Salmonella* spp, and blood culture isolates from calves with bacteremia. Validation of susceptibility testing as being predictive of treatment outcome for calves with diarrhea is currently lacking.

Susceptibility testing in calf diarrhea has focused on using fecal isolates, although this approach is fundamentally flawed. There do not appear to be any data demonstrating that fecal bacterial flora is representative of small intestinal bacterial flora, which is the site of infection in the intestinal tract of calves with enterotoxigenic *E. coli*.[13] Moreover, the predominant strain of *E. coli* in the feces of a scouring calf usually changes during the diarrhea episode,[34,35] and 45% (9/20) of diarrheic calves have different strains of *E. coli* isolated from the upper and lower small intestine.[34] In other words, fecal *E. coli* strains should not be considered to be representative of small intestinal *E. coli* strains.

A clear bias present in most antimicrobial susceptibility studies conducted on fecal *E. coli* isolates is that data is usually obtained from dead calves, which are likely to be treatment failures. Calves that die from diarrhea are likely to have received multiple antimicrobial treatments, and preferential growth of antimicrobial resistant *E. coli* strains starts within three hours of antimicrobial administration.[27] Similar to susceptibility testing of mastitis pathogens, the agar diffusion break points for susceptibility testing of fecal isolates are not based on achievable antimicrobial concentrations in the small intestine and blood of calves, but on achievable antimicrobial concentrations in the plasma of humans.

The only study to statistically test the predictive ability of fecal antimicrobial susceptibility results found that the rectal swab was an inaccurate method of predicting clinical outcome.[10] Two reports concluded that a "good correlation" existed between *in vitro* antimicrobial susceptibility of fecal *E. coli* isolates and clinical response to antimicrobial treatment; however, neither study statistically tested the association.[33,36] In contrast, two other studies reported "no correlation" between *in vitro* antimicrobial susceptibility of fecal *E. coli* and *Salmonella* spp isolates and clinical response to antimicrobial treatment,[8,18] although these studies did not differentiate enterotoxigenic and nonenterotoxigenic strains of *E. coli*, and also failed to sta-

tistically test the association. It is clear that we urgently need studies documenting the antimicrobial susceptibility of *E. coli* isolates from the small intestine of untreated calves, based on achievable drug concentrations and dosage regimens. Until these data are available, it appears that antimicrobial efficacy is best evaluated by the clinical response of a number of calves to treatment, with calves randomly assigned to treatment groups. The current evidence does not support performing *in vitro* antimicrobial susceptibility testing on fecal *E. coli* isolates.

Agar diffusion has more clinical relevance for predicting the clinical response to antimicrobial treatment when applied to blood isolates than fecal isolates. This is because the MIC breakpoints are based on achievable antimicrobial concentrations in human plasma and MIC_{90} values for human *E. coli* isolates, which provide a reasonable approximation to achievable MIC values in calf plasma and MIC_{90} values for bovine *E. coli* isolates. Unfortunately, susceptibility results are not available for at least 48 hours, and very few studies have documented the antimicrobial susceptibility of blood isolates in calves with diarrhea, and one of these studies observed a clinically significant year to year difference in the results of susceptibility testing that probably reflected different antimicrobial administration protocols on the farm.[17]

Pneumonia

The most important determinant of antimicrobial efficacy in treating pneumonia is obtaining an effective antimicrobial concentration at the site of infection, which is the lower respiratory tract. This is a different requirement to that for metaphylaxis, where the goal is to minimize or prevent proliferation of *Mannheimia hemolytica* in the upper and lower respiratory tract.

Antimicrobial susceptibility testing has frequently been recommended to guide the treatment of respiratory disease in cattle. The utility of periodic susceptibility testing to guide treatment decisions on feedlots has not been verified and is questionable, given that strains of *Mannheimia hemolytica* in a single outbreak of bovine respiratory disease vary between and within an animal.[25] A major difficulty with susceptibility testing is obtaining a representative culture of bacteria from the lower respiratory tract of cattle with pneumonia. The gold standard method is culturing affected anteroventral lung parenchyma at necropsy;[37] however, cattle dying of pneumonia have usually been treated with antimicrobial agents, which increases the percentage of resistant isolates.[2,20,22] Necropsy sampling is therefore strongly biased towards treatment failures. Practical methods for obtaining a representative culture of the lower respiratory tract bacteria in untreated cattle are therefore needed.

Antemortem culture of the bovine respiratory tract has used guarded nasopharyngeal swabs, guarded tracheal swabs, bronchoalveolar lavage (BAL) and transtracheal washes. Currently, endoscopic assisted BAL and transtracheal wash provide gold standard methods for obtaining a lower respiratory tract culture in live cattle. Unfortunately, both techniques are rarely performed because they are time consuming and require specific training and appropriate restraint of the animal or expensive and fragile equipment. Nasopharyngeal swabs are commonly used to collect samples from cattle in the field because the technique is rapid and inexpensive;[37] however, nasal swabs should not be used to identify the presence of lower respiratory pathogens in individual cattle.[1]

A nasopharyngeal swab is obtained by clearing the muzzle of accumulated secretions using a disposable towel, and then advancing a guarded swab (20 cm in length) through the nose to sample the caudal nasal passage.[16] A tracheal swab is obtained by restraining cattle using a head gate and two halters to minimize lateral movement, and by applying nose tongs to elevate and extend the head. A laryngoscope or speculum is inserted into the buccal cavity, the dorsum of the tongue is depressed, the larynx is visualized, and a guarded equine uterine swab (83 cm length) is advanced through the larynx and a tracheal swab obtained.[16] Neither the nasal pharyngeal or tracheal swab cultures the lower respiratory tract of adult cattle, although a long tracheal swab (83 cm) can theoretically reach the lower respiratory tract in recently weaned cattle. Studies have clearly shown that bacterial populations in the upper respiratory tract differ from those in the lower respiratory tract,[1,37] which is the site of infection in cattle with pneumonia. It is currently unknown whether the long tracheal swab provides a similar sample to that obtained from lung parenchyma, transtracheal wash, or BAL. However, 30% (8/27) of *Mannheimia hemolytica* isolated from both nasal and tracheal swabs in the same animal differ on the basis of ribotyping, and 37% (10/27) differ on the results of antimicrobial susceptibility testing.[16] These findings clearly indicate that nasal and tracheal swabs culture different bacterial populations

The blind BAL technique requires similar restraint to that for the tracheal swab, but a longer flexible sterile tube (100 to 240 cm in length) is passed through the outer sleeve of a guarded equine uterine swab (after the swab has been removed) positioned in the tracheal lumen. The long flexible tube is then advanced deep into the lower respiratory tract so that the tube wedges into a bronchus.[37] Approximately 50 mL of sterile buffer is then injected through the tube and aspirated, providing a lavage of the bronchoalveolar unit. In the endoscopic assisted BAL technique, an endoscope is passed through the nasal passages and down the trachea to

the carina, and the right apical lobe bronchus identified for sampling.[1] The transtracheal wash technique starts with aseptic preparation of the skin in the ventral mid cervical region, percutaneous passage of a sterile cannula into the tracheal lumen, and passage of a sterile flexible catheter through the cannula towards the carina. Alternatively, the sterile flexible tube is advanced through the outer sleeve of a guarded equine uterine swab in a similar manner to BAL.[25] Once positioned in the region of the carina, sterile phosphate buffered saline in injected through the catheter, reaspirated and the recovered fluid submitted for bacterial culture. The transtracheal wash and endoscopic assisted BAL culture the anteroventral section of the lung, which is the predilection site for bacterial pneumonia. Transtracheal wash is theoretically preferable to a blind BAL procedure because the latter technique cultures the diaphragmatic lung lobes, which are affected only in severe cases of pneumonia.

Studies have conclusively shown that nasal swabs are inaccurate indicators of lung parenchymal *Mycoplasma* species in cattle.[37] It is therefore difficult to interpret the results of most pneumonia studies where nasal swabs are used to monitor susceptibility. For instance, the findings in one study that a poor correlation existed between the MIC values for *Mannhemia hemolytica* and response to treatment with penicillin, oxytetracycline, or trimethoprim-sulfadoxine susceptibility[23] may not have represented the findings if culture samples had been obtained using a transtracheal wash or endoscopic assisted BAL In other words, therapeutic strategies should not be based on the antimicrobial susceptibilities of nasal or tracheal bacterial isolates, because these have not been validated as representing lower respiratory tract infection.

We do not currently have adequate databases of *in vitro* MIC values for bacterial isolates from cattle with pneumonia, because almost all isolates were obtained from nasal swabs of live animals[29] or lung parenchyma of dead animals that represented treatment failures.[29,42] In some studies the source of the isolates was not stated.[32] All the pivotal studies for FDA approval of tilmicosin, spectinomycin and enrofloxacin used pretreatment nasal swabs and necropsy lung swabs (treatment failures) to characterize the susceptibility profile, whereas the pivotal studies for ceftiofur and florfenicol utilized pretreatment nasal swabs, transtracheal washes and lung swabs (treatment failures) obtained at necropsy (www.fda.gov/cvm/efoi). The use of nasal swabs for susceptibility testing therefore casts doubt on the accuracy of NCCLS recommended breakpoints for antibiotics used to treat bacterial pneumonia in cattle. In summary, the results of susceptibility testing should not be currently used to guide treatment decisions in cattle with pneumonia because the break points have not been appropriately validated, and because bacteria are usually cultured from nasal or tracheal swabs, which have not been shown to represent lower respiratory tract bacterial populations, or cultured from dead animals, which represent treatment failures.

Conclusions

Antimicrobial susceptibility testing costs up to $20 per test. Because the cattle industry is economically driven, any diagnostic test should be validated, have appropriate sensitivity and specificity, and an acceptable economic return on the cost of testing before it can be routinely recommended. Antimicrobial susceptibility testing has not been adequately validated for the treatment of mastitis, calf diarrhea and pneumonia. The use of susceptibility testing to guide treatment decisions for individual cattle is therefore not recommended. However, because the results of susceptibility testing are repeatable, the results of population susceptibility testing do provide useful information on the development or loss of antibiotic resistance characteristics for pathogens in a population over time. In calf diarrhea, bacterial isolates for susceptibility testing should be obtained from the small intestine of untreated calves and confirmed to be enterotoxigenic strains of *E. coli*. In cattle with pneumonia, bacterial isolates for susceptibility testing should be obtained from untreated animals using a transtracheal wash or endoscopic assisted BAL, or possibly a tracheal swab in low body weight cattle. Finally, because zone diameter is the measured variable in the agar diffusion test, it is recommended that studies using the results of agar diffusion (Kirby-Bauer test) to describe antimicrobial susceptibility report the measured value (zone diameter) as well as the interpretation of the zone diameter (susceptible, intermediate, resistant). This recommendation is made because the interpretive criteria for agar diffusion and MIC breakpoints will obviously change when the breakpoints are appropriately validated.

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