APPENDIX A
ARA METHOD

Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals; Approved Standard

1 Introduction

A variety of laboratory techniques can be used to measure the *in vitro* susceptibility of bacteria to antimicrobial agents. These include disk diffusion and broth and agar dilution techniques. This document includes a series of recommendations to help standardize the way these tests are actually performed. The performance, applications, and limitations of the currently recommended methods are described. Recommendations by the International Collaborative Study (ICS) and initial regulations proposed by the U.S. Food and Drug Administration have been reviewed, and the appropriate sections have been incorporated into this standard.[1-3]

The current methodology described in this document is applicable only to the therapeutic and control uses of antibiotics as described in Section 2. The subcommittee has determined that the development of new or modified *in vitro* testing procedures to breakpoints or zone interpretive criteria to accommodate field usage practices in livestock was not realistic for several reasons.

First, there is not an apparent variable which can be easily modified in the current procedure to reflect a key factor which will correlate to *in vivo* efficacy. For example, any alteration in the inoculum (which might be reflective of an initial low infectious dose early in an infectious process) would need to be validated with efficacy studies in animals.

Furthermore, redesigning the current methodology would require the development of new quality control guidelines; possibly disks with lowered antibiotic content, or extended dilutions on MIC dilution panels. Second, even if such a procedure were developed, how realistic is it to expect a laboratory to use it and explain the outcome to a veterinarian or other client?

The subcommittee will remain open to alternatives to new procedural development, such as revisions to interpretive criteria. With respect to growth promoters, since the beneficial effects of antibiotics can neither be entirely ascribed to effects on the microbial gut flora, nor correlated to physiological effects on the animal, new *in vitro* techniques for prediction of clinical outcome should be considered only when a better understanding of the mode of action of antibiotics in this situation become available.

1.1 Performance Standards for Disk Diffusion Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically

In most veterinary diagnostic laboratories, the agar disk diffusion method is used for testing the common rapidly growing bacterial pathogens. If results are to be reliable, the technical details of such procedures must be carefully standardized and controlled. Agar disk diffusion methods based solely on the presence or absence of a zone of inhibition, without regard to its magnitude as an indicator of the organism's susceptibility to the drug being tested, are not acceptable.

The standardized method currently recommended by the NCCLS Subcommittee on Veterinary Antimicrobial Susceptibility Testing for agar disk diffusion tests is based on that described by Bauer et al.[4] This is the most completely described method for which interpretive standards have been developed and supported by clinical and laboratory data.

Reliable results can be obtained with disk diffusion tests that use standardized methodology and zone diameter measurement correlated with minimum inhibitory concentration (MIC) and the behavior of strains among clinically susceptible and resistant species.

1.2 Performance Standards for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically

Both broth and agar dilution techniques may be used to measure quantitatively the *in vitro* activity of an antimicrobial agent against a given bacterial culture. Basically, a series of tubes, wells, or plates is prepared with an agar or broth medium to which various concentrations of the antimicrobial agents are added. The tubes, wells, or plates are then inoculated with a suitably standardized suspension of the test organism. After overnight incubation at 35 °C, the tests are examined and the MIC is determined. The final result is influenced greatly by methodology, which must be carefully controlled if reproducible results (intralaboratory and interlaboratory) are to be achieved. Therefore, for the sake of standardization, there must be a reference method to which all other techniques can be compared.

This document describes such standard agar dilution and standard broth dilution (macrodilution and microdilution) techniques. These techniques are based largely on information gathered from the International Collaborative Study in Human Medicine.[1] Although these methods are primarily standard reference methods, some are sufficiently practical to warrant their use both in clinical laboratories and in the research laboratory. If one of these tests is to be adopted as the routine method in an institution where it has not been previously used, an educational program for the clinicians and the staff should be carried out before the sole use of MICs is initiated.

This document describes currently recommended techniques for antimicrobial agent disk and dilution susceptibility tests. When future improvements in methodology are developed, changes will be incorporated into the standard and distributed as informational supplements.

2 Selection of Antimicrobial Agents for Routine Testing and Reporting

Selection of the most appropriate antimicrobial agents to test and to report is a decision best made by each laboratory in accordance with the needs of its clientele. The lists in Table 1 comprise agents of proven clinical efficacy for treatment of systemic infections in that animal group, and which show acceptable *in vitro* test performance. Tests on selected agents can be useful for infection-control purposes. Agents listed are currently approved for treatment or control of disease. Agents used for other purposes such as prevention or growth promotion are not listed because the correlation of antimicrobial susceptibility tests for these uses cannot be established (see Section 2.1).

The subcommittee believes that it is both appropriate and necessary to provide the rationale used to limit the scope of this document to therapeutic and control uses of antibiotics in veterinary medicine. For the most part, these limitations reflect antibiotic uses in livestock used for food production, and do not necessarily apply to equine, or small animal medicine. Currently antibiotics are approved by the U.S. FDA Center for Veterinary Medicine for certain indications; treatment (i.e., therapy), control or prevention of disease, or growth promotion. The subcommittee has used the following definitions of antibiotic uses, which are consistent with current regulatory interpretations in its deliberations:

- therapeutic: administration of an antibiotic to an animal, or group of animals, which exhibit frank clinical disease;

- control: administration of an antibiotic to animals, usually as a herd or flock, in which morbidity and/or mortality has exceeded baseline norms, i.e., early in the course of the onset of disease in the population. It must be recognized that the initial treatment might be made empirically based on the urgency of the situation, and the clinical judgment including the most likely etiological agent, and experience of the veterinarian. However, a pretreatment culture and subsequent susceptibility information should be obtained as soon as possible to guide the appropriate use of an antibiotic. Control does not necessarily consider the health status of a given individual in the population, as that will vary depending upon the disease progression. In other words, a range of clinical manifestations from mortality to overt illness, to normal will be present in a group of animals. The objective is to control the dissemination of the disease within the

group while treating those with clinical signs. Certain exceptions, such as medication of one-day-old chicks, will be discussed later.

- prevention/prophylaxis: administration of an antibiotic to exposed healthy animals prior to expected onset of disease and for which no etiologic agent has yet been cultured. Generally the usage is in a herd or flock situation and not an individual animal.

- growth promotion: administration of an antibiotic, usually as a feed additive, over a period of time to growing animals that results in improved physiologic performance (i.e., weight gain, feed conversion, etc.). Although this has been sometimes referred to as "subtherapeutic" use, this is actually a misnomer since there is no disease claim associated with this use.

The claims for therapeutic and control use, but not prevention, are similar enough to be grouped together in Table 1 without a distinction between their field usage for the following reasons: the only difference between control and therapy is the timing of the administration, i.e., "early" in the course of a disease outbreak vs. "later." In other words, the number of animals affected at a particular stage in the disease progression is the only difference. The similarities are that in each case there are clinical signs (morbidity and/or mortality), a cultured-confirmed pathogen, and an antibiogram available. Since the control mode implies that there are animals already in the clinical stage of disease, the use of therapeutic breakpoints is appropriate. Any differences in doses or durations of the medications used for therapy or control will already have been adjusted during the drug-approval process and do not need to be considered herein.

Since there is no distinction between the two uses with regard to susceptibility data interpretation, it was decided that it was not necessary to have a separate table, or to note control or therapeutic, after the listing. This would simplify the laboratory's responsibility in reporting their recommendations to the practitioner and place the burden of appropriate product use on the veterinarian. Finally, the existing M37 guidelines can be applied to the development of the breakpoints and zone diameter interpretive criteria for control uses.

Prevention use is viewed as different from control/therapeutic because at the time of administration: 1) there is not a recovered pathogen; 2) there is no antibiogram for the pathogen; 3) there is no certainty that a disease outbreak will occur; and 4) it is possible that antibiotics would be used when they are not necessary. Thus, the application of therapeutic interpretations of susceptibility data was not justified. While a retrospective documentation of a given pathogen and susceptibility profile in a particular setting might provide guidance to a practitioner for future use of a particular antibiotic, the laboratory cannot be directly involved in recommending the use of a product in a manner not within the scope of this subcommittee. In other words, the laboratory can provide data to the veterinarian once an agent has been cultured and in this way help to establish a database over time. However, since there is no certainty that the same agent will be the cause of the next outbreak, it is the responsibility of the veterinarian, not the laboratory, to make a decision on whether to use a product in prevention mode.

An exception to the above is the situation with day-old chicks wherein an isolated etiologic agent and antibiogram would not be available and the administration of an antibiotic is actually more of a prevention mode. The subcommittee viewed this situation as an exception where the preventative aspect, along with the flock isolate history, and a high degree of certainty of an outbreak occurring with the known pathogen would allow the veterinarian to use clinical judgment to make a decision on which antibiotic to administer. The role of the laboratory in this case would be to provide the veterinarian with retrospective information obtained on isolates from the flock. This would serve as the historical database for the next outbreak. The laboratory would not be expected to make a recommendation on which antibiotic to administer.

Growth promotion uses of antibiotics have been determined to be outside the scope of the subcommittee and should not be included in laboratory reports. So far as is known, there is no correlation between a specific species of

bacteria, or group of bacteria, their physiological effect on the animal, and how that is affected by antibiotics. There is a complex microbial ecology issue involved in the growth promotion use of antibiotics, which quite likely involves dynamic populations of bacteria. In other words, there is no one bacterial species that can be tested to predict whether an antibiotic will enhance or inhibit the performance of a group of animals. Some antibiotics are used for both therapeutic or control purposes as well as growth promotion within the same animal species. Since there can be no *in vitro* testing for growth promoter uses, the control or therapeutic uses are to be evaluated as above. The testing of growth promotion antibiotics against specific species of enteric bacteria was recognized as perhaps having research value in monitoring for antibiotic resistance, but in no case could the results be correlated to a clinical outcome.

For antimicrobial agents with enteric or diarrheal disease indications, there are a substantial number of antibiotics used to treat a variety of enteric infections in animals and every attempt to include them within M31 should be made in order to guide practitioners in the proper selection of these agents. The interpretive criteria and breakpoints can now be included in the M31 tables and footnoted to indicate that the antibiotic is used to treat enteric infections.

Given the limited number of antimicrobial agents approved for use in some animal species, "extra-label" use of antimicrobial agents is commonly practiced. The U.S. FDA has defined extra-label use as the "use of an animal drug in a food-producing animal in a manner that is not in accordance with drug labeling. This includes, but is not limited to: use in species or for indications (disease or conditions) not listed in the labeling; use at a dosage level higher than those stated in the label; use of routes of administration other than those stated in the labeling; and failure to observe the stated withdrawal time."[5] While laboratory personnel should be familiar with extra-label use of antimicrobial agents in animals, it is the responsibility of the laboratory client (veterinarian/producer/herd owner) to use the compound appropriately in the animal.

2.1 Routine and Selective Reporting

The lists of agents in Table 1 constitute current recommendations for testing and for reporting that are considered appropriate. The lists are based on various considerations, including microbiological, clinical, and pharmacological factors, as well as clinical indications and efficacy.

To avoid misinterpretation, routine reports to the laboratory client should include only those drugs appropriate for therapeutic and control use, as suggested in Table 1. Agents may be added to or removed from these basic lists as conditions demand.

Compounds listed in Table 1, Group A and Group B, are approved for use in the indicated animal. It is the responsibility of the laboratory client to use the agent appropriately for the various animal types or category (e.g., calves, lactating dairy cattle). The laboratory client assumes all responsibility for efficacy, safety, and residue avoidance with extra-label uses of antimicrobial agents.

Compounds listed in Table 1, Group C are not approved but are frequently used in an extra-label manner in the indicated animal. It is the responsibility of the laboratory client to use the compound appropriately in the indicated animal. The laboratory client assumes all responsibility for efficacy, safety, and residue avoidance with extra-label uses of antimicrobial agents.

Drugs other than those approved for use in therapy may be tested to provide taxonomic data and epidemiological information. These results should not be reported in the clinical report.

2.2 Nonproprietary Names

To minimize confusion, all antimicrobial agents should be referred to by nonproprietary names.

2.3 Selection Guidelines

To make the routine susceptibility test relevant and practical, the number of agents tested should be limited. In general, routine tests should include only one representative of each group of related drugs with activity against a nearly identical spectrum of organisms and for

which interpretive results would be nearly always the same. The laboratory should report the specific drug tested and footnote other compounds represented by that drug.

Some of the selection boxes presented in Table 1 include certain agents or groups of agents with comparable clinical indications and activity against a similar but not identical spectrum of pathogens. Table 1 lists those drugs that should fulfill the basic requirements for routine use in most clinical veterinary laboratories.

The following guidelines are to be used for the selection and reporting of agents from some of the larger groups to be tested routinely:

2.3.1 β-Lactams

β-lactam antimicrobial agents all share a common, central, four-membered β-lactam ring. The principal mode of action is inhibition of cell wall synthesis. Additional ring structures or substituent groups added to the β-lactam ring determine whether the agent is a penicillin, cephem (cephalosporin), carbapenem, or monobactam. The substitutions affect the antibacterial spectrum and the pharmacokinetics of the individual compounds.

2.3.1.1 Penicillins

The spectrum of penicillin is directed primarily against non-β-lactamase-producing gram-positive and some gram-negative bacteria. Ampicillin and amoxicillin (the aminobenzylpenicillins) have activity against more gram-negative species, including non-β-lactamase-producing members of the Enterobacteriaceae. Carbenicillin and ticarcillin (carboxy-penicillins) as well as mezlocillin and piperacillin (ureidopenicillins) have a considerably expanded gram-negative spectrum, including activity against many *Pseudomonas* spp. Penicillinase-resistant penicillins (PRPs) (cloxacillin, dicloxacillin, methicillin, nafcillin, and oxacillin) are used specifically for treating the penicillinase-producing, methicillin-susceptible staphylococci.

Staphylococci should be tested against penicillin G and may be screened for β-lactamase production, especially in strains with borderline MICs (0.06 to 0.25 µg/mL; see Section 7). One PRP should be tested; usually, oxacillin is preferable to methicillin because of its storage stability and better reliability in the detection of methicillin-resistant *Staphylococcus aureus* (MRSA; see Section 4.3.7). Other penicillins need not be tested against staphylococci.

Test either penicillin or ampicillin against streptococci: testing for both is not necessary. Testing of secondary agents, such as cephalosporins, erythromycin, clindamycin, or tetracycline can be useful in the treatment of the penicillin-allergic animal.

For testing non-β-lactamase-producing enterococci, susceptibility to penicillin G predicts susceptibility to ampicillin, ampicillin analogues, and amoxicillin. For blood and cerebrospinal fluid (CSF) isolates, a β-lactamase test using an inoculum of $\geq 10^7$ colony-forming units per milliliter (CFU/mL) (or direct colony growth) is also recommended.

To provide synergistic killing activity, combination therapy with penicillin G or ampicillin plus an aminoglycoside is indicated for serious enterococcal infections in companion animals. Enterococci, which exhibit high-level aminoglycoside resistance are not reliably subject to synergy with that agent; such strains are detected by screening with high levels of streptomycin (1,000 µg/mL with broth or 2,000 µg/mL with agar) or gentamicin (500 µg) with agar or broth dilution tests.

For enterococci, tests against other agents, such as cephalosporins and clindamycin should not be reported because of lack of clinical correlation. Vancomycin is the accepted agent for treatment of enterococcal infection in the human penicillin-allergic patient. However, data concerning the efficacy of vancomycin in veterinary medicine are lacking and extra-label use of glycopeptides in food producing animals has been specifically prohibited by the U.S. FDA. Because of this, a footnote could be used to simply indicate that vancomycin is indicated for penicillin-allergic companion animal patients.

2.3.1.2 β-Lactam/β-Lactamase Inhibitor Combinations

These antimicrobial combinations include a penicillin and a second agent that has minimal antimicrobial activity but functions as an

inhibitor of some β-lactamases. For some organisms, the results of tests of only the penicillin portion of the combination might not be predictive of susceptibility to the two-drug combination.

2.3.1.3 Cephalosporins and Other Cephems

The different cephalosporins and other cephem antimicrobial agents often have a slightly different spectrum of activity against the gram-positive and gram-negative bacteria. These agents are often referred to as "first-," "second-," "third-," or "fourth-" generation cephalosporins, depending in large part on the extent of their activity against the more resistant, gram-negative bacteria. Because of the differences in activities of some members of these groups, representatives of each group may be selected for routine testing.

Staphylococci are usually susceptible to the cephalosporins, except for MRSA. Although *in vitro* tests with methicillin-resistant staphylococci can suggest susceptibility, they should be considered resistant. The current information with coagulase-negative staphylococci is conflicting and, thus, this approach is a conservative one.

Enterococci are considered resistant to the cephalosporins, although some can appear to be susceptible under some test conditions, and some cephalosporins have produced clinical cures in some infection sites. Currently, the results of tests with enterococci against the cephalosporins should not be reported routinely.

2.3.1.4 Carbapenems

Carbapenems differ slightly in structure from penicillins and are much more resistant to β-lactamase hydrolysis, which provides them with broad-spectrum activity against many gram-positive and gram-negative bacteria.

2.3.1.5 Monobactams

Monobactam antimicrobial agents are structurally unique and show significant activity only against aerobic, gram-negative bacteria. The only monobactam approved for use in human medicine is aztreonam. None are approved for use in veterinary medicine.

2.3.2 Tetracyclines

These compounds inhibit bacterial protein synthesis at the ribosomal level. Drugs in this group are closely related and, with rare exception, only tetracycline should be tested routinely (see table footnotes).

2.3.3 Aminoglycosides/Aminocyclitols

This group of chemically related drugs includes, among others, streptomycin, amikacin, apramycin, gentamicin, kanamycin, netilmicin, tobramycin and spectinomycin. Members of this group inhibit bacterial protein synthesis at the ribosomal level. This group includes members that are affected in various ways by aminoglycoside-inactivating enzymes, which results in some differences in the spectrum of activity between agents. They are used primarily to treat aerobic gram-negative infections or in synergistic combinations with cell-wall active compounds against some resistant gram-positive bacteria. Aminoglycosides, such as gentamicin, have the potential to produce extended residue times in some animals, i.e., the bovine; therefore, their use should be monitored carefully.

2.3.4 Macrolides

Macrolides are structurally related antimicrobial agents that inhibit protein synthesis at the ribosomal level. This group includes erythromycin, clarithromycin, azythromycin, tylosin, and tilmicosin. Only erythromycin and tilmicosin should be tested.

2.3.5 Lincosamides

The lincosamides (lincosaminides) inhibit protein synthesis at the ribosomal level. This group includes lincomycin, clindamycin, and pirlimycin. Clindamycin should be tested routinely instead of lincomycin. However, clindamycin can be more active than lincomycin against some strains of *S. aureus*. Pirlimycin should be tested separately.

2.3.6 Quinolones

This group of antimicrobial agents functions primarily by inhibiting DNA-gyrase activity of many gram-positive and gram-negative bacteria. This group of compounds includes the

older quinolones (i.e., nalidixic acid) and the newer fluoroquinolones, such as enrofloxacin, sarafloxacin, orbifloxacin, and difloxacin. Currently, only nalidixic acid, enrofloxacin, sarafloxacin, orbifloxacin, and difloxacin are approved for use in veterinary medicine. Both enrofloxacin and sarafloxacin are approved for use in food animals. Recently, the U.S. FDA has banned extra-label use of fluoroquinolones in veterinary medicine.

2.3.7 Sulfonamides and Trimethoprim/Ormetoprim

This group of compounds encompasses a wide variety of chemotherapeutic agents with similar spectra of activity resulting from inhibition of bacterial folate metabolism. Sulfachlorpyridazine and sulfisoxazole are the most commonly used sulfonamides in veterinary medicine and thus can be the appropriate selection for *in vitro* testing. Sulfadimethoxine is the only sulfa drug approved for use in dairy animals over 20 months of age and should be included in a panel if the species and age of an animal is unknown.

When sulfonamides are combined with trimethoprim, two sequential steps in folate metabolism are inhibited. Although trimethoprim/sulfadiazine is the usual combination in veterinary medicine, trimethoprim/sulfamethoxazole may be used for susceptibility testing. Ormetoprim/sulfadimethoxine activity is tested with trimethoprim/sulfamethoxazole.

2.3.8 Chloramphenicol and Related Analogs

Chloramphenicol, florfenicol, and thiamphenicol are a group of compounds that are broad spectrum, bacteriostatic agents which inhibit bacterial growth by blocking the transfer of soluble ribonucleic acid to ribosomes. While chloramphenicol should not be tested, or reported, for any isolate from food animals, florfenicol has been approved for treating pathogens associated with acute bovine respiratory disease. Thiamphenicol is not approved for use in the United States, but is approved for use in Europe.

2.3.9 Single Drug Classes

Currently, vancomycin, rifampin, novobiocin, tiamulin, polymyxin, and nitrofurantoin represent antimicrobial agents for which there are no related compounds that are appropriate for *in vitro* testing. Metronidazole and nitrofurantoin should not be reported for any isolate from food animals.

2.4 Guidelines for Routine Reporting

As listed in Table 1, agents in Group A and B are considered appropriate for inclusion in a routine primary panel for food and companion animals. Compounds in both Groups A and B may be reported routinely or selectively as outlined in Section 2.5.

Group C comprises agents that are frequently used in an extra-label fashion in the indicated animal species. It is the laboratory client's responsibility to use these compounds appropriately in the host animal species.

Compounds with prevention or growth promotion claims are not listed.

2.5 Guidelines for Selective Reporting

Each laboratory should decide which agents (Table 1) to report routinely from each group in accordance with the needs of their clientele. Selective reporting should help improve the clinical relevance of test reports and it should help to minimize the selection of multiresistant strains by overuse of broad-spectrum agents.

2.5.1 Examples

Examples of possible guidelines for selective reporting, as outlined for agents listed in Table 1, are as follows:

- Testing of an enteric bacillus susceptible to ampicillin should not include carbenicillin or ticarcillin, mezlocillin or piperacillin, amoxicillin/clavulanic acid, ampicillin/ sulbactam or ticarcillin/clavulanic acid.
- Testing of an enteric bacillus susceptible to a first-generation cephalosporin should not include a second-, third-, or fourth-generation cephalosporin.
- Testing of an enteric bacillus susceptible to gentamicin should not include tobramycin or amikacin.
- Testing of enterococci from nonurinary sites should not include agents other than

penicillin or ampicillin, except vancomycin as requested for penicillin-allergic animals.

- Staphylococci resistant to methicillin/oxacillin should not include any cephalosporins, amoxicillin/clavulanic acid, imipenem, ampicillin/ sulbactam, or other β-lactam antibiotic, regardless of *in vitro* results.
- Fluoroquinolones should not be reported for extra-label use in food animals.

2.6 Interpretive Categories

2.6.1 Susceptible

This category implies that an infection due to the strain of bacterium can be inhibited by achievable serum or tissue levels of the dosage of antimicrobial agent recommended for that type of infection and infecting species, unless otherwise contraindicated.

2.6.2 Intermediate

This category provides a "buffer zone." This buffer zone should prevent small, uncontrolled technical factors from causing discrepancies in interpretations (e.g., a resistant organism being categorized as susceptible [termed a *very major error*] or a susceptible organism being categorized as resistant [termed a *major error*]), especially for drugs with narrow pharmacotoxicity margins.

This category includes strains with MICs that approach or can exceed usually attainable blood or tissue levels but do not have flexible labeling; and for which response rates can be lower than for strains in the "susceptible" category. These strains can be inhibited by attainable concentrations of certain antimicrobial agents:

In body sites, such as the urinary tract, where drugs are physiologically concentrated (e.g., quinolones, β-lactams).

- Provided the drug has a wide pharmacotoxicity margin and is administered at maximal dosage (e.g., β-lactams).

If the organism is not susceptible to alternative clinically feasible drugs, if the site of infection is not one where the drug is concentrated, or if the high dose cannot be used, the test should be repeated.

2.6.3 Flexible Label

Indicates the availability of U.S. FDA-approved flexible labeling; this organism could be considered susceptible if appropriate dosing modifications found in the product packaging insert are applied.

2.6.4 Resistant

Resistant strains are not inhibited by the usually achievable systemic concentrations of the agent with normal dosage schedules and/or fall in the range where specific microbial resistance mechanisms are likely (e.g., β-lactamases), and clinical efficacy has not been reliable in treatment studies.

3 Susceptibility Testing

3.1 Indications for Performing Susceptibility Tests

3.1.1 Limitations of Disk Diffusion and Dilution Methods

The disk diffusion and dilution methods described in this document have been standardized for testing the rapidly growing pathogens, which include *Staphylococcus* spp., *Enterococcus* spp., the Enterobacteriaceae, *P. aeruginosa*, and *Acinetobacter* spp., and they have been modified for testing some fastidious organisms such as *S. pneumoniae* as outlined in Table 5. Studies are not yet adequate to develop reproducible, definitive standards to interpret disk tests with other microorganisms not listed in the tables that could require different media, different atmospheres, or indicate marked strain-to-strain variation in growth rate. Such organisms should not be tested by the disk diffusion method because the results cannot be interpreted reliably.

Dangerously misleading results can occur when certain antimicrobial agents are tested against specific organisms. These combinations include, but might not be limited to the following: first- and second-generation cephalosporins and aminoglycosides against *Salmonella* spp.; all β-lactam antimicrobial agents (except oxacillin, methicillin, and nafcillin) against

methicillin-resistant staphylococci; cephalosporins, aminoglycosides (except high-level testing for resistance), clindamycin, and trimethoprim/sulfamethoxazole against enterococci; and cephalosporins against *Listeria* spp.

In addition to the above pitfalls, some antimicrobial agents are associated with the emergence of resistance during prolonged therapy. Therefore, isolates that are initially susceptible can become resistant within three to four days after initiation of therapy. This occurs most frequently in *Enterobacter* spp., *Citrobacter* spp., and *Serratia* spp. with third-generation cephalosporins; in *P. aeruginosa* with all antimicrobial agents; and in staphylococci with quinolones.

Detection of β-lactamase production in enterococci is possible only by a direct β-lactamase test. Members of the Enterobacteriaceae, *Pseudomonas* spp., and other aerobic gram-negative bacilli are not to be tested routinely for β-lactamase production because the results might not be predictive of susceptibility to the β-lactams most often used for therapy. Recommended β-lactamase test methods are indicated in Section 7.

Any organism identified as a causal agent is a candidate for susceptibility testing. This may not be necessary if the identity of the organism suggests a choice of chemotherapeutic agents. Etiologic organisms will, however, normally be identified at the same time the susceptibility test is performed. Susceptibility tests are most often indicated when the causative organism is thought to belong to a species known to be capable of exhibiting resistance to commonly used antimicrobial agents (e.g., *Staphylococcus* spp. and the Enterobacteriaceae). Some organisms have predictable susceptibility to antimicrobial agents. Susceptibility tests are seldom necessary when the infection is due to a microorganism that is susceptible to a highly effective drug (e.g., the universal susceptibility of Group C and Group G streptococci and *Arcanobacterium* [*"Actinomyces"*] *pyogenes* to penicillin). Erythromycin, vancomycin, and tetracycline may be tested against streptococci from animals that are allergic to penicillin to detect those strains resistant to these alternative drugs.

Isolated colonies of each type of organism that might be playing a pathogenic role should be selected from primary agar plates and tested for susceptibility. Identification procedures may be performed at the same time. Mixtures of different types of microorganisms should not be tested on the same susceptibility test plate. The practice of conducting susceptibility tests directly with clinical material should be avoided. When the nature of the infection is not clear and the specimen contains mixed growth or normal flora, in which the organisms probably bear little relationship to the infectious process being treated, susceptibility tests are often not necessary; the results can even be grossly misleading.

4 Disk Diffusion Susceptibility Tests

4.1 Zone Size Interpretive Standards

Table 2 shows interpretive guidelines for the sizes of the zones of inhibition for use with agar disk diffusion susceptibility tests and MIC breakpoints for use with dilution susceptibility tests. For those agents for which veterinary-specific guidelines are not available, the use of these values in relation to veterinary bacterial isolates must be done with caution for three reasons. First, the categories listed in Table 2 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-range schedules 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.

4.2 Equivalent MIC Breakpoints

Disk diffusion zone diameters correlate inversely with MICs from standard dilution tests, usually broth microdilution. Table 2 lists 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.[6] However, the zone

(5) Just before use, agitate this turbidity standard vigorously on a mechanical vortex mixer.

(6) Replace standards or recheck their densities three months after preparation.

4.3.4 Inoculation of Test Plates

4.3.4.1 Growth Method Inoculum Preparation

The steps of the standard method are as follows:

(1) Select at least four to five well-isolated colonies of the same morphological type from an agar plate culture. Touch the top of each colony with a wire loop (or nontoxic cotton or Dacron swab) and transfer the growth to a tube containing 4 to 5 mL of a suitable broth medium, such as tryptic-soy broth.

(2) Allow the broth culture to incubate at 35 °C until it achieves or exceeds the turbidity of the standard described in Section 4.3.3 (usually two to eight hours).

(3) Adjust the turbidity of the active growing broth culture with sterile saline or broth to obtain a turbidity visually comparable to that of the 0.5 McFarland turbidity standard described in Section 4.3.3. To perform this step properly, use adequate light, and to aid in the visual comparison, read the tube against a white background with contrasting black lines.

(4) Within 15 minutes after adjusting the turbidity of the inoculum suspension, dip a sterile nontoxic swab on an applicator into the adjusted suspension. Rotate the swab several times, pressing firmly on the inside wall of the tube above the fluid level to remove excess inoculum from the swab.

(5) Inoculate the dry surface of a Mueller-Hinton agar plate by streaking the swab over the entire sterile agar surface. Repeat this streaking procedure two more times, and rotate the plate approximately 60° each time to ensure an even distribution of inoculum. Replace the plate top and allow three to five minutes, but no longer than 15 minutes, for any excess surface moisture to be absorbed before applying the drug-impregnated disks. If the plate is streaked satisfactorily, and the inoculum was correct, the zones of inhibition will be uniformly circular and there will be a confluent—or an almost confluent—lawn of growth. If only isolated colonies grow, the inoculum was too light and the test should be repeated.

NOTE: Avoid extremes in inoculum density. Never use undiluted overnight broth cultures for streaking plates.

4.3.4.2 Direct Colony Suspension Method Inoculum Preparation

For routine susceptibility tests, the inoculum is standardized by making a direct saline or broth suspension of colonies that are selected from an 18- to 24-hour agar plate (a nutrient, nonselective medium, such as blood agar, must be used).[8, 9] The suspension is adjusted to match the turbidity standard, as outlined in Section 4.3.3. This approach is the method of choice for testing species that fail to grow satisfactorily in broth media (i.e., *Actinobacillus pleuropneumoniae and Streptococcus* spp. [see Sections 4.3.5 and 4.3.6]) or for testing *Staphylococcus* spp. for methicillin resistance (see Section 4.3.7).

When testing trimethoprim/sulfamethoxazole disks using this method, colonies obtained from the nonselective medium can carry over enough antagonistic substances to produce a haze of growth inside a zone of inhibition produced by susceptible strains.

Devices that permit direct standardization of inocula without adjustment of turbidity and without preincubation in a broth medium have been found to be acceptable for routine testing purposes.[10, 11]

4.3.4.3 Test Procedure for All Methods of Inoculation

The test procedure for all methods of inoculation is as follows:

(1) Place the appropriate drug-impregnated disks on the surface of the agar plate inoculated by one of the methods described in Section 4.3.4.1 or 4.3.4.2. With sterile

forceps or needle tip, gently press down each disk to ensure complete contact with the agar surface. The disks may be placed individually or with a dispensing apparatus, but they must be distributed evenly so that they are no closer than 24 mm from center to center. *No more than 12 disks should be placed on one 150-mm plate or more than five disks on a 100-mm plate.* Because some of the drug diffuses almost instantaneously, a disk is not to be moved once it has come in contact with the agar surface.

(2) Invert the plates and place them in an incubator at 35 °C within 15 minutes after the disks are applied. The plates should not be incubated under an increased concentration of CO_2, because the interpretive standards were developed by using aerobic incubation and, with some agents (i.e., macrolides, lincosaminides, tetracyclines), CO_2 will significantly alter the size of the inhibitory zones due to pH changes. Organisms requiring CO_2 for standardized testing, such as *Haemophilus somnus* and *Actinobacillus pleuropneumoniae* should be incubated in an environment of 3 to 7% CO_2. If a CO_2 incubator is not available, a candle extinction jar is an acceptable alternative. Control strains, which are tested concurrently, will indicate whether the pH has changed unacceptably. Only those antimicrobial agents for which acceptable quality control values have been achieved should be reported.

(3) After 16 to 18 hours of incubation, examine each plate and measure the diameters of the zones of complete inhibition (as judged by the unaided eye), including the diameter of the disk. Measure the zones to the nearest whole millimeter, using sliding calipers, a ruler, or a template prepared for this purpose. Hold the measuring device onto the back of the petri plate illuminated with a reflected light against a black, nonreflecting background. If the test organism is a *Staphylococcus* or *Enterococcus* spp., 24 hours of incubation are required and transmitted light is used to examine the oxacillin and vancomycin zones for light growth of methicillin- or vancomycin-resistant colonies, respectively, within apparent zones of inhibition. If blood is added to the agar base, measure the zones from the surface illuminated with reflected light and with the cover removed.

(4) The end point should be taken as the area showing no obvious, visible growth that can be detected with the unaided eye, not including faint growth or tiny colonies that can be detected only with difficulty at the edge of the zone of inhibited growth. Large colonies growing within a clear zone of inhibition should be subcultured, reidentified, and retested. Strains of *Proteus mirabilis* and *Proteus vulgaris* may swarm into areas of inhibited growth around certain antimicrobial agents. The zones of inhibition are usually clearly outlined and the veil of swarming growth is ignored. With trimethoprim and the sulfonamides, antagonists can allow some growth; therefore, with these drugs, disregard slight growth (i.e., the density of the growth is 20% or less than that seen outside the zone of inhibition), and measure the margin of heavy growth (80 to 100% of the density seen outside the zone of inhibition) to determine the zone diameter.

(5) Interpret the sizes of the zones of inhibition by referring to Table 2, and report the organism to be susceptible, intermediate, or resistant.

4.3.5 Fastidious Organisms

4.3.5.1 Indications for Testing Fastidious Organisms

Mueller-Hinton medium described above for the rapidly growing aerobic pathogens is not adequate for fastidious organisms. If susceptibility testing is to be done with fastidious organisms, the medium, quality control procedures, and interpretive criteria must be modified to fit each organism.

4.3.5.2 Disk Diffusion Testing of *Haemophilus somnus and Actinobacillus pleuropneumoniae*

The procedures described here have been shown to be accurate and provide reproducible results in accordance with the quality control guidelines outlined in Section 6. The medium of choice for performing disk diffusion testing with these organisms is chocolate Mueller-

Hinton. One liter of this medium consists of the following ingredients:

Mueller-Hinton agar (38.0 g):	
+ Beef Extract	2.0 g (from 300 g of beef infusion)
+ Acid Hydrolysis of Casein	17.5 g
+ Starch	1.5 g
+ Agar	17.0 g
Hemoglobin (10.0 g)	
Nutritional Supplement (10.0 mL):[12]	
+ Vitamin B_{12}	0.01 g
+ L-Glutamine	10.00 g
+ Adenine	1.00 g
+ Guanine HCL	0.03 g
+ p-Aminobenzoic Acid	0.013 g
+ NAD	0.25 g
+ Thiamine pyrophosphate	0.10 g
+ Ferric Nitrate - $8H_2O$	0.02 g
+ Thiamine HCL	0.003 g
+ L-Cysteine HCL	25.90 g
+ L-Cystine	1.10 g
+ Dextrose	100.00 g

The Mueller-Hinton agar is mixed with 60% of the total water volume and steam sterilized. The hemoglobin is mixed with 40% of the total water volume and steam sterilized. The two mixtures are then combined at >50 °C. The mixture is cooled to 48 °C with constant stirring, at which time the nutritional supplement is added aseptically. The medium is allowed to mix for ten minutes and then poured into flat-bottomed petri dishes on a level, horizontal surface to a uniform depth of approximately 4 mm, in accordance with Section 4.3.1.4 of this document.

4.3.5.3 Test Procedure

The direct colony suspension procedure should be used when testing *H. somnus* and *A. pleuropneumoniae*. Using colonies taken directly from an overnight (preferably 20 to 24 hours) chocolate agar culture plate, a suspension of the test organism is prepared in sterile Mueller-Hinton broth, water or 0.9% saline. The suspension should be adjusted to a turbidity equivalent to a 0.5 McFarland standard. This suspension will contain approximately 1 to 4 x 10^8 CFU/mL. Care must be exercised in preparing this suspension because higher inoculum concentrations may lead to false-resistant results. Within 15 minutes after adjusting the turbidity of the inoculum suspension, it should be used for plate inoculation.

The procedure for the disk test should be followed as described, beginning with Section 4.3.4 for nonfastidious bacteria, except that no more than nine disks should be applied to the surface of a 150-mm plate or no more than four disks on a 100-mm plate.

Plates are incubated at 35 °C in an atmosphere of 5% CO_2 for 20 to 24 hours before measuring the zones of inhibition.

The innermost zone of obvious growth inhibition should be regarded as the zone margin.

4.3.5.4 Quality Control Testing of *Haemophilus somnus* and *Actinobacillus pleuropneumoniae*

The antimicrobial agents for which quality control testing has been established for *H. somnus* and *A. pleuropneumoniae* are indicated in Table 3A. Disk diffusion testing of these isolates with other agents is not recommended until appropriate quality control parameters have been established.

4.3.6 ***Streptococcus* spp.**

4.3.6.1 Agar Medium

The recommended medium for testing *S. pneumoniae* and other streptococci is Mueller-Hinton agar supplemented with 5% defibrinated sheep blood.

4.3.6.2 Test Procedure

(1) The direct colony suspension procedure should be employed as follows: growth from an overnight (16- to 18-hour) sheep blood agar plate is suspended in sterile Mueller-Hinton broth, water, or 0.9% saline to a density equivalent to the turbidity of the 0.5 McFarland standard. Within 15 minutes after adjusting the turbidity of the inoculum suspension, it should be used for plate inoculation.

(2) The disk diffusion procedure steps described, beginning with Section 4.3.4, should be followed except that not more than nine disks should be placed on a 150-mm agar plate or four disks on a 100-mm plate.

(3) The plates are incubated at 35 °C in an atmosphere of 5% CO_2 for 20 to 24 hours before measuring the zones of inhibition.

4.3.6.3 Zone Diameter Interpretive Criteria

The zone interpretive criteria to be used when testing *S. pneumoniae* and other streptococci are included in Table 2.

NOTE: Isolates of *S. pneumoniae* with oxacillin zone sizes of ≥ 20 mm are susceptible (MIC ≤ 0.06 µg/mL) to penicillin. Because zones of ≤ 19 mm with the oxacillin disk screening test occur with penicillin resistant, intermediate, and certain susceptible strains, a penicillin MIC should be determined on isolates of *S. pneumoniae* with oxacillin zones of ≤ 19 mm.[13] Isolates should not be reported as penicillin resistant or intermediate based solely on an oxacillin zone of ≤ 19 mm. Oxacillin disk testing for the purpose of determining penicillin susceptibility of streptococci other than *S. pneumoniae* is not recommended. A penicillin MIC should be determined on isolates of viridans group streptococci from normally sterile body sites (e.g., cerebrospinal fluid, blood, bone, etc.). Penicillin (and oxacillin) disk diffusion testing is not reliable with viridans group streptococci.

4.3.7 Methicillin-Resistant Staphylococci[14]

Problems associated with detection of methicillin-resistant (heteroresistant) staphylococci when using the disk diffusion and dilution tests have recently emerged. Some of these problems could be related to changes in the organisms, some to change in test culture media, and some to the limitations of the method itself. The most evident problems now are difficulties in detecting cross-resistance among the penicillinase-resistant penicillins (PRP) and failure of the older interpretive criteria to correlate with the genotypic profiles of organisms (mecA genes). The subcommittee makes the following recommendations:

- Currently, because the oxacillin test is more likely to detect cross-resistance than methicillin, oxacillin is recommended unless it is shown that the hospital-endemic, PRP-resistant staphylococcal population is one that is detected better with methicillin. Nafcillin should not be used if blood or blood products are in the test medium.
- To prepare the inoculum from an 18-to-24-hour agar plate, use the alternative direct method rather than the standard method.[10, 11]
- The user should be aware that most heteroresistant (oxacillin-resistant) staphylococci are usually resistant to multiple antimicrobial agents, including β-lactams, aminoglycosides, macrolides, clindamycin, and tetracycline; and this should be used as a clue to PRP-resistance. Another clue to PRP heteroresistance is the presence of a "film" of growth within a zone of inhibition around a methicillin, oxacillin, nafcillin, or cephalothin disk.
- Methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-resistant coagulase-negative staphylococci should be reported as resistant to all β-lactams regardless of the *in vitro* test result. The justification for this is that, in most cases of documented MRSA infection, the patient has responded poorly to the cephalosporin therapy or clinical data have yet to be derived confirming clinical efficacy (imipenem and sulbactam and clavulanic acid combinations). Methicillin-resistant, coagulase-negative *Staphylococcus* spp. also appear not to respond well to these drugs, but fewer data are available than with MRSA and the breakpoint for mecA positive strains (PCR) may be lower for oxacillin (e.g. ≤ 0.25 or ≤ 0.5 µg/mL).
- See Table 5 for a method for tests of organisms with PRPs. This includes the recommendation for addition of 2% NaCl to the medium.
- Intrinsic oxacillin resistance in *S. aureus* can be confirmed by inoculating the organism onto Mueller-Hinton agar that has been supplemented with 4% NaCl and 6 µg oxacillin/mL.[15] The agar is inoculated as a "spot"; or a quadrant of the plate is streaked using a cotton swab that has been dipped into a suspension equivalent to a 0.5 McFarland standard and from which the excess fluid has been expressed. It is incubated at 35 °C for 24 hours and

observed for the presence of any colony formation that indicates intrinsic oxacillin resistance. This method (oxacillin agar screen plate) has been used extensively with acceptable predictive value for *S. aureus*. Experience with coagulase-negative staphylococci is less extensive. *Staphylococcus* epidermidis appears to react differently with a lower breakpoint MIC,[16,17] and may require 48 hours of incubation using the oxacillin agar screen plate.

4.3.8 Other Organisms

4.3.8.1 *Listeria* spp.

Listeria spp. should be tested with the broth microdilution method using Mueller-Hinton broth supplemented with 2 to 5% lysed horse blood. Due to the slow growth rate of these organisms, the results of the disk diffusion test are to be considered "invalid."

4.3.8.2 *Arcanobacterium ("Actinomyces") pyogenes*

Because *A. pyogenes* is routinely susceptible to penicillin, susceptibility testing should not be necessary. This organism should be tested by a microdilution MIC method. Due to the slow growth rate of the organism, the results of the disk diffusion test are to be considered "invalid." When isolated from a mixed infection, combination therapy including penicillin can be warranted. Isolates from penicillin-allergic animals can respond to drugs in the macrolide class of antimicrobial agents.

4.3.9 Anaerobic Organisms

Susceptibility testing of anaerobic bacteria isolated from animals should be performed in accordance with procedures outlined in the most current edition of NCCLS document M11— *Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria*. The interpretive categories and correlative minimal inhibitory concentrations (MICs) for antimicrobial agents appropriate to treat infections caused by anaerobic bacteria are listed in Table 2A.

5 Broth and Agar Dilution Susceptibility Testing

5.1 Indications for Performing Broth and Agar Dilution Susceptibility Tests

Clinically, an *in vitro* antimicrobial agent susceptibility test is useful as a guide for determining chemotherapy whenever the susceptibility of a pathogen is unpredictable or when an infection has not responded to therapy that otherwise appears appropriate. The MIC obtained by a dilution test can give a clinician an indication as to the concentration of antimicrobial agent needed at the site of infection to eradicate or inhibit the infecting organism. The MIC, however, does not represent an absolute value. The "true" MIC is somewhere between the lowest test concentration that inhibits the organism's growth (i.e., the "observed" MIC) and the next lower test concentration. If, for example, twofold dilutions were used and the "observed" MIC was 16 µg/mL, the "true" MIC would be between 8 and 16 µg/mL. Even under the best of controlled conditions, a dilution test might not yield the same end point each time it is performed. Generally, the acceptable reproducibility of the test is within one twofold dilution of the actual end point. To avoid greater variability, the dilution test must be standardized and carefully controlled.

MICs have been determined using concentrations derived traditionally from serial twofold dilutions of a starting concentration. In recent years, this starting concentration usually has been chosen to be an integral power of 2 (e.g., 128 µg/mL). Other dilution schemes have been used, including use of as few as two widely separated concentrations, and results from these alternative methods can be equally useful clinically.[18] When MICs are reported, however, it is important that the user know or be able to determine what dilution scheme was used. For example, when there is inhibition of growth at the lowest concentration tested, the true numerical result cannot be accurately determined and should be interpreted as equal to or less than the lowest concentration tested. The frequency of indeterminate results increases as fewer dilutions (concentrations) are tested. In tests in which four or fewer consecutive concentrations are tested, or in which nonconsecutive concentrations are tested, it is advisable

to report a qualitative result (i.e., susceptible, intermediate, or resistant) but the MIC range can be reported if desired.

Dilution susceptibility tests can play a role in:

- The study of new antimicrobial agents.
- Defining intermediate levels of susceptibility when higher doses of antimicrobial agent might be feasible or preferable to conventional doses of potentially toxic agents, or when the agent might be physiologically concentrated (as in the urinary tract).

The techniques described in this document are intended primarily for testing commonly isolated bacteria that will grow well after overnight incubation in Mueller-Hinton medium, either unsupplemented or supplemented, as described in Section 5.2.3.[19] For some more fastidious and special problem pathogens that can require MIC tests, alternative methods are included in Table 5. These are not intended to be standard methods, but they are acceptable alternatives with which members of the subcommittee have had substantial experience. In some of these methods, lysed horse blood was used as a blood additive because it contained thymidine phosphorylase (thus making media more suitable for sulfonamide and trimethoprim testing), but it is likely that either lysed sheep or rabbit blood could be used instead. For tests with staphylococci, 2 g/L NaCl should be added only to the wells containing oxacillin or methicillin because it interferes with the growth of some organisms and alters the results obtained with aminoglycosides (2 g/L NaCl should be used in the agar-based MIC method). Although organisms other than staphylococci might not grow adequately in those wells, it is of little significance because these antibiotics are primarily antistaphylococcal drugs. NCCLS document M11 is an agar dilution reference standard for anaerobic bacteria.

Section 5.2.3.4 describes commercially available microdilution systems that contain either frozen or dried antimicrobial agents. These may also be used as a reference method as long as they adhere to standard NCCLS methodologies.

5.1.1 Reporting of Results

For laboratories utilizing dilution-MIC methods, the interpretive category should always be reported and if desired, the specific MIC can be reported along with the interpretive result. (This lessens the chances of clinical errors from mis- or overinterpretation of exact MICs.)

5.1.2 Rapid β-lactamase Test

A rapid β-lactamase test can clarify the susceptibility test results of staphylococci to penicillin determined by broth microdilution, especially in strains with borderline MICs (0.06 to 0.25 μg/mL). Detection of β-lactamase production in enterococci is possible only by a direct β-lactamase test. Do not test members of the Enterobacteriaceae, *Pseudomonas* spp., and other aerobic gram-negative bacilli because the results might not be predictive of susceptibility to the β-lactams most often used for therapy. Recommended β-lactamase test methods are indicated in Section 7.

5.2 Methodologies

5.2.1 Antimicrobial Agents

5.2.1.1 Source

Antimicrobial agent standards or reference powders can be obtained commercially, directly from the drug manufacturer, or from the United States Pharmacopeia (12601 Twinbrook Parkway, Rockville, MD 20852). Pharmacy stock or other clinical preparations are *not* to be used. Acceptable powders bear a label that states the drug's nonproprietary name, its assay potency (usually expressed in micrograms [μg] or International Units [IU] per milligram [μg] of powder), and its expiration date. The powders are to be stored as recommended by the manufacturers, or at -20 °C or below in a desiccator (preferably in a vacuum). When the desiccator is removed from the freezer, it should be warmed to room temperature before it is opened (to avoid condensation of water).

5.2.1.2 Weighing Antimicrobial Agent Powders

All antimicrobial agents are assayed for standard units of activity. The assay units can differ widely from the actual weight of the powder, and they often differ within a drug production lot. Thus, a laboratory must standardize its antimicrobial agent solutions

based on assays of the lots of antimicrobial agent powders that are being used. Either of the following formulae may be used to determine the amount of powder or diluent needed for a standard solution:

$$\textit{Weight}\ (\text{mg}) = \frac{\textit{Volume}\ (\text{mL}) \cdot \textit{Concentration}\ (\mu\text{g/mL})}{\textit{Assay Potency}\ (\mu\text{g/mg})}$$

or

$$\textit{Volume}\ (\text{mL}) = \frac{\textit{Weight}\ (\text{mg}) \cdot \textit{Assay Potency}\ (\mu\text{g/mg})}{\textit{Concentration}\ (\mu\text{g/mL})}$$

The antimicrobial agent powder should be weighed on an analytical balance that has been calibrated with weights traceable to approved standard reference weights. If possible, more than 100 mg of powder is to be weighed. Usually, it is advisable to weigh accurately a portion of the antimicrobial agent in excess of that required and to calculate the volume of diluent needed to obtain the concentration desired.

Example: To prepare 100 mL of a stock solution containing 1,280 µg/mL of antimicrobial agent with antimicrobial agent powder that has a potency of 750 µg/mg, 170 to 200 mg of the antimicrobial agent powder is weighed accurately. If the actual weight is 182.6 mg, the volume of diluent needed is then:

$$\textit{Volume} = \frac{\underbrace{182.6\ \mu\text{g}}_{\text{(Actual Weight)}} \cdot \underbrace{750\ \mu\text{g/mg}}_{\text{(Potency)}}}{\underbrace{1{,}280\ \mu\text{g/mL}}_{\text{(Desired Concentration)}}}$$

Therefore, the 182.6 mg of antimicrobial agent powder is to be dissolved in 107.0 mL of diluent.

5.2.1.3 Preparing Stock Solutions

Antimicrobial agent stock solutions are to be prepared at concentrations of at least 1,000 µg/mL (for example: 1,280 µg/mL) or 10 times the highest concentration to be tested, whichever is greater. There are some antimicrobial agents, however, of limited solubility that can require lower concentrations.[18-20] In all cases, consider directions provided by the drug manufacturer as part of determining solubility.

Some drugs must be dissolved in solvents other than water. In such cases, it is necessary to:

(1) Use only enough solvent to solubilize the antimicrobial agent powder.

(2) Dilute to the final stock concentration with water or appropriate buffer as indicated in Table 6.[20]

Since contamination is extremely rare, solutions that have not been sterilized may be used. If, however, sterilized solutions are desired, they are to be filtered through a membrane filter. Paper, asbestos, or sintered glass filters, which may adsorb appreciable amounts of certain antimicrobial agents, are *not* to be used. Whenever filtration is used, it is important that the absence of adsorption by appropriate assay procedures be documented.

Small volumes of the sterile stock solutions are dispensed into sterile polypropylene or polyethylene vials, sealed carefully, and stored (preferably at -70 °C or colder). Vials are to be removed as needed and used the same day. Any unused drug is to be discarded at the end of the day. Stock solutions of most antimicrobial agents can be stored at -70 °C or colder for six months or more without significant loss of activity. In all cases, directions provided by the drug manufacturer are to be considered as a part of these general recommendations. Any deterioration of an antimicrobial agent may be ascertained from the results of susceptibility testing using quality control strains.

5.2.1.4 Number of Concentrations Tested

The concentrations to be tested for a particular antimicrobial agent should, at a minimum, encompass the breakpoints shown in Table 2. Unusual concentrations may be tested for special purposes (e.g., high concentrations of streptomycin and gentamicin may be tested to indicate whether either drug might have a synergistic effect with a penicillin against an enterococcus).

5.2.2 Agar-Dilution Procedure

The agar-dilution method for determining antimicrobial agent susceptibility is well established.[1, 21] The antimicrobial agent is

incorporated into the agar, with each plate containing a different concentration of the agent. The inoculum can be applied rapidly and simultaneously to the agar surfaces using an inoculum-replicating apparatus.[22] Commercial replicators that are currently available usually transfer 32 to 37 inocula to each plate.

If agar dilution tests are to be performed with fastidious organisms, the medium, quality control procedures and interpretive criteria must be modified to fit each organism. Agar dilution testing of *Haemophilus somnus* and *Actinobacillus pleuropneumoniae* has been shown to be reliable when using Veterinary Fastidious Medium (VFM). It is important to note that the direct inoculum suspension method of preparing the test inoculum must be used with these species. The direct inoculum suspension method consists of making a direct broth or saline suspension of colonies selected from 18- to 24-hour agar plates (a nonselective medium, such as blood agar or chocolate agar should be used). The suspension is adjusted to match the 0.5 McFarland turbidity standard as outlined in Section 5.2.2.6. The medium and important technical aspects of testing these fastidious species are described in relevant sections above and outlined in Table 5. The quality control ranges for these two fastidious organisms are indicated in Table 4A.

5.2.2.1 Reagents and Materials

Mueller-Hinton agar is recommended for routine susceptibility testing of aerobic and facultative anaerobic bacteria. To avoid some of the problems with this medium in supporting growth of some fastidious organisms, various supplements are added (see Table 5). New lots of Mueller-Hinton agar are to be performance tested before use as outlined in Section 6. Media are used that have been tested according to, and meet the acceptance limits described in NCCLS document M6—*Protocols for Evaluating Dehydrated Mueller-Hinton Agar.*

Test media are to be prepared from a dehydrated base in accordance with the manufacturer's recommendations. After the agar is autoclaved, it is cooled to 48 to 50 °C in a water bath before adding antimicrobial agent solutions and unautoclaved supplements. The agar is then poured into the appropriate plates. The liquid agar may also be dispensed into smaller sterile containers, these containers sealed, and the agar allowed to equilibrate to 48 to 50 °C in a water bath before antimicrobial agent solutions are added and the agar is poured into the plates.

The pH of each batch of test medium is to be checked after it is sterilized. If a pH is out of range, the procedure for preparing the medium should be investigated. To ensure that the pH of the medium is between 7.2 and 7.4 at 25 °C, the pH of the medium is measured after it gels. The measurement is made with a standard combination pH electrode or with a surface electrode. If a standard combination electrode is used, it is necessary to:

(1) Submerge it in a small amount of gelled medium that has been macerated, or

(2) Allow a small amount of liquid agar in a small beaker to solidify around the electrode.

In either case, the electrode is supported so that its measuring tip does not press against the container walls. A thermometer is placed into the agar to determine the temperature.

Unlike the broth method, supplemental cations, (e.g., Ca^{++} and Mg^{++}) are not to be added to Mueller-Hinton agar. Mueller-Hinton agar may be supplemented with 5% (v/v) defibrinated sheep, rabbit, bovine or horse blood. The pH is checked after addition of the supplements. Autoclaving is not to be performed after these additions are made. These supplemented media are used only for:

- Testing those organisms that do *not* grow on the unsupplemented medium
- Appropriate control strains, which must be tested at the same time.

Information on dilution tests for some important fastidious or problem organisms is presented in Table 5.

5.2.2.2 Preparing Agar Plates

The procedure for preparing agar plates includes the following steps:

(1) Add appropriate dilutions of antimicrobial agent solution to molten test media that

have been equilibrated in a water bath to 48 to 50 °C.

(2) After mixing the agar and antimicrobial agent solution thoroughly, pour the mixture into petri dishes on a level surface as quickly as possible to prevent cooling and partial solidification in the mixing container (the agar should be approximately 4-mm deep [see Section 4.3.1.4]).

(3) Allow the agar to solidify at room temperature and use the plates immediately, or store them in sealed plastic bags at 2 to 8 °C for up to five days for reference work, or longer for routine tests (see Section 4.3.1.4). (It cannot be assumed that new antimicrobial agents will maintain their potency under these storage conditions. The user should evaluate the stability of these agar plates from results obtained with reference strains and should develop applicable shelf-life criteria. Data generated by drug manufacturers should be consulted for this information if it is available.)

(4) After storage, allow the agar plates to equilibrate to room temperature before using them.

(5) Ensure that the agar surface is dry before inoculating the plates (see Section 4.3.1.6).

5.2.2.3 Dilution Scheme for Reference Method

Although several dilution schemes can be appropriate for routine agar dilution testing, a scheme in which one part of antimicrobial solution is added to nine parts of liquid agar should be used for the reference method (Table 7).

5.2.2.4 Reference Work

For reference work, the agar plates should be used within five days. However, the appropriate reference control strains (Table 4) are to be tested each time tests are performed to ensure that no deterioration of antimicrobial agents has occurred. β-lactams and tetracyclines are generally the drugs most susceptible to deterioration. For routine tests, it is not necessary to limit storage to this time period as long as reference strains are used concurrently to control the tests as described in Section 6.5.

5.2.2.5 Control Agar Plates

Drug-free control agar plates (with or without supplements) are to be prepared.

5.2.2.6 Growth Method Inoculum Preparation

Because variations in the density of inoculum can alter the end points by one or more concentrations or dilutions, the preparation of a standardized inoculum is critical. It is necessary to:

- Select at least four or five well-isolated colonies of the same morphological type from the agar plate. Touch the top of each colony with a wire loop (or nontoxic cotton or dacron swab) and transfer growth to a tube containing 4 to 5 mL of a suitable broth medium, such as tryptic soy broth. (For *H. somnus* and *A. pleuropneumoniae,* prepare the inoculum in accordance with the procedures described in Section 4.3.5.3.) Allow the broth culture to incubate at 35 °C until it achieves or exceeds the turbidity of the standard described in Section 4.3.3. Adjust the density of this culture to a turbidity equivalent to that of a 0.5 McFarland standard by adding sterile broth, saline, or distilled water. Staphylococci should be tested by the direct inoculum procedure.
- Make the adjustment with an adequate light source and, to aid in the visual comparison, examine the fluid in the tubes against a white background with contrasting black lines. Turbidity adjusted to this standard contains approximately 1 to 2 x 10^8 CFU/mL. Most inoculum replicators deposit approximately 0.1 (1 mm pin) to 2.0 (3 mm pin) µL on the agar surface. If a 3-mm pin is used, dilute the adjusted suspensions 1:10 in sterile broth or saline to obtain the desired inoculum concentration of 1.0 x 10^7 CFU/mL. The final inoculum on the agar will then be approximately 1.0 x 10^4 (for the 1 mm pin) to 2.0 x 10^4 (for the 3 mm pin) CFU/mL in an agar area of 2 to 5 mm in diameter.
- If desired, use of photometric or other alternative systems to adjust the inoculum density of the culture has been demon-

so that, if another broth were to be selected, most of the previous studies would have to be repeated. Mueller-Hinton broth may be supplemented to support the growth of fastidious bacteria. Blood or blood constituents may be added for testing certain streptococci. Recommendations for susceptibility testing of *A. pleuropneumoniae* and *H. somnus* are in Section 5.2.3.2.

MIC performance and chemical characteristics of the broth are to be monitored routinely. The pH of each batch of Mueller-Hinton broth is to be checked with a pH meter when the medium is prepared; the pH should be between 7.2 and 7.4 at room temperature (25 °C). MIC performance characteristics of each batch of broth are evaluated using a standard set of quality control organisms (see Section 6).

Unless Mueller-Hinton broth has the correct concentrations of the divalent cations Ca^{++} and Mg^{++} (20 to 25 mg of Ca^{++}/L and 10 to 12.5 mg of Mg^{++}/L), MICs of aminoglycosides for *P. aeruginosa* and MICs of tetracycline for all bacteria will be different from those obtained on Mueller-Hinton agar.[15] Many manufacturers provide Mueller-Hinton broth that has already been adjusted. Therefore, the instructions for cation adjustments (see Section 5.2.3.1.1) should be followed only when the initial Mueller-Hinton broth has been certified by the manufacturer or measured by the user to contain no or inadequate amounts of Ca^{++} and Mg^{++} when assayed for total divalent cation content by atomic absorption spectrophotometry. Adding excess cations to Mueller-Hinton broth may result in erroneous results. Reliable susceptibility test results are achievable by disk diffusion or agar dilution with Mueller-Hinton agar that meet performance criteria specified in NCCLS document M6.

If a new lot of broth does not yield the expected MICs, the cation content is investigated or another lot is obtained. To determine the suitability of the medium for sulfonamide and trimethoprim tests, MICs are performed with *Enterococcus faecalis* ATCC® 29212 or *Enterococcus faecalis* ATCC®33186 (a more thymidine-sensitive strain). The end points should be easy to read (either as no growth or approximately 80% reduction as compared to the control). If MICs obtained with thymidine-free medium are ≤0.5 µg/mL, the medium is probably adequate for clinical use. If needed, thymidine phosphorylase or lysed horse blood (which contains thymidine phosphorylase) may be added to the medium to aid in tests with sulfonamides.[23]

In a routine setting, such as the clinical microbiology laboratory, some antimicrobial agent-broth combinations may require more frequent quality control tests than once weekly because of relatively rapid degradation of the drug.

5.2.3.1.1 Cation Adjustments[15]

The procedure for preparing magnesium stock solution is to dissolve 8.36 g of $MgCl_2 \cdot 6H_2O$ in 100 mL of deionized water. This solution will contain 10 mg of Mg^{++}/mL.

For preparing calcium chloride stock solution, the procedure is to dissolve 3.68 g of $CaCl_2 \cdot 2H_2O$ in 100 mL of deionized water. This solution will contain 10 mg of Ca^{++}/mL.

Stock solutions are sterilized by membrane filtration and stored at 4 °C.

Mueller-Hinton broth is prepared as the manufacturer directs, autoclaved, and chilled overnight at 4 °C or in an ice bath if it is to be used the same day.

With stirring, 0.1 mL of chilled Ca^{++} or Mg^{++} stock solution per liter of broth is added for each desired increment of 1 mg/L in the final concentration of Ca^{++} or Mg^{++} in the adjusted Mueller-Hinton broth. This medium is designated cation-adjusted Mueller-Hinton broth (CAMHB). Adjustments of Ca^{++} or Mg^{++} or both are not necessary when Mueller-Hinton broth from the manufacturer already contains the correct concentrations (20 to 25 mg of Ca^{++}/L and 10 to 12.5 mg of Mg^{++}/L) of divalent cations.

5.2.3.2 Broth Dilution Susceptibility Testing of Fastidious Organisms

The Mueller-Hinton broth used to test rapidly growing aerobic pathogens is not adequate for testing many of the fastidious organisms encountered in veterinary medicine. When a medium has been defined for testing a fastidious organism, quality control procedures

and interpretive criteria must be established for testing that organism in that medium.

5.2.3.2.1 Microdilution Broth Testing of *Haemophilus somnus* and *Actinobacillus pleuropneumoniae*

The medium of choice for microdilution broth testing of these organisms is veterinary fastidious medium (VFM). One liter of this medium consists of the following ingredients:

Mueller-Hinton broth (22.0 g)	
+ Beef Extract	3.0 g (from 300 gm of beef infusion)
+ Acid Hydrolysis of Casein	17.5 g
+ Starch	1.5 g
Yeast Extract (20.0 g)	
Lysed horse blood (20.0 mL)	
Supplement C™[b] (yeast concentrate) (20.0 mL)	

Unless the Mueller-Hinton broth has the correct concentrations of divalent cations, Ca^{++} and Mg^{++}, appropriate salts need to be added to provide 20 to 25 mg per liter of calcium and 10 to 12.5 mg per liter of magnesium.

The Mueller-Hinton broth, with yeast extract (water-soluble portion of autolyzed yeast containing vitamin B complex), is mixed with 95.5% of total water volume and steam sterilized. It is then cooled to 8°C and the lysed horse blood and Supplement C™ are added aseptically. The blood is laked by three cycles of alternating freezing (at -20° or lower) and thawing followed by centrifugation at 3000 x g for 20 minutes for clarification.

5.2.3.2.2 Standardizing the Inoculum for Broth Dilution testing of *Haemophilus somnus* and *Actinobacillus pleuropneumoniae.*

Direct colony suspensions should be used when testing. Using colonies taken directly from an overnight (20 to 24 hours) chocolate agar culture plate, a suspension of the test organism is prepared in sterile Mueller-Hinton broth, water or 0.9% saline. The turbidity should be adjusted to a turbidity equivalent to a 0.5 McFarland standard using a photometric or commercial volumetric inoculum-preparation device. When a volumetric inoculum device is used, the manufacturer's instructions should be followed explicitly. This suspension may then be diluted as described in Section 5.2.2.6 to obtain a desired final concentration of 5×10^5 CFU/mL in each well or tube. Inoculum concentrations higher than 5×10^5 CFU/ml often lead to inappropriately high MICs with certain cephalosporin antimicrobial agents. Because of this inoculum effect determination of colony forming units should be performed on a regular basis (e.g., once weekly) to ensure that the final inoculum concentration is approximately 5×10^5 CFU/mL. The procedure for determining colony-forming units may be found in Section 5.2.3.5 (3).

Acceptable quality control ranges for these two organisms are indicated in Table 4A.

5.2.3.3 Methicillin-Resistant Staphylococci

See Section 4.3.7 for testing of methicillin-resistant staphylococci.

5.2.3.4 Preparing and Storing Diluted Antimicrobial Agents

- Macrodilution (Tube) Broth Method

For this method, it is necessary to:

- Use sterile 13 x 100-mm test tubes to perform the tests.
- Use a control tube containing broth without antimicrobial agent for each organism tested.
- Close the tubes with cotton plugs, loose screw caps, or plastic or metal caps.

When twofold dilutions are used, they may be prepared volumetrically in broth. The subcommittee recommends consulting the schedule in Table 7 when running a small number of tests. The total volume of each antimicrobial agent dilution to be prepared depends upon the number of tests to be performed. A minimum of 1 mL of each antimicrobial agent dilution is needed for each antimicrobial agent tested. A single pipet is used to prepare all diluents and then for adding the stock antimicrobial agent solution to the first tube. A separate pipet is used for each remaining dilution in that set. Because there will be a 1:2 dilution of the drugs when an equal volume of inoculum is

[b] Supplement C is a registered trademark of Difco.

used, the antimicrobial agent solutions are prepared double strength.

- Microdilution Broth Method

This method is named "microdilution" because it involves the use of small volumes of broth. Sterile plastic microdilution trays that have round or truncated-v bottom wells, each containing 0.05 to 0.1 mL of broth are used. The drugs may be diluted as described in Section 5.2.1 and in Table 7.

Microdilution trays can be made in the laboratory, or purchased commercially ready to use. If made in the laboratory, one of the available systems that automatically fills the wells can be obtained. In these systems, the desired media and antimicrobial agents are made in larger volumes and a dispenser automatically fills each well of the tray with the desired volume. Two types of commercial trays have been made. In one, the media-antimicrobial solutions are dispensed into trays and frozen for delivery to the user.[24] The user stores them in the frozen state (preferably at -70 °C or colder) and thaws them as needed. The other kind of commercial tray has dried antimicrobial agents in its wells.[25] The agents are put into solution by adding diluent and inoculum together, or separately, to each well.

The antimicrobial dilutions are to be made so that each concentration is contained in at least 10 mL of broth and, with a dispensing device, the wells of the microdilution trays are filled with 0.1 (± 0.02) mL each from these tubes. If the inoculum is to be added by pipet as described in Section 5.2.3.6, the antimicrobial agent solution is prepared double strength and the wells are filled with 0.05 mL instead of 0.1 mL. The filled trays are sealed in plastic bags and immediately placed in a freezer at -70 °C or colder until needed. Although the antimicrobial agents in frozen trays usually remain stable for several months, if they are to be used for reference work, they are to be used within four weeks after they are prepared. Trays are not to be stored in a self-defrosting freezer or thawed solutions refrozen; repeated freeze-thaw cycles accelerate the degradation of some antimicrobial agents, particularly β-lactams.

5.2.3.5 Standardizing Inoculum for Broth Dilution Testing

To standardize inoculum, follow these procedures:

(1) With the exception of *A. pleuropneumoniae, H. somnus,* and possible methicillin-resistant staphylococci, adjust the turbidity of the inoculated broth culture (see Section 5.2.2.6) to obtain a turbidity visually comparable to that of the 0.5 McFarland turbidity standard. Dilute the adjusted culture in broth (macrodilution method) or sterile water, saline, or broth (microdilution method) so that, after inoculation, each well or tube contains approximately 5 x 10^5 CFU/mL. The dilution procedure to obtain this final inoculum will vary according to the method and must be calculated for each system. The exact inoculum volume delivered to the wells must be known before this calculation can be done. For example, if the volume of medium in the well is 0.1 mL and the inoculum is 0.05 mL, then dilute the adjusted culture (10^8 CFU/mL) 1:100 with water, or saline, to yield 10^6 CFU/mL. When 0.05 mL of this suspension (10^6 CFU/mL) is inoculated into the broth, the final concentration of bacteria will be approximately 5 x 10^5 CFU/mL (or 5 x 10^4 CFU/well).

(2) With *A. pleuropneumoniae* and *H. somnus*, prepare a suspension of test organism in sterile Mueller-Hinton broth, water or 0.9% saline using colonies taken directly from an overnight (20 to 24 hour) culture. This suspension should be adjusted to a turbidity equivalent to a 0.5 McFarland standard using a photometric or commercial volumetric inoculum-preparation device. When a volumetric inoculum device is used, the manufacturer's instructions should be followed explicitly. The precise concentration of organisms in the starting suspension will depend on the conditions of incubation of the overnight blood agar culture; in particular, the length of incubation. The user should be aware that some *A. pleuropneumoniae* strains are adherent and, thus, it can be difficult to obtain a consistent inoculum.

The desired final inoculum concentration of 5 x 10^5 CFU/mL in each well or tube is achieved from the initial inoculum suspension as described in Section 5.2.3.5.(1). Inoculum concentrations higher than 5 x 10^5 CFU/mL often lead to inappropriately high MICs with certain cephalosporin antimicrobial agents.

(3) Laboratories are encouraged to determine colony-forming units on inoculum suspensions periodically to ensure that the final inoculum concentration routinely obtained approximates 5 x 10^5 CFU/mL. This can be easily accomplished by removing a 0.01-mL aliquot from the growth-control well or tube immediately after inoculation and diluting it in 10 mL of 0.9% saline. After mixing, a 0.1-mL aliquot is spread over the surface of a suitable agar medium. Following incubation, the presence of approximately 50 colonies would indicate an inoculum density of 5 x 10^5 CFU/mL.

5.2.3.6 Inoculating Broth

- Macrodilution (Tube) Broth Method

Before adjusting the inoculum, 1 mL of the various antimicrobial agent concentrations is placed in 13 x 100-mm tubes (a growth control without antimicrobial agent is included). Within 15 minutes after the inoculum has been standardized, 1 mL of the adjusted inoculum is added to each tube in the dilution series and mixed. This results in a 1:2 dilution of each antimicrobial agent concentration and a 1:2 dilution of the inoculum.

- Microdilution Broth Method

Each well of a freshly prepared or thawed tray is inoculated with a 0.05-mL pipet dropper or an inoculum replicator. As in macrodilution, the inoculum is diluted and the broth is inoculated within 15 minutes after the inoculum is standardized. If the volume of the inoculum exceeds 10% of the volume of the well, the diluting effect of the inoculum on the antimicrobial agent must be taken into account. If a pipet is used to inoculate the broth in a well, the resulting dilution is usually 1:2 (0.05 mL antimicrobial solution + 0.05 mL inoculum); but if a replicator is used, the resulting dilution is usually negligible (generally <5 µL of inoculum in 0.1 mL antimicrobial solution). To prevent drying, each tray is sealed in a plastic bag, with plastic tape, or with a tight-fitting plastic cover before it is incubated.

- Incubation

In both the macrodilution and the microdilution broth methods, the tubes or trays are incubated at 35 °C for 16 to 20 hours in a forced air incubator except with *H. somnus, A. pleuropneumoniae* and methicillin-resistant staphylococci (see Table 5). When testing *H. somnus* and A. *pleuropneumoniae,* tubes or trays should be incubated for a total of 20 to 24 hours before determining results. With methicillin-resistant staphylococci, the direct inoculum method is recommended, and incubation should be for a full 24 hours. To maintain the same incubation temperature for all cultures, microdilution trays are not to be stacked more than four high.

- Interpreting Results

The MIC is the lowest concentration of antimicrobial agent that completely inhibits growth of the organism as detected by the unaided eye. Viewing devices that make it easier to read microdilution tests are available. The amount of turbidity in the wells or tubes containing the antimicrobial agent is compared with the amount of turbidity in the growth-control wells or tubes (no antimicrobial agent) used in each set of tests. Microdilution MICs for gram-negative bacilli tend to be the same or one $\log_2$ dilution lower than the comparable macrodilution MICs.[8]

6 Quality Control Guidelines

6.1 Purpose

The goals of a quality control program are to monitor the following:

- The precision and accuracy of the susceptibility test procedure.
- The performance of reagents and the viability of the microorganisms used in the test.
- The performances of persons who carry out the tests and interpret the results.

The goals are best realized by, but not limited to, the use of reference strains selected for their genetic stability and for their usefulness in the particular method being controlled.

6.2 Quality Control Responsibilities

6.2.1 Responsibilities of the Manufacturer (Commercial and/or "In-house" Products)

The following are the responsibilities of the manufacturer (commercial and/or in-house products):

- Antimicrobial agent stability
- Antimicrobial agent identification
- Potency of antimicrobial agent
- Compliance with good manufacturing practices
- Integrity of product
- Accountability and traceability to consignee
- Batch-to-batch media uniformity
- Media preparation
- Potency of antimicrobial agent stock solutions.

Manufacturers should design and recommend a quality control program that allows the user to evaluate those variables (e.g., inoculum levels, storage/shipping conditions) that most likely will cause user performance problems and to determine that the assay is performing accurately when used according to directions for use.

6.2.2 Responsibilities of the Laboratory (User)

The following are the responsibilities of the laboratory (user):

- Storage (drug deterioration)
- Personnel proficiency
- Adherence to procedure (e.g., inoculum, incubation conditions [time, temperature, and atmosphere])
- Media storage

6.2.3 Batch or Lot Control

For batch or lot control, the procedure is as follows:

(1) Test each new batch or lot of media, lot of disks, macrodilution tubes, microdilution trays, or agar dilution plates with the appropriate reference strains to determine if zone sizes or MICs obtained with the media/batch fall within the expected range (see Table 3); if they do not, reject the media/batch.

(2) Incubate overnight at least one uninoculated tube, microdilution tray, or agar plate from each batch to ascertain the sterility of the medium.

(3) Variations in divalent cations, principally magnesium and calcium, will affect results with aminoglycosides and tetracyclines with *P. aeruginosa* strains. Excessive cation content will reduce zones sizes, whereas low cation content may result in unacceptably large zones of inhibition. Performance tests with each lot of Mueller-Hinton agar and broth must conform to the control limits in Tables 3 and 4 using *Pseudomonas aeruginosa* ATCC® 27853. Most media manufacturers are aware of the need for cation supplementation and have adjusted their medium accordingly. Such supplementation is indicated on the medium label. However, if a medium requires an increase in cation concentration, follow the procedure outlined in Section 5.2.3.1.1.

(4) Keep a record of the lot numbers of all of the materials and reagents used in these tests.

6.3 Agar Disk Diffusion Tests

6.3.1 Frequency of Testing

Each time a new lot of Mueller-Hinton agar or a new lot of susceptibility disks is introduced, it must be tested with appropriate control strains. Ideally, testing appropriate control strains each day that susceptibility tests are performed is the preferred method of analyzing the overall performance of susceptibility testing. Because this is not always practical, the frequency of test monitoring can be performed less frequently if the laboratory can document that its susceptibility testing procedures are reproducible. To document that the laboratory can provide reproducible susceptibility testing

results,. the laboratory should perform daily quality control tests. A form is provided in the Appendix that can be used to record susceptibility test results for the quality control organisms. To demonstrate the ability to generate reproducible susceptibility testing results, the following procedure should be sed:

(1) Test all applicable control strains for 30 consecutive test days.

(2) For each drug/microorganism combination, no more than 3 of the 30 zone diameters (i.e., zone diameters obtained from one drug/microorganism combination for 30 consecutive test days) may be outside the accuracy control limits stated in Table 3.

Once the laboratory demonstrates the reproducibility of their susceptibility testing procedure by the above criteria, it may begin testing each quality control strain on a weekly basis. When using once-a-week testing, if a zone diameter falls outside the acceptable control limits for any drug/microorganism combination, the laboratory must determine the cause of such an error. If the out-of-control result is due to an obvious error, e.g., use of the wrong disk, the wrong control strain, obvious contamination of the strain, or incorrect atmosphere of incubation, the quality control test should be repeated. If however, the aberrant result is not due to an obvious error, the laboratory should return to daily control testing long enough to define the source of the aberrant results and to document resolution of the problem. Resolution of the problem may be documented by the following actions:

Testing with appropriate quality control strains for five consecutive test days.

Documenting that, for each of these drug/microorganism combinations, the five zone diameters (i.e., zone diameters obtained from one drug/microorganism combination for five consecutive test days) were within the acceptable ranges, as defined in Table 3.

If the problem cannot be resolved immediately (i.e., at least one zone diameter is still observed outside the acceptable control limits), daily control tests must be continued until final resolution of the problem can be achieved. To return to weekly quality control testing in the future will require documentation of satisfactory performance for another 30 consecutive test days.

Results for individual antimicrobial agents for which the quality control strain values are outside the control ranges should not be reported. The tests should be repeated and results reported only when quality control organisms yield values within the accepted ranges.

6.3.2 Zone Size Limits

Listed in Table 3 are the minimum and maximum zone diameters that should be observed with a single control test. In general, when performing susceptibility testing, either on a daily or weekly basis, 1 out of 20 consecutive tests for each drug/microorganism combination might be out of control (i.e., outside the stated accuracy control limits). Any more than one out-of-control result in 20 consecutive control tests requires corrective action (i.e., if a second out-of-control result occurs, corrective action must be taken).

6.3.3 Reference Strains

To control the precision and accuracy of the test procedure, the procedure is as follows:

(1) Maintain stock cultures (see Section 6.5.2) of *Staphylococcus aureus* ATCC® 25923, *Escherichia coli* ATCC® 25922, and *Pseudomonas aeruginosa* ATCC® 27853 from the ATCC or derivatives of these cultures obtained from reliable sources.

(2) Test these reference organisms by the previously described procedure using antimicrobial disks representative of those used to test clinical isolates.

(3) A β-lactamase producing *Escherichia coli* ATCC® 35218 has been designated for quality control of disks containing combinations of β-lactams and β-lactamase inhibitors. When used in conjunction with *Escherichia coli* ATCC® 25922, both components of the combination disks can be monitored (see Section 7).

in a series of 20 tests for each drug/microorganism combination might be out of control (i.e., outside the stated range). If two out-of-control results occur within the series of 20 consecutive tests, corrective action must be taken. When corrective action is taken, the count of 20 begins again.

6.4.3 Other Control Procedures

6.4.3.1 Growth Control

Each microdilution broth tray, macrodilution broth series, and agar dilution series should include a growth control of basal medium without antimicrobial agent to assess viability of the test organisms. With the broth tests, the growth control also serves as a turbidity control for determining end points.

6.4.3.2 Purity Control

A sample of each inoculum is streaked on a suitable agar plate and incubated overnight to detect mixed cultures and to provide freshly isolated colonies in case retesting proves necessary. This step is particularly important for broth dilutions where mixed cultures are likely to be unrecognized.

6.4.3.3 Inoculum Control

Plate counts are performed on representative inocula periodically to ensure that the $BaSO_4$ standard and the procedures for standardizing and diluting inoculum remain under control. Samples for plate counts are removed immediately from the positive control well of microdilution trays, the positive control tube of a macrodilution series, or (in agar dilution) from a random reservoir well of the replicator seed plate.

6.4.3.4 End Point Interpretation Control

End point interpretation is monitored periodically to minimize variation in the interpretation of MIC end points among observers. All laboratory personnel who perform these tests should read independently a selected set of dilution tests. The results from each person's readings are recorded and compared. Where aberrant results occur, the source of these results should be determined and corrected.

6.5 Suggested Reference Strains

6.5.1 Selecting Reference Strains

Reference strains are obtained from a reliable source (for example, from the American Type Culture Collection (ATCC), reliable commercial sources, or institutions with demonstrated reliability to store and use the organisms correctly).

A full set of quality control strains that have adequate or optimal zone sizes or end points for all the commonly used antibiotics is not yet available. There are however, a number of strains that have been tested repeatedly over the years and have proved to be genetically stable.

- Many reference strains used in the standard agar diffusion method (see Section 6.3.3) have also been used often as reference strains for dilution susceptibility tests.[27] The *Staphylococcus aureus* ATCC® 25923 strain, however, is of little value in dilution testing because of its extreme susceptibility to the drugs it is used to monitor. The *Pseudomonas aeruginosa* ATCC® 27853 strain develops resistance to carbenicillin after repeated transfers onto laboratory media, but this problem can be circumvented by removing a new culture from storage at appropriate intervals, or whenever the strain begins to show resistance. Additional strains that may be used for the dilution tests include *Escherichia coli* ATCC® 25922 and *Staphylococcus aureus* ATCC® 29213, which is a weak β-lactamase-producing strain.
- Currently, the subcommittee recommends the following reference strains for controlling susceptibility tests:

— *Escherichia coli* ATCC® 25922

— *Escherichia coli* ATCC® 35218

— *Pseudomonas aeruginosa* ATCC® 27853

— *Staphylococcus aureus* ATCC® 29213 (dilution)

— *Staphylococcus aureus* ATCC® 25923 (disk diffusion)

- *Enterococcus faecalis* ATCC® 29212
- *Enterococcus faecalis* ATCC® 33186
- *Actinobacillus pleuropneumoniae* ATCC® 27090
- *Haemophilus somnus* ATCC® 700025.

Tables 3 and 4 indicate the expected zone sizes and MICs (obtained by either agar dilution or CAMHB dilution) of various antimicrobial agents for these strains. With repeated testing, more than 95% of the zone sizes and MICs should fall within the ranges reported in Tables 3 and 4. Most of the MICs should be at values close to the center of the pertinent range. *Escherichia coli* ATCC® 35218 is recommended as the control organism for β-lactamase inhibitor combinations, such as those containing clavulanic acid or sulbactam. As an alternative, *Acinetobacter baumanii* (ATCC® pending) might be more appropriate for some β-lactam and β-lactamase inhibitor combinations.

Use such cultures to monitor precision and accuracy as long as there is no significant change in the mean zone diameter or MIC that cannot be attributed to methodology. Such a significant change indicates contamination or change in the organism's inherent susceptibility. Obtain fresh cultures if such changes occur.

When sulfonamides, trimethoprim, or trimethoprim/sulfamethoxazole are tested routinely, monitor each new lot of Mueller-Hinton agar for unsatisfactory levels of inhibitors. Perform the tests with *Enterococcus faecalis* ATCC® 29212 or *Enterococcus faecalis* ATCC® 33186 (a more thymidine-sensitive strain). This is especially important for some veterinary pathogens such as *Staphylococcus hyicus* or *Pasteurella haemolytica*. Satisfactory media will produce a zone of inhibition of 20 mm or more (see Section 4.3.1.2).

When selecting reference strains for dilution testing, select strains that have MICs that fall near the midrange of the concentration for all antimicrobial agents tested. An ideal control strain is inhibited at the fourth dilution of a seven-dilution $\log_2$ series, but strains with MICs at either the third or fifth dilution are acceptable. When three or fewer adjacent doubling dilutions of an antimicrobial agent are tested with these methods, quality control methods must be altered. One possible alternative is to use one control organism whose modal MIC is equal to or no less than one doubling dilution of the lower concentration and a second control organism whose modal MIC is equal to or no greater than one doubling dilution of the higher concentration. The combination of these two results provides for at least one on-scale end point. For commercial systems, this strategy might be best used selectively by testing the most labile agents (e.g., clavulanic acid combinations, methicillin, imipenem, and cefaclor) included in the panels. Before a strain is accepted as a reference, it is to be tested for as long as is necessary to demonstrate that its antimicrobial agent susceptibility pattern is stable genetically (see NCCLS document M37—*Development of In Vitro Susceptibility Testing Criteria and Quality Control Parameters for Veterinary Antimicrobial Agents*). The strains listed in Tables 3 and 4 have proven useful for quality control.

6.5.2 Storing Reference Strains

Reference strains are stored to minimize the possibility of mutation in the organism. There are two preferred methods for prolonged storage of reference strains.[28]

- One is to suspend the organisms in a stabilizer (for example, defibrinated whole blood, 50% fetal calf serum in broth, or 10% glycerol in broth) and store them in a freezer at a temperature of lower than −20 °C (preferably -60 °C or lower) or in liquid nitrogen.
- The other preferred method is to lyophilize the organism.

An alternative method, which is used for short-term storage of stock cultures, is to subculture the organisms on tryptic soy agar slants, and store them at 2 to 8 °C. Fresh slants are prepared at two-week intervals. Whenever aberrant results occur, a new stock culture is obtained.

To prepare strains for storage, it is necessary to perform the following steps:

performance for another 30 consecutive test days, as outlined in Section 6.3.1 of this document.

7 β-Lactamase Tests

7.1 Purpose

A positive result to a β-lactamase test can predict resistance to penicillin as well as amino-, carboxy-, and ureido-penicillins with staphylococci and enterococci.

7.1.1 Selecting a β-Lactamase Test

Chromogenic cephalosporin-based tests (either liquid or paper disk formats) or equivalent methods provide reliable results with staphylococci and enterococci.[29, 30] Acidimetric penicillinase tests have produced unacceptably high error rates generally when used to test staphylococci, *Moraxella catarrhalis*, and some anaerobic bacteria.

The β-lactamase test is preferred to the disk diffusion and the agar dilution tests for detection of β-lactamase-mediated resistance among enterococci.

8 Cumulative Antimicrobial Susceptibility Profile

Clinicians can benefit greatly from periodic summaries of selected microbiological results. Tables summarizing the antimicrobial agent susceptibility profiles of the most commonly isolated pathogens and selected fastidious or slow-growing (difficult to test) isolates, by anatomic site, are useful in guiding empiric therapy and in following general trends in antimicrobial agent susceptibility within clinical practice. If cumulative antimicrobial agent susceptibility profiles are to be constructed, it is recommended that the percentage of isolates *susceptible* to each antimicrobial agent be used. To avoid introducing biases in the data, care should be taken to exclude identical strains from the same animal or large numbers of isolates from a single herd. Inclusion of antibiotic cost information can also be appropriate as an aid in the reduction of the expense of antimicrobial therapy.

Table 1. Suggested Groupings of Antimicrobial Agents That Should Be Considered for Routine Testing by Veterinary Microbiology Laboratories

		Cattle[a]	Swine	Horses	Dogs	Poultry[c]	Bovine Mastitis[d]
Group A – (US-FDA Approved Veterinary Specific Interpretive Criteria)	Primary Test and Report	Ceftiofur	Ceftiofur		Enrofloxacin[b]	Enrofloxacin[m,n]	Penicillin/novobiocin
		Tilmicosin			Spectinomycin		
Group B – (US-FDA Approved; NCCLS Approved Provisional Veterinary or Human Interpretive Criteria)	Primary Test and Report	Ampicillin[e] ✓	Ampicillin[e] ✓	Amikacin ✓	Amikacin ✓	Ceftiofur[n]	Ampicillin[e] ✓
		Erythromycin	Erythromycin	Ampicillin[e] ✓	Ampicillin[e] ✓	Erythromycin	Cephalothin[f,g]
		Penicillin	Gentamicin ✓	Ceftiofur	Amoxicillin/clavulanate	Gentamicin ✓	Erythromycin
		Sulfamethazine	Lincomycin[i]	Gentamicin ✓	Ceftiofur	Penicillin	Penicillin
		Sulfadimethoxine	Penicillin	Penicillin	Cephalothin[g]	Sarafloxacin[m,n]	Pirlimycin
		Sulfachlorpyridazine	Sulfadimethoxine	Sulfadimethoxine	Clindamycin[i]	Spectinomycin	Oxacillin[f]
		Tetracycline[k] ✓	Tetracycline[k] ✓	Trimethoprim/ ✓ sulfamethoxazole[j]	Chloramphenicol[h] ✓		Tetracycline[k] ✓
					Erythromycin	Sulfadimethoxine	
					Gentamicin ✓	Tetracycline ✓	
					Kanamycin ✓		
					Penicillin		
					Sulfadimethoxine		
					Tetracycline[k] ✓		
					Trimethoprim/ ✓ sulfamethoxazole[j]		
Group C – Supplemental	(Extra Label)	Amikacin ✓	Amikacin ✓	Erythromycin	Cefazolin	Trimethoprim/ ✓ sulfamethoxazole[j]	
		Gentamicin[l] ✓	Sulfamethoxine	Imipenem	Cefoxitin		
		Trimethoprim/ ✓ sulfamethoxazole[j]	Trimethoprim/ ✓ sulfamethoxazole[j]	Oxacillin	Imipenem		
					Tobramycin		
				Rifampin	Vancomycin		
				Tetracycline[k] ✓			
				Ticarcillin			
				Ticarcillin/clavulanic acid			
				Vancomycin			

Table 1. (Continued)

NOTE 1: This table contains those compounds used for therapeutic and control treatment of animal diseases. Compounds with prophylactic or prevention indications are not listed.

NOTE 2: Selection of the most appropriate antimicrobial agent to test and to report is a decision best made by each veterinary laboratory in consultation with pharmacy and veterinarians. Compounds listed in Group A and B are those compounds approved by the U.S. FDA for use in the United States for diseases in the indicated host animal. It is the responsibility of the laboratory client to ensure that compounds are used appropriately for host categories for each animal (e.g., lactating cows, calves) in accordance with the approved indication. Compounds listed in Group C are not approved but are frequently used in an extra-label manner in the indicated animal. The laboratory client assumes all responsibility for efficacy, safety, and residue avoidance with extra-label use of antimicrobial agents.

NOTE 3: Information in boldface type is considered provisional.

Footnotes

a. Does not include goats or sheep.
b. Includes cats.
c. Includes chickens and turkeys.
d. Compounds listed are for lactating dairy cattle only.
e. The results of ampicillin susceptibility tests may be used to predict susceptibility to amoxicillin and hetacillin.
f. Oxacillin is used to detect methicillin-resistant staphylococci and is preferred to methicillin or cloxacillin. Methicillin-resistant staphylococci should be reported as resistant to all β-lactams, including cephalosporins and β-lactamase inhibitor combinations despite apparent *in vitro* susceptibility.
g. Cephalothin can be tested to represent the first-generation cephalosporins, such as cephapirin and cefadroxil. Cefazolin should be tested separately against the Enterobacteriaceae.
h. Chloramphenicol must not be reported with any food-producing animal.
i. Trimethoprim/sulfamethoxazole can be tested to represent the potentiated sulfonamides, including trimethoprim/sulfadiazine and ormetoprim/sulfamethoxine.
j. Clindamycin is tested as the class representative for both lincomycin and clindamycin.
k. Tetracycline is tested as the class representative for chlortetracycline and oxytetracycline.
l. Due to extended residue times, use of gentamicin in cattle should be carefully monitored.
m. The U.S. FDA has recommended that fluoroquinolones not be reported for extra-label use.
n. Approved for enteric disease treatment.

Table 2. Zone Diameter Interpretive Standards and Minimum Inhibitory Concentration (MIC) Breakpoints for Veterinary Pathogens

Antimicrobial Agent	Disk Content	Zone Diameter (mm)				MIC Breakpoint (µg/mL)			
		Susceptible	Intermediate	Flexible Label	Resistant	Susceptible	Intermediate	Flexible Label	Resistant
β-Lactams Penicillins									
Ampicillin[a,d]									
Enterobacteriaceae	10 µg	≥ 17	14-16		≤ 13	≤ 8	16		≥ 32
Staphylococci*	10 µg	≥ 29	—		≤ 28	≤ 0.25	—		≥ 0.5
Enterococci*	10 µg	≥ 17	—		≤ 16	≤ 8	—		≥ 16
Streptococci (not *S. pneumoniae*)*	10 µg	≥ 26	19-25		≤ 18	≤ 0.25	0.5-4		≥ 8
*Listeria monocytogenes**	—	—	—		—	≤ 2	—		≥ 4
Oxacillin[b,g]									
Staphylococci*	1 µg	≥ 13	11-12		≤ 10	≤ 2	—		≥ 4
Penicillin G[g]									
Staphylococci*	10 units	≥ 29	—		≤ 28	≤ 0.12	—		≥ 0.25
Enterococci*	10 units	≥ 15	—		≤ 14	≤ 8	—		≥ 16
S. pneumoniae[i]	1 µg oxacillin	≥ 20	—		—	≤ 0.06	0.12-1		≥ 2
Streptococci (not *S. pneumoniae*)*[i]	10 units	≥ 28	20-27		≤ 19	≤ 0.12	0.25-2		≥ 4
*Listeria monocytogenes**	—	—	—		—	≤ 2	—		≥ 4
Ticarcillin									
*Pseudomonas aeruginosa**	75 µg	≥ 15	—		≤ 14	≤ 64	—		≥ 128
Gram-negative enteric organisms*	75 µg	≥ 20	15-19		≤ 14	≤ 16	32-64		≥ 128
β-Lactam/β-Lactamase Inhibitor Combinations									
Amoxicillin/clavulanic acid									
Canine (dermal, UTI)									
Staphylococci*	20/10 µg	≥ 20	—		≤ 19	≤ 4/2	—		≥ 8/4
Other organisms*	20/10 µg	≥ 18	14-17		≤ 13	≤ 8/4	16/8		≥ 32/16
Ticarcillin/clavulanic acid									
*Pseudomonas aeruginosa**	75/10 µg	≥ 15	—		≤ 14	≤ 64/2	—		≥ 128/2
Gram-negative enteric organisms*	75/10 µg	≥ 20	15-19		≤ 14	≤ 16/2	32-64		≥ 128/2
Cephalosporins									
Cephalothin*[c,g]	30 µg	≥ 18	15-17		≤ 14	≤ 8	16		≥ 32
Cefazolin*	30 µg	≥ 18	15-17		≤ 14	≤ 8	16		≥ 32
Cefoxitin*	30 µg	≥ 18	15-17		≤ 14	≤ 8	16		≥ 32

Table 2. (Continued)

Antimicrobial Agent	Disk Content	Zone Diameter (mm)				MIC Breakpoint (µg/mL)			
		Susceptible	Intermediate	Flexible Label	Resistant	Susceptible	Intermediate	Flexible Label	Resistant
Ceftiofur									
Cattle (Bovine Respiratory Disease)	30 µg	≥ 21	18-20		≤ 17	≤ 2	4		≥ 8
Pasteurella haemolytica									
Pasteurella multocida									
Haemophilus somnus									
Swine (Swine Respiratory Disease)	30 µg	≥ 21	18-20		≤ 17	≤ 2	4		≥ 8
Actinobacillus pleuropneumoniae									
Pasteurella multocida									
Salmonella choleraesuis									
Streptococcus suis									
Carbapenems									
Imipenem	10 µg	≥ 16	14-15		≤ 13	≤ 4	8		≥ 16
Aminoglycosides									
Amikacin*	30 µg	≥ 17	15-16		≤ 14	≤ 16	32		≥ 64
Gentamicin*	10 µg	≥ 15	13-14		≤ 12	≤ 4	8		≥ 16
Kanamycin*	30 µg	≥ 18	14-17		≤ 13	≤ 16	32		≥ 64
Macrolides									
Erythromycin*									
Organisms other than streptococci	15 µg	≥ 23	14-22		≤ 13	≤ 0.5	1-4		≥ 8
Streptococci[f]	15 µg	≥ 21	16-20		≤ 15	≤ 0.25	0.5		≥ 1
Tilmicosin[i]									
Cattle (Bovine Respiratory Disease)	15 µg	≥ 14	11-13		≤ 10	≤ 8	16		≥ 32
Pasteurella haemolytica									
Tetracyclines									
Tetracycline*,44									
Organisms other than streptococci	30 µg	≥ 19	15-18		≤ 14	≤ 4	8		≥ 16
Streptococci[f]	30 µg	≥ 23	19-22		≤ 18	≤ 2	4		≥ 8
Quinolones									
Enrofloxacin									
Canine and feline (dermal, URI, UTI)	5 µg	≥ 23		17-22	≤ 16	≤ 0.5		1-2	≥ 4
Gram-negative enteric bacilli									
Staphylococcus spp.									
Other susceptible organisms									

Table 2. (Continued)

Antimicrobial Agent	Disk Content	Zone Diameter (mm)				MIC Breakpoint (µg/mL)			
		Susceptible	Intermediate	Flexible Label	Resistant	Susceptible	Intermediate	Flexible Label	Resistant
Tiamulin[k]									
Swine Respiratory Disease		—	—		—	≤ 8	—		≥ 16
Actinobacillus pleuropneumoniae									
Sulfonamides[a]	250 or 300 µg	≥ 17	13-16		≤ 12	≤ 256	—		≥ 512
Trimethoprim/Sulfamethoxazole[a,f]									
Organisms other than *S. pneumoniae*	1.25/23.75 µg	≥ 16	11-15		≤ 10	≤ 2/38	—		≥ 4/76
Streptococcus pneumoniae	**1.25/23.75 µg**	**≥ 19**	**16-18**		**≤ 15**	**≤ 0.5/9.5**	**1/19-2/38**		**≥ 4/76**
Vancomycin[a]									
Enterococci	30 µg	≥ 17	15-16		≤ 14	≤4	8-16		≥ 32
Streptococci[j]	30 µg	≥ 17	—		—	≤1	—		—
Other gram-positive organisms	30 µg	≥ 12	10-11		≤ 9	≤4	8-16		≥ 32

NOTE 1: Zone interpretive criteria and MIC breakpoints for antimicrobial agents with an asterisk and/or gray shading are human data taken from M2 and M7.
NOTE 2: Veterinary-specific interpretive guidelines are valid only for indicated organisms.
NOTE 3: Interpretive guidelines in bold are provisional.
NOTE 4: **Flexible label indicates the availability of FDA-approved labeling; this organism should be considered susceptible if appropriate dosing modifications found in the packaging insert are applied.**

Footnotes

a. Ampicillin is used to test for susceptibility to amoxicillin and hetacillin.
b. Oxacillin is used to test for susceptibility to methicillin, nafcillin, and cloxacillin.
c. Cephalothin is used to test all first-generation cephalosporins, such as cephapirin and cefadroxil. Cefazolin should be tested separately with the gram-negative enteric organisms.
d. Tetracycline is used to test for susceptibility to chlortetracycline, oxytetracycline, minocycline, and doxycycline.
e. Zone interpretive criteria for tiamulin are not available for 6-mm paper disks.
f. Trimethoprim/sulfamethoxazole is used to test for susceptibility to trimethoprim/sulfadiazine and ormetoprim/sulfadimethoxine. A breakpoint of ≤2/38 should be used for isolates from urinary tract infections. For systemic disease, isolates with MICs of ≤0.5/9/5 should be considered susceptible.
g. Available as an infusion product for treatment of bovine masititis during lactation.
h. Clindamycin is used to test for susceptibility to clindamycin and lincomycin. However, clindamycin can be more active against some staphylococcal strains.
i. Tilmicosin interpretive criteria are also applicable to *P. multocida*.
j. **The interpretive standards for *Streptococcus* spp. including *S. pneumoniae* only apply to disk susceptibility testing performed using Mueller-Hinton agar supplemented with 5% CO_2 and broth with 2 to 5% lysed horse blood.**
k. For control use, only in chickens and turkeys.

Table 3. Control Limits for Monitoring Antimicrobial Disk Susceptibility Tests With Veterinary Pathogens; Zone Diameter (mm) Limits for Individual Tests on Mueller-Hinton Medium Without Blood or Other Supplements

Antimicrobial Agent	Disk Contents	*Escherichia coli* ATCC® 25922	*Staphylococcus aureus* ATCC® 25923	*Pseudomonas aeruginosa* ATCC® 27853	*Escherichia coli* ATCC® 35218	*Streptococcus pneumoniae* ATCC® 49619
Amikacin	30 µg	19–26	20–26	18–26	—	—
Amoxicillin/clavulanic acid	20/10 µg	19–25	28–36	—	18–22	—
Ampicillin	10 µg	16–22	27–35	—	—	30–36[c]
Apramycin	**15 µg**	**15–20**	**17–24**	**13–18**	**—**	**—**
Cefazolin	30 µg	23–29	29–35	—	—	—
Cefoxitin	30 µg	23–29	23–29	—	—	—
Ceftiofur	**30 µg**	**26–31**	**27–31**	**14–18**	**—**	**—**
Cephalothin	30 µg	15–21	29–37	—	—	26–32[c]
Chloramphenicol	30 µg	21–27	19–26	—	—	23–27[c]
Clindamycin	2 µg	—	24–30	—	—	19–25[c]
Enrofloxacin	**5 µg**	**32–40**	**27–31**	**15–19**	**—**	**—**
Erythromycin	15 µg	—	22–30	—	—	25–30[c]
Gentamicin	**10 µg**	**19–26**	**19–27**	**16–21**	**—**	**—**
Imipenem	10 µg	26–32	—	20–28	—	—
Kanamycin	30 µg	17–25	19–26	—	—	—
Oxacillin	1 µg	—	18–24	—	—	8–12[c]
Penicillin G	10 units	—	26–37	—	—	—
Penicillin/novobiocin	10 units/30 µg	—	30–36	—	—	24–30[c]
Pirlimycin	2 µg	—	20–25	—	—	—
Premafloxacin	**1 µg**	**26–34**	**29–37**	**11–17**	**—**	**—**
Rifampin	5 µg	8–10	26–34	—	—	25–30[c]
Tetracycline	30 µg	18–25	24–30	—	—	27–31[c]
Ticarcillin	75 µg	24–30	—	22–28	—	—
Ticarcillin/clavulanic acid	75/10 µg	25–29	29–37	20–28	21–25	—
Tilmicosin[a]	15 µg	—	17–21[a]	—	—	—
Sarafloxacin	**5 µg**	**30–36**	**25–30**	**23–29**	**—**	**—**
Spectinomycin	**100 µg**	**21–25**	**13–17**	**10–14**	**—**	**—**
Sulfisoxazole	250 or 300 µg	15–23	24–34	—	—	—
Trimethoprim/ Sulfamethoxazole (1/19)[b]	1.25/23.75 µg	24–32	24–32	—	—	22–27[c]
Vancomycin	30 µg	—	17–21	—	—	20–27[c]

Table 3. (Continued)

NOTE 1: Information with gray shading is taken from NCCLS document M2.
NOTE 2: Information in boldface type is considered provisional.
NOTE 3: To determine whether the Mueller-Hinton medium contains excessive levels of thymine, an *E. faecalis* (ATCC® 29212 or 33186) should be tested with trimethoprim, sulfa compounds, or trimethoprim/sulfamethoxazole especially when testing *Staphylococcus hyicus* or *Pasteurella haemolytica*. An inhibition zone of ≥ 20 mm that is free of fine colonies is acceptable.

Footnotes

a. Quality control guidelines for tilmicosin were developed using Mueller-Hinton agar supplemented with 5% defibrinated sheep blood and incubated aerobically.

b. Very medium-dependent, especially with enterococci.

c. **These quality control ranges for *Streptococcus pneumoniae* ATCC® 49619 are applicable only to tests performed by disk diffusion using Mueller-Hinton agar supplemented with 5% defibrinated sheep blood, incubated in 5% CO_2.**

Table 4. (Continued)

Antimicrobial Agent	*Staphylococcus aureus* ATCC® 29213	*Enterococcus faecalis* ATCC® 29212	*Escherichia coli* ATCC® 25922	*Pseudomonas aeruginosa* ATCC® 27853	*Escherichia coli* ATCC® 35218	*Streptococcus pneumoniae*[b] ATCC® 49619
Premafloxacin	0.002-0.008	0.008-0.03	0.004-0.03	0.5-2	—	—
Rifampin	0.004-0.015	0.5-4	4-16	16-64	—	0.015-0.06
Sarafloxacin	0.06-0.25	0.5-2	0.008-0.03	0.12-1	—	—
Spectinomycin	64-256	64-256	8-64	256->512	—	—
Tetracycline	0.25-1	8-32	0.5-2	8-32	—	0.12-0.5
Ticarcillin	2-8	16-64	2-8	8-32	—	—
Ticarcillin/clavulanic acid	0.5/2-2/2	16/2-64/2	2/2-8/2	8/2-32/2	4/2-16/2	—
Tilmicosin	1-4[a]	≥ 32	≥64	>64	—	—
Tylosin	0.5-4	0.5-4	>32	>32	—	—
Tiamulin	0.5-2	—	—	—	—	—
Sulfisoxazole	32-128	32-128	8-32	—	—	—
Trimethoprim/ Sulfamethoxazole (1/19)	≤0.5/9.5	≤0.5/9.5	≤0.5/9.5	8/152-32/608	—	0.12/2.4-1/19
Vancomycin	0.5-2	1-4	—	—	—	0.12-0.5

NOTE 1: Information with gray shading is taken from NCCLS document M7.

NOTE 2: Information in boldface type is considered provisional.

NOTE 3: To determine whether the Mueller-Hinton medium contains excessive levels of thymidine or thymine, an *E. faecalis* (ATCC® 29212 or 33186) should be tested with trimethoprim, sulfa compounds, or trimethoprim/sulfamethoxazole especially when testing *Staphylococcus hyicus* or *Pasteurella haemolytica*. An inhibition zone of ≥ 20 mm that is free of fine colonies is acceptable. If excessive thymidine is present, an expected MIC within the susceptible category (trimethoprim/sulfamethoxazole MIC ≤ 0.5/9.5 µg/mL) will shift to the resistant category (trimethoprim/sulfamethoxazole MIC >4/76 µg/mL).

Footnotes

a. An MIC for tilmicosin of 0.5 or 8 µg/mL may indicate a medium or pH problem. The methodology should be reexamined.

b. These quality control ranges for *Streptococcus pneumoniae* ATCC® 49619 are applicable only to tests performed by the broth microdilution method using cation-adjusted Mueller-Hinton broth with 2 to 5% lysed horse blood.

Appendix. Antimicrobial Susceptibility Quality Control Record

ORGANISM TESTED_____ ATCC #_____

TEST METHOD: BROTH DILUTION/DISK DIFFUSION

ANTIMICROBIAL AGENT	DISK DIFFUSION CONTROL LIMITS*	DISK DIFFUSION TEST RESULTS	BROTH DILUTION CONTROL LIMITS*	BROTH DILUTION TEST RESULTS

*CONTROL LIMITS AS DEFINED IN TABLES 3, 3A, 4, and 4A OF M31.

TECHNOLOGIST_____ DATE TESTED_____