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

This document provides the currently recommended techniques for antimicrobial agent disk and dilution susceptibility testing, criteria for quality control testing, and interpretive criteria for veterinary use.

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Abstract

If the susceptibility of a bacterial pathogen to antimicrobial agents cannot be predicted based on the identity of the organism alone, in vitro antimicrobial susceptibility testing of the organism isolated from the disease processes in animals is indicated. Susceptibility testing is particularly necessary in those situations where the etiologic agent belongs to a bacterial species for which resistance to commonly used antimicrobial agents has been documented, or could arise.

A variety of laboratory techniques can be used to measure the in vitro susceptibility of bacteria to antimicrobial agents. This document describes the standard agar disk diffusion method, as well as standard broth dilution (macrodilution and microdilution) and agar dilution techniques. It also includes a series of procedures designed to standardize test performance. The performance, applications, and limitations of the current NCCLS-recommended methods are described.

The tabular information in this document presents the most current information for drug selection, interpretation, and quality control. Only a few compounds have veterinary-specific interpretive criteria; so, human interpretive criteria are used for the majority of compounds. As more veterinary-specific information becomes available, these changes will be incorporated into future revisions of this document.

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Foreword

This approved revision of the M31 standard represents a continuation of the collective efforts of the Subcommittee on Veterinary Antimicrobial Susceptibility Testing to produce a useful consensus document for standardized in vitro susceptibility testing of veterinary pathogens. The subcommittee has worked diligently to improve M31-A—Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals; Approved Standard, by incorporating relevant updates derived from NCCLS documents M2—Performance Standards for Antimicrobial Disk Susceptibility Tests, and M7—Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically, as well as referencing other relevant NCCLS documents. The subcommittee recognizes the ongoing NCCLS efforts that are necessary to maintain M2 and M7 as “state of the art” and expresses its appreciation to the Subcommittee on Antimicrobial Susceptibility Testing for its contributions. In addition, it is also appropriate to acknowledge the users of M31-A for their continued support and application of M31-A in their daily work routine.

In the interval between the issuance of M31-A and the finalization of this revision, rapid and dynamic global events have placed even more importance on the essential role of antimicrobial susceptibility testing of bacteria isolated from animals. For example, judicious use guidelines (consensus directions for the appropriate use of antimicrobial agents in animals) have been developed worldwide by veterinary, industry, and public health organizations for both food and companion animals, that emphasize the critical need for obtaining susceptibility data using standardized test methods and interpretive criteria. M31-A2 is updated to meet these needs.

Also, several additional antimicrobial agents have been reviewed and received veterinary-specific interpretive criteria approval from the subcommittee. M31-A2 features these new antimicrobial agents in Table 1, Group A (FDA-approved, NCCLS-approved, veterinary-specific interpretive criteria). Further revisions were made to designate FDA-approved, human interpretive criteria (Group B); FDA-approved drugs with no interpretive criteria (Group C); and, finally “AMDUCA-use” (Animal Medicinal Drug Use Clarification Act) products (Group D). Additionally, refinements to flexible label usage, and definitions for “susceptible” and “resistant” designations are now included. In conjunction with the elaboration of veterinary guidelines, a number of antimicrobial resistance monitoring programs throughout the world have begun to apply NCCLS standardized test methods to their work, with the expectation that susceptibility data can be compared across regions. As a first step toward worldwide applications of susceptibility testing using NCCLS methods, a new separate section lists quality control strain entries in various international repositories.

Additionally, a glossary of antimicrobial agents and resistance mechanisms, and a listing of antimicrobial resistance tests have been assembled for the convenience of investigators interested in this area. Development of specific test methods and quality control values for several key agents effective against campylobacter have also been developed with the expectation that they will contribute to improved monitoring efforts. Finally, a flow sheet for quality control testing is now available that should aid laboratories to track their performance for increased proficiency.

The NCCLS Subcommittee on Veterinary Antimicrobial Susceptibility Testing has revised the document to meet the need for specific testing standards, and to serve as an educational resource. It is our collective hope that diagnostic laboratory personnel, veterinarians, students, and allied professionals will benefit from this document. The user should be aware that, while M31 is primarily derived from NCCLS M2 and M7 which are “human” documents, this version incorporates more veterinary-specific information than before, although there is still ample opportunity for more. This standard should not be considered a static document; it will change as improved methods for testing veterinary pathogens become available and in response to changes in antimicrobial agent usage in veterinary medicine. The subcommittee is committed to making every effort to incorporate the latest information into future versions of the M31 standard. It is anticipated that future revisions will encompass those antimicrobial
agents used for enteric disease therapy or control. Until now, the inclusion of interpretive criteria was limited to systemic disease and mastitis; however, the subcommittee has now allowed the opportunity for inclusion of products that are useful in enteric diseases by incorporating new review criteria in NCCLS document M37—Development of In Vitro Susceptibility Testing Criteria and Quality Control Parameters for Veterinary Antimicrobial Agents. As before, prevention and growth promotion uses are not covered in M31-A2 with respect to interpretive criteria. The subcommittee anticipates that, as a result of the efforts of several working groups, additional fastidious pathogens will be included in future editions, such as the intestinal spirochetes, *Haemophilus parasuis*, and mycoplasma. A separate NCCLS working group is developing antimicrobial susceptibility testing guidelines for aquaculture pathogens. Additionally, the inclusion of antimicrobial agents not currently marketed in the United States is welcomed for future editions. As mentioned before, the inclusion of international entries in culture collections of quality control strains is viewed as the first step in this process, so that leading laboratories might begin to use NCCLS methods and compare them with their own national methods. Perhaps the most important future revision will be to shift antimicrobial agents from Group C or B, to A. Because approvals of new antimicrobial agents for veterinary medicine are foreseen to be a rare event, the subcommittee will shift its focus to the Generics Working Group to develop data in conformance with the M37 guideline in order to continue to advance generic drugs into Group A. As chairholder, I encourage and welcome input regarding ways to improve the document.

In closing, I recognize the efforts of the Subcommittee on Veterinary Antimicrobial Susceptibility Testing in producing this revised document. I would like to particularly acknowledge the individual members of the Editorial Working Group. Their willingness to sacrifice significant amounts of their personal time for the editing process and to address controversial topics demonstrates a real commitment to the NCCLS process and the advancement of the veterinary and microbiology professions. In particular, I thank Jeff Watts for his pioneering leadership that was instrumental in developing M31-A and, now, in leading the revision process. I thank Bob Walker for his tremendous work in developing quality control values for susceptibility testing of campylobacter and serving on nearly all of the working groups that contributed the “nuts and bolts” of methods and quality control processes to the document. I thank Clyde Thornsberry and Ron Jones for allowing us to benefit from their experience in developing standards for testing antimicrobial agents for human use. I thank David White for contributing his expertise on resistance mechanisms. Finally, I would like to express my sincere appreciation to the members of the NCCLS Executive Offices’ staff for their ongoing support with the countless revisions, meetings, phone calls, and e-mails that were necessary to produce this document.

Thomas R. Shryock, Ph.D., Chairholder, Subcommittee on Veterinary Antimicrobial Susceptibility Testing

Key Words

Agar diffusion, agar dilution, antimicrobial agent, antimicrobial susceptibility, susceptibility testing, veterinary

Mission Statement

To develop and promote performance standards and interpretive criteria for *in vitro* antimicrobial susceptibility testing of bacteria isolated from animals.
Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals; Approved Standard—Second Edition

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.\(^1-^3\) This document describes current methodology applicable to therapeutic uses of antimicrobial agents used in veterinary medicine for diseases of animals as described in Section 2.

It is the opinion of the subcommittee that the development of new, or modified, *in vitro* testing procedures to determine breakpoints or zone of inhibition interpretive criteria to accommodate specific field usage practices in livestock was not realistic for two reasons:

First, there is no 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 antimicrobial 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 continue to consider new developments in test methodologies and procedures, as well as revisions to interpretive criteria for therapeutic agents. With respect to antimicrobial agents used to promote or enhance animal growth, the beneficial effects of antimicrobial agents can neither be entirely ascribed to effects on the microbial gut flora, nor correlated to physiological effects on the animal. Thus, new *in vitro* techniques for prediction of clinical outcome will be considered only when a better understanding of the mode of action of antimicrobial agents in this situation becomes available.

1.1 Scope

This document provides veterinary diagnostic laboratories with currently recommended antimicrobial agent disk and dilution susceptibility test methods for bacteria isolated from animals; criteria for quality control testing; and interpretive criteria. The interpretive criteria are intended only to support therapeutic label claims for animal antimicrobial agent use and do not apply to label claims for disease prevention or performance enhancement.

Relatively few antimicrobial agents have had veterinary-specific interpretive criteria established, therefore human interpretive criteria are used for those antimicrobial agents lacking veterinary-specific interpretive criteria (see NCCLS documents M2—Performance Standards for Antimicrobial Disk Susceptibility Tests, M7—Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically, and M11—Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria). As more veterinary-specific information becomes available, changes in the listing of the agents will be incorporated into future revisions of this document.
Additionally, the document provides a brief overview of the various antimicrobial classes and mechanisms of resistance to them, including specific tests for antimicrobial resistance.

1.2 Indications for Performing Susceptibility Testing

Susceptibility testing is indicated for any bacterial pathogen that contributes to an infectious process requiring antimicrobial agent intervention, if its susceptibility cannot be reliably predicted from knowledge of the organism’s identity. Susceptibility tests are most often indicated when the causative organism is thought to belong to a bacterial species capable of possessing resistance mechanisms to commonly used antimicrobial agents. Mechanisms of resistance include production of drug-inactivating enzymes, alteration of drug targets, and altered uptake or efflux. Some organisms still have predictable susceptibility to antimicrobial agents, and empiric therapy is widely recognized. Susceptibility tests are seldom necessary when the infection is due to a microorganism known to be highly susceptible to a specific drug and to which resistance has not been reported (e.g., the continued susceptibility of *Arcanobacterium pyogenes* to penicillin). Susceptibility tests are also important in studies of the epidemiology of resistance and in studies of new antimicrobial agents.

Colonies of each type of bacterial pathogen isolated from an infectious disease process should be selected from primary agar plates and tested for susceptibility. A statistical model for assessing sample size for bacterial colony selection has been proposed. Identification procedures are often performed at the same time as *in vitro* susceptibility tests. 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 (e.g., normally sterile body fluids and urine) 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 unnecessary, and the results may be misleading and result in incorrect therapy.

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

In many 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 testing was adapted from those methods in NCCLS document *M2—Performance Standards for Antimicrobial Disk Susceptibility Tests*, originally described by Bauer, et al. 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. These results may be correlated with minimal inhibitory concentration (MIC) among clinically susceptible and resistant species. Caution is urged if one attempts to use a regression line analysis to extrapolate MIC values from measurements of zones of inhibition because, in some cases, the relationship, while mathematically correct, cannot always be considered comparable to an MIC derived by actual dilution testing for a given isolate (see NCCLS document *M37—Development of In Vitro Susceptibility Testing Criteria and Quality Control Parameters for Veterinary Antimicrobial Agents*).
1.4 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 taken from NCCLS document M7—*Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically* based largely on information gathered from the International Collaborative Study in Human Medicine.⁴ 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 to enable the clinicians and the staff to understand how to use the quantitative results should be carried out before the sole use of the test is initiated.

The MIC obtained using a dilution test provides insight as to the concentration of antimicrobial agent needed at the site of infection to 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 (that is, the MIC reading) and the next lower test concentration. For example, using twofold dilutions if the MIC was determined to be 16 µg/mL, the “true” MIC would be between 16 and 8 µg/mL. Even under the best of controlled conditions, a dilution test may 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 as described herein.

MICs have been determined using concentrations derived traditionally from serial twofold dilutions indexed to the log base 2 (e.g., 1, 2, 4, 8, 16 µg/mL, etc). Other dilution schemes have also been used, including use of as few as two widely separated or “breakpoint” concentrations between the usual values (e.g., 4, 16 µg/mL) or alternative dilution schemes (e.g., 100, 25, 12.5, 6.25, 3.12, 1.56 µg/mL, etc). The results from these alternative methods may be equally useful clinically; however, some are more difficult to control. When there is inhibition of growth at the lowest concentration tested, the true MIC value cannot be accurately determined, and this should be reported as equal to or less than the lowest concentration tested. To apply interpretive criteria when concentrations between the usual dilutions are tested, results falling between serial twofold dilutions should be rounded up to the next highest concentration (e.g., an MIC of 6 µg/mL would become 8 µg/mL).

1.5 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 (including *Staphylococcus* spp., *Pasteurella* spp., and the Enterobacteriaceae) and modified for testing some fastidious organisms, such as *A. pleuropneumoniae* and *Haemophilus* spp. (see Table 7). Tentative agar dilution QC ranges for *Campylobacter jejuni* in Table 5A have been approved by the NCCLS Subcommittee on Veterinary Antimicrobial Susceptibility Testing until full compliance of NCCLS guidelines M23—*Development of In Vitro Susceptibility Testing Criteria and Quality Control Parameters* and M37—*Development of In Vitro Susceptibility Testing Criteria and
Quality Control Parameters for Veterinary Antimicrobial Agents have been met. 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 fluoroquinolones.

2 Selection of Antimicrobial Agents for Routine Testing and Reporting

2.1 Definitions*

The selection of the most appropriate antimicrobial agents to test and to report is a decision best made by each laboratory in consultation with the pharmacy and veterinarians. The lists in Table 1 comprise agents of proven clinical efficacy for treatment of infections in that animal group, and which show acceptable *in vitro* test performance. 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. Tests on selected agents can also be useful for epidemiological or research purposes.

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 antimicrobial agents in veterinary medicine.

For the most part, these limitations reflect antimicrobial uses in livestock and poultry used for food production, and do not necessarily apply to equine or small-animal medicine. Currently antimicrobial agents 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 antimicrobial uses, which are consistent with current regulatory interpretations in its deliberations:

**Control, n** - Administration of an antimicrobial 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; NOTES: a) It must be recognized that the initial treatment might be made empirically based on the urgency of the situation, 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 antimicrobial; b) 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 may be present in a group of animals. The objective is to control the dissemination of the disease within the group while

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*a Some of these definitions are found in NCCLS document NRSL8—Terminology and Definitions for Use in NCCLS Documents. For complete definitions and detailed source information, please refer to the most current edition of that document.*
treating those with clinical signs. Certain exceptions, such as medication of one-day-old chicks, will be discussed later.

**Growth promotion, n** - Administration of an antimicrobial, 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.); **NOTE:** Although this has been sometimes referred to as “subtherapeutic” use, it implies only a lower dosage and longer duration of medication than for a therapeutic use of a feed additive.

**Interpretive criteria//Breakpoint, n** - MIC or zone diameter value used to indicate susceptible, intermediate, and resistant as defined in M2—*Performance Standards for Antimicrobial Disk Susceptibility Tests*; M7—*Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically*; and M11—*Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria.*

For example, for antimicrobial X with interpretive criteria (µg/mL) of:

<table>
<thead>
<tr>
<th>MIC (µg/mL)</th>
<th>Zone Diameter (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Susceptible *</td>
<td>≤ 4</td>
</tr>
<tr>
<td>Intermediate</td>
<td>8-16</td>
</tr>
<tr>
<td>Resistant †</td>
<td>≥ 32</td>
</tr>
</tbody>
</table>

*“Susceptible breakpoint” is 4 µg/mL or 20 mm.
†“Resistant breakpoint” is 32 µg/mL or 14 mm.

**Minimal inhibitory concentration (MIC), n** - The lowest concentration of an antimicrobial agent that prevents visible growth of a microorganism in an agar or broth dilution susceptibility test.

**Prevention/prophylaxis, n** - Administration of an antimicrobial to exposed healthy animals considered to be at risk, but prior to expected onset of disease and for which no etiologic agent has yet been cultured; **NOTE:** Generally the usage is in a herd or flock situation and not an individual animal.

**Therapeutic, n** - Administration of an antimicrobial to an animal, or group of animals, which exhibit frank clinical disease.

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 reason: 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 dose or duration 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, nor to note “control” or “therapeutic” after the listing. This would simplify the laboratory’s responsibility in reporting their recommendations to the veterinarian 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 antimicrobial agents 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 antimicrobial, 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 pathogen 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 antimicrobial 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 antimicrobial 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 antimicrobial to administer.

Growth promotion uses of antimicrobial agents 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 among a specific species of bacteria (or group of bacteria), their physiological effect on the animal, and how that is affected by antimicrobial agents. There is a complex microbial ecology issue involved in the growth promotion use of antimicrobial agents, 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 antimicrobial will enhance or inhibit the performance of a group of animals. Some antimicrobial agents are used for 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 antimicrobial agents against specific species of enteric bacteria was recognized as perhaps having research value in monitoring for antimicrobial 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 antimicrobial agents used to treat a variety of enteric infections in animals, and every attempt to include them within NCCLS document M31 should be made in order to guide veterinarians 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 antimicrobial is used to treat enteric infections.

### 2.2 Selection of Antimicrobial Agents

The lists of antimicrobial agents in Table 1 constitute current NCCLS recommendations for testing and 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, Group B, and Group C are approved for use in the indicated animal species by the Food and Drug Administration-Center for Veterinary Medicine (FDA-CVM). However, the subcommittee feels that it is most prudent to report those compounds with veterinary-specific interpretive criteria (Group A) preferentially over those using human interpretive criteria. These
compounds have demonstrated an acceptable level of agreement between susceptibility test results and clinical outcome. While compounds using human guidelines (Group B) may perform adequately, the relationship for all approved veterinary applications has not been demonstrated.

Compounds listed in Table 1 - Group C are FDA-CVM-approved for use in the specific animal species but have neither veterinary-specific nor human-specific, NCCLS-approved interpretive criteria. These agents may be approved for use in other animal species and have veterinary-specific interpretive criteria in those animals. However, the "porting" of interpretive criteria from one animal species to another is not recommended due to differences in dosages and pharmacokinetics. Thus, these agents should be reported selectively before extra-label use agents (Group D) but after agents in Group B. Again, 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.

Given the limited number of antimicrobial agents approved for use in some animal species, “extra-label” use of antimicrobial agents is commonly practiced and Group D lists agents that commonly fall into this category. The U.S. Congress, in the Animal Medicinal Drug Use Clarification Act (AMDUCA), 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.”6,7 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.

It is the responsibility of the laboratory client to use the agent appropriately for the various animal types or categories (e.g., calves, lactating dairy cattle). The laboratory client assumes all responsibility for efficacy, safety (including public health issues of antimicrobial-resistant, food-borne bacteria), and residue avoidance with extra-label uses of antimicrobial agents. The laboratory should be prepared to offer advice to the veterinarian to enable appropriate decisions to be made. Although the laboratory may choose to modify the list of antimicrobial agents it tests and reports, on the basis of public health concerns, it would need to be done in consultation with appropriate experts, based on good clinical judgment, and in accordance with recognized principles of judicious use, such as those advocated by the American Veterinary Medical Association.6,8,9

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.

### 2.3 Nonproprietary Names

To minimize confusion, all antimicrobial agents should be referred to by nonproprietary names. To emphasize the relatedness of the many currently available drugs, they may be grouped together within classes as described below in Section 2.5 and in Glossary 1.

### 2.4 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 (class) 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.

2.5 Antimicrobial Classes

The following antimicrobial agent overview is to be used for the selection and reporting of agents from some of the larger classes to be tested routinely. For additional in-depth information on the specific agents, the reader is referred to other texts. Antimicrobial class, subclass designations, and resistance mechanisms are listed in Glossary 1, and abbreviations for antimicrobial agents incorporated into disks or susceptibility panels are listed in Glossary 2.

2.5.1 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.

2.5.1.1 Penicillins

The spectrum of penicillin is directed primarily against non-β-lactamase-producing, gram-positive, and some gram-negative bacteria. Ampicillin and amoxicillin (the aminobenzyl-penicillins) 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 (ureido-penicillins), have a considerably expanded gram-negative spectrum, including activity against many *Pseudomonas* spp. Penicillinase-resistant penicillins (PRPs) such as cloxacillin, dicloxacillin, methicillin, nafcillin, and oxacillin are used specifically for treating the penicillinase-producing, methicillin-susceptible staphylococci.

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 6.

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 6). 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 6.5.1). 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.

Detection of β-lactamase production in enterococci is possible only by a direct β-lactamase test. 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 ≤ 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.

2.5.1.2 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. Currently, three β-lactamase inhibitors are in use in human medicine: clavulanic acid, sulbactam, and tazobactam. 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.

2.5.1.3 Cephalosporins and Cephemycins

The different cephalosporins and other cepham antimicrobial agents can have a somewhat 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, based on the extent of their activity against the more antimicrobial-resistant, gram-negative bacteria. Not all representatives of a specific group or generation necessarily have the same spectrum of activity. Because of these differences in activities, representatives of each group may be selected for routine testing. An exception is with the first-generation cephalosporins, where cephalothin is tested as the class representative. However, cefazolin may also be tested against Enterobacteriaceae.

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.

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.

2.5.1.4 Carbapenems

Carbapenems differ slightly in structure from penicillins, are much more resistant to β-lactamase hydrolysis, and have a broad spectrum of activity against many gram-positive and gram-negative bacteria. None are approved for use in veterinary medicine.

2.5.1.5 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.
2.5.2 **Tetracyclines**

These compounds inhibit bacterial protein synthesis at the ribosomal level. Drugs in this group are closely related (tetracycline, oxytetracycline, chlortetracycline, minocycline, and doxycycline). Although minocycline and doxycycline are generally more active against gram-positive organisms, only tetracyclines that are approved for use in animals should be tested.

2.5.3 **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, such as enterococci. Aminoglycosides, such as gentamicin, have the potential to produce extended residue times in some animals, e.g., the bovine; therefore, their use should be monitored carefully.

2.5.4 **Macrolides**

Macrolides are structurally related, bacteriostatic antimicrobial agents that inhibit protein synthesis at the ribosomal level. This group includes erythromycin, tylosin, and tilmicosin, which are used in animals; and clarithromycin, azithromycin, dirithromycin, and telithromycin, which are used in human medicine. Only erythromycin and tilmicosin should be tested.

2.5.5 **Lincosamides**

The lincosamides (lincosaminides) are bacteriostatic antimicrobial agents that 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, and only against isolates from bovine milk.

2.5.6 **Fluoroquinolones**

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, orbifloxacin, marbofloxacin, and difloxacin. Currently, only nalidixic acid, enrofloxacin, marbofloxacin, orbifloxacin, and difloxacin are approved for use in veterinary medicine. Only enrofloxacin is approved for use in food animals. Recently, the U.S. FDA has banned extra-label use of fluoroquinolones in food animals.12

2.5.7 **Sulfonamides and Potentiated Sulfonamides**

This group of compounds encompasses a 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 sulfonamide 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. Trimethoprim-sulfamethoxazole may be used to predict susceptibility to ormetoprim-sulfadimethoxine.

### 2.5.8 Phenicols

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.

### 2.5.9 Antimicrobial Agents

Currently, vancomycin, rifampin, linezolid, quinupristin-dalfopristin, nitrofurantoin, and fosfomycin are approved for use in human medicine; whereas bacitracin, novobiocin, and polymyxin are used in both groups, and tiamulin is used only in animals. These drugs must be tested individually to determine their activity against clinical isolates.

### 2.6 Guidelines for Routine Reporting

As listed in Table 1, agents in Group A are considered appropriate for inclusion in a routine primary panel for food and companion animals. Compounds in Groups A, B, and C may be reported routinely or selectively as outlined in Section 2.7.

Group D comprises agents that are frequently used in an AMDUCA–use or 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.

Data for agents with prevention or growth promotion claims should not be reported. Chloramphenicol should not be reported for food animals. Fluoroquinolones and glycopeptides (vancomycin) should not be reported as Group D compounds in food animals. Aminoglycosides that will be administered parenterally to cattle should not be reported for cattle.

### 2.7 Guidelines for Selective Reporting

Each laboratory, in consultation with the pharmacy and veterinarians, should decide which agents (Table 1) to report routinely from each group. 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.

#### 2.7.1 Examples

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

- If an enteric bacillus is susceptible to ampicillin, do not report carbenicillin or ticarcillin, piperacillin, amoxicillin-clavulanic acid, ampicillin-sulbactam, or ticarcillin-clavulanic acid.

- If an enteric bacillus is susceptible to a first-generation cephalosporin, do not report a second-, third-, or fourth-generation cephalosporin.

- If an enteric bacillus is susceptible to gentamicin, do not report tobramycin or amikacin.
When testing enterococci from nonurinary sites, do not include agents other than penicillin or ampicillin, except vancomycin as requested for penicillin-allergic companion animals.

If staphylococci are resistant to oxacillin, they should be considered resistant to all of the cephalosporins, amoxicillin-clavulanic acid, imipenem, ampicillin-sulbactam, or other β-lactam antimicrobial, regardless of in vitro results.

Fluoroquinolones and glycopeptides are prohibited from AMDUCA extra-label use in food animals. ¹²

Metronidazole and nitrofurantoin should not be reported for any isolate from food animals.

### 2.8 Interpretive Categories

#### 2.8.1 MIC and Zone-Size Interpretive Criteria

Table 2 shows interpretive criteria 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 interpretive criteria are not available, the use of these values in relation to veterinary bacterial isolates must be done with caution for three reasons. First, the value listed in the gray shaded areas 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.

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

Whenever MIC results are reported to veterinarians to direct therapeutic use, an interpretive category (i.e., susceptible, intermediate, or resistant) should accompany the MIC result based on the criteria outlined in the tables. When tests in which four or fewer consecutive concentrations are tested, or when nonconsecutive concentrations are tested, an interpretive category result must be reported. The MIC range may also be reported if desired.

#### 2.8.2 Susceptible

This category implies that there is a high likelihood of a favorable clinical outcome when the drug is administered at label dosage, because of adequate pharmacodynamic parameters relative to the MIC of the causative organism.

#### 2.8.3 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); and

- 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.

### 2.8.4 Flexible Label

This category 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.

### 2.8.5 Resistant

This category implies that there will not be a favorable clinical outcome, because the achievable systemic concentrations of the agent will be lower than the MIC of the causative organism with normal dosage schedules and/or fall in the range or where specific microbial resistance mechanisms are likely (e.g., β-lactamases), and clinical efficacy has not been reliable in treatment studies.

### 3 Disk Diffusion Susceptibility Tests

#### 3.1 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. However, the zone diameters may not correspond precisely to the listed MIC breakpoints due to differences in the methodologies and the original databases. Thus, the information provided in Table 2 cannot be used to convert zone diameters to absolute MIC values. See also Section 1.3.

#### 3.2 Methodologies

##### 3.2.1 Agar Media

3.2.1.1 Mueller-Hinton Agar

Of the many media available, the subcommittee considers Mueller-Hinton agar the best medium for routine susceptibility tests for the following reasons:

- It results in good batch-to-batch reproducibility for susceptibility testing.
• It is low in sulfonamide, trimethoprim, and tetracycline inhibitors.

• It results in satisfactory growth of most bacterial pathogens.

• A large amount of data has been collected concerning susceptibility tests performed with this medium.

Although Mueller-Hinton agar is generally reliable for susceptibility testing, results obtained with some batches may, on occasion, vary significantly. If a batch of medium does not support adequate growth of the test organism, zones obtained in a diffusion test will usually be larger, can be outside the quality control limits, and can yield erroneous results. Media that have been tested according to and meet the acceptance limits described in the most current edition of NCCLS document M6—Protocols for Evaluating Dehydrated Mueller-Hinton Agar are to be used.

3.2.1.2 Effects of Thymidine or Thymine

Media containing excessive amounts of thymidine or thymine can reverse the inhibitory effect of sulfonamides and of trimethoprim, thus yielding smaller and less distinct zones, or even no zone at all, which can result in false-resistant reports. Addition of thymidine phosphorylase or lysed horse blood can improve the clarity of zones and the reliability of sulfonamide and trimethoprim testing of most common pathogens, except enterococci.15, 16 However, Mueller-Hinton agar that is as thymidine-free as possible should be obtained; it is available from most commercial sources.

To evaluate a new lot of Mueller-Hinton agar, a standard control strain of Enterococcus faecalis American Type Culture Collectionb (ATCC®) 29212 or Enterococcus faecalis ATCC® 33186 (a more sensitive indicator of thymidine levels) is tested with trimethoprim-sulfamethoxazole disks. Satisfactory media will provide essentially clear zones of inhibition, 20 mm or more in diameter.

3.2.1.3 Effects of Variation in Divalent Cations

Variation in divalent cations, principally magnesium and calcium, will affect results of aminoglycoside tests with Pseudomonas aeruginosa strains and tetracycline tests with numerous species. Excessive cation content will reduce zone sizes, whereas low cation content may result in unacceptably large zones of inhibition. Excess zinc ions may reduce zone sizes of carbapenems. (Performance tests using aminoglycosides and tetracycline with each lot of Mueller-Hinton agar must conform to the control limits listed in Table 4.)

3.2.1.4 Preparation of Mueller-Hinton Agar

Following is the procedure for preparing Mueller-Hinton agar:

(1) Prepare Mueller-Hinton agar from a dehydrated base according to the manufacturer's recommendations.

(2) Immediately after autoclaving, allow it to cool in a water bath at 48 to 50 °C (see Section 4.2.2.2).

(3) Pour the freshly prepared and cooled medium into petri dishes on a level, horizontal surface to give a uniform depth of approximately 4 mm. This corresponds to 60 to 70 mL of medium for 150-mm (15-cm) plates, and 25 to 30 mL for 100-mm (10-cm) plates. Either glass or plastic petri dishes may be used, but the bottom half of the plate should be flat.

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b ATCC is a registered trademark of the American Type Culture Collection.
(4) Allow the agar medium to cool to room temperature and, unless the plate is used the same day, store in a refrigerator (2 to 8 °C). Plates should be used within seven days after preparation unless adequate precautions, such as wrapping in plastic, have been taken to minimize evaporation and unless they have been shown to perform correctly with the control organism as specified in Section 5.

(5) Test samples of each batch of plates for sterility by incubating at 35 °C for 24 hours or longer; discard these samples.

3.2.1.5 pH

The pH of each batch of Mueller-Hinton agar is to be checked when the medium is prepared. The exact method used will depend largely on the type of equipment available in the laboratory. The agar medium should have a pH between 7.2 and 7.4 at room temperature and must therefore be checked after gelling. The pH can be checked by:

- macerating a sufficient amount of agar to submerge the tip of a pH electrode;
- allowing a small amount of agar to solidify around the tip of a pH electrode in a beaker or pH meter cup; and
- using a properly calibrated surface electrode.

3.2.1.6 Moisture

If, just before use, excess surface moisture is present, the plates are to be put in an incubator (35 °C) with lids ajar until excess surface moisture is lost by evaporation (usually 10 to 30 minutes). The surface should be moist, but no droplets of moisture should be on the surface of the medium or on the petri dish covers when the plates are inoculated.

3.2.2 Storage of Antimicrobial Agent Disks

Generally, cartridges containing paper disks specifically for susceptibility testing are supplied in separate containers. Methods for packaging must ensure appropriate anhydrous conditions. For long-term storage, it is necessary to:

- Refrigerate the containers (maintain at 8 °C or below) or freeze at -14 °C or below until needed, according to the manufacturer's recommendations.
- To maintain their potency, freeze disks that contain drugs from the β-lactam family, except for a small working supply, which can be refrigerated for up to one week.
- Remove the unopened containers from the refrigerator or freezer one to two hours before use of the disks, and allow them to equilibrate to room temperature before opening. This procedure minimizes the condensation that would occur when warm air reaches the cold containers.
- When using a filled disk-dispensing apparatus, it should be fitted with a tight cover and supplied with an adequate indicator desiccant; allow it to warm to room temperature before opening.
- Avoid excessive moisture by replacing the desiccant when the indicator changes color.
- When not in use, always refrigerate the dispensing apparatus containing the disks.
- Use only those disks that have not reached the manufacturer's expiration date stated on the label. Discard disks on that date.

### 3.2.3 Turbidity Standard

To standardize the inoculum density, a BaSO₄ turbidity standard is used (0.5 McFarland standard). The procedure is as follows:

1. Prepare this turbidity standard by adding 0.5 mL of 0.048 mol/L barium chloride (1.173g BaCl₂•2H₂O; 1.175% w/v) to 99.5 mL of 0.18 mol/L (0.36 N; 1% v/v) H₂SO₄.

2. Verify the correct density of the turbidity standard by using a spectrophotometer with a 1-cm light path and matched cuvettes to determine the absorbency. The absorbency at 625 nm should be 0.08 to 0.10 for the 0.5 McFarland standard.

3. Distribute 4 to 6 mL into screw-cap tubes of the same size as those used in growing or diluting the broth culture inoculum.

4. To prevent evaporation, seal these tubes tightly and store them at room temperature.

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.

### 3.2.4 Inoculation of Test Plates

#### 3.2.4.1 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 polyester textile fiber 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 3.2.3 (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 3.2.3. 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.

### 3.2.4.2 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). The suspension is adjusted to match the turbidity standard, as outlined in Section 4.2.3.5. 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 3.2.5.3 and 3.2.6]) or for testing *Staphylococcus* spp. for methicillin resistance (see Section 6.5).

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.

There are some devices that permit direct standardization of inocula without adjustment of turbidity and without preincubation in a broth medium that have been found to be acceptable for routine testing purposes.

### 3.2.4.3 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 3.2.4. 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 nor 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 CO₂, because the interpretive standards were developed by using aerobic incubation and, with some agents (i.e., macrolides, lincosamminides, tetracyclines), CO₂ will significantly alter the size of the inhibitory zones due to pH changes. Organisms requiring CO₂ for standardized testing (e.g., *Haemophilus somnus* and *Actinobacillus pleuropneumoniae*) should be incubated in an environment of 3 to 7% CO₂. If a CO₂ 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 acceptably. 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.

### 3.2.5 Fastidious Organisms

#### 3.2.5.1 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.

#### 3.2.5.2 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 5. 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)¹⁹
  - +Vitamin B₃₂ 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 - 8H₂O 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 3.2.1.4 of this document.

### 3.2.5.3 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 3.2.4.3 for non-fastidious 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% CO₂ 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.

### 3.2.5.4 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 6. Disk diffusion testing of these isolates with other agents is not recommended until appropriate quality control parameters have been established.

### 3.2.6 *Streptococcus* spp.

#### 3.2.6.1 Agar Medium

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

#### 3.2.6.2 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 3.2.4.3, should be followed, except that not more than nine disks should be placed on a 150-mm agar plate nor more than four disks on a 100-mm plate.

3. The plates are incubated at 35 °C in an atmosphere of 5% CO₂ for 20 to 24 hours before measuring the zones of inhibition.
3.2.6.3 Zone Diameter Interpretive Criteria

The zone interpretive criteria to be used when testing streptococci, including *S. pneumoniae*, are included in Table 2. Additional information specific to *S. pneumoniae* testing is in NCCLS document M100—*Performance Standards for Antimicrobial Susceptibility Testing*.

NOTE: A penicillin MIC should be determined on isolates of viridans streptococci from normally sterile body sites (e.g., cerebrospinal fluid, blood, bone, milk, etc.). Penicillin (and oxacillin) disk diffusion testing is not reliable with viridans streptococci.

3.2.7 Other Organisms

3.2.7.1 *Listeria* spp.

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

3.2.7.2 *Arcanobacterium (Actinomyces) pyogenes*

Because *A. pyogenes* is routinely susceptible to penicillin, susceptibility testing should not be necessary. However, if 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.

3.2.8 *Campylobacter jejuni* and Related Species

Due to the difficulty in determining accurate and reproducible zone sizes, disk diffusion testing is not valid for testing *Campylobacter jejuni* and related species. (Refer to Section 1.5.)

3.2.8.1 Medium

The procedure described here has been shown to produce intra- and interlaboratory reproducible results in accordance with the quality control guidelines outlined in Section 5. The medium of choice for testing this organism is Mueller-Hinton agar supplemented with 5% defibrinated sheep blood. Fresh blood may be used, since the age of the blood has not been shown to be critical.

3.2.8.2 Test Procedure

Agar dilution is the method of choice for testing *Campylobacter* isolates at this time. Studies are underway to investigate broth microdilution and concentration gradient testing methods. However, until those studies have been performed in multiple laboratories, on multiple lots of media, to demonstrate their intra- and interlaboratory reproducibility, in accordance with the guidelines described in NCCLS documents M37—*Development of In Vitro Susceptibility Testing Criteria and Quality Control Parameters for Veterinary Antimicrobial Agents* or M23—*Development of In Vitro Susceptibility Testing Criteria and Quality Control Parameters*, only the agar dilution testing method should be used.

The direct colony suspension procedure should be used when testing *C. jejuni* and related species. Using colonies taken directly from a blood agar plate, incubated in an atmosphere appropriate to enhance the growth of *Campylobacter*-like organisms (see below) for 24 hours at 42 °C or 48 hours at 37 °C, a suspension of the test organism is prepared in sterile Mueller-Hinton broth, water, or 0.85% 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 10⁸ CFU/mL. Care must be exercised in preparing this suspension, because higher inoculum concentrations may lead to false-resistant results. Within 30 minutes after adjusting the turbidity of the inoculum suspension, it should be used for plate inoculation.

The agar-dilution method for determining antimicrobial agent susceptibility is well established. The procedure for performing the agar-dilution test is described in Section 4.2.2. The medium to be used is Mueller-Hinton agar supplemented with 5% defibrinated sheep blood.

Plates are to be incubated at 35 to 37 °C in a 10% CO₂ incubator; sealable plastic pouches containing 10% CO₂, 5% O₂, and 85% N₂; or atmospheres generated by commercially available systems; these have all been shown to produce equivalent results using the agar dilution testing method.

3.2.8.3 Quality Control Testing of *Campylobacter jejuni* and Related Organisms

The antimicrobial agents for which quality control testing has been established for *Campylobacter jejuni* are indicated in Table 5A. *In vitro* susceptibility testing of this organism with other agents is not recommended until appropriate quality control parameters have been established.

3.2.9 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 use of disk diffusion tests is not recommended.

4 Broth and Agar Dilution Susceptibility Testing

4.1 Indications for Performing Broth and Agar Dilution Susceptibility Tests

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 Sections 4.2.2.1 and 4.2.3.1.¹⁰ For some more fastidious and special problem pathogens that can require MIC tests, alternative methods are included in Table 7. 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 antimicrobials are primarily antistaphylococcal drugs. NCCLS document M11—*Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria* is an agar dilution reference standard for anaerobic bacteria.

Section 4.2.3.4 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 (See NCCLS document M23—*Development of In Vitro Susceptibility Testing Criteria and Quality Control Parameters* for further details on plate format comparisons). Only those results for which appropriate quality control values have been achieved should be reported.
4.1.1 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.)

4.2 Methodologies

4.2.1 Antimicrobial Agents

4.2.1.1 Source

Antimicrobial agent standards or reference powders can be obtained commercially, directly from the drug manufacturer, or from the United States Pharmacopoeia (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).

4.2.1.2 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:

\[
\text{Weight (mg)} = \frac{\text{Volume (mL)} \times \text{Concentration (µg/mL)}}{\text{Assay Potency (µg/mg)}}, \quad \text{or}
\]

\[
\text{Volume (mL)} = \frac{\text{Weight (mg)} \times \text{Assay Potency (µg/mg)}}{\text{Concentration (µg/mL)}}
\]

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:

\[
\text{Volume} = \frac{182.6 \text{ µg} \times 750 \text{ µg/mg}}{1,280 \text{ µg/mL}} = \frac{(\text{Actual Weight}) \times (\text{Potency})}{(\text{Desired Concentration})}
\]

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

Antimicrobial agent stock solutions are to be prepared at concentrations of at least 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. 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.

During preparation and storage of stock drug solutions, the solution should be protected from excessive light. Ideally, dilution activities in Sections 4.2.1.3, 4.2.2.2, and 4.2.3.4 should have minimal light exposure.

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.

4.2.1.4 Number of Concentrations Tested

The concentrations to be tested for a particular antimicrobial agent should, at a minimum, encompass both the QC range and the breakpoints shown in Table 2 (e.g., serial twofold centering on 1 µg/mL [i.e., 0.5, 1, 2, 4 µg/mL]). 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 penicillin against an Enterococcus spp.).

4.2.2 Agar Dilution Procedure

The agar dilution method for determining antimicrobial agent susceptibility is well established. The antimicrobial agent is incorporated into the agar, with each plate containing a different concentration of the agent in log2 doubling dilutions. The inoculum can be applied rapidly and simultaneously to the agar surfaces using an inoculum-replicating apparatus. 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 chocolate Mueller-Hinton agar as described in Section 3.2.5.2 and Table 7. 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 4.2.2.4. The medium and important technical aspects of testing these fastidious species are described in relevant sections above and outlined in Table 7. The quality control ranges for these two fastidious organisms are indicated in Table 6.

4.2.2.1 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 7). New lots of Mueller-Hinton agar are to be performance tested before use as outlined in Section 5. 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; and

• 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 7.

4.2.2.2 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 3.2.1.4]).

(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 3.2.1.4). (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 3.2.1.6).

4.2.2.3 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. For example, a log2 doubling dilution scheme would begin with a stock concentration of 5,120 µg/mL. A 2-mL aliquot, when mixed with 18 mL molten agar (1:10), will give a final agar concentration of 512 µg/mL (log2 9). Subsequent 1:2 dilutions of the antimicrobial agent are diluted as before with molten agar until the lowest desired concentration is reached.

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 Sections 5.3 and 5.3.3.

4.2.2.4 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 polyester textile fiber 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 3.2.5.3.) Allow the broth culture to incubate at 35 °C until it achieves or exceeds the turbidity of the standard described in Section 3.2.4. 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. 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.

- If desired, use of photometric or other alternative systems to adjust the inoculum density of the culture has been demonstrated to yield equivalent results. A study has shown that inocula of the proper concentration prepared with bacteria from colonies on an overnight agar plate (direct inoculum method) yield results equivalent to those obtained with the standard method described here. Staphylococci should be tested by the direct inoculum procedure. (See Table 9C for MRSA.)
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/spot in an agar area of 2 to 5 mm in diameter.

4.2.2.5 Inoculating Agar Plates

To inoculate agar plates, it is necessary to perform the following steps:

1. Dry the surface of the medium before inoculating it (see Section 3.2.1.6). If necessary, place the plates with the lids ajar in an incubator. Avoid excessive drying. Arrange the tubes containing the adjusted and diluted bacterial suspensions (1.0 x 10^7 CFU/mL) in order in a rack. Place an aliquot of each suspension into the corresponding well in the replicator seed block.

2. Mark the agar plates for orientation of the inocula.

3. Apply 0.1 to 2 µL of each inoculum to the agar surface either by the use of standardized loops or, preferably, with an inoculum-replicator device.

4. Inoculate a control agar plate (no antimicrobial agent) first; then, starting with the lowest concentration, inoculate the plates containing the different antimicrobial concentrations. Inoculate a second control agar plate last to ensure that there was no contamination or antimicrobial agent carry-over during the inoculation. (In addition, a quadrant agar plate may be streaked with each isolate tested to enhance the potential for detection of a mixed culture.)

4.2.2.6 Incubating Agar Plates

To incubate agar plates, it is necessary to perform the following steps:

1. Let the inoculated agar plates remain at room temperature until the moisture in the inoculum spots is absorbed into the agar; that is, until the spots are dry. Invert the plates and incubate them at 35 °C for 16 to 20 hours. (See Table 9C for MRSA.)

2. Avoid incubating the plates routinely in an atmosphere with increased CO₂, because the surface pH can be altered. However, organisms requiring increased CO₂, such as H. somnus and Haemophilus parasuis, should be incubated in an environment of 3 to 5% CO₂. If a CO₂ incubator is not available, a candle extinction jar is an acceptable alternative. Control strains tested concurrently will indicate whether the pH has changed unacceptably. Only drugs for which the quality control strains yield acceptable values should be reported.

4.2.2.7 Determining End Points

To determine end points, it is necessary to perform the following steps:

1. Lay the agar plates on a dark, nonreflecting surface to visually observe them. Record the MIC as the lowest concentration of antimicrobial agent that completely inhibits colony formation; disregard a single colony or a faint haze caused by inoculum.

2. Subculture to check purity, and repeat the test if two or more colonies persist in concentrations of the agent beyond an obvious end point, or there is no colony formation at lower concentrations, but there is growth at higher concentrations.
4.2.2.8 Dilution Testing of *Campylobacter jejuni*

The information presented here is to enhance monitoring programs that are testing *Campylobacter jejuni* and related species by providing a standardized testing method, including a quality control organism and quality control ranges for seven antimicrobial agents. It is not intended for, nor can it be used for, predicting clinical efficacy of antimicrobial agents tested against these organisms, since there are currently no NCCLS interpretive criteria for the *in vitro* antimicrobial susceptibility testing of *Campylobacter jejuni* and related species.

4.2.3 Broth Dilution Procedures

4.2.3.1 Broth Medium

Mueller-Hinton broth is recommended as the medium for susceptibility testing of commonly isolated, rapidly replicating pathogens in both NCCLS document M2—*Performance Standards for Antimicrobial Disk Susceptibility Tests* and in the report of the ICS.1 Mueller-Hinton broth demonstrates fairly good batch-to-batch reproducibility for susceptibility testing; is low in sulfonamide, trimethoprim, and tetracycline inhibitors; and yields satisfactory growth of most pathogens. In addition, a large volume of information about tests performed with this medium is available 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. somnis* are in Section 3.2.5.2.

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 5).

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.10, 25 Many manufacturers provide Mueller-Hinton broth that has already been adjusted. Therefore, the instructions for cation adjustments (see Section 4.2.3.1.1) 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 meets performance criteria specified in NCCLS document M6—*Protocols for Evaluating Dehydrated Mueller-Hinton Agar*.

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.26

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.27
4.2.3.1 Cation Adjustments

The procedure for preparing magnesium stock solution is to dissolve 8.36 g of MgCl₂•6H₂O 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 CaCl₂•2H₂O 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.

4.2.3.2 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.

4.2.3.2.1 Broth Microdilution Testing of Haemophilus somnus and Actinobacillus pleuropneumoniae

The medium of choice for broth microdilution 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™ (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 autolysed 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 °C or lower) and thawing followed by centrifugation at 3000 x g for 20 minutes for clarification.

 Supplement C is a registered trademark of BD Diagnostics.
4.2.3.2.2 Standardizing the Inoculum for Broth Dilution Testing of *Haemophilus somnus* and *Actinobacillus pleuropneumoniae*

Direct colony suspensions should be used when testing these organisms. 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 4.2.2.4 to obtain a desired final concentration of $5 \times 10^5$ CFU/mL in each well or tube. Inoculum concentrations higher than $5 \times 10^5$ 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 $5 \times 10^5$ CFU/mL. The procedure for determining colony-forming units may be found in Section 4.2.3.5(3).

Acceptable quality control ranges for these two organisms are indicated in Table 6.

4.2.3.3 Methicillin-Resistant Staphylococci

See Section 6.5 for testing of methicillin-resistant staphylococci.

4.2.3.4 Preparing and Storing Diluted Antimicrobial Agents

- **Broth Macro dilution (Tube) 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. Because there will be a 1:2 dilution of the drugs when an equal volume of inoculum is used, the antimicrobial agent solutions are prepared in double-strength medium. 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.

- **Broth Micro dilution 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 4.2.1.

  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. 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. 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 4.2.3.6, 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 six 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 nor thawed solutions refrozen; repeated freeze-thaw cycles accelerate the degradation of some antimicrobial agents, particularly β-lactams.

4.2.3.5 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 4.2.2.4) 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 4.2.2.4. 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.
4.2.3.6 Inoculating Broth

• Broth Macrodilution (Tube) 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 in broth, 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. (See Section 4.2.3.5(1).)

• Broth Microdilution 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 broth microdilution 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 7). 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 log₂ dilution lower than the comparable macrodilution MICs.

5 Quality Control Guidelines

5.1 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; and

• 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.

Multiple test parameters are monitored by following the quality assurance guidelines described in this standard. Acceptable results derived from testing quality control strains do not guarantee accurate results with all patient isolates. When atypical or inconsistent results are encountered with a patient’s isolates, repeat testing and/or repeat identification procedures should be performed in an effort to ensure accurate results. Each laboratory should develop its own policies for verification of atypical antimicrobial susceptibility test results.

5.2 Quality Control Responsibilities

5.2.1 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; and
- 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.

5.2.2 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; and
• maintenance of a quality control test log (see Appendix A for sample form).

5.2.3 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 Tables 4 and 5); 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 \textit{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 4 and 5 using \textit{Pseudomonas aeruginosa} ATCC® 27853. Most media manufacturers are aware of the need for cation supplementation and have adjusted their media 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 4.2.3.1.1.

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

5.3 Suggested Reference Strains

5.3.1 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 antimicrobials 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 5.3.3) have also been used often as reference strains for dilution susceptibility tests.\textsuperscript{10} The \textit{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 \textit{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 \textit{Escherichia coli} ATCC® 25922 and \textit{Staphylococcus aureus} ATCC® 29213, which is a weak β-lactamase-producing strain.

• The reference strains currently recommended by the subcommittee for controlling susceptibility tests are provided in Table 3. The QC guidelines have been developed using ATCC reference strains. Strains from other collections, while derived from ATCC strains and indicated as equivalent to the ATCC strains, should be tested for equivalency. If discrepancies occur and other sources of error have been excluded, ATCC strains should be obtained.
Tables 4 and 5 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 4 and 5. 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 baumannii (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 Mannheimia haemolytica. Satisfactory media will produce a zone of inhibition of 20 mm or more.

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 log₂ 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.

5.3.2 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.

- 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 -70 °C or lower) or in liquid nitrogen.

- The other preferred method is to lyophilize the organism.

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

1. Incubate the organisms overnight on a tryptic-soy agar plate (fastidious organisms can require supplemented tryptic-soy or chocolate agar).
(2) Select several colonies and perform the appropriate susceptibility tests to demonstrate the expected 
MIC results (see Table 5 for expected MICs of some reference strains).

(3) Subculture strains yielding expected results onto an agar plate and incubate for 16 to 18 hours.

(4) Observe the colonies on the agar plate to determine the purity of the culture. A gram stain can provide 
additional information as to the purity of the culture.

(5) Suspend the colonies from the agar plate in the stabilizing fluid to make a heavy suspension (or if 
lyophilizing, suspend the growth in the appropriate medium).

(6) Distribute the turbid suspension in small volumes (one or two drops) into several small containers.

(7) Place these containers in a -70 °C freezer or a liquid nitrogen box.

Stocks so prepared can remain indefinitely without significant risk of alteration in antimicrobial agent 
susceptibility patterns. When the supply of containers is nearly exhausted, this process is repeated to 
prepare a new supply.

5.3.3 Routine Working Control Cultures: Storage and Use of Reference Strains

For routine use of reference strains, it is necessary to perform the following procedure:

(1) Remove a container of the culture from the freezer or from a lyophilized vial.

(2) Thaw the frozen mixture at room temperature, or rehydrate the lyophilized culture.

(3) Subculture onto trypticase-soy (nonfastidious) or chocolate (fastidious) agar plates and incubate at 
35 °C for 16 to 18 hours.

(4) Remove four to five colonies, subculture to broth for the appropriate susceptibility tests, and then 
subculture onto trypticase-soy or chocolate agar slants.

(5) After incubating the strains overnight, store them at 2 to 8 °C.

(6) Subculture from the slant to an agar plate.

(7) Always perform susceptibility tests on colonies from overnight plates. These agar slants may be used 
as working stock cultures. Fresh slants of working cultures are prepared at weekly intervals, and new 
working cultures are prepared monthly. Whenever unexplained, aberrant results suggest a change in 
the organism’s inherent susceptibility, a new stock culture is obtained.

5.4 Common Sources of Error

Review the following common sources of error whenever a zone diameter or MIC is outside the accuracy 
control limits stated in Tables 4 and 5:

- clerical error in transcribing the control data;

- reader error in measuring zone diameters (usually occurs in multiples of 5 mm) or MIC;

- contamination or other changes in the control strain;
• inoculum adjusted too heavy or too light;

• failure to vortex the turbidity standard;

• variability in the performance of Mueller-Hinton agars or broth (each new lot should have been checked before being used);

• loss of disk potency during handling or storage in the laboratory; and

• improper storage of microtiter trays.

The first four types of errors can be readily resolved by carefully reexamining the test plates when a result outside the accuracy control limits is first noted. If no obvious deviations are found for the standard method, daily control tests should be resumed for at least five consecutive test days. If all control test results during the five consecutive test days are within the accuracy control limits, and the zone range is no larger than the maximum allowable range for precision, weekly accuracy control tests may be resumed. If, however, at least one zone diameter is observed outside the accuracy control limits, or the zone range is larger than the maximum allowable range for precision, daily control tests must be continued. To return to weekly testing in the future will require documentation of satisfactory performance for another 30 consecutive test days, as outlined in Section 5.6.3 of this document.

5.5 Disk Diffusion Tests

5.5.1 Disk Diffusion Quality Control Limits

Acceptable zone diameter quality control limits for a single quality control test (single-drug/single-organism combination) are listed in Tables 4 and 6. The overall performance of the test system should be monitored using these ranges by testing the appropriate control strains each day the test is performed or, if satisfactory performance is documented (see Section 5.6.3), testing may be done weekly (see below).

5.6 Frequency of Quality Control Testing (Also refer to Appendix B)

5.6.1 Daily Testing

When testing is performed daily for each antimicrobial agent/organism combination, 1 out of every 20 consecutive results may be out of the acceptable range (based on 95% confidence limits, 1 out of 20 random results can be out of control). Any more than 1 out-of-control result in 20 consecutive tests requires corrective action (see Section 5.6.4).

5.6.2 Weekly Testing

Weekly quality control testing may be performed once satisfactory performance has been documented (see Section 5.6.3). Perform quality control testing once per week and whenever any reagent component of the test (e.g., a new lot of agar or a new lot of disks from the same or a different manufacturer) is changed. If any of the weekly quality control results is out of the acceptable range, corrective action is required (see Section 5.6.4)

If a new antimicrobial agent is added, it must be tested for 30 consecutive days and satisfactory performance documented before it can be tested on a weekly schedule. Thirty days of testing is also required if there is a major change in the method of reading test results, such as conversion from manual zone measurements to an automated zone reader.
5.6.3 Demonstrating Satisfactory Performance for Conversion from Daily to Weekly Quality Control Testing

Test all applicable control strains for 30 consecutive test days and document results.

To convert from daily to weekly quality control testing, no more than 3 out of the 30 zone diameters for each antimicrobial agent/organism combination may be outside the acceptable zone diameter limits stated in Tables 4 and 6.

5.6.4 Corrective Action

5.6.4.1 Out-of-Control Result Due to an Obvious Error

If there is an obvious reason for the out-of-control result, including:

- use of the wrong disk,
- use of the wrong control strain,
- obvious contamination of the strain, or
- inadvertent use of the wrong incubation temperature or conditions,

document the reason and retest the strain on the day the error is observed. If the repeated result is within range, no further corrective action is required.

5.6.4.2 Out-of-Control Result Not Due to an Obvious Error

5.6.4.2.1 Immediate Corrective Action

If there is not an obvious reason for the out-of-control result, immediate corrective action is required.

Test the implicated antimicrobial agent/organism combination on the day the error is observed, and monitor for a total of five consecutive test days. Document all results.

If all five zone diameter measurements for the antimicrobial agent/organism combination are within the acceptable ranges, as defined in Tables 4 and 6, no additional corrective action is necessary.

If any of the five zone diameter measurements are outside the acceptable range, additional corrective action is required (see Section 5.6.4.2.2).

Daily control tests must be continued until final resolution of the problem can be achieved.

5.6.4.2.2 Additional Corrective Action

When immediate corrective action does not resolve the problem, it is likely due to a system versus a random error. The following common sources of error should be investigated to verify that:

- zone diameters were measured and transcribed correctly;
- the turbidity standard has not expired, is stored properly, meets performance requirements, and was adequately mixed prior to use;
• all materials used were within their expiration date and stored at the proper temperature;
• the incubator is at proper temperature and atmosphere;
• other equipment used (e.g., pipettors) are functioning properly;
• disks are stored desiccated and at proper temperature;
• the control strain has not changed and is not contaminated;
• inoculum suspensions were prepared and adjusted correctly;
• inoculum for the test was prepared from a plate incubated for the correct length of time and in no case more than 24 hours old.

It may be necessary to obtain a new quality control strain (either from freezer storage or a reliable source) and new lots of materials (including new turbidity standards), possibly from different manufacturers. If the problem appears to be related to a manufacturer, the manufacturer should be contacted. It is also helpful to exchange quality control strains and materials with another laboratory using the same method. Until the problem is resolved, it may be necessary to use an alternate test method. Once the problem is corrected, in order to return to weekly quality control testing, documentation of satisfactory performance for another 30 consecutive days is required (see Section 5.6.3).

5.6.5 Reporting Patient Results When Out-of-Control Tests Occur

Whenever an out-of-control result or corrective action is necessary, careful assessment of whether to report patient results should be made on an individual basis, taking into account if the source of the error, when known, is likely to have affected relevant patient results. Options that may be considered include suppressing the results for an individual antimicrobial agent; retrospectively reviewing individual patient or cumulative data for unusual patterns; and using an alternate test method or a reference laboratory until the problem is resolved.

5.7 Dilution Susceptibility Tests

5.7.1 Dilution Quality Control Limits

Acceptable MIC quality control limits for a single quality control test (single-drug/single-organism combination) are listed in Tables 5 and 6. The overall performance of the test system should be monitored using these limits by testing the appropriate control strains each day the test is performed or, if satisfactory performance is documented (see Section 5.9.1), testing may be done weekly (see below).

5.7.2 Frequency of Quality Control Testing (also refer to Appendix C)

The weekly quality control testing option outlined below is applicable to routine MIC tests only. Quality control testing should be performed each test day for MIC tests performed infrequently.

5.8 Daily Testing

When testing is performed daily for each antimicrobial agent/organism combination, 1 out of every 20 consecutive results may be out of the acceptable range (based on 95% confidence limits, 1 out of 20 random results can be out of control). Any more than 1 out-of-control result in 20 consecutive tests requires corrective action (see Section 5.10).
5.9 Weekly Testing

5.9.1 Demonstrating Satisfactory Performance for Conversion from Daily to Weekly Quality Control Testing

Test all applicable control strains for 30 consecutive test days and document results.

To convert from daily to weekly quality control testing, no more than 3 out of the 30 MICs for each antimicrobial agent/organism combination may be outside the acceptable MIC limits stated in Tables 5 and 6.

5.9.2 Implementing Weekly Quality Control Testing

Weekly quality control testing may be performed once satisfactory performance has been documented (see Section 5.9.1).

Perform quality control testing once per week and whenever any reagent component of the test (e.g., a new lot of broth from the same manufacturer) is changed.

If any of the weekly quality control results is out of the acceptable range, corrective action is required (see Section 5.10).

If a new antimicrobial agent is added or a different broth manufacturer used, it must be tested for 30 consecutive days and satisfactory performance documented before it can be tested on a weekly schedule. In addition, 30 days of testing is required if there is a major change in the method of reading test results, such as converting from a visual reading of MICs to an instrument reading.

These guidelines can also be used for testing systems in which an MIC is determined using three or fewer adjacent doubling dilutions of an antimicrobial agent.

For some drugs, quality control records may indicate the need for testing to be done more frequently than once a week because of the relatively rapid degradation of the drug. Since screening tests (e.g., agar screen for detection of VRE) for special situations are generally not performed routinely, it is recommended that appropriate quality control strains be included on each day of testing for these kinds of tests.

5.10 Corrective Action

5.10.1 Out-of-Control Result Due to an Obvious Error

If there is an obvious reason for the out-of-control result, including:

- use of the wrong control strain,
- obvious contamination of the strain or the medium, or
- inadvertent use of the wrong incubation temperature or conditions,

document the reason and retest the strain on the day the error is observed. If the repeated result is within range, no further corrective action is required.
5.10.2 Out-of-Control Result Not Due to an Obvious Error

5.10.2.1 Immediate Corrective Action

If there is not an obvious reason for the out-of-control result, immediate corrective action is required.

Test the implicated antimicrobial agent/organism combination on the day the error is observed, and monitor for a total of five consecutive test days. Document all results.

If all five MICs for the antimicrobial agent/organism combination are within the acceptable ranges, as defined in Tables 5 and 6, no additional corrective action is necessary.

If any of the five MICs is still outside the acceptable range, additional corrective action is required (see Section 5.10.2.2).

Daily control tests must be continued until final resolution of the problem can be achieved.

5.10.2.2 Additional Corrective Action

When immediate corrective action does not resolve the problem, it is likely due to a system versus a random error. The following common sources of error should be investigated to verify that:

- the turbidity standard has not expired, is stored properly, meets performance requirements, and was adequately mixed prior to use;
- all materials used were within their expiration date and stored at the proper temperature;
- the incubator is at proper temperature and atmosphere;
- other equipment used (e.g., pipettors) are functioning properly;
- plates were stored at proper temperature;
- the control strain has not changed and is not contaminated;
- inoculum suspensions were prepared and adjusted correctly; and
- inoculum for the test was prepared from a plate incubated for the correct length of time and in no case more than 24 hours old.

It may be necessary to obtain a new quality control strain (either from freezer storage or a reliable source) and new lots of materials (including new turbidity standards), possibly from different manufacturers. If the problem appears to be related to a manufacturer, the manufacturer should be contacted. It is also helpful to exchange quality control strains and materials with another laboratory using the same method.

Until the problem is resolved, it may be necessary to use an alternate test method.

Once the problem is corrected, in order to return to weekly quality control testing, documentation of satisfactory performance for another 30 consecutive days is required (see Section 5.9.1).
5.11 Reporting Patient Results When Out-of-Control Tests Occur

Whenever an out-of-control result occurs or corrective action is necessary, careful assessment of whether to report patient results should be made on an individual basis, taking into account if the source of the error, when known, is likely to have affected relevant patient results. Options that may be considered include suppressing the results for an individual antimicrobial agent; retrospectively reviewing individual patient or cumulative data for unusual patterns; and using an alternate test method or a reference laboratory until the problem is resolved.

6 Specific Antimicrobial Resistance Tests

6.1 Purpose

In some cases it is necessary to perform specialized susceptibility tests to detect resistance to a given antimicrobial agent, rather than use interpretive criteria from the tables. This section describes the performance and interpretation of these tests for key organisms and antimicrobial agents. Additional information is found in Tables 9A thru 9D.

6.2 β-Lactamase Tests

6.2.1 Purpose

A rapid β-lactamase test may yield clinically relevant information earlier than the results of an MIC test with \textit{Haemophilus} spp., \textit{N. gonorrhoeae}, and \textit{Moraxella catarrhalis}; it is the only reliable test for detecting β-lactamase-producing \textit{Enterococcus} spp. β-lactamase testing may also 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).

A positive β-lactamase test result predicts the following:

- resistance to penicillin, ampicillin, and amoxicillin among \textit{Haemophilus} spp. and \textit{M. catarrhalis}; and
- resistance to penicillin as well as amino-, carboxy-, and ureidopenicillins among staphylococci and enterococci.

A negative β-lactamase test result does not rule out resistance due to other mechanisms. Do not test members of the Enterobacteriaceae, \textit{Pseudomonas} spp., and other aerobic, gram-negative bacilli, because the results may not be predictive of susceptibility to the β-lactams most often used for therapy.

6.3 Selecting a β-Lactamase Test

Chromogenic, cephalosporin-based (such as nitrocefin) tests are the preferred method for testing \textit{Haemophilus} spp., \textit{M. catarrhalis}, staphylococci, enterococci, and anaerobes.\textsuperscript{10,31,32} Acidimetric β-lactamase tests have generally produced acceptable results with \textit{Haemophilus} spp. and staphylococci. Accurate detection of β-lactamase in staphylococci may require induction of the enzyme and incubation of a chromogenic, cephalosporin-based test for up to one hour. Induction can be easily accomplished by testing the growth from the zone margin surrounding an oxacillin disk test. Care must be exercised when using these assays to ensure accurate results, including testing of known positive and negative control strains at the time clinical isolates are examined.
6.4 Detection of Extended-Spectrum, β-Lactamase-Producing, Gram-Negative Bacilli

Extended-spectrum β-lactamases (ESBLs) are newly described enzymes that arise by mutations in genes for common plasmid-mediated β-lactamases, such as TEM-1, TEM-2, and SHV-1. ESBLs may confer resistance to penicillins, cephalosporins, and aztreonam in clinical isolates of *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Escherichia coli*, and a few other genera of the Enterobacteriaceae family that are usually susceptible to these agents. Some of these strains will show MICs above the normal susceptible population but below the standard breakpoints for certain extended-spectrum cephalosporins or aztreonam; such strains may be screened for potential ESBL production by using the screening breakpoints listed in the table at the end of Tables 9A and 9B. Other strains may test intermediate or resistant by standard breakpoints to one or more of these agents. In all strains with ESBLs, the MICs for one or more of the extended spectrum cephalosporins or aztreonam should decrease in the presence of clavulanic acid (see ESBL table at the end of Tables 9A and 9B). For all ESBL-producing strains, the test interpretation should be reported as resistant for all penicillins, cephalosporins, and aztreonam. For current recommendations for further testing and reporting, see Tables 9A and 9B.

6.5 Detection of Resistant Staphylococci

6.5.1 Methicillin/Oxacillin Resistance

Historically, resistance to the antistaphylococcal, β-lactamase-stable penicillins has been referred to as “methicillin resistance,” thus the acronyms “MRSA” (for methicillin-resistant *S. aureus*) or “MRS” (for methicillin-resistant staphylococci) are still commonly used, even though methicillin is no longer the agent of choice for testing or treatment. In this document, resistance to these agents may be referred to using several terms, e.g., “MRS,” “methicillin resistance,” or “oxacillin resistance.” Problems associated with detection of MRS continue to be experienced by some laboratories. For best recognition of these strains, the following points should be considered:

- Tests incorporating oxacillin are more likely to detect resistance than those using methicillin or nafcillin. Therefore, oxacillin is the preferred agent to test for methicillin/oxacillin resistance.

- The addition of NaCl (2% w/v; 0.34 mol/L) to the medium for both agar and broth dilution is recommended for testing of the penicillinase-stable penicillins.

- The inoculum should be prepared using the direct method of colony suspension (Section 3.2.4.2) rather than the inoculum growth method (Section 3.2.4.1).

- Tests to detect MRS must be incubated for a full 24 hours (rather than 16 to 20 hours) at 33 to 35 ºC (do not exceed 35 ºC).

- Microbiologists should be aware that methicillin-resistant staphylococci are often resistant to multiple antimicrobial agents, including other β-lactams, aminoglycosides, macrolides, clindamycin, and tetracycline. The observation of multiple resistance should be a clue to the possibility of methicillin resistance.

- If the MIC test result is in doubt with a possible methicillin-resistant *Staphylococcus aureus*, perform additional confirmatory tests, such as the oxacillin-salt agar-screening test as described below and in Table 9C.
• Methicillin-resistant \emph{S. aureus} and coagulase-negative staphylococci should be reported as resistant to all cephalosporins and other β-lactams, such as amoxicillin-clavulanic acid, ampicillin-sulbactam, ticarcillin-clavulanic acid, piperacillin-tazobactam, and imipenem, regardless of \textit{in vitro} test results with those agents. This is because most cases of documented MRS infections have responded poorly to β-lactam therapy, or because convincing clinical data have yet to be presented that document clinical efficacy for those agents.

### 6.5.2 Oxacillin Screening Plates

The oxacillin salt-agar screening-plate procedure can be used in addition to the dilution methods described above for the detection of MRSA. The test is performed by inoculating an \emph{S. aureus} isolate onto Mueller-Hinton agar that has been supplemented with NaCl (4% w/v; 0.68 mol/L) and that contains 6 µg oxacillin/mL. The agar is inoculated as either a spot or streak using a cotton swab that was dipped into a direct colony suspension equivalent to a 0.5 McFarland standard. The plate is incubated no higher than 35 ºC for 24 hours and examined carefully with transmitted light for evidence of small colonies (>1 colony) or a light film of growth, indicating oxacillin resistance.34 See Table 9C for details.

### 6.5.3 Reduced Susceptibility to Vancomycin

Strains of coagulase-negative staphylococci with intermediate and resistant MICs to vancomycin and teicoplanin have been described.35, 36 The first occurrence of strains of \emph{S. aureus} with decreased susceptibility to vancomycin (MICs 4 to 8 µg/mL) was reported from Japan in 1997,37 followed by reports from the U.S. and France.38 The exact mechanism of resistance that results in these elevated MICs is unknown, although it likely involves alterations in the cell wall and hyperexpression of penicillin-binding proteins (PBPs). To date, all of these \emph{S. aureus} strains appear to have developed from MRSA.

In order to recognize strains with vancomycin MICs of 4 to 8 µg/mL, MIC testing must be performed, or the vancomycin agar screen test described for enterococci may be successfully used to detect these isolates,39 incubating the plates for a full 24 hours at 35 ºC. Use of a negative quality control strain, such as \emph{S. aureus} ATCC® 29213, is critical to ensure specificity. Until further data on the prevalence or clinical significance of these isolates is known, laboratories may choose to examine MRSA strains more carefully for elevated MICs to vancomycin.

### 6.6 Detection of Resistant Enterococci

#### 6.6.1 Penicillin-Ampicillin Resistance

Enterococci may be resistant to penicillin and ampicillin because of production of low-affinity, penicillin-binding proteins (PBPs) or, less commonly, because of the production of β-lactamase. The agar or broth dilution test can accurately detect isolates with altered PBPs, but it will not reliably detect β-lactamase-producing strains.17 The rare β-lactamase-producing strains are detected best by using a direct, nitrocefin-based β-lactamase test (see Section 6.3). Certain penicillin- or ampicillin-resistant enterococci may possess high-level resistance (i.e., penicillin MICs ≥ 128 µg/mL). For enterococci recovered from blood and CSF, the laboratory should determine the actual MIC for penicillin or ampicillin, since \emph{E. faecium} strains with normal lower-level resistance (penicillin MICs ≤ 64 µg/mL and ampicillin ≥ 32 µg/mL) should be considered potentially susceptible to synergy with an aminoglycoside (in the absence of high-level aminoglycoside resistance); whereas higher-level resistance may be resistant to such synergy.21, 40, 41
6.6.2 Vancomycin Resistance

Accurate detection of vancomycin-resistant enterococci by the agar or broth dilution test requires incubation for a full 24 hours (rather than 16 to 20 hours) and that the plates, tubes, or wells be examined carefully for evidence of faint growth. A vancomycin agar screen test may also be used, as described in Table 9D.

6.6.3 High-Level Aminoglycoside Resistance

High-level resistance to aminoglycosides is an indication that an enterococcal isolate will not be affected synergistically by a combination of a penicillin or glycopeptide plus an aminoglycoside.11 Agar or broth high-concentration gentamicin (500 µg/mL) and streptomycin (1,000 µg/mL with broth microdilution; 2,000 µg/mL with agar) tests can be used to screen for this type of resistance (see Table 9D). Quality control of these tests is also explained in Table 9D. Other aminoglycosides need not be tested, because their activities against enterococci are not superior to gentamicin or streptomycin.

7 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 or flock. Inclusion of antimicrobial cost information can also be appropriate as an aid in the reduction of the expense of antimicrobial therapy. Procedures for compiling and reporting cumulative susceptibility data can be found in NCCLS document M39—Analysis and Presentation of Cumulative Antimicrobial Susceptibility Test Data.
References


8. AVMA Judicious Therapeutic Use of Antimicrobials. *JAVMA.* 1999;214:168


## Appendix A. Antimicrobial Susceptibility Quality Control Record

**ORGANISM TESTED** ________________________________  **ATCC #** ____________

**TEST METHOD:** BROTH DILUTION/DISK DIFFUSION

<table>
<thead>
<tr>
<th>ANTIMICROBIAL AGENT</th>
<th>DISK DIFFUSION CONTROL LIMITS*</th>
<th>DISK DIFFUSION TEST RESULTS</th>
<th>BROTH DILUTION CONTROL LIMITS*</th>
<th>BROTH DILUTION TEST RESULTS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

*CONTROL LIMITS AS DEFINED IN TABLES 4, 5, 5A, AND 6 OF M31.

**TECHNOLOGIST** ____________________________  **DATE TESTED** _________________

- Test daily (Section 5.6.1)

  - ≤ 1 out of 20 tests out of range
    - Continue daily testing
  - > 1 out of 20 tests out of range
    - Corrective Action (Section 5.6.4)
      - Reason for error obvious
        - Retest the same day
          - Results in range—continue daily testing
      - Reason for error not obvious
        - Immediate Corrective Action (Section 5.6.4.2.1)
          - Retest the same day and monitor for 5 consecutive days
            - All results in range
              - Continue daily testing
            - Any results out of range
              - Additional Corrective Action (Section 5.6.4.2.2)
                - Investigate possible sources of error

Demonstrating Satisfactory Performance
(Section 5.6.3)

≤ 3 out of 30 results out of range

Implement weekly testing

Any result out of range

Corrective Action
(Section 5.6.4)

Reason for error obvious
Retest the same day

Reason for error not obvious

Immediate Corrective Action
(Section 5.6.4.2.1)

Retest the same day and monitor for 5 consecutive days

≤ 3 out of 30 results out of range
Implement weekly testing

Results in range
Return to weekly testing

Any results out of range

Additional Corrective Action
(Section 5.6.4.2.2)

Investigate possible sources of error

All results in range
Return to weekly testing

Results out of range

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Appendix C1. Aerobic Dilution Daily Quality Control Testing Protocol

Test daily (Section 5.8)

≤ 1 out of 20 tests out of range

Continue daily testing

Reason for error obvious

Retest the same day

Results in range—continue daily testing

All results in range

Continue daily testing

> 1 out of 20 tests out of range

Corrective Action (Section 5.10)

Reason for error not obvious

Retest the same day and monitor for 5 consecutive days

Any results out of range

Additional Corrective Action (Section 5.10.2.2)

Investigate possible source of errors

Reason for error obvious

Retest the same day

Results in range—continue daily testing

All results in range

Continue daily testing

Reason for error not obvious

Retest the same day and monitor for 5 consecutive days

Any results out of range

Additional Corrective Action (Section 5.10.2.2)

Investigate possible source of errors

Demonstrate Satisfactory Performance
(Section 5.9.1)

≤ 3 out of 30 results out of range

Implement weekly testing

Any result out of range

Corrective Action
(Section 5.10)

Reason for error obvious
Retest the same day

Reason for error not obvious

Results in range
Return to weekly testing

Results out of range
Immediate Corrective Action
(Section 5.10.2.1)

Retest the same day and monitor for 5 consecutive days

All results in range
Return to weekly testing

Any results out of range
Additional Corrective Action
(Section 5.10.2.2)

Investigate possible source of errors
Table 1. Comprehensive List of Antimicrobial Agents\(^1\) That Could Be Considered for Routine Testing by Veterinary Microbiology Laboratories

<table>
<thead>
<tr>
<th>Group A — US-FDA-CVM-Approved Veterinary-Specific Interpretive Criteria</th>
<th>Swine</th>
<th>Cattle(^a)</th>
<th>Bovine Mastitis(^b)</th>
<th>Poultry(^c)</th>
<th>Horses</th>
<th>Dogs(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ceftriaxone</td>
<td>Ceftriaxone</td>
<td>Penicillin-novobiocin</td>
<td>Enrofloxacin(^e)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tiamulin</td>
<td>Enrofloxacin</td>
<td>Pirlimycin</td>
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</tr>
<tr>
<td></td>
<td>Tilmicosin</td>
<td>Florfenicol</td>
<td>Spectinomycin</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Tilmicosin</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group B — US-FDA-CVM-NCCLS-Approved Human Interpretive Criteria</th>
<th>Amoxicillin</th>
<th>Amoxicillin</th>
<th>Ampicillin(^f)</th>
<th>Erythromycin</th>
<th>Ampicillin(^g)</th>
<th>Amikacin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ampicillin(^h)</td>
<td>Ampicillin(^h)</td>
<td>Cephalothin(^h, i)</td>
<td>Gentamicin</td>
<td>Ceftriaxone</td>
<td>Amoxicillin-clavulanic acid</td>
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<tr>
<td></td>
<td>Erythromycin</td>
<td>Erythromycin</td>
<td>Cefoxitin</td>
<td>Penicillin</td>
<td>Gentamicin</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gentamicin</td>
<td>Penicillin</td>
<td>Erythromycin</td>
<td>Spectinomycin</td>
<td>Imipenem</td>
<td>Ampicillin(^g)</td>
</tr>
<tr>
<td></td>
<td>Penicillin</td>
<td>Sulphonamides</td>
<td>Penicillin</td>
<td>Sulfadimethoxine</td>
<td>Nitrofurantoin</td>
<td>Cefazolin</td>
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<tr>
<td></td>
<td>Sulfadimethoxine</td>
<td>Tetracycline(^j)</td>
<td>Tetracycline(^j)</td>
<td>Tetracycline(^j)</td>
<td>Sulfadimethoxine</td>
<td>Cefoxitin</td>
</tr>
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<td>Tetracycline(^j)</td>
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<td></td>
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</tbody>
</table>

\(^1\) NCCLS global consensus standard. © NCCLS. All rights reserved.
### Table 1. (Continued)

<table>
<thead>
<tr>
<th>Group C — US-FDA-CVM-Approved</th>
<th>Primary Test, Selectively Report</th>
<th>Swine</th>
<th>Cattle&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Bovine Mastitis&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Poultry&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Horses</th>
<th>Dogs</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Apramycin</td>
<td>Neomycin</td>
<td>Novobiocin</td>
<td>Bacitracin</td>
<td>Ceftiofur</td>
<td>Ceftiofur</td>
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<tr>
<td></td>
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<td>Bacitracin</td>
<td>Streptomycin</td>
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<td>Ceftiofur</td>
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<td>Lincomycin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lincomycin</td>
<td>Tyllosin</td>
<td></td>
<td>Lincomycin</td>
<td>Neomycin</td>
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<tr>
<td></td>
<td></td>
<td>Neomycin</td>
<td>Virginiamycin</td>
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<td></td>
<td>Spectinomycin</td>
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<td></td>
<td>Spectinomycin</td>
<td>Streptomycin</td>
<td>Virginiamycin</td>
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<td></td>
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<td>Sulfachlorapyridazine</td>
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<td></td>
<td></td>
<td>Sulfamethazine</td>
<td>Tyllosin</td>
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<td></td>
<td></td>
<td>Virginiamycin</td>
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</tr>
<tr>
<td>Group D — Supplemental (AMDUCA-use)</td>
<td>Selectively Test, Selectively Report</td>
<td>Amikacin</td>
<td>Amikacin&lt;sup&gt;m&lt;/sup&gt;</td>
<td></td>
<td>Erythromycin</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sulfamethoxine</td>
<td>Gentamicin&lt;sup&gt;n&lt;/sup&gt;</td>
<td></td>
<td>Imipenem</td>
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<tr>
<td></td>
<td></td>
<td>Trimetoprim-</td>
<td>Trimetoprim-</td>
<td></td>
<td>Oxacillin</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>sulfamethoxazole</td>
<td>sulfamethoxazole</td>
<td></td>
<td>Rifampin</td>
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<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>Tetracycline&lt;sup&gt;j&lt;/sup&gt;</td>
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<td></td>
<td></td>
<td></td>
<td>Ticarcillin</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>Ticarcillin-clavulanic acid</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**NOTE 1:** This table contains those compounds used for therapeutic and control treatment of animal diseases. Compounds with prophylactic, prevention, or growth promotion indications are not listed. Some of these drugs do not have interpretive criteria (Table 2) and QC guidelines (Tables 4 through 6).

**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 Groups A, B and C 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 D are not approved but are frequently used in an extralabel manner in the indicated animal. The laboratory client, or veterinarian, assumes all responsibility for efficacy, safety, and residue avoidance with extra-label use of antimicrobial agents.

**Footnotes**

a. Does not include goats or sheep.
b. Only compounds approved for use in lactating dairy cattle by intramammary infusion are listed.
c. Includes chickens and turkeys.
d. Includes cats.
e. The U.S. FDA has recommended that fluoroquinolones and glycopeptides not be reported for AMDUCA use in food animals.
f. Clindamycin is also used to test for susceptibility to lincomycin.
g. The results of ampicillin susceptibility tests may be used to predict susceptibility to amoxicillin and hetacillin.
h. 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.
i. Cefazolin can be tested to represent the first-generation cephalosporins, such as cephapirin and cefadroxil. Cefazolin should be tested separately against the Enterobacteriaceae.
j. Tetracycline is tested as the class representative for chlortetracycline doxycycline, minocycline, and oxytetracycline.
k. Trimethoprim-sulfamethoxazole can be tested to represent the potentiated sulfonamides, including trimethoprim-sulfadiazine and ormetoprim-sulfamethoxine.
l. Chloramphenicol must not be reported with any food-producing animal.
m. Due to extended residue times, use of gentamicin in cattle should be avoided, and if used, carefully monitored.
Table 2. Zone Diameter Interpretive Standards and Minimal Inhibitory Concentration (MIC) Breakpoints for Veterinary Pathogens

<table>
<thead>
<tr>
<th>Antimicrobial Agent</th>
<th>Disk Content</th>
<th>Zone Diameter (mm)</th>
<th>MIC Breakpoint (µg/mL)</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>S</td>
<td>I</td>
<td>F</td>
</tr>
<tr>
<td>Aminoglycosides/Aminocyclitols</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amikacin</td>
<td>30 µg</td>
<td>≥ 17</td>
<td>15-16</td>
<td>≤ 14</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>10 µg</td>
<td>≥ 15</td>
<td>13-14</td>
<td>≤ 12</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>30 µg</td>
<td>≥ 18</td>
<td>14-17</td>
<td>≤ 13</td>
</tr>
<tr>
<td>Spectinomycin</td>
<td>100 µg</td>
<td>≥ 14</td>
<td>11-13</td>
<td>≤ 10</td>
</tr>
<tr>
<td>Bovine (Respiratory Disease)</td>
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<td></td>
<td></td>
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<tr>
<td>Mannheimia haemolytica</td>
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<tr>
<td>Pasteurella multocida</td>
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<td></td>
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<tr>
<td>Haemophilus somnus</td>
<td></td>
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<tr>
<td>β-Lactam/β-Lactamase Inhibitor Combinations</td>
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<tr>
<td>Amoxicillin-clavulanic acid</td>
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<tr>
<td>Staphylococci</td>
<td>20/10 µg</td>
<td>≥ 20</td>
<td>—</td>
<td>≤ 19</td>
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<tr>
<td>Other organisms</td>
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<td>≥ 18</td>
<td>14-17</td>
<td>≤ 13</td>
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<td>Ticarcillin-clavulanic acid</td>
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<td>≥ 15</td>
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<td>≤ 14</td>
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<td>Pseudomonas aeruginosa</td>
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<td>15-19</td>
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<td>Gram-negative enteric organisms</td>
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<td>β-Lactams Penicillins</td>
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<td>Ampicillin</td>
<td>10 µg</td>
<td>≥ 17</td>
<td>14-16</td>
<td>≤ 13</td>
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<tr>
<td>Enterobacteriaceae</td>
<td>10 µg</td>
<td>≥ 29</td>
<td>—</td>
<td>≤ 28</td>
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<tr>
<td>Staphylococci</td>
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<td>≤ 16</td>
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<tr>
<td>Enterococci</td>
<td>10 µg</td>
<td>≥ 26</td>
<td>19-25</td>
<td>≤ 18</td>
</tr>
<tr>
<td>Streptococci (not S. pneumoniae)</td>
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<tr>
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<td>1 µg</td>
<td>≥ 13</td>
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<tr>
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<tr>
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<td>≥ 15</td>
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<td>≤ 14</td>
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<tr>
<td>S. pneumoniae</td>
<td>1 µg oxacillin</td>
<td>≥ 20</td>
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<td>≤ 0.06</td>
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<td>S. pneumoniae</td>
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<td>Ticarcillin</td>
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<td>Pseudomonas aeruginosa</td>
<td>75 µg</td>
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<td>75 µg</td>
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<td>15-19</td>
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Ampicillin is used to test for susceptibility to amoxicillin and hetacillin.

Oxacillin is used to test for susceptibility to methicillin, nafcillin, and cloxacillin.

The interpretive standards for Streptococcus spp. including S. pneumoniae only apply to disk susceptibility testing performed using Mueller-Hinton agar supplemented with 5% CO₂ and broth with 2 to 5% lysed horse blood.
### Table 2. (Continued)

<table>
<thead>
<tr>
<th>Antimicrobial Agent</th>
<th>Disk Content</th>
<th>Zone Diameter (mm)</th>
<th>MIC Breakpoint (µg/mL)</th>
<th>Comments</th>
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<td>Penicillin-novobiocin</td>
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<tr>
<td>Bovine mastitis</td>
<td>10 units/30 µg</td>
<td>≥ 18</td>
<td>15-17</td>
<td>≤ 14</td>
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<tr>
<td><em>Staphylococcus aureus</em></td>
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<tr>
<td><em>Streptococcus agalactiae</em></td>
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<tr>
<td><em>Streptococcus dysgalactiae</em></td>
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<td>Cefiofur</td>
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<td>≤ 17</td>
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<tr>
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<tr>
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<td>≤ 16</td>
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<td>Canine and feline (dermal, URI, UTI)</td>
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<tr>
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<td>17-22</td>
<td>≤ 16</td>
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<tr>
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<td>MIC Breakpoint (µg/mL)</td>
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<td>5 µg</td>
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<td>17-20</td>
<td>≤ 16</td>
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<td><em>Pasteurella multocida</em></td>
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<td><em>Haemophilus somnus</em></td>
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<tr>
<td>Difloxacin</td>
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<tr>
<td>Orbifloxacin</td>
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<td>≤ 17</td>
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<td>Gram-negative enteric bacilli</td>
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<td><em>Staphylococcus</em> spp.</td>
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<td>Clindamycin</td>
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<td>—</td>
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<td><em>Streptococcus agalactiae</em></td>
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<td><em>Streptococcus dysgalactiae</em></td>
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<td><em>Streptococcus uberis</em></td>
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<td>Macrolides</td>
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<td>Erythromycin</td>
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<td>15 µg</td>
<td>≥ 23</td>
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<td>≤ 13</td>
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<td>15 µg</td>
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<td>16-20</td>
<td>≤ 15</td>
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<tr>
<td>Tilmicosin</td>
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<td>Bovine (Respiratory Disease)</td>
<td>15 µg</td>
<td>≥ 14</td>
<td>11-13</td>
<td>≤ 10</td>
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<tr>
<td>Swine (Respiratory Disease)</td>
<td>15 µg</td>
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<td>—</td>
<td>≤ 10</td>
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<tr>
<td><em>Actinobacillus pleuroneumoniae</em></td>
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### Table 2. (Continued)

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<tr>
<th>Antimicrobial Agent</th>
<th>Disk Content</th>
<th>Zone Diameter (mm)</th>
<th>MIC Breakpoint (µg/mL)</th>
<th>Comments</th>
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<td>S I F R</td>
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<td><strong>Phenics</strong></td>
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<td>30 µg</td>
<td>≥ 18 13-17 ≤ 12</td>
<td>≤ 8 16 ≥ 32</td>
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<tr>
<td>S. pneumoniae</td>
<td>30 µg</td>
<td>≥ 21 — ≤ 20</td>
<td>≤ 4 — ≥ 8</td>
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</tr>
<tr>
<td>Streptococci (not S. pneumoniae)</td>
<td>30 µg</td>
<td>≥ 21 18-20 ≤ 17</td>
<td>≤ 4 8 ≥ 16</td>
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<td><strong>Florfenicol</strong></td>
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<tr>
<td>Bovine (Respiratory Disease)</td>
<td>30 µg</td>
<td>≥ 19 15-18 ≤ 14</td>
<td>≤ 2 4 ≥ 8</td>
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</tr>
<tr>
<td>Mannheimia haemolytica</td>
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<td></td>
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<tr>
<td>Pasteurella multocida</td>
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<td>Haemophilus sominus</td>
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<td><strong>Pleomutilins</strong></td>
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<tr>
<td>Tiamulin</td>
<td>30 µg</td>
<td>≥ 9 — ≤ 8</td>
<td>≤ 16 — ≥ 32</td>
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<tr>
<td>Swine (Respiratory Disease)</td>
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<tr>
<td>Actinobacillus pleuropneumoniae</td>
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<tr>
<td><strong>Potentiated Sulfonamides</strong></td>
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<tr>
<td>Trimethoprim-sulfamethoxazole</td>
<td>1.25/23.75 µg</td>
<td>≥ 16 11-15 ≤ 10</td>
<td>≤ 2/38 — ≥ 4/76</td>
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<tr>
<td>Organisms other than S. pneumoniae</td>
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<tr>
<td>Streptococcus pneumoniae</td>
<td>1.25/23.75 µg</td>
<td>≥ 19 16-18 ≤ 15</td>
<td>≤ 0.5/9.5 1/19-2/38 ≥ 4/76</td>
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<td><strong>Ansamycins</strong></td>
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<td>Rifampin</td>
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<tr>
<td>Organisms other than streptococci</td>
<td>5 µg</td>
<td>≥ 20 17-19 ≤ 16</td>
<td>≤ 1 2 ≥ 4</td>
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<tr>
<td>Streptococcus pneumoniae</td>
<td>5 µg</td>
<td>≥ 19 17-18 ≤ 16</td>
<td>≤ 1 2 ≥ 4</td>
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<tr>
<td><strong>Sulfonamides</strong></td>
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<tr>
<td>Sulfisoxazole</td>
<td>250 or 300 µg</td>
<td>≥ 17 13-16 ≤ 12</td>
<td>≤ 256 — ≥ 512</td>
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<td><strong>Tetracyclines</strong></td>
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<tr>
<td>Organisms other than streptococci</td>
<td>30 µg</td>
<td>≥ 19 15-18 ≤ 14</td>
<td>≤ 4 8 ≥ 16</td>
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<tr>
<td>Streptococci</td>
<td>30 µg</td>
<td>≥ 23 19-22 ≤ 18</td>
<td>≤ 2 4 ≥ 8</td>
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</tbody>
</table>

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.

Tetracycline is used to test for susceptibility to chlortetracycline, oxytetracycline, minocycline, and doxycycline.

The interpretive standards for Streptococcus spp. including S. pneumoniae only apply to disk susceptibility testing performed using Mueller-Hinton agar supplemented with 5% CO₂ and broth with 2 to 5% lysed horse blood.
Table 2. (Continued)

<table>
<thead>
<tr>
<th>Antimicrobial Agent</th>
<th>Disk Content</th>
<th>Zone Diameter (mm)</th>
<th>MIC Breakpoint (g/mL)</th>
<th>Comments</th>
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<tr>
<td><strong>Glycopeptides</strong></td>
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<tr>
<td>Vancomycin</td>
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<td>Enterococci</td>
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<td>≥ 17</td>
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<td>—</td>
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<tr>
<td>Streptococci</td>
<td>30 µg</td>
<td>≥ 17</td>
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</tr>
<tr>
<td>Other gram-positive organisms</td>
<td>30 µg</td>
<td>≥ 12</td>
<td>10-11</td>
<td>≤ 9</td>
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</table>

NOTE 1: Zone interpretive criteria and MIC breakpoints for antimicrobial agents with gray shading are human data taken from M100-S12 supplements to M2 and M7.

NOTE 2: Veterinary-specific interpretive criteria are valid only for indicated organisms.

NOTE 3: Flexible label (F) indicates the availability of FDA-approved labeling; this organism should be considered susceptible if appropriate dosing modifications found in the packaging insert are applied.

NOTE 4: Certain veterinary specific disks may or may not be commercially available. Disks not commercially available may be obtained from the pharmaceutical sponsors as indicated:

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<th>Antibiotic</th>
<th>Sponsor</th>
<th>Address</th>
<th>Phone</th>
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<td>Apramycin</td>
<td>Elanco</td>
<td>Greenfield, IN</td>
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<td>Tilmicosin</td>
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<td>Ceftiofur</td>
<td>Pharmacia</td>
<td>Kalamazoo, MI</td>
<td>800-793-0596</td>
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<td>Penicillin-Novobiocin</td>
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<td>Pirlimycin</td>
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<td>Spectinomycin</td>
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<td>Overland Park, KS</td>
<td>800-685-5656</td>
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<td>Bayer</td>
<td>Shawnee Mission, KS</td>
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<tr>
<td>Florfenicol</td>
<td>Schering-Plough</td>
<td>Union, NJ</td>
<td>800-521-5767</td>
</tr>
<tr>
<td>Orbifloxacin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tiamulin</td>
<td>Boehringer Ingelheim Vetmedica, Inc.</td>
<td>St. Joseph, MO</td>
<td>800-325-9167</td>
</tr>
</tbody>
</table>
Table 3.  Culture Collection Numbers for Organisms Used for Quality Control of Antimicrobial Susceptibility Tests

<table>
<thead>
<tr>
<th>Organism</th>
<th>ATCC(^1)</th>
<th>DSM(^2)</th>
<th>UKNCC(^3)</th>
<th>JCM(^4)</th>
<th>CIP(^5)</th>
<th>CCUG(^6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Escherichia coli</td>
<td>25922</td>
<td>1103</td>
<td>12241</td>
<td>5491</td>
<td>7624</td>
<td>17620</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>35218</td>
<td>5923</td>
<td>11954</td>
<td></td>
<td>102181</td>
<td>30600</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>27853</td>
<td>1117</td>
<td>10896</td>
<td></td>
<td>76110</td>
<td>17619</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>29213</td>
<td>2569</td>
<td>2874</td>
<td></td>
<td>103429</td>
<td>15915</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>25923</td>
<td>1104</td>
<td>12702</td>
<td>2413</td>
<td>7625</td>
<td>17621</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>43300</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enterococcus faecalis</td>
<td>29212</td>
<td>2570</td>
<td>12697</td>
<td>2875</td>
<td>103214</td>
<td>9997</td>
</tr>
<tr>
<td>Enterococcus faecalis</td>
<td>33186</td>
<td></td>
<td>12756</td>
<td></td>
<td>100750</td>
<td></td>
</tr>
<tr>
<td>Enterococcus faecalis</td>
<td>51299</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actinobacillus pleuropneumoniae</td>
<td>27090</td>
<td></td>
<td></td>
<td></td>
<td>100916 T</td>
<td></td>
</tr>
<tr>
<td>Haemophilus somnus</td>
<td>700025</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Campylobacter jejuni</td>
<td>33560</td>
<td>11351</td>
<td></td>
<td></td>
<td>70.2 T</td>
<td></td>
</tr>
<tr>
<td>Streptococcus pneumoniae</td>
<td>49619</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>700603</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^1\)American Type Culture Collection (www.atcc.org)
\(^2\)Deutsche Sammlung von Mikroorganismen und Zellkulturen (www.dsmz.de)
\(^3\)United Kingdom National Culture Collections (includes National Collection of Type Cultures [NCTC] and National Collection of Industrial and Marine Bacteria [NCIMB]) (www.uknc.co.uk)
\(^4\)Japan Collection of Microorganisms (www.jcmriken.go.jp)
\(^5\)Collection of Bacterial Strain of Institut Pasteur
\(^6\)Swedish Reference Group for Antibiotics (www.srga.org)
### Table 4. Acceptable Quality Control Ranges of Antimicrobial Disk Susceptibility Test Zone Diameters (mm) for Reference Strains on Mueller-Hinton Medium Without Blood or Other Supplements

<table>
<thead>
<tr>
<th>Antimicrobial Agent</th>
<th>Disk Content</th>
<th>Escherichia coli ATCC® 25922</th>
<th>Staphylococcus aureus ATCC® 25923</th>
<th>Pseudomonas aeruginosa ATCC® 27853</th>
<th>Streptococcus pneumoniae ATCC® 49619</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amikacin</td>
<td>30 µg</td>
<td>19-26</td>
<td>20-26</td>
<td>18-26</td>
<td>—</td>
</tr>
<tr>
<td>Amoxicillin-clavulanic acid</td>
<td>20/10 µg</td>
<td>18-24</td>
<td>28-36</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>10 µg</td>
<td>16-22</td>
<td>27-35</td>
<td>—</td>
<td>30-36</td>
</tr>
<tr>
<td>Apramycin</td>
<td>15 µg</td>
<td>15-20</td>
<td>17-24</td>
<td>13-18</td>
<td>—</td>
</tr>
<tr>
<td>Cefazolin</td>
<td>30 µg</td>
<td>21-27</td>
<td>29-35</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>30 µg</td>
<td>23-29</td>
<td>23-29</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Cefotiofur</td>
<td>30 µg</td>
<td>26-31</td>
<td>27-31</td>
<td>14-18</td>
<td>—</td>
</tr>
<tr>
<td>Cephalothin</td>
<td>30 µg</td>
<td>15-21</td>
<td>29-37</td>
<td>—</td>
<td>26-32</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>30 µg</td>
<td>21-27</td>
<td>19-26</td>
<td>—</td>
<td>23-27</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>2 µg</td>
<td>—</td>
<td>24-30</td>
<td>—</td>
<td>19-25</td>
</tr>
<tr>
<td>Difloxacin</td>
<td>10 µg</td>
<td>28-35</td>
<td>27-33</td>
<td>16-22</td>
<td>—</td>
</tr>
<tr>
<td>Enrofloxacin</td>
<td>5 µg</td>
<td>32-40</td>
<td>27-31</td>
<td>15-19</td>
<td>—</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>15 µg</td>
<td>—</td>
<td>22-30</td>
<td>—</td>
<td>25-30</td>
</tr>
<tr>
<td>Florfenicol</td>
<td>30 µg</td>
<td>22-28</td>
<td>22-29</td>
<td>—</td>
<td>24-31</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>10 µg</td>
<td>19-26</td>
<td>19-27</td>
<td>16-21</td>
<td>—</td>
</tr>
<tr>
<td>Imipenem</td>
<td>10 µg</td>
<td>26-32</td>
<td>—</td>
<td>20-28</td>
<td>—</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>30 µg</td>
<td>17-25</td>
<td>19-26</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Orbifloxacin</td>
<td>10 µg</td>
<td>29-37</td>
<td>24-30</td>
<td>16-22</td>
<td>—</td>
</tr>
<tr>
<td>Oxacillin</td>
<td>1 µg</td>
<td>—</td>
<td>18-24</td>
<td>—</td>
<td>≤ 12c</td>
</tr>
<tr>
<td>Penicillin</td>
<td>10 units</td>
<td>—</td>
<td>26-37</td>
<td>—</td>
<td>24-30</td>
</tr>
<tr>
<td>Penicillin-novobiocin (1/3)</td>
<td>10 units/30 μg</td>
<td>—</td>
<td>30-36</td>
<td>—</td>
<td>24-30</td>
</tr>
<tr>
<td>Pirlimycin</td>
<td>2 µg</td>
<td>—</td>
<td>20-25</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Rifampin</td>
<td>5 µg</td>
<td>8-10</td>
<td>26-34</td>
<td>—</td>
<td>25-30</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>30 µg</td>
<td>18-25</td>
<td>24-30</td>
<td>—</td>
<td>27-31</td>
</tr>
<tr>
<td>Tiamulin</td>
<td>30 µg</td>
<td>—</td>
<td>25-32</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Ticarcillin</td>
<td>75 µg</td>
<td>24-30</td>
<td>—</td>
<td>21-27</td>
<td>—</td>
</tr>
<tr>
<td>Ticarcillin-clavulanic acid</td>
<td>75/10 µg</td>
<td>24-30</td>
<td>29-37</td>
<td>20-28</td>
<td>—</td>
</tr>
<tr>
<td>Tilmicosin</td>
<td>15 µg</td>
<td>—</td>
<td>17-21</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Spectinomycin</td>
<td>100 µg</td>
<td>21-25</td>
<td>13-17</td>
<td>10-14</td>
<td>—</td>
</tr>
<tr>
<td>Sulfisoxazole</td>
<td>250 or 300 µg</td>
<td>15-23</td>
<td>24-34</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Trimethoprim-Sulfamethoxazole</td>
<td>1.25/23.75 µg</td>
<td>23-29</td>
<td>24-32</td>
<td>—</td>
<td>20-28</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>30 µg</td>
<td>—</td>
<td>17-21</td>
<td>—</td>
<td>20-27</td>
</tr>
</tbody>
</table>
Table 4. (Continued)

a. 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% CO₂.

b. The QC range for *E. coli* ATCC 35218 is 17-22 mm.

c. Deterioration in oxacillin disk content is best assessed with QC organism *Staphylococcus aureus* ATCC® 25923, with an acceptable zone diameter of 18 to 24 mm.

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

e. Very medium-dependent, especially with enterococci.

**NOTE 1:** The dash (—) indicates that no range has been established.

**NOTE 2:** 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 *Mannheimia haemolytica*. An inhibition zone of ≥ 20 mm that is free of fine colonies is acceptable.

**NOTE 3:** Certain veterinary specific disks may or may not be commercially available. Disks not commercially available may be obtained from the pharmaceutical sponsors as indicated:

<table>
<thead>
<tr>
<th>Drug</th>
<th>Company</th>
<th>Address</th>
<th>Phone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apramycin</td>
<td>Elanco Animal Health</td>
<td>Greenfield, IN</td>
<td>800-428-4441</td>
</tr>
<tr>
<td>Tilmicosin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>Pharmacia Animal Health</td>
<td>Kalamazoo, MI</td>
<td>800-793-0596</td>
</tr>
<tr>
<td>Penicillin-Novobiocin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pirlimycin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spectinomycin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Difloxacin</td>
<td>Fort Dodge Animal Health</td>
<td>Overland Park, KS</td>
<td>800-685-5656</td>
</tr>
<tr>
<td>Enrofloxacin</td>
<td>Bayer Animal Health</td>
<td>Shawnee Mission, KS</td>
<td>800-422-9874</td>
</tr>
<tr>
<td>Florfenicol</td>
<td>Schering-Plough Animal Health</td>
<td>Union, NJ</td>
<td>800-521-5767</td>
</tr>
<tr>
<td>Orbifloxacin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tiamulin</td>
<td>Boehringer Ingelheim Vetmedica, Inc.</td>
<td>St. Joseph, MO</td>
<td>800-325-9167</td>
</tr>
</tbody>
</table>
### Table 5. Acceptable Quality Control Ranges of MICs (µg/mL) for Reference Strains

<table>
<thead>
<tr>
<th>Antimicrobial Agent</th>
<th>Staphylococcus aureus ATCC® 29213</th>
<th>Enterococcus faecalis ATCC® 29212</th>
<th>Escherichia coli ATCC® 25922</th>
<th>Pseudomonas aeruginosa ATCC® 27853</th>
<th>Streptococcus pneumoniae ATCC® 49619</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amikacin</td>
<td>1-4</td>
<td>64-256</td>
<td>0.5-4</td>
<td>1-4</td>
<td>—</td>
</tr>
<tr>
<td>Amoxicillin-clavulanic acidb</td>
<td>0.12/0.06-0.5/0.25</td>
<td>0.25/0.12-1.0/0.5</td>
<td>2/1-8/4</td>
<td>—</td>
<td>0.03/0.016-0.12/0.06</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>0.5-2</td>
<td>0.5-2</td>
<td>2-8</td>
<td>—</td>
<td>0.06-0.25</td>
</tr>
<tr>
<td>Apramycin</td>
<td>2-8</td>
<td>—</td>
<td>2-16</td>
<td>2-16</td>
<td>—</td>
</tr>
<tr>
<td>Cefazolin</td>
<td>0.25-1</td>
<td>—</td>
<td>1-4</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>1-4</td>
<td>—</td>
<td>2-8</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Ceftiofur</td>
<td>0.25-1.0</td>
<td>0.25-1.0</td>
<td>16-64</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Cephalothin</td>
<td>0.12-0.5</td>
<td>—</td>
<td>4-16</td>
<td>—</td>
<td>0.52</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>2-8</td>
<td>4-16</td>
<td>2-8</td>
<td>—</td>
<td>2-8</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>0.06-0.25</td>
<td>4-16</td>
<td>—</td>
<td>—</td>
<td>0.03-0.12</td>
</tr>
<tr>
<td>Difloxacin</td>
<td>0.06-0.5</td>
<td>1-4</td>
<td>0.015-0.12</td>
<td>1-8</td>
<td>—</td>
</tr>
<tr>
<td>Enrofloxacin</td>
<td>0.03-0.12</td>
<td>0.12-1</td>
<td>0.008-0.03</td>
<td>1-4</td>
<td>—</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>0.25-1</td>
<td>1-4</td>
<td>—</td>
<td>—</td>
<td>0.03-0.12</td>
</tr>
<tr>
<td>Florfenicol</td>
<td>2-8</td>
<td>2-8</td>
<td>2-8</td>
<td>&gt;16</td>
<td>1-4</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>0.12-1</td>
<td>4-16</td>
<td>0.25-1</td>
<td>0.5-2</td>
<td>—</td>
</tr>
<tr>
<td>Imipenem</td>
<td>0.016-0.06</td>
<td>0.5-2</td>
<td>0.06-0.25</td>
<td>1-4</td>
<td>0.03-0.12</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>1-4</td>
<td>16-64</td>
<td>1-4</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Orbifloxacin</td>
<td>0.25-2</td>
<td>1-8</td>
<td>0.015-0.12</td>
<td>2-16</td>
<td>—</td>
</tr>
<tr>
<td>Oxacillin</td>
<td>0.12-0.5</td>
<td>8-32</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Penicillin</td>
<td>0.25-2</td>
<td>1-4</td>
<td>—</td>
<td>—</td>
<td>0.25-1</td>
</tr>
<tr>
<td>Penicillin-novobiocin</td>
<td>0.015/0.03-0.06/0.12</td>
<td>0.25/0.5-2/4</td>
<td>8/16-&gt;8/16</td>
<td>&gt;8/16</td>
<td>—</td>
</tr>
<tr>
<td>Pirlimycin</td>
<td>0.25-1.0</td>
<td>2-8</td>
<td>&gt;16</td>
<td>&gt;16</td>
<td>—</td>
</tr>
<tr>
<td>Rifampin</td>
<td>0.004-0.016</td>
<td>0.5-4</td>
<td>4-16</td>
<td>16-64</td>
<td>0.015-0.06</td>
</tr>
<tr>
<td>Spectinomycin</td>
<td>64-256</td>
<td>64-256</td>
<td>8-64</td>
<td>256-&gt;512</td>
<td>—</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>0.12-1</td>
<td>8-32</td>
<td>0.5-2</td>
<td>8-32</td>
<td>0.12-0.5</td>
</tr>
<tr>
<td>Tiamulin</td>
<td>0.5-2</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0.5-4</td>
</tr>
<tr>
<td>Ticarcillin</td>
<td>2-8</td>
<td>16-64</td>
<td>4-16</td>
<td>8-32</td>
<td>—</td>
</tr>
<tr>
<td>Ticarcillin-clavulanic acidb</td>
<td>0.5/2-2/2</td>
<td>16/2-64/2</td>
<td>4/2-16/2</td>
<td>8/2-32/2</td>
<td>—</td>
</tr>
<tr>
<td>Tilmicosin</td>
<td>1-4b</td>
<td>≥ 32</td>
<td>≥ 64</td>
<td>&gt;64</td>
<td>—</td>
</tr>
<tr>
<td>Tylosin</td>
<td>0.5-4</td>
<td>0.5-4</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>—</td>
</tr>
<tr>
<td>Sulfisoxazole</td>
<td>32-128</td>
<td>32-128</td>
<td>8-32</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Trimethoprim-Sulfamethoxazole (1/19)</td>
<td>≤ 0.5/9.5</td>
<td>≤ 0.5/9.5</td>
<td>≤ 0.5/9.5</td>
<td>8/152-32/608</td>
<td>0.12/2.4-1/19</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>0.5-2</td>
<td>1-4</td>
<td>—</td>
<td>—</td>
<td>0.12-0.5</td>
</tr>
</tbody>
</table>
**NOTE 1:** 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 *Mannheimia haemolytica*. 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. 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.

b. The QC ranges for *E. coli* 35218 for amoxicillin-clavulanic acid and ticarcillin-clavulanic acid are 4/2-16/8 and 4/2-16/2 µg/mL, respectively.

c. An MIC for tilmicosin of 0.5 or 8 µg/mL may indicate a medium or pH problem. The methodology should be reexamined.
Table 5A. Tentative Quality Control Ranges for the Agar Dilution Method of Testing *Campylobacter jejuni* (ATCC® 33560)

<table>
<thead>
<tr>
<th>Antimicrobial Agent</th>
<th>MIC (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ciprofloxacin</td>
<td>0.12–1</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>0.5–2</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>1–8</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>0.5–4</td>
</tr>
<tr>
<td>Meropenem</td>
<td>0.004–0.015</td>
</tr>
<tr>
<td>Nalidixic Acid</td>
<td>8–32</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>1–4</td>
</tr>
</tbody>
</table>
### Table 6. Acceptable Quality Control Ranges for *Haemophilus somnus* and *Actinobacillus pleuropneumoniae*

<table>
<thead>
<tr>
<th>Antimicrobial Agent</th>
<th>Disk Content</th>
<th>Zone Diameter</th>
<th>MICs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>Haemophilus somnus</em> ATCC®700025</td>
<td><em>Actinobacillus pleuropneumoniae</em> ATCC®27090</td>
</tr>
<tr>
<td>Ceftiofur</td>
<td>30 µg</td>
<td>36-46</td>
<td>34-42</td>
</tr>
<tr>
<td>Enrofloxacin</td>
<td>5 µg</td>
<td>32-38</td>
<td>31-38</td>
</tr>
<tr>
<td>Florenicol</td>
<td>30 µg</td>
<td>34-44</td>
<td>31-40</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>10 µg</td>
<td>14-22</td>
<td>15-19</td>
</tr>
<tr>
<td>Penicillin</td>
<td>10 units</td>
<td>35-44</td>
<td>29-36</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>30 µg</td>
<td>27-33</td>
<td>23-30</td>
</tr>
<tr>
<td>Tiamulin</td>
<td>30 µg</td>
<td>-</td>
<td>12-19</td>
</tr>
<tr>
<td>Tilmicosin</td>
<td>15 µg</td>
<td>8-13</td>
<td>8-13</td>
</tr>
<tr>
<td>Trimethoprim-</td>
<td>1.25/23.75 µg</td>
<td>26-32</td>
<td>28-32</td>
</tr>
<tr>
<td>sulfamethoxazole</td>
<td>(1/19)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**NOTE 1:** Zone diameter quality control ranges are applicable only to tests performed by disk diffusion using chocolate Mueller-Hinton agar, incubated in 5 to 7% CO₂ for 20 to 24 hours.

**NOTE 2:** MIC quality control ranges are applicable only to tests performed by broth microdilution procedures using veterinary fastidious medium (VFM).
Table 7. Suggested Modifications of Standard Methods for Susceptibility Testing of Some Fastidious and Special Problem Veterinary Pathogens

<table>
<thead>
<tr>
<th>Organism</th>
<th>Method</th>
<th>Medium</th>
<th>Incubation</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staphylococcus hyicus</em></td>
<td>Broth Microdilution</td>
<td>CAMHB + thymidine phosphorylase (0.2 IU/mL); for sulfonamides and trimethoprim only</td>
<td>35 °C/18-24 h</td>
<td>Wegener, et al.b</td>
</tr>
<tr>
<td><em>Haemophilus somnus</em> and <em>Actinobacillus pleuropneumoniae</em></td>
<td>Disk Diffusion</td>
<td>Chocolate Mueller-Hinton agar</td>
<td>35 °C/5-7% CO₂/20-24 h</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Broth Microdilution</td>
<td>Veterinary Fastidious Medium (VFM)</td>
<td>35 °C/5-7% CO₂/20-24 h</td>
<td></td>
</tr>
<tr>
<td><em>Enterococci</em></td>
<td>Broth Microdilution</td>
<td>CAMHB</td>
<td>35 °C/16-24 h</td>
<td></td>
</tr>
<tr>
<td><em>Streptococcus pneumoniae</em></td>
<td>Broth Microdilution</td>
<td>CAMHB + LHB (2-5% v/v)</td>
<td>35 °C/20-24 h</td>
<td>The final volume of broth in the well of the microdilution plate should be 100 µL.</td>
</tr>
<tr>
<td><em>Listeria spp.</em></td>
<td>Broth Microdilution</td>
<td>CAMHB + LHB (2-5% v/v)</td>
<td>35 °C/18 h</td>
<td></td>
</tr>
</tbody>
</table>

**NOTE 1:** These are acceptable alternatives with which some members of the subcommittee have had substantial experience.

CAMHB, Cation-adjusted Mueller-Hinton broth. 
LHB, Lysed horse blood.

**Footnotes**

a. To prepare LHB, freeze-thaw 3-4 times until blood is thoroughly lysed. Aseptically mix equal volumes of LHB and sterile distilled water (now 50% LHB). To be used in the broth test, the combination of broth and LHB must be clear; this can be done by centrifuging the blood at 16,000 x g for 20 minutes. Decant the supernatant; recentrifuge if necessary. Add appropriate amounts of the 50% LHB to the broth medium to yield a final concentration of 2 to 5% LHB.

### Table 8. Solvents and Diluents for Preparation of Stock Solutions of Antimicrobial Agents Requiring Solvents Other Than Water

<table>
<thead>
<tr>
<th>Antimicrobial Agent</th>
<th>Solvent</th>
<th>Diluent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin, clavulanic acid, and ticarcillin</td>
<td>Phosphate buffer, pH 6.0, 0.1 mol/L</td>
<td>Phosphate buffer, pH 6.0, 0.1 mol/L</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>Phosphate buffer, pH 8.0, 0.1 mol/L</td>
<td>Phosphate buffer, pH 6.0, 0.1 mol/L</td>
</tr>
<tr>
<td>Cephalothin</td>
<td>Phosphate buffer, pH 6.0, 0.1 mol/L</td>
<td>Water</td>
</tr>
<tr>
<td>Chloramphenicol, erythromycin, florfenicol, tylosin, tilmicosin</td>
<td>95% ethanol</td>
<td>Water</td>
</tr>
<tr>
<td>Difloxacin</td>
<td>1/2 volume of water, then add 1 mol/L NaOH dropwise to dissolve</td>
<td>Water</td>
</tr>
<tr>
<td>Enrofloxacin</td>
<td>1/2 volume of water, then add 1 mol/L NaOH dropwise to dissolve</td>
<td>Water</td>
</tr>
<tr>
<td>Imipenem</td>
<td>Phosphate buffer, pH 7.2, 0.01 mol/L</td>
<td>Phosphate buffer, pH 7.2, 0.01 mol/L</td>
</tr>
<tr>
<td>Nitrofurantoin</td>
<td>Phosphate buffer, pH 8.0, 0.1 mol/L</td>
<td>Phosphate buffer, pH 8.0, 0.01 mol/L</td>
</tr>
<tr>
<td>Orbifloxacin</td>
<td>1/2 volume of water, then add 1 mol/L NaOH dropwise to dissolve</td>
<td>Water</td>
</tr>
<tr>
<td>Rifampin</td>
<td>Methanol</td>
<td>Water (with stirring)</td>
</tr>
<tr>
<td>Sulfonamides</td>
<td>1/2 volume hot water and minimal amount of 2.5 mol/L NaOH to dissolve</td>
<td>Water</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>0.05N (0.05 mol/L) lactic or hydrochloric acid, 10% of final volume</td>
<td>Water (can require heat)</td>
</tr>
</tbody>
</table>

### Footnotes

a. These solvents and diluents can be further diluted as necessary in water or broth. The products known to be suitable for water solvents and diluents are amikacin, carbenicillin, ciprofloxacin, clindamycin, gentamicin, methicillin, novobiocin, oxacillin, penicillin, pirlmycin, tetracyclines, tiamulin (if hydrogen fumarate), trimethoprim (if lactate), and vancomycin.

b. All other cephalosporins and cephems except ceftiofur not listed above are solubilized (unless otherwise indicated by the manufacturer) in phosphate buffer, pH 6.0, 0.1 mol/L, and further diluted in sterile distilled water. Ceftiofur can be solubilized in water or broth.

c. Alternatively, nitrofurantoin, tylosin, and tilmicosin can be dissolved in dimethyl sulfoxide (DMSO).

d. These compounds are potentially toxic. Consult the Material Safety Data Sheets (MSDS) available from the product manufacturer before using any of these materials.
Table 9A. Screening and Confirmatory Tests for Expanded-spectrum-β-lactamases (ESBLs) in *Klebsiella pneumoniae*, *K. oxytoca*, and *Escherichia coli* (Zone Diameter)

<table>
<thead>
<tr>
<th>Method</th>
<th>Initial Screen Test</th>
<th>Phenotypic Confirmatory Test</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Medium</strong></td>
<td>Mueller-Hinton Agar</td>
<td>Mueller-Hinton Agar</td>
</tr>
<tr>
<td><strong>Antimicrobial Disk Concentration</strong></td>
<td>Cefpodoxime 10 µg or 30 µg</td>
<td>cefazidime 30 µg or 30/10 µg</td>
</tr>
<tr>
<td></td>
<td>ceftazidime 30 µg or 30/10 µg</td>
<td>and cefotaxime 30 µg or 30/10 µg</td>
</tr>
<tr>
<td></td>
<td>aztreonam 30 µg or 30/10 µg</td>
<td>cefotaxime-clavulanic acid</td>
</tr>
<tr>
<td></td>
<td>cefotaxime 30 µg or 30/10 µg</td>
<td>(Confirmaory testing requires use of both cefotaxime and ceftazidime, alone and in combination with clavulanic acid.)</td>
</tr>
<tr>
<td></td>
<td>ceftriaxone 30 µg</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(The use of more than one antimicrobial agent for screening improves the sensitivity of detection.)</td>
<td></td>
</tr>
<tr>
<td><strong>Inoculum</strong></td>
<td>Standard disk diffusion recommendations</td>
<td>Standard disk diffusion recommendations</td>
</tr>
<tr>
<td><strong>Incubation conditions</strong></td>
<td>Standard disk diffusion recommendations</td>
<td></td>
</tr>
<tr>
<td><strong>Incubation length</strong></td>
<td>Standard disk diffusion recommendations</td>
<td></td>
</tr>
<tr>
<td><strong>Results</strong></td>
<td>Cefpodoxime zone ≤ 17 mm</td>
<td>A ≥ 5 mm increase in a zone diameter for either antimicrobial agent tested in combination with clavulanic acid versus its zone when tested alone = ESBL (e.g., ceftazidime zone = 16; ceftazidime-clavulanic acid zone = 21)</td>
</tr>
<tr>
<td></td>
<td>Ceftazidime zone ≤ 22 mm</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Aztreonam zone ≤ 27 mm</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cefotaxime zone ≤ 27 mm</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ceftriaxone zone ≤ 25 mm</td>
<td></td>
</tr>
<tr>
<td></td>
<td>= may indicate ESBL production</td>
<td></td>
</tr>
<tr>
<td><strong>QC Recommendations</strong></td>
<td>E. coli ATCC® 25922 (see control limits in Table 4)</td>
<td>E. coli ATCC® 25922: ≤ 2 mm increase in zone diameter for antimicrobial agent tested alone versus its zone when tested in combination with clavulanic acid</td>
</tr>
<tr>
<td></td>
<td><em>Klebsiella pneumoniae</em> ATCC® 700603:</td>
<td><em>Klebsiella pneumoniae</em> ATCC® 700603:</td>
</tr>
<tr>
<td></td>
<td>cefpodoxime zone 9-16 mm</td>
<td>≥ 5 mm increase in ceftazidime-clavulanic acid zone diameter;</td>
</tr>
<tr>
<td></td>
<td>ceftazidime zone 10-18 mm</td>
<td>≥ 3-mm increase in cefotaxime-clavulanic acid zone diameter.</td>
</tr>
<tr>
<td></td>
<td>aztreonam zone 9-17 mm</td>
<td></td>
</tr>
<tr>
<td></td>
<td>cefotaxime zone 17-25 mm</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ceftriaxone zone 16-24 mm</td>
<td></td>
</tr>
</tbody>
</table>

**Footnote**

a. Preparation of ceftazidime-clavulanic acid (30 µg/10 µg) and cefotaxime-clavulanic acid (30 µg/10 µg) disks. Using a stock solution of clavulanic acid at 1,000 µg/mL (either freshly prepared or taken from small aliquots that have been frozen at -70 °C), add 10 µL of clavulanic acid to ceftazidime (30 µg) and cefotaxime (30 µg) disks. Use a micropipette to apply the 10 µL of stock solution to the ceftazidime and cefotaxime disks within one hour before they are applied to the plates, allowing about 30 minutes for the clavulanic acid to absorb and the disks to be dry enough for application. Use disks immediately after preparation or discard; do not store.
### Table 9B. Screening and Confirmatory Tests for ESBLs in *Klebsiella pneumoniae*, *K. oxytoca*, and *Escherichia coli* (MIC)

<table>
<thead>
<tr>
<th>Method</th>
<th>Initial Screen Test</th>
<th>Phenotypic Confirmatory Test</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Medium</strong></td>
<td>CAMHB</td>
<td>CAMHB</td>
</tr>
<tr>
<td><strong>Antimicrobial Concentration</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cefpodoxime</td>
<td>4 µg/mL or</td>
<td>cefazidime</td>
</tr>
<tr>
<td>cefazidime</td>
<td>1 µg/mL or</td>
<td>0.25-128 µg/mL</td>
</tr>
<tr>
<td>aztreonam</td>
<td>1 µg/mL or</td>
<td>cefazidime-clavulanic acid</td>
</tr>
<tr>
<td>cefotaxime</td>
<td>1 µg/mL or</td>
<td>0.25/4-128/4 µg/mL</td>
</tr>
<tr>
<td>ceftriaxone</td>
<td>1 µg/mL</td>
<td>cefotaxime</td>
</tr>
<tr>
<td></td>
<td>(The use of more than one antimicrobial agent for screening will improve the sensitivity of detection.)</td>
<td>(Confirmatory testing requires use of both cefotaxime and cefazidime, alone and in combination with clavulanic acid.)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Inoculum</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubation Conditions</td>
<td>Standard broth dilution recommendations</td>
</tr>
<tr>
<td>Incubation Length</td>
<td></td>
</tr>
<tr>
<td>Results</td>
<td>Growth = may indicate ESBL production (i.e., MIC ≥ 2 µg/mL for cefazidime, aztreonam, cefotaxime or ceftriaxone or MIC ≥ 8 µg/mL for cefpodoxime).</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>QC Recommendations</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli ATCC® 25922</td>
<td>No growth (also refer to control limits listed in M7 Table 3)</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em> ATCC® 700603</td>
<td>Growth:</td>
</tr>
<tr>
<td>cefpodoxime</td>
<td>MIC ≥ 8 µg/mL</td>
</tr>
<tr>
<td>cefazidime</td>
<td>MIC ≥ 2 µg/mL</td>
</tr>
<tr>
<td>aztreonam</td>
<td>MIC ≥ 2 µg/mL</td>
</tr>
<tr>
<td>cefotaxime</td>
<td>MIC ≥ 2 µg/mL</td>
</tr>
<tr>
<td>ceftriaxone</td>
<td>MIC ≥ 2 µg/mL</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em> ATCC® 700603:</td>
<td>≥3 twofold concentration decrease in an MIC for an antimicrobial agent tested in combination with clavulanic acid versus its MIC when tested alone</td>
</tr>
</tbody>
</table>
Table 9C. Oxacillin-Salt Agar Screening Test for *Staphylococcus aureus*

<table>
<thead>
<tr>
<th>Screen Test</th>
<th>Oxacillin Resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>MHA with NaCl (4% w/v; 0.68 mol/L)</td>
</tr>
<tr>
<td>Antimicrobial concentration</td>
<td>6 µg/mL oxacillin</td>
</tr>
<tr>
<td>Inoculum</td>
<td>Direct colony suspension to obtain 0.5 McFarland turbidity</td>
</tr>
<tr>
<td></td>
<td>Using a 1-µL loop that was dipped in the suspension, spot an area 10 to 15 mm in diameter. Alternatively, using a swab dipped in the suspension and expressed, spot a similar area or streak an entire quadrant.</td>
</tr>
<tr>
<td>Incubation conditions</td>
<td>35 ºC; ambient air</td>
</tr>
<tr>
<td>Incubation length</td>
<td>24 hours</td>
</tr>
<tr>
<td>Results</td>
<td>&gt;1 colony = resistant</td>
</tr>
<tr>
<td></td>
<td>Examine carefully with transmitted light for &gt;1 colony or light film of growth.</td>
</tr>
<tr>
<td>QC Recommendations</td>
<td><em>Staphylococcus aureus</em> ATCC® 29213 - Susceptible</td>
</tr>
<tr>
<td></td>
<td><em>Staphylococcus aureus</em> ATCC® 43300 - Resistant</td>
</tr>
</tbody>
</table>

MHA = Mueller-Hinton Agar
Table 9D. Screening Tests for High-Level Aminoglycoside Resistance (HLAR) and Vancomycin Resistance in *Enterococcus* spp.

<table>
<thead>
<tr>
<th>Screen Test</th>
<th>Gentamicin HLAR</th>
<th>Streptomycin HLAR</th>
<th>Vancomycin Resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Medium</strong></td>
<td>BHI broth or agar</td>
<td>BHI broth or agar</td>
<td>BHI agar</td>
</tr>
<tr>
<td><strong>Antimicrobial concentration</strong></td>
<td>500 µg/mL</td>
<td>Broth: 1000 µg/mL Agar: 2000 µg/mL</td>
<td>6 µg/mL</td>
</tr>
<tr>
<td><strong>Inoculum</strong></td>
<td>Growth method or direct colony suspension to obtain 0.5 McFarland turbidity</td>
<td>Growth method or direct colony suspension to obtain 0.5 McFarland turbidity</td>
<td>Growth method or direct colony suspension to obtain 0.5 McFarland turbidity</td>
</tr>
<tr>
<td></td>
<td>Agar — 10 µL of a 0.5 McFarland suspension spotted onto agar surface</td>
<td>Agar — 10 µL of a 0.5 McFarland suspension spotted onto agar surface</td>
<td>1-10 µL of a 0.5 McFarland suspension spotted onto agar surface</td>
</tr>
<tr>
<td></td>
<td>Broth — standard broth dilution recommendations</td>
<td>Broth — standard broth dilution recommendations</td>
<td>Broth — standard broth dilution recommendations</td>
</tr>
<tr>
<td><strong>Incubation conditions</strong></td>
<td>35 ºC; ambient air</td>
<td>35 ºC; ambient air</td>
<td>35 ºC; ambient air</td>
</tr>
<tr>
<td><strong>Incubation length</strong></td>
<td>24 hours</td>
<td>24 - 48 hours (if susceptible at 24 hours, reincubate)</td>
<td>24 hours</td>
</tr>
<tr>
<td><strong>Results</strong></td>
<td>Agar: &gt;1 colony = resistant Broth: any growth = resistant</td>
<td>Agar: &gt;1 colony = resistant Broth: any growth = resistant</td>
<td>&gt;1 colony = presumptive resistance</td>
</tr>
<tr>
<td></td>
<td>Resistant — will not be synergistic with cell-wall-active agent (e.g., ampicillin, penicillin, vancomycin)</td>
<td>Resistant — will not be synergistic with cell-wall-active agent (e.g., ampicillin, penicillin, vancomycin)</td>
<td>Perform vancomycin MIC and tests for motility and pigment production to distinguish species with acquired resistance (VanA and VanB) from those with intrinsic, intermediate-level resistance to vancomycin (VanC) such as <em>E. gallinarum</em>, <em>E. casseliflavus</em>, or <em>E. flavescens</em>, which often grow on the vancomycin screen plate. In contrast to other enterococci, <em>E. casseliflavus</em>, <em>E. flavescens</em>, and <em>E. gallinarum</em> with vancomycin MICs of 8-16 µg/mL (intermediate) differ from vancomycin-resistant enterococci for infection control purposes.</td>
</tr>
<tr>
<td></td>
<td>Susceptible — will be synergistic with cell-wall-active agent that is also susceptible (e.g., ampicillin, penicillin, vancomycin)</td>
<td>Susceptible — will be synergistic with cell-wall-active agent to which the isolate is susceptible (e.g., ampicillin, penicillin, vancomycin)</td>
<td></td>
</tr>
<tr>
<td><strong>QC Recommendations</strong></td>
<td><em>E. faecalis</em> ATCC® 29212 — Susceptible</td>
<td><em>E. faecalis</em> ATCC® 29212 — Susceptible</td>
<td><em>E. faecalis</em> ATCC® 29212 — Susceptible</td>
</tr>
<tr>
<td></td>
<td><em>E. faecalis</em> ATCC® 51299 — Resistant</td>
<td><em>E. faecalis</em> ATCC® 51299 — Resistant</td>
<td><em>E. faecalis</em> ATCC® 51299 — Resistant</td>
</tr>
</tbody>
</table>

**BHI** = Brain heart infusion

**Footnote**

a Even though not as widely available, dextrose phosphate agar and broth have been shown in limited testing to perform comparably.
## Glossary 1. Antimicrobial Class, Antimicrobial Agents, and Antimicrobial Resistance Mechanisms

<table>
<thead>
<tr>
<th>Antimicrobial Class</th>
<th>Antimicrobial Agents</th>
<th>Antimicrobial Resistance Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aminocyclitols</td>
<td>spectinomycin, apramycin</td>
<td>Modifying enzymes Efflux</td>
</tr>
<tr>
<td>Aminoglycosides</td>
<td>streptomycin, kanamycin, gentamicin, amikacin, tobramycin, neomycin</td>
<td>Modifying enzymes Decreased permeability Target resistance (ribosome) Efflux</td>
</tr>
<tr>
<td>Ansamycin</td>
<td>Rifampin</td>
<td>Target resistance (RNA polymerase)</td>
</tr>
<tr>
<td>ß-lactam antibiotics:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Penicillins</td>
<td>penicillin, ampicillin, oxacillin, ticarcillin, piperacillin, amoxicillin</td>
<td>Reduced permeability Altered penicillin-binding proteins ß-lactamases, cephalosporinases Efflux</td>
</tr>
<tr>
<td>ß-lactam/B-lactamase inhibitors</td>
<td>amoxicillin-clavulanic acid, ticarcillin-clavulanic acid, piperacillin-tazobactam,</td>
<td></td>
</tr>
<tr>
<td>Cephalosporins and cephamycins:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1&lt;sup&gt;st&lt;/sup&gt; generation (narrow spectrum)</td>
<td>cephalothin, cefazolin, cephalpirin, cephalexin, cefadroxil</td>
<td></td>
</tr>
<tr>
<td>2&lt;sup&gt;nd&lt;/sup&gt; generation (expanded spectrum)</td>
<td>cefotetan, cefoxitin (cephamycin), cefaclor</td>
<td></td>
</tr>
<tr>
<td>3&lt;sup&gt;rd&lt;/sup&gt; generation (expanded spectrum)</td>
<td>ceftiofur, cefotaxime, ceftazidime, ceftriaxone</td>
<td></td>
</tr>
<tr>
<td>4&lt;sup&gt;th&lt;/sup&gt; generation (expanded spectrum)</td>
<td>cefepime, cefquinome</td>
<td></td>
</tr>
<tr>
<td>Carbapenems</td>
<td>imipenem, meropenem</td>
<td></td>
</tr>
<tr>
<td>Monobactams</td>
<td>aztreonam</td>
<td></td>
</tr>
<tr>
<td>Folate pathway inhibitors</td>
<td>sulfonamides, trimethoprim, ormetoprim, trimethoprim-sulfamethoxazole</td>
<td>Decreased permeability Production of drug-insensitive enzymes</td>
</tr>
<tr>
<td>Glycopeptides</td>
<td>vancomycin, teicoplanin</td>
<td>Target resistance (cell wall)</td>
</tr>
<tr>
<td>Lincosamides</td>
<td>lincomycin, clindamycin, pirlimycin</td>
<td>Decreased ribosomal binding</td>
</tr>
<tr>
<td>Macrolides:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14-membered rings</td>
<td>erythromycin</td>
<td>Decreased ribosomal binding</td>
</tr>
<tr>
<td>15-membered rings</td>
<td>azithromycin</td>
<td>Decreased permeability</td>
</tr>
<tr>
<td>16-membered rings</td>
<td>spiramycin, tylosin, clarithromycin, tilmicosin</td>
<td>Modifying enzymes Efflux</td>
</tr>
</tbody>
</table>

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### Glossary 1. (Continued)

<table>
<thead>
<tr>
<th>Antimicrobial Class</th>
<th>Antimicrobial Agents</th>
<th>Antimicrobial Resistance Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitroimidazoles</td>
<td>metronidazole</td>
<td>Altered drug activation enzymes</td>
</tr>
<tr>
<td>Oxazolidinones</td>
<td>linezolid</td>
<td>Target resistance (ribosome)</td>
</tr>
<tr>
<td>Phenicols</td>
<td>flornofenicol, chloramphenicol</td>
<td>Decreased ribosomal binding Decreased permeability Modifying enzymes Efflux</td>
</tr>
<tr>
<td>Polypeptides</td>
<td>bacitracin, polymyxin</td>
<td>Lipopolysaccharide modification</td>
</tr>
<tr>
<td>Quinolones and fluoroquinolones</td>
<td>nalidixic acid ciprofloxacin, enrofloxacin, difloxacin, orbifloxacin, marbofloxacin</td>
<td>Target resistance (DNA gyrase, Topoisomerase IV) Decreased permeability Efflux</td>
</tr>
<tr>
<td>Streptogramins:</td>
<td>streptogramin A, pristinamycin IIA, virginiamycin M, mikamycin A, synergistin A</td>
<td>Decreased ribosomal binding Modifying enzymes Efflux</td>
</tr>
<tr>
<td>Streptogramins A</td>
<td>streptogramin B, virginiamycin S, pristinamycin IB, mikamycin B, synergistin B</td>
<td></td>
</tr>
<tr>
<td>Streptogramins B</td>
<td>quinupristin-dalfopristin, virginiamycin</td>
<td></td>
</tr>
<tr>
<td>Combinations</td>
<td>doxycycline, minocycline, tetracycline, chlortetracycline, oxytetracycline</td>
<td>Target resistance (ribosome) Drug detoxification Efflux</td>
</tr>
<tr>
<td>Tetracyclines</td>
<td>tiamulin</td>
<td>Target resistance (ribosome) Decreased permeability</td>
</tr>
<tr>
<td>Others</td>
<td>fosfomycin, nitrofurantoin, novobiocin</td>
<td></td>
</tr>
</tbody>
</table>
## Glossary 2. Abbreviations for Antimicrobial Agents Incorporated into Disks or Susceptibility Panels

<table>
<thead>
<tr>
<th>Antimicrobial Agents</th>
<th>Agent Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amikacin</td>
<td>AN, AK, Ak, AMI, AMK</td>
</tr>
<tr>
<td>Amoxicillin-clavulanic acid</td>
<td>AMC, A/C, AXC, Aug, AUG, XL, AML, AMOX/CA</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>AM, Am, AMP, AP, ABPC</td>
</tr>
<tr>
<td>Apramycin</td>
<td>AP, APR</td>
</tr>
<tr>
<td>Cefazolin</td>
<td>CZ, CFZ, Cfz, FAZ, KZ, CEZ, CFAZ</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>FOX, CX, Cfx, FX, CFX, CFOX, COX</td>
</tr>
<tr>
<td>Ceftiofur</td>
<td>XNL, FUR, CEF, ACC</td>
</tr>
<tr>
<td>Cephalothin</td>
<td>CF, Cf, CEP, CE, KF, CET, CEF, CTIN, CTN</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>C, CHL, CL, CP, CMP</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>CC, CD, CM, Cd, CLI, DA, CLDM</td>
</tr>
<tr>
<td>Difloxacin</td>
<td>DIC, DIF, DF</td>
</tr>
<tr>
<td>Enrofloxacin</td>
<td>ENO, ENR, ENF, EFX, ENRO, EF</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>E, ERY, EM</td>
</tr>
<tr>
<td>Florfenicol</td>
<td>FFC</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>GM, GMS, HLG, Gm, GmS, GEN, CN</td>
</tr>
<tr>
<td>Imipenem</td>
<td>IPM, IMI, Imp, IP, IMIP</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>K, KAN, HLK, KM</td>
</tr>
<tr>
<td>Orbifloxacin</td>
<td>OBX, ORB</td>
</tr>
<tr>
<td>Oxacillin</td>
<td>OX, OXS, Ox, OXA, MPIPC, OXAC</td>
</tr>
<tr>
<td>Penicillin</td>
<td>P, PEN, PG, PCG, PENG</td>
</tr>
<tr>
<td>Penicillin-novobiocin</td>
<td>P/NB, P/NOV, P/N</td>
</tr>
<tr>
<td>Pirlimycin</td>
<td>PRL, PIRL</td>
</tr>
<tr>
<td>Rifampin</td>
<td>RA, RI, RIF, Rif, RD, RP, RFP, RAM</td>
</tr>
<tr>
<td>Spectinomycin</td>
<td>SPT, SPE, SC, SH, SPC</td>
</tr>
</tbody>
</table>
### Glossary 2. (Continued)

<table>
<thead>
<tr>
<th>Antimicrobial Agent</th>
<th>Designations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfisoxazole</td>
<td>G, FIS, SF</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>Te, TE, TET, TC, T</td>
</tr>
<tr>
<td>Tiamulin</td>
<td>TIA</td>
</tr>
<tr>
<td>Ticarcillin</td>
<td>TIC, TC, Ti, TI, TIPC, TICC</td>
</tr>
<tr>
<td>Ticarcillin-clavulanic acid</td>
<td>TIM, T/C, TCC, Tim, TlC, TICC/CA, TIL</td>
</tr>
<tr>
<td>Tilmicosin</td>
<td>TIL, TMS</td>
</tr>
<tr>
<td>Trimethoprim-sulfamethoxazole</td>
<td>SXT, T/S, STG, TS, ST</td>
</tr>
<tr>
<td>Tylosin</td>
<td>TYL, TYLB, TYLT, TY, TLS</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>Va, VA, VAN, VCM</td>
</tr>
</tbody>
</table>

Note: Designations for antimicrobial agents that are also used in human medicine can be found in the most current edition of M100—*Performance Standards for Antimicrobial Susceptibility Testing.*
NCCLS consensus procedures include an appeals process that is described in detail in Section 9 of the Administrative Procedures. For further information contact the Executive Offices or visit our website at www.nccls.org.

Summary of Comments and Subcommittee Responses

M31-A:  *Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals; Approved Standard*

**General**

1. Page 25, Section 5.2.3.6. Macrodilution-tube broth method. If the inoculum is prepared in broth (as listed in this section) and 1 ml is added to the 1 ml of antimicrobials, which have been dissolved and diluted in water..., the broth is only at half-strength and could result in poor growth of some organisms.

   - **Section 4.2.3.4** addresses the oversight by requiring double-strength medium to be used as the aqueous diluent.

2. Page 36, Table 2, line- Amox/Clav...Staph - There is no value listed for the MIC breakpoint in the “resistant” column. Page 36, Table 2, line- Amox/Clav...other organisms The value listed in the MIC breakpoint "susceptible" column is the same as the “resistant” column; both are at 8/4.

   - These errors have been corrected and conform to M100-S12 values.

3. Page 36, Table 2, line-Ticar/Clav - There is a value in the MIC breakpoint “resistant” column that is just floating there, not for any organism — where does it belong??? with the amox/clav???

   - These errors have been corrected and conform to M100-S12 values.

4. Page 36, Table 2, , line-Ticar/Clav - The clavulanic values are all at 2 —- shouldn't they increase like the clavulanic values increase with the amoxicillin? Page 36, Table 2, line- Ticar/Clav ... gram-negative enteric organisms the MIC breakpoint “intermediate” value is 32-64..., the clav isn't added? Shouldn't it be 32/4 - 64/8??

   - A fixed concentration of 2 µg of clavulanate is used with all dilutions of ticarcillin. The intermediate values are now designated as 32/2 – 64/2 to conform to M100-S12.

5. I found a minor error in the M31-A document that should be corrected during its next version. Page 20, Section 5.2.2.6. the second bullet: the final inoculum on the agar is incorrectly stated as CFU/mL. It should be CFU/spot.

   - The wording has been changed to CFU/spot.

6. It should be interesting to state the natural resistance of the major bacterial species from animal origin. The natural resistance is useful for bacterial species identification and interpretive reading of results (recommendation from the CA-SFM).

   - It is expected that veterinary microbiologists are aware of intrinsic resistance in various bacteria to specific agents in order to select the appropriate agents to test. For example, one would not test a lincosamide or macrolide compound against an enteric bacillus as these organisms are intrinsically resistant to these compounds.
7. It is recommended to store the antimicrobial stock solution at –70 °C. Several antimicrobial agents did not support several thawing/dethawing cycles and a reference strain is not a good indicator to control antimicrobial stability. For example, florfenicol stock solution at 1280 µg/ml in 10% methanol/water, stored at –20 °C lost 30% of activity after 6 months of storage while MIC value of control strain did not differ during the same period. We recommend indicating in a table the conservation procedure and stability of antimicrobial on the basis of the literature.

- The comments above describe several deviations from the procedures described in the referenced section. Section 4.2.1.3 indicates that that stock solutions are to be stored at –70 °C which would minimize degradation of the agent. Also, it is indicated that stock solutions are to removed as needed, used the same day, and any unused portion discarded. This avoids problems associated with freeze/thaw cycles. Finally, a properly performed susceptibility test with the appropriate quality control organism should readily detect a 30% loss in activity in antimicrobial agent.

Glossary

8. 1) Thiamphenicol is approved for veterinary use internationally (Europe). Should this be added to the list for phenicols? 2) Should the new human drug, linezolid, be listed under "Others"?

- Thiamphenicol has been added to the phenicol group. Linezolid has been added as a separate group under "Oxazolidinones."

9. This table is interesting but is it very useful, especially since certain mechanisms do not apply to some of the specific drugs?

- This table is intended to provide an overview of the major resistance mechanisms to each class of drug and is not intended to address the specific agents within each class.
Summary of Delegate Comments and Subcommittee Responses

M31-A2: Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals; Approved Standard—Second Edition

General

1. The M31-A2 document cannot be accepted, because some information was not correct: especially on marbofloxacin. The manufacturer has provided as an attachment quality control and interpretive criteria information, as well as supporting information. The manufacturer would like to rectify the information on marbofloxacin and could send the necessary documents to evaluate the work performed.

• The sole basis for the reject vote appears to be the lack of inclusion of a particular sponsor's antimicrobial agent, not the inclusion of incorrect data. There does not appear to be any substantive reason for rejection that relates to the methodology or other statements in NCCLS document M31-A2. To date, the sponsor has not submitted marbofloxacin data to be reviewed by the subcommittee; thus, it has not been included.

The most current edition of NCCLS document M37—Development of In Vitro Susceptibility Testing Criteria and Quality Control Parameters for Veterinary Antimicrobial Agents outlines the process for a sponsor to present information to the subcommittee to request approval for quality control ranges, interpretive criteria, and other listings in M31. The sponsor has been encouraged to follow M37 to compile marbofloxacin data and request that it be reviewed at the next subcommittee meeting. The NCCLS consensus process does not allow for any data to be placed in a document without appropriate subcommittee review; thus, the sponsor's request to amend M31-A2 cannot be granted.
Related NCCLS Publications*

M2-A7 Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved Standard—Seventh Edition (2000). This document provides current recommended techniques for disk susceptibility testing, and new frequency criteria for quality control testing.


M7-A5 Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard —Fifth Edition (2000). This document provides updated reference methods for the determination of minimal inhibitory concentrations (MICs) for aerobic bacteria by broth macrodilution, broth microdilution, and agar dilution.

M11-A5 Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria; Approved Standard—Fifth Edition (2001). This document provides reference methods for the determination of minimal inhibitory concentrations (MICs) of anaerobic bacteria by broth macrodilution, broth microdilution and agar dilution. Interpretive and quality control tables are included.

M23-A2 Development of In Vitro Susceptibility Testing Criteria and Quality Control Parameters; Approved Guideline—Second Edition (2001). This document addresses the required and recommended data needed for the selection of appropriate interpretative standards and quality control guidelines for new antimicrobial agents.

M37-A2 Development of In Vitro Susceptibility Testing Criteria and Quality Control Parameters for Veterinary Antimicrobial Agents; Approved Guideline—Second Edition (2002). This document addresses the recommended data needed for selection of appropriate interpretative standards and quality control guidelines for new veterinary antimicrobial agents.

M39-A Analysis and Presentation of Cumulative Antimicrobial Susceptibility Test Data; Approved Guideline (2002). This document describes methods for the recording and analysis of antimicrobial susceptibility test data, consisting of cumulative and ongoing summaries of susceptibility patterns of epidemiologically significant microorganisms.

M100-S12 Performance Standards for Antimicrobial Susceptibility Testing; Twelfth Informational Supplement (2002). This document provides updated tables for the NCCLS antimicrobial susceptibility testing standards M2-A7 and M7-A5.

NRSCSL8-A Terminology and Definitions for Use in NCCLS Documents; Approved Standard (1998). This document provides standard definitions for use in NCCLS standards and guidelines, and for submitting candidate reference methods and materials to the National Reference System for the Clinical Laboratory (NRSCSL).

* Proposed- and tentative-level documents are being advanced through the NCCLS consensus process; therefore, readers should refer to the most recent editions.