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# Disk diffusion method

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# CHAPTER 2

# Disk Diffusion Method

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## PRINCIPLE

This method is based on the principle that antibiotic-impregnated disk, placed on agar previously inoculated with the test bacterium, pick-up moisture and the antibiotic diffuse radially outward through the agar medium producing an antibiotic concentration gradient. The concentration of the antibiotic at the edge of the disk is high and gradually diminishes as the distance from the disk increases to a point where it is no longer inhibitory for the organism, which then grows freely. A clear zone or ring is formed around an antibiotic disk after incubation if the agent inhibits bacterial growth.

## MEDIA

The disk diffusion method is performed using Mueller-Hinton Agar (MHA), which is the best medium for routine susceptibility tests because it has good reproducibility, low in sulfonamide, trimethoprim, and tetracycline inhibitors, and gives satisfactory growth of most bacterial pathogens.

The inoculum for the disk diffusion method is prepared using a suitable broth such as tryptic soy broth. This medium is prepared according to manufacturer's instructions, dispensed in tubes at 4-5 ml and sterilized. Sterile 0.9% salt solution may also be used.

Media are supplemented with 1-2% sodium chloride (NaCl) if intended for marine organisms.

### Preparation of agar medium

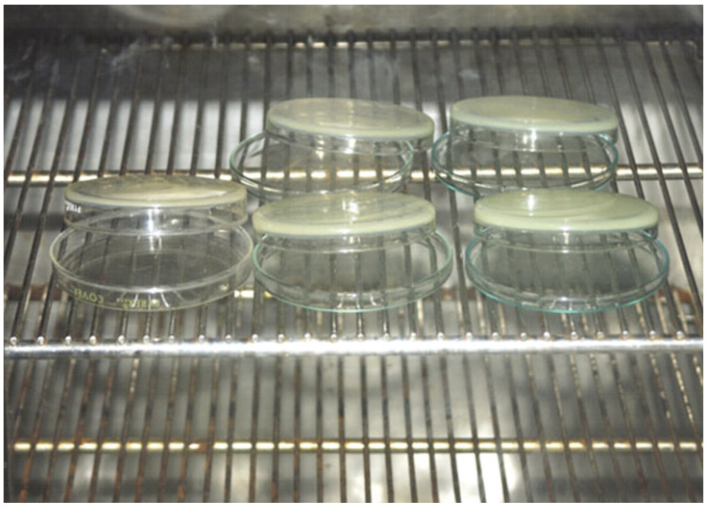
1. Prepare MHA from the dehydrated medium according to the manufacturer's instructions. Media should be prepared using distilled water or deionized water.

2. Heat with frequent agitation and boil to dissolve the medium completely. Sterilize by autoclaving at 121°C for 15 min.

3 Check the pH of each preparation after it is sterilized, which should be between 7.2 and 7.4 at room temperature. This is done by macerating a small amount of medium in a little distilled water or by allowing a little amount of medium to gel around a pH meter electrode.

4 Cool the agar medium to 40-50°C. Pour the agar into sterile glass or plastic petri dish on a flat surface to a uniform depth of 4 mm.

5 Allow to solidify.



6 Prior to use, dry plates at 30-37°C in an incubator, with lids partly ajar, for not more than 30 minutes or until excess surface moisture has evaporated. Media must be moist but free of water droplets on the surface. Presence of water droplets may result to swarming bacterial growth, which could give inaccurate results. They are also easily contaminated.

## Storage

1 If plates are not to be immediately used, they may be stored in the refrigerator inside airtight plastic bags at 2-8°C for up to 4 weeks.

2 Unpoured media may be stored in airtight screw-capped bottles under the conditions specified by the manufacturer.

### Control

Before use, check the ability of the agar to support the growth of control strains (listed in the Introduction) by streaking bacterial cultures on the agar medium. It is also advisable to check the ability of each batch of media to support the growth of a representative member of the species to be tested.

## INOCULUM

### Preparation

1 From a pure bacterial culture (not more than 48 hours, old except for slow growing organisms), take four or five colonies with a wire loop.

2 Transfer colonies to 5 ml of Trypticase soy broth or 0.9% saline.

**3** Incubate the broth at 30°C or at an optimum growth temperature until it achieves or exceeds the turbidity of 0.5 MacFarland standard (prepared by adding 0.5 ml of 0.048 M $BaCl_2$ to 99.5 ml of 0.36 $NH_2SO_4$; commercially available).

**4** Compare the turbidity of the test bacterial suspension with that of 0.5 MacFarland (vigorously shaken before use) against a white background with contrasting black line under adequate light. Arrow points to tube with correct turbidity.

**5** Reduce turbidity by adding sterile saline or broth.

**NOTE:** *Standardized inoculum has a concentration of 1-2 × $10^8$ cfu/ml.*

## Inoculation of plates

1 Dip a sterile cotton swab into the standardized bacterial suspension.

2 Remove excess inoculum by lightly pressing the swab against the tube wall at a level above that of the liquid.

3 Inoculate the agar by streaking with the swab containing the inoculum.

4 Rotate the plate by 60° and repeat the rubbing procedure. Repeat two times. This will ensure an even distribution of the inoculum.

5 Allow the surface of the medium to dry for 3-5 minutes but not longer than 15 minutes to allow for absorption of excess moisture.

## ANTIMICROBIAL DISKS

### Selection

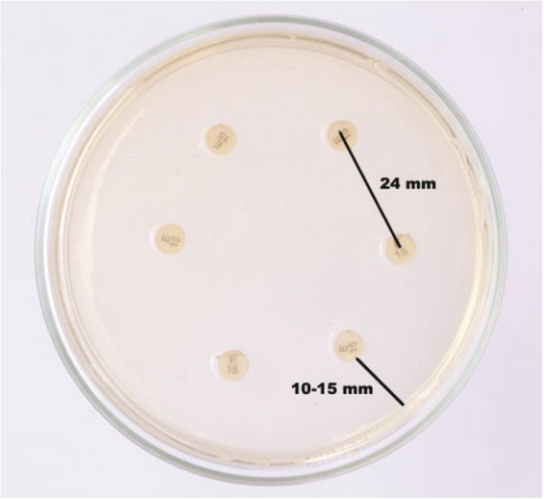
The number of antimicrobial agents to be tested should be limited. To make the test practical and relevant, include only one representative of each group of related drugs; those indicated for veterinary use to control or prevent disease, and those that can be useful for epidemiological or research purposes.

Use antibiotic disks purchased from a reputable manufacturer. The disk diameter is approximately 6 mm. Disks should be properly stored in a tightly sealed container with desiccant at 2-8°C. Expired disks should not be used.

### Application

1 Using sterile forceps or disk dispenser, place antibiotic disk on the surface of the inoculated and dried plate.

2 Immediately press it down lightly with the instrument to ensure complete contact between the disk and the agar surface. Do not move a disk once it has come into contact with the agar surface since some diffusion of the drug occurs instantaneously.




3 Position disks such that the minimum center - center distance is 24 mm and no closer than 10 to 15 mm from the edge of the petri dish. A maximum of six disks may be placed in a 9-cm petri dish and 12 disks on a 150 mm plate. Reduce the number of disks applied per plate if overlapping zones of inhibition are encountered.

## CONTROL PLATE

Include one plate inoculated with a control strain (Appendix 2.1) for every set of plates and incubate together.

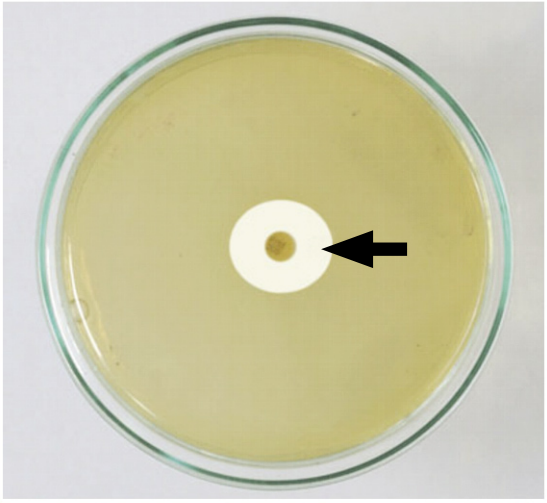
## INCUBATION

1 Incubate plates in an inverted position at 30°C or at an optimum growth temperature.

2 Observe for the zone of inhibition after 16 to 18 hours. Slow growing organisms may require longer incubation period.

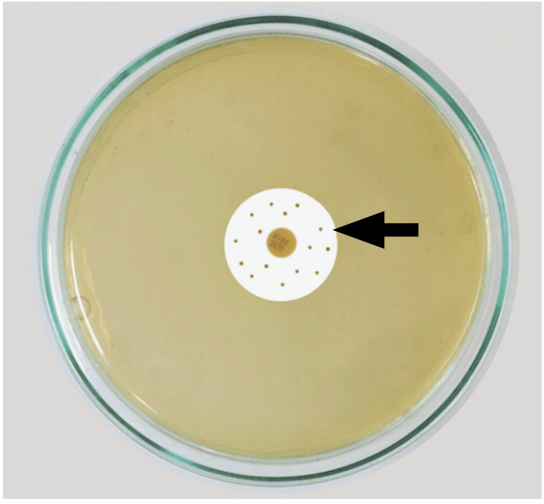
## READING AND MEASUREMENT OF ZONES OF INHIBITION

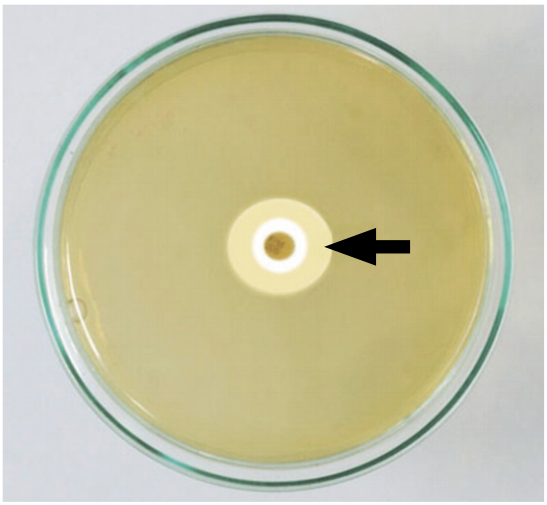
### Description



1 The zone of inhibition (arrow) is the point at which no growth is visible to the unaided eye.

2 Record the presence of individual colonies (arrow) within zones of inhibition.

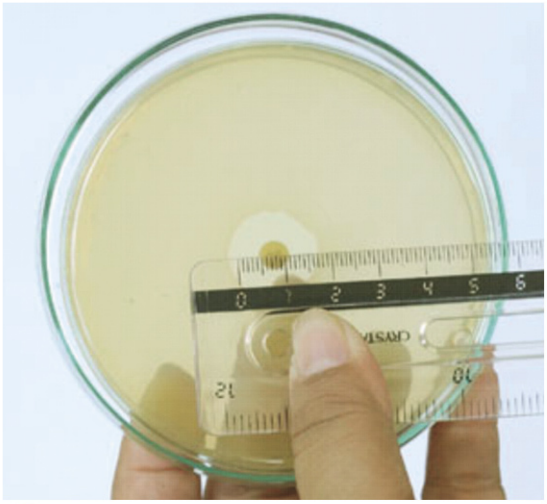




3 Record occurrence of fuzzy zones (arrow). In measuring the zone diameter, the fuzzy portion of the zone should be ignored as much as possible. The zone limit is the inner limit of the zone of normal growth.

## Reading

1 Read and record the diameter of the zones of inhibition using a ruler graduated to 0.5 mm.



2 Round up the zone measurement to the nearest millimeter.

## INTERPRETATION OF RESULTS

1. Compare the diameter of the zone of inhibition of the test isolates with those in the chart of interpretative standard for veterinary pathogens (Appendix 2.2).

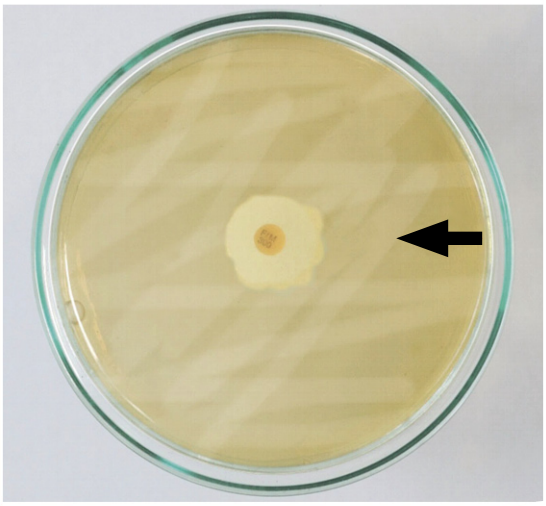
2. Report result as Resistant (R), Intermediate (I) or Susceptible (S).

   Example

   | | |
   |---|---|
   | Disk used: | Chloramphenicol, 30 $\mu$g (C-30) |
   | Zone of inhibition: | 16 mm |
   | Result/ interpretation: | Intermediate → based on the zone diameter interpretative chart (Appendix 2.2) |

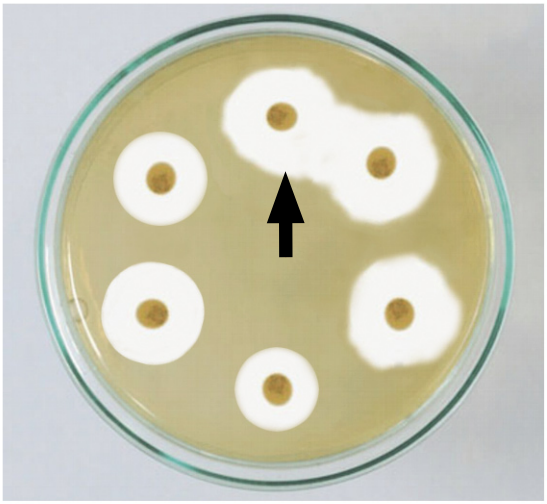
3. Susceptibility test results using agents other than those listed in the chart are interpreted on the basis of the presence or absence of a definite zone of inhibition and is considered only as qualitative until such time as interpretative zones have been established.

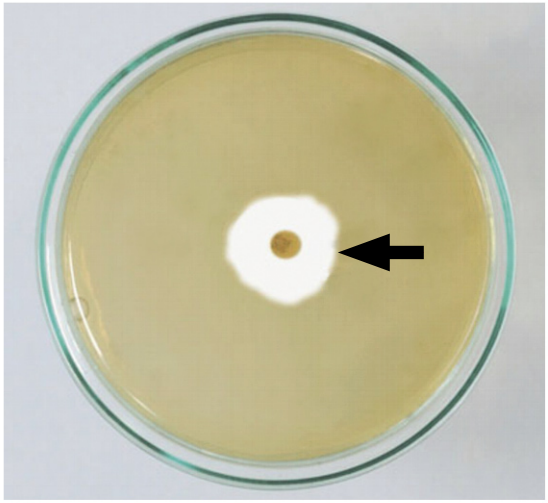
## REJECTION CRITERIA



1. Do not read plates on which growth of test bacteria have isolated colonies or less than semi-confluent growth (arrow).

2 Do not read zones of inhibition of two adjacent disks that overlap (arrow) to the extent that measurement of the zone diameter cannot be made.





3 Do not read zones showing distortion from circular (arrow).

4 Reject all data collected in a particular set if the zones of inhibition produced on plate inoculated with a control strain are not within the tolerance limits set.

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Prescott, L.M., J.P. Harley and D.A. Klein. 1993. Microbiology, Second Edition. Wm C Brown Publishers, England, pp. 325-343.

**APPENDIX 2.1.** Acceptable inhibitory zone diameter (mm) limit of control strains recommended for use in the disk diffusion test of antimicrobial sensitivity testing of bacteria isolated from animals.

| Antimicrobial | Disk | *Escherichia coli* | *Staphylococcus aureus* | *Pseudomonas aeruginosa* | *Streptococcus pneumoniae*[a] |
|---|---|---|---|---|---|
| | | | | | |
| **Agent** | **Content** | **ATCC 25922** | **ATCC 25923** | **ATCC 27853** | **ATCC 49619** |
| | | | | | |
| Amikacin | 30 μg | 19-26 | 20-26 | 18-26 | - |
| Amoxicillin- | 20/10μg | 18-24 | 28-36 | - | - |
| Clavulanic acid[b] | | | | | |
| Ampicillin | 10μg | 16-22 | 27-35 | - | 30-36 |
| Cefazolin | 30μg | 21-27 | 29-35 | - | - |
| Cefoxitin | 30μg | 23-29 | 23-2 | | - |
| Cephalothin | 30 μg | 15-21 | 29-37 | - | 26-32 |
| Chloramphenicol 30μg | 21-27 | 19-26 | - | 26-32 | |
| Clindamycin | 2 μg | - | 24-3 | - | 19-25 |
| Erythromycin | 15μg | - | 22-30 | - | 25-30 |
| Gentamicin | 10μg | 19-26 | 19-27 | 16-21 | - |
| Imipenem | 10μg | 26-32 | - | 20-28 | - |
| Kanamycin | 30μg | 17-25 | 19-26 | - | - |
| Oxacillin | 1μg | - | 18-24 | - | ≤12[c] |
| Penicillin | 10 units | - | 26-37 | - | 24-30 |
| Rifampin | 5μg | 8-10 | 26-34 | - | 25-30 |
| Tetracycline | 30μg | 18-25 | 24-30 | - | 27-31 |
| Ticarcillin | 75μg | 24-30 | - | 21-27 | - |
| Ticarcillin- | 75/10μg | 24-30 | 29-37 | 20-28 | - |
| Clavulanic acid | | | | | |
| Spectinomycin | 100 μg | 21-25 | 13-17 | 10-14 | - |
| Sulfisoxazole | 250 μg or 300 μg | 15-23 | 24-34 | - | - |
| Trimethoprim- | 1.25/ | 23-29 | 24-32 | - | 20-28 |
| Sulfamethoxazole[d] | 23.75 μg | | | | |
| Vancomycin | 30μg- | | 17-21 | - | 20-27 |

* Adapted from M31-A2 NCCLS. 2002. Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals; Approved Standard-Second Edition. NCCLS document M31-A2 (ISBN 1-56238-461-9). NCCLS, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898, USA.

\- no established range.

a applicable only using Mueller-Hinton Agar supplemented with 5% defibrinated sheep blood, incubated in 5% $CO_2$.

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

c best assessed using Staphylococcus auresus ATCC 25923 with acceptable zone diameter of 18-24 mm.

d very medium-dependent specially with enterococci.

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**APPENDIX 2.2.** Zone diameter interpretative standard for veterinary pathogens.

| | | Zone Diameter (mm) | | | |
|---|---|---|---|---|---|
| **Antimicrobial Agent** | **Disk Content** | **S** | **I** | **F** | **R** |
| Amikacin* | 30μg | ≥17 | 15-16 | | ≤ 14 |
| Gentamicin* | 10μg | ≥ 15 | 13-14 | | ≤ 12 |
| Kanamycin* | 30μg | ≥18 | 14-17 | | ≤ 13 |
| Spectinomycin | 100μg | ≥ 14 | 11-13 | | ≤ 10 |
| Amoxicillin-clavulanic acid* | | | | | |
| Staphylococci | 20/10μg | ≥ 20 | - | | ≤ 19 |
| Other organisms | 20/10μg | ≥18 | 14-17 | | ≤ 13 |
| Ticarcillin-clavulanic acid* | | | | | |
| *Pseudomonas aeruginosa* | 75/10μg | ≥ 15 | - | | ≤ 14 |
| Gram(-)enteric organisms | 75/10μg | ≥20 | 15-19 | | ≤ 14 |
| Ampicillin* | | | | | |
| Enterobacteriaceae | 10μg | ≥ 17 | 14-16 | | ≤ 13 |
| Staphylococci | 10μg | ≥ 29 | - | | ≤ 28 |
| Enterococci | 10μg | ≥17 | - | | ≤ 16 |
| Streptococci (not *S. pneumoniae*) | 10μg | ≥ 26 | 19-25 | | ≤ 18 |
| Oxacillin* | | | | | |
| Staphylococci | 1 μg | ≥ 13 | 11-12 | | ≤ 10 |
| Penicillin* | | | | | |
| Staphylococci | 10 units | ≥ 29 | - | | ≤ 28 |
| Enterococci | 10 units | ≥ 15 | - | | ≤ 14 |
| *S. pneumoniae* | 1μg oxacillin | ≥ 20 | - | | - |
| Streptococci (not *S. pneumoniae*) | 10 units | ≥ 28 | 20-27 | | ≤ 19 |
| Ticarcillin* | | | | | |
| *Pseudomonas aeruginosa* | 75μg | ≥ 15 | - | | ≤ 14 |
| Gram (-) enteric organisms | 75μg | ≥ 20 | 15-19 | | ≤ 14 |
| Penicillin-novobiocin | 10 units/30 μg | ≥ 18 | 15-17 | | ≤ 14 |
| Imipenem* | 10μg | ≥ 16 | 14-15 | | ≤ 13 |
| Cephalothin* | 30μg | ≥ 18 | 15-17 | | ≤ 14 |
| Cefazolin* | 30μg | ≥ 18 | 15-17 | | ≤ 14 |
| Ceftiofur | 30μg | ≥ 21 | 18-20 | | ≤ 17 |
| Enrofloxacin (canine/feline) | 5μg | ≥ 23 | - | 17-22 | ≤ 16 |
| Enrofloxacin (chickens/turkeys) | 5μg | ≥ 23 | 17-22 | | ≤ 16 |
| Enrofloxacin (bovine) | 5μg | ≥ 21 | 17- 20 | | ≤ 16 |
| Difloxacin | 10μg | ≥ 21 | 18- 20 | | ≤ 17 |
| Orbifloxacin | 10μg | ≥ 28 | - | 18-22 | ≤ 17 |

## **APPENDIX 2.2.** Continuation

| | | Zone Diameter (mm) | | | |
|---|---|---|---|---|---|
| **Antimicrobial Agent** | **Disk Content** | **S** | **I** | **F** | **R** |
| Clindamycin | 2μg | ≥ 21 | 15-20 | | ≤ 14 |
| Pirlimycin | 2μg | ≥ 13 | - | | ≤ 12 |
| Erythromycin* | | | | | |
| Streptococci | 15μg | ≥ 21 | 16-20 | | ≤ 15 |
| Organisms other than Streptococci | 15μg | ≥ 23 | 14-22 | | ≤ 13 |
| Tilmicosin (Bovine) | 15μg | ≥ 14 | 11-13 | | ≤ 10 |
| Tilmicosin (Swine) | 15μg | ≥ 11 | | | ≤ 10 |
| Chloramphenicol* | | | | | |
| Streptococci (not *S. pneumoniae*) | 30μg | ≥ 21 | 18-20 | | ≤ 17 |
| *S. pneumoniae* | 30μg | ≥ 21 | - | | ≤ 20 |
| Organisms other than Streptococci | 30μg | ≥ 18 | 13-17 | | ≤ 12 |
| Florfenicol | 30μg | ≥ 19 | 15- 18 | | ≤ 14 |
| Tiamulin | 30μg | ≥ 9 | - | | ≤ 8 |
| Trimethoprim-sulfamethoxazole* | | | | | |
| *Streptococcus pneumoniae* | 1.25/23.75μg | ≥ 19 | 16-18 | | ≤ 15 |
| Organisms other than *S. pneumoniae* | 1.25/23.75μg | ≥ 16 | 11-15 | | ≤ 10 |
| Rifampin* | | | | | |
| *Streptococcus pneumoniae* | 5 | ≥ 19 | 17-18 | | ≤ 16 |
| Organisms other than Streptococci | 5 | ≥ 20 | 17-19 | | ≤ 16 |
| Sulfisoxazole* | 250 or 300 | ≥ 17 | 13- 16 | | ≤ 12 |
| Tetracycline* | | | | | |
| Streptococci | 30 | ≥ 23 | 19-22 | | ≤ 18 |
| Organisms other than Streptococci | 30 | ≥ 19 | 15-18 | | ≤ 14 |
| Vancomycin* | | | | | |
| Enterococci | 30 | ≥ 17 | 15-16 | | ≤ 14 |
| Streptococci | 30 | ≥ 17 | - | | - |
| Other gram-positive organisms | 30 | ≥ 12 | 10-11 | | ≤ 9 |

* human data taken from M100-S12 supplements to M2 and M7
**S** Susceptible
**I** Intermediate
**R** Resistant
**F** Flexible; should be considered susceptible if appropriate dosing modifications specified in the packaging insert are applied

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