GLOBAL TASK FORCE ON CHOLERA CONTROL

Laboratory Methods for antimicrobial susceptibility testing (AST) for *Vibrio cholerae*: points of particular attention

This document discusses a few critical points regarding the laboratory procedures for phenotypic antimicrobial susceptibility testing by diffusion methods, using antibiotic-impregnated discs (Kirby-Bauer method) and test strips, as presented in Job Aid "Antimicrobial susceptibility testing for treatment and control of cholera". Other methods exist for this phenotypic detection, dilution methods including broth microdilution and agar dilution, but these are more expensive and difficult to implement therefore not generally recommended by the GTFCC.

Standard protocols for antimicrobial susceptibility testing are described in detail in the Antibiogram guidelines and references (CLSI*, EUCAST**, CA-SFM EUCAST ***)

Safety Compliance. Testing of *Vibrio cholerae* and other potentially infectious specimen should always be performed in compliance with laboratory safety policies and procedures. Personal protective equipment (PPE) should be worn when handling potentially infectious materials. Always consult individual SDS (safety data sheet) forms for reagent-specific information.

Quality Control Strains (QC). According to Antibiogram guidelines and references[1,2,3], QC strains should be included to monitor the AST system to ensure accurate and reproducible results and always be set up in parallel with test strains. Selection is based on what is needed to cover each drug being tested, see below. However other available laboratory strain carefully characterized and with known susceptibility to the antimicrobial agents tested can be used.

Azithromycin (MIC) *S. aureus* ATCC 29213

Erythromycine (diameter) *S. aureus* ATCC 29213

Pefloxacine (diameter) *E. coli* ATCC 25922

Ciprofloxacin (MIC) *E. coli* ATCC 25922

Tetracycline (diameter) *E. coli* ATCC 25922

Doxycycline (MIC) *E. coli* ATCC 25922

Quality and preparation of Medium. Mueller-Hinton agar medium is the only susceptibility test medium that has been validated by international guidelines[1,2,3]. Commercial ready-to-use dehydrated Mueller-Hinton media are recommended. Agar should be poured into flat-bottom glass or plastic petri dishes on a level pouring surface to a uniform depth of 4 mm ± 0.5 mm. **Greater or lesser depths affect the diffusion of antimicrobial agents and drug activity may be affected.** Each new batch of medium should be tested for sterility, ability to support growth of the target pathogen(s), ability to produce appropriate antibioresistance pattern with the control strain. Store the poured Petri dishes at 4–8 °C according to the manufacturer's instruction. The agar surface must be dry before use but be careful not to dry out the agar.

Inoculum preparation. The antibiogram must be realized **using a freshly sub-cultured strain** (16–18 h at 35 °C ± 2 °C) on suitable non-selective media such as Mueller Hinton Agar, Brain Heart Infusion Agar or Trypticase Soy Agar, inoculated so as to have isolated colonies. Also streak the ATCC QC strains needed for disk diffusion testing and incubate in the same manner. Make a bacterial suspension in saline **from several isolated colonies** (to avoid selecting an atypical variant) to achieve a turbidity equivalent to that of the McFarland 0.5 standard. Adjust the density by adding either saline solution or bacteria.

Inoculating agar plates. Bacterial inoculum should ideally be used within 15 min of preparation but within 60 min at the latest. Dip a sterile cotton swab into the bacterial suspension and remove excess liquid by rotating the swab on the walls of the tube. If several agar plates are to be inoculated with the same inoculum, it is necessary to correctly reload the swab between each agar plate. Streak the entire surface of the plate 3 times, rotating 60 degrees each time,ensuring that there are no gaps between streaks. **Discs or test-strips should be deposited within 15 min of inoculation**, if plates are left too long at laboratory temperature before discs or strips are deposited, the bacteria may start to grow, leading to a false decrease in the size of the inhibition zones.

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Antimicrobial disks. Refrigerate sealed cartridges at ≤8 °C or freeze at ≤-14 °C in non-self-defrosting freezer until use according to manufacturer's recommendations. To avoid condensation, allow discs or dispenser containers to return to room temperature before use. Use a disk dispenser, if not available find a sterile container such as an empty petri plate to put the disks in and some sterile forceps to handle the disks with (1). Use no more than 6 disks if using small (90 or 100-mm) agar plates. Place the discs firmly on the surface of the dry, inoculated agar. **Once the discs have been placed, they must not be moved, as antibiotic diffusion is very rapid.**

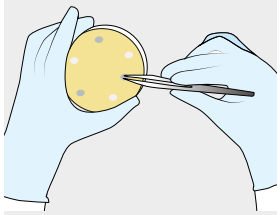
Test-strips. Allow strip containers to return to room temperature, approximately 30 min. Manually or with forceps, remove sufficient number of Test-strips from storage. Only handle the strip above the black line in the area where the antibiotic code is shown. Do not touch the surface of the strip opposite the MIC scale. Once the strips are down, they must stay down. **Strips cannot be moved because of the instantaneous release of antibiotic into the agar.**

Incubation. Incubate the plates at 35 ± 2 °C for 18 ± 2h in an inverted position of stacks no higher than five, ideally **within 15 min of disc deposition, but no longer than 30 min**.

Reading. Correct inoculum should result in a confluent culture spread over the entire surface of the agar. The presence of isolated colonies indicates that the inoculum is too low and the test must be repeated. After incubation period, measure the diameter of inhibition (including the disk) to the nearest millimeter using a sliding caliper or ruler held on the back of the plate (2). Check that the diameters of the zones of inhibition of quality control strains are within acceptable limits. Refer to *Antibiogram guidelines* documents [3,4,5] for interpretation. The standards are reviewed on a regular basis, please check that you are using an updated version.

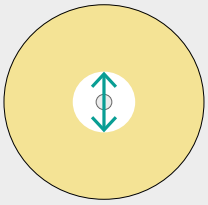
Read the MIC value where the edge of the inhibition ellipse intersects the side of the strip (3); if the ellipse intersected between two MIC values, the higher of the two values is reported (4); if the strip has been placed upside down it is an invalid result, repeat the test (5).

Troubleshooting. Out-of-range QC tests are often due to the contamination or the use of an incorrect QC strain. Corrective action should first include repeating the test with a pure culture of a freshly sub-cultured QC strain. If the issue is unresolved, refer to the Antibiogram guidelines and references trouble shooting guide (CLSI M100[1]) or Frequently Asked Questions (Eucast[4]). According to EUCAST reading guide for disk diffusion[6], in case of distinct colonies within an inhibition zone, check purity and repeat the test if necessary. If cultures are pure, colonies within zones should be taken into account when measuring the diameter. In case of double zones, check for purity and repeat the test, if necessary. If cultures are pure, read the inner zone.



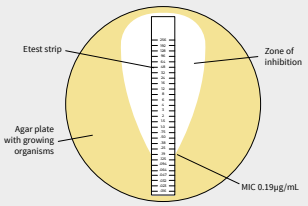
1

Use a sterile container and forceps



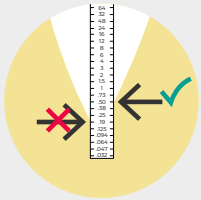
2

Measure the diameter of inhibition



3

Read the MIC value



4

Report the higher of the two MIC values

5

Do not place the strip upside down

References

1. https://clsi.org/standards/products/microbiology/documents/m100/, CLSI M100 Performance Standards for Antimicrobial Susceptibility Testing, 34th Edition
2. https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Disk_test_documents/2024_manuals/Manual_v_12.0_EUCAST_Disk_Test_2024.pdf
3. https://www.sfm-microbiologie.org/wp-content/uploads/2023/06/CASFM2023_V1.0.pdf
4. https://www.eucast.org/clinical_breakpoints
5. https://clsi.org/all-free-resources/ (CLSI M45 ED3:2016TABLE 20)
6. https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Disk_test_documents/2023_manuals/Reading_guide_v_10.0_EUCAST_Disk_Test_2023.pdf

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