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Original article

Deviations from Standard Disk Diffusion Protocols for Antimicrobial Susceptibility Testing in Medical Laboratories in Gharyan

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Deviations, Standard Disk Diffusion, Protocols, Antimicrobial Susceptibility Testing, Medical Laboratories.

A B S T R A C T
Antimicrobial Susceptibility Testing (AST) is essential for guiding appropriate antibiotic therapy. The disk diffusion method is widely used in Libya due to its low cost, yet deviations from standardized protocols can compromise result accuracy, affecting clinical decisions and patient outcomes. This study evaluated the performance of the disk diffusion AST method in medical laboratories in Gharyan, Libya, using EUCAST guidelines (version 12.0) as a benchmark. Seventy-five AST plates representing 122 clinical specimens were randomly collected from three pathology centers between September and November 2025. Plates were analyzed for the type of culture media, number of specimens per plate, number of antibiotic disks, and readability of results. All plates were 90 mm in diameter. Nutrient agar was used for 118 specimens, and blood agar for two; none used Mueller-Hinton agar as recommended. Only 17% of plates tested a single specimen per plate; the majority tested one specimen on half or a quarter of a plate. The number of antibiotic disks per specimen often exceeded EUCAST limits (up to 20 disks per plate), and only 8% of plates yielded readable results. Widespread deviations from EUCAST protocols—including inappropriate media, specimen overloading, and excessive disks—compromise AST reliability. These errors may lead to incorrect antibiotic prescriptions, delayed recovery, and increased antimicrobial resistance. Strict adherence to standardized guidelines is urgently required to ensure accurate, clinically meaningful AST results. Moreover, raising awareness of the importance of following standard guidelines is crucial for correcting the course.

Introduction

Antimicrobial susceptibility testing (AST) is an essential laboratory procedure used to determine the effectiveness of specific antibiotics against bacterial pathogens. However, several errors can compromise the validity and reliability. The accuracy of these results directly influences clinical decision-making, patient recovery, and antimicrobial stewardship. Typically, pathogenic bacteria are isolated from clinical specimens such as blood, urine, stool, sputum, tissue, cerebrospinal fluid, or mucus from the nose, throat, or genitals etc. The specimens might be examined microscopically and/or sub-cultured to allow the pathogen to thrive. After microbial (bacterial or fungal) growth is observed, the pathogen can be tested later to identify it and determine its susceptibility to antibiotics [1]. Generally, conventional AST methods require one to three days; Therefore, in many cases, a broad-spectrum antibiotic is prescribed immediately, and only patients with complications or chronic infections are referred to clinical laboratories for AST [2].

There are three main conventional methods for testing the antimicrobial susceptibility, including: Disk diffusion antimicrobial susceptibility test, Macro or Micro-dilution antimicrobial susceptibility assay, agar dilution antimicrobial susceptibility assay. In Libya, the disk diffusion method is widely used to diagnose the sensitivity of bacterial clinical isolates to antibiotics, especially in the private sector, due to its low cost (personal communication). The main institutional guidelines that describe the disk diffusion method for AST are published by major authorities, including the Clinical and Laboratory Standards Institute (CLSI) [3], European Committee on Antimicrobial Susceptibility Testing (EUCAST) [4], and British Society for Antimicrobial Chemotherapy [5], which considers EUCAST as a main guideline. EUCAST offers the guidelines for free, which makes the information available to laboratories in resource-limited countries.

Briefly, the main criteria for the EUCAST disk diffusion method guideline 4 are as follows: the medium should be Mueller-Hinton (MH) agar, with a level depth of 4.0 ± 0.5 mm in each plate, or MH + 5% mechanically defibrinated horse blood + 20 mg/L β-NAD, depending on the genus or species of the bacteria. The inoculum should be prepared by the direct colony suspension method in saline to the density of a 0.5 McFarland turbidity standard, which corresponds to approximately 1-2 x 108 CFU/ml for *Escherichia coli.* One specimen for one agar plate. Inoculation of agar plates is performed by swabbing in three directions or

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by using an automatic plate rotator. The maximum number of disks on a plate should be 6 and 12 disks on a 90- and 150-mm circular plate, respectively, to avoid overlapping of zones and interference between agents. In addition, choosing the antibiotic disk concentration is not random. The incubation temperature is 35 ± 1°C for 18 ± 2 h with some exceptions (e.g., *Brucella melitensis* should be incubated for 48 ± 2 h). The incubation should be in air or 4-6% CO2 in air, depending on the genus and species. The diameter of the inhibition zone around the disk is measured after 24-48 hrs. Then, EUCAST Breakpoint tables for interpretation of MICs and zone diameters should be used to report the results as susceptible (S), Susceptible, increased exposure (I), and Resistant (R) [6].
Clinical breakpoints are used in clinical microbiology laboratories to classify microorganisms as clinically susceptible (S), intermediate (I), or resistant (R) based on the quantitative antimicrobial susceptibility indicated by the MIC values obtained from a standardized testing system. The laboratory report, denoting S, I, or R for each antimicrobial agent, provides clinicians with guidance regarding the potential application of these agents in patient treatment. Consequently, clinical breakpoints should distinguish between patients who are likely or unlikely to respond to antimicrobial therapy [7]. This study utilizes the EUCAST disk diffusion method version 13.0 guideline [5] as a standard to evaluate the quality and validity of the protocols employed by several medical laboratories in Gharyan for conducting AST tests.

Methods

Images of 75 Petri dishes were collected from three pathology centres in Gharyan city during the 17th of September 2025, to the 23rd of November 2025, at random intervals. The AST results for 122 specimens are shown on these plates. The type of media, the quantity of clinical specimens on each plate, the quantity of antibiotic disks, and the readability of the results were all photographed and assessed.

Results

All the collected AST plates were circular with a diameter of 90mm. The isolates were diagnosed as bacterial strains according to the microbiologist in the lab (personal communication). Seventy-five plates presented the results of 122 specimens, and each specimen belonged to one person (patient). The plates were examined for the type of culture media used, the number of clinical specimens on each plate, the number of antibiotic disks, and the readability of the results.

The culture media used for ASTs

Regarding the culture media used for ASTs, nutrient agar was used to culture 120 specimens; while the remaining two specimens were cultured on a single blood agar plate.

The number of specimens and antibiotic disks on each plate

Only 21 plates carried a single specimen per plate: 8 of these plates were tested using 6 disks (Fig. 1), while the remaining 13 plates were tested using 7–20 disks (Fig. 2). There were 97 specimens; each specimen was cultivated on one half of a plate, and 45 plates carried two specimens instead of one. Six additional plates carried a single specimen on one half of a plate (fig. 3 and 4). The number of the antibiotic disks used to perform the ASTs for each specimen ranged from 5 to 16 disks per specimen, all of which were cultivated on one half of a plate. To illustrate, 49 specimens were tested using 5-7 disks (Fig.3), 48 specimens were tested using 8-11 disks (Fig. 4), and two specimens were tested using 16 disks for a single specimen in one half of a plate (specimens no. 107 & 103 in Fig. 4). Interestingly, there are two plates in which two specimens were cultivated, each on one quarter of the plate. The specimens on one quarter of the plate were examined using 6 or 11 disks per specimen (Fig.5).

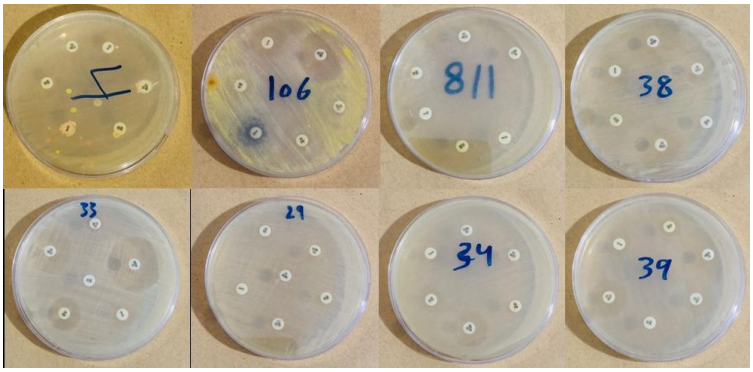

Figure 1. Six disks were used to test one specimen on each of the antimicrobial susceptibility test plates (Ø = 90 mm).

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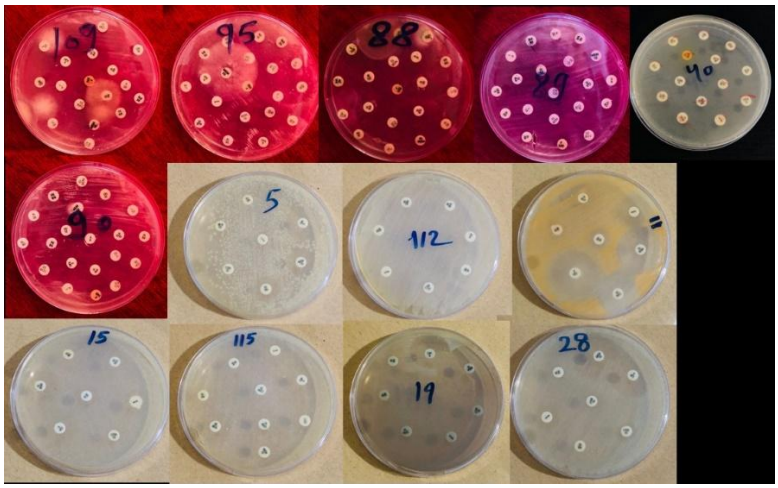

Figure 2. Seven to twenty disks were used to test one specimen on each of the antimicrobial susceptibility test plates (Ø = 90 mm).

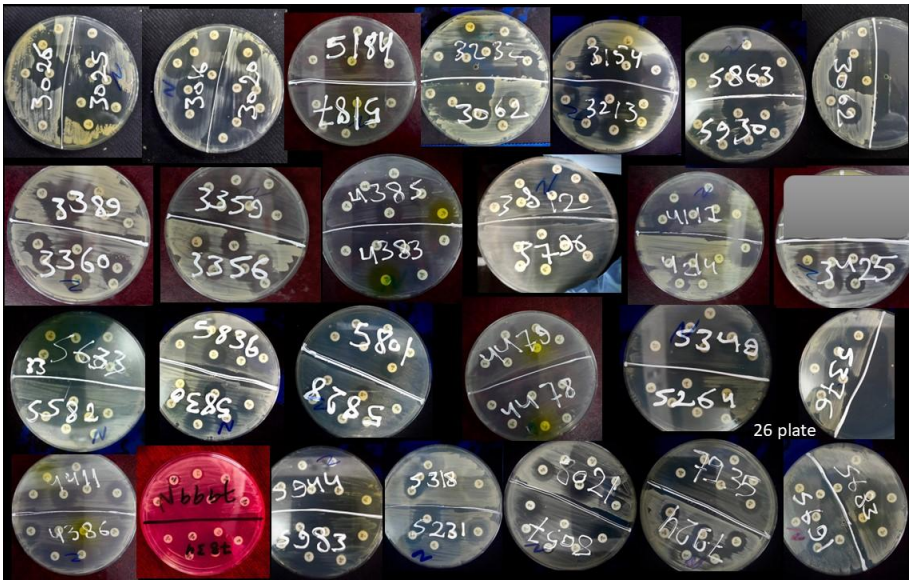

Figure 3. Five to seven disks were used to test one specimen on one half of the antimicrobial susceptibility test plates (Ø = 90 mm).

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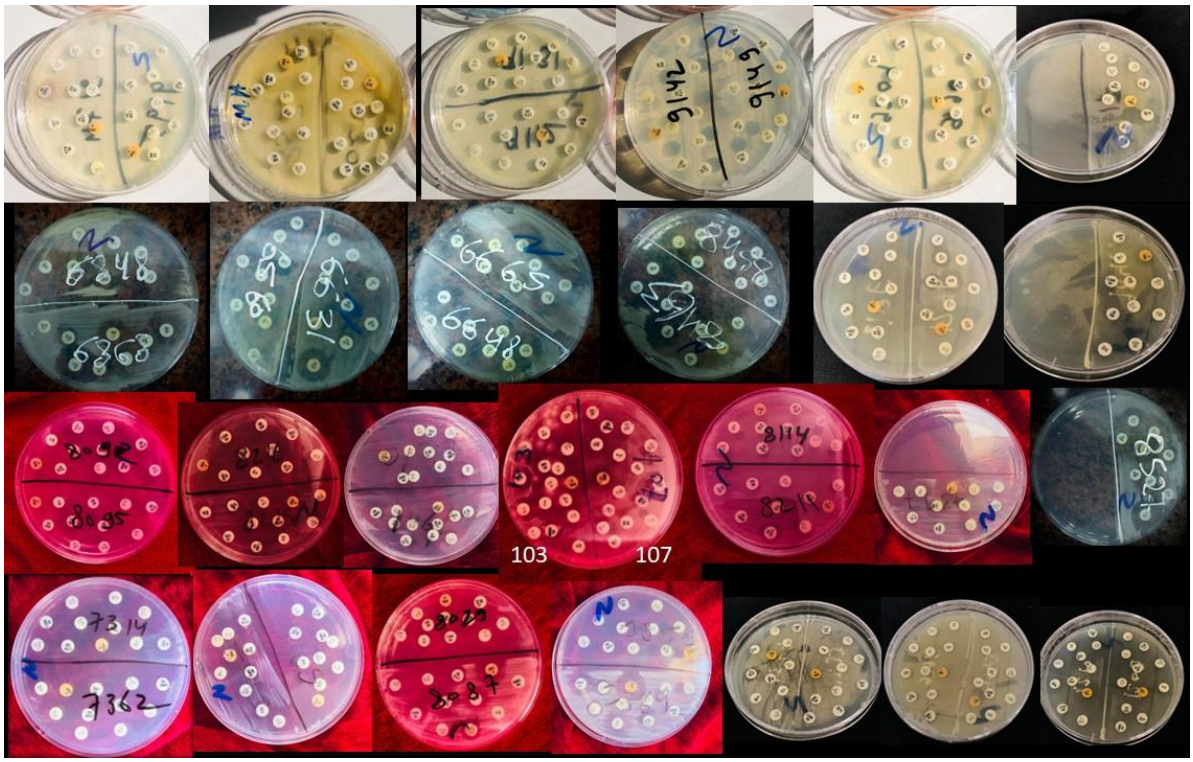

Figure 4. Eight to sixteen disks were used to test one specimen on one half of the antimicrobial susceptibility test plates (Ø = 90 mm). specimens no. 103 and 107 contain 16 disks.

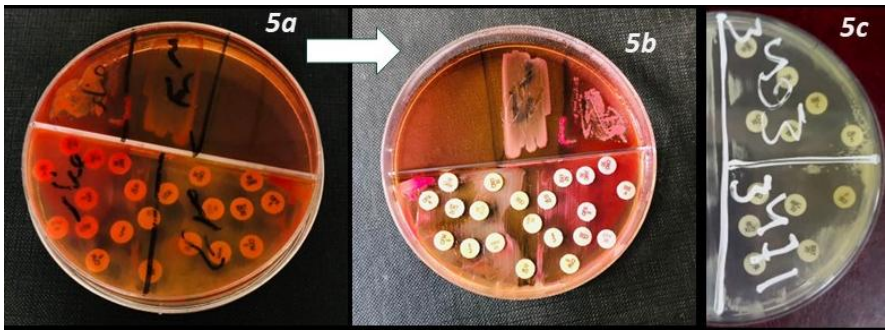

Figure 5. Six to eleven disks were used to test one specimen on one quarter of the antimicrobial susceptibility test plates (Ø = 90 mm). Two plates are shown, 5a is an image for the bottom of the first plate that shows names for two specimens, and 5b is an image for the top of the first plate. 5c is the second plate.

Readability of the results

Placing more than 7 disks on one half of a plate and, in some cases, on one quarter of a plate, interferes with the readability of the results. As seen in the images, it is not possible to accurately measure the diameter of the clear zones surrounding the antibiotic disks in most of the plates because the disks are placed too closely together. This causes the overlap between growth-inhibiting regions. However, only about 8% of the plates show readable results, as these plates contain only one specimen tested using 6 or 7 disks. In general, all the plates in the collection were 90mm in diameter. 100% of the ASTs did not use Muller Hinton Agar (MHA) nor MH + 5% mechanically defibrinated horse blood + 20 mg/L β-NAD (MH-F). Additionally, 82% of the plates had more than one specimen cultivated over the whole plate. Furthermore, 93.3% of the plates had more than 6 disks (Fig.6).

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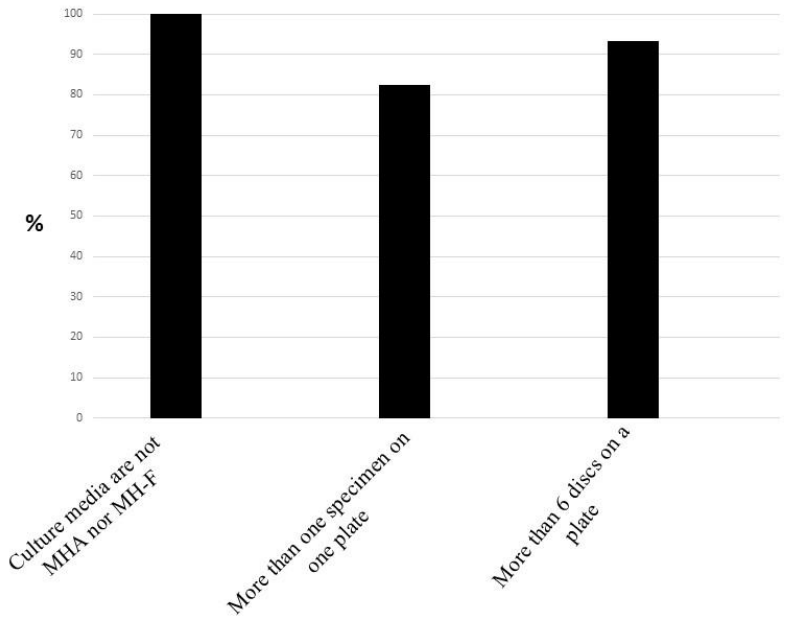

Figure 6. Bar graph presents the rates of the plates which do not contain MHA nor MH-F, the plates which contain more than one specimen, and the plates which contain more than 6 disks.

Discussion

The categorisation of antimicrobial agents in laboratory reports as S, I, or R guides clinicians regarding the potential use of these agents for patient treatment. The clinical breakpoints should therefore distinguish between patients who are likely or unlikely to respond to antimicrobial treatment. In Europe, clinical breakpoints are established by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) following a specified protocol [6]. This includes the assessment of efficacy in experimental and clinical studies to establish pharmacodynamic targets, the pharmacokinetic characteristics of the agent, Monte Carlo simulations to estimate antimicrobial agent exposure level in the target patient population, and the dosing regimens most commonly used. Therefore, setting breakpoints does not depend only on the MIC value or inhibition zone diameter, but also considers several factors, including wildtype MIC distributions for relevant species of organisms, antimicrobial dosing, pharmacokinetic (PK), pharmacodynamic (PD) aspects, and clinical results from various types of studies [7]. Clinical outcome is dependent on the triangular relationship between MIC, exposure, and efficacy. In turn, exposure of the microorganism to the agent in the patient is dependent on the dose administered and the pharmacokinetic properties of the drug. For many agents, the efficacy of the non-protein-bound, free (f) agent in serum is correlated with the area under the concentration-time curve (AUC) and inversely correlated with the MIC [8].

All of this demonstrates how much information and work go into the standardized published tables of the breakpoints, which are made available by the authorized committees or organizations, and how important it is to follow their procedures rather than relying on random protocols or explanations of the results. Neither CLSI nor EUCAST guidelines use nutrient agar or blood agar to test the antimicrobial susceptibility of bacteria. Instead, Mueller-Hinton agar or MHA supplemented with 5% defibrinated horse blood and 20 mg/L β-NAD (MH-F) is described. All the collected AST plates in this study used only nutrient or blood agar, making the interpretation of the results unreliable.

Utilizing one plate to perform AST for one specimen has been emphasized by both CLSI [3] and EUCAST [4]. The results in (Figure 1) show that only 17.5% of the specimens were tested on one plate, whereas the rest were tested on half or a quarter of a plate. which affects the reliability of the results enormously? The number of the applied antibiotic disks was far more than the number specified by EUCAST and CLSI. According to these organisations, 6 and 12 disks are the maximum possible number on a 90- and 150-mm circular plate (3,4), respectively. In contrast, the specimens in this study were tested using up to 20 disks on one plate or 6-11 disks to test one sample on half or quarter of a plate, which causes difficulty in measuring the diameter of the inhibition zones, not to mention the interaction between the antibiotics due to the diffusion and the short distances between them. The interaction can be synergistic, antagonistic, or indifferent/additive. A good example shows that the interaction between the effects of two close antibiotic disks is manipulated in the D test for *Staphylococcus aureus* [9].

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The way of interpreting the results is based on how big the clear zone is; the bigger, the more effective (personal communication), which is a totally incorrect approach. Referring to the breakpoint tables [6], bacterial species that exhibit a larger clear area are not necessarily sensitive to those antibiotics. All of these violations contribute to incorrect diagnosis of the bacterial susceptibility to the tested antibiotics, which may affect the life of the patient or delay the recovery. Other aspects should be investigated, which could not be traced from the obtained images, such as the accurate identification of the tested microorganism, the inoculum size, the type and concentration of each tested antibiotic, measuring the diameter of the clear zone, and the interpretation of the results. All of these aspects should be considered because of their direct impact on the results. Incorrect performance of ASTs leads to misleading the clinical decision-maker to prescribe the proper antibiotic, which affects the recovery rate of the patients. Moreover, incorrect choices of prescribing antibiotics lead to an increased trend of antimicrobial resistance locally and worldwide.

Conclusion

In conclusion, this study presents a sample of incorrect performance of AST in some medical microbiology laboratories in a small region in Libya. Although 122 specimens might be statistically insignificant, it represent the lives of 122 patients for whom incorrect AST results could lead to delayed recovery, complications, or even death as a result of inappropriate antibiotic prescriptions. Raising awareness of the importance of following standard guidelines is crucial for correcting the course.

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Conflict of interest

There is no conflict of interest.

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Originality and plagiarism

The views and opinions expressed in this article are those of the authors and are the product of professional original research.

References

1. Sánchez-Romero MI, García-Lechuz Moya JM, González López JJ, Orta Mira N. Collection, transport and general processing of clinical specimens in Microbiology laboratory. Enferm Infecc Microbiol Clin (Engl Ed). 2019 Feb;37(2):127-134.
2. Chen C, Hong W. Recent development of rapid antimicrobial susceptibility testing methods through metabolic profiling of bacteria. Antibiotics (Basel). 2021 Mar 16;10(3):311.
3. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial disk susceptibility tests. 14th ed. CLSI standard M02. Wayne, PA: Clinical and Laboratory Standards Institute; 2024.
4. The European Committee on Antimicrobial Susceptibility Testing. EUCAST disk diffusion method for antimicrobial susceptibility testing. Version 13.0. 2025. Available from: https://www.eucast.org/fileadmin/eucast/pdf/disk_test_documents/2025/Manual_v_13.0_EUCAST_Disk_Test_2025.pdf
5. BSAC. BSAC methods for antimicrobial susceptibility testing. Version 12. 2013. Available from: https://bsac.org.uk/wp-content/uploads/2012/02/Version-12-Apr-2013_final.pdf
6. The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 15.0. 2025. Available from: https://www.eucast.org
7. Mouton JW, Ambrose PG, Canton R, Drusano GL, Harbarth S, MacGowan A, et al. Conserving antibiotics for the future: new ways to use old and new drugs from a pharmacokinetic and pharmacodynamic perspective. Drug Resist Updat. 2011 Apr;14(2):107-17.
8. Mouton JW, Brown DF, Apfalter P, Cantón R, Giske CG, Ivanova M, et al. The role of pharmacokinetics/pharmacodynamics in setting clinical MIC breakpoints: the EUCAST approach. Clin Microbiol Infect. 2012 Mar;18(3):E37-45.
9. Shrestha B, Rana SS. D test: a simple test with big implications for Staphylococcus aureus macrolide-lincosamide-streptogramin B resistance pattern. Nepal Med Coll J. 2014 Mar;16(1):88-94.

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