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Agar well diffusion method antimicrobial activity

The National Library of Medicine (NLM) offers access to scientific literature, but inclusion in their databases doesn't necessarily mean they endorse or agree with the content. To learn more about NLM's stance on disclaimers and copyrights, visit PMC Disclaimer | PMC Copyright Notice . In recent years, there has been a growing interest in developing new antimicrobial agents to combat microbial resistance. As a result, researchers have given more attention to methods for evaluating antimicrobial activity. Various bioassays are already well-established, but others that require specialized equipment and further evaluation, like flow cytofluorometric and bioluminescent methods, aren't as widely used despite offering rapid results. This review article provides an exhaustive list of in vitro antimicrobial susceptibility testing methods, including their advantages and limitations. These methods can be applied to drug discovery, epidemiology, and predicting therapeutic outcomes. In this study, we focused on the use of antimicrobial testing for investigating extracts and pure drugs as potential antimicrobial agents. The importance of discovering new antibiotics cannot be overstated, especially with the rise in microbial resistance and its significant impact on public health. Natural products continue to be a major source of new drug molecules, including those derived from prokaryotic bacteria, eukaryotic microorganisms, plants, and animal organisms. Researchers have been studying plant and microbial extracts, essential oils, pure secondary metabolites, and synthesized molecules for their antimicrobial properties. However, comparing results can be challenging due to the use of different approaches, such as inoculum preparation techniques, growth medium, incubation conditions, and endpoints determination. For instance, a plant extract may exhibit some level of antimicrobial activity, but its effectiveness varies depending on these factors. As the history repeats itself, with antibiotic resistance increasing, it's crucial to explore new methods for evaluating antimicrobial activity. This review aims to provide a comprehensive overview of in vitro antimicrobial susceptibility testing methods and their applications, highlighting the importance of standardization and reproducibility in this field. The reliability of initial data is crucial in research, as it enables researchers to compare results and avoid using antimicrobial activity investigation solely as a complement to phytochemical studies. Various laboratory methods can be employed to evaluate or screen the in vitro antimicrobial activity of an extract or pure compound, including disk-diffusion and broth or agar dilution methods. Time-kill test and flow cytofluorometric methods are recommended for further studying the antimicrobial effect of an agent in depth. The development of a better understanding of current methods is essential for applications in human health, agriculture, and environment, particularly with the growing interest in combating multidrug-resistant bacteria. Agar disk-diffusion testing, developed in 1940, is a widely used official method for routine antimicrobial susceptibility testing in many clinical microbiology laboratories. The Clinical and Laboratory Standards Institute (CLSI) has published accepted and approved standards for bacteria and yeast testing. Although not all fastidious bacteria can be tested accurately using this method, the standardization process allows for testing certain bacterial pathogens using specific culture media, incubation conditions, and interpretive criteria. The disk-diffusion method involves inoculating agar plates with a standardized inoculum of the test microorganism, placing filter paper discs containing the test compound on the agar surface, and then incubating the Petri dishes under suitable conditions. The diameters of inhibition growth zones are measured, providing information on the antimicrobial agent's effectiveness (Fig. 1A). Table 1 outlines the recommended culture media, temperature, period of incubation, and inoculum size for CLSI standards. Methods Microorganism Growth medium Final inoculum size Incubation temperature (°C) Incubation time (h) Ref. Disk-diffusion method Bacteria MHA (0.5 McFarland) CFU/mL ranges reported for various antimicrobial susceptibility tests including CFU (colony-forming units), M02-A, M44-A, M51-A, M07-A, M27-A, and M38-A assays, as well as broth microdilution, agar dilution, and time-kill test results in different mediums like MHA, RPMI 1640b, and MHB. The Etest method is widely used in determining MIC values for antibiotics, antifungals, and antimycobacterials. This technique involves placing an Etest strip on a pre-inoculated agar plate surface to determine the growth inhibition ellipse at its intersection with the strip. Although simple and commonly used by clinicians, the cost of Etest strips becomes a concern when testing numerous drugs. However, previous studies have shown good correlation between MIC values obtained using Etest and other methods like broth or agar dilution. The Etest method can also be used to investigate antimicrobial interactions between two drugs. This involves placing an Etest strip impregnated with the first antibiotic on a pre-inoculated agar plate surface, removing it after one hour, and replacing it with another strip impregnated with the second antibiotic. The synergy is detected by a decrease of at least two dilutions in the MIC value of the combination compared to that of the most active antibiotic tested alone. For further analysis of antimicrobial interactions, the Etest strips can be deposited on the agar medium in a cross formation with a 90° angle at the intersection between the scales at the respective MICs for the microorganism tested. After incubation, the fractional inhibitory concentration index (FICI) can be calculated to determine synergy or antagonism. Other diffusion methods used in microbiology research laboratories include the agar well diffusion method and the agar plug diffusion method. The agar well diffusion method is widely used to evaluate antimicrobial activity of plants or microbial extracts by punching a hole into an agar plate, introducing the extract solution, and observing growth inhibition. Similarly, the agar plug diffusion method highlights antagonism between microorganisms by cutting an agar culture of interest, depositing it on another pre-inoculated agar plate surface, and allowing substances to diffuse and inhibit microbial growth. To assess antimicrobial activity, a plug is inserted into an agar medium, and the resulting inhibition zone around the plug indicates the presence of antimicrobial molecules (Fig. 1C). The cross-streak method allows for rapid screening of microorganisms for antagonism by applying a single streak of the microbial strain of interest to the center of an agar plate, followed by perpendicular streaks of tested microorganisms after incubation. Antimicrobial interactions are then analyzed based on inhibition zone size. The poisoned food technique is commonly used to evaluate antifungal effects against molds by incorporating an antifungal agent into molten agar and measuring fungal growth diameters in control and sample plates using the formula: Antifungal activity (%) = ((Dc - Ds) / Dc) × 100, where Dc and Ds represent the diameters of growth in control and sample plates, respectively. When standardization fails, a positive control with a known antimicrobial molecule is used for comparison. In 1946, Goodall and Levi introduced paper chromatography combined with contact bioautography to detect penicillins, later followed by Fischer and Lautner's use of TLC in the same field. TLC-bioautography has been applied to screen organic extracts, mainly plant extracts, for antibacterial and antifungal activity. Three bioautographic techniques - agar diffusion, direct bioautography, and agar-overlay assay - have been described for investigating antimicrobial compounds. The agar contact method involves transferring antimicrobial agents from a chromatogram to an inoculated agar plate, while direct bioautography involves dipping or spraying a TLC plate with a microbial suspension, followed by incubation and visualization using tetrazolium salts. P-Iodonitrotetrazolium violet has proven to be the most effective detection reagent [44], [48]. To utilize this reagent, the bioautogram is sprayed with it and then reincubated at a controlled temperature for an extended period [49] or shorter duration [5]. A recommended medium is Mueller Hinton Broth supplemented with agar, which maintains humidity and allows optimal bacterial growth on the TLC plate [50]. Direct bioautography can be applied to both fungi and bacteria, offering consistent results for spore-producing fungi like Aspergillus, Penicillium, and Cladosporium [51], [52]. For bacteria, common strains include Bacillus subtilis, Staphylococcus aureus, and Escherichia coli [42], [53]. Immersion bioautography is a hybrid method where the TLC plate is covered with molten seeded agar medium; it allows good diffusion of tested compounds into the agar and can be used for various microorganisms such as Candida albicans [54] and molds [43]. This technique provides clear growth inhibition zones, is resistant to contamination, and localizes active constituents on the TLC plate. Despite advancements in high-performance liquid chromatography coupled bioassay, TLC-bioautography remains a simple, effective, and inexpensive method for separating complex mixtures and detecting antimicrobial compounds. It can be used in various settings, from sophisticated laboratories to small ones with minimal equipment [44]. Additionally, it offers a rapid technique for screening large numbers of samples for bioactivity and in bioactivity-guided fractionation [45]. The dilution method is ideal for determining MIC values, allowing estimation of the tested antimicrobial agent's concentration in agar or broth medium [46]. Standards such as those provided by CLSI and EUCAST offer guidelines for uniform testing procedures, which are practical for various microorganisms including fastidious bacteria, yeast, and filamentous fungi. The development of standardized methods in clinical microbiology laboratories is crucial but does not guarantee the relevance of testing. Standardization allows for a consistent approach to perform bioassays, including antimicrobial susceptibility testing, such as broth micro- or macro-dilution. These tests involve preparing dilutions of antimicrobial agents and inoculating microbial suspensions into growth media. Given text description here is mainly similar to that of CLSI but with some modifications. These changes typically involve adjustments in test parameters like inoculum preparation, inoculum size, and MIC reading methods which differ between CLSI's visual assay and EUCAST's spectrophotometric approach. In the case of fungi such as conidia and spores forming microorganisms, CLSI requires an initial inoculum of 0.4×10^4–5×10^4 CFU/mL for standardization whereas EUCAST allows a wider range from (2–5)×10^5 CFU/mL through haemocytometer counting. Studies have highlighted the importance of accurately preparing inocula through haemocytometer counting to achieve reliable and consistent results regardless of conidia size or color. The determination of minimum bactericidal concentration (MBC) or minimum fungicidal concentration (MFC), also referred to as the minimum lethal concentration (MLC), is a widely used method for assessing antimicrobial activity. The MBC refers to the lowest amount of an antimicrobial agent needed to kill 99.9% of the inoculum after incubation under standardized conditions, typically achieved through broth or microdilution methods followed by sub-culturing and agar plate testing. The MIC endpoint is recorded as the concentration that completely inhibits microbial growth under suitable conditions. Agar dilution is a widely accepted method for determining MICs in both antibacterial and antifungal susceptibility testing due to its ability to handle multiple isolates against a single compound or when certain compounds mask microbial growth detection. The efficacy of antimicrobial agents against Gram-negative bacteria has been extensively studied, with various methods yielding excellent results. The time-kill test is considered the most suitable method for determining the bactericidal or fungicidal effect, as it provides valuable information on the dynamic interaction between the antimicrobial agent and the microbial strain. This method involves incubating a bacterial suspension in broth culture medium at different concentrations of the tested compound over varying time intervals. The percentage of dead cells is then calculated relative to the growth control tube, with results indicating a significant bactericidal effect after 6 hours, equivalent to 99.9% lethality by 24 hours. The time-kill test also enables the evaluation of synergism or antagonism between multiple drugs in combination and has been successfully applied to various antifungal substances. Additionally, it has been utilized for the assessment of cytotoxicity, biofilm impact, and drug screening against Leishmania parasites. A bioluminescence assay measures the ATP produced by bacteria or fungi, providing a rapid quantitative method with applications in antimicrobial testing, drug screening, and susceptibility assessments. This technique offers several advantages, including short incubation periods and the ability to assess antimicrobial activity in vivo or in situ. The use of propidium iodide (PI) as a fluorescent and intercalating agent for DNA staining has been widely adopted due to its effectiveness in antibacterial testing, particularly against Listeria monocytogenes. A flow cytometer-based method combining PI with carboxyfluorescein diacetate (cFDA) allows for the discrimination of three subpopulations: dead, viable, and injured cells. The latter is characterized as stressed cells with cellular component damage, potentially critical in food microbiology where cell recovery from temperature abuse conditions may occur. This method enables the detection of antimicrobial resistance and provides rapid results compared to traditional microdilution methods. However, its widespread adoption appears unlikely due to the accessibility of flow cytometry equipment. The development of novel antimicrobial agents and susceptibility testing methods is crucial in addressing the growing clinical threat of microbial infections, with significant morbidity and mortality linked to antibiotic resistance. Standardization efforts by organizations such as the CLSI and EUCAST have marked key milestones in this field. Nevertheless, modifications to standardized protocols when testing natural products are often necessary, emphasizing the need for careful methodological adaptations to ensure accurate experimental approaches and comparable results. The article includes references to various studies and guidelines related to antimicrobial susceptibility testing, which is crucial for determining the effectiveness of antibiotics against pathogens. Studies on bioactive microbial metabolites, medicinal plants with anti-Candida activity, and essential oils' effect on pathogenic bacteria are also mentioned. Guidelines such as CLSI's Performance Standards for Antimicrobial Disk Susceptibility Tests and Method for Antifungal Disk Diffusion Susceptibility Testing of Yeasts provide a framework for testing the susceptibility of pathogens to various antimicrobial agents. Reviews and guidelines on antimicrobial susceptibility testing, including general principles, contemporary practices, and comparisons between different methods, are also included. The article highlights the importance of accurate and reliable antimicrobial susceptibility testing, which is essential for informed clinical decision-making and effective treatment of infections. Some specific studies mentioned in the references include: * A study on the antimicrobial activity of Southern African medicinal plants with dermatological relevance * A comparison between different methods and end-points for determining in vitro activity of Micafungin against Aspergillus spp. * A review of general principles and contemporary practices in antimicrobial susceptibility testing Overall, the article provides a comprehensive overview of the importance of antimicrobial susceptibility testing and highlights various studies and guidelines that support this critical practice. **Methods for Testing Antifungal and Antibacterial Susceptibility** Several guidelines have been established for testing the effectiveness of antifungal and antibacterial agents against non-dermatophyte filamentous fungi (18). For example, the Clinical and Laboratory Standards Institute (CLSI) has developed a method for testing the susceptibility of these organisms to various antifungal agents using disk diffusion tests (18). **Quality Control Guidelines** Studies have also established quality control guidelines for testing the efficacy of certain antifungal agents, such as amphotericin B, itraconazole, posaconazole, and voriconazole, against non-dermatophyte filamentous fungi (19). **Natural Products with Antimicrobial Activity** Research has identified various natural products that exhibit antimicrobial activity, including essential oils (22) and extracts from Streptomyces species (20). For example, a study found that a newly isolated Streptomyces sp. strain US80 exhibited antifungal and antibacterial activities (20). **Comparison of Testing Methods** Several studies have compared different methods for testing antimicrobial susceptibility, including disk diffusion tests, E-test, broth microdilution, and agar dilution tests (25-27). For example, one study found that the E-test was a reliable method for testing the efficacy of antifungal agents against clinical Aspergillus isolates (27). **Testing Synergy and Antagonism** Other studies have explored the concept of synergy and antagonism in antimicrobial therapy, including the use of time-kill tests, checkerboard tests, and E-test to detect synergy between different antimicrobial agents (28, 29). A list of references to scientific articles and studies on various topics, including: * The antimicrobial properties of essential oils in combination (Stenotrophomonas maltophilia) * Methods for testing the susceptibility of microorganisms to antimicrobials * The use of bioautography as a method for detecting antimicrobial compounds * Studies on the antimicrobial activities of actinomycete strains, Bacillus species, and Streptomyces sp. * Research on the antagonistic activity of plant extracts against dermatophytes and fungi * Investigations into the effects of temperature on the biocontrol potential of Trichoderma sp. on Sclerotium rolfsii These studies were published in various scientific journals between 1946 and 2015, including Molecules, Int. J. Infect. Dis., Mycoses, and more. **Studies on Antimicrobial Compounds** Several researchers have investigated the detection and identification of antimicrobial compounds using various methods. A study by Grzelak et al. (2011) developed a novel direct bioautography-thin-layer chromatography test to detect gram-negative bacteria, Escherichia coli. Another study by Brantner (1997) explored how different parameters affect the evaluation of antibacterial compounds using bioautographic TLC assay. **Plant Extracts and Antimicrobial Activity** The antimicrobial activities of various plant extracts have been studied. Silva et al. (2005) investigated the in vitro activity of Physalis angulata L. fraction and Physalin B, highlighting the importance of assay determination. Shahat et al. (2008) examined the essential oil from Enterolobium contortisiliquum seeds for its antimicrobial properties. **Bioautography on Thin-Layer Chromatograms** Homans and Fuchs (1970) first described direct bioautography on thin-layer chromatograms as a method for detecting fungitoxic substances. Hamburger and Cordell (1987) developed a direct bioautographic TLC assay for compounds possessing antibacterial activity. **Other Studies** Various other studies have investigated the antimicrobial activities of different extracts, including Bacillus spp. isolated from Calotropis procera AIT. Rhizosphere against Candida albicans (Balouiri et al., 2015). Pfaller et al. (2004) discussed the need for standardization in determining fungicidal activities against yeasts and molds. Note: The text has been paraphrased to make it more readable, but the original meaning and references have been preserved. Research has been conducted on various methods for testing the susceptibility of microorganisms to antifungal agents. Several studies have explored the use of high-throughput screening, flow cytometry, and other techniques to quickly identify which compounds can effectively kill or inhibit the growth of fungi and bacteria. One study used resazurin to screen microbial natural extracts against Aspergillus fumigatus, a fungus that can cause serious infections in people with weakened immune systems. Another study compared different methods for testing the susceptibility of Mycobacterium tuberculosis, a bacterium that causes tuberculosis. Other research has focused on the chemical composition of plant-based essential oils and their potential antibacterial and antifungal properties. For example, one study found that the essential oil from citrus plants had antioxidant and antibacterial effects. Additionally, several studies have investigated the use of resazurin broth microdilution assays to detect drug susceptibility in rapidly growing mycobacteria. Another study explored the effect of different inoculum sizes on the minimum inhibitory concentrations (MICs) of antifungal agents against pathogenic filamentous fungi. These studies highlight the importance of developing reliable and efficient methods for testing the efficacy of antifungal agents, particularly against pathogens that can cause serious diseases in humans. The following references discuss testing methods for antifungal susceptibility, specifically for Aspergillus spp. and filamentous fungi that are pathogenic to humans. The EUCAST methodology was used to test the antifungal susceptibility of Aspergillus spp., while inoculum standardization was tested as a method for antifungal susceptibility testing. Two different methods for preparing inocula were compared, with one showing better results. A study on the minimum fungicidal concentrations of amphotericin B for bloodstream Candida species found that the drug was effective against most strains. Other studies discussed the testing conditions for determining minimum fungicidal concentrations and the optimal testing conditions for determining MICs (minimum inhibitory concentrations) and minimum fungicidal concentrations. The CLSI (Clinical and Laboratory Standards Institute) provides guidelines for antimicrobial dilution and disk susceptibility of infrequently isolated or fastidious bacteria, including filamentous fungi. Additionally, studies examined the efficacy of antifungal drugs such as fluconazole and itraconazole in treating oral candidiasis in HIV patients. New methods were developed to assess the susceptibility of Aspergillus isolates to caspofungin. The susceptibility of dermatophytes that cause tinea capitis was also tested, as well as the susceptibility of Fusarium clinical isolates to antifungal drugs. Overall, these references provide information on various testing methods and antifungal susceptibility tests for different types of fungi. The study examines the effects of caspofungin on different Candida species, including Candida albicans, Candida parapsilosis, and Candida glabrata. The researchers used a simultaneous time-kill and postantifungal-effect experiment to analyze the effects of the antifungal drug. Additionally, the study also references several other studies that have used bioluminescence assays to measure antimicrobial activity. These studies include: * A proposal for standardized methods for testing antifungal drugs * The use of ATP bioluminescence as a measure of cell proliferation and cytotoxicity * The application of bioluminescence assays in various fields, such as evaluating the impact of biofilms on porous media hydraulic properties, screening antimicrobial agents against Leishmania, and determining the antibacterial properties of cyclodextrin-antiseptics complexes. * The use of flow cytometry to assess antimicrobial activity and compare it with the NCCLS broth microdilution test. Overall, these studies demonstrate the value of bioluminescence assays in evaluating antimicrobial activity and providing insights into the effects of antifungal drugs on different Candida species. LY303366 showed rapid and strong antifungal activity in the flow cytometric test for yeast viability as reported in Mann et al. (1999).

Agar well diffusion technique. What is agar diffusion method. What is agar well diffusion method. Diffusion method for antimicrobial activity. Agar well diffusion method for antimicrobial activity. Agar well diffusion assay. Agar diffusion method for antimicrobial activity. Agar well diffusion method definition.