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Review Article

INSIGHT INTO ANTIMICROBIC EVALUATION TECHNIQUES AND THEIR ROLE IN BETTER ASSESSMENT OF ANTIMICROBIC AGENTS: A REVIEW

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ABSTRACT

At present, researchers have a keen interest in creating advanced antimicrobic agents to overcome microbial resistance from various new sources either from plants or microbes. For this, robust attention has been given to the advancement in the rapid antimicrobic assessment strategies and methods to develop antimicrobic agents. Some of the antimicrobic techniques such as well diffusion using broth and agar and disc diffusion are more prominent in assaying antimicrobial testing while techniques such as bioluminescence, flow cytofluorometry methods are less used due to specific equipment's, high calibration and evaluation processes. Thus, the information of precise antimicrobial techniques is must to the new researchers for antimicrobic testing. In this review article, various antimicrobic techniques with their advantages and limitations are being reported which are currently being carried out for antimicrobial testing.

Keywords: Antimicrobic, Assessment, Diffusion, Susceptibility, Techniques.

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INTRODUCTION

After the uprising of the golden era, when all the significant antibiotics (macrolides aminoglycosides, cephalosporins, and tetracyclines) were determined and along with it, the chief complications of chemotherapy were secured during the 1960s, the past recaps itself nowadays. All the present compounds are in danger of breakdown of their efficacy on account of the development of microbial insusceptibility [1]. Hence, the exploration of modernized antibiotics is a crucial objective. Natural items are yet one of the vital origins of medication these days. They are advanced from unicellular microbes (prokaryotes), plants, eukaryotes, and various creatures. The output obtained from plant and microbial products is one of the major parts of the antimicrobial compounds that evolved till now [2]. Freshly, innumerable researchers have been concentrating on the examination of pure secondary metabolites, essential oils, plant and microbial extracts, new synthesized molecules as an antimicrobial agent [3-5]. The antimicrobic action is exhibited by the plant extract but the introductory portion ought to befactual and the scientists should look forward to the outcomes. The researchers should not only use them as an accessory measure but also to study the antimicrobic screening and the phytochemical study as well. To detect the antimicrobial action of an unadulterated compound or an extract can be assessed in the research facility by various techniques. The most appropriate techniques are disk diffusion and broth or agar dilution strategies. On the high-level range to examine, the antimicrobial impacts of a flow cytofluorometric of an agent and time-kill test methods are suggested as they give the information on the restraint impact (bactericidal or bacteriostatic) (time- or dosage-based) and injury to cell to test the microbe. Because the in-vitro antimicrobial action of a compound or a concentrate can be surveyed in the research facility by various techniques, current properties of disinfectant substances like the resistance of multidrug-safe microbes, it is exceptionally fundamental to raise a better thought than the accessible techniques for screening and estimate the antimicrobic impact of a concentrate from a purified compound and execute it in social well-being. This review elaborates on the various techniques for the estimation of antimicrobial activity.

ANTIMICROBIAL USING DISPERSION STRATEGIES

Disk-diffusion agar technique

In 1940, the disk-diffusion technique was evolved [6]. It is the most used method in various microbiology labs. It is exercised for the practicing of the antimicrobial susceptibility testing. Presently, several consented guidelines are announced by the Clinical and Laboratory Standards Institute (CLSI) for bacteria and yeast testing [7,8]. All the bacteria cannot be examined with this technique; the uniformity has been made to examine some fastidious bacterial microbes such as *Haemophilus influenzae*, *Streptococci*, *Neisseria meningitides*, *Neisseria gonorrhoeae,* and *Haemophilus parainfluenzae*, utilizing definite culture media, different conditions mandatory for the incubation and interpretive standards for inhibition zones [7].

The Aforementioned, prominent mechanism is followed by the inoculation of agar plates with the methodized inoculums of the analyzed microbe. Next, filter paper discs of about 6 mm in diameter having the appropriate concentration of the tested compound are settled over agar surface. Below optimum conditions, the Petri dishes are incubated. Usually, the antimicrobic agent is scattered in the agar. This inhibits the growth and progression of the examined microorganism. There is the evaluation of the diameter of the zone of inhibition (Fig. 1a). Table 1 demonstrates the various requirements by the standards of the CLSI, particularly that of the temperature, inoculum size, the time of incubation and the growth media.

Antimicrobial susceptibility tests furnish methodological outcomes by classifying microorganisms as resistant, sensitive or moderate [9]. Thus, it is a mechanism to categorize the bacteria on the basis of the phenotypic resistance of the microbial strain being screened; its consequences also direct medico felicitous preference of early experimental analysis and antitoxins exercised for particular sufferer in specific circumstances [10]. Nevertheless, because the bacterial development inhibition doesn't conclude the downfall of the microbes, this procedure fails to demarcate among bactericidal and bacteriostatic impacts.

Table 1: Antimicrobial susceptibility testing methods recommended by CLSI (culture techniques, inoculum size and incubation conditions)

Techniques	Growth medium	Microorganism	Incubation time (h)	Final inoculum size	Incubation temperature (°C)	Ref.
Disk-diffusion	Non- supplemented MHA	Molds	-	(0.4–5) × 10^6 CFU/mL	-	M51-A [18]
	MHA	Bacteria	16–18	(0.5 McFarland) (1–2) × 10^8 CFU/mL	35 ± 2	M02-A [9]
	MHA+GMB[a]	Yeast	20–24	(0.5 McFarland) (1-5) × 10^6 CFU/mL	35 ± 2	M44- A [10]
Broth microdilution	RPMI 1640[b]	Molds	48	(0.4–5) × 10^4 CFU/mL	35	M38-A [70]
	MHB	Bacteria	20	5×10^5 CFU/mL	35 ± 2	M07-A [56]
	RPMI 1640[b]	Yeast	24–48	(0.5–2.5) × 10^3 CFU/mL	35	M27-A [69]
Broth macrodilution	RPMI 1640[b]	Molds	48	(0.4–5) × 10^4 CFU/mL	35	M38-A [70]
	MHB	Bacteria	20	5×10^5 CFU/mL	35 ± 2	M07-A [56]
	RPMI 1640[b]	Yeast	46–50	(0.5–2.5) × 10^3 CFU/mL	35	M27-A [69]
Agar dilution	MHA	Bacteria	16–20	10^4 CFU/spot	35 ± 2	M07-A [56]
Time-kill test	MHB	Bacteria	0, 4, 18 and 24	5×10^5 CFU/mL	35 ± 2	M26-A [75]

MHA: Mueller Hinton Agar. MHB: Mueller Hinton Broth. [a]GMB: the medium was supplemented with 2% glucose and 0.5 mg/mL methylene blue. [b]RPMI 1640: Roswell Park Memorial Institute medium (with glutamine, without bicarbonate, and with phenol red as a pH indicator) was 1640, buffered to pH 7.0 with MOPS (morpholine propane sulfonic acid) at 0.165 M

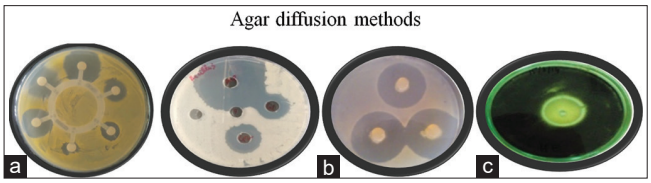

Fig. 1: Agar diffusion methods for anti-microbial testing. (a) Disk-diffusion method of microbial extract using *Bacillus subtilis* as test microorganism. (b) Agar-plug diffusion method of *Bacillus* sp. against *C. albicans*. (c) Agar-well diffusion method of essential oil using *Aspergillus niger* as test microorganism.

Besides, this technique depicts to be highly unsuitable for the examination of the minimum inhibitory concentration (MIC) as it is considered to be a pointless option to assess the yield of the antimicrobic agent diffused into the agar medium. However, in general practically definite MIC can be assessed for certain microorganisms and antimicrobial by the examination of the zones of inhibition with calculations [11].

However, disk-diffusion tests offer generous merits as compared to the other alternative techniques such as clarity, less expensive, test for the demonstration of enormous microbes and antimicrobial agents and the results are obtained at last of the experiment. Furthermore, the recent studies by researchers have been elaborated the fact that the sufferers suffering from the infections caused by bacteria of the pharmaceutical measures mainly emphasize the antibiotic-resistant drugs of the causative specialist [12]. It is certainly because of the phenomenon that there is an excellent correspondence intervened with the microdialysis [10].

Before its uniformity, the method of disk-diffusion turned out to be antiquated to estimate Posaconazole contrary to the filamentous fungi [13], micafungin and caspofungin against *Fusarium* [14] and against *Aspergillus* and *Fusarium*, respectively [15]. Nowadays, for the demonstration of non-dermatophyte filamentous fungi, this method is widely used for the advancement in the antifungal disk-diffusion method [16]. The conditions for incubation, size of the inoculum and the culture media are put forth in Table 1 as mentioned [17].

The merits that are acknowledged prior have been imparted for the access to the antibiogram screening of the essential oils, plants extracts and drugs [18-21].

Antimicrobial gradient method (Etest)

In the contemplation of the MIC concentration, the antimicrobial slope procedure is amalgamated with the standards of the weakening strategies. The definition that exists behind this technique is the likelihood for the creation of the focus inclination of the bactericide on the agar surface. The Etest is the practical release of this framework. The cycle is done as a strip is infused with the rising captivation of the antimicrobic agent across the sides and is put over the agar surface that was once infused with the microorganism examined.

Such methods are highly utilized for the assurance of MIC of fungicides, antimicrobials, and antimycobacterial by specialists [22]. The estimation of MIC can be calculated by the junction of the layer and the growth inhibition circle. Although, Etest strips are worth $2-3 each, it is easy to execute and therefore, it is usually applicable in clinics by the clinicians. Thus, it could be highly expensive to afford this method if we need to test wide range of drugs [9].

For the best interrelationship among the MIC assay, Etest is utilized and the others attained by dilution of broth or agar [23-25]. This method is helpful for the study of antimicrobial interaction between two drugs [26]. To commingle any two anti-infection agents, an Etest strip is saturated with a first antibiotic. And later-on, positioned on a pre-infused surface of agar plate. Following a range of 60 min, it is uncovered and substituted with another microbicide. There is an analysis for the reduction of the MIC of the cooperated two drugs. The minimum dilutions should be two as compared to the highly efficient antibiotic [27]. Moreover, for a similar aim to be accomplished, the Etest strips possibly covered on the medium of agar slanted at 90 between the scales at the MICs for the respective pathogen that is to be examined [28]. Later on, when the incubation is accomplished, the fractional inhibitory concentration index (FICI) can be evaluated with the application of formula as follows:

$$\sum \text{FICI} = \text{FIC(A)} + \text{FIC(B)}$$

Where FIC (A) = MIC (A) in combination/MIC (A) alone and FIC (B) = MIC (B) in combination/MIC (B) alone

Synergy was described by FICI ≤0.5 and antagonism by FICI ≥4. The FICI between 0.5 and 1 was interpreted as addition and between 1 and 4 as indifference [29].

Other diffusion methods

In addition to these methods, there are some more techniques that are used for the screening of extracts, fractions or pure substances in the

microbiological laboratories to demonstrate antimicrobial potency. The highly recommendable and widely used methods are discussed as under:

Well diffusion agar method

This technique is widely used for the demonstration of the antimicrobial activity of various organisms [30,31]. The procedure is quite identical to the disc-diffusion technique; the inoculation of the plate with agar is done with the diffusion of the inoculums of microbes on the entire surface of the agar. Later on, a pierce is punched that has the measurement of roughly 6–8 mm with the sterile cork borer or a tip. Then, an optimum concentration of the antimicrobial agent by the specialist is permitted to fill the well. After this, incubation of the agar plates is allowed to be done. The agar medium with the presence of the antimicrobial agent diffuses and hinders the amplification of the bacterial strain (Fig. 1b).

Plug diffusion method

Agar plug diffusion is generally used to determine for the enmity of the microorganisms [32,33]. The procedure behind this technique is like that of the disk diffusion technique. The process involves the preparation of the agar culture of the strain by the tight streaks on the agar surface. With time, while the growth is examined, the microbial cells derive particles that get scattered in the medium of agar. Then, an agar-plot is made aseptically with a cork borer that is sterilized and collected over the surface of the other agar plate that was inoculated by the microorganism. Then, at that point, the assessment of the antimicrobial property is exhibited by the perception of the zone of inhibition around the agar plug (Fig. 1c).

Cross streak technique

The Cross streak technique is mostly applicable while the screening of antimicrobial activity of the microorganisms is carried out [34]. The microbial strain is to be filled in the center of the agar plate. At the point when the incubation time frame is finished, the plate is treated with the microorganisms examined by the single streak opposite to that of the focal streak. The antimicrobial action is analyzed by the examination of the magnitude of the inhibition zone.

Poisoned food technique

The poisoned food technique is highly applicable for the evaluation of the antifungal activity against molds [35-37]. The extract or the antifungal agent is fused with the agar that is in molten state and thoroughly mixed. Later, the medium is spurted over the petri plates. After a period of a whole night of pre-incubation, inoculums can be cultivated by the mycelia disc which maybe of 2–5 mm. This mycelia disc is placed centrally on the plate. Afterward, when the incubation is allowed to be done, the widths of the parasitic development or the growth of fungus in control and the sample plates are examined. The antifungal impact is determined by utilizing this equation:

$$\text{Antifungal action (\%)} = ((Dc\text{-}Ds)/Dc) \times 100$$

Where Dc is the distance across fungal development in control plate and Ds is the measurement of growth in the plate containing verified antifungal specialist or agent. Sporulation of fungus can likewise measure up to the control.

As a general rule, when the strategy is ineffectively advanced, the specialist should convey a positive control with the natural antimicrobial particle to differentiate between the outcomes and declare the methodology toward the trial.

THIN-LAYER CHROMATOGRAPHY (TLC)–BIOAUTOGRAPHY

To examine different penicillin's, (1946) Goodall and Levi [38] merged the (paper chromatography [PC] method) using the contact chromatography. Thenceforth, Fischer and Lautner [39] presented thin layer chromatography in the similar ground. It also associates thin layer chromatography with both organic and synthetic recognition strategies. Various distributions have been finished on the screening of the plant extracts for the antibacterial and antifungal movement by the utilization of TLC bioautography [40,41]. As under reference, the three following procedures, that is, agar overlay, agar dispersion and direct bioautography examination have been explained for the investigation of the antimicrobic properties of the specific concentrate or the compound.

Diffusing agar

This is also termed as agar contact method. This is the least preferred method to test the antimicrobic activity. It involves diffusion of the agent that possesses antimicrobial characteristics from the chromatogram (PC or TLC) to the plate of agar formerly inoculated with the examined microorganism. Eventually, the chromatogram is taken off and the incubation of the agar plate is done. There is an appearance of zone of the inhibition in which the compounds that are antimicrobic in nature shows proximity to the agar layer [42].

Direct bioautography

The widely administered method out of the three methods is direct autobiography. The process is carried out as the TLC plate is sprinkled by suspension of the microbe and at 25°C; the incubation is accomplished for about 48 h and possesses moist conditions [43]. Tetrazolium salts are often used for visualizing microbial growth. The conversion of these salts takes place to intense colored formazan by the dehydrogenases of the living cells [44,45]. The reagent which is most commendable for the detection is p-Iodonitrotetrazolium violet [42,46]. The bioautogram is splashed with the salts and afterward again incubated for around 24 h at 25°C [47] or 37°C for 3–4 h [5]. For the best adherence to the TLC plate, The Mueller Hinton Broth enhanced with agar has been prescribed to give an adequate liquid and humidity for optimum development of the bacteria [48].

This method could be useful for either fungi or bacteria. It is the simplest way to detect the antifungal substances. It offers constant outcomes for the spore production fungi such as *Cladosporium, Penicillium, and Aspergillus* [49,50]. The most often used bacterial strains are *Escherichia coli*, *Staphylococcus aureus,* and *Bacillus subtilis* [40,51].

Overlaying agar bioassay

It is designated as immersion bioautography. This method deals with the combination of the earlier two discussed techniques as above. The first step involves the concealing of the TLC plate with the liquefied media of agar. The plates are kept at low temperature a few hours earlier the incubation is done so that there is proper dispersion of the analyzed mixtures in the agar medium. Staining should be possible with tetrazolium color after incubation under ideal conditions. Like direct bioautography, this technique is effective for most microbes like *Candida albicans* [52] and molds [41]. This technique is not prone to infection and comes up with well-marked growth inhibition zones [42].

It is correct to specify that TLC-bioautography is an easy, valid, successful and low-cost procedure recommended for the segregation of the complex mixture and it limits the elements on the TLC plate. Along these lines, it tends to be done with less equipment as well as well-equipped laboratories [42]. Although this technique obtains a successful grade of results for the application as a strategy for decision for a last organizing up of extractive portions to get the complete mixtures, the TLC method suggests a moment screening of an enormous various measure of the examples for bioactivity-directed fractionation [43]. It is an effective strategy for the discovery of antimicrobials in the methodology for the discovery of new antimicrobial drugs, environmental and food samples.

DILUTION METHODS

The highly effective way to obtain the MIC values and the dilution methods are applicable as they provide the probability for the approximate concentration of the microbial agent in the agar (agar

dilution) or broth dilution (microdilution or macrodilution). Any of the two methods can be applied for assessing and evaluating the controlled *in vitro* antimicrobial activity in the case of fungi and bacteria. MIC is described as the minimum concentration of the chemical agent that prevents the advancement of the particular microbe to the extent that there is no visible growth of the microbe and is measured in mg/L or μg/mL. Mostly, dilution method recommendations are being proposed for the tests that are practiced for the examination of the antimicrobic properties of the filamentous fungi, yeast, a fastidious or non-fastidious bacterium. The supreme criterion is established by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and CLSI. According to the proposed protocol, they provide a consistent process for testing in many laboratories. However, it does not concede in performing the biological assay in a proper perspective to demonstrate the clinical results [53].

Diluting broth method

The highly efficient method that is recommended for antimicrobial susceptibility techniques is the diluting broth technique. The mechanism comprises the preparation of two-fold dilutions of the antimicrobic agents (for example 1, 2, 4, 8, 16 and 32 mg/mL) in a medium which is allocated in the chambers with some measure of 2 mL (microdilution) or with more modest focuses utilizing 96-well microtitration plate (microdilution) (Fig. 2). Afterward, inoculation of every well or tube is arranged in the comparative medium after dilution of microbial suspension acclimated to 0.5 McFarland scale (Fig. 3). At the point when well-blending is done, the inoculated cylinders or the 96-well microtitration plate are incubated (usually without stirring) under the ideal conditions (Table 1). The test approaches to perform precisely the microdilution is schematized in Fig. 4.

Fig. 2: Broth micro-dilution method of plant extract against *B.subtilis* using resazurin dye as growth pointer

MIC is the minimal value of concentration that implies the hindrance to the development of the microbe in the tubes or the microdilution wells [54]. On the contrary to the microdilution method, the demerit of the microdilution is that it is tedious, high probability of making mistakes in the framing of the antimicrobial aspects for each trial and requires an enormous number of space and elements [9]. The basic merit of the microdilution method involves the infinitesimal period for the test and there is the safe saving of money and replicability. However, the conclusion that is obtained (intralaboratory and interlaboratory) by the end must be cautiously administered [54]. The use of the viewing devices can be helpful for the reading of the microdilution tests and the results with appropriate results to foresee the growth in the wells and therefore we can determine the MIC endpoint. Furthermore, huge progress has been made in the application of dye reagents for the various calorimetric methods. To demonstrate the antibacterial and antifungal microdilution assays, and 2,3-bis {2-methoxy-4-nitro-5-[(sulfenylamino) carbonyl]-2H-tetrazolium-hydroxide} (XTT) and Tetrazolium salts, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) are employed in the MIC endpoint [55-58]. The indicator that is applicable for the growth for this objective is resazurin-dye (The Alamar blue dye) [59-62].

One of the important aspects to consider is to know the size of the inoculum [63-65], the technique for the preparation of the inoculum and the incubation time [66] and the inoculum preparation method can affect the values of the MIC [67,68]. Hence, the method that is successfully approved by CLSI is the broth dilution and it can be used for the aerobic growth [54], filamentous fungi [68] and yeast [67]. There are similar characteristics between the EUCAST broth dilution method and the CLSI in various aspects; that is the size of inoculum, preparation of the inoculum and the MIC technique by spectrophotometry and the CLSI assay that are proposed by the guidelines of the EUCAST [69].

In respect to the spore-forming fungi and the fungi that form conidia, the microdilution requires the spores that are inoculated altered by spectrophotometrically to 0.4×10^4 –5×10^4 CFU/mL. Nevertheless, by

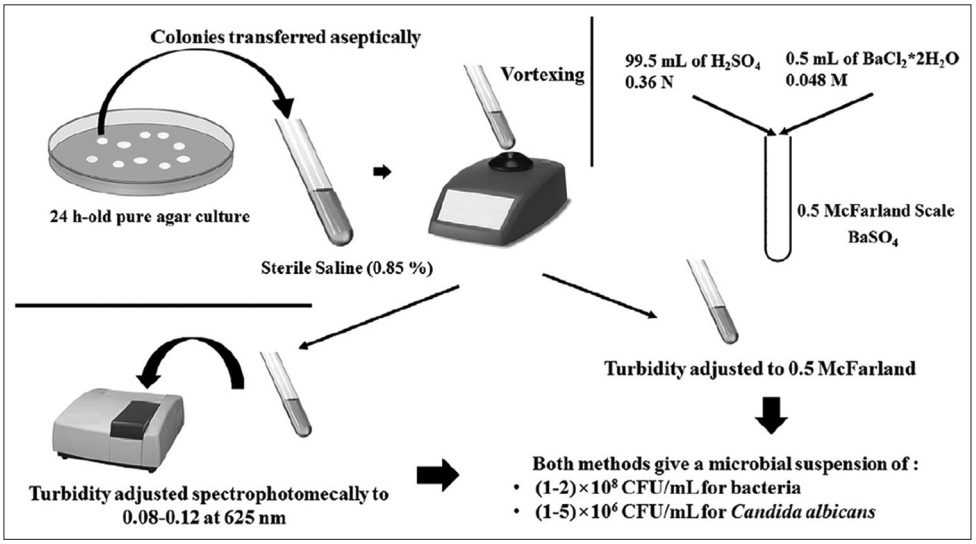

Fig. 3: 0.5McFarland microbial inoculums preparation by the direct colony suspension as recommended by CLSI guidelines. [Adopted from Balouiri *et al*., 2016]

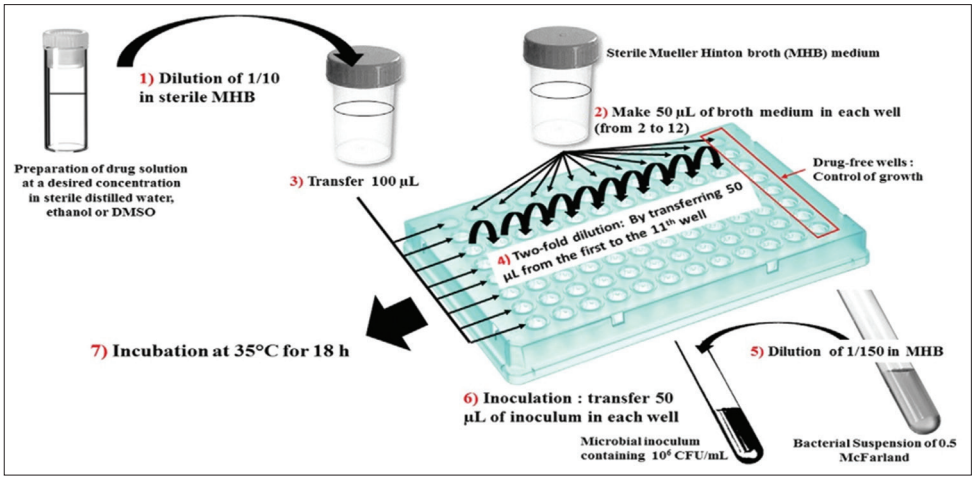

Fig. 4: Broth microdilution for antibacterial testing as recommended by CLSI protocol. (Adopted from Balouiri *et al*., 2016)

acknowledgement through the undertaking of EUCAST, the inoculum can be reclaimed by hemocytometer counting (2–5) × 10^5 CFU/mL [70]. Various studies have been revealed out the significance of the preparation of the inoculum by hemocytometer count and allow us to obtain the remarkable results in the size of the conidia and color [66,71,72].

The minimum bacterial concentration (MBC) or the minimum fungicidal concentration (MFC) is also designated as the minimum lethal concentration that is applicable for the reviewing fungicidal or bactericidal activity. Under the complete set of data in document M26-A, the minimum concentration of the antimicrobial agent that is required to kill 99.9% of the final inoculum after the incubation of about 24 h under standard conditions is termed as MBC [73]. Hereby, we can evaluate MBC after macrodilution or broth dilution by performing the subculturing of the sample from the wells or tubes, and it obtains a negative microdilution growth on the surface of non-selective agar plates. This helps us to evaluate the surviving cells (CFU/mL) after 24 h of incubation. We can define MFC as the minimum concentration of the chemical agent that gives 98–99.9% cidal effect as compared to the initial inoculum [69]. Many researchers are working on the evaluation of a different test for parameters of MFC of numerous drugs against *Candida* isolates [74], *Aspergillus* [75], and other molds [76].

Agar dilution technique

The technique of the agar dilution requires different concentration for the inclusion with antimicrobial agent within the medium of agar. Most commonly, it deals with the two-fold dilutions and the next step involves the inoculation by the particular inoculum on the agar plate. The MIC is described as the concentration at the minutest form of antimicrobial agent's which fully hinders the development under optimum situations (Table 1).

This method is applicable for the antimicrobial susceptibility of antifungal and antibacterial testing. At present, inoculant replicators are widely accessible and may displace around 32–60 inocula of bacteria in the direction of every plate of agar. This is a highly accessible method for fastidious bacteria [76] like *Helicobacter* species and anaerobes. This technique is practiced as a drug counter to *Aspergillus* [77], *Candida sp*., dermatophytes and *Fusarium* and as an antifungal-agent [78-81].

This procedure put forth an excellent association besides the antimicrobial testing by Etest against both Gram-negative and Gram-positive bacteria. Further, this method elaborates a clear contrast of broth microdilution, disk-diffusion and agar dilution practices that yields best outcomes [23].

T-KILL TEST (TIME-KILL CURVE)

One of the best techniques that are applicable to examine the antibacterial result is the Time-kill test. This technique is considered to be highly effective for the gathering up of the data between the interface of the microbial strain and the antimicrobial agent. This test elaborates tedious or effects that are antimicrobic and concentration-dependent also [53].

According to the information in the M26-A document of CLSI, this test proves to be recommendable for the bacterium [73]. The procedure is carried out by the preparation of the medium made from the broth in the three tubes with the suspension of bacteria of about 5 × 10^5 CFU/mL. With the range as 0.25 × MIC and 1 × MIC in the first and second test tubes respectively, the third one is referred as the growth control. Under particular conditions, the incubation is followed for diverse time gaps (0, 4, 8, 12 and 24 h) [19,53]. The next step involves calculating the percentage of the dead cells with the growth control by the estimation of the number of living cells (CFU/mL) of every glassware by the exercising the agar plate count technique. Usually, the antibacterial result is acquired by the lethality rate of 90% for 6 h that proves to be alike with 99.9% lethalness in 24 h [19]. It is also applicable for the examination of the synergism or antagonism between any chemical agents, maybe two or more [26,53]. Likewise, various substances that had antifungal substances were examined by the application of this method [82,83].

BIOLUMINESCENCE-ATP ASSAY

The bioluminescence- Adenosine triphosphate (ATP) assay is mainly focused over the ability to evaluate the ATP generated for the bacteria and fungi. The chemical form to produce energy in all the respective living cells is the ATP that is available almost in every cell. Hence, the analysis of its consumption helps us to determine the population of the microbe in a sample. With the presence of ATP, it possibly converts D-luciferin by enzyme luciferase to oxyluciferin which further access to develop light. A luminometer can be applicable for the examination of the light that is illuminated by it. It is measured in terms of relative light unit (RLU) which can be converted to RLU/mole of ATP. Here exists a direct relationship between measured luminescence and cell viability.

The assay of bioluminescence has a huge array for the operations like the trial for the cytotoxicity [84], unsaturation for the estimation of the drug screening on *Leishmania* [86] and effect of microbes [85]. Furthermore, this method is practiced by numerous researchers worldwide for the testing of antibacterial activity [87], antimycobacterial testing [88,89], antifungal against yeast [90] and molds [91]. According to the recent study about this technique, it might be concluded that this method is the major initiative that is highly applicable in antimicrobial testing *in vivo* or *in situ* [92].

FLOW CYTOFLUOROMETRIC METHOD

It's been long ago when the flow-cytometry was proposed for the testing of antimicrobial susceptibility of various pathogens. Hence, researchers

even studied about the antifungal and antibacterial activities of the chemical agents by the practice of this method [96]. By the application of this technique, we can have an instant finding of the cells that are damaged by the utility of the appropriate dyes that are recommendable for the staining [96,97]. So, the most often used DNA stains are an intercalating agent, propidium iodide (PI), and fluorescent. Specific research regarding the efficiency of flow-cytometer as a mechanism followed to investigate the antimicrobic susceptibility of oils that are essential against *Listeria monocytogenes* along with the staining of PI for the carboxyfluorescein diacetate and damaging of the membrane for esterase activity detection [93]. Hence, in addition to this, we can distinguish between the three subpopulations (viable, injured cells and dead). Such cells that are injured are designated as the stressed cells which indicate the damaging of the cellular components and therefore, lead to the growth [96]. The calculation of the damaged cells shows a broad effect over the food microbiology as statistics might be analytical if the recovery of the cell becomes possible like in temperature, abuse condition during the food storage [93]. Flow cytofluorometric method accesses us to detect the antimicrobial protection and evaluates the molecular influence that was confirmed about the damaged cell and feasibility of the confirmed microscopic organism [95]. This also provides the best results in few periods of 2–6 h compared to 24–72 h for the microdilution technique [94]. But the wide application of the procedure to test the antimicrobic activity in the present era is very rare because of the access of the flow cytometric apparatus in different laboratories.

CONCLUSION

At present, microbial infections play a crucial role in the clinical threat with notable diseases and the death rate because there is an advancement of the resistance against microbes to the familiar agents that exhibit the antimicrobic properties. Hence, various techniques are certainly useful for antimicrobial susceptibility by the use of the respective antimicrobial agents. Some of the methods were recommended by the CLSI and EUCAST which had laid a major foundation in this field. One needs to be cautious enough to avoid any sort of alteration in the basics of the biological sciences with the dilution of the culture media and the highly concentrated media. Eventually, we may consider the fact of the microbes that by exercising the solvents may mark the growth of the bacteria that are tested, we may conclude that there are inconsequential procedural variations to standardize the procedures to ensure the accuracy in the experiments and gives the access to the researchers for the comparability of the respective outcomes.

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AUTHORS CONTRIBUTION

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CONFLICTS OF INTERESTS

Authors have no conflicts of interest.

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