Global Scientific JOURNALS

GSJ: Volume 8, Issue 12, December 2020, Online: ISSN 2320-9186
www.globalscientificjournal.com

A REVIEW OF CURRENT *IN-VITRO* ANTIMICROBIAL SUSCEPTIBILITY TESTING METHODS AND FUTURE DIRECTIONS

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KeyWords
Agar diffusion assay, antimicrobial agents, antimicrobial gradient method, antimicrobial susceptibility testing, bioautography, broth dilution, infectious diseases, microbes, minimum inhibitory concentration, time-kill test.

ABSTRACT

The increasing emergence of antimicrobial resistance and the failure of many currently available antimicrobials in combatting disease-causing pathogens have led to a surge in research by scientists to identify new and/or modify available compounds in order to have an upper hand in the battle against disease-causing pathogens. Various efforts have been geared toward obtaining bioactive compounds from natural sources but to test for their microbial susceptibility, antimicrobial susceptibility testing methods are relevant. Currently available conventional methods of susceptibility testing provide accurate and reliable results despite being slow, labor-intensive, and time-consuming. Therefore, there is an increasing trend toward the integration of automated systems in susceptibility testing, which provides quicker results and is easy to use. This study reviews the current knowledge of the available methods of *in-vitro* susceptibility testing, highlighting the advantages and limitations, the challenges with the use of automated systems, and areas to be focused on in future progress.

1.0 INTRODUCTION

The twentieth century saw the advent of antimicrobial agents for combatting infectious diseases as one of the most relevant accomplishments. Notwithstanding, microbes with acquired resistance appeared shortly after the introduction of these antimicrobial agents [1]. Resistance to nearly all antimicrobial agents has been observed. This occurrence is not new neither is it unexpected, but the rate at which resistance is emerging is a problem. This has become a problem at the global level that has been acknowledged by the World Health Organization (WHO), Food and Agriculture Organization (FAO), and the World Organization for Animal

Health (OIE) [2]. The ultimate effect has always been treatment failure. Disease-causing microbes acquire resistance by (1) modifying the target to which the antimicrobial agent binds (2) upregulating efflux pumps that extrude the agent from the cell (3) producing enzymes that modify or inactivates the antimicrobial agent and (4) altering an outer membrane protein channel necessary for antimicrobial entry into the cell [3].

For this reason, scientists have been forced to identify new compounds of therapeutic worth that can be used as novel antimicrobial agents with a novel mechanism of action in combatting infectious diseases as well as curb the resistance problem associated with currently accessible antimicrobial agents [4]–[6]. In the search, several recent studies have focused on the analysis of the potential antimicrobial agents of plants and microbial extracts, essential oils, pure secondary metabolites, and newly synthesized molecules [7].

Several methods have been developed to determine the antimicrobial activity of various extracts or pure compounds. The choice of selection is dependent on factors which include practicality, flexibility, cost, accuracy, and individual preference [2]. While current standard methods, accepted by various bodies such as the Clinical and Standards Laboratory Institute (CLSI), the British Society for Antimicrobial Chemotherapy (BSAC), and the European Committee for Antimicrobial Susceptibility Testing (EUCAST), exist for guidelines for conventional drug antimicrobial susceptibility testing, they may not be directly applicable and may need modification. This article reviews the current knowledge on the various methods available and commonly used for *in-vitro* antimicrobial susceptibility testing, the advantages and limitations as well as future trends.

2.0 Dilution methods

Dilution methods are used to quantitatively evaluate antimicrobial susceptibility by determining the minimum inhibitory concentration (MIC) values. The MIC is defined as the lowest concentration of the antimicrobial agent that will inhibit the growth of the test microorganism [8]. Dilution methods are used as reference methods which other antimicrobial susceptibility testing methods are calibrated [9][9], [10]. The use of MIC methods allows for the comparative study of new agents. In dilution tests, the microorganisms are tested for their ability to grow on a variety of agar plates (agar dilution) or in broth (broth macrodilution and broth microdilution) containing dilutions of the antimicrobial agent. The minimum concentration of the antimicrobial agent that after 16 to 24 hours of incubation, inhibits the growth of the test microorganism is recorded as the MIC, usually in mg/L or μg/L [11][7].

2.1 Agar dilution

In agar dilution, varying desired concentrations of the antimicrobial agent (usually twofold serial dilutions) are prepared and integrated into a molten form of agar before it solidifies. The test microbe is then inoculated from a standard suspension onto the agar plate surface and then the plate is incubated for 20 to 24 hours under suitable conditions. After incubation, the lowest concentration of the antimicrobial agent that completely inhibited the growth of the microbes is recorded as the MIC [2], [7]. This method as described by the Clinical and Laboratory Standards Institute (CLSI), can be easily reproduced [12]. Moreover, there is the potential to enhance the identification of the MIC endpoints and also to broaden the concentration range of the antimicrobial agent with this method [2]. The antimicrobial activity of hydrophobic extracts and essential oils can be determined with agar dilution because it has been demonstrated [13][14] that the problem of emulsion stability associated with essential oils in liquid media is solved when agar is added as a stabilizer. Another advantage of the agar dilution method is that since the method depends on the ability to form visible growth on the agar medium, even with the unaided eye it could be easily detected. Therefore, for strong coloring compounds, the determination of bacterial growth on the agar surface is much simpler and clearer [15]. However, it has been reported that agar dilution is not commonly used because large amounts of the test compound (antimicrobial agent) are required. Besides, this method is labor-intensive and time-consuming, and not suited for routine laboratory use [16]–[19].

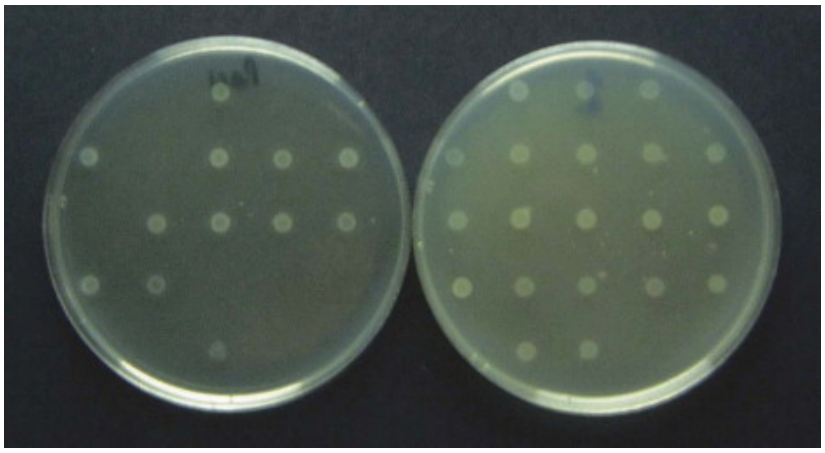

Figure 1: Agar dilution assay plates showing different test sample concentrations.

2.2 Broth dilution

Broth dilution is a susceptibility technique in which containers containing equivalent volumes of broth with an antimicrobial solution are inoculated with a standardized number of microbes at geometrically increasing concentrations (usually a twofold dilution series). The test can be carried out in either tube with a minimum volume of 2mL (macrodilution) or smaller volumes using microtitre plates (microdilution) [11], [20], [21].

In broth macrodilution, serial dilutions of the antimicrobial agent are dispensed into tubes containing a volume of at least 2mL of nutrient broth and a standard microbial suspension of microbes that have been adjusted to 0.5 McFarland scale. The tubes are incubated under suitable conditions, after which the concentration of the antimicrobial agent that completely inhibited microbial growth in the tubes is recorded as the MIC [11], [16].

The broth microdilution uses a 96-well microtitre plate. In this procedure, serial dilutions of the test samples are dispensed into the wells of the microtitre plate followed by the addition of standard suspension and nutrient broth across the rows of the microtitre plate. The plate is incubated under suitable conditions after which the lowest concentration that completely inhibited microbial growth is recorded as the MIC. Viewing devices can promote the reading of microdilution tests and record results with high capacity to detect growth in the wells to determine the MIC endpoint. Also, some colorimetric methods have been developed based on the use of dye reagents. The common ones include Alamar blue dye (resazurin) and tetrazolium salts like 3-(4,5-dimethylthiazoyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 2,3-bis{2-methoxy-4-nitro-5-[(sulfenylamino) carbnonyl]-2H-tetrazolium-bromide} (XTT) [7], [22].

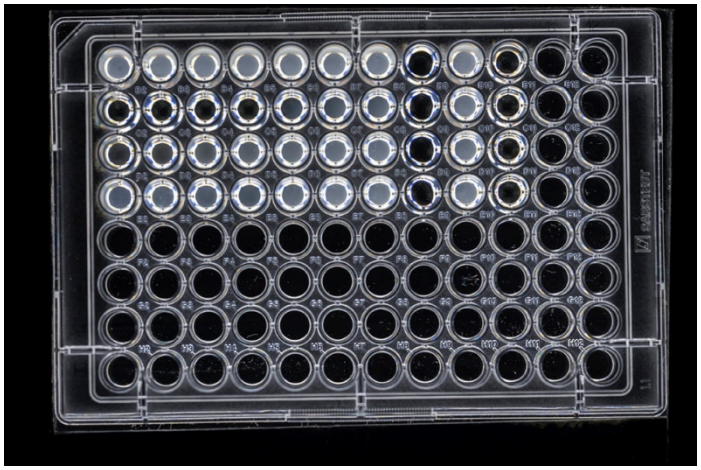

Figure 2: A 96-well microtitre plate used for broth microdilution.

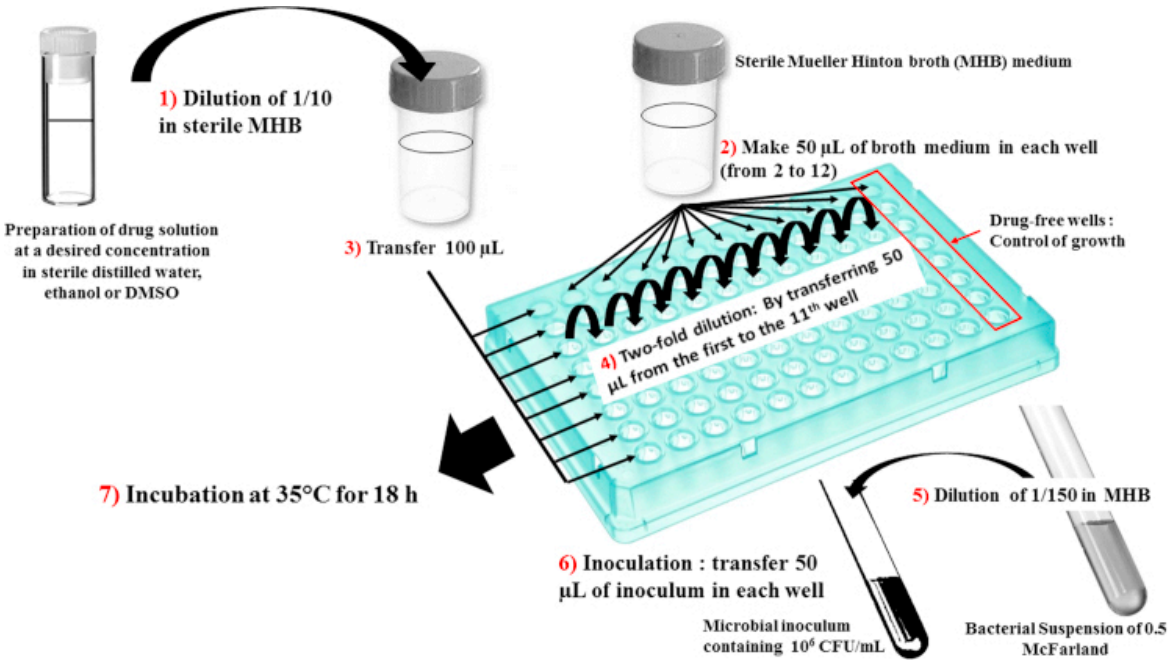

Figure 3: Broth microdilution for antibacterial testing as recommended by CLSI protocol.

It is important to include control tests for every batch of MIC determinations to verify that the susceptibility outcomes are correct. The test is accurate and valid only if the positive control indicates growth and the negative control indicates no growth [16], [21]. The major disadvantages of the macrodilution method are the repetitive manual undertaking, the possibility of errors in preparing the antimicrobial solution for each test, and the relatively large quantity of reagents and space needed [23]. The method is also time-consuming and it is laborious [24]. Microdilution on the other hand is less laborious and inexpensive as smaller quantities of the medium, reagents, and test compounds are required. This method has also been reported to be unsuitable for determining the MIC of volatile, highly non-polar compounds and extracts [15], [25], [26]. An automated and simpler approach has been used in many studies where an automated panel reader was used to generate computerized reports [23], [27].

3.0 Diffusion methods

3.1 Agar disk diffusion

Agar disk diffusion assay, introduced in 1956 [28], is the official method utilized in numerous clinical microbiology laboratories for antimicrobial susceptibility testing. In this popular procedure, test microorganisms from a standard inoculum suspension that has been adjusted to 0.5 McFarland scale are inoculated onto an agar plate. Then, filter paper disks (about 6mm in diameter), impregnated with the desired concentrations of the test compound (antimicrobial agent) are put on the surface of the agar. The plates are then incubated under suitable conditions for 18 to 24 hours after which the activity is observed as zones of inhibition [7], [10], [21]. This method is based on the principle that a disk impregnated with test compound (antimicrobial agent) and put on agar surface pick up moisture and diffuse radially outward through the medium producing a concentration gradient. Therefore, the concentration of the test compound (antimicrobial agent) at the edge of the disk is high and eventually decreases as the distance from the disk increases to a point where it has no inhibitory effect on the microbe. If the test compound can inhibit the growth of the microbe, a

clear zone or ring is observed around the disk [29]. The zone of inhibition is measured (usually in mm) to categorize isolate as susceptible, intermediately susceptible, or resistant by comparing with a standard interpretation chart [21], [28], [30].

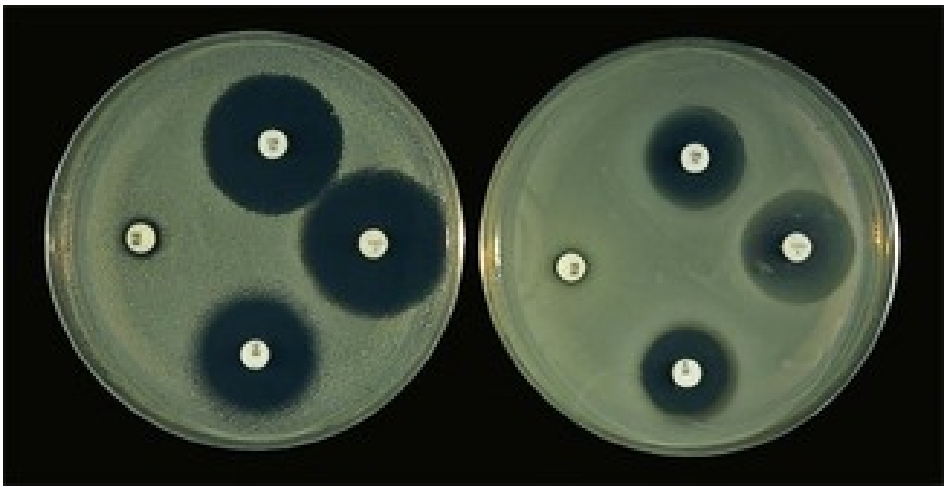

Figure 4: Agar disk diffusion assay showing zones of inhibition.

The agar disk diffusion assay, although reported to be labor-intensive and time-consuming is commonly used because it is simple and not expensive. With the use of this method, large numbers of isolates can be screened against a test compound (antimicrobial agent) and results are also easily interpreted. However, this method cannot be used to determine MIC, as it is a qualitative test. Therefore, it only categorizes the isolate as susceptible, intermediate, or resistant [31]–[34]. For certain microorganisms and antibiotics, an estimated MIC can be determined by comparing the inhibition zones with stored algorithms [7]. The disk test is not easily automated or mechanized [23]. It has been reported that when this method is used for mixtures (such as plant extracts) other than pure compounds, unreliable results may be obtained because they diffuse slowly in agar media. Even some pure compounds such as vancomycin, colistin, and macrolides diffuse very slowly in agar media due to the specific physicochemical properties of the molecules and as such, the zone of inhibition may not be a true reflection of the activity. Also, due to the volatile components of essential oils, it makes it difficult in determining their antimicrobial activity by the agar disk diffusion method [10], [15], [35], [36].

3.2 Antimicrobial gradient method (Etest)

This diffusion method uses the concept of establishing an antimicrobial gradient in the agar medium as a means to assess susceptibility. The Etest uses a commercially produced plastic test strip infused with a specific antibiotic concentration that steadily decreases. A numerical scale that corresponds to the antibiotic concentration present therein is also shown in the strip. In the procedure, a strip impregnated from one end to the other with an increasing concentration of the test compound is placed on the surface of an agar plate that has been inoculated with the test microorganism. The plates are then incubated after which the MIC is read from the strips. The intersection of the lower part of the ellipse-shaped growth inhibition region with the test strip defines the MIC [7], [21], [23].

Figure 5: A *Staph. aureus* isolate tested by the Etest gradient diffusion method with vancomycin (VA), daptomycin (DM), and linezolid (LZ) on Mueller-Hinton agar.

Due to its simplicity and reproducibility, this test is very attractive. The strips have a long shelf life and simple storage. Etest has a finer gradation and a wider range of MIC values, which are easy to read [37]–[39]. Etest has been used to determine the susceptibility of fungi and bacteria in many studies [40]–[43]. This approach, however, becomes expensive if more than a few compounds are tested because each compound requires a separate test strip [7], [23]. Generally, it has been established that there is a good correlation between the MIC values obtained from the Etest method and that of agar and broth dilution methods [38], [44], [45].

3.3 Agar well diffusion method

This method is generally used to determine the antimicrobial activity of microbial and plant extracts. In this assay, a volume of inoculum suspension is spread over the whole surface of an agar plate and holes are created aseptically with a borer or sharp tool. Volumes of the test compound of desired concentrations are dispensed into the holes after which the plate is incubated under appropriate conditions. Similar to the agar disk diffusion, the test samples diffuse into the agar medium to inhibit the growth of the test microorganism and the activity is observed as zones of inhibition [46]–[48]. This assay consumes time and it is labor-intensive like the agar disk diffusion, nevertheless, it is mostly used because of its simplicity and its suitability for routine laboratory use [49].

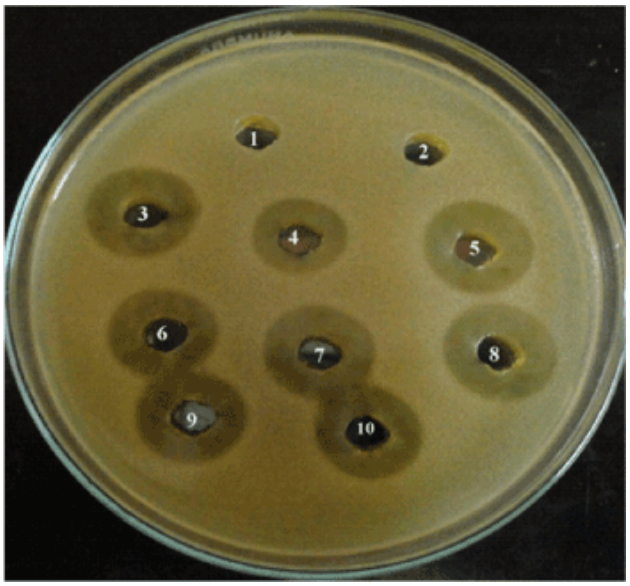

Figure 6: Agar well diffusion assay showing zones of inhibition.

4.0 Time Kill method

This assay is used to study the effectiveness of an antimicrobial agent to a specific microbial strain. It is the most effective method for bactericidal or fungicidal effect determination. The time-kill test shows either a time-dependent effect or a concentration-dependent effect. In this assay, a standard inoculum suspension of the microbial strain is cultured in the presence of the antimicrobial agent (usually in tubes) in varying concentrations. A growth control test is also conducted where the microbial strain is cultured in the absence of the antimicrobial agent. The tubes are then incubated over a 24-hour time course, during which the number of viable cells is counted at varying time intervals (e.g. 0, 3, 6, 12, and 24). This is done by performing serial dilutions on an aliquot removed at various time intervals. The results are then used to make a plot, known as the *time-kill curve* [7], [50], [51]. This time-kill curve can be used to study drug interactions, synergism, or antagonism in combinations between drugs [52]–[55]. This test offers data on both inhibition and killing but it requires a lot of effort and time [56]–[58].

5.0 Bioautographic methods

Bioautographic methods are classified as qualitative techniques as they only indicate the presence or absence of substances with antimicrobial activity [59]. In bioautographic methods, the technique is similar to that used in the agar dilution methods. The difference is that the compounds being examined diffuse from a chromatographic layer to the inoculated agar medium [60]. In 1946, Goodal and Levi first used paper chromatography – bioautography to estimate the purity of penicillin. This became known as contact bioautography. Fisher and Lautner introduced thin-layer chromatography – bioautography in 1961. In 1973, Betina wrote the first review paper covering both paper and thin-layer chromatography – bioautography. The key advantage in the use of bioautographic methods is that it offers details on the antimicrobial activity of substances isolated from a mixture. Thus, it is very suitable for evaluating extracts [61], [62]. Several works have been done and reported in the literature on the use of bioautography to detect antimicrobial compounds effective against plant and human pathogenic bacteria and fungi [63]–[67]. There are three bioautographic methods namely, contact bioautography, direct bioautography, and immersion bioautography.

Generally, bioautographic methods are simple, effective, and economical. As such, they can be carried out in advanced laboratories as well as in small laboratories that have access to a minimum of equipment [7].

However, the techniques work best with water-soluble compounds that quickly diffuse through water-based microbial media [68]. These techniques are also less reproducible when compared to dilution methods [69].

5.1 Contact bioautography

In contact bioautography (also known as agar diffusion), there is a transfer by diffusion of the antimicrobial agent from a TLC plate or paper into an agar plate that has been inoculated with the test microorganism. The chromatogram is put face down on the inoculated agar layer and left for several minutes or hours to allow diffusion. Then the chromatogram is removed and the agar plate is incubated for 16-24 hours under suitable conditions after which the zones of inhibition are observed in the positions where the antimicrobial spots are stuck to the agar [70], [71]. The drawbacks of contact bioautography have been the difficulties in obtaining full contact between the agar and the plate and the adherence of adsorbent to the agar surface. By adding silicon acid-glass fibre sheets, chromAR, for chromatography, these deficiencies were prevented. Nevertheless, the basis of the technique was the same and it was necessary to transfer antimicrobials from the sheet to agar, resulting in their loss and dilution [62].

5.2 Direct bioautography

In direct bioautography, a developed plate is dipped in a suspension of test microorganisms growing in a suitable broth, or the test microorganism is sprayed onto the plate. The plate is incubated in a humid atmosphere and the microorganisms grow on it directly. Therefore, the separation, preconditioning, incubation, and visualizations are carried out directly on the plate. Visualization is normally done by spraying the plate with tetrazolium salt such as MTT (3-(4,5-dimethylthiazoyl-2-yl)-2,5-diphenyltetrazolium bromide). Tetrazolium salt is converted into purple formazan by the dehydrogenases of living microorganisms. Creamy spots, known as the zones of inhibition, which appear against the purple background, point to the presence of antimicrobial agents [72]–[74]. This method is the most used bioautography method [75].

5.3 Immersion bioautography

This method, also known as agar overlay, is a combination of direct bioautography and contact bioautography. In the procedure, the chromatogram is covered with a molten seeded agar medium. Before incubation, the plate may be placed at a low temperature for a few hours to allow the tested compounds to diffuse well into the agar medium. After incubation under appropriate conditions, visualization can be carried out by staining with tetrazolium salt [76]–[80].

6.0 Future Directions

It is now an accepted fact that there is an increased resistance against the prevailing antimicrobials and our armory against disease-causing pathogens is running out at a very quick pace. Data gathered from research and documented in literature points to the prediction that new diseases and infections will and continue to emerge. Also, current treatment methods against existing infections will no longer be effective due to antimicrobial resistance. The ultimate effect of this phenomenon is treatment failure leading to increased mortality. To withstand this challenge, there is the need to develop new antimicrobials, which itself is a great challenge. According to the report of the workshop organized by the Committee on New Directions in the Study of Antimicrobial Therapeutics, held in Washington DC (2005), it was made clear that the phenomenon of antimicrobial resistance is inevitable and that it is worthwhile to identify research that would help surmount the problem of resistance or at least slow its emergence [81].

For drug discovery and resistance surveillance, antimicrobial susceptibility methods are required. Even if new classes of drugs are identified or modifications are made to currently available drugs, there is still the need to test for their susceptibility. The use of currently available conventional antimicrobial susceptibility testing

methods provides accurate and reliable results. However, there are drawbacks to their use [82]. They are labor-intensive, time-consuming, and takes a long time to obtain results. In a typical clinical setting, performing tests before treatments is a problem as results take time. Also, results obtained from using these techniques are not always comparable. Therefore, the development of susceptibility methods that are rapid and easy to use is of high priority as we need to succeed in fighting antimicrobial resistance.

Technological advances have led to the development and integration of automated systems into antimicrobial susceptibility testing and it is not a misstatement of fact that susceptibility testing has been improved by being rapid and less laborious. Results are quickly obtained and they are accurate as well. For some tests, results can be obtained within hours. It is economical to some extent due to lower reagent costs and relatively reduced labor requirements. Some automated systems include the Vitek System (bioMerieux), Phoenix, Sensititre, Micro-scan Walk Away, and Micronaut [83]. These approaches are designed to reduce technical mistakes and long preparation times. However, a major drawback for laboratories is the cost of the initial purchase, operation, and maintenance of machinery, thereby making their usage for analysis limited to advanced laboratories. Moreover, when compared with the current conventional approaches, automated systems lack reproducibility, sensitivity, and reliability [84]. Because these systems provide quicker results, improving upon them or developing new systems that will save money by lower reagent costs and reduced labor requirements will be a milestone that will contribute significantly to the fight against antimicrobial resistance. The focus should be drawn to improving the reproducibility and reliability of the systems.

Cassell and Mekalanos (2001) pointed out that, there will be three variables influencing the future impact of infectious diseases. The first is the relationship between rising microbial resistance and success in the production of vaccines and antibiotics. The second is the course of developing and transitional economies, especially the basic quality of life of the poorest groups in these countries. The third is the degree of effectiveness of global and national initiatives to develop successful surveillance and response systems [85]. It is without dispute that susceptibility testing is required to succeed in the first and third variables, hence an improvement in susceptibility testing methods will support the course to surmount the problem of resistance.

CONCLUSION

Current treatment methods against existing infections will no longer be effective in the near future due to antimicrobial resistance. The ultimate effect of this phenomenon is treatment failure leading to increased mortality. For drug discovery and resistance surveillance, antimicrobial susceptibility methods are required. Conventional testing methods have proved to provide accurate and reliable results, however, they are labor-intensive, time-consuming, and takes a long time to obtain results. The development and integration of automated systems have greatly improved antimicrobial susceptibility testing because they are rapid and easy to use, but, they are expensive. As we need to succeed in fighting antimicrobial resistance, new automated methods that will save money, ensure accuracy, and be easily accessible to small and advanced laboratories. An improvement in susceptibility testing methods will support the course to surmount the problem of antimicrobial resistance.

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