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EVALUATING THE ACTIVITY OF NOVEL ANTIMICROBIAL COMPOUNDS : A REVIEW OF METHODS AND TECHNIQUES

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ABSTRACT

It is well evident that antimicrobial resistance is increasing progressively and resulting in mortality and morbidity due to failures in treatment and continuous increase in health care costs. Although if appropriate antimicrobial drugs are used for treatment, then benefits gained will be unquestionable. Therefore a great attention is now paid towards screening and evaluating methods for antimicrobial activity. Several techniques like Disk Diffusion, Gradient Well Diffusion are discussed that provide cost savings as well as flexibility. The most commonly used methods include Broth Dilution, Agar Dilution methods etc. This review paper throws light on various techniques like Agar Disk Diffusion Method, E-Test Method, Cross Streak Method, Broth Dilution Method, Agar Dilution Method etc. and a detailed idea of advantages and limitations of these methods are discussed.

Key words: Antimicrobial activity, Agar Dilution Methods, Broth Dilution Methods.

INTRODUCTION

ANTIMICROBIAL ACTIVITIES:

There is a history of coexistence of pathogens with animals and human beings [1-5]. In early times of around hundred years back, there were not the effective means and methods with human beings to combat these pathogenic microorganisms. In early time medicines were primarily focused on relieving pain without dealing with the cause of condition i.e. source of infection in case of infectious diseases. Robert Koch and Henle [6] had given a set of postulates that is known as germ theory of diseases. This theory suggests that for each disease there is a specific agent causing it. At present we have the knowledge that the list of such pathogens causing diseases is quite large and includes many bacteria, viruses, fungi etc. [7]. Since the antibiotics were discovered and used as chemotherapeutic agents, the medical group was believing that it would lead towards the gradual eradication of diseases caused by pathogen. Although, antibiotics are used as the main therapy of infections caused by bacteria. However, over use of antibiotics became the main cause of emergence of multidrug resistant strains of pathogens [8]. The worldwide exposure of *Escheria coli*, *Haemophilus*, *Klebsiella pneumoniae* etc. has become a serious threat before human beings.

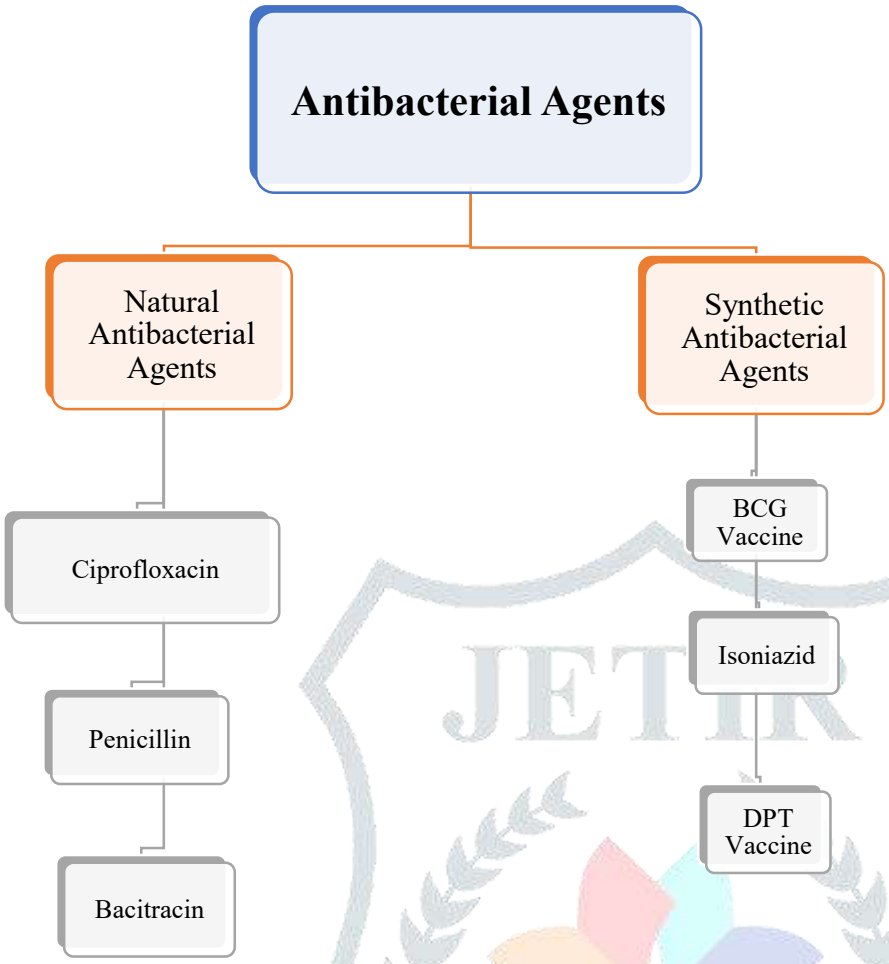
K. pneumoniae and *E. coli* are the bacterial strains that are multidrug-resistant and causes community infection [9,10]. *Candida albicans* also causes hospital acquired infections. It has been reported to cause 50-70% of invasive candidiasis [11]. In past time cases of hospital acquired infection candidemia has increased sharply [12]. These all has caused several serious consequences including increase in the cost of medicines as well as mortality rate of patients.

Since there is a rapid growth of drug-resistant bacterias, thus, there is an urge to search new antimicrobial agents. Although, the past record showing continuous resistance to newly synthesized antimicrobial agents shows that even if we develop the new families of antimicrobial agents, these will also have a short life expectancy [13]. Thus there is a need to develop new drugs that can work effectively against multidrug resistant (MDR) microbe strains.

Antibacterials are the substances that either kill the bacteria or inhibit its growth. For example, Ciprofloxacin, BCG vaccine, Penicillin etc. The antibacterial agents inhibit the functioning of bacterial cell membrane, DNA synthesis, Protein synthesis or RNA synthesis.

Depending on the origin, the antibacterial agents are categorised as natural and synthetic antibacterial agents. Natural antibacterials were extracted from animal and plant sources. However synthetic antibacterials were synthesized compounds.

CLASSIFICATION OF ANTIBACTERIAL AGENT



1. **Natural antibacterial agents:-** Ciprofloxacin [14] and Penicillin[15] are used for treating infections caused by *Bacillus anthracis*.

Bacitracin [16] being highly toxic, cannot be used for human clinical use. But it is used in diagnostic laboratories for *Streptococcus pyogenes*.

Rifampicin [17] acts against mitochondrial RNA polymerase by inhibiting RNA metabolism.

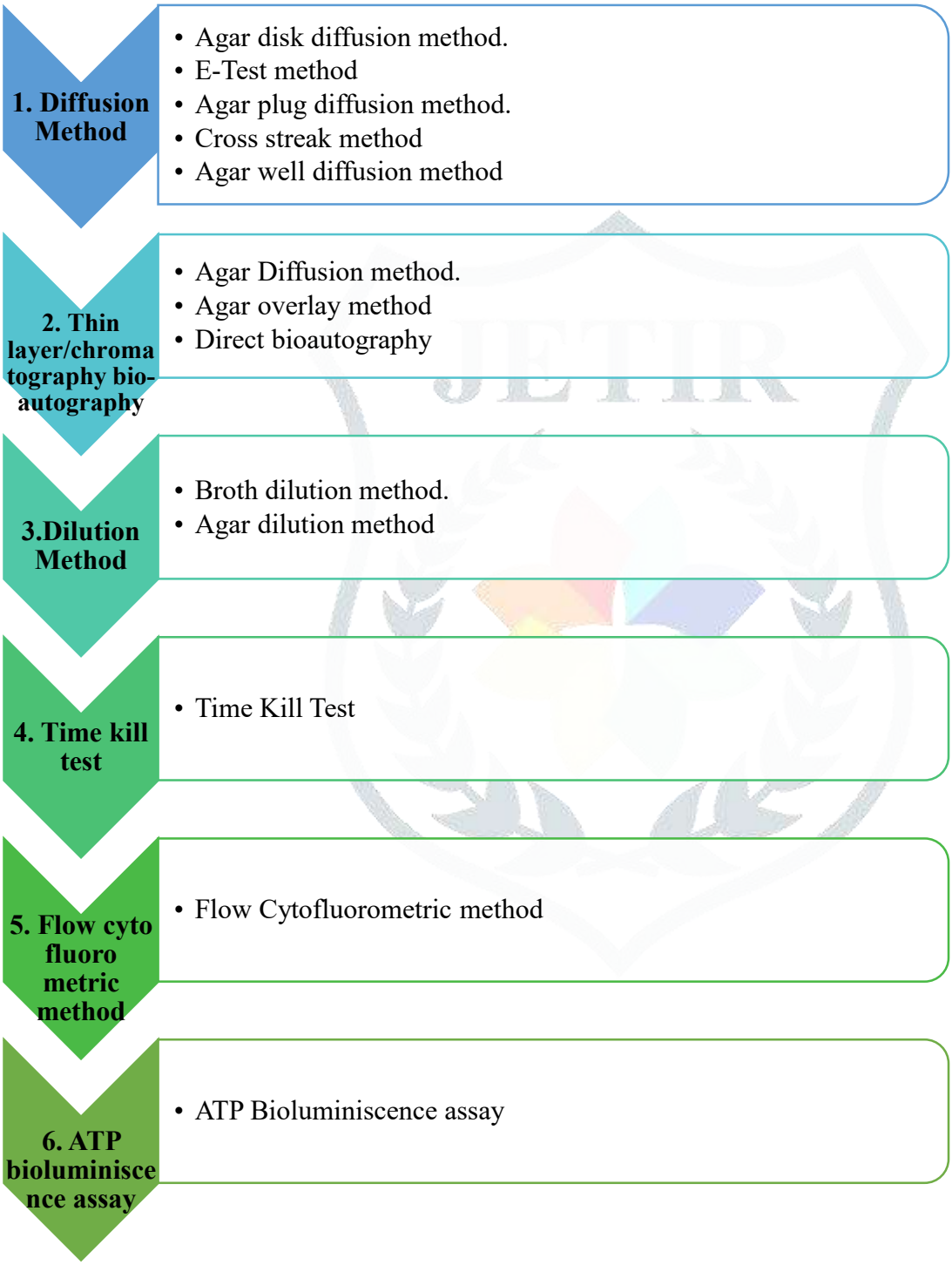
2. **Synthetic antibacterial agents:-** BCG vaccine and Isoniazid [18] are used for treating *Mycobacterium tuberculosis*.

DPT vaccine [19] is used for treating *Clostridium*.

Screening Methods for evaluating Antimicrobial Activity of Pure Compound:

Along with the growing interest in developing new antibacterial agents, great attention is also given to evaluate and screen the newly discovered antimicrobial compound, to test its effectiveness against a microbe. Various laboratory methods can be used to screen the antimicrobial activity of a pure compound. The most commonly used methods are disc diffusion method and agar or broth dilution. To know the activity of a compound in detail the other methods like time kill test, flow cyto flurorometric methods are also used:-

These methods provide the knowledge regarding the inhibitory effect of antibacterial agent i.e. either it is bactericidal or bactiriostatic. As recently there is a great need of antimicrobial agents against multidrug resistant bacteria, therefore it is quite important to first get a better understanding of the available methods for evaluating antibacterial properties of a pure compound, so that the best suited method can be opted for evaluating antibacterial activity of the synthesized compound. These methods are discussed in detail as follows:-



Different Methods for evaluating Antimicrobial Activity of a Compound

1. Diffusion Methods:

1.1 Disk-diffusion method:-

In many microbiology laboratories antibacterial testing is done by using agar disc diffusion method that was developed in 1940 [20]. Clinical and laboratory standards Institute (CLSI) had published many standards for testing of bacteria [21]. Although this test cannot test all the scrupulous bacteria accurately, but it is standardized to test certain such bacteria like *Haemophillus influenza*, *Neisseria gonorrhoeae*, *Haemophillus parainfluenzae*, by the use of particular conditions for incubation, using particular culture media, Interpreting inhibition zones [21].

Disc diffusion method is also known as Kirby Bauer disk method (Fig.1). It is a simple and practical method. It uses the disk impregnated with antibacterial agent to be tested to find out whether a particular bacteria is vulnerable to the antibacterial agent or not [22-24]. In this method a sterile petridish agar plate is inoculated by uniformly spreading suitable bacterial inoculum with the help of a sterile cotton swab. Then filter paper discs were impregnated with the appropriate concentration of test compound. These discs were placed on the inoculated agar plate. This step was done with the help of sterile forceps. The antibacterial agent diffuses from the disk into the agar medium. Its amount keeps on decreasing as it diffuses away from the disk. When concentration of antibacterial agent becomes considerably low, it can no longer inhibit test bacterial growth. At this point zone of inhibition is marked. The zone of inhibition depends on the concentration of antibacterial compound in disk, diffusion rate of antibacterial agent as well as on the sensibility of bacteria chosen. It is a qualitative method as it gives the results by categorizing bacteria as:-

1. **Susceptible:-** If bacterial growth is inhibited by generally recommended dose of antibacterial agent.

2. **Intermediate:-** If the greater concentration of antibacterial agent is required to inhibit the growth of bacteria than required in case of susceptible category.

3. **Resistant:-** If the bacterial growth is not inhibited by generally recommended concentrations of antibacterial agents.

This method cannot be used to differentiate between bactericidal or bacteriostatic effect of antibacterial agent since inhibition of growth of bacteria cannot be considered as death of bacteria.

Moreover Agar disk diffusion method cannot be used to determine minimum inhibitory concentration (MIC) appropriately, because it is impossible to measure the amount of antibacterial agent that is diffused into agar medium plate.

Still this method has many advantages as compare to other methods like simplicity, low cost, reproducibility, can be used to test numbers of micro-organisms, easy interpretation of results. Due to the advantages of this method discussed above, specially ease to perform and low cost, it is commonly and preferably used to study the antibacterial activity of drugs [25-28].

1.2 Antimicrobial gradient method E-Test:-

E-Test is also known as Epsilmeter test. It is based on combination of principle of dilution as well as diffusion method. It is used to determine the MIC value of antibiotics, anti-fungals and anti-mycobacterials [29].

In this method a strip is impregnated with antibacterial agent in increasing concentration gradient from one end to other. This impregnated strip is then deposited on the agar surface that is already inoculated with the test bacteria. There is an immediate release of antibacterial agent/drug. Then it is kept for overnight incubation. An inhibition eclipse is produced (Fig.2). The MIC value is find out at the point of intersection of growth inhibition eclipse and strip. A good correlation is reported between the results of E-test and broth dilution or agar dilution method [30-33].

E-Test has been used to find out the MIC for scrupulous micro-organism like β-hemolytic Streptococci, S.pneumoniae Haemophilus etc. It can also be used for testing Pseudomonas sp. And Burkholderia pseudomallei [34-36].

This method is costly as compared to disk diffusion method. But it is simple, reliable and accurate and can also be used to find out Minimum inhibitory concentration (MIC) of antimycobacterial and antifungal agents [37].

1.3 Agar well diffusion method:-

This method is used to check the antimicrobial activity of microbial extracts [38-39]. In this method first of all a agar plate is prepared when the medium is solidified then it is inoculated by spreading bacterial suspension over the agar surface with the help of a cotton swab. Then a 6-8 mm hole is punched with the help of a sterile cork borer. This hole is called as well. These wells are then filled with 20-50uL of the solution of antibacterial compound to be tested. The plates are then incubated under favorable conditions as per the micro-organism under observation. Incubation is done at 35-37°C for nearly 18-24hr. The antibacterial agent under examination diffuses into the agar medium and the growth of micro-organism is inhibited (Fig.3). The activity of antimicrobial agent is calculated by using the formula:-

Zone of Inhibition = Total diameter of inhibited growth zone – diameter of the well.

1.4 Agar plug diffusion method:-

The procedure of Agar plug diffusion method is same to that of disk diffusion method. This method is generally used to show the enmity between the micro-organisms [40,41]. In this method an agar culture of strain under observation is made on the surface of plate. This is allowed to grow, when the molecules secreted from this microbial strain will diffuse into the agar medium. Then this plate is incubated. Once the incubation is done then a plug is cut with the help of a sterile cork borer and placed on the surface of second agar plate that is already inoculated with the micro-organism under observation. The substance from this plug diffuses into the medium of this second plate. The appearance of inhibition zone near the agar plug shows the antibacterial activity of the molecules secreted [Fig.4].

1.5 Cross streak method:-

This method is also used to find out the enmity between the micro-organism [42]. In this method the strain of microbe concern is seeded as a single stripe at the center of the plate having agar medium. It is then incubated. After proper incubation the test micro-organism is applied on the same plate as single stripe perpendicular to the centre stripe. It is again incubated. The antimicrobial activity is considered by the observation of inhibition zone size.

2. Dilution methods:-

Dilution methods are quantitative methods. These methods involve both dilution and diffusion. These methods are used to determine minimal inhibitory concentration (MIC) of the antibacterial agent which is just sufficient to either inhibit the growth of bacteria or to kill the bacteria. MIC is expressed as mg/L or µg/ml. In this method the antimicrobial agent/antibacterial agent is diluted either in agar or broth medium. MIC does not always give the absolute value. Any laboratories using the dilution methods should maintain a standard procedure for testing with accuracy.

2.1 Broth dilution method:-

In this method a suspension of test bacteria of a particular concentration is prepared. It is then tested against different concentrations of antibacterial agents. The concentrations of antibacterial agents are generally taken as two fold dilutions i.e. 1,2,4,8,16,32 and 64 ug/ml. in the liquid medium in a test tube. The broth dilution method can either be performed by macro dilution method in which a volume of 2ml is taken in tubes (Fig.5) or by micro dilution method in which smaller volumes are taken in 96-well micro titration plates (Fig.6). Then inoculation of each tube or well is done with a microbial inoculums. This microbial inoculum is prepared by diluting microbial suspension to a standard range. After well mixing incubation of the tubes or the micro titration plates are done.

The minimum concentration of antimicrobial(antibacterial) agent that is required to completely inhibit the growth of micro-organisms under observation either in tubes or in micro titration plates is the MIC value of the antibacterial agent. It is detected by unaided eye [43].

In comparison to micro dilution method, macro dilution methods are more exhausting, involve risk of error and comparatively larger amount of reagent and space is needed [44]. The advantage of the method is to provide quantitative results.

2.2 Agar dilution method:-

In agar dilution method different concentration of antimicrobial agent is incorporated into the agar medium. Concentrations are generally varied as two fold dilutions i.e. 1, 2, 4, 8, 16, 32 and 64 µg/ml. After it a particular bacterial inoculum is applied on to the agar plate with the help of a cotton swab [45-47]. The plates are then incubated at 35-37°C for 18-24 hr. After it the plates are examined for the zones of growth inhibition.

The results of Agar dilution method are taken as the most reliable results for measuring the MIC values for particular bacteria.

The advantage of agar dilution method is that many organisms can be tested on the same plate.

The disadvantage of the method is that it is quite laborious for making agar plate and once the plates are made their life is very short.

3. Thin layer chromatography-bioautography:-

Goodall and Levi [48] in 1946 used paper chromatography by combining it with contact bioautography to detect different penicillins. After them Fischer and Lautner [49] used TLC for the same purpose. This technique involves TLC combined with chemical and biological detection methods. Various works were done on the listing of plant extracts for their antimicrobial activity by using TLC bioautography [50-51]. It includes three methods for testing of antimicrobial activity of compound. These methods are Agar diffusion, Direct bioautography and Agar overlay bioassay.

3.1 Agar Diffusion:-

This method is also known as agar Contact method. In this method an agar plate is first inoculated with the test micro-organism. Then the antimicrobial agent is transferred from the chromatogram to this plate by diffusion. After some minutes or hours, when the diffusion has occurred, the chromatogram is removed. Incubation of agar plate is done. Appearance of zone of growth inhibition occurs where the agar layer comes in contact with antimicrobial compounds [52]. It is least applied method.

3.2 Direct bioautography:-

It is most applied method among the three TLC bioautography method. In this method the developed TLC is either sprayed or dipped into the microbial suspension. Then incubation of this bioautogram is done for 48hr at 25°C in humid condition [53]. Visualization of growth of microbes is one by spraying with suitable salts on the bioautogram.

This method can be used for evaluation of both antibacterial or antifungal activity. Bacillus subtillis, Slaphy Clococcus aureus and Escheria Coli are generally used bacteria for testing antibacterial Compounds [50, 54].

3.3 Agar Overlay bioassay:-

This method is also known as immersion bioautography. This is a combination of Agar diffusion and direct bioautography method. In this method TLC plate is covered with the agar medium in molten seeded form. Before Incubation, plates are kept at low temperature for few hours, so that the compound under examination can diffuse properly into the agar medium. After incubation, visualization can be made by using tetrazolium dye. This method can also be applied to all micro-organisms like molds [51] Candida albicans [55]. The best part of this method is that it is not sensitive for contamination [52] and provides clear growth inhibition zone.

TLC bioautography is an effective simple and inexpensive technique. It can be used both in simple small laboratories and in sophisticated laboratories for detection of antimicrobial agents.

4. Time kill test:-

Time kill test is considered as most appropriate method for evaluating bactericidal effect of a Compound. It gives information of interaction between the microbe and microbial agent. This method informs about the concentration related or time related effect of microbial agent [56].

It is done in broth medium, In this method three tubes are taken having bacterial suspension of concentration 5×105 CFU/ml. In first tube 0.25×MIC of tested Compound is taken and in second tube 1×MIC of tested Compound is taken while the third tube is of growth Control.

Incubation is then done with variation of time as 0, 4, 6, 8, 10, 12, 24hr [56,26]. Then dead cells percentage is calculated relative to growth control. The bactericidal effect is generally found to be a lethality percentage of 90% in 6h.

5. ATP bioluminescence assay:-

This method is based on the principal of linear relation between viability of bacterial cell and measured luminescence emitted by cell. This method is used by many researchers for antibacterial testing [57]. The main advantage of this technique is that it is very rapid. It takes 3-5 days for giving results of antibacterial tests [58,59]. However the dilution methods need 3-4 weeks for incubation.

6. Flow cyto flurometric method:-

This method was used by many authors for investigating the antibacterial and antifungal activities of various drugs. This method detect damaged cells rapidly by using particular dyes staining [60,61]. Propidium Iodide is used as DNA stain [60]. This method rapidly gives reproducible results (2-6 hr) as compared to 24-72 hr of microdilution method [60].

Since bacterial infections have become a serious threat due to multiple drug resistant strains against existing antibacterial agent. Therefore there is a great need for discovering novel antibacterial agents and continuous use of methods for evaluating them is required.

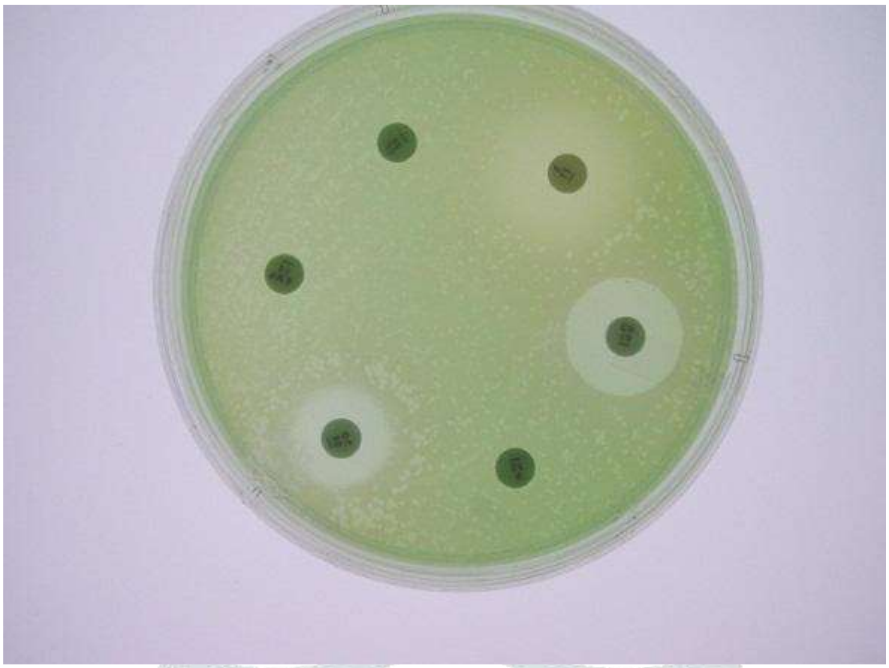


Figure 1: Kirby Bauer disk disc diffusion method

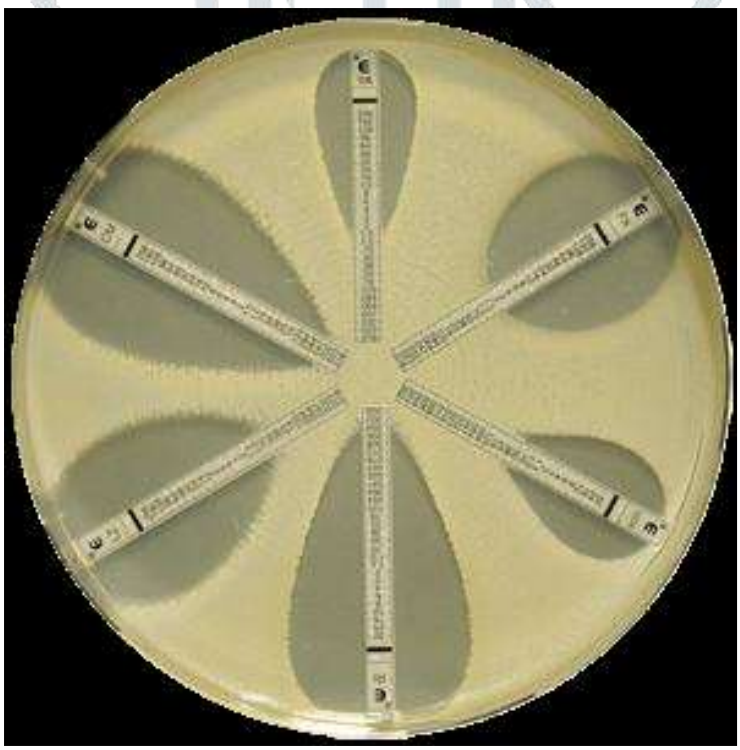


Figure 2: E Test gradient diffusion method

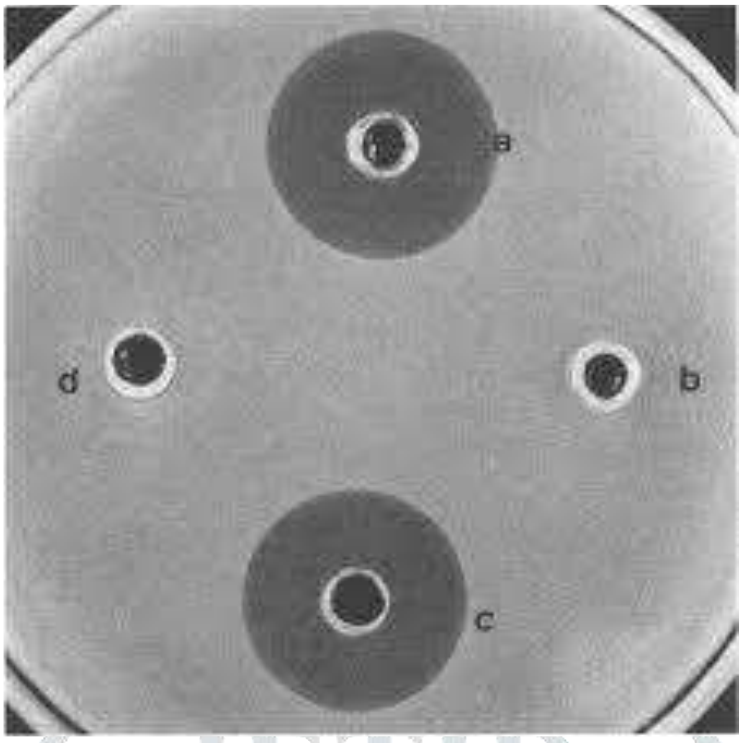


Figure 3: Agar well diffusion method

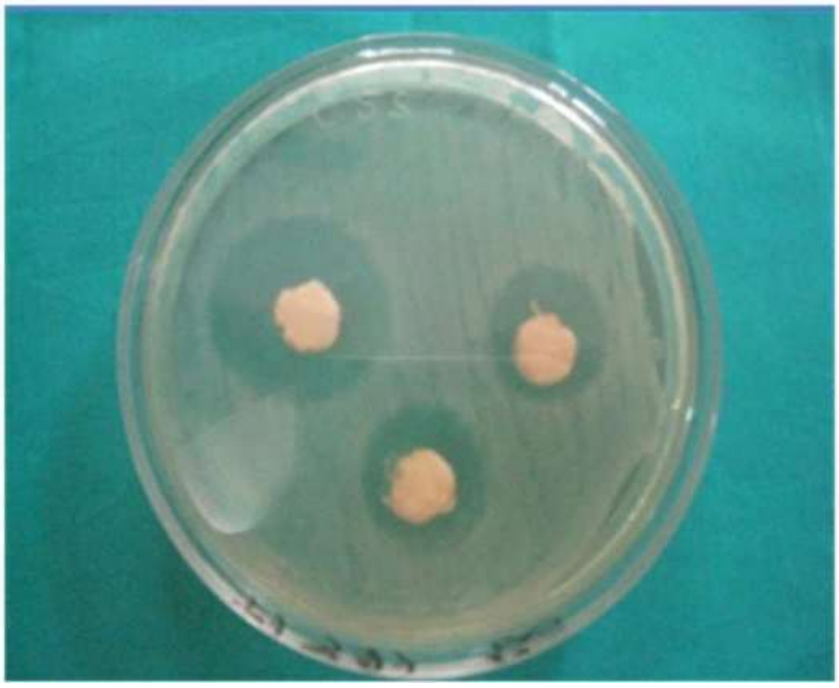


Figure 4: Agar Plug Diffusion method

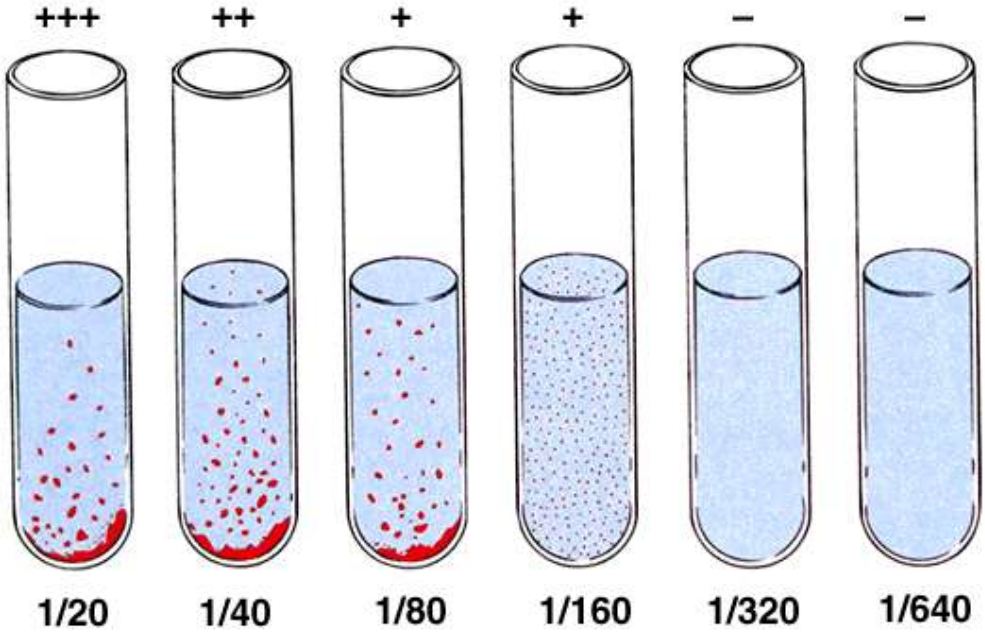


Figure 5: Tube or Macro broth dilution test

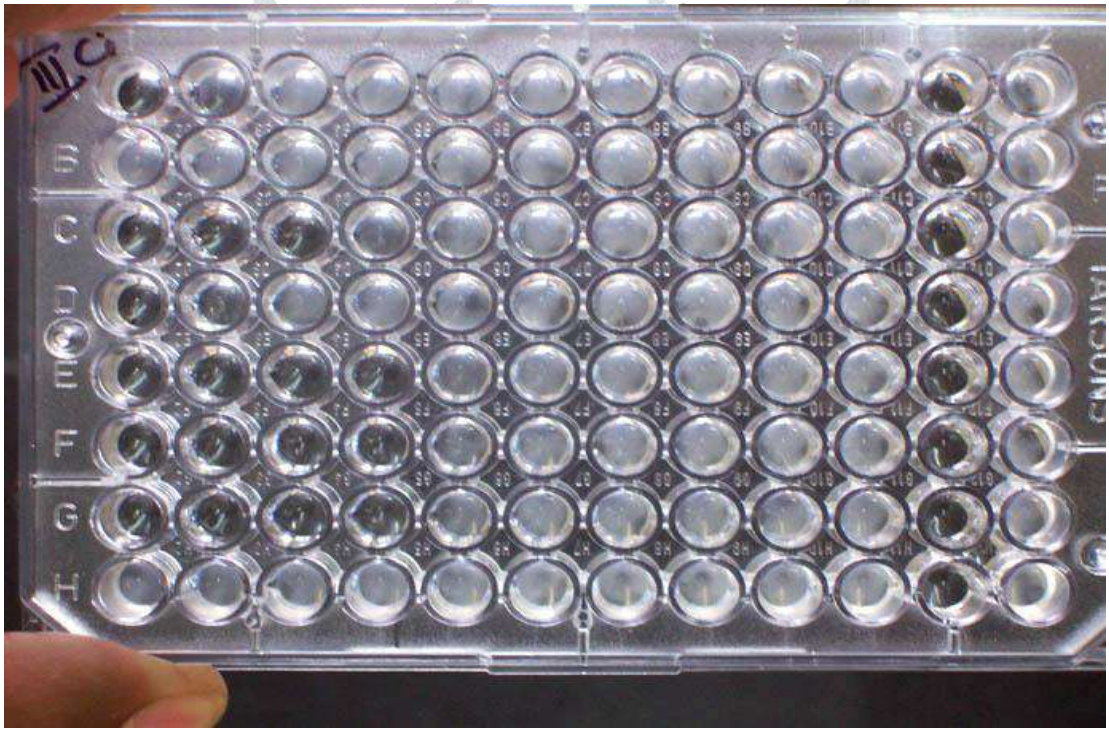


Figure 6: A broth microdilution panel containing 96 wells

CONCLUSION:

Recently in clinical industry microbial infections have become a great threat due to increasingly growing antimicrobial drug resistance. Therefore there is a regular urge among researchers to develop novel compounds and test their antimicrobial activities. This paper helps the researchers to know about the best suitable method that can be used by them for testing their newly synthesized compounds. Moreover the

researchers can be attracted for the development of new more reliable , appropriate, advanced, cost effective, antimicrobial testing methods.

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