



Review on Antimicrobial Susceptibility Testing: Principle, Common Methods and Limitation

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Abstract

Antimicrobial susceptibility test (AST) is an essential test provided by clinical laboratories to guide clinicians in selecting the most effective antimicrobial therapy for bacterial infections. Then this can be explained in clinical microbiology as a principle that the ability of AST to measure antimicrobial agent to inhibit bacteria growth is estimated by different common methods like dilution and diffusion method. To begin, AST do have two broad methodology and types which are phenotypic and genotypic AST method, and qualitative and quantitative (which determine the minimum inhibitory concentration or MIC) type respectively but most widely used method are phenotypic AST method which contain both qualitative and quantitative types of AST methods. Although AST are graceful method, they are not perfect and they do have shortcoming such as report of falsely susceptibility, requirement of substantial economy or equipment are expensive, and inflexible. The reason why incited to go over this AST test method is to have detail comprehend and also to have in common in antimicrobial susceptibility testing principle, common methodologies, limitation and how to determine microbial sensitivity or resistant to antimicrobial agent and to find out the right treatment options.

Keywords: Antimicrobial susceptibility test, Limitation, Methodology, Minimum inhibitor concentration, Principle

1. Introduction

Many bacteria have become resistant to multiple antimicrobial agents; the war has seemingly escalated in favor of the bacteria. So Resistance to antimicrobial agents has become a major source of morbidity and mortality worldwide. Those Microbial species that are not susceptible with synthesize antimicrobial agent are such as extended spectrum beta-lactamase producing Enterobacteriaceae, Streptococcus pneumoniae, Pseudomonas sp., Staphylococcus sp. and

Enterococcus sp and the like. Impact of increasing in antimicrobial resistance contributors to higher morbidity and mortality as well as added healthcare costs (PMC, 2018).

Antimicrobial susceptibility test (AST) is an essential test provided by clinical laboratories to guide clinicians in selecting the most effective antimicrobial therapy for bacterial infections. In this age of increasing antibiotic resistance, treatment is becoming more complex and options for therapy are often limited. A variety of

antibiotic susceptibility test method age used in clinical microbiology laboratories. These range from simple diffusion-based methods to more complex broth-based automated systems. Antibiotic susceptibility test can be performed in either a quantitative or qualitative manner. Quantitative methods determine the lowest concentration of antibiotic that is required to inhibit the growth of a certain organism *in vitro* (Mahon *et al.*, 2011).

The performance of Antimicrobial susceptibility test by the clinical microbiology laboratory is important to confirm susceptibility to chosen empirical antimicrobial agents, or to detect resistance in individual bacterial isolates. Empirical antibiotic therapy continues to be effective for some bacteria: for example, there has not yet been any reported resistance of *Streptococcus pyogenes* to penicillin (Jorgensen *et al.*, 2009).

Antimicrobial susceptibility test methods are available to determine bacterial susceptibility to antimicrobials. The selection of a method is based on many factors such as practicality, flexibility, automation, cost, reproducibility, accuracy, and individual preference. Standardization and harmonization of AST methodologies, used in epidemiological surveillance of antimicrobial drug resistance, are critical if data are to be compared among national or international surveillance/monitoring programmes of OIE Members. It is essential that AST methods provide reproducible results in day-to-day laboratory use and that the data be comparable with those results obtained by an acknowledged 'gold standard' reference method. In the absence of standardized methods or reference procedures, susceptibility results from different laboratories cannot be reliably compared. (OIE, 2012). The standards of interpretation are published by authorities such as the European Committee on Antimicrobial Susceptibility Testing EUCAST (2019) and the Clinical and Laboratory Standards Institute (CLSI 2019) located in the USA. In the case of routine methods for Antimicrobial susceptibility test, it usually takes at least 24 h to obtain growth of bacterial colonies and an additional 24 h to obtain isolate characterization,

namely biochemical identification and phenotypic AST (Faro *et al.*, 2016).

The methods generally used afford qualitative results, using the susceptible, intermediate or resistant categories, but some of them provide quantitative results, expressed as minimum inhibitory concentration (MIC) (Jenkins and Schuetz 2012). Some of the phenotypic AST methods, which detect phenotypic resistance by measuring bacterial growth in the presence of the antimicrobials being tested, have survived the development of modern diagnostic tools in the clinical microbiology laboratories, such as disc diffusion and broth microdilution methods (Idelevich and Becker 2019). The reason why selected to review this AST test method is to have detail comprehend and also to have in common in antimicrobial susceptibility testing principle, common methodologies, limitation and how to determine microbial sensitivity or resistant to antimicrobial agent and to find out the right treatment options (CLSI, 2013).

1.2. Objectives of a Review

1.2.1 General objective

1.2.2 Specific objective

- To review on antimicrobial susceptibility test: Principles, Common Method and Limitations.
- To highlight the principles of antimicrobial susceptibility test.
- To give a brief revision on common method of antimicrobial susceptibility test
- To list out the shortcomings of antimicrobial susceptibility test.

2. Common method of antimicrobial susceptibility test

2.1. Principles of Antimicrobial Susceptibility Test

Antimicrobial Susceptibility Test method measures the ability of an antibiotic or other antimicrobial agent to inhibit bacteria growth in

vitro (CLSI, 2013). Susceptibility testing is to predict the likely outcome of treating a patient's infection with a particular antimicrobial agent. The two important and related functions of susceptibility testing are the detection of frank resistance and the quantitative measurement of susceptibility to antimicrobial agents with some species that may have direct therapeutic relevance (Jorgensen *et al.*, 2009). This ability may be estimated either by the dilution method or diffusion method. Susceptibility is based on the concentration required to reduce growth below the visible level (CLSI, 2013).

2.2. Phenotypic Antimicrobial Susceptibility Test Method

The most widely used methods for characterizing antibiotic resistance in clinical isolates detect phenotypic resistance by measuring bacterial growth in the presence of the antibiotic being tested. These techniques include broth microdilution, antimicrobial gradient methods (e.g. Etest strips), disc diffusion and various commercially available automated systems (e.g. the MicroScan WalkAway system from Siemens, the Phoenix Automated Microbiology System from BD Diagnostics and the Vitek systems from bioMérieux). In addition to their high sensitivity for detecting antibiotic resistance, they have been highly standardized, a process that has been facilitated by the elaboration of international guidelines for AST, such as those published by CLSI and EUCAST (Marina *et al.*, 2013).

2.2.1. Disk diffusion

The disk diffusion method is the gold standard for confirming the susceptibility of bacteria (Zeeshan *et al.*, 2019). Disk diffusion refers to the diffusion of an antimicrobial agent of a specified concentration inoculum isolated in a pure culture. Disk diffusion is based on the determination of an inhibition zone proportional to the bacterial susceptibility to the antimicrobial present in the disk (OIE, 2012).

The disc diffusion test provides qualitative results by categorizing the bacterial susceptibility as susceptible, intermediate or resistant, and is not

appropriate for determination of MIC (Benkova *et al.*, 2020). This method consists of inoculating the isolated bacteria onto a Mueller-Hinton agar plate, followed by placing antibiotic-impregnated paper disks on the surface of the agar. By incubating this plate, antibiotics will diffuse into the agar in a gradient; the antibiotic concentration will decrease as the distance from the disk increases (Lisette *et al.*, 2020).

In this method, the isolated bacterial colony is selected, suspended into growth media, and standardized through a turbidity test. The standardized suspension is then inoculated onto the solidified agar plate, and the antibiotic-treated paper is tapped on the inoculated plate. The disc containing the antibiotic is allowed to diffuse through the solidified agar, resulting in the formation of an inhibition zone after the overnight incubation at 35°C. Thereafter, the size of the inhibition zone formed around the paper disc is measured; the size of the inhibition zone corresponds to the concentration of antibiotic (Zeeshan *et al.*, 2019). This is performed manually using a sliding caliper or a ruler which is held on the back of the inverted agar plate. However, the diffusibility of a given compound must be taken into account (Benkova *et al.*, 2020). Antibiotic susceptibility is determined by measuring the diameter of the zones of bacterial inhibition around the antibiotic disks and comparing the diameter with disk diffusion interpretive criteria (Lisette, *et al* 2020).

Generally, the larger the zone of inhibition, the lower the concentration of antimicrobial required to inhibit the growth of the organisms. However, this depends on the concentration of antibiotic in the disk and its diffusibility. Note: Disk diffusion tests based solely on the presence or absence of a zone of inhibition without regard to the size of the zone of inhibition are not acceptable AST method (OIE, 2012).

The principle of the method is that when an antibiotics (present in form of disc) is plated in agar medium, it diffuses in the surrounding area causing possible inhibition of the bacteria growth in this zone. At the end of incubation period, a clear zone called inhibition zone appears around the antibiotics while the rest of plate show growth turbidity (CLSI, 2013).



Figure 1: A disk diffusion test with an isolate of *Escherichia coli* from a urine culture. (Jorgensen *et al.*, 2009).

2.2.2. Dilution

Dilution was one of the earliest tools in microbiological practice, starting in the early 1870s, and it allows the growth and identification of bacterial populations in suspension. Pasteur, Lister, Koch, and Ehrlich were listed as the pioneers in the field of bacteriology, and they worked on the concept of macrodilution. William Roberts and John Tyndall further contributed to the macrodilution method and observed bacterial growth in a diluted medium. The two basic types of dilution are microdilution and macrodilution, wherein broth and agar are the most commonly used mediums (Zeeshan *et al.*, 2019).

The Dilution method is used to determine the MIC of an antimicrobial to inhibit or kill microorganism. The purpose of the tests is to check the effectiveness of a drug against a

bacterium and to select best drug that acts against the bacterium (Enass, 2020). In this test a control strain set with known susceptibility is made separately. The tubes are incubate at a specific temperature, depending on the optimal growth temperature of bacteria or fungi, and for a period of time. The end points are taken as MIC i.e., amount of antimicrobial agent required to inhibit growth or MBC i.e., amount of antimicrobial agent required to kill the bacteria. Generally, there exists a positive control i.e., antibiotic, maximum growth of bacteria/fungi, and minimum growth of bacteria/fungi with the presence of antibiotic, as controls. An antibiotic control is necessary and the results are accepted only if the control plate shows good growth. No growth of the test organism indicates that there is susceptibility at the antimicrobial concentration incorporated in the medium (Sofia *et al.*, 2014).

Broth dilution

Broth dilution methods may be used to quantitatively measure the in vitro activity of an antimicrobial agent against a given bacterial isolate. To perform the tests, a series of tubes are prepared using a broth medium to which serial concentrations of the antimicrobial agents are added. The tubes are then inoculated with a standardized suspension of the test organism. Following incubating at the appropriate temperature for the appropriate time interval, the tests are read, the MIC is determined, and the results are interpreted using approved clinical breakpoints. The final result is significantly influenced by methodology, which must be carefully controlled if reproducible results (i.e. Intra-and interlaboratory) are to be achieved (CLSI, 2020).

The MIC value of an antimicrobial agent can also be determined with dilution methods. Each tube is inoculated with a known number of test microorganisms. When EOs (Ethylene oxide) are investigated with broth dilution test (BDT), the main difficulty is their low water solubility. Therefore, solvents (eg, dimethyl sulfoxide or ethanol) or detergents (eg, Tween 20) are added to the tubes containing EO to be tested. However, these materials may influence the result and the MIC value especially when their concentration is >5% (v/v) (Györgyi and Béla, 2016).

A broth-dilution method for performing AST on anaerobic bacteria has been proposed. Medium used in the test was Schaedler broth, with incubation in a glove box with an Atmosphere of 5% CO₂, 10% H₂, and 85% N₂, or in the GasPak system. MIC for selected antibiotics were determined, under these conditions, by using a conventional twofold dilution scheme for the antibiotics and a categorization three-tube method “in which two or three clinically significant concentrations of each antibiotic were used. MIC obtained by both Methods were very similar. The categorization method could be used routinely to test the antimicrobial susceptibility of anaerobic bacteria (Donald and Clybc, 1974).

Broth macrodilution

One of the earliest methods of AST was the macrobroth or tube-dilution method. This procedure involves preparing two-fold dilutions of antibiotics (e.g 1mg/mL, 2mg/mL, 4mg/mL, 8mg/mL, or 16mg/mL) liquid bacterial growth medium. The antibiotic-containing tubes are inoculated with a standardized bacterial suspension of 5×10^5 cfu/ML in each tube. After overnight incubation at 35°C the tubes are examined for macroscopically visible evidence of bacterial growth in the form of turbidity. The lowest concentration of antibiotic that prevents visible growth represents the endpoint of the test (Jorgensen and Ferraro, 1998).

The lowest concentration of the antimicrobial agent where no visible evidence of bacterial growth (no turbidity) represents the MIC. The precision of broth macrodilution was considered to be plus/minus one twofold dilution concentration, due to the manual preparation of the dilutions (Benkova *et al.*, 2020).

By this method Susceptibility testing was performed in RPMI 1640 medium (American Biorganics, Inc., Niagara Falls, N.Y.), With L-glutamine and without sodium bicarbonate, and buffered at pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS) and adjusted pH 7.0, contained 2g of glucose. Drug dilutions were pre-pared at 10 times the strength of the final drug concentration by additive drug dilution schemes for minimizing systematic pipetting errors. Drug dilutions were stored at -70°C until used. (Francesco *et al.*, 1994).

Fluconazole, ketoconazole, itraconazole and amphotericin B were obtained as reagent-grade powders from their respective manufacturers. Amphotericin B, ketoconazole and itraconazole were dissolved in dimethyl sulfoxide at a concentration of 320 mg/mL, and fluconazole was dissolved in 0.85% saline at a concentration of 2560 mg/mL. Stock solutions were stored at -70°C until used. For the azoles, itraconazole, ketoconazole and fluconazole, the MIC was established as the lowest antifungal concentration that inhibited 80% of the control growth (0.2 mL

of growth control plus 0.8 mL of uninoculated RPMI). For amphotericin B, endpoints were determined visually by recording the lowest concentration of the agent that prevented the appearance of visible growth (Maria do *et al.*, 2002).

Broth microdilution

The broth microdilution MIC method is used to measure (semiquantitatively) the in vitro activity of an antimicrobial agent against a bacterial isolate. A sterile multiwell plastic tray containing various concentrations of antimicrobial agents is inoculated with a standardized number of test bacteria. After overnight incubation at $35 \pm 2^\circ\text{C}$, the MIC is determined by observing the lowest concentration of an antimicrobial agent which will inhibit visible growth of the bacterium. MICs obtained are interpreted as susceptible, intermediate, or resistant, based on the criteria listed in the CLSI MIC standard (Leber, 2016).

Due to the miniaturization of the test, the method became more practical and popular. This technique is performed by using small sterile disposable polystyrene microtitration plates, usually containing 96 wells (Benkova *et al.*, 2020). It generally performed in a 96 well format allowing up to 12-15 antibiotics to be tested simultaneously. The tests may be read manually

or in an automated fashion using specialized machines (Matthew, 2015).

Principle of the procedure: The aim of this procedure is to determine the MIC of a bacterial strain to a particular antimicrobial. This is done by making several dilution of an antimicrobial in a 96 well plate format (CLSI, 2013).

Antimicrobial susceptibility test was performed by then broth microdilution method using cation-supplemented Mueller-Hinton broth, according to CLSI guidelines (CLSI broth microdilution method). The colorimetric broth microdilution method with STC (STC broth microdilution) was identical to the broth microdilution method described by CLSI in terms of reagents, medium preparation, inoculum, drug concentrations, and incubation time, with one exception, namely, that STC was added to the cation-supplemented Mueller-Hinton broth with antimicrobial agents to a final concentration of 50 g/ml. Levels of agreement between the two STC and CLSI broth microdilution methods with or without STC were calculated for each strain drug-MIC endpoint and were expressed as percentages at the same MIC or as MICs within one or two 2-fold dilutions. The rates of agreement were calculated as the percentage of isolates with MICs in the range of two 2-fold dilutions (Lee, *et al.*, 2007).

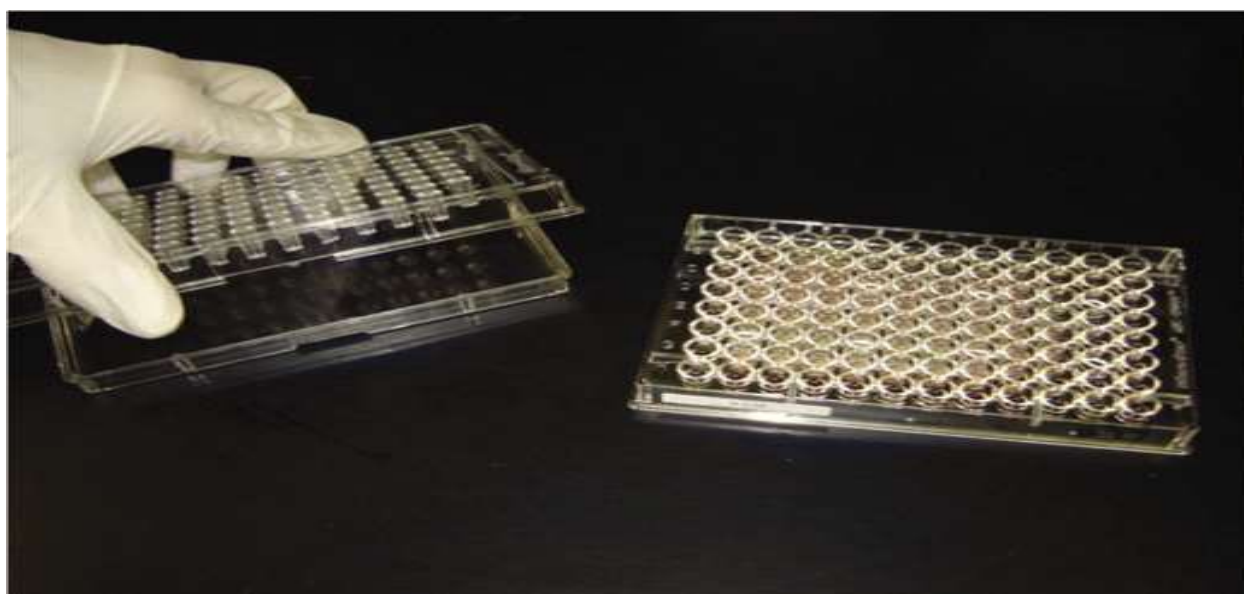


Figure 2: A broth microdilution susceptibility panel containing 96 reagent wells and a disposable tray inoculator. (Jorgensen *et al.*, 2009).

2.2.3. Agar dilution

Agar dilution susceptibility test is regarded as the golden standard for all other susceptibility testing methods (Rene and Hendriksen 2003). Represents one of the earliest methods used for determining bacterial susceptibility to antibiotics (Jorgensen and Ferraro 1998). The AD method is a manual method not frequently used in the researched literature. This technique is standardized by the National Committee for Clinical Laboratory Standard. The method is performed by incorporation of different concentrations of the antimicrobial agent into a molten agar medium, inoculum to the surface of the agar plate (Benkova, *et al.*, 2020). The inoculum can be applied effortlessly with an inoculator or manually. Note to use the plates either immediately or store them in sealed plastic bags at 2-8°C for up to five days for reference work, or longer for routine tests (Lisette, *et al.*, 2020).

Quantitative method for determining the MIC of antimicrobial agent against the test organism and Mueller–Hinton agar is used in this method. The concentration at which bacterial growth is completely inhibited is considered as the MIC of the antibiotic and the organisms are reported sensitive, intermediate, or resistant by comparing the test MIC values with that given in CLSI guidelines. The main aid of this method is that a number of organisms can be tested simultaneously on each plate containing an antibiotic solution (Enass, 2020).

These results are often considered as the most reliable for the determination of an MIC for the test bacterium/antimicrobial combination (OIE, 2012). Principle of agar dilution method involve incorporation of the antimicrobial agent in doubling solution into agar medium followed by application of standardized numbers of cells to plate. Plates are read by observing the lowest concentration that inhibit visible bacteria growth (BIOMIC, 2021).

2.2.4. Epsilonometer testing

Epsilonometer testing (Etest) is another significant development for the routine analysis of wide

spread antibiotic resistance in bacteria (Zeeshan *et al.*, 2019). Etest is commercial product based on the principle of establishment of an antimicrobial concentration gradient in an agar medium as a means of determining susceptibility (Jorgensen *et al.*, 2009). The antimicrobial gradient diffusion method provides quantitative MIC results but is based on diffusion of antibiotics into solid media much like the Kirby Bauer method (Matthew, 2015). the method combines the principles of the dilution and diffusion methods (Benkova, *et al.*, 2020).

A Predefined stable antibiotic gradient is present on a thin inert carrier strip. Test is used to determine the on-scale MIC (Enass, 2020). Typically, MIC values determined by Etests are within one doubling dilution of those determined by conventional broth or agar based methods. Like the Kirby Bauer Assay, Etests are useful in determining the susceptibility of fastidious organisms because the strips can be placed atop plates that are more nutrient rich (Matthew, 2015).

The ability of convenient interpretations of MIC under diverse physical conditions made the Etest a preferential method over standardized disk diffusion and dilution techniques in clinical laboratories for AST. One of the significant aim of the Etest is its sensitivity; it can detect extended-spectrum beta-lactamase (ESBL), even in a trace amount. Additionally, accurate resistance strains can easily be easily quantified in laboratories/hospitals due to the stable concentration .gradient of antibiotics marked on the Etest strip (zeeshan *et al.*, 2019).

2.3. Genotypic Antimicrobial Susceptibility Test Methods

Molecular or genotypic AST are the effective direct methods that eliminate tedious bacterial Cultures, long incubation, chances of contamination, and the spreading of deadly infections (Zeeshan *et al.*, 2019).

2.3.1. Polymerase chain reaction-based methods

Polymerase chain reaction (PCR)-based genotypic techniques, both conventional and real time, are commercially available as assays and automated machines. PCR relies on the amplification of DNA sequences that are specific for a particular pathogen and its drug susceptibility or resistance. PCR was initially used for the rapid identification and quantification of the pathogen but thereafter, with increased knowledge of the genetic basis of resistance to antibiotics, PCR-based approaches have been developed to detect the presence of genetic determinants of resistance to different antimicrobial agents (Benkova *et al.*, 2020).

Polymerase chain reaction is one of the most efficient and rapid molecular tools for quantification and profiling of bacterially infectious genes. The general method of PCR includes cycles of denaturation, annealing of the primers, and elongation of the primers by Thermo stable DNA polymerase in a compatible buffer containing nucleotides, ions, and so on. Each cycle of amplification doubles the target DNA molecule. The amplified target can be confirmed for the presence of resistance genes through electrophoresis, southern blotting, restriction fragment-length polymorphism, single-strand conformation polymorphism (SSCP), DNA fingerprinting, molecular beacons, and other DNA sequencing analysis methods (Zeeshan *et al.*, 2019).

The basic principle of polymerase chain reaction is amplification of resistance factors (gene). It is biochemical technology to amplify a single or a few copies of a piece of DNA to generate millions of copies of certain DNA sequence (CLSI, 2013).

3. Limitations of antimicrobial susceptibility test

The shortcomings of rapid susceptibility testing methods are some sacrifice in the ability to detect certain inducible or otherwise subtle antimicrobial resistance mechanisms. The instruments most notable for such problems are no longer marketed, and the manufacturers of the two remaining

instruments have made significant strides toward correcting earlier problems. However, it is important to emphasize that accuracy should not be sacrificed in an effort to generate a rapid susceptibility result. A significant problem with commercial micro-dilution or automated systems is the inflexibility of the standard antibiotic batteries or test panels (Jorgensen, 1993).

Although some protocols may allow for testing directly with clinical samples, phenotypic AST methods generally require pure cultures for susceptibility testing to be performed (Marina *et al.*, 2013). Unfortunately, these elegant methods are not flawless, and reports of falsely susceptible (less frequently, falsely resistant) bacteria are by no means rare in daily clinical practice. Many resistance mechanisms are not detectable with this routine test, and this is particularly true for the production of some beta-lactamases (Susan *et al.*, 2019).

Some other limitations of this technique are, they are very laborious and require substantial economic and technical resources and the purchase of antimicrobial associated equipment may be expensive, some AST methodology may not be feasible for some laboratories (OIE, 2012). Another limitation of this test related to the inaccurate and inconsistent behavior for certain antibacterial agents, such as Penicillin, ciprofloxacin, ofloxacin, and rifampicin. Some of the general restrictions of the AST genotypic method are the individual antimicrobial agents to be tested need a specific assay for detection, only potential/key resistance genes can be detected, which are often not relevant due to coincidental mutations; there is a lack of sensitivity towards the patients with latent infections, or when only a few organisms are present in a sample; the genetic mechanism/profile for the resistance of all bacteria is not yet defined; the occurrence of false-positive results due to contamination of the test sample might be expected; they require expensive reagents and machinery with specific maintenance conditions; and most importantly all the tools have a prerequisite of skilled personnel (Zeeshan *et al.*, 2019).

4. Conclusion and Recommendations

Antibiotic susceptibility testing is an essential test provided by clinical laboratories to guide clinicians in selecting the most effective antimicrobial therapy for bacterial infections. *In-vitro* antimicrobial susceptibility testing can be performed using a variety of formats, the most common being disk diffusion, agar dilution, broth macrodilution, broth microdilution, and E-test. Each of these procedures requires the use of specific testing conditions and methods, including media, incubation conditions and times, and the identification of appropriate quality control organisms along with their specific QC ranges. In the absence of standardized methods or reference procedures, antimicrobial susceptibility/resistance results from different laboratories cannot be reliably compared. Meanwhile the results can be determined as MIC and susceptibility, intermediate or resistant quantitative and qualitative AST method respectively. Despite the new influx of genotypic tests however, standardized phenotypic AST methods will still be required in the near future to detect emerging resistance mechanisms among bacterial pathogens. Although AST method aid in Rapid identification of pathogen and its resistance to antimicrobial drugs, they are not efficient, they do have shortcoming.

Therefore, based on the above conclusion the following recommendations were forwarded:

- In case of phenotypic antimicrobial susceptibility test method it is advisable to use automated method to overcome laborious steps in processing errors and forgetfulness.
- Attention should paid to each and every principle and procedure of AST methods in order not to come up with falsely resistant report.
- It is suggestible to interpret the result, if AST method is qualitative type as susceptibility, intermediate or resistant where as if it is quantitative type as MIC.
- It is feasible to minimize the limitation of AST method through interpreting result following international guideline and performing accurately the procedure and principle.

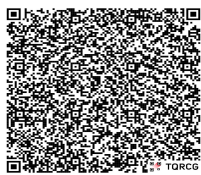
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