Review Article

Phenotypic Tests of Bacterial Antimicrobial Susceptibility Testing: A Systematic Review

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Abstract

Background: Although a variety of methods exist, the goal of *in-vitro* antimicrobial susceptibility testing is the same; to provide a reliable predictor of how a microorganism is likely to respond to antimicrobial therapy in the infected host. This type of information aids the clinician in selecting the appropriate antimicrobial agent, provides data for surveillance and aids in developing antimicrobial use policies. The objective of this review was to review phenotypic tests of bacterial antimicrobial susceptibility testing and to offer guidance in selecting the appropriate method of testing.

Result: In this review, we summarized the different phenotypic antimicrobial susceptibility tests including the principles, advantages and disadvantages. In addition, susceptibility testing of fastidious bacteria, anaerobic bacteria and actinomycets are separately discussed. *In-vitro* antimicrobial susceptibility testing can be performed using a variety of forms, the most common being disk diffusion, agar dilution, broth macro dilution, broth micro dilution, and a concentration gradient test.

Conclusion: The choice of antimicrobial susceptibility testing depends on different factors including the target organism, antimicrobial agent and testing intensions. The use of up-to-date interpretation breakpoints and regular quality control mechanisms is mandatory to maintain the reliability and reproducibility of test results and to draw the trends of antimicrobial susceptibility. Because phenotypic tests are time consuming and technically demanding, clinical laboratories should look for rapid, easy and accurate automated methods of antimicrobial susceptibility testing.

Introduction

Antimicrobial is an agent that kills micro-organisms or inhibits their growth. *Streptococcus pneumonia* developed up to 55% resistance to penicillin in some regions, *Salmonella typhi* (outbreaks of multi-resistant strains in 11 countries) [1]. Multi-Drug-Resistant Tuberculosis (MDR-TB) has emerged as a challenge to global Tuberculosis (TB) control and remains a major public health concern in many countries [2]. Historically, medical practitioners and veterinarians selected antimicrobials to treat bacterial infectious diseases based primarily on past clinical experiences. However, with the increase in bacterial resistance to traditionally used antimicrobials, it has become more difficult for clinicians to empirically select an appropriate antimicrobial agent [3].

As a result, *in vitro* Antimicrobial Susceptibility Testing (AST) of the relevant bacterial pathogens, from properly collected specimens, uses to validate methods.

Phenotypes are observable characteristics of cells. They can be easily observed, scored, and measured without requiring expensive technology. Antimicrobial susceptibility testing is screening of microbial presence, grow and identify organism and test for anti-microbial susceptibility or to predict the *in vivo* success or failure of antibiotic therapy. In combination; phenotypic testing of bacterial antimicrobial resistance; can be widely used in clinical and diagnostic microbiology laboratories [4].

These assays also are essential for new resistance discovery. In the United States dilution and disc diffusion tests are two basic methodologies that are standardized by the Clinical and Laboratory Standard Institute (CLSI), formerly known as a National Committee for Clinical Laboratory Standards (NCCLS). The lowest concentration that inhibits the visible growth of an organism is the MIC (Minimum Inhibitory Concentration) value [5]. Minimum Inhibitory Concentrations (MICs), the clinical laboratory standard guidelines provides for the interpretative criteria that give the value of MICs or growth inhibition zone sizes to determine the categories of susceptible, intermediate and resistant [5].

The performance of antimicrobial susceptibility testing is important to confirm susceptibility to chosen empirical antimicrobial agents or to detect resistance in individual bacterial isolates and to offer guidance to physician in selecting effective antimicrobial therapy for a pathogen in a specific body site [6]. Therefore, the objective of this review was to review phenotypic tests of bacterial antimicrobial susceptibility testing and to offer guidance in selecting the appropriate testing method.

Methodology

In this review, related research articles, review articles, textbooks and standard guidelines of known organizations such as CLSI were collected from PubMed and Google scholar based on the keywords phenotypic tests, antimicrobial susceptibility, and review. A total of 250 related literatures have been downloaded; the importance of each material was determined based on the objectives of this review and the 210 were not found significant for this review. Finally, 40 of the 250 literatures were systematically reviewed and sited for this output.

Results and Discussion

Selection of antimicrobials

Selecting the appropriate antimicrobials for susceptibility testing can be difficult due to vast numbers of agents available.

The following guidelines are noted when selecting antimicrobials. Antimicrobials in the same class may have similar *in-vitro* activities against selected bacterial pathogens. Certain microorganisms can be intrinsically resistant to particular antimicrobial classes. Periodic review of microorganisms that are currently predictably susceptible to certain antimicrobial agents is recommended to ensure that emergent, unexpected resistance is detected. Emerging resistance may also be suspected following poor response to a standard antimicrobial treatment regime [7].

The CLSI provides tables that list the antimicrobial agents appropriate for testing members of the *Entrobacteriaceae*, *Pseudomonas*, and other bacteria like *Staphylococcus*, *Enterococcus*, *Streptococcus*, *Haemophilus* species, etc.

The listings include recommendations for agents that are important to test routinely, and those that may be tested or reported selectively based on the institution's formulary [8]. Generally, labs choose 10-15 antibiotics to test susceptibility for Gram positive organisms and another 10-15 for Gram negative organisms; too many choices can confuse physicians and be too expensive. Primary objective is; use the least toxic, most cost-effective, and most clinically appropriate agents that refrain from more costly, broader-spectrum agents.

Antimicrobial susceptibility testing methods

Ease of performance, flexibility, adaptability to automated or semi-automated systems, cost, reproducibility, reliability and accuracy are factors affecting selection of AST methods [7].

The following methods (Dilution method, broth micro dilution, disk diffusion method, gradient diffusion and automated instrument methods) can be consistently providing reproducible and repeatable results when followed correctly [3,9].

Dilution method

This is quantitative assays used to determine Minimum Inhibitory Concentration (MIC) of the antibiotic. Serial dilutions of the antibiotic in broth or in agar are inoculated by standardized suspension of the microorganisms (105-106 bacteria/ml). Drugs at the lowest concentration of each antibiotic that inhibits visible growth of organisms designated as the Minimum Inhibitory Concentration (MIC). Ranges should encompass the concentrations used to define the interpretive categories (susceptible, intermediate, and resistant) of the antimicrobial agent [10]. The Mueller-Hinton medium used for the testing of frequently encountered pathogens (members of the family *Entrobacteriaceae*, *Staphylococci*, *Enterococci*, and some nonfermentative gram-negative *bacilli*, such as *Acinetobacter baumannii* and *Pseudomonas aeruginosa*) due to the flexibility of dilution methods [10].

Breakpoints derived by regulatory bodies and professional groups are frequently similar. Technical factors including incubation temperature and atmosphere, inoculums size, and test medium formulation, can affect MICs, justifying different breakpoints. Antimicrobial susceptibility dilution methods appear to be more reproducible and quantitative than agar disk diffusion [5].

Agar dilution

Agar testing is one of the standardized antimicrobial testing methods Mueller Hinton Agar (MHA) is used for testing nonfastdious aerobes and facultative anaerobic that requires no special supplement for growth [10]. To prevent the interference for drug activity, any calcium and magnesium containing supplement is not added.

Oxacillin MIC for *Staphylococcus* spp. carrying the mecA gene (is a gene that codes for a PBP (Penicillin-Binding Protein) that does not bind beta-lactam antibiotics) are detected with increased sensitivity by the agar containing NaCl [11].

The test method has the ability to test multiple bacteria, except bacteria that swarm, on the same set of agar plates at the same time and has the potential to improve the identification of MIC endpoints and extend the antibiotic concentration range. However; agar dilution is often recommended as a standardized AST method for fastidious organisms, such as anaerobes, *Campylobacter* and *Helicobacter* species.

Broth dilution method

Broth dilution is a technique in which a suspension of bacterium of a predetermined optimal or appropriate concentration is tested against varying concentrations of an antimicrobial agent (usually serial twofold dilutions) in a liquid medium of predetermined, documented formulation [7]. The antibiotic-containing tubes are inoculated with a standardized bacterial suspension of 1-5×105 CFU/ml. Following overnight incubation at 35˚C, the tubes are examined for visible bacterial growth as evidenced by turbidity [12].

The broth dilution method can be performed either in tubes containing a minimum volume of 2ml (macrodilution) or in smaller volumes using microtitration plates (microdilution). The broth macrodilution method is both reliable and well standardized and is of particular utility in research studies and in testing of a single antimicrobial agent for one bacterial isolate.

The method is, however, both laborious and time intensive and, because of the ready commercial availability of convenient micro-dilution systems, is not generally considered practical for routine use in clinical microbiology laboratories [13].

Standard trays contain 96 wells, each containing a volume of 0.1mL that allows approximately 12 antibiotics to be tested in a range of 8 two-fold dilutions in a single tray. Microdilution panels are typically prepared using dispensing instruments that aliquot precise volumes of pre-weighed and diluted antibiotics in broth into the individual wells of trays from large volume vessel test results may be determined either visually or through the use of semi automated or automated instruments [14]. However; the macro dilution method is tedious, manual task of preparing the antibiotic solutions for each test, the possibility of errors in preparation of the antibiotic solutions, and the relatively large amount of reagents and space required for each test.

Advantage of micro dilution: The generation of MICs, the reproducibility and convenience of having prepared panels, the economy of reagents and space that occurs due to the miniaturization of the test and assistance in generating computerized reports if an automated panel reader is used.

Disadvantage of micro dilution: Less flexible than agar dilution or disk diffusion in adjusting to the changing needs of the surveillance/monitoring programme and the purchase of antimicrobial plates and associated equipment is be costly; this methodology may not be feasible for some laboratories.

Agar disk diffusion

The disk diffusion susceptibility method is simple and practical and has been well-standardized. The test is performed by applying a bacterial inoculum of approximately 1-2×108 CFU/mL to the surface of a large (150 mm diameter) Mueller-Hinton agar plate. Up to 12 commercially-prepared, fixed concentrations, paper antibiotic disks are placed on the inoculated agar surface. Plates are incubated for 16-24 h at 35°C prior to determination of results [15,16].

It is routinely used for the testing of common, rapidly growing, and some fastidious bacterial pathogens.

The positive results of the disk diffusion test are "qualitative," in that a category of susceptibility (i.e., susceptible, intermediate, or resistant) is derived from the test rather than an MIC. However, some commercially-available zone reader systems claim to calculate an approximate MIC with some organisms and antibiotics by comparing zone sizes with standard curves of that species and drug stored in an algorithm [17]. With this testing method, commercially prepared filter paper disks impregnated with specified predetermined concentrations of the antibiotics to be assessed are applied to the surface of a defined agar medium previously inoculated with the bacterial pathogen.

The disk diffusion method for AST is standardized primarily for commonly encountered, rapidly growing bacterial pathogens and is applicable to neither anaerobes nor fastidious species that demonstrate marked variability in growth rate from strain to strain [18].

Although not all fastidious or slow growing bacteria can be accurately tested by this method, the disk test has been standardized

for testing *Streptococci*, *Haemophilus influenzae*, and *Neisseria meningitidis* through use of specialized media, incubation conditions, and specific zone size interpretive criteria [14].

Advantages: It is technically easy to perform and results are reproducible, the reagents and supplies are inexpensive, it does not require the use of expensive equipment, it generates categorical interpretive results well understood by clinicians and it allows for considerable flexibility in the selection of antibiotics for testing.

Disadvantage: A limited number of bacterial species can be tested using this method, is inadequate for detection of vancomycin-intermediate *Staphylococcus aureus* and It provides only a qualitative result, whereas a quantitative MIC result that indicates the degree of susceptibility may in some cases be required.

Gradient diffusion method

The antimicrobial gradient diffusion method uses the principle of establishment of an antimicrobial concentration gradient in an agar medium as a means of determining susceptibility. The E-test is a commercially available, it employs thin plastic test strips that are impregnated on the underside with a dried antibiotic concentration gradient and are marked on the upper surface with a concentration scale. Several strips containing different antimicrobial agents may be applied in a radial arrangement to the surface of large round plates, or they may be placed in opposite directions on large rectangular plates [19].

The MIC is determined by the intersection of the lower part of the ellipse shaped growth inhibition area with the test strip. The assays are performed in a manner similar to that for disk diffusion using a suspension of test organism equivalent in turbidity to that of a 0.5 McFarland standard to inoculate the surface of an agar plate. This method is best suited to situations in which an MIC for only 1 or 2 drugs is needed or when a fastidious organism requiring enriched medium or special incubation atmosphere is to be tested (eg, penicillin and ceftriaxone with pneumococci) [20]. Generally, E-test results have correlated well with MICs generated by broth or agar dilution methods.

Advantages: the ability to generate quantitative MIC results for infrequently tested antimicrobial agent and the option to test fastidious and anaerobic organisms, for which reliable disk diffusion methods and/or commercial systems are not available, through the use of specific enriched media. Gradient diffusion strips are, however, considerably more expensive than the paper disks used for diffusion testing.

Automated instrument methods

Use of instrumentation can standardize the reading of end points and often produce susceptibility test results in a shorter period than manual readings because sensitive optical detection systems allow detection of subtle changes in bacterial growth. There are different types of automated instruments (Micro Scan Walk away, BD phoenix, Trek Sensititere and Vitek 1 and Vitek 2). They can generate susceptibility test results within (3.5-16 hours) but the fourth one is overnight system. Gram-negative susceptibility test panels containing fluorogenic substrates can be read within 3.5-7 hours. Separate gram-positive and gram-negative panels read using turbidimetric end points are ready in 4.5-18 hours [21,22].

Advantage: Increased reproducibility decreased labor costs and issued rapid results.

Disadvantage: These are not available widely in developing country including Ethiopia.

Susceptibility testing of fastidious bacteria

Clinical microbiology laboratories are faced with the challenge of accurately detecting emerging antibiotic resistance among several important bacterial pathogens. Certain of these are fastidious organisms that require enriched media and modified growth conditions for reliable susceptibility testing (e.g. *S. pneumoniae*) [23]. Many fastidious bacterial species do not grow satisfactorily using standard *in vitro* susceptibility testing with unsupplemented media.

For several of more frequently encountered pathogens (eg. *S. pneumoniae, Streptococcus* spp. other than *S. pneumoniae, N. gonorrhoeae, N. meningitidis, H. influenzae* and *H. parainfluenzae*), modification is made to the standard CLSI. The CLSI has published guidelines for AST of the fastidious and/or infrequently recovered bacteria (*Aeromonas* spp., *Bacillus* spp. other than *Bacillus anthracis, Campylobacter coli, Campylobacter jejuni* and *Corynebacterium* spp. [24].

Susceptibility testing of anaerobic bacteria

Most anaerobic infections are caused by penicillin-sensitive bacteria, with the exception of infections originating in the intestinal tract or the vagina. Such infections generally contain *Bacteroides fragilis*, which produces β-lactamase and is resistant to penicillins, ampicillins and most cephalosporin. The importance of anaerobic bacteria as participants in and causes of significant infections and the need for specific antibiotic therapy for bacteremia and surgical prophylaxis against anaerobes are well documented. If practical, individual hospitals should establish antibiograms for the more frequently recovered anaerobes on a periodic basis and test individual patient isolates as needed to assist in patient care [25].

The agar dilution susceptibility testing method, which uses Brucella blood agar as the medium, is designated the reference method by the CLSI anaerobe working group. Because of the time-consuming, labor-intensive nature of this method, it is not generally considered practical for routine use in most clinical microbiology laboratories but serves as the reference method to which other more practical testing methods can be compared.

Alternative testing methods currently used include BMD (Broth Micro Dilution; is only standardized for members of the *Bacteroides fragilis* group) limited agar dilution, and gradient strip diffusion assays, such as E-test [14].

Susceptibility testing of *Nocardia* species and other aerobic actinomycetes

Nocardia asteroides, the most commonly recognized aerobic actinomycete, causes significant disease in immunocompromised patients. Other species associated with human disease include *Nocardia brasiliensis, Nocardia otitidiscaviarum, Nocardia farcinica, Nocardia nova* and *Nocardia transvalensis* [26]. Susceptibility testing results serve to guide initial therapeutic choices and may document emergence of drug resistance. No commercially available broth systems is cleared by the FDA (Food and Drug Administration) for

Nocardia spp. or other aerobic actinomycetes recommended drugs for primary testing are amikacin, amoxicillin-clavulanate, ceftriaxone, ciprofloxacin, clarithromycin, imipenem, linezolid, minocycline, moxifloxacin, trimethoprim-sulfamethoxazole, and tobramycin. Second-line drugs for testing include cefepime, cefotaxime, and doxycycline [27].

Susceptibility testing of *Mycobacteria*

Mycobacterial susceptibility testing is important for the management of patients with tuberculosis and those with disease caused by certain nontuberculous mycobacteria. According to Centers for Disease Control and Prevention report mycobacterial susceptibility testing guidelines, initial isolates from patients with tuberculosis should be tested for susceptibility to isoniazid, rifampin, ethambutol, and pyrazinamide [28].

Emerging and spread of drug resistance TB has encountered as a great challenge in Africa egion, Sub-Saharan Africa in particular. Information on the extent of MDR-TB from Africa region is very limited, probably due to poor laboratory facilities, poor surveillance mechanisms and reporting procedures, outdated databases and sub-optimal coverage of the infrequent surveys. Sub-Saharan Africa stands the burden of both very high TB incidence and the highest HIV prevalence rates in the world, and represents 14 % of the global burden of new MDR-TB cases [29]. In tuberculosis *bacilli*, resistance is by means of genetic mutations: codon 531 of the *rpoB* gene (rpoB531) is found to be the most frequent mutation associated with rifampicin resistance and codon 315 of the *katG* gene (katG315) is found to be the most frequent mutation associated with isoniazid resistance [30].

This guidance overrides the prior practice of performing susceptibility testing for only 3 drugs (isoniazid, rifampin, and ethambutol) and then only when a pulmonary or infectious disease clinician requested it. Current guidelines also state that susceptibility testing should be repeated after 3 months if the patient remains culture-positive despite appropriate therapy. However, susceptibility testing may be performed earlier if the patient appears to be failing to respond to therapy or if intolerance to the drug regimen is evident.

First-line susceptibility test results should be available for isolates of the Mycobacterium tuberculosis complex within 15 to 30 days of original receipt of the specimen in the laboratory [28]. However; ideally, susceptibility results should be available within 7 to 14 days of specimen receipt. If resistance to any of the 4 initially tested agents is discovered, testing of secondary drugs should be performed as soon as possible. If the isolate is resistant only to pyrazinamide, *Mycobacterium bovis* should be ruled out because most *M. tuberculosis* isolates are susceptible to pyrazinamide [27].

Both the agar proportion method and the radiometric method define resistance as growth of more than 1% of the inoculum of bacterial cells in the presence of an anti tubercular drug. The anti tubercular drugs are inoculated at specific *in vitro* concentrations, the values of which correlate to clinical responsiveness. If more than 1% of the bacterial population grows in the presence of a drug, that particular drug will not be of therapeutic utility [27]. The agar proportion method is used primarily to confirm results from commercial liquid broth systems and to test additional drugs that may not be available for testing using other systems. Susceptibility testing of Non-Tuberculous Mycobacteria (NTM) should be performed on isolates considered clinically significant.

The American Thoracic Society criteria for clinical significance of NTM are positive cultures from at least two sputum specimens or one bronchial wash or bronchial lavage specimen. Alternatively, a transbronchial or lung biopsy with histopathologic findings consistent with *Mycobacteria* and positive on culture for NTM is sufficient to be interpreted as clinically significant. However, accurate susceptibility predictions for other slowly growing Mycobacteria cannot be made. The standard susceptibility testing method for NTM is BMD [27]. The macrolides are the only antimicrobial agents that should be tested against *M. avium* complex because they are the only agents for which correlations have been demonstrated between *in vitro* susceptibility tests and clinical response [31].

Because the mutation leading to resistance is the same for clarithromycin and azithromycin, only one drug need to be tested. Generally, clarithromycin is tested because azithromycin demonstrates poor solubility. Commercially available broth systems have not yet been cleared by the FDA for slowly growing NTM.

Quality control and quality assurance

Adequate quality control or quality assurance systems should be established in AST performing laboratories: quality control refers to the operational techniques that are used to ensure accuracy and reproducibility of AST. Strict adherence to specified and documented techniques in conjunction with quality control (i.e. assurance of performance and other critical criteria) of media and reagents, record keeping, the appropriate reference microorganism(s) should be strictly performed and reference microorganisms must be obtained from a reliable source; for example, from the American Type Culture Collection (ATCC) [32].

Susceptibility testing of *Klebsiella pneumoniae* and *Staphylococcus aureus*

Klebsiella pneumoniae (*K. pneumoniae*) are ubiquitously present and reported worldwide. In recent years, *K. pneumoniae* has become important pathogens in nosocomial infections [4]. The importance of *K. pneumoniae* species in the ever increasing number of gram negative aerobic bacillary nosocomial infections in the United States and India has been well documented. Epidemic and endemic nosocomial infections caused by *K. pneumoniae* species are leading causes of morbidity and mortality [33]. In addition to being the primary cause of respiratory tract infections like pneumonia, rhinoscleroma, ozaena, sinusitis and otitis, it also causes infections of the alimentary tract like enteritis, appendicitis and cholycystitis [34].

Staphylococcus aureus has long been recognised as an important pathogen in many diseases, for example the toxic shock syndrome, vasculitis and glomerulonephritis [35]. Therapy of infection has become problematic due to an increasing number of Methicillin-Resistant Strains (MRSA). The difference between MRSA and methicillin-susceptible strains is that MRSA is resistant to *β*-lactamase stable *β*-lactam antibiotics. Often this is also associated with resistance to many other antibiotics, which limits the therapeutic options. The prevalence of MRSA has also increased world-wide and new therapeutic agents, optimization of infection control measures and introduction of new medical devices with a reduced risk of infection are being investigated [36].

External proficiency testing

To ensure that reported antimicrobial susceptibility data are accurate; member Countries should initiate an inter-laboratory proficiency testing programme. External proficiency testing can be carried out on a national basis. Laboratories in member Countries are also encouraged to participate in international inter-laboratory comparisons (e.g. Enter-Net). All bacterial species subjected to AST should be included. Countries should appoint or establish designated reference or national laboratories that are responsible for: monitoring the quality assurance programmes of laboratories participating in surveillance and monitoring of antimicrobial resistance, characterizing and supplying to those laboratories a set of reference microorganisms and creating managing, and distributing samples to be used in external proficiency testing [37].

Future directions in antimicrobial susceptibility testing

The antimicrobial susceptibility testing methods provides reliable results when used according to the procedures defined by the CLSI or by the manufacturers of the commercial products. However, there is considerable opportunity for improvement in the area of rapid and accurate recognition of bacterial resistance to antibiotics.

There is a need for development of new automated instruments that could provide faster results and also save money by virtue of lower reagent costs and reduced labor requirements.

The use of genotypic methods for detection of antimicrobial resistance genes is promoted as a way to increase the rapidity and accuracy of susceptibility testing [38]. Numerous DNA-based assays are being developed to detect bacterial antibiotic resistance at the genetic level. The newest and perhaps most state-of-the-art approach is to predict antimicrobial resistance phenotypes via identification and characterization of the known genes that encode specific resistance mechanisms. Methods that employ the use of comparative genomics, genetic probes, microarrays, nucleic acid amplification techniques (e.g. Polymerase Chain Reaction (PCR) and DNA sequencing offer the promise of increased sensitivity, specificity and speed in the detection of specific known resistance genes [38,39].

There are hundreds of *β*-lactamases, and numerous mutations, acquisitions, and expression mechanisms that result in fluoroquinolone, aminoglycoside, and macrolide resistance; too many to be easily detected by current molecular techniques [40]. However, despite the new influx of genotypic tests, documented and agreed upon phenotypic AST methods will still be required in the near future to detect emerging resistance mechanisms among bacterial pathogens.

Conclusion

Even though there are various methods, choice of antimicrobial susceptibility testing depends on different factors including the target organism, antimicrobial agent and testing intensions. Above all, the growth requirement or fastidious nature of the organism highly determines the selection of a particular method of susceptibility testing. The use of up-to-date interpretation breakpoints and establishing regular quality control mechanisms is mandatory to maintain the reliability and reproducibility of test results and to draw the trends of antimicrobial susceptibility. Because phenotypic tests are time consuming and technically demanding, clinical laboratories should look for rapid, easy and accurate automated methods of

antimicrobial susceptibility testing. The implementation of advanced genotypic methods enables detection of drug resistance of a particular microbe at genetic level.

Authors' contribution

AH: Conceived, designed, proposed the review article idea, collecting important materials, systematically reviewed the review article and prepared the manuscript. MT and DG: prepared the initial and final version of the manuscript for publication. All authors read and approved the final version of the manuscript.

Acknowledgement

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Citation: Atsbaha AH, Tedla DG and Shfare MT. Phenotypic Tests of Bacterial Antimicrobial Susceptibility Testing: A Systematic Review. SM J Clin Med. 2017; 3(1): 1020.

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