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DETECTION OF ANTIMICROBIAL RESISTANCE IN COMMON GRAM NEGATIVE AND GRAM POSITIVE BACTERIA ENCOUNTERED IN INFECTIOUS DISEASES – AN UPDATE

Resistance to antimicrobial agents (AMR) has resulted in increased morbidity and mortality from treatment failures and increased health care costs. Although defining the precise public health risk and estimating the increased costs is not a simple undertaking, there is little doubt that emerging antibiotic resistance is a serious global problem. The present write-up enumerates various methods for detecting the resistance of common bacteria to various antimicrobial agents as available today.

Prudent Use of Antibiotics

Appropriate antimicrobial drug use has unquestionable benefit, but physicians and the public frequently use these agents inappropriately. Inappropriate use results from physicians unnecessarily prescribing expensive broad spectrum antimicrobial drugs to treat viral infections, using inadequate criteria for diagnosis of infections that potentially have a bacterial etiology, and not following established recommendations for using chemoprophylaxis.

Widespread antibiotic usage exerts a selective pressure that acts as a driving force in the development of antibiotic resistance. The association between increased antimicrobial use and resistance has been documented for nosocomial infections as well as for resistant community acquired infections. As resistance develops to first-line antibiotics, therapy with new (Table I) broad spectrum, more expensive antibiotics

Table I. Newer antibiotics tested for antimicrobial susceptibility for cofmmonly occurring pathogenes in infectious diseases hospitals.

β-lactams

Cephalosporins: cefazolin, cefotaxime, ceftazidime, ceftriaxone and cefuroxime, cefepime (1998),

Newer penicillins: nafcillin (1996): anti staphylococcal

Carbapenems: etrapenem (2002), imipenem and meropenem (1998), feropenem

Monobactams: aztreonam.

Fluoroquinolones: ciprofloxacin, levofloxacin, moxifloxacin, gemifloxacin (2000) for pneumococci, trovafloxacin (1999), norfloxacin and ofloxacin.

Aminoglycosides: amikacin, gentamicin, netilmicin and tobramycin, arbekacin (2000) for MRSA, streptogramin, quinapristine/dalfopris (1999) for VRE (*E. faecium*)

Glycopeptides: teicoplanin and vancomycin.

Macrolides: telithromycin (2000), azithromycin, erythromycin, tigecycline.

Other agents: linezolid (2000) against VRE, daptomycin (1999), doxycycline, minocycline, tetracycline, TMP-SMX, metronidazole, clindamycin, nitrofurans, chloramphenicol, rifampicin, *etc* are the older antibiotics which are in use.

increases. It is in turn followed by development of resistance to the new class of drugs also. Over the last 20 years, β-lactam antibiotics (penicillins, cephalosporins, carbapenems, *etc*.) are the most commonly used drugs.

Role of Laboratory

The bacteriology laboratory must provide facilities for culture and identification of relevant pathogens as also the antibiotic sensitivities of these pathogens to a wide range of antibiotics. Moreover, a clinical microbiologist must be aware of local strains and their sensitivity patterns to help the clinicians.

How resistance develops?

The main mechanisms of development of resistance are as follows:

Mutation

Mutation occurs spontaneously at fairly constant rates, usually in the range of 10^2-10^{10} cell division (*eg* in *M. tuberculosis, Streptococcus pneumoniae, etc)*.

Plasmid Mediated Drug Resistance

Conjugation between the commensal-commensal, commensal-pathogen and pathogen-pathogen are responsible for the development of resistance in bacteria. Acquisition of resistance by transduction is common in gram positive bacteria like Staphylococcus (penicillinase plasmid); where as in gram negative bacteria conjugation is a major mechanism of transfer of drug resistance and can occur in unrelated genera. Transferable drug resistance in enterobacteriaceae involves all antibiotics in common use. Bacteria containing R-plasmid can spread from animal to human. Hence indiscriminate use of antibiotics in human and animals or in animal feed can increase spread of plasmid mediated drug resistance.

Currently new resistance mechanisms like class A ESBL (Extended Spectrum β-lactamases), class B MBLs (Metallo-β-lactamases), carbapenemases and class C AmpC β-lactamases (cephalosporinases) have also been commonly seen.

Selection Pressure

It is not an independent mechanism. Antibiotic pressure is an attribute. Selection pressure increases the growth of resistance bacteria.

Multidrug resistance (MDR) is another challenge faced by the clinicians which occurs due to inappropriate use of antibiotics. Multidrug-resistant pathogens travel not only locally but also globally, and newly introduced pathogens are spreading rapidly in susceptible hosts. Antibiotic resistance patterns may vary locally and regionally, so surveillance data need to be collected from selected sentinel sources. Patterns can change rapidly and they need to be monitored closely because of their implications for public health and as an indicator of appropriate or inappropriate antibiotic usage by physicians in that area.

The results of *in vitro* antibiotic susceptibility testing (AST) guide clinicians in the appropriate selection of initial empiric regimens and, drugs used for individual patients in specific situations. The selection of an antibiotic panel for susceptibility testing is based on the commonly observed susceptibility patterns, and is revised periodically.

Various methods[1-14] are available for the susceptibility testing. Disc diffusion methods are suitable for organisms that grow rapidly at 35-37^{0}C. The techniques are technically simple, cheap and reliable, but there is no single internationally accepted method of disc diffusion testing. In the United Kingdom majority of laboratories use the modification of Stoke's disk diffusion method (originally started to use for primary cultures where inoculum standardisation could not be ensured) and BSAC (British Society of Antimicrobial Chemotherapy) methods. However, agar disc diffusion is accepted as a standard operating procedure (SOP). Many countries including France, Germany, Sweden, and United States have adopted disc diffusion method of CLSI (Clinical and Laboratory Standards Institute)[12,14] and the updates are published every 2-3 years which are accepted world-wide including the laboratories in India. However, there is a difference in methods used in USA and UK. The main difference between BSAC[6] (UK) and CLSI[12,14] (USA) guidelines being the number of isolates (250 vs 500) tested respectively. Further, for methicillin resistant *Staphylococcus aureus* (MRSA) detection in BSAC guidelines methicillin is used where as in CLSI oxacillin is used.

For fastidious organisms like *S. pneumococci* and *Neisseria gonorrhoeae*, resistance is expressed as MIC (minimum inhibitory concentration) and data is available by regression line analysis[9]. The antibiotic sensitivity report is recorded as sensitive, intermediate or resistant as per the therapeutic successes. The MIC breakpoint does vary between BSAC and CLSI methods with some antibiotics like gentamicins in enterobacteriaceae. However, in

Haemophilus and *Pseudomona*s CLSI has achieved more acceptance. In India both the methods are used, but CLSI methods are commonly used in most Indian laboratories. Further, according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) only MIC breakpoints are revised for clinically resistant, clinically susceptible and clinically intermediates isolates.

Effectilveness of antibiotics will depend on various parameters. Formulary should be available to the prescriber as well as in the hospital. Aggressive hospital infection control will reduce the development of the resistance. For this, a simple, concise operative procedure in the laboratory is described in this write-up.

In UK, test of organisms of unknown sensitivity and control organism of known sensitivity are set up at the same time by modified Stoke's method in one plate. Interpretation includes comparison of size of inhibition zone. Analysis of data from UK National External Quality Control reveals that major errors are common, particularly reporting of false susceptibility and failure to use control organisms. In the USA a modified Kirby-Bauer method using the control in a separate plate is used. This method is recommended by CLSI[14] and the World Health Organization[13]. Standardization of techniques, controls, variations and interpretation is done by comparison of inhibition zones with published tables of critical zone diameter in both the methods.

PRINCIPLE OF ANTIMICROBIAL SUSCEPTIBILITY TESTING

The principles of determining the effectivity of a noxious agent to a bacterium have been well enumerated[1-14]. The discovery of antibiotics rendered these tests(or their modification)too cumbersome for the large number of tests necessary to be put up as a routine. The ditch plate method of agar diffusion used by Alexander Fleming was the forerunner of a variety of agar diffusion methods devised by workers in this field[1]. The Oxford group used these methods initially to assay the antibiotics contained in blood by allowing the antibiotics to diffuse out of reservoirs in the medium in containers placed on the surface.

With the introduction of a variety of antimicrobials it became necessary to perform the antimicrobial susceptibility test as a routine. For this, the antimicrobial contained in a reservoir was allowed to diffuse out into the medium and interact in a plate freshly seeded with the test organisms. Even now a variety of antimicrobial containing reservoirs (commercially available) are used but the antimicrobial impregnated absorbent paper disc is by far the commonest type used[9]. The disc diffusion method of antimicrobial susceptibility testing is the most practical method and is still the method of choice in most of laboratories. Automated methods may force disk diffusion out of the diagnostic laboratory but in this country as well as in the smaller laboratories of even advanced countries, it will certainly be the most commonly carried out microbiological test for many years to come. It is, therefore, imperative that microbiologists understand the principles of the test well and keep updating the information as and when necessary. All techniques involve either diffusion of antimicrobial agent in agar or dilution of antibiotics in agar or broth. Even automated techniques are variations of the above methods.

Basic sets of antimicrobials for routine susceptibility tests and guidelines for groups of antimicrobials for common hospital pathogens along with zone of inhibition for sensitive/intermediate/resistant are summarized in table IIa. Quality control (QC) strains and antimicrobials for interpretation are given in table IIb. These QC strains are available wilth most of the laboratory using these methods. Reporting of sensitivity pattern, suggested antibiotics on the basis of sensitivity (table III) and serum concentration per ml should be given at the bottom of the report.

ENTEROBACTERIACEAE AND PSEUDOMONAS

This family consists of gram-negative bacilli that can be found in the normal flora of human and animal intestinal tracts. When present in the environment they are usually in the form of fecal flora. Some members of this family are pathogenic to humans and animals and generally infect through ingestion *e.g. Shigella, Salmonella, E. coli, Enterobacter, Klebsiella, etc.*

The most important mechanism of antimicrobial resistance in enterobacteriaceae is production of β-lactamases. Other mechanisms of resistance include modification of the outer membrane proteins, alteration of the target sites of antibacterial activity, and active efflux pump that results in the expulsion of the antibiotic out of the bacterial cell.

Routine Methods for Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing methods are divided into three types based on the principle applied in each system. These include: (a) disk diffusion; (b) dilution; and (c) diffusion and dilution

Table II. Guidelines for antimicrobial susceptibility testing for commonly occurring pathogens (CLSI, 2006)[14]

(a) Suggested battery of antibiotics for susceptibility testing

Staphylococcus[1] *sp*	Gram negative bacilli	*Streptococcus (Enterococcus*[2]*, Pneumococcus)*	*Haemophilus sp.*	*N. gonorrhoeae*	*Pseudomonos*
Penicillin	Ampicillin*	Penicillin	Ampicillin	Penicillin	Piperacillin*
Oxacillin*	Piperacillin**	Oxacillin	Amoxycillin/ Clavulanic acid	Cefazolin	Gentamicin*
Cephalothin	Cephalothin*	Ampicillin/ Amoxycillin	Cefuroxime	Ceftriaxone	Tobramycin*
Gentamicin**	Cefotaxime**	Cefotaxime**	Cefotaxime**	Chloramphenicol	Amikacin**
Netilmicin	Ceftazidime	Erythromycin	Tetracycline	Ciprofloxacin	Ciprofloxacin**
Amikacin**	Gentamicin*	Chloramphenicol	Erythromycin		Ceftazidime**
Chloramphenicol*	Netilmicin	Tetracycline	Chloramphenicol		Imipenem***
Tetracycline*	Amikacin**	Vancomycin	Ciprofloxacin**		Meropenem***
Erythromycin*	Chloramphenicol*	Teicoplanin**			Ertapenem***
Co-trimoxazole**	Tetracycline*	Levofloxacin**			
Clindamycin**	Co-trimoxazole*	Imipenem***			
Ofloxacin	Nalidixic Acid*	Meropenem***			
Rifampicin	Ciprofloxacin**				
Vancomycin	Ofloxacin***				
Teicoplanin	Nitrofurantoin				
Nitrofurantoin**	Imipenem***				
	Meropenem***				
	Sulphonamide*				
	Trimethoprim				

1. Coagulose negative *Staphylococcus.* 2. Vancomycin resistant enterococci.

*First line choice of drugs, **Second line choice of drugs, ***Third line choice of drugs and is used in hospitals where *Pseudomonas* or *Acinetobacter* are suspected. Ertapenem is used for infection due to ESBLs.

Only first line choice should be reported. Second and third line choice only if asked for and/or if first line choice is resistant. Chloramphenicol should be reported only in case of typhoid fever (*Salmonella typhi/paratyphi*) and CNS involvement like meningitis. Newer antimicrobials (*e.g.* azithromycin, spectinomycin) are generally sensitive if the older groups (like erythromycin) are sensitive.

Table II. Guidelines for antimicrobial susceptibility testing for commercially occurring pathogens: (b) Control limits for monitoring antimicrobial disk susceptibility test[12,13,14]

Antimocrobial agent	Disk content	*E. coli* ATCC 25922	*Staphylococcus aureus* ATCC 25922	*Pseudomonas aeruginosa* ATCC 27853
Amikacin	30µg	19-26	20-26	18-26
Amoxicillin/Clavulanic acid	20/10 µg	19-25	28-36	--
Ampicillin	10 µg	16-22	27-35	--
Ampicillin/sulbactam	10/10 µg	20-24	29-37	--
Azithromycin	15 µg	--	21-26	--
Aztreonam	30 µg	28-36	--	23-29
Carbenicillin	100 µg	23-29	--	18-24

contd...

Antimocrobial agent	Disk content	*E. coli* ATCC 25922	*Staphylococcus aureus* ATCC 25922	*Pseudomonas aeruginosa* ATCC 27853
Cefaclor	30 µg	23-27	27-31	--
Cefamandole	30 µg	26-32	26-34	--
Cefazolin	30 µg	23-29	29-35	--
Cefepine	30 µg	29-35	23-29	24-30
Cefaxime	5 µg	23-27	--	--
Cefmatazole	30 µg	26-32	25-34	--
Cefaperazone	75 µg	28-34	24-33	23-29
Cefotaxime	30 µg	29-35	25-31	18-22
Cefoxitin	30 µg	23-29	23-29	--
Cefpodoxime	10 µg	23-28	19-25	--
Ceftazidime	30 µg	25-32	16-20	22-29
Ceftibuten	30 µg	27-35	--	--
Ceftizoxime	30 µg	30-36	27-35	12-17
Ceftriaxone	30 µg	29-35	22-28	17-23
Cefuroxime	30 µg	20-26	27-35	--
Cefalothin	30 µg	15-29	29-37	--
Chloramphenicol	30 µg	21-27	19-26	--
Ciprofloxacin	5 µg	30-40	22-30	25-33
Clarithromycin	15 µg	--	26-32	--
Clindamycin	2 µg	--	24-30	--
Doxycycline	30 µg	18-24	23-29	--
Erythromycin	15 µg	--	22-30	--
Fleroxacin	5 µg	28-34	21-27	12-20
Gentamicin	10 µg	19-26	19-27	16-21
Imepenem	10 µg	26-32	--	20-28
Levofloxacin	5 µg	29-37	25-30	19-26
Linezolid	30 µg	--	27-31	--
Lomefloxacin	10 µg	27-33	23-29	22-28
Lorcarbef	30 µg	23-29	23-31	--
Meropenem	10 µg	28-34	29-37	27-33
Methicillin	5 µg	--	17-22	--
Minocycline	30 µg	19-25	25-30	--
Moxalactam	30 µg	28-35	18-24	17-25
Nalidixic Acid	30 µg	22-28	--	--
Netilmicin	30 µg	22-30	22-31	17-23
Nitrofurantoin	300 µg	20-25	18-22	--
Norfloxacin	10 µg	28-35	17-28	22-29
Ofloxacin	5 µg	29-33	24-28	17-21
Oxacillin	1 µg	--	18-24	--
Penicillin	10 µg	--	26-37	--

contd...

Antimocrobial agent	Disk content	*E. coli* ATCC 25922	*Staphylococcus aureus* ATCC 25922	*Pseudomonas aeruginosa* ATCC 27853
Piperacillin	100 µg	24-30	--	25-33
Piperacillin/Tazobactem	100/10 µg	24-30	27-36	25-33
Rifampacin	5 µg	8-10	26-34	--
Sparfloxacin	5 µg	30-38	27-33	21-29
Sulfisoxazole	250/300 µg	15-23	24-34	--
Teicoplanin	30 µg	--	15-21	--
Tetracycline	30 µg	18-25	24-30	--
Ticarcillin	75 µg	24-30	--	22-28
Ticarcillin/Clavulanic acid	75/10 µg	25-29	29-37	20-28
Tobramycin	10 µg	18-26	19-29	19-25
Trimethoprim	5 µg	21-28	19-26	--
Trimethoprim/sulfamethoxazole	1.25/23.75	24-32	24-32	--
Trovafloxacin	10 µg	29-36	29-35	21-27
Vancomycin	30 µg	--	17-21	--

– An *Enterococcus faecalis* (ATCC 29212 or 33186) may be tested with trimethoprim/sulfamethoxazole disks. An inhibition zone of > 20 mm that is essentially free of fine colonies indicates a sufficiently low level of thymine and thymidine in Muller Hinton agar.

– Control strains may be taken from the nearby reference laboratory or else may be requsitioned from (CRI, Kasauli, India), NCTC (National Collection of Type Cultures) Colindale, UK or ATCC (American Type Culture Collection), CDC, Atlanta, USA.

Table III. Sensitivity reporting to commonly used groups of antimicrobials for the bacteria

Test/Report Group	Antimicrobial agent	Disk content (Cone/ml)	Zone diameter (Nearest whole mm)			Equivalent MIC breakpoints (µg/ml)	
			R	I	S	R	S
Penicillins							
A	Ampicillin	10 µg	≤13	14-16	≥17	≥32	≤8
B	Piperacillin	100 µg	≤17	18-20	≥21	≥128	≤16
B	Ticarcillin	75 µg	≤14	15-19	≥20	≥128	≤16
U	Carbenicillin	100 µg	≤19	20-22	≥23	≥64	≤16
β-lactam/β-lactamase inhibitor combinations							
B	Amoxicillin/Clavulanic acid	20/10 µg	≤13	14-17	≥18	≥16/8	≤8/4
B	Ampicillin/Sulbactam	10/10 µg	≤11	12-14	≥15	≥32/16	≤8/4
B	Piperacillin/tazobactam	100/10 µg	≤17	18-20	≥21	≥128/4	≤16/4
B	Ticarcillin/Clavulanic acid	75/10 µg	≤14	15-19	≥20	≥128/2	≤16/2
Cephems							
A	Cefazolin	30 µg	≤14	15-17	≥18	≥32	≤8
A	Cephalothin	30 µg	≤14	15-17	≥18	≥32	≤8
B	Cefamandole or	30 µg	≤14	15-17	≥18	≥32	?8
B	Cefonicid or	30 µg	≤14	15-17	≥18	≥32	≤8
B	Cefuroxime (oral) or	30 µg	≤14	15-22	≥23	≥32	≤4
B	Cefuroxime(Parenteral)	30 µg	≤14	15-17	≥18	≥32	≤8
B	Cefepine	30 µg	≤14	15-17	≥18	≥32	≤8
B	Cefmetazole	30 µg	≤12	13-15	≥16	≥64	≤16
B	Cefoperazone	75µg	≤15	16-20	≥21	≥64	≤16

contd...

Test/Report Group	Antimicrobial agent	Disk content (Cone/ml)	Zone diameter (Nearest whole mm)			Equivalent MIC breakpoints (µg/ml)	
			R	I	S	R	S
B	Cefotetan	30 µg	≤12	13-15	≥16	≥64	≤16
B	Cefoxicitin	30 µg	≤14	15-17	≥18	≥32	≤8
B	Cefotaxime or	30 µg	≤14	15-22	≥23	≥64	≤8
B	Ceftizoxime or	30 µg	≤14	15-19	≥20	≥32	≤8
B	Ceftriaxone	30 µg	≤13	14-20	≥21	≥64	≤8
C	Ceftazidime	30 µg	≤14	15-17	≥18	≥32	≤8
O	Cefaclor	30 µg	≤14	15-17	≥18	≥32	≤8
O	Cefpodoxime	10µg	≤17	18-20	≥21	≥32	?
O	Moxalactam	30 µg	≤14	15-22	≥23	≥64	≤
Carbapenems							
B	Imipenem or	10µg	≤13	14-15	≥16	≥16	≤4
B	Meropanam	10µg	≤13	14-15	≥16	≥16	≤4
Monobactams							
C	Aztreonam	30µg	≤15	16-21	≥22	≥32	≤8
Amonoglycosides							
A	Gentamicin	10 µg	≤12	13-14	≥15	≥8	≤4
B	Amikacin	30µg	≤14	15-16	≥17	≥32	≤16
C	Kanamycin	30µg	≤13	14-17	≥18	≥25	≤6
C	Netilmicin	30µg	≤12	13-14	≥15	≥32	≤12
C	Tobramicin	10 µg	≤12	13-14	≥15	≥8	≤4
Tetracyclines							
C	Tetracycline	30µg	≤14	15-18	≥19	≥16	≤4
C	Doxycycline	30µg	≤12	13-15	≥16	≥16	≤4
O	Minocycline	30µg	≤14	15-18	≥19	≥16	≤4
Quinolones			≤				
B	Ciprofloxacin or	5µg	≤15	16-20	≥21	≥4	≤1
B	Levofloxacin	5µg	≤13	14-16	≥17	≥8	≤2
U	Lomefloxacin or	10µg	≤18	19-21	≥22	?8	≤2
U	Norfloxacin or	10µg	≤12	13-16	≥17	≥16	≤4
U	ofloxacin	5µg	≤12	13-15	≥16	≥8	≤2
O	Nalidixic Acid	30µg	≤13	14-18	≥19	≥32	≤8
Others							
B	Trimethoprim/ sulfamethoxazole	1.25/23.75µg	≤10	11-15	≥16	≥8/152	≤2/38
C	Chloramphenicol	30µg	≤12	13-17	≥18	≥32	≤8
U	Nitrofuratoin	300µg	≤14	15-16	≥17	≥128	≤32
U	Trimethoprim	5µg	≤ 12	11-15	≥16	≥16	≤4

For isolates of *Salmonella* and *Shigella spp.* only ampicillin, a quinolone, and trimethoprim/sulfamethoxazole should be tested and reported routinely. In addition, chloramphenicol and a third generation cephalosporin should be tested and reported for extraintestinal isolates of *Salmonella spp.*

ESBL producing strains may be resistant clinically to all cephems and aztreonam.

For *Salmonella spp*., and *Shigella spp*., aminoglycosides may appear active *in vitro* but are ineffective clinically and should not be reported as susceptible.

Tetracycline is the representative for all tetracyclines and the results can be applied to doxycycline and minocycline. Certain organisms, however, may be more susceptible to minocycline and doxycycline than tetracycline.

Disk Diffusion

Reagents for the disk diffusion test

Mueller-Hinton agar medium

Of the many media available, Mueller-Hinton agar is considered to be the best for routine susceptibility testing of non-fastidious bacteria for the following reasons:

- It shows acceptable batch-to-batch reproducibility for susceptibility testing.
- It is low in sulphonamide, trimethoprim, and tetracycline inhibitors.
- It gives satisfactory growth of most non-fastidious pathogens.
- A large body of data and experience has been collected concerning susceptibility tests performed with this medium.

Although Mueller-Hinton agar is generally reliable for susceptibility testing, results obtained with some batches may, on occasion, vary significantly. If a batch of medium does not support adequate growth of a test organism, zones obtained in a disk diffusion test will usually be larger than expected and may exceed the acceptable quality control limits. Only Mueller-Hinton medium formulations that have been tested according to, and that meet the acceptance limits described in CLSI document M62-A7- Protocols for evaluating dehydrated Mueller-Hinton agar should be used[12,14].

Antibiotic stock solutions

Antibiotics may be received as powders or tablets. It is recommended to obtain pure antibiotics from commercial sources, and not use injectable solutions. Powders must be accurately weighed and dissolved in the appropriate diluents to yield the required concentration, using sterile glassware. Standard strains of stock cultures should be used to evaluate the antibiotic stock solution.

Preparation of dried filter paper disks

Whatman filter paper no. 1 is used to prepare discs approximately 6 mm in diameter, which are placed in a petri dish and sterilized in a hot air oven.

Turbidity standard for inoculum preparation

To standardize the inoculum density for a susceptibility test, a BaSO4 turbidity standard, equivalent to a 0.5 McFarland standard or its optical equivalent *(e.g.*, latex particle suspension), should be used.

Methodology

The Kirby-Bauer and Stokes' methods are usually used for antimicrobial susceptibility testing[6,12-14]. The accuracy and reproducibility of these tests are dependent on maintaining a standard set of procedures. Interpretative criteria of CLSI[14] are developed based on international collaborative studies which correlate with MIC's and with clinical data. CLSI interpretative criteria are revised frequently based on the results of non-resistance studies.

Growth method is not used routinely for inoculum preparation. As a convenient alternative to the growth method, the inoculum can be prepared by making a direct broth or saline suspension of isolated colonies selected from an 18- to 24-h agar plate (a nonselective medium, such as blood agar should be used). The suspension is adjusted to match the 0.5 McFarland turbidity standards, using saline and a vortex mixer. This is also a recommended method for testing the fastidious organisms, *Haemophilus spp., Neisseria gonorrhoeae,* and streptococci, and for testing staphylococci for potential methicillin or oxacillin resistance.

Inoculation of test plates

The sterile cotton swab is dipped into the adjusted suspension, rotated several times and pressed firmly on the inside wall of the tube above the fluid level. This will remove excess inoculum from the swab. The dried surface of a Müeller-Hinton agar plate is inoculated by streaking the swab over the entire sterile agar surface. This procedure is repeated by streaking two more times.

Application of discs to inoculated agar plates

The predetermined battery of antimicrobial discs is dispensed onto the surface of the inoculated agar plate. The plates are inverted and placed in an incubator set to 35^0C within 15 min after the discs are applied. With the exception of *Haemophilus spp*., streptococci and *N.gonorrhoeae,* the plates should not be incubated in an increased Co_2 atmosphere, because the interpretive standards were developed by using ambient air incubation, and Co_2 will significantly alter the size of the inhibitory zones of some agents.

Reading and Interpretation of results

Quality control strains: *E. coli* ATCC 25922, *E. coli* ATCC 35218 (for β-lactam/β-lactamase inhibitor combinations)

- The diameters of the zones of complete inhibition (as judged by the unaided eye) are measured, including the diameter of the disc. If the test organism is a *Staphylococcus* or *Enterococcus spp.* 24 h of incubation are required for vancomycin and oxacillin, but other agents can be read at 16 to 18 h. Transmitted light (plate held up to light) is used to examine the oxacillin and vancomycin zones for light growth of methicillin- or vancomycin- resistant colonies, respectively, within apparent zones of inhibition. Any discernable growth within zone of inhibition is indicative of methicillin or vancomycin resistance.
- When using blood-supplemented medium for testing streptococci, the zone of growth inhibition should be measured, not the zone of inhibition of hemolysis. With trimethoprim and the sulfonamides, antagonists in the medium may allow some slight growth; therefore, slight growth (20% or less of the lawn of growth) should be disregarded and more obvious margin need to be measured to determine the zone diameter.
- The sizes of the zones of inhibition are interpreted by referring to tables III and the organisms are reported as either susceptible, intermediate, or resistant to the agents that have been tested. Some agents may only be reported as susceptible, since only susceptible breakpoints are given.

Dilution

Minimum inhibitory concentration

Dilution susceptibility testing methods are used to determine the minimal concentration of antimicrobial to inhibit or kill the microorganism. This can be achieved by dilution of antimicrobial in either agar or broth media. Antimicrobials are tested in $\log_2$ serial dilutions (two fold). When equivocal results are obtained or in prolonged serious infection *e.g.* bacterial endocarditis, the quantitation of antibiotic action vis-a-vis the pathogen needs to be more precise. Also the terms susceptible and resistant can have a realistic interpretation. Thus when in doubt, the way to a precise assessment is to determine the minimum inhibitiory concentration of the antibiotic to the organisms concerned.

Broth dilution method

The broth dilution method is a simple procedure for testing a small number of isolates, even single isolate. It has the added advantage that the same tubes can be used for testing minimum bactericidal concentration (MBC). Doubling dilution of the antibiotics in overnight culture is tested in this method.

Reading and interpretation of results

MIC is expressed as the lowest dilution, which inhibited growth judged by lack of turbidity in the tube. Standard strain of known MIC value run with the test is used as the control to check the reagents and conditions. Because very faint turbidity may be given by the inoculum itself, the inoculated tube kept in the refrigerator overnight may be used as the standard for the determination of complete inhibition.

Minimum bactericidal concentrations

The main advantage of the broth dilution method for the MIC determination lies in the fact that it can readily be converted to determine the MBC.

To determine MBC, the tubes are incubated overnight. MIC has already been checked for the correctness of the drug concentration as well as the lowest concentration inhibiting growth of the organism, but the tubes not showing visible growth must be subcultured and comparison may be made for the amount of growth from the control tube before incubation, which represents the original inoculum. The test must include a second set of the same dilutions inoculated with an organism of known sensitivity. These tubes are not subcultured; the purpose of the control is to confirm by its MIC that the drug level is correct, whether or not this organism is killed is immaterial.

Reading and interpretation of results

These subcultures may show

- Similar number of colonies–indicating the antibiotic to be bacteriostatic only.
- A reduced number of colonies–indicating a partial or slow bactericidal activity.
- No growth–if the whole inoculum has been killed indicating bactericidal activity.
- The highest dilution showing at least 99% inhibition is taken as MBC.

Diffusion and Dilution

E-Test

E test is a commercially available rapid test for MIC. E-test strips are drug impregnated strips that contain a gradient of antibiotic on one end and ceftazidime plus clavulanic acid on the other end. After inoculating the Muller Hinton agar plates with the standard inoculums using 0.5 McFarland, E-test strips should be placed over the plate and then the plates should be incubated overnight at 37°C. After overnight incubation reading should be performed.

PREVALENCE OF ESBL, AMPC (CEPHALOSPORINASES) AND CARBAPENEM HYDROLYZING β-LACTAMASES

Every day new β-lactamases seems to be detected for resistance in gram negative bacteria. Extensive use of antimicrobials led to the evolution of resistance due to ESBL, AmpC and carbapenem hydrolyzing β-lactamases. Wide spread dissemination of these mechanism of drug resistance has important clinical and therapeutic implilcations in isolates of enterobacteriaceae. Testing for all these has not become popular because of lack of awareness. Extended spectrum β-lactamases) are the result of mutation in the ubiquitous class A TEM or SHV β-lactamases. TEM-1 accounts for 75% of all plasmid encoded amoxicillin resistance throughout the world and for the majority of all β-lactamase mediated resistance. The later generation of cephalosporins were developed in part to overcome the effect of mutations in the TEM and SHV molecules provided with nearly 30 new enzymes that can confer resistance to nearly all cephalosporins including cefotaxime and ceftazidime. All common extended spectrum β-lactamases remain sensitive to inhibition by clavulanic acid. Therefore any strain that is resistant to cefotaxime or ceftazidime, becomes sensitive in presence of clavulanic acid, is likely to contain ESBL. This can be readily demonstrated by a double disc test, but no discs of clavulanic acid are commercially available. However, it is possible to use clavulanic acid content of co-amoxiclav disc.

Organisms over expressing AmpC β-lactamases (cephalosporinases) are a major clinical concern because these organisms are usually resistant to all β-lactam drugs, exept cefepime, cefpirome and the carbapenems. Constitutive over expression of AmpC β-lactamases in gram negative organisms occurs either by deregulation of the ampC chromosomal gene or by acquisition of a transferable ampC gene on a plasmid or other transferable element.

Recently, the numbers of carbapenem resistant clinical isolates which belong to the family enterobacteriaceae or pseudomonads have been increasing worldwide. Based on molecular studies, two types of carbapenem hydrolyzing enzymes have been described: serine enzymes possessing a serine moiety at the active site, and metallo-β-lactamases (MBLs), requiring divalent cations, usually zinc, as metal cofactors for enzyme activity.

By routine method of detecting AMR it is not possible to detect resistance in ESBL producing organisms. The activity of extended spectrum cephalosporins or aztreonam against ESBL producing organisms will be enhanced by the presence of clavulanic acid. The basic strategy to detect ESBL is to use an indicator cephalosporin to screen for likely producers, and then these should be subjected to a confirmatory test.

Methodology for ESBL Resistance

ESBL resistance detection methods fall into two main categories: those used to screen for ESBLs and those used to confirm that the observed reduction in susceptibility is due to an ESBL production. Screening methods include both disk diffusion and broth dilution methods. Phenotypic confirmatory test currently relies on demonstrating an increase in the size of the inhibition zone around a disk containing an extended spectrum cephalosporin with β-lactamase inhibitor (clavulanic acid), or a decrease in MIC to an extended spectrum cephalosporin presence of β-lactamase inhibitor (clavulanic acid). A > 5mm increase in zone diameter or any reduction in the cephalosporin MIC of more than 3 doubling dilution steps, for the agent tested alone verses its MIC when tested in combination with clavulanic acid, indicates the presence of an ESBL. These tests distinguish AmpC β-lactamases (not inhibited by β-lactamase inhibitors) from ESBLs.

Phenotypic tests

Disk diffusion method

This is carried out for all the enterobacteriaceae isolates using Muller Hinton agar medium with ceftazidime (30µg), cefotaxime (30µg), ceftriaxone (30µg) and cefpodoxime (10µg) antibiotic disks to screen the isolates for potential ESBL production. The discs are so arranged that the distance between them is approximately twice the radius of the inhibition zone produced by the cephalosporins tested on its own. The results are interpreted using the following zone of inhibition to screen potential ESBL

producers – ceftazidime ≤ 22mm, Cefotaxime ≤ 27mm ceftriaxone ≤ 25mm, cefpodoxime ≤ 17mm. *K. pneumoniae* ATCC 700603 and *E. coli* ATCC 25922 are taken as positive control (ESBL producer) and negative control (ESBL non-producer), respectively.

Disk potentiation method

The strains are confirmed as an ESBL producer by disk potentiation method using ceftazidime (30µg) and cefotaxime (30µg) antibiotic disks with and without clavunalic acid (10µg).

Double disk approximation method

The antibiotic disks used in this method are ceftazidime (30µg), cefotaxime (30µg), ceftriaxone (30µg) and cefpodoxime (10µg). This is a confirmatory test to see the ESBL production by the bacterial isolates.

Broth micro dilution test

Phenotypic confirmatory testing can also be performed by broth microdilution assays using ceftazidime (0.25 to 128µg/ml), ceftazidime plus clavulanic acid (0.25/4 to 128/4 µg/ml), and cefotaxime plus clavulanic acid (0.25/4 to 64/4µg/ml).

Disk-on-disk test

In this test cefotaxime and ceftazidime disks are tested against test organism both alone and in combination with co-amoxiclavulanic acid disk being placed on top of the cephalosporin disk. The interpretation is done on the basis of enhancement of inhibition zone when they are used on its own.

AmpC β-lactamase detection test

Screening of AmpC β-lactamase can be done by modified double disk approximation method (MDDAM)[12] and spot inoculation method (Amp C disk test) using only cefoxitin disc inhibition zone.

Blunting of zone of inhibition between cefotaxime and/or ceftazidime and cefoxitin or reduced susceptibility to ceftazidime or cefotaxime and cefoxitin suggest production of AmpC β-lactamase.

A negative test will have no distortion. Carbapenem resistance detection by disc diffusion are considered if the zone of inhibition for resistant was <13mm, for intermediate 14-15mm and for sensitive ≥16mm (Table III).

Commercially available methods for ESBL detection

MIC by E-test ESBL strips

A positive test for an ESBL is A ≥3 dilution reduction in the MIC of ceftazidime in the presence of clavulanic acid.

ESBL cards

Laboratories using conventional cards risk incorrectly reporting ESBL-producing organisms as susceptible to cephalosporins when MICs are < 8µg/ml. A specific card, which includes tests for ESBL production has now been FDA approved. The ESBL test utilizes cefotaxime and ceftazidime alone and in combination with clavulanic acid. Inoculation of cards is identical to that performed for regular Vitek cards. Analysis of all wells is performed automatically once the growth control well has reached a set threshold (4 to 15 h of incubation). A predetermined reduction in the growth of the cefotaxime or ceftaziodime wells containing clavulanic acid, compared with the level of growth in the well with the cephalosporin alone incites a positive result. Sensitivity and specificity of the month exceed 90%. False-negative results have been observed in *K. pneumoniae* isolates producing both an ESBL and an AmpC-type β-lactamase.

Automated microbiology system

The Phoenix ESBL test uses growth response to cefpodoxime, ceftazidime, ceftriaxone, and cefotaxime with or without clavulanic acid, to detect the production of ESBLs. Results are usually available within 6 h. It has been found to detect ESBL production in greater than 90% of strains genotypically confirmed to produce ESBLs. The method correctly detects ESBL production by *Enterobacter, Proteus*, and *Citrobacter* spp besides *Klebsiella* and *E. coli.*

Molecular methods of ESBL detection

Molecular methods assess variation in the genomes of bacterial isolates with respect to composition (*e.g.* presence or absence of plasmids), overall structure (*e.g.* restriction endonuclease profiles, number and positions of repetitive elements), or precise nucleotide sequence (of one or more genes or intergenic regions). Basic genetic analysis of the molecular event(s) (acquisition, multiplication, mutation, deletion, insertion) associated

with pattern variation is the preferred approach to measuring inter-strain relatedness.

ESBL strain should be characterized genotypicallly to know the ESBL types such as TEM, SHV, OXA, CTX-M, its epidemiological pattern and point mutation in their plasmids.

Methodology to characterize ESBLs in enterobacteriaceae

DNA probes

Early detection of ß-lactamase genes is performed using DNA probes that are specific for TEM and SHV enzymes. The first ESBLs studied with probes belong to the TEM family. Using of DNA probes can sometimes be labor intensive.

Polymerase chain reaction

PCR with oligonucleotide primers that are specific for a ß-lactamase gene is the easiest and most common molecular methods used to detect the presence of a ß-lactamase belonging to a family of enzymes. However, PCR will not discriminate among different variants of TEM or SHV.

Oligotyping

Several molecular methods that will aid in the detection and differentiation of ESBLs without sequencing have been suggested. The oligotyping method was used to discriminate between TEM-1 and TEM-2. This method used oligonucleotide probes that are designed to detect point mutations under stringent hybridization conditions. Several new TEM variants were identified using this method. These probes are less sensitive for the detection of mutations which are responsible for the extended substrate range. In some cases these mutations lead to the appearance or disappearance of restriction sites. Amplification of the relevant part of the gene by PCR followed by restriction enzyme analysis can thus indicate the presence or absence of specific TEM or SHV derived ESBLs.

PCR-SSCP and PCR-RFLP

PCR-single-strand conformation polymorphism (PCR-SSCP) has also been applied to the study of ESBLs with satisfactory results. This method has been used to detect a single base mutation at specific location within the beta-lactamase gene. The combination of PCR-SSCP with PCR-restriction fragment length polymorphism (PCR-RFLP) allows the identification of newer SHV variants.

Ligase chain reaction

The ligase chain reaction (LCR) is used for the identification of SHV genes. LCR allows the discrimination of DNA sequences that differ by a single base pair. A novel sequence-specific peptide nucleic acid provides an accurate means of identification of *bla* (GES-2) compared to the standard PCR and the gene sequencing techniques.

Nucleotide sequencing

Nucleotide sequencing remains the standard for determination of the specific ß-lactamase gene present in a strain. However, this too can give variable results depending on the method used.

STAPHYLOCOCCI

Staphylococcus aureus and coagulase negative *Staphylococcus* (CONS) is a leading cause of nosocomial and community acquired infection. Currently majority of the *Staphylococcus aureus* strains are β-lactamase producers and resistant to penicillin and ampicillin. However, these strain are susceptible to β-lactam antibiotics such as methicillin or oxacillin and nafcillin. Methicillin resistance in hospital acquired infection is due to resistance to all β-lactam antibiotics, and cephalosporins when community acquired *Staphylococcus aureus i*s multi drug resistant except for β-lactamase resistance. Resistance to other antibiotics like macrolides and vancomycin are also known.

Methicillin-Resistant Staphylococcus Aureus

The methicillin resistant *Staphylococcus aureus (MRSA)* has been defined as resistant to all penicillinase-stable penicillins; thus the acronym MRSA is still commonly used even though methicillin is no longer the agent of choice for treatment.

Apart from doing routine susceptibility tests for identifying MRSA strains following modifications are required *viz* modification of *in vitro* testing condition to enhance the expression of oxacillin resistance by the preparation of inocula using the direct inoculum suspension procedure, incubation of tests at temperatures no greater than 35^0C, obtaining final test readings after a full 24 h of incubation, by examining the test plates using transmitted light, (any other growth must be considered significant) and addition of 5% NaCl to the medium.

Laboratory Detection of MRSA

Disk diffusion method

The method is same as in enterobacteriaceae using MHA with 2% NaCl as medium and control sensitive strain is *S. aureus* ATCC 25923 or NCTC 6571 while resistant control is *S. aureus* ATCC 43300 or *S. aureus* NCTC 12493. The anti-microbial discs are methicillin 5 μg or oxacillin 1 μg and cefoxitin 30 μg per disk. The plate is incubated for 24 h at 30-35^{0}C in air. Zones are measured to the nearest millimeter and examined carefully in good light to detect colonies within the zone; some of these may be very small. Any colonies within zones may be indicative of hetroresistance, but to exclude the possibility of a mixed culture, should be identified and retested.

Interpretation

For *S. aureus* and CONS: oxacillin/methicillin- R ≤10mm, I 11-12, S ≤–13 mm, cefoxitin- R ≤19mm, S ≤20mm; ATCC 25923 (sensitive control) 17-22mm; *S. aureus* ATCC 43300 or *S. aureus* NCTC 12493 (oxacillin/methicillin resistant) ≤10mm/no zone.

Several phenotyping laboratory techniques are available. All oxacillin/methicillin intermediate strains should be reported as sensitive or resistant based on cefoxcitin results if facilities for other confirmatory methods such as mecA or PBP2a detection are not available. However, for MRSA screening cefoxitin disc diffusion and oxacillin disc diffusion method (MHOX) are comparable with oxacilllin MIC results hence these two methods can be used. These methods are comparable with the detection of mecA gene by mecA PCR for both. MHOX method with 6 g/ml of oxacillin and 4% NaCl is best single method for oxacillin resistance in CONS species.

SUSCEPTIBILITY OF FASTIDIOUS BACTERIA

Disc Diffusion Methods For Fastidious Organisms

Antibiotic Susceptibility Testing of *S. pneumoniae*

Standardization of inoculum

The media used is Mueller Hinton sheep blood agar. The inocula for seeding the susceptibility media with *S. pneumoniae* is prepared from fresh pure cultures (grown overnight on chocolate agar). Cell suspensions of the bacteria to be tested are prepared in sterile saline or Mueller-Hinton broth by transferring a portion of the fresh growth with a swab or inoculating loop to the suspending medium, using caution when mixing the cells with the suspending medium so as not to form bubbles. The suspension is then compared to the McFarland standard by holding the suspension and McFarland standard in front of a light against a white background with contrasting black lines and comparing the turbidity[9]. If the turbidity is too heavy, the suspension should be diluted with additional suspending medium. If the turbidity is too light additional cells should be added to the suspension.

For *S. pneumoniae* direct colony suspension is made in normal saline and turbidity adjusted to 0.5 McFarland standard. Within 15 min after adjusting the turbidity of the suspension the plate should be inoculated.

Inoculation of the susceptibility test media

After proper turbidity is achieved, a new sterile swab (cotton or dacron) is submerged in the suspension, lifted out of the broth, and the excess fluid is removed by pressing and rotating the swab against the wall of the tube. The swab is then used to inoculate the entire surface of the supplemented Mueller Hinton agar plate three times, rotating the plate 60 degrees between each inoculation. The inoculum is allowed to dry (usually taking only a few min but no longer than 15 min) before the discs are placed on the plates. The discs should be placed on the agar with sterile forceps and tapped gently to ensure the adherence to the agar. The plates containing the disks are incubated at 35^{0}C for 16 to 18 h in an inverted position in a 5% Co_2 incubator. A candle extinction jar may be used if a Co_2 incubator is not available.

Estimating the sensitivity of the strains

After overnight incubation, the diameter of each zone of inhibition is measured. The zones of inhibition on the media containing blood are measured from the top surface of the plate with the top removed. Care should be taken not to touch the disk or surface of the agar. In all measurements, the zones of inhibition are measured from the edges of the last visible colony-forming growth. The results are recorded in millimeters (mm) and interpretation of sensitivity is obtained by comparing the results to the standard zone sizes. For *S. pneumoniae* the zone measurement is from top of plate with the lid removed. Faint growth of tiny colonies that may appear to fade from the more obvious zone should be ignored in the measurement.

Each zone size is interpreted as sensitive (S), intermediate (I) and resistant (R)[12,14].

Antibiotic Susceptibility Testing of Enterococci

There are two types of antimicrobial resistance in enterococci. Intrinsic/inherent resistance that is chromosomal mediated. Enterococci are intrinsically resistant to cephalosporin, semi-synthetic penicillin (oxacillin, nafcillin) clindamycin, trimethoprim, sulfamethoxazole and have low level of resistance to aminoglycosides. Acquired resistance arises secondary to a mutation of existing DNA or acquisition of new DNA. The most troublesome resistance in enterococci is resistance to vancomycin.

Vancomycin resistant enterococci

Those enterococci that are resistant to vancomycin at a concentration greater than or equal to 32 mg/l[14] are considered as vancomycin resistant (VRE). The vancomycin resistance phenotypes which have been described include VanA, VanB and VanC (occur frequently) as well as VanD, VanE and VanG (occur rarely).

Mechanism of resistance

Glycopeptides (vancomycin and teicolainin) inhibit cell wall synthesis by forming complexes with the peptidyl-D-alanine (D-ala-D-ala) termini of peptidoglycan precursors at the cell surface. Resistance is due to synthesis of peptidoglycan precursor ending in the desipeptide D-alanyl-D-lactate (D-ala-D-lac) in VanA and B and D-ala-D-Ser in VanC that binds to glycopeptides with reduced affinity.

Methods for testing

Disk diffusion test done is same as in enterobacteriaceae except that the plates should be incubated at 35 ± 2^0C in ambient air for full 24 h. Acceptable limits for quality control strain (*S. aureus* ATCC 25923) should be used to monitor accuracy of disc diffusion testing (Table IV).

Some of fully automated methods (commercially available) of testing enterococci for resistance to vancomycin are unreliable specially in detecting VRE isolates containing the Van B resistant determinant. In a study evaluating the accuracy of eight currently available susceptibility test methods (Agar dilution, disk diffusion, E-test, agar screen plate, GPS-TA and GPS 101, and Microscan overnight and rapid panels), it was shown that vanA VRE were detected by all methods but vanB VRE were often not detected by Vitek GPS-TA and microscan rapid (sensitivities, 47 and 53% respectively). The new GPS-101 shows improved sensitivity compared to the GPS-TA without significant loss of specificity.

If detection of VanA, VanB, VanC1, and VanC2 mediated resistance in enterococci is required then VRE agar screening test is the most reliable method for routine screening. Enterococci may also be tested for vancomycin resistance by using PCR assays designed to detect the genes responsible for glycopeptides resistance in these organisms.

Antibiotic Susceptibility Testing of *Haemophilus* species

The medium of choice for disc diffusion testing of *Haemophilus sp*. is haemophilus test medium (HTM). Mueller-Hinton chocolate agar is not recommended for routine testing of *Haemophilus spp.*

In its agar form,HTM consists of (i) Mueller-Hinton agar, (ii) 15 g/ml β-NAD, (iii) 15 g/ml bovine hematin, and (iv) 5 mg/ml yeast extract.

To make HTM, first a fresh hematin stock solution is prepared by dissolving 50 mg of bovine hematin powder in 100 ml of 0.01 mol/L NaOH with heat and stirring until

Table IV. Interpretation of glycopetide sensitivity test

Antimicrobial agent	Disk content (per ml)	Zone diameter			*S. aureus* ATCC 25923 zone diameter (mm)
		Resistant (mm)	Intermediate (mm)	Sensitive (mm)	
Vancomycin	30µg	≤14	15-16	≤17	17-21
Teicoplanin	30µg	≤10	11-13	≤14	15-21

Organisms with intermediate zones should be tested by MIC method.

the powder is thoroughly dissolved. Thirty ml of the hematin stock solution are added to 1 L of MHA with 5 g of yeast extract. After autoclaving and cooling to 45 to 50°C, 3 ml of an NAD stock solution (50 mg of NAD dissolved in 10 ml of distilled water and filter sterilized) are also aseptically added. The pH should be 7.2 to 7.4.

Test procedure

The direct colony suspension procedure should be used when testing *Haemophilus sp*. Using colonies taken directly from an overnight (preferably 20 to 24 h) chocolate agar culture plate, a suspension of the test organism is prepared in Müeller-Hinton broth or 0.9% saline. The suspension should be adjusted to a turbidity equivalent to a 0.5 McFarland standard using a photometric device. This suspension will contain approximately 1 to $4x10^8$ CFU/ml. Care must be exercised in preparing this suspension, because higher inoculum concentrations may lead to false-resistant results with some β-lactam antibiotics, particularly when β-lactamase producing strains of *H. influenzae* are tested. Within 15 min after adjusting the turbidity of the inoculum suspension, it should be used for plate inoculation.

The procedure for the disc test should be followed as described for nonfastidious bacteria, except that, in general, no more than 9 discs should be applied to the surface of a 150-mm plate or no more than 4 discs on a 100-mm plate. Plates are incubated at 35°C in an atmosphere of 5% Co_2 for 16 to 18 h before measuring the zones of inhibition. The zone margin should be considered as the area showing no obvious growth visible with the unaided eye. Faint growth of tiny colonies that may appear to fade from the more obvious zone should be ignored in the measurement.

Zone diameter interpretive criteria

The antimicrobial agents suggested for routine testing of *Haemophilus sp*. are indicated in table I. Each zone size is interpreted as sensitive, intermediate and resistant[12,14]. Disc diffusion testing of *Haemophilus sp*p. with other agents is not recommended.

Antibiotic Susceptibility Testing for *N.gonorrhoeae*

The recommended medium for testing *N.gonorrhoeae* consists of GC agar to which a 1% defined growth supplement is added after autoclaving. Cysteine-free growth supplement is not required for disc testing. Enriched chocolate agar is not recommended for susceptibility testing of *N. gonorrhoeae*.

Test procedure

The direct colony suspension procedure should be used when testing *N. gonorrhoeae*. Using colonies taken directly from an overnight chocolate agar culture plate, a suspension equivalent to that of the 0.5 McFarland standards is prepared in either Mueller-Hinton broth or 0.9% saline. Within 15 min after adjusting the turbidity of the inoculum suspension, it should be used for plate inoculation.

The disc diffusion test procedure steps, as described for nonfastidious bacteria, should be followed. No more than 9 antimicrobial discs should be placed onto the agar surface of a 150-mm agar plate not more than 4 discs onto a 100-mm plate. However, when testing some agents (*e.g.* quinolones) which produce extremely large zones, fewer discs may need to be tested per plate. The plates are incubated at 35°C in an atmosphere of 5% Co_2 for 20 to 24 h before measuring the zones of inhibition.

Zone diameter interpretive criteria

The antimicrobial agents suggested for routine testing of *N. gonorrhoeae* are indicated in table I. Each zone size is interpreted as sensitive, intermediate and resistant. Disc diffusion testing of *N. gonorrhoeae* with other agents is not recommended. Organisms with 10 μg penicillin disc zone diameters of < 19 mm generally produce β-lactamase. However, β-lactamase tests are faster and are therefore preferred for recognition of this plasmid-mediated resistance. Organisms with plasmid-mediated resistance to tetracycline also have zones of inhibition (30 μg tetracycline discs) of < 19 mm.

Determination of MIC for Fastidious Organisms

The inoculum should be an actively growing culture diluted in saline to 10^4 to 10^5 microrganism per ml.

For *S.pneumoniae, N. gonorrhoeae* and *H.influenzae,* direct colony suspension from a 12-15 h culture from TSBA medium is to be used. The colonies are suspended in 0.5ml of normal saline and the opacity adjusted to McFarland 0.5. A 1/10 dilution of this suspension is made and within 15 min of making the diluted suspension the test plates should be inoculated with either a platinum loop calibrated to deliver 0.001ml or multipoint inoculator.

Inoculation of Test Plate

In general the inoculum should be applied as a spot that covers a circle about 5-8mm in diameter. A platinum loop calibrated to deliver 0.001ml of the inoculum is used to spot inoculate the cultures. Appropriate ATCC quality control organism(s) should be included along with each test. Inoculated plates are left undisturbed until the spots of inoculum have dried.

Incubation

After the spots of inoculum have dried, the plates are incubated at 35°C for 16 to 18 h in an inverted position in a 5% Co_2 incubator. A candle extinction jar may be used if a Co_2 incubator is not available.

Reading

The control plate should show the growth of the QC test organism. The MIC of the quality control strain should be in the expected quality control range. The end point is the lowest concentration of antibiotic that completely inhibits growth. A barely visible haziness or single colony should be disregarded. Results are reported as the MIC in micrograms or units/ml. Interpretation is made in accordance to the guidelines laid down[12] as susceptible, intermediate and resistant.

MIC Test for VRE

MIC and test for motility to distinguish species with acquired resistance (Van A and Van B) from those with intrinsic intermediate level resistance to vancomycin (van C such as *E. gallinarum* and *E. casseliflavus*) which often grow on the vancomycin screen plate but appear to be sensitive on disc diffusion testing should be performed. MIC can be determined by agar dilution, agar gradient dilution, broth microdilution or manual broth microdilution and by E-test strips on unsupplemented MHA plates and interpreted as sensitive (≤4 µg/ml) intermediate (8-16 µg/ml) and resistant (≥32 µg/ml).

Conclusions

The increased prevalence of microorganisms resistant to antibiotics has made susceptibility testing a crucial aspect of the treatment of serious bacterial illness. There is a need for antibiotic policy in a health care setting which depends on the changing or addition of newer antibiotics, their spectrum of activity, pharmacokinetics and pharmacodynamics.

There is a definite need among the clinicians and clinical microbiologist for knowing the susceptibility testing of the organism locally in their hospitals, clinics and communities. Besides, the local physician should take leadership in raising awareness, the national medical associations should urge the government that the antimicrobial agents be available only through prescription provided by licensed and qualified health care professionals. Thus initiation of a national media campaign explaining the disadvantages of irrational use of antibiotics is the need of the hour. National data collection on antimicrobial resisance surveillance in the form of national task force should be started so that individual government can cooperate with the world Health Organization and enhance global network. It is therefore, important for the clinicians, public health as well as the laboratory personnel to understand the basic principles and procedures of the standard testing protocols. These protocols need to be updated every 2-3 years, in view of the rapidly changing antibiotic resistant pattern. The present article has reviewed the currently available procedures which should be used by all the laboratories in India to maintain the uniformity of the information in our health care system. This would help, in future, transparency and better networking of the laboratories. Quality control and the accreditation of the laboratories is also need of the hour.

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ICMR NEWS

The following meetings of various technical committees/ groups of the Council were held:

Meetings of the Task Forces(TFs)/Technical Advisory Groups (TAGs)/Core Committees

TF on Immunophenotyping of Hematolymphoid Neoplasma	December 2, 2008 (at New Delhi)
TF on Acute Coronary Event Registries	December 3, 2008 (at New Delhi)
TAG on Environmental and Occupational Health	December 29, 2008 (at Chennai)
Core Committee on Oral Health	December 30, 2008 (at New Delhi)
TF on Jai Vigyan Mission Mode Project on Control of RF/RHD	January 7, 2009 (at New Delhi)
EG to Discuss the Strategies for Sustaining Eradication after Cessation of Wild Poliovirus	January 20, 2009 (at New Delhi)
EG on Meningococcal Disease Outbreak in Meghalaya and Its Control	February 4, 2009 (at New Delhi)
TF on Chronic Kidney Diseases	February 13, 2009 (at New Delhi)
TF on Registry of People with Diabetes on Young Age at Onset	February 20, 2009 (at New Delhi)

Meetings of Project Review Committees (PRC) held at New Delhi

PRC on Oncology	December 16, 2008
PRC on Oral Health	December 30, 2008
PRC on Viral Diseases	January 2, 2009
PRC on North-East Projects	January 5, 2009
PRC on Neurology	January 20, 2009
PRC on Gastroenterology	January 27, 2009
PRC on Cardiovascular Diseases	January 27, 2009
PRC on Leprosy, and Tuberculosis and Other Chest Diseases	January 27, 2009
PRC on Urology	February 12, 2009
PRC on Ophthalmology	February 25, 2009

Participation of ICMR Scientists in Scientific Events

Dr. N. Arunachalam, Scientist F, Centre for Research in Medical Entomology (CRME), Madurai, participated in the International Ecohealth Forum 2008 at Merida, Mexico (December 1-5, 2008).

Dr. B.K. Tyagi, Scientist F and Officer-in-Charge, CRME, Madurai, participated in the TDR BL5 Genetically Modified Vector Projects Coordination Meeting at Bamako, Mali (December 1-7, 2008).

Dr. A.C. Mishra, Director, National Institute of Virology (NIV), Pune, participated in the meeting on Strengthening the WHO Global Influenza Surveillance Network at Barcelona (December 2-5, 2008).

Dr. A.R. Risbud and Dr. S.P. Tripathy, Scientists F, National AIDS Research Institute (NARI), Pune, participated in the AIDS Clinical Trials Group Leadership Retreat at Baltimore (December 4-6, 2008).

Dr. Neena Valecha, Scientist F, National Institute of Malaria Research (NIMR), Delhi, participated in the Symposium on Dihydartemisinin/Piperaquine: An Innovative ACT for the Treatment of *P. falciparum* Malaria at New Orleans (December 7-11, 2008).

Dr. Neeru Singh, Director, Regional Medical Research Centre (RMRC) for Tribals, Jabalpur and Dr. S.K. Sharma, Scientist E, NIMR, Delhi, participated in the LVII Annual Conference of the American Society of Tropical Medicine and Hygiene at New Orleans (December 7-11, 2008).

Dr. P. Jambulingam, Director, Vector Control Research Centre (VCRC), Puducherry, participated in the XII WHOPES Working Group Meeting at Geneva (December 8-11, 2008).

Dr. Dipika Sur and Dr. Byomkesh Manna, Scientists E, National Institute of Cholera and Enteric Diseases (NICED), Kolkata, participated in the Annual Meeting of American Society of Tropical Medicine and Hygiene at New Orleans (December 8-11, 2008).

Dr. N.S. Chatterjee, Scientist D; and Dr. S. Ganguly,Dr. Sulagna Basu, Dr. Santasabuj Das and Dr. Mamta Chawla Sarkar, Scientists C, NICED, Kolkata, participated in the Asian African Research Forum on Emerging and Reemerging Infections at Sapporo (December 14-15, 2008).

Dr. N. Selvakumar, Scientist F, Tuberculosis Research Centre (TRC), Chennai, participated in the II SAARC Conference on Tuberculosis, HIV/AIDS and Respiratory Diseases at Kathmandu (December 15-18, 2008).

Dr. G.K. Medhi, Scientist C, RMRC for North-East Region, Dibrugarh, participated in the XII International Symposium on HIV Medicine at Bangkok (January 14-16, 2009).

Prof. A.P. Dash, Director, Dr. S.K. Sharma and Dr. K. Raghavendra, Scientists E, NIMR, Delhi, participated in the WHO Meeting on Insecticide Resistance at Geneva (February 2-3, 2009). Prof. Dash, Dr. Raghavendra along with Dr. P. Jambulingam, Director, VCRC, Puducherry, also participated in the Technical Consultations on Combining Long Lasting Insecticidal Nets and Indoor Residual Spraying, at Geneva (February 4-6, 2009).

Dr. A.C. Mishra, Director, NIV, Pune, participated in the II Workshop to Further Develop the Emerging and Dangerous Pathogens Laboratory Network for Outbreak Response and Readiness at Geneva (February 5-6, 2009).

Dr. Soumya Swaminathan, Scientist F, TRC, Chennai, participated in the XVI Conference on Rotaviruses and Opportunistic Infections-2009 at Montreal (February 8-11, 2009).

Dr. Dipika Sur, Scientist E and Dr. Suman Kanungo, Scientist B, NICED, Kolkata, participated in the XII Annual Scientific Conference of International Centre for Diarrhoeal Diseases Research Bangladesh, at Dhaka (February 9-12, 2009).

Dr. U.D. Gupta, Scientist E, National JALMA Institute for Leprosy and Other Mycobacterial Diseases (NJIL&OMD), Agra, participated in the International Meeting on Emerging Diseases and Surveillance at Vienna (February 13-16, 2009).

Dr. S.M. Mehendale, Scientist F, NARI, Pune, participated in the Consultations to Review Ethical Issues Associated with HIV Testing in the Context of Surveillance Surveys at Geneva (February 23-24, 2009).

Dr. P. Jambulingam, Director, VCRC, Puducherry, participated in the Training Course on Management of Malaria Field Operations 2009 at Kanchanaburi, Thailand (February 26-27, 2009).

Dr. V.A. Arankalle, Scientist F, NIV, Pune, participated in the JSPS-DST Exploratory Exchange under the Japan-India Cooperative Science Progarmme at Tokyo (February 27 - March 5, 2009).

Training

Dr. Anil Prakash, Scientist E, RMRC for N.E. Region, Dibrugarh, participated in the Training on Laboratory Biosafety and Biosecurity Aspects in Relation to Genetically Modified Vectors for Disease Control at Mali (December 3-7, 2008).

Shri G.N. Sapkal, Scientist B, NIV Pune, underwent Training on Standard Protocols and Testing of PSM Samples using PRNT Procedures at Mahidol (January 11-24, 2009).

Dr. V. Chandrasekaran, Scientist B, TRC, Chennai, proceeded to undergo Advanced Training in HIV/TB Biostatistics at Rhode Island for 6 months w.e.f. January 19, 2009.

INDIAN COUNCIL OF MEDICAL RESEARCH

Health Systems Research Cell

Research Proposals are invited by the Director General, Indian Council of Medical Research on the following broad areas of Health Systems Research. The Research projects should be initially submitted as Concept Proposal as per Format of ICMR. If found interesting, one need to submit 15 copies of full proposal in the ICMR prescribed Application Format which can either be downloaded from the ICMR Website:http:/www.icmr.nic.in or can be obtained from Dr. T.P.Ahluwalia, Scientist F, Health Systems Research Cell, 2nd Floor Hall, Indian Council of Medical Research, Ansari Nagar, New Delhi-110029.E-mail: ahluwaliatp@icmr.org.in; Telephone:26589277). The last date for submitting the Concept project proposals would be 17th August, 2009. Approved research proposals would be supported by the Council for funding, subject to fund availability.

1. Public, Public-Private and NGO Partnership for the delivery of reproductive and child health (RCH) services.
2. Health insurance for the population living in rural areas and urban slums.
3. Strengthening research capacity and effective knowledge utilization.
4. Reduction of gap in the health system manpower and service delivery.
5. Reducing gender discrimination and improving adolescent health.

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