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Advances in Rapid Detection and Antimicrobial Susceptibility Tests

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ABSTRACT

The rise of antibiotic resistance is an emerging problem of the millennium. Clinical microbiology plays an important role in combating the problem by facilitating diagnostics and therapeutics thus managing infection in patients. Diagnostic failures are a major limiting factor during bacterial infection that causes inappropriate use of antibiotics, delay in start up of treatment and decrease in the survival rate during septic conditions. Thus rapid and reliable detection is highly relevant during such bacterial infections and also at the time of disease outbreak as many such pathogens can be used as biothreat agents or bioweapons affecting human health and posing risk to national security. The importance of various methods for fast pathogen detection and antimicrobial susceptibility determination is highlighted. These methods have the potential to provide very precise and rapid ways for bacterial screening and identifying the correct antibiotics to cure infection.

Keywords: Antimicrobial susceptibility testing; Antibiotic resistance; Rapid pathogen detection

NOMENCLATURE

AR	Antibiotic resistance
ITMC	Isothermal microcalorimetry
AFM	Atomic force microscopy
AST	Antibiotic susceptibility testing
POC	Point of care
SPR	Surface plasmon resonance
LAMP	Loop-mediated isothermal amplification
SAW	Surface acoustic waves
NMR	Nuclear magnetic resonance
LC-ESI	Liquid chromatography - electrospray ionisation tandem mass spectrometry
MALDI-TOF MS	Matrix-assisted laser desorption ionisation–time of flight mass spectrometry
SERS	Surface-enhanced raman scattering
TOF-SIMS	Time-of-flight secondary ion mass spectrometry
$DiBAC_4$	bis-(1,3-dibutylbarbituric acid) trimethine oxonol
RST	Respirometric screening technology
DEP	Dielectrophoresis
RI	Refractive index

1. INTRODUCTION

Antibiotics are natural, synthetic or semi-synthetic substances used to treat patients suffering from multiple microbial infections to reduce the pace of associated mortality and morbidity. The golden era of antimicrobial therapy started with the introduction of the β-lactam antibiotics for the treatment in 1940s. Among various countries, India is at the top in antibiotic consumption followed by China and USA[1]. However, during recent years antibiotic resistance is increasing which is a very complex and challenging problem in various sectors like medical healthcare centres, hospitals and in communities as it increases the number of patients and adds a significant increase in cost associated with treatment[2-3]. There is a need for regular antibiotic stewardship as effectiveness of antibiotics is continuously decreasing in contrast to the increasing infectivity of pathogens. The causes of resistance development are multifactorial some of which are highlighted in Fig.1.

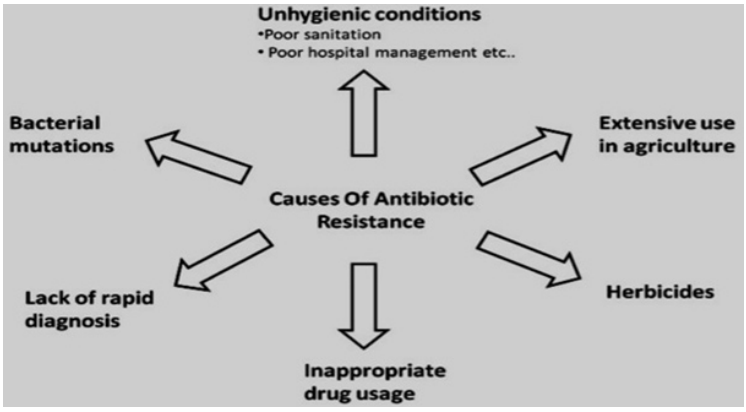

Figure 1. Major causes of resistance development in bacterial strains.

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Among the conventional methods for antimicrobial susceptibility testing most commonly used include disc diffusion and broth microdilution which are used as reference methods[4]. Other conventional methods include E-test, biochemical assays and microscopy[5-6]. Although conventional methods are inexpensive, they lack rapidity for effective clinical use, selectivity for fastidious and non-fastidious microrganisms, time and requirement of trained diagnostician to perform the test. Early stage diagnosis followed by effective antibiotic treatment has the potential to decrease the emergence of antibiotic resistance and provide a means for judicious use of antibiotics. Thus the review summarises the recent advances in the pathogen identification and antimicrobial susceptibility testing methods which are developed to decrease the time duration, increase sensitivity and specificity with the potential to be used in point of care settings. These methods may provide possible alternatives to the existing conventional methods in near future and proves to be an important aspect of measures to control infectious diseases[7]. Rapid diagnosis is also very important in the context of a biological warfare[8]. Such a need also arises in ICUs and hospitals where a direct relationship has been demonstrated between antibiotic consumption and emergence of multidrug resistance in patients infected with non-infectious agents[9]. Inappropriate initial antibiotics for pneumonia infection are usually linked to extended intensive care unit stay and are associated with an increased risk of mortality[10]. This emphasise the relevance of rapid diagnostic platforms that offers fast result with high sensitivity, specificity and low cost. Table 1 highlights the underlying principle of various methods described in the manuscript.

Table 1. Different techniques for detection of microbes and principle involved

Technique	Basic principle
ITMC	Changes in heat profile of live and dead bacteria due to various metabolic activities
Real-Time Microscopy	Observes morphological changes in bacteria on antibiotic exposure
PCR	Amplification of resistance factors/gene
AFM Cantilevers	Changes in pattern of vibrations of cantilevers due to presence of viable cells
Spectroscopic detection	Monitors the intensity of specific biomarkers or observes changes in spectral properties
Electrochemical detection	Change in electrical properties of culture media like impedence, conductance, capacitance
Luminescence based detection	Changes in fluorescence/luminescence intensity of certain dyes in medium containing viable cells
Flow cytometric detection	Changes in scattering of light due to changes in cell interior under various conditions
Respirometric detection	Monitoring of cellular respiration by O_2 consumption or monitoring O_2 concentration in media
DEP	Changes in dielectrophoretic force between live and dead cells
SPR	Changes in RI due to changes in of biochemical charecteristics of bacteria subjected to antibiotics
LAMP	Amplification of resistance factors but difference lies in primers and it eliminates the need for costly PCR machines
SAW	Change in pattern of propagating acoustic waves in presence of bacteria in culture environment

2 RAPID DETECTION METHODS

2.1 Isothermal Microcalorimetry

Isothermal Microcalorimetry (IMC) is a non-specific, label-free analytical technique that measures heat in microwatt or lower range[11]. The technique can be used to detect persistence of infections and AST by monitoring thermodynamic and kinetic properties of the biological system within viable cells. Heat is generated as a by-product of various biological processes. It can also be used for determination of drug susceptibility and MICs of against various gram negative, gram positive and *Mycobacterium* sp.[12-13]. Certain advantages of this technique includes its higher senstivity, efficiency, rapidness, versatility and compatibility with solid and liquid media. The method requires a standard multichannel microcalorimetry instrument.

2.2 Real-time Microscopy

This method is based upon morphological phenotyping that requires commercial high-resolution camera-based systems. An optical high throughput POC instrument called ocelloscope that relies on time-lapse microscopy is in use for AST in 3 hour duration[14]. Fluorescence microscopy can also be used for susceptibility testing based upon cellular changes on antibiotic exposure using dyes like DAPI, SYTOX Green and WGA-647. The results can be observed within 2 hr providing a rapid, robust, accurate and flexible method of evaluation[15]. Baltekina[16], *et al.* have developed a 30 min POC test that monitors growth rate of bacterial cells captured from urine samples using custom-designed microfluidic chip. McLaughlin[17], *et al.* have performed rapid susceptibility testing in *B. anthracis* using time-lapse microscopic observation of growth and morphology in presence of antibiotics. Kalashnikov[18], *et al.* have monitored cell death under the influence of antibiotics using automated microscopy. Choi[19], *et al.* have used microscopic detection for early estimation of antibiotic susceptibility profile of positive blood cultures by mixing them directly with agarose and spreading on a plastic microchip containing lyophilised antibiotics.

2.3 PCR Based Detection

PCR based techniques rely on amplification of known resistance genes i.e. resistance profiling against tetracycline resistance genes offers an advantage in disease control and management as it is an effective antibiotic against pathogenic microbes including number of potential biowarfare agents[8].

This method has also been used to detect presence of various foodborne pathogens on the basis of toxin secreting gene[20]. Strategies based on PCR are promising, highly sensitive, fast, specific and offers an advantage for slow growing and unculturable bacteria[21-22]. However the major drawback lies in its inability to discriminate between viable and dead cells. This drawback was removed by the use of DNA intercalators that prevents amplification of DNA from non-viable cell and free cellular DNA i.e. propidium monoazide (PMA) and ethidium monoazide (EMA) that selectively enters damaged cells and blocks the DNA for PCR amplification via photoactivation[23-24]. Real-time PCR (RT-PCR) is also widely used for detection of various microorganisms in clinical and environmental samples. Recently a procedure based on RT-PCR, monitors highly conserved 16s rRNA gene to determine pathogenic load in blood samples indicating susceptibility against various antimicrobial drugs[25].

2.4 Cantilever Based Detection

Cantilevers are mainly used in AFM that contains a very sharp tip to scan the surface of the sample and an attractive force is excerted between the tip and the surface that causes deflections in the cantilever that are detected using a LASER beam. Surface imaging via AFM does not require staining, fixation or cell labelling[26]. Change in the weight of the bacterial cells after antibiotic exposure causes vibrational changes in cantilevers. Apart from predicting about the success of antimicrobial it also indicates its MIC within less time and at lower cell concentrations (less than even 10^5 cells). The technology has also been used in microfluidics for highthroughput susceptibility profiling and detection of bacterial pathogens like *Listeria monocytogenes* using receptor functionalised microchannels embedded in bimaterial microcantilever[27-28].

2.5 Spectroscopy Based Detection

Various spectroscopic techniques include NMR, IR spectroscopy, LC-ESI MS, SERS, MALDI-TOF MS and TOF-SIMS. In a recent study, a handheld SERS based system have been used for bacterial detection and identification[29]. The decrease in intensity of specific biomarkers in SERS spectra after 2 hr of antibiotic exposure leading to rapid susceptibility and MIC determination[30]. SERS profile are very sensitive and stable and have been used for detection, classification, quantification, discrimination of different strains including various biothreat agents[31].

2.6 Electrochemical Detection

Monitoring electrochemical parameters like impedence, capacitance and conductance of cells in growth medium are used for susceptibility testing against antibiotics. Bacterial entrapment influences the electrical properties of electrode thus changes in such properties indicates presence of pathogens[32]. Webster[33], *et al*. evaluated the susceptibility of antibiotics on *Pseudomonas aeruginosa* biofilms by electrochemical monitoring an electroactive pyocyanin, a virulence factor. Electrochemical techniques like cyclic voltammetry[34] and differential pulse Voltammetry are used for selection of antibiotics against pathogens[35] and are highly compatible with microfluidics and integrated circuit technology.

2.7 Fluorescence and Luminescence Based Detection

Fluorescence based detection relies upon the use of fluorescent labels, dyes or probes whose emission spectra are detected using fluorescence spectrophotometer. ATP based bioluminescence is an important indicator of bacterial populations which is also used as an indicator of bacterial contamination in health care centres[36]. Hunter and Lim[37] used the method for development of an immunoassay using pathogen specific antibodies to capture target bacterial cells from a sample matrix followed by incubation with reagent (Bactitre glo) that converts cellular ATP into an output luminescent signal with a limit of detection of 10^4 cfu/ml for *S typhimurium* and *E coli* O157:H7.

2.8 Flow Cytometry Based Detection

Flow Cytometry (FC) is being used in microbiology since the late 1970s and was firstly used to determine bacterial DNA and protein[38]. Cohen[39], *et al* also detected pathogen in clinical samples within 2 hr and tested the effect of amikacin on positive samples in 1 hr. $DiBAC_4$ was also used as a fluorescence probe for evaluating the effects of antibiotics on *E coli* by visualising changes in membrane potential[40]. Several other dyes used for FC based detection include SYTO-9, SYTO-X green I, SYTO 16, SYBR green I and Acridine Orange[41]. However the technique only assess cellular damage on antibiotic exposure and can't distinguish between static or cidal effect of antibiotics.

2.9 Respirometry Screening Technology

RST allows high-throughput analysis of consumption of oxygen by viable cells, tissues, isolated mitochondria, organisms and enzymes, and is therefore a very convenient and highly valued technique with promising future potential[42]. Level of oxygen is an indicator of presence of viable bacteria. Sensors based on monitoring changes in molecular O_2 level have been used to confirm the presence of live cells[43-44].

2.10 Dielectrophoresis

Dielectrophoresis (DEP) is the translational movement of a cell in a non-uniform electric field due to it's interaction with the electric field gradient. Here manipulation of cells occur at microscale levels[45]. DEP has also been used for nucleic acids, proteins and other biomolecules apart from bacteria, spores, yeast, viruses and other eukaryotic cells. Exposure to antibiotics at their MIC value causes a change in morphology and frequency of migration of the cells causing a significant elongation of the gram negative rod shaped microbe[46]. These changes are observed by their dielectrophoretic behavior. At a definite alternating current frequency, bacteria which resist the antibiotic exposure does not migrate and get arranged at the center due to negative force while elongated once get adsorbed at the edges due to positive dielectrophoretic force. The phenomenon is more commonly used as a strategy for seperation of microbes from non-biological samples and concentrating the bacteria present in clinical and industrial samples thus decreasing the time required for screening[47].

Table 2. Biosensor strategies used in pathogen detection and susceptibility testing

Biosensor type	Bacterial strain	Functional role	Range of detection	Ref.
ATP-bioluminescence	Antibodies immobilised onto fiberglass membrane sandwiched between polypropylene components used to capture uropathogenic micro-organisms	Pathogen identification and susceptibility testing	1×10^3 to 1×10^5 cfu /ml	58
Phase-Shift Spectroscopy	*E. coli*	Susceptibility testing	10^3 cfu/ml in 2–3 hr	59
Colorimetric immunoassay	*Vibrio parahaemolyticus*	Pathogen detection	110 to 10^5 cfu/ml	60
Positive Dielectrophoresis focusing	*Escherichia coli* in drinking water based on impedance measurement	Pathogen detection	300 cfu/ml	61
Optical biosensor	E. coli and E. carotovora, biorecognition monitored by ATR-FTIR and confocal microscopy	Pathogen detection	10^3-10^4 cfu /ml	62
Amperometric biosensor	Efficacy of different antibiotics on E. coli, S. aureus and S. choleraesuis Based on electrochemical monitoring of glucose consumption	Detection identification and susceptibility testing	6.5x6 10^2 or 6.5 cfu/ml 3 or 7 hr	63
Electrochemical biosensor	Electrochemical detection of 16S rRNA of pathogens in Clinical Samples	Pathogen detection, identification and susceptibility testing	$5x10^5$ to $1x10^9$ cfu/ml (3.5 hr)	64
Electrokinetics enhanced electrochemical biosensing	*E. coli* in clinical isolates in blood	Pathogen detection and susceptibility testing	-	65
Acoustic biosensor	Detection of 3 most important plant pathogens using QCM multiplexed with PCR	Pathogen detection	10^2−10^3 cfu/ml	66
Impedance biosensor	Detection based upon electroactive metabolite, pyocyanin secreted from - *Pseudomonas aeruginosa* that alters the impedance of growth media	*Pseudomonas aeruginosa* detection	10^6 cfu/ml in 24 hr	67
Dual Response (Impedimetric/LAMP) Biosensors	LAMP amplifies the *Tuf* gene in *E. coli* after lysis of *E. coli* cells bound to bacteriophage used as bioreceptor, followed by detection using linear sweep voltammetry	Quantification, screening,identification and viability detection of *Escherichia coli*	8×10^2 cfu/ml to 10^2 cfu/ml (within 1hr)	68
SAW based sensor	Uses SH-SAW, fabricated using the complementary metaloxide-semiconductor (CMOS) method	Pathogen sensing	-	69
Optical photodiode array (PDA) based biosensor	*E. coli* O157:H7 using an integrated circuit of PDA	Pathogen detection	-	70
Electrical biosensor	*E. coli and* MRSA, pathogen captured on plastic microchips with printed electrodes	Susceptibility testing	10^6 cfu/ml	71

2.11 Surface Plasmon Resonance

SPR is an advanced optical, label-free, ultra-senstive method for AST based upon light scattering phenomenon. The technique requires relatively small amount of sample. Gold (Au) thin film coated chemically with poly-L-lysine commonly used to trap bacteria after its purification from mixed cell population. Change in the pattern of resonance of the surface plasmons is revealed on the detector through changes in the angle of resonance. Thus SPR shows the difference between resistant and senstive strain[48]. Nguyen[49], *et. al.* detected changes

in RI of tested bacteria subject to antibiotics in real time.

2.12 Loop-Mediated Isothermal Amplification

LAMP is a highly simple, specific, sensitive, rapid, reliable and affordable DNA amplification assay for detection of drug resistance and microbial identification introduced by Notomi[50]. This assay requires 4 to 6 primer designed using LAMP specific primer designing software and having specificity for distinct target region to form a loop structure that undergo autocycling rather than thermal cycling for strand displacement followed by nucleic acid amplification at isothermal conditions of 60 °C - 65 °C. The amplification process requires a water bath instead of a costly device. The reaction product need not be resolved on agarose gel electrophoresis as in conventional PCR, instead product can be visualised by turbidity measurement, flourescence of reaction mixture or directly in form of precipitate. The quantity of product formed is relatively larger[51-52].

2.13 Surface Acoustic Waves

SAWs are basically sound waves, a longitudinal mechanical wave travelling parallel to the surface of a material exhibiting elasticity[53]. Its application include all areas of sensing including thermal, optical, pressure, torque, acceleration and also for detection of pathogens. It requires a piezoelectric substrate and interdigital transducers (IDTs). IDTs affect the propagation of waves mainly due to dispersion from live bacterial cells, mass load, osmotic pressure, conductivity changes of the medium, etc[54].

3. ROLE OF BIOSENSORS IN EARLY DETECTION AND ANTIBIOTIC SUSCEPTIBILITY ESTIMATION

Biosensors are analytical tools for converting a biorecognition event into a detectable signal[55]. They can be used to reduce limit of detection and to increase senstivity by employing various micro and nanoscale technologies. The technology offers a more specific, label free, rapid, real-time, portable and cost-effective strategy apart from the ease of miniaturisation to form robust and portable POC devices[56]. Apart from their diverse role in medical field they have shown their potential at the times of outbreaks for detection of various infectious diseases (virus, bacteria, parasite) thus globally preventing issues related to bioterrorism[57]. Most of the methods discussed like SAW, SPR, cantilever, LAMP, electrical and electrochemical sensing can be used for biosensor development to detect pathogens in food, water and clinical samples. Table 2 highlights some biosensing based strategies for pathogen detection and susceptibility testing.

4. CONCLUSIONS

Bacterial septicemia is a life-threatening condition that requires immediate antibiotics prescription**.** Although culture based techniques are the gold standards for pathogen detection and ASTs, they offers some limitations especially in terms of time duration due to long culture steps. Thus the advances in technologies for the rapid detection and AST of pathogens can be used as a measure to reduce the havoc caused due to increasing antibiotic resistance (summarised as in Fig. 2). These offer potential for specific, more reliable, real-time, cost effective, point of care, high-throughput ASTs. Most of the methods discussed are based upon metabolic activities or biorecognition events hence are rapid and can be miniaturised to point of care device for medical use in hospitals and remote areas. The techniques alone or in conjugation with other technologies can be used for highthroughput quantification and detection of pathogenic bacteria directly from samples.

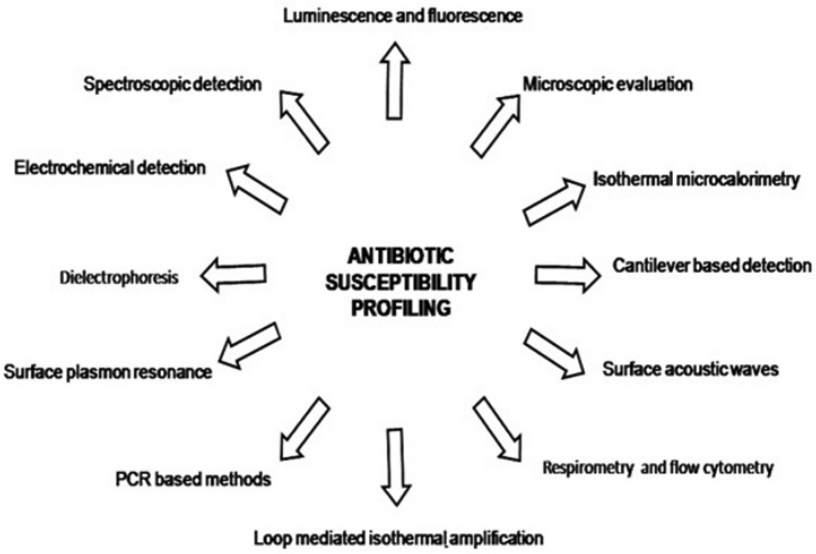

Figure 2. Methodologies for the rapid detection and AST of pathogens.

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CONFLICT OF INTEREST

The authors declares no conflict of interest.

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