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Review

Emerging antimicrobial susceptibility methods in monitoring colistin-resistant *Enterobacteriaceae*

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Abstract

One of the most important essential pillars in the fight against antibiotic resistance is to optimize antibiotic treatment by developing and optimizing appropriate methods to establish the antibiotic susceptibility profiles of a specific microbial strain. Moreover, this will contribute to the surveillance and limitation of antimicrobial resistance transmission and spread. Therefore, it is also imperative to harmonize different approaches and techniques and to perform suitable antimicrobial susceptibility tests in microbiology laboratories to achieve precise, reproducible, and comparable results. However, the conventional methods for antimicrobial susceptibility testing are usually based on bacterial culture methods, which are time-consuming, complicated, and labor-intensive. Therefore, other approaches are needed to address these issues. In this mini-review, we will present the common and future perspectives in antimicrobial susceptibility testing. Microfluidic technology and electrochemical devices have recently gained significant attention in infection management. These advantages include rapid detection, high sensitivity and specificity, highly automated assay, simplicity, low cost, and potential for point-of-care testing in low-resource areas.

Keywords Antimicrobial resistance, susceptibility tests, microfluidics, single-cell

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Introduction

The alarming increase in antimicrobial resistance leads to the urgent need to harmonize different approaches and techniques and to perform suitable antimicrobial susceptibility tests (ASTs) in microbiology laboratories (PEELING & al [1]).

Quantitatively, bacterial drug resistance is established by measuring a parameter called minimum inhibitory concentration (MIC), the lowest concentration of a drug that prevents the growth of bacteria. MIC measurements are the basis for establishing resistance breakpoints by agencies such as CLSI or EUCAST. A breakpoint is a drug concentration against which a patient sample is tested – if there is growth, the bacterial strain is resistant; if there is no growth, the strain is susceptible. Setting breakpoint values by medical agencies is based on MIC and pharmacokinetics and pharmacodynamics (PK/PD) of an antibiotic (POSTEK & al [2]). Clinicians use breakpoint values based on the MIC but not the MIC itself. Breakpoint values, although highly useful, do not convey the information that a MIC screen personalized to a given patient would: *e.g.*, a breakpoint does not necessarily take into account a wild-type resistance distribution, which can lead to both false positives and false negatives, or there is a possibility that a tested bacterium does have a resistance mechanism but is still below the breakpoint (CAMA & al [3]).

Since 1976, The Clinical and Laboratory Standards Institute (CLSI) has sought to find the most appropriate values for polymyxin in the clinic by introducing polymyxin disk diffusion breakpoints (NCCLS, 1976). Today, the threat of MDR *Acinetobacter spp*. and *Pseudomonas aeruginosa* persists, and carbapenem-resistant Enterobacterales (CRE) have become significant global health challenges. CRE are endemic in the U.S., Latin America, Asia, Greece, Italy, and Israel, with high rates in some countries (LOGAN & al [4]; TAMMA & al [5]). In 2013, considering this explosion of resistance phenomenon, CLSI and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) reviewed colistin breakpoints for Enterobacterales, *Acinetobacter* spp., and *P. aeruginosa*.

Common AST methods

The use of an accurate method for testing antimicrobial susceptibility (AST) for colistin is critical and urgent, given the continuing increase in the number of multi-resistant strains. EUCAST and CLSI have recommended broth microdilution (BMD) as the reference for identifying MIC (minimum inhibitory concentration) breakpoints in clinical settings. However, the BMD of colistin has some limitations in methodology, which is why it is rarely used in clinical laboratories. First, colistin can bind to polystyrene trays, solving this problem by adding a surfactant (KAUR & al [6]). This situation was analyzed by a joint working group, which recommended that the testing be done using sulfate salt of colistin and standard polystyrene trays, given that the surfactant does not improve the performance of the method. Also, BMD requires antibiotic solutions prepared extemporaneous or frozen solutions; it is a laborious method that consumes time and requires experience to interpret the results correctly (HU & al [7]; RANJAN & al [8]).

Consequently, most laboratories have focused on using automated susceptibility testing systems and disc-diffusion methods. However, international committees have expressed concern about the variable results obtained from these rapid tests. Due to colistin molecules' large size and cationic nature, disc-diffusion and gradient diffusion methods have proved unfeasible. Kulengowski et al. compared the results obtained after performing the BMD and E-test methods in 70 CRE strains. The authors found a considerable discordance between the E-test and BMD (a significant error of 88%).

Most importantly, E-test poorly predicted the polymyxin B MIC for isolates exhibiting elevated polymyxin B MICs by BMD (KULENGOWSKI & al [9]). In another study aiming to analyze these rapid methods, the gradient tests generally underestimated colistin MICs, resulting in many false susceptible results, a significant mistake in the clinic (MATUSCHEK & al [10]). Kananizadeh et al. obtained significantly higher MICs for colistin using the BMD method associated with brain-heart infusion (BHI) medium, Luria-Bertani (LB) broth, tryptic soy broth (TSB), or cation-adjusted Mueller-Hinton broth CA-MHB supplemented with casein, tryptone or peptone. These results suggest that the BMD method using BHI is beneficial when performed with the BMD method using CA-MHB to detect *mcr-9*-positive isolates (KANANIZADEH & al [11]).

Although the BMD has some limitations, EUCAST and CLSI recommend this method as the gold standard for colistin's antimicrobial susceptibility testing. However, considering that many laboratories rarely use this method in clinical routine, alternative AST methodologies are highly desirable.

Emerging AST methods

Microfluidic-based diagnostic is one of the most promising technologies for AST. Microfluidics is an expanding field based on using fluids in micro-volume to obtain a controllable environment in an *in vitro* system characterized by portability, cost-effectiveness, and reproducibility (POSTEK & al [2]; LI & al [12]). Integrated microfluidic devices are based on micro-total analysis systems and are used successfully in molecular biology (QIN & al [13]). Given that the amount of biological samples has been a problem over time, using a minimum amount of samples in microfluidic technology makes this system a perfect candidate for solving this problem. Currently, microfluidic systems can analyze a single cell and the interaction of the cell in the signaling network that exists within the cells in culture. However, as mentioned earlier, the techniques commonly used to achieve AST are laborious, time-consuming, high risk of cross-contamination, and require resources that limit their use in certain developed regions (GAJIC & al [14]). Microfluidics systems can be a solution for addressing these shortcomings (KLEIN & DIETZEL [15]).

Another possible strategy for improving AST is to couple microfluidic devices with an optical sensor to detect MIC values within a few hours. Recent studies on single-cell analysis have shown that microfluidic optical sensor-based can detect MIC breakpoints in 30 minutes (QIU & NAGL [16]; HUANG & al [17]).

ATP bioluminescence assay is a luciferase-mediated enzymatic reaction that converts the luciferin substrate to oxyluciferin in the presence of ATP, leading to the emission of a quantum of light (WANG & al [18]). Dong and Zhao analyzed the susceptibility of 13 strains associated with urinary tract infections using this phenomenon. The analysis was performed against eight antibiotics on a microfluidic plate. The resistance is transposed into a bioluminescence phenomenon when the bacteria grow in an antibiotic's presence while the sensitive strains remain neutral. This method provides MIC breakpoints that could be detected in 6-8 hours (DONG & ZHAO [19]).

Another research direction in improving AST is the use of electrochemical devices. One of the most significant studies utilized AC electrokinetic fluid motion and Joule heating-induced temperature elevation for the electrochemical sensing of bacterial 16S rRNA, providing essential information on the analysis of susceptible bacteria (LIU & al [20]). The latest electrochemical biosensor can achieve AST in about 90 minutes and isolate bacteria from blood samples (SAFAVIEH & [21]; ZHANG & al [22]).

Diep et al. combined inexpensive portable components for microbial cytometry to establish the feasibility of rapidly monitoring bacterial motility in the presence of antibiotics. They investigated whether the 3D-printed OpenFlexure microscope using a low-cost Raspberry Pi v2 camera has sufficient magnification and resolution to monitor bacterial motility in microdevices. Adequate magnification and contrast were achieved to view motile bacteria and allowed differences in behavior to be observed in the presence of antibiotics above the organisms' minimum inhibitory concentration (MIC) for that antibiotic. The authors demonstrated that the OpenFlexure microscope combined with microfluidic systems allows rapid antibiotic resistance detection. (DIEP & al [23]).

Lin et al. present a microfluidic device that generates a concentration gradient for antibiotics produced by diffusion in the laminar flow regime along a series of lateral microwells to encapsulate bacteria for antibiotic treatment. All the AST preparation steps were performed in a single chip. After the antibiotic treatment, the viable bacterial cells in each microwell are then quantified by their surface-enhanced Raman scattering (SERS) signals acquired after placing a uniform SERS-active substrate in contact with all the microwells. The authors demonstrated the AST performance of this system on ampicillin (AMP)-susceptible and -resistant *E. coli* strains (LIN & al [24]).

Yamagishi et al. used the drug susceptibility testing microfluidic device (DSTM) to achieve the rapid screening of extended-spectrum β-lactamases (ESBLs) and metallo-β-lactamases (MBLs). β-lactams and β-lactamase inhibitors were pre-fixed in the DSTM for use, and a bacterial suspension in Mueller-Hinton broth was introduced into the device. The effects of β-lactamase inhibitor on morphological changes caused by β-lactam were evaluated after three hours of incubation. The authors conclude that the DSTM method allows rapid detection of β-lactamases and may be a valuable replacement for the disc diffusion method (YAMAGISHI & al [25]).

Future perspectives

The current AST design challenges are the inoculum size and the need to select only a few isolated colonies. The first step in performing AST is to culture bacteria from the original sample on primary inoculum plates. Subsequently, only a few isolated colonies are selected to prepare an inoculum, followed by incubation for 16-18 hours. Performing AST starting from the original sample's inoculation on antibiotic screening flat agar, antibiotic resistance is detected only for certain bacteria in the inoculum. Therefore, resistant bacteria will be at a low frequency, making it impossible to detect them by conventional AST, which is a significant error in clinical settings. This situation is caused by the current standardization of the amount of inoculum and the selection of a small number of individual colonies, reducing bacterial diversity (BRUKNER & OUGHTON [26]). An alternative to the problem of selecting individual colonies is to perform population-based AST via qPCR in the context of the original clinical sample. These amplification tests can detect species-specific growth rates of bacteria in the original samples (MAXSON & al [27]; BRUKNER & OUGHTON [26]).

Microfluidics-based studies of single-cell growth in static chambers are relatively rare, although any static chamber device for population-level studies could be repurposed for single cells (KLEIN & al [28]). However, single-cell approaches could be placed on a distinct niche (research only) and not clinically applicable now due to the need for finding resistant individual cells in the highly-dense bacterial population from clinical samples (e.g., sensitive *P. aeruginosa* and meropenem-resistant *Escherichia coli*). Additionally, single-cell approaches do not capture fine inter- and intra-species communications, allowing certain bacteria to co-exist under selective antibiotic pressure in a complex clinical sample. Thus, this communication between resistant bacteria is missed at the single-cell level, leading to a loss of clinically valuable information. Therefore, clinical microbiology has to implement these bacterial interactions into the predictive models and overcome individual cell approaches.

Author contributions

S.I.T., I.G.B. and I.C.B. conceived and corrected the manuscript. R.E.C., M.C., S.I.T. and I.C. contributed to the literature survey and revised the manuscript. C.O.V. drafted the manuscript. All authors have read and agreed to the published version of the manuscript. The authors have contributed equally to this work and share first authorship.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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