Progress on the development of rapid methods for antimicrobial susceptibility testing

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Antimicrobial susceptibility testing is essential for guiding the treatment of many types of bacterial infections, especially in the current context of rising rates of antibiotic resistance. The most commonly employed methods rely on the detection of phenotypic resistance by measuring bacterial growth in the presence of the antibiotic being tested. Although these methods are highly sensitive for the detection of resistance, they require that the bacterial pathogen is isolated from the clinical sample before testing and must employ incubation times that are sufficient for differentiating resistant from susceptible isolates. Knowledge regarding the molecular determinants of antibiotic resistance has facilitated the development of novel approaches for the rapid detection of resistance in bacterial pathogens. PCR-based techniques, mass spectrometry, microarrays, microfluidics, cell lysis-based approaches and whole-genome sequencing have all demonstrated the ability to detect resistance in various bacterial species. However, it remains to be determined whether these methods can achieve sufficient sensitivity and specificity compared with standard phenotypic resistance testing to justify their use in routine clinical practice. In the present review, we discuss recent progress in the development of methods for rapid antimicrobial susceptibility testing and highlight the limitations of each approach that still remain to be addressed.

Keywords: susceptibility testing, antibiotic resistance, bacteria

Introduction

The administration of appropriate antibiotic therapy for many types of bacterial infection requires that a microbiological diagnosis, consisting of identification of the causative agent of infection and its resistance profile, is obtained. In routine clinical practice, this process can take between 24 and 72 h, during which time empirical therapy is selected based on the suspected causative organism and local epidemiology. The use of methods that are able to rapidly detect antibiotic resistance in bacterial isolates therefore has the potential to reduce the duration of empirical therapy and facilitate the early initiation of targeted treatment with proven antibiotic activity against the causative agent of infection. This is important in light of studies that have demonstrated that the rapid availability of antimicrobial susceptibility test results can improve patient outcomes1,2 and that a delay in the initiation of appropriate antibiotic therapy is associated with higher patient mortality in certain bacterial infections.3,4 In addition, rapid antimicrobial resistance testing may contribute to reducing healthcare costs, given studies showing that the early availability of antibiotic susceptibility information can result in the ordering of fewer laboratory tests, a decrease in the number of invasive procedures performed and reduced hospital stay.3,5 An additional foreseeable benefit of the rapid availability of antibiotic susceptibility test results is its potential to contribute to antimicrobial stewardship efforts, which include the administration of appropriate antibiotic therapy once susceptibility test results are available.6 In this context, the early initiation of adequate therapy with the narrowest spectrum needed for appropriate treatment of the infecting bacteria could play a role in reducing the emergence and transmission of resistant strains. In the present review, we give an overview of the techniques that have been developed for the rapid detection of antibiotic resistance in bacterial pathogens (excluding Mycobacterium, a topic that has recently been reviewed7,8) and provide examples of how these techniques have been employed for detecting resistance in a variety of bacterial pathogens. In addition, we discuss questions that still remain to be addressed regarding these approaches.

Currently used methods

The most widely used methods for characterizing antibiotic resistance in clinical isolates detect phenotypic resistance by measuring bacterial growth in the presence of the antibiotic being tested. These techniques include broth microdilution, antimicrobial gradient methods (e.g. Etest strips), disc diffusion and various commercially available automated systems (e.g. the MicroScan WalkAway system from Siemens, the Phoenix...
Automated Microbiology System from BD Diagnostics and the Vitek systems from bioMerieux). In addition to their high sensitivity for detecting antibiotic resistance, a major advantage of these techniques is that they have been highly standardized, a process that has been facilitated by the elaboration of international guidelines for antimicrobial susceptibility testing, such as those published by CLSI and EUCAST.\(^9,10\) For each of these methods, a variety of commercial suppliers provide reagents in ready-to-use formats that facilitate their use in clinical microbiology laboratories with high workloads. In the cases of broth microdilution and antimicrobial gradient methods, an MIC value is obtained, which provides information on the concentration of antibiotic necessary for inhibiting bacterial growth. However, despite their broad use and high sensitivity, there are some limitations associated with these approaches. These methods generally require pure cultures for susceptibility testing to be performed, although some protocols may allow for testing directly with clinical samples. In addition, because these techniques measure bacterial growth in order to detect resistance, incubation times that are sufficient for differentiating susceptible from resistant strains must be employed. In the following sections, we comment on the advantages and disadvantages of techniques and protocols that are being developed for the rapid detection of antibiotic resistance with respect to these commonly used methods.

**PCR-based techniques**

PCR-based techniques (both conventional and real time) rely on the sequence-specific amplification of nucleic acids. For this reason, PCR was initially used in microbiological testing for the rapid identification and quantification of causative agents of infections through the amplification of sequences specific to a particular pathogen.\(^1\) With increased knowledge of the genetic basis of antibiotic resistance, PCR-based approaches have been developed for detecting the presence of genetic determinants of resistance to a variety of antibiotics for a number of different bacterial species. One salient example has been the use of PCR to identify methicillin-resistant Staphylococcus aureus (MRSA) through the detection of the \(mecA\) gene, which encodes a modified penicillin-binding protein with reduced affinity for \(\beta\)-lactam antibiotics. Many PCR assays (both conventional and real time) have been described for detecting \(mecA\) and/or associated sequences,\(^12–16\) including commercial systems that in some cases have the ability to detect MRSA directly from clinical samples in \(<2\) h.\(^17–19\) Examples of commercialized systems that employ real-time PCR for detection are the BD GeneOhm MRSA assay from Becton Dickinson and the GeneXpert system from Cepheid, the latter of which showed high sensitivity (\(>93\)% compared with routine methods for detecting MRSA in a multicentre study.\(^20\) A recent study demonstrating that the clinical implementation of a test for detection of the \(mecA\) gene reduced the time to receiving optimal antibiotic therapy by an average of 25.4 h in patients with \(S.\) aureus bacteraemia illustrates the potential utility of this method.\(^21\) PCR-based approaches have also been developed for detecting vancomycin resistance associated with the \(vanA\) and \(vanB\) genes and have primarily been used for detecting resistance in Enterococcus species. The sensitivity and specificity of these assays varies depending on the study; however, a number of these studies have reported high false-positive rates for the detection of the \(vanB\) gene, resulting in reduced specificity.\(^22–25\) Assays for detecting the presence of resistance genes in Gram-negative bacteria have also been developed. Most notably, this has included assays for identifying numerous cephalosporinase- and carbapenemase-encoding genes, including, but not limited to, KPC, NDM, IMP, VIM, AmpC, TEM, SHV and the OXA carbapenemases in Acinetobacter baumannii, Pseudomonas aeruginosa, Klebsiella pneumoniae and Escherichia coli.\(^26–32\)

The major advantage of these PCR-based approaches is that they can be carried out in a relatively short period of time, in some cases using clinical samples without the need for purity culture. PCR thus clearly has the potential to significantly reduce turnaround times and rapidly provide information on antibiotic resistance. The major limitation of this approach, however, is that the presence of resistance genes may not always correlate with phenotypic resistance. This is less worrisome in cases where the presence of a genotype is highly associated with phenotypic resistance, such as the presence of the \(mecA\), \(vanA\) and \(vanB\) genes. However, other cases are not so clear cut, such as the presence of carbapenemases in Gram-negative bacteria. There are dozens of distinct carbapenemases for which phenotypic resistance may depend not only on the presence of the gene, but on its level of expression. One example is the gene encoding OXA-51 in A. baumannii, which only produces phenotypic resistance if it is highly expressed, e.g. due to the integration of mobile insertion sequences in the gene’s promoter region that increase expression.\(^33,34\) The development of techniques that rely on the measurement of gene transcripts (RNA levels) instead of the presence of a gene may provide a potential solution to this problem. PCR-based techniques that detect the presence of resistance genes are also unable to detect novel or uncharacterized mechanisms of resistance for which the genetic determinant is unknown. This may be especially important in the case of carbapenemases in Gram-negative bacteria, given the continuous emergence of new variants. This is a critical point, as the inability of a technique to detect resistance would lead to the inappropriate classification of a resistant isolate as susceptible, an error that could lead to the administration of ineffective therapy. A final consideration is that these methods do not provide MIC values, which can be useful for guiding clinical decisions regarding therapy.

In addition to using PCR for detecting the presence of genetic determinants of resistance, the ability of real-time PCR to accurately quantify the number of copies of a specific nucleic acid in a sample has led to the development of approaches that employ this method for measuring bacterial growth. This approach monitors the number of bacterial genome copies present during growth of the isolated bacteria in the presence of the antibiotic being tested. Since quantitative real-time PCR can provide precise information regarding genome copy numbers, very short incubation times can be used for differentiating susceptible from resistant strains. One recent example is the detection of resistance to imipenem, ciprofloxacin and colistin in clinical isolates of A. baumannii using a real-time PCR assay targeting highly conserved sequences of the \(ompA\) gene.\(^35\) As shown in Figure 1, growth of a resistant strain in the presence of antibiotics could be detected with relatively short incubation times. This approach has been applied to a number of bacterial species with various antibiotics.\(^36,37\) One advantage of this approach with respect to the PCR-based
approaches described above is that it does not depend on the mechanism of resistance and that the assay is indirectly measuring phenotypic resistance by detecting growth in the presence of antibiotic. The major disadvantage is that, unlike PCR-based approaches that detect the presence of resistance determinants, this approach requires previous culture and cannot be used directly with clinical samples.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS)

MALDI-TOF MS identifies molecules based on their time of flight through a vacuum tube after laser irradiation of a matrix that is co-crystallized with the sample. The time of flight allows for determination of the mass/charge ratio (m/z) of the ions present and a spectrum of the sample is generated. The spectrum is then compared with a reference database in order to identify the analyte. Similar to PCR, MALDI-TOF MS was originally introduced into clinical microbiology laboratories for the identification of pathogens and has only recently been applied to the detection of resistance. The use of MALDI-TOF MS to detect resistance most commonly aims to differentiate spectra from resistant and susceptible isolates using whole cells or crude extracts. Comparison of fingerprints from E. coli ATCC 700926 and the same strain carrying the β-lactamase-producing plasmid pUC19 determined just one discriminatory peak corresponding to a β-lactamase. However, no pattern was found that could reliably identify β-lactamase resistance in clinical isolates of E. coli, K. pneumoniae and P. aeruginosa. The detection of vancomycin-resistant enterococci has been recently demonstrated by identifying discriminatory peaks between vanB-positive Enterococcus faecium isolates and those lacking vanB. In the case of MRSA, different results have been obtained in individual studies. Some studies have reported measurable differences in spectra when MRSA were compared with methicillin-susceptible S. aureus isolates whereas other authors affirm that these differences are due to differences in clonality between the resistant and susceptible strains. The major advantages of the use of MALDI-TOF MS for the identification of resistant strains based on differences in spectra are that it is extremely rapid and highly automated. However, similar to PCR-based techniques, the results obtained using this approach may not always directly correlate with phenotypic resistance and differences between strains that are not related to resistance can complicate the interpretation of results.

MALDI-TOF MS has also been employed to detect the hydrolysis of antibiotics during incubation with a bacterial isolate in order to detect degradation products. Two reports describing the detection of β-lactamase activity using this approach were published in 2011. Hrabak et al. validated this method in Enterobacteriaceae and P. aeruginosa using 124 strains by detecting carbapenemase activity through meropenem degradation, reporting a sensitivity and specificity of 96.67% and 97.87%, respectively. Moreover, the same authors reported an improvement in the technique by adding SDS to the reaction in order to decrease the incubation time and the number of bacteria necessary for the assay. Carbapenem resistance has also been analysed in K. pneumoniae, E. coli, P. aeruginosa and Citrobacter freundii using ertapenem. In separate studies, the presence of β-lactamases was detected by showing hydrolysis of various antibiotics, including penicillin G, ampicillin, imipenem and ceftoxin in lysates of E. coli, ampicillin, piperacillin, ceftazidime, cefotaxime, ertapenem, imipenem and meropenem in supernatants from E. coli and K. pneumoniae. The hydrolysis of ertapenem has also been shown directly in K. pneumoniae-positive blood cultures. Recently published results have determined that the detection of class D β-lactamase activity requires a longer incubation time than that seen with classes A and B in Acinetobacter spp. Also, a study with 106 A. baumannii strains determined that the use of imipenem to identify carbapenem resistance showed a sensitivity and specificity of 100%. The main advantage of this method is the detection of activity without considering the type of enzyme involved.

Microarrays

Microarrays identify the presence of specific nucleic acid sequences using complementary oligonucleotides. Owing to the fact that these oligonucleotides can be assembled onto solid supports in very close proximity, this method has the ability to detect numerous (i.e. thousands of) sequences in a single assay. For this reason, this method offers the possibility of creating arrays with the ability to detect a broad range of resistance genes present in bacterial isolates. One example is the use of microarrays for the detection of β-lactamase genes present in bacterial isolates. Numerous studies have employed microarrays for the detection of β-lactamases in Gram-negative bacteria, some of which can provide results in one working day. In a recently published study, real-time PCR was combined with a microarray in order to identify respiratory pathogens causing ventilator-associated pneumonia and detect the presence of 24 genes associated with resistance to β-lactam antibiotics directly from clinical samples. This technique demonstrated high sensitivity and
specificity for the detection of resistance genes, with a limit of detection of 10–100 DNA copies.

Microarray technology offers the ability to detect vast numbers of different resistance genes in a single assay, as opposed to PCR-based approaches, which can only identify a handful of genes. For this reason, microarrays are ideally suited to bacteria in which there are numerous distinct mechanisms of resistance or variants of a single mechanism, such as the case of β-lactamases in Gram-negative bacteria. However, similar to the approaches described above, data obtained from microarrays may not always correlate with phenotypic resistance and this approach does not provide data on MIC values. In addition, this method may have limited ability to detect resistance in isolates harbouring novel or uncharacterized mechanisms of resistance.

Microfluidics

Advances in bioengineering and nanotechnology have facilitated the miniaturization of molecular assays that can be used for the detection of antibiotic resistance. These so-called ‘lab on a chip’ platforms utilize extremely small volumes of reagent and analyte (picolitres). These devices can incorporate multiple functionalities onto a single chip, including bacterial culture, nucleic acid hybridization and amplification, and cell lysis. The detection methods vary widely, depending on the device employed, and can be based on electrochemical, magnetic and optical detection, among others. A handful of recent studies have illustrated the potential of microfluidic devices for rapidly providing information regarding antibiotic resistance. Choi et al. demonstrated that a device consisting of microfluidic agarose channels could track the growth of single cells using microscopy in the presence of antibiotics. Interestingly, approximate MIC values could be obtained using this approach in only 3–4 h. In another study, the electrochemical quantification of 16S rRNA levels was used to measure bacterial growth in the presence of antibiotics. This method was validated directly with patient urine samples and was able to provide results in 3.5 h with 94% agreement with standard antibiotic susceptibility testing methods. In a separate study, a microfluidic pH sensor was developed that could be used to detect pH changes that occur during bacterial growth in the presence of antibiotics due to the accumulation of metabolic products. With this approach, bacterial growth curves could be generated in as few as 2 h in nanolitre-scale cultures.

In addition to the very small volumes of analyte that are needed for these assays, this approach has the advantage of being highly automated with the potential for providing results extremely rapidly. Owing to their small size, the chips used in these assays can be incorporated into portable devices, which may facilitate antimicrobial susceptibility testing at the point of care. In many cases, microfluidic devices indirectly measure bacterial growth in the presence of antibiotic, making it likely that the results obtained will correlate well with phenotypic resistance. This aspect also makes this approach amenable for use in detecting resistance in bacteria for which the mechanisms of resistance are not well characterized.

Cell lysis-based approaches

Recently, an approach based on detecting bacterial cell lysis after incubation with the antibiotic being tested has been described. The bacterial isolate is first incubated with the desired concentrations of the antibiotic being tested and then immobilized in an agarose microgel. The immobilized bacteria are subsequently immersed in a lysing solution, which results in disruption of the nucleoid in bacteria that have been affected during incubation with the antibiotic in the previous step. The preparation is incubated with a DNA-specific fluorescent stain and the nucleoid fragmentation is visualized by microscopy. As can be seen in Figure 2, which shows ampicillin-susceptible and -resistant strains of Enterococcus faecalis processed as described above, nucleoid fragmentation is evident in the susceptible strain, whereas the resistant strain maintains an intact nucleoid. This approach has been validated for the detection of quinolone and ampicillin resistance in E. coli and recently for detecting carbapenem resistance in A. baumannii. The procedure could be carried out in 100 min and showed good correlation with microdilution and Etest data. Interestingly,
although it has not been rigorously determined experimentally, this approach may have the ability to provide approximate MIC values, since nucleoid fragmentation is visualized after incubation with different concentrations of the antibiotics being tested. The studies described to date have only assessed this technique using culture-purified bacteria and it remains to be determined if this approach can be used directly with clinical samples. An advantage of this approach is that a result is obtained regardless of the mechanism that is producing the resistance.

Whole-genome sequencing

Advances in DNA sequencing technology have made it possible to sequence entire bacterial genomes extremely rapidly. These methods, coupled with bioinformatic tools that can quickly assemble and analyse the massive amount of data obtained from these sequencing runs, open the possibility of using these techniques for detecting antibiotic resistance. A number of studies describing whole-genome sequencing of small numbers of clinical isolates in order to characterize the genetic determinants of antibiotic resistance have been described.67–70 The objective of these studies has primarily been to characterize strains with interesting phenotypic resistance profiles. In a recent study, whole-genome sequencing was used to characterize the resistance profiles of 200 bacterial isolates from four bacterial species to a variety of antibiotics and the results were compared with those obtained using phenotypic susceptibility testing.71 High concordance (99.74%) was observed between the two techniques, demonstrating that data obtained from genome sequences can correlate well with phenotypic resistance in some cases. For these studies, the speed with which the sequencing runs and subsequent analyses are carried out are of less importance than would be the case if this technology were to be applied in the clinical setting for susceptibility testing. Taking into account the turnaround times necessary for whole-genome sequencing, and the elevated cost compared with traditional and other emerging techniques, the use of whole-genome sequencing for routine susceptibility testing may not yet be practical.72 Although in its current form it may not be suited for routine testing, whole-genome sequencing has demonstrated its utility in tracking outbreaks of clinically important strains, as was demonstrated during a hospital outbreak of MRSA in the UK73 and during an outbreak of E. coli O104:H4 in Germany.74–76

Undoubtedly, genome sequencing will increasingly be employed for a variety of applications in the clinical microbiology laboratory as the cost of sequencing decreases and the speed of sequencing and analysis increases. However, it should be noted that similar to the microarrays and PCR-based approaches described above, DNA sequencing relies on the identification of the genetic determinants of resistance, raising the possibility that a limitation of this approach may be the detection of novel or uncharacterized mechanisms of resistance.

Conclusions

The methods described here all aim to shorten the time necessary for detecting resistance in bacterial pathogens; however, in many cases it remains to be determined whether these approaches provide sufficient sensitivity and specificity. In addition, although the methods discussed here have undergone microbiological testing to determine whether they give similar results to those obtained using standard methods, studies providing information on the clinical validation of these approaches have yet to be performed in many cases. For a method to be approved by the US FDA, the very major error rate (the classification of a resistant isolate as susceptible) must be <1.5% and the major error rate (the classification of a susceptible isolate as resistant) must be <3.0%.77 One of the key limitations that must be addressed regarding many of these methods, in order to ensure they meet these criteria, is how often there is discordance between the presence of a resistance determinant and phenotypic resistance. This is of particular importance with the use of techniques that rely solely on the detection of resistance determinants, such as PCR for gene detection or MALDI-TOF MS. How these tests will deal with novel or uncharacterized resistance mechanisms must also be considered, since the inability of a test to identify resistance will lead to very major errors. Additional aspects that will need to be addressed on a case-by-case basis are whether or not the tests being developed require highly qualified personnel, the added expense associated with the test and the acceptance of test results by clinicians. However, given the potential benefits in terms of improving patient outcomes by reducing the period of time during which empirical therapy is administered and in terms of reducing healthcare costs, the continued development of these approaches is warranted.

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None to declare.

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