



Enfermedades Infecciosas y Microbiología Clínica

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Continuing medical education: Methods of rapid diagnosis

Rapid methods for detection of bacterial resistance to antibiotics[☆]

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ARTICLE INFO

Article history:

Received 22 November 2016

Accepted 16 December 2016

Keywords:

Rapid antibiotic susceptibility test

Direct antibiotic susceptibility test

Susceptibility

ABSTRACT

The most widely used antibiotic susceptibility testing methods in Clinical Microbiology are based on the phenotypic detection of antibiotic resistance by measuring bacterial growth in the presence of the antibiotic being tested. These conventional methods take typically 24 h to obtain results. Here we review the main techniques for rapid determination of antibiotic susceptibility. Data obtained with different methods such as molecular techniques, microarrays, commercial methods used in work routine, immunochromatographic methods, colorimetric methods, image methods, nephelometry, MALDI-TOF mass spectrometry, flow cytometry, chemiluminescence and bioluminescence, microfluids and methods based on cell disruption are analysed in detail.

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Métodos rápidos para la detección de la resistencia bacteriana a antibióticos

RESUMEN

Los métodos más frecuentemente utilizados en Microbiología Clínica para la determinación de la sensibilidad de las bacterias a los antibióticos se basan en un estudio fenotípico, observando el crecimiento bacteriano de una cepa incubada en presencia del antibiótico a estudiar. Estos métodos requieren normalmente un tiempo de unas 24 h para la obtención de resultados. En esta revisión se exponen el fundamento y los resultados de las principales técnicas instrumentales que proporcionan un antibiograma rápido. De manera pormenorizada se exponen datos relativos a técnicas moleculares, *microarrays*, métodos comerciales utilizados en el trabajo de rutina, técnicas inmunocromatográficas, métodos colorimétricos, métodos de imagen, nefelometría, espectrometría de masas MALDI-TOF, citometría de flujo, quimioluminiscencia y bioluminiscencia, microfluidos y métodos de lisis bacteriana.

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Palabras clave:

Antibiograma rápido

Antibiograma directo

Sensibilidad

Introduction

To achieve a favourable clinical course in patients who suffer from infections, the clinical microbiology laboratory should report the agent causing the infection as quickly as possible. If it is a bacterium that does not have uniform sensitivity to antibiotics, the antibiogram should also be reported, since the significant increase in antimicrobial resistance represents an obstacle to empirical treatment in some cases.

Routine antibiogram techniques are based on a phenotypic study in which microbial growth is observed in the presence of different antibiotics. These techniques include agar dilution (the gold standard for the antibiogram), broth macrodilution and microdilution, and strips with an antibiotic gradient. They yield results in around 17 h. To shorten this time, it would be desirable to have fast and reliable antibiogram results. To evaluate reliability, according to the US Food and Drug Administration (FDA),¹ the results of a rapid antibiogram are classified, compared to the antibiogram obtained through the gold standard, as agreements (concordance), minor errors (erroneous intermediate sensitivity result), major errors (false resistance) and very major errors (false sensitivity).

There are many instrumental techniques that allow an antibiogram to be made quickly. Notable among these are molecular techniques, microarrays, commercial methods used in

[☆] Please cite this article as: March-Rosselló GA. Métodos rápidos para la detección de la resistencia bacteriana a antibióticos. *Enferm Infecc Microbiol Clin.* 2017;35:182–188.

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routine work, immunochromatographic techniques, colourimetric methods, imaging methods, nephelometry, MALDI-TOF mass spectrometry, flow cytometry, chemiluminescence and bioluminescence, microfluids and bacterial lysis methods. The basis of each of these techniques and the results obtained are presented below.

Molecular techniques

Molecular techniques enable the detection of genetic material, both deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). Polymerase chain reaction (PCR) is the molecular technique that has acquired the greatest diagnostic value, since it not only allows the infectious agent to be accurately identified, but is also the leading method to characterise its resistance and virulence genotypes. Conventional PCR requires approximately 12 h to perform and consists of 3 steps. The first step consists of extraction of genetic material. The second step, performed in a thermocycler, consists of DNA amplification. The thermocycler reaches the optimal temperatures required for each of the 3 steps comprising an amplification cycle (denaturation of the DNA to be used as a mould, ringing of synthetic primers and extension catalysed by the polymerase DNA of the primers) to take place. Amplification is repeated a certain number of times, generally 25–35. Each time, the number of product molecules (amplicons) is duplicated. Thus a high number of amplicons is synthesised, which allows very small initial amounts of DNA to be detected.² The third and final step of PCR consists of detection of amplicons through agarose gel electrophoresis. Real-time PCR was designed to shorten the time to diagnosis of conventional PCR. In real-time PCR, amplification and detection of the amplicons synthesised take place at the same time through different methods. Thus, real-time PCR yields results in a few hours.²

Several real-time PCRs which identify several pathogenic agents and genes that directly confer antibiotic resistance based on different samples have been marketed. These PCRs are fully automated: samples are processed in just a few minutes. The Verigene[®] system (Nanosphere) is based on grown blood culture bottles. Detection of amplicons takes place through hybridisation with synthetic specific oligonucleotides marked with gold nanoparticles. In Gram-positive bacteria, it detects 9 species and 4 genera of bacteria, as well as the resistance genes *mecA* and *vanA/B*, in less than 3 h with a sensitivity and a specificity very close to 100%.³ In Gram-negative bacteria, it detects 5 species and 4 genera of bacteria, as well as the resistance genes that encode CTX-M extended-spectrum beta-lactamases (ESBLs) and KPC, NDM, VIM, IMP and OXA carbapenemases, with the same response time and with a sensitivity and a specificity in excess of 93%.⁴ The FilmArray Blood Culture Identification Panel system (BioFire Diagnostics) is also applied to the grown blood culture bottle. In this case a nested PCR is performed. First, a region of DNA that contains the target segment is amplified. Next, this amplification product is used as a mould for a second PCR which takes place in a matrix with wells that contain the primers for the different assays. Finally, the instrument uses the fluorochrome LCGreen[®] Plus (BioFire Diagnostics) to evaluate the fusion curve of the DNA in each well of the matrix to determine whether a PCR product appears in said well. In an hour, this system detects 11 species and 15 genera of Gram-positive and Gram-negative bacteria and 5 species of yeast. It also detects the resistance genes *mecA*, *vanA/B* and *KPC*. Sensitivity ranges from 83% to 100%, and specificity is more than 99%, depending on the pathogen studied.³ The GeneXpert[®] system performs real-time PCR in single-use disposable cartridges. There are many cartridges for performing different analyses. To detect *Staphylococcus aureus* and its methicillin resistance in clinical samples, 2 cartridges are available: the Xpert[®] MRSA/SA BC cartridge, which uses grown blood culture bottles, and the Xpert[®] MRSA/SA SSTI cartridge, which uses

swabs to diagnose skin and soft-tissue infections. Both tests yield results in an hour and have a sensitivity and a specificity very close to 100%.^{3,5} The Xpert MTB/RIF[®] cartridge detects *Mycobacterium tuberculosis* and its rifampicin resistance in sputum and biological fluids in 2 h. In this case a semiquantitative nested PCR takes place. It has been observed that in sputum and bronchoalveolar lavage, the test has a sensitivity and a specificity of 86.8% and 93.1%, respectively, thereby improving sputum smear microscopy results.⁶

Many PCRs – “in-house”, commercial and automated – and PCR kits that accurately detect, with a sensitivity and a specificity of practically 100%, a large number of genes that confer antibiotic resistance have been described in the literature.⁷ It should be noted that these methodologies do not provide microbial identification and that they are applied, in the majority of cases, to colonies grown on isolation plates.

The main commercial automated real-time PCRs are detailed below. The NucliSENS EasyQ[®] KPC platform (bioMérieux) detects genes that encode KPC carbapenemases in 2 h.⁸ The Xpert[®] Carba-R cartridge from GeneXpert[®] detects genes that encode KPC, NDM, VIM, IMP and OXA-48 carbapenemases in an hour.⁹ The eazyplex[®] system (Amplex Biosystems GmbH) consists of a platform that performs nucleic acid amplification through the loop-mediated isothermal amplification (LAMP) technique. This technique is based on DNA amplification through chain displacement at a constant temperature; thus, the system does not require a thermocycler. To carry out amplification, the DNA extracted is inserted in a tube containing a lyophilisate with the reagents required to perform nucleic acid amplification; next, this tube is inserted in the Genie[®] II apparatus (OptiGene), which keeps the tube at a constant temperature and detects the amplicons formed in real time. For this platform, several kits have been marketed that detect genes that confer antibiotic resistance in less than 30 min. Notable among them is the eazyplex[®] SuperBug CRE which detects genes that encode CTX-M ESBL and VIM, NDM, KPC and OXA-48 carbapenemases, both in colonies grown on isolation plates and directly from urine samples and grown blood culture bottles.¹⁰ AID Autoimmun Diagnostika GmbH has marketed a PCR based on line probe assays. In this case, once the PCR has been performed, reverse hybridisation of the amplicons takes place with complementary probes anchored to a nitrocellulose strip. Hybridisation is detected using a biotin marker present in the primers used in the PCR. Thus a band pattern is obtained which is interpreted visually or with a scanner. Notable among antibiotic resistance kits is the AID ESBL, which detects genes that encode TEM, SHV and CTX-M ESBLs and KPC carbapenemases in 5 h.¹¹

Notable among the kits marketed to detect genes that confer bacterial resistance to antibiotics are the following: LightMix (Roche Diagnostics) and Check-Direct CPE (Check-Points Health B.V.). The LightMix kit, using the LightCycler[®] 480 Instrument II platform (Roche Diagnostics), detects KPC, NDM, VIM, IMP and OXA-48 carbapenemases in an hour and a half. It should be noted that this kit may also be applied to grown blood culture bottles.¹² The Check-Direct CPE kit may be used on several real-time PCR platforms, such as the ABI 7500 (Applied), CFX96[™] (Bio-Rad), LightCycler[®] 480 system I & II (Roche), Rotor-Gene Q (Qiagen) and BD MAX[™] (Becton Dickinson) platforms. This kit includes the reagents required to detect genes that encode KPC, NDM, VIM and OXA-48 carbapenemases in 2 h.⁹ In addition, some kits to perform a ligation-mediated PCR have been marketed. Briefly, this PCR uses 2 DNA probes that recognise one of the 2 strands of the gene to be detected. If hybridisation occurs, the probes remain adjacent and a DNA ligase binds them, thereby generating a double-chain DNA. This ligation step is performed in the MyCycler apparatus (Bio-Rad). Finally, real-time PCR takes place on the ABI 7500 (Applied) platform using some universal primers. Notable among the kits that detect genes that encode bacterial resistance to antibiotics

through ligation-mediated PCR are one kit for CTX-M, TEM and SHV ESBLs¹³ and another kit for KPC, NDM, IMP, VIM, and OXA-48 carbapenemases.¹⁴ Both kits yield results in 4 h and a half.

Finally, it should be noted that the commercial molecular methods mentioned above have a limited percentage of false negatives as these methods do not detect all allelic variants of genes that confer antibiotic resistance.¹⁵

Microarrays

This method is based on using an image analysis to detect hybridisation of a target molecule to a specific probe immobilised on a solid base. Microarrays detect a large number of resistance genes in a single assay given that these probes, which are normally oligonucleotides, are attached very close to one another. Several microarrays have been marketed, such as the Check-MDR CT102, the Check-MD CT103 and the Check-MDR CT103 XL (Check points Health BV). These microarrays detect a large number of genes that encode different beta-lactamases (ESBLs, AmpCs and carbapenemases) based on colonies grown on isolation plates. These 3 microarrays require a first step of a PCR with a pair of universal primers marked with biotin. Next, the amplicons are classified through hybridisation with the oligonucleotide probes. Finally, the manufacturer's software program detects hybridisation using the biotin marker, automatically translates the data and expresses the results in the form of the presence or absence of a gene. These microarrays yield results in 8 h and have a sensitivity and a specificity of practically 100%.^{16–18}

Commercial antibiogram methods

Different commercial antibiogram methods used in routine clinical microbiology laboratory work have been applied directly based on different clinical samples. Commercial strips with an antibiotic gradient have been used to make a direct antibiogram based on respiratory samples. To do this, the sample is inoculated in Mueller Hinton agar plates and the strips with antibiotic are added. The plates are incubated for 24 h and, once the colonies have grown, the MIC is obtained. Boyer et al.¹⁹ obtained a rate of agreements of 88.9%, a rate of very major errors of 1.5% and a rate of major errors of 9.6%. Bouza et al.²⁰ obtained a rate of agreements of 96.44%, a rate of major errors of 1.98% and a rate of minor errors of 1.56%.

Semiautomated broth microdilution methods (MicroScan, VITEK2 and Phoenix) allow the bacteria to be identified and the antibiogram to be obtained directly based on the grown blood culture bottle. The bacteria are identified in 3 h, with poor results in Gram-positive bacteria and acceptable results in Gram-negative bacteria, and the antibiogram is obtained in 14 h, with good results in both Gram-positive and Gram-negative bacteria.²

Immunochromatographic techniques

These techniques yield results in around 20 min. They are affordably priced and require neither instrumentation nor expert staff. This means that they may be performed in any laboratory. In antibiotic resistance, they detect bacterial enzymes that hydrolyse the antibiotic. The procedure consists of suspending the bacterium in a diluent. Next, a few drops of this diluent are deposited on one end of the strip (normally made of nitrocellulose), and the bacteria move by means of capillarity towards the other end of the strip. If a coloured band appears on the test position of the strip, where an antibody that recognises the antigen of the bacteria is fixed, this means that the test is positive. Two immunochromatography systems that detect OXA-48 and KPC carbapenemases (Coris Bio-Concept) with a sensitivity and a specificity of practically 100%

have been marketed.²¹ The BinaxNOW PBP2a test (Alere) has been marketed to detect the methicillin resistance of *S. aureus*. This test shows a sensitivity and a specificity of practically 100%. It was also applied to grown blood culture bottles and offered a sensitivity of 95.4% and a specificity of practically 100%.³

Colourimetric methods

Several kits have been marketed to detect carbapenemases, such as the RAPIDEC® CARBA NP kit (bioMérieux) and the Rapid CARB Screen® kit (Rosco Diagnostica A/S). These kits yield results in around 2 h with a sensitivity and a specificity very close to 100%.²² In these tests, the bacterium is incubated in the presence of the antibiotic. If the bacterium possesses a carbapenemase, the antibiotic is hydrolysed and there is a change in the pH of the medium which is detected through a change in the colour of the indicator. These tests do not characterise the type of carbapenemase. However, in a phenotypic study, they can detect any carbapenemase variant that is being expressed.

Resazurin, added to a bacterial suspension resistant to the antibiotic with which it is incubated, is reduced by the metabolic activity of the bacterium. If the bacterium is sensitive to the antibiotic, its metabolism stops, and consequently resazurin remains in its oxidised form. Given that the reduced form of resazurin is stable and red in colour, and photometrically distinguishable from the oxidised form, which is blue in colour, cell viability may be monitored through spectrophotometry, colourimetry or fluorimetry. In 6 h, Coban et al.^{23,24} determined the sensitivity of *S. aureus* to methicillin and vancomycin. March et al.^{25,26} made an antibiogram for staphylococci, enterococci and enterobacteria in 2 h. However, the disadvantage of resazurin is that it is not metabolised by non-fermenting Gram-negative bacilli. Another colourimetric method consists of measuring the activity of the bacterial enzyme nitrate reductase. When the bacterium is incubated in the presence of NO₃[–] ions and antibiotic, if the bacterium is resistant to the antibiotic, NO₂[–] ions are going to be generated that, with Griess reagents, yield a compound that is red in colour. In around 6 h, this method determines the sensitivity of *S. aureus* to methicillin and vancomycin.^{23,24} These 2 colourimetric methods have also been applied in mycobacteria and the antibiogram has been obtained in 7–14 days.²⁷ In all cases, the rate of correlation with the reference methods that was obtained was close to 100%.

Imaging methods

Based on grown blood culture bottles, the ACCELERATE pheno™ SYSTEM apparatus (Accelerate Diagnostics) identifies 10 species and 6 genera of bacteria through the fluorescence *in situ* hybridisation (FISH) technique in 1 h. To make the direct antibiogram, the bacterial growth of a strain incubated in the presence of different concentrations of antibiotic is monitored through imaging. Thus, in 5 h this piece of equipment reports the MIC and the phenotypes for high-level resistance to gentamicin and streptomycin in enterococci and for induction of clindamycin resistance by erythromycin in staphylococci. Depending on the pathogen studied, the sensitivity obtained has a rate of agreement of 92–100% with that obtained through broth microdilution.²⁸

Nephelometry

Nephelometry is a technique that measures the intensity of scattered radiation that is generated when a beam of light passes through a suspension of particles. Given that light diffusion is proportional to particle concentration, this instrument-based technique allows microorganisms to be quantified.² The Alfred 60

apparatus (Alifax) performs a urine sample screening to diagnose urinary tract infections. To do this, an aliquot of urine is spiked in a tube with liquid enrichment medium and bacterial growth is monitored through nephelometry for 3 h. With this piece of equipment, by monitoring bacterial growth in marketed tubes that contained a liquid enrichment medium and antibiotic together with an aliquot of the sample, an antibiogram directly based on urine and bottles of blood culture was made in 5 h.^{29,30}

MALDI-TOF mass spectrometry

The MALDI-TOF system uses a protein analysis to identify bacteria (including mycobacteria), yeasts and filamentous moulds in minutes.² Regarding the rapid antibiogram, the MALDI-TOF system predicts whether the bacteria produce enzymes that hydrolyse the antibiotics, such as carbapenemases and ESBLs, in less than 3 h. To do this, the microorganisms are incubated for a while with the antibiotic. Centrifugation is performed and the supernatant obtained is analysed using MALDI-TOF. If the microorganism possesses the enzyme that degrades the antibiotic, the peak corresponding to the antibiotic will be seen to disappear and new peaks corresponding to the metabolites resulting from the rupture of the antibiotic will be seen to appear. If the bacterium does not hydrolyse the antibiotic, only the peak corresponding to the antibiotic will be observed. This methodology has yielded sensitivities of practically 100%, both based on colonies grown on isolation plates³¹ and based on grown blood culture bottles from patients.³² It is also possible to predict resistance to chloramphenicol and clindamycin by detecting the 16S ribosomal RNA methylation performed by methyltransferases.³³ Moreover, both methicillin-sensitive and methicillin-resistant *S. aureus* strains have been observed to yield specific identification peaks; thus, from a database that is normally prepared in the laboratory itself, it is possible to distinguish between methicillin-sensitive and methicillin-resistant *S. aureus* strains in a few minutes based on colonies grown on isolation plates.³⁴ Similarly, the MALDI-TOF system also discriminates between strains of vancomycin-resistant and vancomycin-sensitive enterococci.³⁵ Antibiotic sensitivity may also be studied through incubation of microorganisms in the presence of antibiotic in a medium with isotope-marked molecules. If the microorganism is resistant, it will incorporate the marked molecules which may be detected by MALDI-TOF.³⁶ Jung et al.³⁷ developed an antibiogram based on a semiquantitative analysis. They incubated an aliquot from the grown blood culture bottle in a liquid medium, both in the presence and in the absence of antibiotic. The latter was the control group. The mass spectra were obtained from the sediments and the relative intensity of the peaks and the area under the curve (AUC) were calculated. For purposes of interpreting the antibiogram, a strain was considered to be resistant to the antibiotic if it yielded a peak intensity and AUC similar to those of the control group when incubated with the antibiotic. By contrast, a strain was considered to be sensitive to the antibiotic if, when incubated with antibiotic, it provided a peak intensity and AUC lower than those of the control group. An antibiogram was thus obtained based on the grown blood culture bottle in 4 h, with a 2% discrepancy rate compared to the sensitivity obtained by the E-test.

Flow cytometry

Flow cytometry is a technique based on the formation of a flow of particles (generally cells) arranged in a row with an intensity of 500–4000 particles/second. Thanks to this alignment, the technique allows multiple characteristics of a single cell to be measured simultaneously such that it is possible to characterise, separate and

quantify the different cell subpopulations that are included in a set. In addition, various bacterial parameters (membrane potential, cell size, enzyme activity, cell membrane integrity and microbial concentration) which provide information about the sensitivity of microorganisms to antibiotics may be studied using various fluorochromes.²

Through histograms which represented scattered light at a 90-degree angle (side scatter channel [SSC]) to the fluorescence signal, Shrestha et al.³⁸ managed to distinguish between methicillin-resistant and methicillin-sensitive *S. aureus* strains following an incubation period of 4 h in the presence of the antibiotic. The Sysmex UF-1000i flow cytometry apparatus (Sysmex Corporation) is used in microbiology laboratories to perform a urine screening using the bacterial count. This piece of equipment was used to obtain 2 antibiograms by comparing the counts obtained from strains incubated in a liquid medium with antibiotics to the counts obtained for the same strains incubated without antibiotic (control group). A strain was considered to be resistant to the antibiotic if it yielded a microbial count very similar to that of the control group when incubated with the antibiotic. By contrast, a strain was considered to be sensitive to the antibiotic if it yielded a microbial count lower than that of the control group when incubated with the antibiotic. Broeren et al.³⁹ calculated the MIC of amoxicillin, gentamicin and piperacillin in *Escherichia coli*, *Pseudomonas aeruginosa* and *S. aureus*. In 4 h they obtained an antibiogram with a rate of agreement of 100% compared to the antibiogram obtained through the VITEK2, E-test and broth macrodilution systems. March et al.⁴⁰ made an antibiogram based on 100 grown blood culture bottles and obtained, in just 2 h, a rate of agreement of 98% compared to the sensitivity obtained through the E-test, MicroScan and VITEK2 commercial methods. Faria-Ramos et al.⁴¹ managed to distinguish between ESBL-producing and non-ESBL-producing strains in less than 3 h based on bacteria incubated in the presence of ceftazidime and cefotaxime with and without clavulanic acid, using the fluorochrome DiBAC₄(3), which only penetrates non-viable bacteria. Gauthier et al.⁴² made an antibiogram through flow cytometry based on urine. They used 2 fluorochromes, DiBAC₄(3) and propidium iodide, which also only penetrates non-viable bacteria. They analysed 114 urine samples and, by measuring the fluorescence signal after incubating the aliquots of urine for 2 h in the presence of different antibiotics, obtained a 2% discrepancy rate compared to the sensitivity obtained through broth microdilution.

In yeasts, it is possible to obtain an antifungigram in 6 h through the use of propidium iodide.^{43,44} In mycobacteria, Pina-Vaz et al.⁴⁵ determined the sensitivity of *M. tuberculosis* to streptomycin, isoniazid, rifampicin and ethambutol. After 3 days of incubation in the presence of the antituberculosis drug in the BACTEC MGIT 960 system (Becton Dickinson), the microorganisms were stained with the fluorochrome SYTO 16, which only penetrates microorganisms with a cell membrane abnormality. By comparing the intensity of the fluorescence signal obtained from these microorganisms to that obtained from microorganisms incubated during the same time without the presence of the antituberculosis drug, they distinguished between sensitive, intermediate and resistant strains and obtained results that showed excellent agreement with those obtained from 3 weeks of incubation in the BACTEC MGIT 960 system. Kirk et al.⁴⁶ determined the sensitivity of *M. tuberculosis* by studying the capacity of the mycobacteria to hydrolyse, through esterases, the substrate fluorescein diacetate, which turns into fluorescein, a compound which emits fluorescence when it is excited with light from a suitable wavelength. If the mycobacterium is sensitive to the antituberculosis drug, the hydrolytic capacity of the mycobacterium decreases, and therefore the fluorescence signal detected also decreases. By comparing the fluorescence signal and the scattered light at 90 degrees obtained through flow cytometry based on mycobacteria without contact with the antituberculosis

drug to the signals obtained from mycobacteria with an incubation period of 24 h with isoniazid, ethambutol and rifampicin, they obtained rates of agreement of 95%, 92% and 83%, respectively, compared to the antibiogram obtained through the method of proportions.

Chemiluminescence and bioluminescence

Chemiluminescence is a process of light emission that occurs in certain chemical reactions when excited molecules return to the ground state. To obtain light, menadione must be added to the microorganism culture. The bacterial membrane is permeable to this molecule. Once menadione penetrates the microorganisms, it is reduced and various compounds are generated that diffuse to the extracellular environment where they self-oxidise and light photons are emitted.² The viability of the microorganisms is established by comparing the chemiluminescence signal of strains incubated with antibiotics to the chemiluminescence signal of the same strains incubated without antibiotics.^{47–50} If the strain is sensitive to the antibiotic, then the culture incubated with antibiotic will yield a much weaker signal than the culture incubated without antibiotic. If the strain is resistant to the antibiotic, the signals obtained from the cultures incubated in the presence and absence of antibiotic will be very similar. With an incubation period of around 4 h, chemiluminescence yields an MIC with a rate of agreement of 88–100% compared to the MICs obtained through broth macrodilution or microdilution.^{47,48,50} In a period of 8 h, it also detects intermediate and vancomycin-heteroresistant strains of *S. aureus*.⁴⁹ Finally, it has been found that chemiluminescence can be used to determine mycobacteria sensitivity in 4 days.⁵¹

Bioluminescence is a form of chemiluminescence that occurs as a result of a chemical reaction that takes place in some living organisms such as fireflies.² Studies conducted on an antibiogram are based on measuring the levels of bacterial ATP of intracellular origin, extracellular origin or overall, by comparing the bioluminescence signal obtained from microorganisms incubated without the presence of antibiotic (control group) to the signal obtained from the same microorganisms incubated in the presence of antibiotic.^{52,53} Regarding the control group, if the intracellular ATP is measured, the strains that are sensitive to the antibiotic studied will yield a decrease in the bioluminescence signal⁵⁴; if the extracellular ATP of the sensitive strains is measured, they will yield an increase in the signal⁵⁵; and if the total ATP of the sensitive strains is measured, they will yield a decrease in the ATP signal.^{52,53,56} By contrast, if the microorganism is resistant to the antibiotic, the control group measurements will be practically identical to those obtained from the strains incubated with antibiotic. With this methodology, it is possible to obtain a reliable antibiogram in just 2 h.⁵⁶ In addition, several studies referring to antibiograms obtained through bioluminescence based on a direct sample have been published. Ivancic et al.⁵⁷ made an antibiogram based on urine by comparing the ATP signal obtained from an aliquot of urine incubated without antibiotic to the ATP signal from another aliquot of the same urine incubated with antibiotic. In 2 h, they achieved a rate of agreement of 91% compared to the MIC obtained from the colony. Dong and Zhao⁵⁸ developed a microfluidic simulator with immobilised antibodies through which, based on urine, the identification of 13 species of bacteria and the antibiogram are obtained in 6 h. In yeasts and mycobacteria, bioluminescence yields a reliable antibiogram in 6 h and 7 days, respectively.^{59,60}

Microfluids

Advances in nanotechnology have made it possible to make antibiograms on platforms or chips using small working volumes to

carry out multiple assays that provide information on the sensitivity of bacteria to antibiotics, such as cell rupture, bacterial culture and hybridisation and amplification of nucleic acids. Tang et al.⁶¹ developed a platform with which, by measuring pH changes that occur as a result of the accumulation of metabolic products, it is possible to detect bacterial growth in 2 h. Mach et al.⁶² made an antibiogram directly based on urine samples using a microfluidic system with a built-in electrochemical sensor to quantify 16S ribosomal RNA. In 3 h and a half, this group obtained a rate of agreement of 94% compared to the sensitivity obtained through broth microdilution.

Bacterial lysis methods

Another methodology for determining sensitivity consists of detecting bacterial lysis. To do this, the bacterium is incubated in the presence of the antibiotic at the desired concentration. Then, the bacterium is immobilised in an agarose microgel and exposed to a lysis solution that causes DNA release. Subsequently, the preparation is incubated with the fluorochrome SYBR Gold and the integrity of the DNA may be studied through observation under a fluorescence microscope. This methodology yields an antibiogram in less than 2 h.⁶³

In conclusion, the methodologies described in this review shorten the time needed to determine the sensitivity of bacteria to antibiotics compared to the usual methods. To interpret the antibiogram, it is essential to be familiar with the species of bacteria that is being studied. In this regard, the rapid identification provided by the MALDI-TOF system allows the methodologies by which a rapid antibiogram is made to be implemented. However, to determine whether these techniques yield a sensitivity and a specificity that are acceptable for clinical practice, the number of bacterial strains and antibiotics studied must be increased. Given the significant benefits that derive from obtaining a rapid result for sensitivity of bacteria to antibiotics (increase in survival, reduction in expenses and delay in selection of resistant bacterial strains), it can be affirmed that the antibiogram in clinical microbiology, far from being definitively established, is continuously evolving.

Conflicts of interest

The author declares that there are no conflicts of interest.

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