Phenotypic and genotypic detection methods for antimicrobial resistance in ESKAPE pathogens (Review)

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Abstract. Antimicrobial resistance (AMR) represents a growing public health problem worldwide. Infections with such bacteria lead to longer hospitalization times, higher healthcare costs and greater morbidity and mortality. Thus, there is a greater need for rapid detection methods in order to limit their spread. The ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp.) are a series of epidemiologically-important microorganisms of great concern due to their high levels of resistance. This review aimed to update the background information on the ESKAPE pathogens as well as to provide a summary of the numerous phenotypic and molecular methods used to detect their AMR mechanisms. While they are usually linked to hospital acquired infections, AMR is also spreading in the veterinary and the environmental sectors. Yet, the epidemiological loop closes with patients which, when infected with such pathogens, often lack therapeutic options. Thus, it was aimed to give the article a One Health perspective.

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1. Introduction

Antimicrobial resistance (AMR) is one of the greatest threats to the progress achieved in medicine in the last century. Its increase in the last decades raises concern about the treatment of regular infections in what the World Health Organization is calling a 'post-antibiotic' era (1). The ESKAPE pathogens (*Enterococcus faecium* (*E. faecium*), *Staphylococcus aureus* (*S. aureus*), *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp.) are a series of bacteria capable of acquiring high levels of resistance and which are responsible for difficult-to-treat infections or for which, no treatment is currently available. When the acronym was first used to describe their dangerous potential more than a decade ago, the ESKAPE pathogens were mainly present in hospital settings (2). At present, the problem is far more extensive as bacteria cannot be contained, spreading in the community but also, in the veterinary sector and the environment (3-5). Rapid detection methods for AMR are crucial in limiting the spread of these strains and in the initiation of the optimal treatment. The aim of the present review was to briefly summarize the AMR mechanisms in these important pathogens and describe the main phenotypic and genotypic methods currently used in diagnosis.

2. Methods

A non-systematic review was performed using PubMed and Google Scholar databases. Key words relevant to each heading were used ('vancomycin-resistant *Enterococcus* spp.', 'vancomycin resistance', 'methicillin-resistant *Staphylococcus aureus*', '*Klebsiella pneumoniae*', '*Acinetobacter baumannii*', '*Pseudomonas aeruginosa*', '*Enterobacter cloacae*', '*Enterobacter* spp.', 'carbapenem resistance', 'carbapenemase', 'extended spectrum β-lactamase', 'ESKAPE', 'colistin resistance'). Articles written in English, published in the last decade, and based on relevance were selected. Conventional inclusion and exclusion criteria were not used.

3. AMR mechanisms

E. faecium. Although they are a part of the normal flora of both humans and animals, enterococci are able to cause a range of infections such as urinary tract infections, intra-abdominal, pelvic or soft tissue infections, and bacteremia or endocarditis, particularly in immunocompromised patients (6). As they normally live in the gastrointestinal tract of mammals, they may also be found in water, soil and food. The majority of infections are caused by two species of enterococci, *Enterococcus faecalis* and *E. faecium*. Both species have a natural low level resistance to aminoglycosides, cephalosporins and macrolides (7). Their spread in the hospital settings and their association with healthcare-associated infections in recent years is caused both by the acquisition of new resistance mechanisms and the ability to produce biofilm (7).

However, the greatest concern regarding treatment is in *E. faecium* as it has a higher intrinsic resistance and the ability to acquire resistance mechanisms towards last resort antibiotics. Its intrinsic low level resistance towards β-lactams is mediated by the production of a low-affinity penicillin-binding protein (PBP5) but may also rarely be caused by the production of β-lactamases (8,9). PBP5 is usually hyper-produced, giving rise to moderate or even high-level resistance towards cephalosporins as well as ampicillin. Nevertheless, in the case of *Enterococcus faecalis* ampicillin remains active (9). *E. faecium* also produces a chromosomal AAC(6')-I enzyme that does not allow synergism between aminoglycosides (except gentamicin, amikacin and streptomycin) and penicillins or glycopeptides (10).

One successful clone that emerged in the hospital setting was revealed by multilocus sequence typing and was designated CC17. The isolated strains were resistant to ampicillin and quinolones and contained a series of antibiotic resistance and virulence genes specific to the hospital environment (11).

Glycopeptides are antibiotics used to treat infections caused by resistant strains or in case of patient allergies. The most frequently encountered mechanism of glycopeptide resistance in enterococci is the reduced binding of glycopeptides to their target due to a VanA or a VanB ligase that replaces the terminal D-Ala in the peptidoglycan with D-Lac. Numerous other Van variants have been described (VanC1/C2/C3, VanD, VanE, VanG, VanL, VanM, VanN) but with a lower prevalence (12).

Previous use of antibiotics appears to increase the colonization rates with enterococci which may lead to ensuing clinical infection (13). Gastro-intestinal colonization of patients leads to the dissemination in the hospital setting and the formation of ecological niches (14).

Staphylococcus aureus (S. aureus). Staphylococci are gram-positive bacteria that naturally colonize the skin of humans and other mammals. *S. aureus* is the predominant human pathogen from this genus, causing a wide range of infections, although 30% of the human population are healthy carriers (15). Although the wild-type staphylococcal strains are susceptible to all β-lactams with the exception of monobactams, their ability to easily acquire resistance genes has transformed them into veritable MDR pathogens. The first strains resistant to penicillin G appeared as a defense and evolutionary mechanism a short time after it started to be used by patients. In a similar way, the first methicillin-resistant *S. aureus* (MRSA) strains appeared after penicillinase-resistant penicillins such as methicillin, oxacillin or cloxacillin were introduced in clinical practice. At present, MRSA causes high levels of morbidity and mortality worldwide (16). Luckily, unlike enterococcal strains, MRSA can still be successfully treated with glycopeptides in most cases, as resistance remains exceptional (17).

Regarding its resistance mechanisms, while in the 1940s infections could easily be treated with penicillin, at present >90% of all staphylococcal strains produce penicillinases (18). The four enzymatic variants of β-lactamases (A, B, C and D) in *S. aureus* have a narrow spectrum, hydrolyzing penicillins such as penicillin G, ampicillin, ticarcillin or piperacillin (19). These enzymes belong to the Ambler class A and are encoded by the *blaZ* gene which is located on a plasmid (20).

However, the main mechanism of β-lactam resistance is the production of a PBP with modified structure. *S. aureus* naturally presents four types of PBPs, numbered from 1 to 4, which are vital for bacterial survival (19). β-Lactams have a high affinity towards them with a pronounced bactericidal effect. In the presence of a *mecA* or *mecC* gene, the strains produce a PBP with low affinity towards β-lactams, called PBP2a or PBP2. Both *mecA* and *mecC* are part of a staphylococcal cassette chromosome (SCC), a mobile genetic element integrated in its chromosome. The SCC contains a *mec* operon and the *ccr* (cassette chromosome recombinase) gene. This complex encodes the site-specific recombinases that allow SCC*mec* mobility (20). There is a great variety of both the *mec* operon and the *ccr* gene (*ccrA*, *ccrB*, *ccrC*). The horizontal transfer of the complex leads to the global clonal dissemination of different MRSA strains. At present there are thirteen different types of SSC*mec* described in the literature (21). Certain of them are responsible only for β-lactam resistance while others also contain certain other resistance genes either on plasmids or transposons.

Klebsiella pneumoniae and Enterobacter spp. Both *Klebsiella pneumoniae* and *Enterobacter* spp. are members of the Enterobacterales family and are gram-negative bacilli capable of inducing great levels of morbidity and mortality (22,23). Due to their high efficacy and safety profile, β-lactams are the primary antibiotics used to treat infections caused by enterobacteria. However, as observed with penicillin and subsequently with oxacillin and *S. aureus* in the 1950s',

resistance towards different members of the β-lactam family was a step-by-step process (24). The first concern arose regarding third generation cephalosporin resistance when the first extended-spectrum β-lactamases (ESBLs) started to spread at a global scale. Infections caused by ESBLs has led to high rates of carbapenem consumption which has continued to increase the microbiologic pressure in different ecological systems all over the world. At present, the most concerning type of resistance is carbapenem resistance which may occur through multiple mechanisms. Carbapenemase-production is the most encountered type of carbapenem-resistance mechanism due to its potential for the rapid spread worldwide as carbapenemases are encoded by mobile genetic elements (plasmids, transposons and integrons). Carbapenemases are enzymes which open the β-lactam ring, therefore inactivating carbapenems. There are two main general classification systems being used to characterize carbapenemases at present, the Ambler and the Jacoby-Bush classification. The Ambler classification divides enzymes into four classes, A-D, based on the amino acid sequence of the enzymes. In order to hydrolyze their substrate (β-lactams), classes A, C and D use a serine residue while class B uses divalent zinc ions (25). The Jacoby-Bush classification attempts to correlate the substrate and inhibitor profiles with the phenotype of clinical isolates (26). The most commonly encountered enzymes in Enterobacterales are *Klebsiella pneumoniae* carbapenemase (KPC) and imipenemase (IMI) from Ambler class A, New-Delhi metallo-β-lactamase (NDM), Verona integron-borne metallo-β-lactamase (VIM) and IMP from Ambler class B and different oxacillinases, particularly OXA-48 from Ambler class D. Their epidemiology varies worldwide depending on the geographical location and between different ecological niches (e.g. different hospital settings) (27,28).

New combinations of β-lactams with β-lactam-inhibitors (ceftazidime-avibactam, ceftolozan-tazobactam and meropenem-varbobactam) show great promise in the treatment of carbapenem-resistant Enterobacterales (CRE). Although their use has yet to be spread worldwide, resistance has already started to be reported (29,30).

In infections that may no longer be treated with β-lactams, last resort antibiotics from other classes are starting to be used such as colistin or tigecycline (31-33). Unfortunately, resistance to these classes has been reported as well. The *mcr* gene which encodes colistin resistance is of great interest as it could also be transferred between bacteria, similar to carbapenemase genes, very often resulting in pan-resistant strains which are difficult, if not impossible, to treat (34).

Depending on the local epidemiology, the antibiotics usage profile and on the type of infection, fluoroquinolones or aminoglycosides may or may not remain active. In strains which exhibit multidrug resistance efflux pumps, multiple classes of antibiotics are actively being removed from the bacterial cells, leaving limited therapeutic options (34).

Acinetobacter baumannii and Pseudomonas aeruginosa. The mechanisms of resistance in non-fermenters are similar to the ones described in Enterobacterales with a few differences. Both microorganisms have high intrinsic resistance to antibiotics caused by their natural impermeability and overproduction of AmpC enzymes. They can also easily acquire other resistance mechanisms, particularly in the hospital environment (35).

In *Acinetobacter baumannii* the main mechanism of β-lactam resistance is the production of β-lactamases. All *Acinetobacter baumannii* strains produce chromosomally encoded AmpC cephalosporinases which confer resistance to extended-spectrum cephalosporins. Carbapenem resistance is mainly caused by the production of class D enzymes different than OXA-48, such as OXA-23-like, OXA-24/40-like, OXA-58-like or OXA-143. Of note, the presence of naturally occurring chromosomal OXA-51 (and its variants), coupled with the presence of IS*Aba1* promoter has been shown to lead to carbapenem resistance. Changes in outer membrane proteins (OMPs), multidrug efflux pumps and alterations in the affinity or expression of penicillin-binding proteins may also be responsible for carbapenem resistance (36).

In *Pseudomonas aeruginosa*, carbapenem resistance is mostly mediated by OprD loss, which primarily confers imipenem resistance. When present, carbapenemase production of class B enzymes such as VIM, IMP or NDM is the most frequently encountered (37). Overproduction of efflux systems such as MexAB-OprM and MexXY-OprM have also been described (38).

4. Phenotypic and genotypic detection of AMR in gram-positive bacteria

Phenotypic methods. Since the main mechanisms of β-lactam resistance in both *S. aureus* and *E. faecium* is the modification of the target, there are few phenotypic detection methods available. The interpretation of the disk diffusion antibiogram is an inexpensive and accurate method to detect β-lactam resistance in gram-positive bacteria. Glycopeptide resistance is rare in *S. aureus* and it is determined by the minimum inhibitory concentration (MIC) value (39,40). In *E. faecium*, genotypic methods may be more useful as they provide a rapid response, particularly since they may be used directly on clinical samples (41).

Enterococcus spp. There are several phenotypic methods used to detect antibiotic resistance in enterococci such as disk diffusion, broth microdilution and the breakpoint agar method particularly for *vanA* strains. VanB-mediated resistance is often harder to detect phenotypically and other methods must be used in such cases. All strains must be correctly identified, ideally by the means of MALDI-TOF. An incorrect identification may lead to confusing results and reporting (for instance *E. gallinarum* and *E. casseliflavus* may be confused with *E. faecium* due to a positive arabinose test). In settings where MALDI-TOF is not available, the European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommends the use of the methyl-α-D-glucopyranoside (MGP) test or a motility test in order to differentiate these species (42).

Regarding *E. faecium*, EUCAST recommends that ampicillin-resistant strains should be reported resistant to all β-lactams, including carbapenems (43). In a similar manner, ampicillin resistance in all enterococci is a predictor of amoxicillin and piperacillin resistance as well. As ampicillin resistance in *Enterococcus faecalis* is rare, EUCAST recommends that it be confirmed using MICs (43).

Perhaps the most important molecules used in the treatment of ampicillin-resistant enterococcal strains are glycopeptides. Vancomycin and teicoplanin may be tested using the disk diffusion method in enterococci. Susceptibility to vancomycin is defined by a sharp-edged zone of inhibition with a diameter of over 12 mm, in the absence of colonies in the inhibition zone or a MIC <4 mg/l. Although the incubation time for enterococcal antibiograms is 24 h, *vanB*-positive strains that present low level resistance to vancomycin, may need a prolonged, 48 h incubation time (43). Usually, vanA-producing strains are both vancomycin and teicoplanin-resistant, whereas vanB-producing strains usually remain susceptible to teicoplanin (10). Semi-automated methods such as VITEK2 have not exhibited superiority compared with the disk diffusion method for the detection of vanB-producing enterococci (44).

The breakpoint agar tests can accurately detect both *vanA*- and *vanB*-positive strains. The test is performed by applying 10 μl of a 0.5 McFarland inoculum on a Brain Heart Infusion agar plate supplemented with 6 mg/l vancomycin. Any growth is considered a positive test (44).

Vancomycin-variable enterococci (VVE) are vancomycin-susceptible enterococci that contain the *vanA* gene that have the ability to revert to a vancomycin-resistant phenotype upon exposure to vancomycin (45). Low-MIC vancomycin-resistant enterococci (VRE) are used to describe *vanB* strains with low expression that have MICs below the clinical breakpoint but which could become phenotypically resistant in case of prolonged exposure to vancomycin (46). Usually, VVE and low-MIC VRE strains can only be determined by molecular methods.

S. aureus. The majority of staphylococci produce a penicillinase that confers resistance towards penicillin G, phenoxymethylpenicillin, aminopenicillins, carboxypenicillins and ureidopenicillins. The *in vitro* marker used to detect its presence is a zone diameter of <26 mm when a disk of penicillin G of one unit is placed on the disk diffusion plate. In the absence of cefoxitin resistance, these strains maintain their susceptibility towards isoxazolyl-penicillins such as oxacillin or cloxacillin, certain cephalosporins and carbapenems (10,43).

A cefoxitin disk of 30 μg that has an inhibition diameter of <22 mm or a cefoxitin MIC of over 4 mg/l could be used as *in vitro* markers for MRSA strains (10,43). These strains are reported to be resistant to all β-lactams with the exception of ceftaroline and ceftobiprole which were particularly designed to treat MRSA strains (47).

A quick slide agglutination test could be performed to detect the presence of PBP2a (the product of the *mecA* gene) which leads to low affinity to β-lactam antibiotics (48).

Last resort antibiotics used to treat MRSA infections are glycopeptides and oxazolidinones. Due to their poor diffusion into agar plates, glycopeptides cannot be tested using the disk diffusion method and must therefore be tested using the MIC. A MIC of >2 mg/l is considered the clinical breakpoint for vancomycin non-susceptibility. Vancomycin-resistant *S. aureus* is defined by a MIC of >8 mg/l but few strains have been reported worldwide (49). However, other variants of vancomycin non-susceptibility have been described such as hVISA (heteroresistant vancomycin-intermediate *S. aureus*) and VISA (vancomycin-intermediate *S. aureus*) (50).

hVISA contains vancomycin-susceptible strains as well as a small population of bacteria with a MIC of >2 mg/l while VISA strains have MICs of 4-8 mg/l. These types of resistance cannot be determined using broth microdilutions and are generally difficult to detect, particularly hVISA. However, a number of screening tests as well as a confirmatory method have been developed. The macro gradient E-test could be used as a screening method but it cannot distinguish between different variants of vancomycin non-susceptibility. The test uses a higher inoculum (2 McFarland) and a different media (Broth Hearth Infusion, BHI) than the standard E-test, a teicoplanin value of >12 mg/l being considered positive. The Glycopeptide Resistance Detection Gradient test uses vancomycin-teicoplanin double-sided gradient test strips on Mueller-Hinton agar supplemented with 5% sheep blood and a 0.5 McFarland inoculum. A result of >8 mg/l for either vancomycin or teicoplanin is considered positive. The use of BHI screening agar plates containing 4 μg/ml vancomycin and 16 g/l casein with 0.5 and 2 McFarland inocula is another potentially useful option (51). Mueller-Hinton agar plates supplemented with 5 mg/l of teicoplanin may be used as a screening method as well by spot-plating 10 μl of a 2 McFarland bacterial inoculum. Growth of colonies at 48 h is suggestive of glycopeptide non-susceptibility.

All screening tests must be confirmed by a test called PAP which analyses the population profile of the isolate on agar plates that contain a range of vancomycin concentrations (52).

Genotypic methods

E. faecium. For the accurate identification and species differentiation of enterococci, the superoxide dismutase gene, *sodA*, is a potential target (53).

As glycopeptide resistance is important to be determined rapidly in order to stop its spread, genotypic methods target the most frequently encountered genes responsible, *vanA* and *vanB*. At present, both genes can be detected directly from clinical samples, with high accuracy (54).

Recently, as whole genome sequencing (WGS) is increasingly being used, it may provide therapy guidance for enterococcal infections as well (55).

S. aureus. MRSA strains contain either *mecA* or *mecC* (56). VRSA strains posess the *vanA* gene which is mediated by the Tn*1546* transposon acquired from glycopeptide-resistant enterococci (57). WGS was useful in epidemiological studies, particularly in the guiding efforts to control MRSA transmission (58).

5. Phenotypic and genotypic detection of AMR in Enterobacterales

Phenotypic methods. In addition to the interpretative reading of the antibiogram, at present there are a few phenotypic tests available which can detect ESBLs, carbapenemase production or colistin resistance in a timely manner.

Carbapenemase production. Although phenotypic methods are not able to differentiate between different types of specific carbapenemases, they may detect the presence of a carbapenem-hydrolyzing enzyme in a given bacteria which

represents an advantage as compared with the majority of genotypic methods where detection is based on already known components. The fact that they are inexpensive compared with the genotypic methods is another advantage, since they could be more readily available in clinical laboratories with limited resources. Certain phenotypic tests which include the use of carbapenemase inhibitors may indicate a certain Ambler class and guide the management of the patient.

The last EUCAST recommendation regarding carbapenemase production confirmation is from 2017. The initial screening starts from the disk diffusion antibiogram. A meropenem diameter of <28 mm is considered the best compromise between sensitivity and specificity in detecting carbapenemase producers. Although ertapenem is the carbapenem most susceptible to hydrolysis, it may give false positive results in ESBL and AmpC producers (59-63). Several schemes of interpretative screening were also developed such as the use of ticarcillin-clavulanate, temocillin and imipenem (recommended by the French Society of Microbiology, CA-SFM) or the use of faropenem-temocillin (64-66). However, they are limited by the individual subjectivity in reading and in regions with a high burden of antibiotic resistance; they may not offer additional information.

Cloxacillin is used to inhibit the class C β-lactamase, AmpC. Inclusion of cloxacillin in the growth medium or its addition to the carbapenem disk allows the monitoring of synergy and establishes a non-carbapenemase-resistant mechanism (67).

A test that is still used in microbiology laboratories but is no longer recommended by the EUCAST nor by the Clinical and Laboratory Standards Institute (CLSI) is the Modified Hodge Test. The test consists of plating a susceptible strain of *Escherichia coli* (*E. coli*) (ATCC 25922) on Mueller-Hinton agar. A meropenem disk is then added on the center of the plate, while strains which are to be evaluated are streaked linearly from the disk to the edges of the plate. A clover-leaf-like indentation caused by the growth of the indicator strain towards the disk is considered to be a positive result (the strain is considered to produce a carbapenemase). The main disadvantages of the test are that results may be susceptible to interpretation and false-positive (for ESBL and AmpC producers) as well as false-negative (for NDM-producing strains) results have been observed (68). Although easy to perform, due to the lack of sensitivity and specificity as well as the fact that other phenotypic methods are currently available it is anticipated that the Modified Hodge Test may soon be replaced in the clinical microbiology laboratories in the near future.

In case of carbapenem resistance, the use of carbapenemase inhibitors [phenyl boronic acid (PBA) for class A, dipicolinic acid (DPA) for class B] which could be used in combination with a carbapenem disc, may indicate the presence of a carbapenemase and the Ambler class. Temocillin may be used as a marker for OXA-48 production (69).

All the colorimetric tests (Carba NP and its derivates including Rapidec Carba NP, Rapid Carb Blue Screen, β Carba test and GoldNano Carb) have the same principle of carbapenemase detection. Each test consists of incubating imipenem with the strain of interest and a pH indicator such as red phenol or bromothymol blue. Imipenem hydrolysis is detected by the change of colour (from red or blue to yellow) caused by the pH drop (70-73).

In 2005, Kim *et al* proposed a new method termed the Carbapenem Inactivation Method. This test consists of incubating one loop of the strain of interest with a disk of meropenem in 400 μl of distilled water for 2 h, the recovered disk being afterwards placed on Mueller-Hinton plated with a highly susceptible strain of *E. coli*. In principle, the meropenem in the disk will be inactivated by a carbapenemase-producing strain, allowing the unhindered growth of the *E. coli*, while a carbapenem-resistant strain by other mechanisms, would have no effect on the meropenem therefore inhibiting the highly susceptible strain. The interpretation can made after at least 6 h, while for certain strains an overnight incubation is required for improved results (74,75).

The CIM test inspired a series of similar tests in the hope of raising the sensitivity and specificity of the method as well as having a more rapid result. A CLSI team modified the protocol by incubating the strains for 4 h in tryptic soy broth, a zone diameter of <15 mm being evaluated as a positive result. The test is termed mCIM and it is currently recommended by the CLSI. The mCIM was adapted as well by using sodium mercaptoacetate for an improved metallo-β-lactamase detection (76).

The main disadvantage of the CIM and mCIM is the 24-h period of incubation. This was addressed by a version of the CIM which could provide results in <3 h, called rapid CIM (rCIM). A total of two 10-μl loopfulls of the strain of interest are incubated with two disks of meropenem for 30 min in 1 ml of distilled water. Following centrifugation, the supernatant is placed over a 0.5 McFarland inoculum of a highly susceptible *E. coli* strain and re-incubated for 2 h. Production of carbapenemases is confirmed by a growth of >0.5 McFarland (77). The rCIM was tested with carbapenemase inhibitors as well, exhibiting favorable results but further studies are required to confirm its utility on different types of carbapenemases (78).

zCIM is a newly described method which consists of the incubation of the strain of interest in distilled water with added $ZnSO_4$ and a meropenem disk. The test revealed high sensitivity and specificity and it is recommended to be used in combination with immunochromatographic tests (79).

Immunochromatographic tests such as the RESIST-4 (Coris BioConcept) or the CARBA-5 (NG Biotech) are rapid, easy to perform and have demonstrated high-performances (79).

With MALDI-TOF-MS technology becoming increasingly available in the clinical microbiology laboratory, a carbapenem-degradation test was proposed for the evaluation of carbapenemase activity. This technique was further evaluated for direct use from blood culture bottles and other biological fluids (80,81).

Colistin resistance. The CLSI and EUCAST both recommend the MIC determined by broth microdilutions as a reference method for testing colistin resistance in Enterobacterales. MICs should be performed in cation-adjusted Mueller-Hinton broth (MHB) with sulfate salts of polymyxins, without additives such as polysorbate 80 and without treated polystyrene trays. Unfortunately, at present, not all microbiology laboratories are able to implement this method and continue to use

the disk and gradient diffusion methods despite the high error rates reported (82).

Certain laboratories use semi-automated systems for MIC determination such as the MicroScan WalkAway (Beckman Coulter, Inc.), Vitek 2 (BioMérieux SA) or BD Phoenix™ (Becton Dickinson; BD Biosciences) (83).

Other tests that use modified versions of the broth microdilution method include the addition of ethylenediaminetetraacetic acid (EDTA) and dipicolinic acid (DPA) which were tested for *mcr-1* to *mcr-5* detection. Neither method was comparable as accuracy with the standard method but DPA inhibition may be useful for *E. coli* strains (84).

As colistin resistance is rising, a number of phenotypic tests besides antibiotic susceptibility testing are being developed for its rapid detection.

The newly described Colistin Broth Disk Elusion Test is a potential alternative to broth microdillution as it requires few materials (MHB and colistin disks) (85). The test consists of incubating 1, 2 and 4 disks of colistin (10 μg) in 10 ml of cation-adjusted Mueller-Hinton which corresponds to the final concentrations of 1, 2 and 4 μg/ml, as well as a growth control tube in which no colistin is added. Following a 30-min incubation period in which colistin diffuses into the media, 50 μl of a 0.5 McFarland suspension of the bacteria are added. Following another 16 to 20 h of incubation, the results are read by using the recommended MIC breakpoints.

Rapid polymyxin NP is a test similar to Carba NP that can detect the bacterial growth based on the pH modification caused by glucose metabolism in the presence of a specific concentration of colistin (3.75 μg/ml/well). The test may be interpreted in 2 h with a reported sensitivity and specificity of upwards of 95% (86).

Similar to the method used for carbapenemase detection, MALDI-TOF can be used for the detection of lipid A modifications caused by polymyxin resistance in <15 min (87).

Recently, a lateral flow immunoassay has been developed and tested for the rapid detection of MCR-1-producing Enterobacteriaceae (88).

Genotypic methods

Carbapenemase production. While there are a number of available methods for the molecular detection of carbapenemase genes, WGS provides the most important information.

Certain genotypic methods are very expensive and require highly trained personnel for data interpretation while others are particularly designed to be easy to use directly from clinical samples. Usually, these benchtop systems are able to detect the five most common carbapenemase genes described (bla_{KPC}, bla_{NDM}, bla_{IMP}, bla_{VIM} and bla_{OXA-48}) (89,90).

Other commercial kits target a larger number of genes, to minor carbapenemases, ESBL (CTX-M) and even colistin resistance (91,92).

Colistin resistance. Colistin resistance can be either chromosomally encoded (by alterations in the *pmrA*, *pmrB*, *phoP*, *phoQ*, *mgrB* and *crrB* genes) or plasmid-mediated (encoded by the *mcr-1* to *mcr-8* genes) (93,94). These genes may be detected using real-time PCR, loop-mediated isothermal amplification (LAMP), microarray techniques or WGS, usually in National Reference Centers (95-98). However, this may not be the optimal method for detecting colistin-resistance as numerous gene modifications are yet to be described.

6. Pseudomonas aeruginosa and Acinetobacter baumannii

Phenotypic methods

Carbapenemase production. All the phenotypic methods described in the Enterobacterales section were also tested or adapted for non-fermenters as well but usually rendering lower overall sensitivities and specificities.

Phenotypic methods include growth-based methods (such as the boronic acid synergy test, the E-test metallo-β-lactamase strips, mCIM), colorimetric tests based on biochemical reactions (such as the Carba NP and its variants) or electrochemical tests (BYG test) (99).

The boronic acid synergy test consists of placing several antibiotics (imipenem, meropenem, ceftazidime) with or without the class B inhibitor boronic acid in two concentrations (300 and 600 μg) on a plate inoculated with a 0.5-McFarland of the strain. A difference >5 mm between the zone diameters is considered the cutoff for resistant isolates (100).

The E-test metallo-β-lactamase consists of double-sided strips impregnated with a seven-dilution range of imipenem or ceftazidime (4 to 256 μg/ml) and imipenem or ceftazidime (1 to 64 μg/ml) with added EDTA or 2-mercaptopropionic acid (MPA) at a constant concentration (101). The test has exhibited high sensitivity and specificity for both *Pseudomonas* and *Acinetobacter* strains (102).

As the mCIM was reported to have a low sensitivity (45.1%) on non-fermenters, the protocol was modified by using a 10-μl loopful instead of a 1-μl, in order to achieve improved results. The overall sensitivity and specificity of the test revealed its utility for testing *Pseudomonas aeruginosa* but not for *Acinetobacter* (103).

The classic CIM test was reported to have a low sensitivity in detecting carbapenemase production in non-fermenters (104). For this reason, a new method termed CIMTris was described with an overall sensitivity of 97.6% and an overall specificity of 92.6% (105). The modified protocol consists of incubating the suspect strain in Tris-HCl instead of distilled water for 2 h.

There are numerous studies which have compared the performances of the colorimetric tests (Carba NP and its derivates including Rapidec Carba NP, Rapid Carb Blue Screen, β Carba test and GoldNano Carb) used for Enterobacterales in non-fermenters. Generally, the non-fermenters exhibited lower performances which has led to the development of new derivates. A modified Carba NP where the lysis buffer was replaced with cetyl trimethyl ammonium bromide revealed 100% sensitivity and specificity (106). Another change in the protocol of the Carba NP test (replacing the lysis buffer with NaCl) was especially created for the improved detection of carbapenemase production in *Acinetobacter baumannii* strains, termed CarbAcineto NP with a sensitivity of 89-95% (107). GoldNano Carb is a test that uses gold nanoparticles as a pH indicator of carbapenemase production. Similar to the other Carba NP variants, the low pH caused by imipenem hydrolysis leads to the aggregation of the gold nanoparticles, causing a color change from red to purple, blue or green (108).

The BYG Carba test is an electrochemical assay which detects the increase of conductivity of a polyaniline-coated electrode, very sensitive to pH modifications and redox activity (109).

Regarding *Acinetobacter baumannii*, to date, the optimal method for carbapenemase detection is represented by assays involving MALDI-TOF MS hydrolysis (110).

Colistin resistance. In addition to the already described Colistin Broth Disk Elusion Test which was validated for non-fermenter testing as well, a resazurin reduction-based assay was recently described for polymyxin resistance in *Acinetobacter* spp. and *Pseudomonas* spp. (111). The test is described to be performed in 4 h, exhibiting high sensitivity and specificity (100 and 92%, respectively).

Genotypic methods

Carbapenemase production. Both, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* have a high genomic diversity. Besides the intrinsic AMR, there is an increase of carbapenem-resistant strains. High-risk clones are incriminated in spreading resistance genes. In these two species the majority of acquired resistance genes exist as gene cassettes in integron, and they are also associated with various horizontally acquired resistance elements. In *Acinetobacter baumannii* the resistance elements are most often clustered in AMR islands and plasmid-borne resistance genes (112).

The association between phenotypic antimicrobial susceptibility testing and whole genome sequencing was synthesized by the EUCAST Subcommittee (112). For *Pseudomonas*, in particular for meropenem and levofloxacin, the sensitivity and specificity were 91 and 94%, respectively, while for amikacin it was 60%. For *Acinetobacter baumannii* in strains with amikacin resistance, the presence of *aphA6* and *armA* has been observed (112, 113).

Detection of AMR based on the presence of acquired and chromosomal resistance-associated mutations may have a high sensitivity and specificity, but the main challenge remains in identifying the chromosomal alterations which lead to changes in the expression, particularly regarding the efflux pumps or outer membrane proteins (112).

Colistin resistance. The Micromax Assay for *Acinetobacter* is a test that can detect DNA fragmentation and cell wall damage in <4 h (114). However, it requires access to fluorescence microscopy which limits its use in the clinical microbiology laboratories.

7. Veterinary implications

At present, the veterinary sector has a unified legislative framework in the European Union compared with the human sector.

Concerning regulations, there are several European agencies, such as the European Food Safety Authority, or the European Medicines Agency, that have provided studies concerning the spread of AMR between animals and humans including the Antimicrobial Advice Ad Hoc Expert Group (AMEG), the Reduction of the Need for Antimicrobials in Food-producing animals and Alternatives (RONAFA) or the Joint Interagency Antimicrobial Consumption and Resistance Analysis (JIACRA) studies.

The AMEG provides a categorization of antimicrobials based on their potential for generating AMR in humans following use in animals. It also advises on the impact of the antimicrobial use in animals, by generating a risk profile. For instance, the AMEG concluded that glycylcycline should be restricted in animals as it was observed that resistance in humans emerged rapidly. In 2016, following the discovery of the *mcr-1* gene, the AMEG also advised for the reduction of colistin sales across the European Union (115).

The RONAFA report included measures to reduce the need to use antimicrobials in animal husbandry in the EU and the impacts on food safety while the JIACRA reports analyzed the potential relationship between the consumption of antimicrobials by humans and animals and the occurrence of AMR (116-119).

The use of the glycopeptide, avoparcin, as a growth promoter in animals is considered to have contributed to the widespread of glycopeptide-resistant enterococci, serving as a reservoir for the human food chain (120). The 'The Danish Integrated AMR Monitoring and Research Programme' (DANMAP) banned its use in 1995, with vancomycin resistance dropping significantly since then (121). In 1997, the use of avoparcin as a growth-promoter was banned all over the European Union. Since 2006, no antimicrobial drug was allowed to be used for growth promotion, including antimicrobial drug classes not used in human medicine (122).

Animal contact (farm or companion animals) was identified as a potential risk factor for carriage or infection with MRSA (100). One of the most notable cases was a community-acquired MRSA strain transmitted to humans from pigs, which was reported in the Netherlands (123).

Concerning colistin resistance, the *mcr-1* gene was detected in chicken meat and other food products (124).

8. Environmental implications

At present, there is a lack in the current understanding of the issue and therefore a lack of a regulatory process regarding the surveillance and control of AMR in the environment.

The use of manure of animal origin as soil fertilizers increases the abundance of antibiotic resistance genes and antibiotics in soil (125).

AMR genes were identified in the soil of 12 organic farms evaluated in Nebraska, most frequently for tetracycline and sulfonamide including *tet*(G), *tet*(Q), *tet*(S), *tet*(X) and *tetA*(P). The samples were collected from two different depths, but this did not influence the presence of identified AMR genes (126).

It is clear that unless the chain of excessive antimicrobial consumption is limited, the impact on the environment will continue to rise.

Until recently the presence of AMR genes in the air had not been appropriately evaluated, however, in one study the presence of 30 gene subtypes was screened in particulate matter in 19 cities and were identified more frequently in San Francisco, for example, than in Bandung; the most often encountered genes were bla_{TEM}, which encodes for β-lactamine resistance and *qepA* (a quinolone resistance gene) (127).

Another study conducted during a severe smog event detected 205 airborne AMR genes most frequently encoding for tetracycline, β-lactam and aminoglycoside resistance (128).

Regarding the AMR genes detected in drinking water sources, a study conducted in Canada identified genes such as *ampC*, *tet*(*A*), *mecA*, β-lactamase genes, such as TEM-type, OXA-1 or CMY-2-type, and carbapenemase genes including OXA-48, IMP, VIM, KPC NDM and GES (129).

In samples obtained from wastewater from hospitals from Rio Grande, New Mexico, in 58% of the samples at least one antibiotic was detected, most frequently ofloxacin, sulfamethoxazole and trimethoprim (130).

9. Conclusions

The ensemble of ESKAPE pathogens are known for their capacity to evade the effects of antimicrobial therapy. While the mechanisms conferring resistance are varied, these pathogens are uniform in the risk of causing difficult, hard-to-treat infections. This leads to the need for new and improved methods to detect AMR, to quickly assess therapeutic options. Genotypic methods may detect resistance directly from clinical samples, however, they are expensive and require specific infrastructure. Phenotypic methods may occasionally offer more general information, usable in the clinical environment. The veterinary sector is, in its own right, a source of antibiotic resistance, due to use of antimicrobials as growth agents. The environmental sector combines resistance from the clinical and veterinary sector through medical waste and the use of fertilizer. There is a need for improved antibiotic use in both the human and veterinary medical sectors as well as a need for constant surveillance of the AMR phenomenon.

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Authors' contributions

MMM and AAM conceived the review, prepared the methodology, wrote the original draft, and reviewed and edited the study. MP also participated in the writing of the original draft. LSCM contributed to the writing of the review and editing. CD contributed to the review and editing. MIP supervised the study, wrote the original draft, contributed to the writing of the review and editing. GLP conceived the review, contributed to the writing of the review and supervised the study. Data authentication is not applicable. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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