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Non-Phenotypic Tests to Detect and Characterize Antibiotic Resistance Mechanisms in *Enterobacteriaceae*

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Abstract

In the past two decades, we have observed a rapid increase of infections due to multidrug-resistant *Enterobacteriaceae*. Regrettably, these isolates possess genes encoding for extended-spectrum β-lactamases (e.g., $bla_{\text{CTX-M}}$, bla_{TEM}, bla_{SHV}) or plasmid-mediated AmpCs (e.g., bla_{CMY}) that confer resistance to last-generation cephalosporins. Furthermore, other resistance traits against quinolones (e.g., mutations in *gyrA* and *parC*, *qnr* elements) and aminoglycosides (e.g., aminoglycosides modifying enzymes and 16S rRNA methylases) are also frequently co-associated. Even more concerning is the rapid increase of *Enterobacteriaceae* carrying genes conferring resistance to carbapenems (e.g., bla_{KPC}, bla_{NDM}). Therefore, the spread of these pathogens puts in peril our antibiotic options. Unfortunately, standard microbiological procedures require several days to isolate the responsible pathogen and to provide correct antimicrobial susceptibility test results. This delay impacts the rapid implementation of adequate antimicrobial treatment and infection control countermeasures. Thus, there is emerging interest in the early and more sensitive detection of resistance mechanisms. Modern non-phenotypic tests are promising in this respect, and hence, can influence both clinical outcome and healthcare costs. In this review, we present a summary of the most advanced methods (e.g., next-generation DNA sequencing, multiplex PCRs, real-time PCRs, microarrays, MALDITOF MS, and PCR/ESI MS) presently available for the rapid detection of antibiotic resistance genes in *Enterobacteriaceae*. Taking into account speed, manageability, accuracy, versatility, and costs, the possible settings of application

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(research, clinic, and epidemiology) of these methods and their superiority against standard phenotypic methods are discussed.

Keywords

PCR; multiplex; real-time; microarray; ESBL; carbapenemases; MALDI-TOF

1. INTRODUCTION

Microorganisms belonging to the family of *Enterobacteriaceae* (*Ent*) are important causes of community- and hospital-acquired infections. *Escherichia coli* is a frequent cause of urinary tract infections (UTIs), *Klebsiella* spp. and *Enterobacter* spp. are important causes of nosocomial pneumonia, and all of the *Ent* have been implicated in bloodstream (BSIs) and intra-abdominal infections (Donnenberg, 2005; Gaynes & Edwards, 2005).

Serious infections due to *Ent* are usually treated with extended-spectrum cephalosporins (ESCs) included in the third- and fourth-generations (Endimiani & Paterson, 2007; Michalopoulos & Falagas, 2010). These drugs target a broad-spectrum of pathogens, possess low toxicity, and demonstrate favorable pharmacokinetics and pharmacodynamics (Grayson, 2010). Unfortuantely, there has been a rapid increase of infections due to ESC-resistant (ESC-R) *Ent* (Coque, Baquero, & Canton, 2008; Meyer, Schwab, Schroeren-Boersch, & Gastmeier, 2010). As a consequence, quinolones and aminoglycosides are the alternative antibiotics considered, but high co-associated resistance rates are also reported (Giamarellou & Poulakou, 2009; Hawser, et al., 2010). Although it is understandable from a clinical perspective to use then carbapenems, it is equally worrisome that this practice leads to the rapid selection of carbapenem-resistant *Ent* (Walsh, 2010). As a result, only a few antimicrobial agents (e.g., colistin, fosfomycin, tigecycline) with an uncertain *in vivo* efficacy and/or reported toxicity are usually left to treat infections due to multidrug-resistant *Ent* (MDR-*Ent*) (Livermore, et al., 2011). Thus, the rapid characterization of the resistance mechanism(s) is essential to establish a correct antibiotic therapy and to implement measures to contain the spread of these life-threatening pathogens.

1.1 Why should we rapidly detect antibiotic resistance mechanisms in Ent

Standard microbiological procedures require several days to isolate the causing pathogen(s) responsible for the syndrome and to provide the antimicrobial susceptibility test (AST) results. For instance, for the diagnosis of bacteremia clinical laboratories usually implement automated systems capable of monitoring microbial growth in blood culture bottles (Riedel & Carroll, 2010). When the bottles are positive (most *Ent* grow within 2 days after incubating), the sample is plated on standard agar media (Endimiani, Tamborini, Luzzaro, Lombardi, & Toniolo, 2002). If colonies grow (usually after overnight incubation), ASTs are performed. Generally, these tests require at least additional 24 hrs to yield results. Hence, for non-MDR-*Ent*, a total time from primary incubation to test results (TTR) of 3-4 days is often required. However, ASTs can be inaccurate for certain MDR-*Ent*(Livermore, et al., 2012)causing mischaracterization of carbapenemase (Poirel, Potron, & Nordmann, 2012), extended-spectrum β-lactamase (ESBL) (Luzzaro, et al., 2006), or plasmid-mediated

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AmpC (pAmpC) producers (Doi & Paterson, 2007) (see also section 1.2). These difficulties can be partially overcome by several adjunctive phenotypic tests that may include: *i*) clavulanate for ESBLs detection (Drieux, Brossier, Sougakoff, & Jarlier, 2008), *ii*) boronic acid for pAmpCs and KPCs detection (Pournaras, Poulou, & Tsakris, 2010), or *iii*) modified Hodge test (mHT) for detecting carbapenemases (Doyle, et al., 2012). Unfortunately, these tests require at least an additional day. Thus, up to 4-6 days are required to provide physicians with an accurate phenotype for MDR-*Ent*.

The delay in identifying and reporting AST results has important clinical implications. Patients with MDR-*Ent* infections have less favorable outcomes than those infected by non-MDR isolates (Schwaber, et al., 2006; Tumbarello, et al., 2007; Tumbarello, et al., 2010). Many studies have shown that: *i*) mortality attributable to BSI is significantly higher in patients infected with ESBL producers than those with non-ESBL producers; and *ii*) treatment failure rate for patients infected with ESBL-producing *Ent* (ESBL-*Ent*) is almost twice as high as that of the patients infected with non-ESBL-producing organisms (Endimiani, et al., 2005; Endimiani, et al., 2004). Hence, a rapid detection of MDR-*Ent* can help to identify patients at risk for poor outcome.

The delay has also economic implications. A rapid reporting system of antibiotic resistance patterns reduces the duration of hospitalization and, as consequence, the healthcare costs. For instance, the number of days using broad-spectrum antibiotics can be shortened (e.g., avoiding the use of the more expensive carbapenems). Lee *et al*. showed that the mean length of hospital stay (MLHS) for patients with ESBL producers was longer than that for patients infected with non-ESBL producers (21 *vs.* 11 days). The additional costs for an infection due to ESBL producers were $16,450 per patient (S. Y. Lee, Kotapati, Kuti, Nightingale, & Nicolau, 2006). Schwaber *et al*. noted that patients with BSIs due to ESBL-*Ent* had a MLHS post-infection of 11 days *vs*. 5 days in those with non-ESBL producers. The average hospital cost for BSIs caused by ESBL producers was $46,970 *vs*. $16,877 for those due to non-ESBL producers (Schwaber, et al., 2006).

The rapid identification of patients colonized with MDR-*Ent* can also assure a prompt implementation of hospital hygiene precautions to prevent the spread (e.g., outbreaks) of these pathogens. However, standard laboratory methods are too slow and may lack the required sensitivity. For instance, screening of intestinal carriers of ESBL- or carbapenemase-producing *Ent* is usually performed with commercially available selective agar plates that have a lower sensitivity compared to PCR-based methods. This is especially true for those isolates expressing low-level MICs for the antibiotics added to the agar (e.g., OXA-48-producing *E. coli*) (Nordmann, Gniadkowski, et al., 2012). In a clinical study, Singh *et al*. showed that a real-time PCR designed to detect bla_{KPC} in rectal swabs demonstrated a sensitivity of 97%, whereas CHROMagar plates were only of 77%; overall, 13% of colonized subjects were not identified with standard cultures (Singh, et al., 2012). Naas T *et al*. demonstrated that a real-time PCR for bla_{NDM} had a limit of detection of 10 CFU/ml, whereas for ChromID ESBL and CHROMAgar KPC plates it was 10^2-10^3 CFU/ml of stool (Naas, Ergani, Carrer, & Nordmann, 2011).

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Based on the above examples, there is no doubt that early and more sensitive detection of resistance mechanisms by non-phenotypic tests has benefits. However, we also emphasize the importance of implementing advanced and rapid methodologies in other settings, such as the research context and/or when large collection of isolates should be screened before further and more specific molecular analyses.

Overview of the main mechanisms of antibiotic resistance in Ent

1.2.1 Resistance to extended-spectrum cephalosporins (ESCs).—Resistance to β-lactams in *Ent* may be due to mutations in the penicillin-binding proteins (PBPs) or reduced permeability of the cell wall (e.g., disruption of outer membrane proteins, OMPs). However, production of β-lactamases is the most frequent mechanism encountered in *Ent*. These periplasmic enzymes can be grouped into four classes (A to D) on the basis of their amino acid sequence homology (Bush & Jacoby, 2009).

The most clinically important class A enzymes are the ESBLs. They confer resistance to penicillins, ESCs and aztreonam but not to carbapenems, and are usually inhibited by the commercially available β-lactamase inhibitors (i.e., clavulanate, tazobactam, sulbactam). TEM, SHV, and CTX-M are the three main families of ESBLs described. While TEM and SHV ESBLs arise via substitutions in strategically positioned amino acids from the natural narrow-spectrum TEM-1/-2 or SHV-1 β-lactamase, all CTX-M variants demonstrate an ESBL phenotype (Gniadkowski, 2008; Paterson & Bonomo, 2005). Until the 1990s, most ESBLs identified were of SHV- and TEM-types. At present, the CTX-M (especially CTX-M-15) are the most prevalent ESBLs worldwide (Livermore, et al., 2007; Rossolini, D'Andrea, & Mugnaioli, 2008; Seiffert, Hilty, Perreten, & Endimiani, 2013). With a limited global, but a significant local impact, other ESBLs (e.g., PER, VEB, and GES-1) have also been reported (Akinci & Vahaboglu, 2010).

Several *Ent* possess genes encoding for class C chromosomal AmpCs (cAmpCs; e.g., *Citrobacter freundii*, *Enterobacter* spp., *Serratia* spp.). Such β-lactamases are under the control of a complex regulon (*ampD*, *ampR*) and confer resistance to third-generation cephalosporins and β-lactam/β-lactamase inhibitor combinations, but not to carbapenems (Bush & Jacoby, 2009; Harris & Ferguson, 2012; Hilty, et al., 2013; Jacoby, 2009). Also, *E. coli* possess a chromosomal bla_{AmpC} but only mutations in the promoter/attenuator region can lead to constitutive hyper-expression of the gene resulting in cephalosporins resistance. However, these strains are only occasionally reported (Jorgensen, Nielsen, Friis-Moller, Fjeldsoe-Nielsen, & Schonning, 2010).

An increasing number of bla_{pAmpC} genes have been observed among *Ent*. These enzymes belong to several families (i.e., CMY, FOX, LAT, MIR, ACT, DHA, ACC, MOX) and are derived from those possessed by the chromosomal producers. So far, CMY-2 is the most prevalent pAmpC in *Ent*, but DHA and FOX have high prevalence in certain geographic regions (Jacoby, 2009; Seiffert, et al., 2013). Unlike class A enzymes, cAmpCs and pAmpCs are poorly inhibited by the commercially available β-lactamase inhibitors but the fourth-generation cephalosporins (e.g., cefepime) usually remain in the susceptible ranges (Harris & Ferguson, 2012; Hilty, et al., 2013).

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1.2.2. Resistance to carbapenems—Carbapenemases are β-lactamases able to hydrolize β-lactam antibiotics, including carbapenems (Papp-Wallace, Endimiani, Taracila, & Bonomo, 2011). Class A carbapenemases can be chromosomally encoded (e.g., SME) or plasmid-encoded (e.g., KPC, GES-5). KPC-types are the most clinically common carbapenemases found in *Ent* and are responsible for hospital outbreaks in many countries (Rapp & Urban, 2012; Walther-Rasmussen & Hoiby, 2007). Class B carbapenemases (metallo-β-lactamases, MBLs) are usually of VIM- and IMP-types, but the recently emerged NDM-types are becoming the most threatening carbapenemases and have spread rapidly among *Ent* in all continents (Nordmann, Naas, & Poirel, 2011; Walsh, 2010). The GIM and SPM MBLs have less global impact, but their prevalence in some countries deserve attention (e.g., South America and Germany, respectively) (Walsh, 2010). In *Ent*, class D carbapenemases are mainly represented by the OXA-48-like enzymes (e.g., OXA-48, -162, and -181). These genes are extensively reported among *E. coli* and *K. pneumoniae* isolates in the European and African Mediterranean countries (Poirel, et al., 2012). Recently, OXA-48 producers have been reported in North America (Lascols, Peirano, Hackel, Laupland, & Pitout, 2013; Mathers, et al., 2013).

It should be noted that carbapenem resistance in *Ent* may also be due to disruption of OMPs. This phenomenon may be observed under carbapenem treatment in *K. pneumoniae* (OmpK-35 and -36) (Endimiani, et al., 2009; Tsai, et al., 2011), *E. coli* (OmpF/C) (Oteo, et al., 2008; Tangden, Adler, Cars, Sandegren, & Lowdin, 2013), and *Enterobacter* spp. (OmpF/C) (Doumith, Ellington, Livermore, & Woodford, 2009).

1.2.3. Resistance to quinolones—Quinolones resistance among *Ent* is usually mediated by chromosomal mutations in the quinolone-resistance determining region (QRDR) that encode DNA gyrase (*gyrA* and *parC*) genes (Hooper, 2001; Jacoby, 2005). Nevertheless, low-level resistance can also arise from the expression of plasmid-mediated quinolone resistance (PMQR) determinants such as:: *i*) *qnrA, -B, -S, -C, -D* genes that encode proteins protecting the DNA gyrase from the quinolones' action; *ii*) an aminoglycoside acetyltransferase encoded by the *aac(6')-Ib-cr* gene that also acetylates quinolones; and *iii*) plasmid-mediated quinolone efflux-pumps (*qepA*) (Strahilevitz, Jacoby, Hooper, & Robicsek, 2009). The prevalence of *qnr* genes in ESBL-*Ent* is estimated around 10% (Karah, et al., 2010; Robicsek, Strahilevitz, Sahm, Jacoby, & Hooper, 2006), whereas that of *aac(6')-Ib-cr* is much higher (15-50%) (Ambrozic Avgustin, Keber, Zerjavic, Orazem, & Grabnar, 2007; C. H. Park, Robicsek, Jacoby, Sahm, & Hooper, 2006; K. S. Park, et al., 2012; Pitout, Wei, Church, & Gregson, 2008). Notably, Hansen *et al*. characterized *oqx*AB (Hansen, Johannesen, Burmolle, Sorensen, & Sorensen, 2004), a plasmid-mediated gene encoding for an efflux pump conferring resistance to chloramphenicol and quinolones (Hansen, Jensen, Sorensen, & Sorensen, 2007) (Kim, et al., 2009; K. S. Park, et al., 2012).

1.2.4. Resistance to aminoglycosides—Aminoglycosides resistance in *Ent* is generally due to enzymatic inactivation, which is mediated by 3 different classes of aminoglycoside-modifying enzymes (AMEs): acetyltransferases, nucleotidyltransferases, and phosphotransferases (Magnet & Blanchard, 2005). More recently, a new

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aminoglycosides resistance mechanism that consists of ribosomal protection through enzymatic methylation of specific residues within the 16S rRNA (impeding binding of drugs to the 30S ribosomal subunits) has been described. These 16S rRNA methylases (ArmA, RmtA, RmtB, RmtC, RmtD, RmtF, RmtG, and NpmA) confer high-levels of resistance to aminoglycosides and can be mobilized among different species (Bueno, Francisco, O'Hara, de Oliveira Garcia, & Doi, 2013; Doi & Arakawa, 2007; Hidalgo, et al., 2013). For instance, the *armA* (the most prevalent methylase gene) can be associated with the bla_{ESBLs} (Galimand, Sabtcheva, Courvalin, & Lambert, 2005; Yan, et al., 2004). More importantly, the bla_{NDM} genes are usually linked with 16S rRNA methylases (Nordmann, Poirel, Walsh, & Livermore, 2011).

1.2.5. Resistance to other antibiotic classes—*Ent* non-susceptible to ESCs and/or carbapenems are also frequently co-resistant to other classes of antibiotics. The most frequent genes conferring such resistance traits are as follows: tetracyclines (*tet* genes), sulfonamides (*sul* genes), trimethoprim (*dfr* genes), and phenicols (*cml*, *cat*, and *floR* genes) (Endimiani, Rossano, Kunz, Overesch, & Perreten, 2012).

1.3 Ideal characteristics of rapid detection methods

On the basis of epidemiological data (section 1.2), some antibiotic resistance traits deserve more attention than others when we design a rapid diagnostic tool (Table 1). In particular, we believe that $bla_{\text{CTX-M}}$, bla_{TEM}, bla_{SHV}, bla_{CMY}, bla_{KPC}, bla_{NDM}, $bla_{\text{OXA-48}}$, the 16S rRNA methylases genes and mutations in *gyrA* and *parC* are the most important targets that should be always tested for *Ent.* However, systems capable to detect further genes (e.g., bla_{PER}, bla_{VEB}, bla_{SPM}, bla_{GIM}, bla_{SME}, and PMQR determinants) with less global epidemiological and/or clinical impact would be welcome. This is important because in some geographic areas the spread of specific resistance genes (e.g., bla_{SPM} and bla_{SME} in America or bla_{GIM} in central Europe) has reached worrisome levels (Bush, et al., 2013; Hamprecht, et al., 2013; Walsh, 2010).

Ideally, a rapid system should investigate all resistance traits in one reaction and should provide easy to interpret results the same day the test is performed. Moreover, the assay should be able to identify specific variants of alleles that encode for proteins with a different impact on the antibiotic resistance phenotype (e.g., TEM and SHV with ESBL spectrum; OXA-48 rather than non-OXA carbapenemases). Finally, the methodology should be easy to perform, relatively cheap, accurate (sensitive and specific), and versatile enough to be regularly updated according to the evolution of the antibiotic resistance traits.

2. END-POINT PCRs

2.1 Single end-point PCRs

The single PCR is the ancestor of the molecular methods used in diagnostic and epidemiological studies for the detection of antibiotic resistance genes. It begins with the amplification of a target gene supposed to confer resistance and then includes an endpoint visualization of the amplification product(s) (Mullis & Faloona, 1987; Predari, Ligozzi, & Fontana, 1991; Zhou, Bordon, Sirot, Kitzis, & Gutmann, 1994). The single PCR allows

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identification of only one gene and requires the design of specific primers for the intended target. This approach is especially useful for the screening of a limited number of isolates and only if the detection of a gene at the generic level is sufficient (Table 1). This method can be appropriate in a known outbreak context when early detection of new cases is required.

Furthermore, by designing degenerate primers, single end-point PCRs can be used to detect specific genes (Table 2). For instance, they allow the rapid detection of all variants of the CTX-M-types enzymes (Boyd, et al., 2004). However, for specific gene characterization, discriminatory analysis of the PCR products (e.g., DNA sequencing) is necessary (see section 3). In some cases, digestion by endonucleases of the relative PCR product may discriminate among variants of a given gene, preventing sequencing. For example, the variant *aac(6)-Ib-cr* lacks the cleavage site for the BstF5I enzyme which is present in the ancestor gene (C. H. Park, et al., 2006).

2.2 Multiplex end-point PCRs

The multiplex end-point PCRs are based on the same principle of the single end-point PCRs, but use multiple primer sets allowing the amplification of different targets in the same reaction. The designing of a multiplex PCR presents some complex requirements: *i*) high specificity of the primer sets to the intended targets; *ii*) no self-reaction of the primers; *iii*) ideally, identical cycling condition for all primer sets; and *iv*) different sizes for the intended amplicons to allow a direct differentiation (Markoulatos, Siafakas, & Moncany, 2002). The use of positive controls is also required, especially when similar PCR product sizes are generated for different targets and when standard agarose gels are implemented for visualization.

To date, several multiplex PCRs able to detect antimicrobial resistance traits have been proposed (Table 2). One example is the multiplex designed more than 10 years ago by Perez-Perez and Hanson for the detection of bla_{pAmpCs}. The DNA template was obtained by boiling the colonies and the TTR was estimated at around 3 hrs. However, cross reaction with the cAmpCs occurred. Moreover, the size of different gene amplicons was too similar to be discriminated by standard gels; thus WAVE DNA analysis was applied for the discrimination (Perez-Perez & Hanson, 2002). Dallenne *et al*. developed a multiplex PCR able to detect a wide panel of *bla* genes. The setup, besides being laborious (i.e., seven multiplex reactions and three different cycling conditions), presents cross hybridization with the chromosomal class A -lactamases of certain producers (e.g., *Kluyvera ascorbata*, *K. georgiana*, and *K. pneumoniae*) and with the cAmpC of *Hafnia alvei*, *Enterobacter asburiae* and *Morganella morganii*. However, direct DNA sequencing of obtained amplicons allowed correct gene identification. The sequencing did not discriminate CTX-M-15 from CTX-M-28 and some VEB and CMY variants, but was able to discern between TEM and SHV ESBLs from non-ESBL variants (Dallenne, Da Costa, Decre, Favier, & Arlet, 2010). This method has found application in numerous studies for the screening of ESBLs and carbapenemase producing strains (Hornsey, Phee, & Wareham, 2011). Voets *et al*. used part of the above setup and implemented it by including the detection of further carbapenemases. They optimized the PCR process to reduce cross reaction with chromosomal β-lactamases

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and simplified the procedure to only one cycling condition (Voets, Fluit, Scharringa, Cohen Stuart, & Leverstein-van Hall, 2011). Ellington *et al*. published a multiplex PCR for the detection of MBLs. DNA extracts obtained by boiling the colonies were implemented with the method and proved to amplify different IMP and VIM variants (Ellington, Kistler, Livermore, & Woodford, 2007). Later, Poirel *et al*. included this approach in a multiplex PCR with a wider range of carbapenemase detection and using DNA templates obtained by alkaline lysis of colonies. The method was specific when primers sets were used either in single or multiplex PCR reactions (Poirel, Walsh, Cuvillier, & Nordmann, 2011). This protocol is easily and rapidly executed (<4 hrs) and is applicable for the screening of large collections (Capone, et al., 2013). More recently, Hong *et al*. proposed a multiplex PCR for the detection of class A carbapenemases. Although the method was tested on a limited number of control strains, which did not comprise several variants of the targeted genes, the procedure is simple and rapid and it could find application in an opportune epidemiological context. This multiplex PCR did not produce false positive results, suggesting high specificity (Hong, et al., 2012) and it has been used for the molecular confirmation of phenotypic analysis (Rojo-Bezares, Martin, Lopez, Torres, & Saenz, 2012; Ruiz, et al., 2012). Doyle *et al*. designed an in-house multiplex PCR for the simultaneous detection of bla_{KPC}, bla_{NDM}, bla_{VIM}, bla_{IMP}, and $bla_{\text{OXA-48}}$-like. The methodology was evaluated against 142 *Ent* obtained from the SMART study (2008-2009) and compared to the performance of phenotypic tests (i.e. mHT, Mastdiscs ID inhibitor combination disks, Rosco Diagnostica Neo-Sensitabs, and MBL Etest). The PCR had 100% sensitivity and specificity, whereas the other methods were 58% and 93%, 78% and 93%, 80% and 93%, and 55% and 100%, respectively (Doyle, et al., 2012).

As anticipated, monitoring of co-resistance to other antibiotics (especially aminoglycosides and quinolones) among ESBL- and/or carbapenemase-producing *Ent* is an important task (Table 1). Cattoir *et al*. designed a multiplex PCR for the detection of several variants of *qnr*A/B/S genes (Cattoir, Poirel, Rotimi, Soussy, & Nordmann, 2007). However, we note that there is no multiplex PCR able to detect all relevant PMQR determinants. Thus, the method proposed by Cattoir *et al*. should be used in association with further single PCRs for the detection of *qepA*, *aac(6′)-Ib-cr* and *oqxAB* (Yamane, Wachino, Suzuki, & Arakawa, 2008; Yue, et al., 2008). Berçot *et al*. developed a protocol for the detection of 16S rRNA methylase genes in NDM-possessing *Ent*. The template for the multiplex reaction was obtained utilizing a commercial DNA extraction kit and the TTR starting from bacterial culture was estimated to be less than 4 hrs. This proposed multiplex PCR is rapid, specific, sensitive, of simple execution, and it has been applied for the screening of strain collections (Bercot, Poirel, & Nordmann, 2011; Williamson, et al., 2012). Diaz *et al.* established a multiplex PCR limited to the detection of the AMEs *aac(3)-IIa* and *aac(3)-IVa* in *Ent* of environmental origin (Diaz, Cooper, Cloeckaert, & Siebeling, 2006). Except for this latter report, no multiplex PCRs detecting AMEs in *Ent* have been engineered, so far. Probably the high diversity of such enzymes and their corresponding sequences hinders the determination of an efficient multiplex protocol. Of note, several investigators have designed single PCRs for the detection of AMEs genes (Table 2).

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2.3 Commercially available multiplex PCRs

Amplex Diagnostics (Germany) has designed three commercial PCR-ELISA kits to rapidly detect clinically important *bla* genes (www.hyplex.info/). The assays are based on specific multiplex PCRs for target genes, reverse hybridization with labeled PCR products in a 96-well plate, addition of conjugate (peroxidase), addition of substrate (tetra-methyl-benzidin), and photometric measurement at 450 nm. In particular, hyplex ESBL ID, hyplex SuperBug ID, and hyplex CarbOXA ID detect respectively: *i*) bla_{TEM}, bla_{SHV}, bla_{CTX-M}, and bla_{OXA}; *ii*) bla_{KPC}, bla_{IMP}, bla_{VIM}, bla_{NDM}, $bla_{OXA-48-like}$; and *iii*) bla_{OXA-23}, bla_{OXA-24}, and $bla_{OXA-51-like}$. Hyplex SuperBug ID was recently evaluated by testing 132 carbapenem-resistant *Ent* which included KPC-, VIM-, IMP-, NDM-, and OXA-48-like-producing isolates. Overall, the agreement with the PCR/sequencing results was 97% (128/132), but three VIM producers were not detected. Starting from buffer lysis of colonies, the TTR is usually of 2.5-4 hrs (Kaase, Szabados, Wassill, & Gatermann, 2012). A prototype of the above platform (i.e., hyplex MBL ID: able to detect only bla_{IMP} and bla_{VIM}) was also evaluated against 326 clinical samples (including blood, urine, bronchial secretions, and wound swabs) of which 73 were positive for VIM-producing *Ent* or nonfermenters. Overall, sensitivity and specificity were 98.6% and 98%, respectively (Avlami, et al., 2010).

2.4 Advantage and disadvantages of PCRs

Both single and multiplex PCRs require a high copy number of the intended target to produce a detectable amplification product. Thus, preliminary bacterial isolation, cultivation and DNA extraction are necessary. In this regard, amplification from colonies represents a rapid alternative to genomic DNA isolation. In contrast, clinical samples may harbor inhibitors (e.g. hemoglobin and heparin in the blood; urates in the urine; polysaccarides in the feces) for the PCR reaction making the test less sensitive (Schrader, Schielke, Ellerbroek, & Johne, 2012).

Depending on the level of details required, results from single and multiplex PCRs can be obtained in less than 4 hrs (i.e., amplification results) to more than 24 hrs (determination of the specific DNA sequence or further analyses). Typically, multiplex PCRs provide a generic identification of targets genes. However, such PCRs detect numerous genes simultaneously and, even if the setup of such protocols is laborious, the execution is simple. The optimization of the method to a few reactions and/or one cycling condition, and the relatively low costs of performance, make multiplex PCRs more appropriate and useful for epidemiological studies focusing on the molecular characterization of the drug resistance genes (Table 4).

3. NEXT-GENERATION DNA SEQUENCING METHODOLOGIES

As previously mentioned, characterizing variants of certain genes is important, thus their DNA sequence must be determined. Two *de novo* sequencing methods were developed in the ′70s, one by Maxam and Gilbert (Maxam & Gilbert, 1977), and the other one by Sanger (Sanger, Nicklen, & Coulson, 1977). The first method is based on the enzymatic digestion of the DNA template. Four digestions in independent reactions are carried out on the PCR product, each of them performing cleavages at one specific base. The resolution by

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electrophoresis of the obtained fragments provides with the sequence of the product. The Sanger method consists of a reaction of DNA synthesis by a DNA polymerase using dNTPs (deoxy-nucleotides tri-phosphates) and ddNTPs (dideoxy-nucleotides tri-phosphates) labeled with four different fluorophores (one fluorophore for each base). The incorporation of ddNTPs, which are terminators of the synthesis, produces fragments of different sizes that can be separated by capillary electrophoresis or analyzed by mass spectrometry (Murray, 1996). So far, this methodology is the most used in clinics for the sequencing of antibiotic resistance genes. However, this method is laborious, requiring several post-PCR procedures such as purification of the products, sequencing reaction and resolution of the sequence products. A common alternative is out-sourcing the process which in turn results in a delay of the final data.

Many efforts have been made in the last years to overcome these limitations giving rise to the so-called “next-generation sequencing” (NGS) methodologies (L. Liu, et al., 2012). Mostly, these methods are based on the “real-time” fluorescence/light detection occurring during the sequencing process. The methods differ for the chemistry of the underling reactions. Ronaghi *et al.* published a sequencing methodology based on the action of four enzymes: a DNA polymerase adds dNTPs to a growing DNA strand, the pyrophosphate group PPi released during the polymerization is the substrate of a sulfurylase which in turn synthetizes ATP, this latter is used by a luciferase for light emission. An apyrase removes excess of dNTPs and ATP improving fidelity of the sequencing, also ensured by the DNA strand conformation. The intensity of light is specific to the base that has been incorporated (Ronaghi, Karamohamed, Pettersson, Uhlen, & Nyren, 1996). This methodology, famous as pyrosequencing, and commercially as “454” (Roche), has found application for antibiotic resistance gene characterization in several studies and is becoming a bench-top tool. For instance, Poirel *et al*. implemented it to characterize bla_{GES} variants encoding carbapenemases from *K. pneumoniae* and *Pseudomonas aeruginosa* (Poirel, Naas, & Nordmann, 2006). Naas *et al.* characterized CTX-M variants in clinical ESBL-producing *E. coli* using pyrosequencing after real-time PCR of $bla_{\text{CTX-M}}$. The five CTX-M groups were discriminated with this technique by 13 bp DNA region analysis and this result was confirmed by the standard DNA sequencing approach (Naas, Oxacelay, & Nordmann, 2007). In one study, the application of pyrosequencing and analysis of single nucleotide polymorphisms (SNPs) for bla_{SHV} and bla_{TEM} detected ESBLs variants of the respective genes in a collection of *Ent*. Notably, this method also enabled the identification of new *bla* variants (Jones, et al., 2009). Interestingly, Haanpera *et al.* used the pyrosequencing for the discrimination of chromosomal SHV from acquired ESBLs types on a collection of *K. pneumoniae* confirming the powerful resolution of this technique (Haanpera, Forssten, Huovinen, & Jalava, 2008). Pyrosequencing was also efficiently applied for the discrimination of the *aac(6)-Ib* from the variant *aac(6)-Ib-cr* (Guillard, et al., 2010), and for the detection of *gyr*A and *par*C mutations conferring quinolones resistance in a *Salmonella enterica* (Hopkins, Arnold, & Threlfall, 2007).

Although yet not applied for the antibiotic resistance characterization, other NGS methods are available. The sequencing by synthesis, commercially referred to as “Illumina”, consists of the neo-synthesis of DNA by a DNA polymerase incorporating dNTPs labeled with

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fluorescent terminators which are detected in real-time (L. Liu, et al., 2012). The sequencing by ligation, implemented by Life-technology with the commercial name “SOLiD”, is based on the action of a DNA ligase which adds fluorescent labeled dNTPs to an anchor probe hybridizing a single strand DNA in proximity of the region to be sequenced (L. Liu, et al., 2012).

The Ion torrent sequencing is another powerful NGS approach recently developed and which might become a bench-top NGS tool. The sequencing by synthesis reaction is performed on a semiconductor chip and is based on the detection of the hydrogen ions released at each addition of a nucleic base. Electrical pulses are then directly converted into a DNA sequence (L. Liu, et al., 2012). This technology has been recently applied to the investigation of an outbreak of *E. coli* infections, allowing not only the detection of CTX-M-15, but providing the genome sequences of these strains (Sherry, et al., 2013). The authors described this method as fast (five days from a positive culture to completion of sequencing) and reasonably cheap (~ US $300 per strain, reagents only). Although development of further bioinformatics for an easier data interpretation is necessary, the advent of NGS methodologies and their commercialization promise to be revolutionary for the improvement of diagnosis and epidemiology of infections due to MDR-*Ent*.

4. REAL-TIME PCRs

The real-time PCR consists of an amplification reaction coupled with the simultaneous detection of the exponentially amplified target and visualization of the reaction phases (Higuchi, Fockler, Dollinger, & Watson, 1993). The number of cycles necessary to reach this exponential phase often depends on the initial quantity of target present in the reaction. The cycle at which the reaction assumes an exponential state is referred to as the quantitative cycle (Cq) because it allows the quantification of the initial DNA target (qPCR). Such quantification can be absolute or relative, the first requires the comparison of the target quantification to a standard with known concentration. This quantification is useful to calculate the number of copies of a target in a genome and/or to compare the amount of a target from two different samples. The relative quantification, using mRNA as template or a retro-transcriptional template (RT-PCR), allows defining the level of expression of a target compared to a constantly expressed (housekeeping) gene (Heid, Stevens, Livak, & Williams, 1996). Furthermore, real-time PCR apparatus are able to quantify the amount of denatured DNA (single strand DNA) at a certain temperature (Tm), thereby providing the melting curve of a DNA fragment. The denaturing temperature is specific to the composition of the DNA fragment, thus a precise measure of the melting curve(s) gives information on even small variations in the DNA sequence (the so called “High Resolution Melting Analysis”) (Erali, Voelkerding, & Wittwer, 2008; Tong & Giffard, 2012).

Monitoring of fluorescence emission occurring during the amplification is the mechanism on which the dynamics of real-time PCR is based (Higuchi, Dollinger, Walsh, & Griffith, 1992). The fluorescence emission can be obtained according to four approaches (Arya, et al., 2005): *i*) generic, implementing a dye (e.g., SYBR Green, FAM, Cal Fluor Red, Hex) which fluoresces when introduced in the double-strand DNA; *ii*) specific, using TaqMan probes harboring a fluorophore at the 5′ and a quencher (preventing fluorescence emission) at the

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3′. TaqMan probes hybridize on a specific DNA target and are depredated by the Taq polymerase exo-nuclease activity during amplification. The removal of the fluorophore from the quencer allows fluorescence emission. Different TaqMan probes labeled with diverse fluorophores can be used in the same PCR mixture; *iii*) specific, utilizing HYB probes, which harbor two different fluorescent labels. They hybridize on the target only during the annealing step in a head-to-tail direction and when they are close to each other emit fluorescence. The probes are removed during the elongation, due to the increase in temperature of the reaction; *iv*) specific, using molecular beacons consisting of the probe flanked by two complementary sequences carrying a fluorophore and a quencer at their respective extremities. This match creates a stem-loop structure where the quencer, close to the fluorophore, prevents fluorescence emission. Appropriate Tm and presence of a complementary sequence for the probe open the stem-loop structure allowing the removal of the fluorophore from the quencer and favoring fluorescence emission. Scorpion probes are designed using a similar principle (Arya, et al., 2005).

The loop-mediated isothermal amplification of DNA (LAMP), based on the detection of fluorescence using SYBR Green and a peculiar engineering of the primer set, allows DNA target amplification at a unique and constant temperature (Notomi, et al., 2000). This method, being cheaper, could represent an alternative to PCR (due to its higher sensitivity) and to real-time PCR. **4.1 Single real-time PCRs**

Single real-time PCRs

One of the first applications of real-time PCR for detecting antibiotic resistance in *Ent* was performed by Hammond *et al*. where a SNP of bla_{SHV} was analyzed to distinguish ESBL from non-ESBL variants in *K. pneumoniae* (Table 3). The methodology also enabled the quantification of copy numbers of the allele and to elucidate the effect of a mobile genetic element on the amplification of the bla_{SHV} in the genome of the strain (Hammond, Schooneveldt, Nimmo, Huygens, & Giffard, 2005). Mroczkowska and Barlow used molecular beacons for detecting single nucleotide polymorphism of bla_{TEM} (Mroczkowska & Barlow, 2008). Using TaqMan probes specific for CTX-M variants, Birkett *et al*. implemented a highly specific method that avoids sequencing costs and provides a faster alternative to conventional end-point PCR (Birkett, et al., 2007). Several other studies have highlighted the higher sensitivity of real-time PCR compared to culture based detection of carbapenemase producers (Table 3). Raghunathan *et al*. compared real-time PCR using the dye SYBR Green with the mHT for the detection of bla_{KPC} in *K. pneumoniae* isolates. Although sensitivity and specificity were comparable, real-time PCR was more rapid (Raghunathan, Samuel, & Tibbetts, 2011). Kruettgen *et al*. designed real-time PCR for the detection of bla_{NDM} and proposed a methodology for synthetic positive controls preparation (Kruttgen, Razavi, Imohl, & Ritter, 2011). Detection of bla_{NDM} using TaqMan showed a detection limit of 10 copies of the target gene per reaction (Manchanda, et al., 2011). Oxacelay *et al*. engineered a HYB probe for the detection of CTX-M variants directly from urine samples. The method was quite sensitive (detection limit of 10^2-10^3 cells/ml of urine) (Oxacelay, Ergani, Naas, & Nordmann, 2009). Naas *et al*. engineered a TaqMan probe for the detection of bla_{NDM-1} from spiked fecal stools. This method had a detection limit of 10^1 CFU/100 mg of feces, while selective media such as ChromoID ESBL and CHROMAgar

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KPC were 10^1-3×10^1 and 10^1-4×10^3 CFU/100 mg of feces, respectively. The method demonstrated 100% sensitivity and specificity, and the protocol was rapidly peformed (4 hrs). Therefore, this method is a valuable tool for rapid detection of NDM-1 producers in clinical samples (Naas, Ergani, et al., 2011). Alternatively, the LAMP methodology has been applied for NDM-1 detection with a very rapid protocol (1 h) and a detection limit of 10.7 pg/ml (W. Liu, et al., 2012). Recently, real-time PCR using a 100% sensitive TaqMan probe for the detection of $bla_{\text{OXA-48}}$ in spiked stool samples was implemented. The method had a detection limit of 10-50 CFU/100 mg of feces, while for ChromID ESBL and SUPERCARBA medium were at 10^1-10^2 and $1\text{-}3\times10^1$ CFU/100 mg of feces, respectively (Naas, Cotellon, Ergani, & Nordmann, 2013).

Real-time PCR can also be used for copy number determination of target genes. Kurpiel and Hanson used SYBR Green for the copy number determination of $bla_{\text{CMY-2,}}$ harbored by clinical *E. coli* isolates, demonstrating tandem duplication of the gene (Kurpiel & Hanson, 2011). Importantly, Landman *et al*. designed two TaqMan probes for the quantification of OmpK35 and -36 in carbapenem-resistant *K. pneumoniae* isolates, confirming the role of OmpK36 expression in the susceptibility profile of these organisms (Landman, Bratu, & Quale, 2009).

4.2 Multiplex real-time PCRs

The analysis of the melting temperature curves allows a specific and easy discrimination of amplified products obtained in a multiplex real-time PCR (Table 3). Bisiklis *et al*. used HYB probe to amplify bla_{VIM} and bla_{IMP}. The analysis of the Tm provided discrimination between the two genes and allowed the detection of gene variants (Bisiklis, Papageorgiou, Frantzidou, & Alexiou-Daniel, 2007). Adapting a previously developed method to detect pAmpCs (Perez-Perez & Hanson, 2002), Brolund *et al*. designed two triplex real-time PCRs using SYBR Green dye with the same cycling conditions. Starting from DNA templates of boiled colonies, the method was rapid (<3 hrs) and the analysis of the melting curves demonstrated high specificity (Brolund, et al., 2010). More recently, Geyer *et al*. developed a real-time TaqMan assay for pAmpCs showing specificity and sensitivity of 100% (Geyer, Reisbig, & Hanson, 2012). Swyne *et al*. designed a TaqMan real-time PCR for the detection of class A and D carbapenemases. The method was specific and the amplicons could be distinguished by melting curve analysis (Swayne, Ludlam, Shet, Woodford, & Curran, 2011). Highly accurate detection of different classes of carbapenemases was also obtained by Monteiro *et al*. by implementing Tm analysis (Monteiro, Widen, Pignatari, Kubasek, & Silbert, 2012). Interestingly, Chen *et al*. combined MLST and bla_{KPC} detection in *K. pneumoniae* using molecular beacons in a unique multiplex mixture. The method was rapid (1 h) and had a detection limit of 4 CFU per reaction (Chen, et al., 2012). The same authors applied a similar technique to distinguish KPC variants (Chen, et al., 2011).

Guillard *et al*. elaborated the first real-time PCR for the detection of PMQR genes using the ResoLight dye for the detection of *qnr* genes and SYBR Green for *qepA* detection. The method was rapid (< 2 hrs) and melting curve analysis guaranteed discrimination of the *qnr* alleles (Guillard, et al., 2011). The same authors developed a single and multiplex PCR coupled with high resolution melting analysis for the detection of PMQR determinants

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(Guillard, et al., 2012). Taking advantage of the high resolution melting curve analysis, Bell *et al*. were able to detect the *aac(6')-Ib-cr* variant after the real-time PCR assay (Bell, Turnidge, & Andersson, 2010).

4.3 Commercially available real-time PCRs

The New NucliSENS EasyQ KPC (bioMérieux) has been shown to be a highly sensitive and specific commercial real-time PCR system for the detection of bla_{KPC} in *K. pneumoniae*. Spanu *et al*. evaluated the performance of the kit on a large collection of *Ent* and compared the results with those obtained with conventional amplification methods. Besides the high sensitivity and specificity, the method was very rapid (2 hrs) making it valuable for surveillance studies and rapid identification of carriers (Spanu, et al., 2012). Check-Points Health BV (the Netherlands) has developed a rapid (3.5 hrs) ligation mediated multiplex real-time PCR (Check-MDR ESBL kit) to detect SHV/TEM variants (ESBL and non-ESBL) and CTX-M groups. Nijhuis *et al*. compared this platform with the phenotypic combination disc method showing sensitivity and specificity of 99% and 92.2%, respectively (Nijhuis, van Zwet, Stuart, Weijers, & Savelkoul, 2012). The company has also developed a multiplex real-time PCR (Check-MDR Carba) to rapidly detect bla_{KPC}, bla_{NDM}, bla_{VIM}, bla_{IMP}, and bla_{OXA-48}. However, the performance and accurateness of the kit has not been evaluated yet.

4.4 Clinical experiences with real-time PCRs

A TaqMan probe design enabled bla_{KPC} detection in DNA extracted directly from rectal/perianal swabs using two automated systems (i.e., NucliSENS easyMAG and MagNA Pure LC). The method was rapid and had a superior analytical sensitivity compared to culture dependent methods (Hindiyeh, et al., 2008). Similarly, Singh *et al*. implemented a single PCR using two TaqMan probes, one designed for bla_{KPC} and one for the 16S gene, for the rectal screening of KPC carriers. The method used overnight cultures enriched with ceftazidime and DNA template was obtained by boiling. The assay had both sensitivity and a specificity of 97% demonstrating a higher sensitivity compared to selective media CHROMAgar (77.3%) (Singh, et al., 2012). Hindiyeh *et al*. adapted the above TaqMan probe protocol for the detection of bla_{KPC} with a multiplex real-time PCR including the RNaseP gene as control. This method was able to detect bla_{KPC} from blood culture bottles with a sensitivity and specificity of 100%. The automated extraction of the DNA improved the performance of the assay and the method was performed in less than 4 hrs (Hindiyeh, et al., 2011).

4.5 Advantage and disadvantages of real-time PCR

Real-time PCR avoids time consuming steps (e.g., electrophoresis analysis) in comparison to end-point PCRs and in many cases it does not require DNA sequencing of the obtained products. For these reasons, the method is rapid and excludes experimental errors linked to post amplification procedures. Moreover, real-time PCR is very sensitive, especially when TaqMan/HYB probes or molecular beacons are used instead of the general fluorescent dyes. Indeed, the latter might intercalate into primer dimers producing ambiguous signals. The selective hybridization of these probes, and the possibility to label them with different fluorophores, allows greater specificity in multiplex conditions and easier resolution of

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amplicons compared to end-point approaches. On the other hand, the setup of a real-time PCR is more complicated because the Tm of primers and probes needs to be taken into consideration. However, probe sequences available from literature and implementation of commercial kits could facilitate the procedure.

Generally, the reproducibility of the results obtained with real-time PCR is the major limitation of this technique (Bustin, et al., 2009). However, some precautions in the experimental setup (especially standardizing the DNA preparation) and in the interpretation of the data make real-time PCR a powerful, sensitive, reliable alternative to end-point PCRs, and a cost effective choice compared to the other currently available molecular approaches.

The overall characteristics of real-time PCR make this molecular approach particularly suitable for diagnostic purposes. This is especially true for multiplex real-time PCRs able to simultaneously detect clinically important drug-resistance traits. In the future, this application could lead to: *i*) the early detection of antibiotic resistance genes in clinical samples, dramatically influencing the effectiveness of therapy; *ii*) a less extensive use of broad-spectrum antibiotics in the empirical treatment of serious infections; and *iii*) the rapid screening of ESBL and/or carbapenemase producers carriers. . This application enormously contributes to the implementation of hygienic measures, necessary for combating the spread of such mechanisms among *Ent* and other MDR Gram-negative pathogens.

5. MICROARRAY

Microarray is an ideal methodology that allows for simultaneous identification and partial characterization of a very large number of genes (even >1000) of interest. This technology consists of pre-designated oligonucleotide probes that are specifically bound and immobilized on the solid surface of an array. If the targeted allele of a pathogen is present, it is labeled and subsequently hybridized to the immobilized probe present on the matrix of the array; such successful reactions are then measured with dedicated scanners (Miller & Tang, 2009; Sibley, Peirano, & Church, 2012).

Several microarrays for the genotyping of *bla* genes have been developed in the past. For instance, Grimm *et al*. designed a rapid DNA microarray (i.e., TTR of ~6 hrs) for genotyping of TEM β-lactamases. The assay was able to identify SNPs of 96% of TEM variants which were related to ESBL phenotypes (Grimm, et al., 2004). The same authors further developed an array for bla_{TEM}, bla_{SHV} and $bla_{\text{CTX-M}}$ implementing 618 probes capable of covering the mutations responsible for 156 key amino acid substitutions. The validity of the tests was demonstrated detecting 58 out of 60 isolates previously characterized as ESBL producers by phenotypic tests (Leinberger, et al., 2010). In an assay designed to study bla_{TEM}, bla_{SHV} and $bla_{\text{CTX-M}}$, Robtsova *et al*. implemented biotin and streptavidin horseradish peroxidase to label the *bla* genes and to detect (by colorimetric reaction) their presence on the matrix, respectively. The system correctly detected 90 ESBL producers previously characterized with phenotypic and molecular methods (Rubtsova, Ulyashova, Edelstein, & Egorov, 2010).

Although the above in-house microarrays demonstrated excellent ability to detect and characterize the main bla_{ESBL} genes, the complete lack of probes able to detect further genes

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(e.g., those for carbapenemases) makes their use insufficient for the present epidemiology of MDR-*Ent*. More importantly, the implementation of those assays in other laboratories appears difficult, especially because of problems related to the standardization of the procedures. The advent of commercially available microarrays addresses this concern.

5.1 Commercially available microarrays

Check-Points has developed several rapid DNA microarray tests based on a ligation-mediated amplification reaction to detect the most important *bla* genes carried by Gram-negative pathogens (www.check-points.com). The results are automatically interpreted by the software provided by the manufacturer and the overall TTR starting from fresh colonies is very rapid (e.g., 50 strains in ~8 hrs) (Endimiani, Hujer, Hujer, Gatta, et al., 2010).

Cohen Stuart *et al*. evaluated the first microarray platform designed by the company to detect bla_{TEM}, bla_{SHV}, and $bla_{\text{CTX-M}}$ in a collection of 212 *Ent* (of which 106 were ESBL producers). In particular, specific probes were designed to distinguish non-ESBL and ESBL variants of TEM and SHV (i.e., SNPs determining substitutions E104K, R164S/C/H, G238S for TEMs and D179A/N/G, G238S/A, E240K for SHVs) and the CTX-M-1, -2, -9, and -8/25 groups. After comparison with the PCR/DNA sequencing data, the sensitivity and specificity of the microarray resulted 95% and 100%, respectively (Cohen Stuart, et al., 2010). In the same period, we evaluated an updated version (Check KPC/ESBL) of the microarray able to further detect the bla_{KPC}. A total of 106 Gram-negative strains (including 57 KPC producers) were tested showing an overall agreement of 91.5% with standard DNA sequencing. The following sensitivity and specificity results were also recorded, respectively: bla_{SHV} (98.8% and 100%), bla_{TEM} (100% and 96.4%), $bla_{\text{CTX-M}}$ and bla_{KPC} (both 100% and 100%) (Endimiani, Hujer, Hujer, Gatta, et al., 2010). Later, consistent results (i.e., sensitivity of 97% and specificity of 98%) were also obtained for 346 clinical *Ent* collected from 31 clinical laboratories in the Netherlands in 2009 (Platteel, et al., 2011), and for 125 Gram-negatives collected in one French hospital (i.e., 95% of agreement) (Naas, Cuzon, Truong, Bernabeu, & Nordmann, 2010). The Check KPC/ESBL was recently implemented to perform the molecular epidemiology of 1,051 *Ent* collected over the world during the longitudinal Study for Monitoring Antimicrobial Resistance Trends (SMART; 2008-2009). The assay demonstrated to be a rapid and very accurate system to screen for bla_{ESBLs} and bla_{KPC} genes and to guide further PCR/sequencing analyses (Lascols, et al., 2012).

More recently, new Check-Points platforms have been designed to detect further clinically relevant *bla* genes. The Check-MDR CT101 assay detects bla_{SHV}, bla_{TEM}, $bla_{\text{CTX-M}}$ and bla_{KPC} as Check KPC/ESBL, but also bla_{pAmpCs} (bla_{CMY}, bla_{DHA}, $bla_{\text{ACT/MIR}}$, bla_{ACC}) and bla_{NDM}. Bogaerts *et al*. evaluated this microarray testing 207 clinical and reference strains of *Ent* showing an agreement with PCR/DNA sequencing of 100% for both bla_{pAmpCs} and bla_{NDM}. As expected, false positive pAmpC results were recorded for cAmpC producers (e.g., *Enterobacter* spp. and *C. freundii*) (Bogaerts, et al., 2011). The Check-MDR CT102 platform detects bla_{SHV}, bla_{TEM}, and $bla_{\text{CTX-M}}$, but also possesses the ability to detect more carbapenemase genes (i.e., bla_{VIM}, bla_{IMP}, and the $bla_{\text{OXA-48-like}}$) rather than only bla_{KPC} and bla_{NDM}. However, it does not contain probes to detect the

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bla$_{pAmpCs}$. Naas *et al*. tested 144 Gram-negative strains possessing various *bla* genes indicating that this assay enables accurate identification of common ESBL and carbapenemase genes (i.e., both sensitivity and specificity of 100%; agreement with standard sequencing of 99%) (Naas, Cuzon, Bogaerts, Glupczynski, & Nordmann, 2011). So far, the Check-MDR CT103 is the most complete microarray platform provided by Check-Points. It merges the characteristics of CT101 and CT102 making the platform able to identify the most important non-ESBL, ESBL, pAmpCs, and carbapenemase genes spreading among *Ent*. Cuzon *et al*. tested 187 well-characterized Gram-negative bacilli showing sensitivity and specificity of both 100% for each single gene tested; moreover, all *bla*$_{CTX-M}$ were classified into the appropriate family group (Cuzon, Naas, Bogaerts, Glupczynski, & Nordmann, 2012).

Batchelor *et al*. developed a miniaturized microarray for the detection of 47 resistance genes in Gram-negative bacteria. In particular, this assay was able to detect traits encoding resistance to aminoglycosides [AMEs: *aadA1/2/4*-like, *aac(3)-Ia*/*-IVa*, *aac(6')-Ib*, *ant(2'')-Ia*], quinolones (*qnr*), trimethoprim (*dfrA1/7/14/17/19*, *dfr12*), sulphonamides (*sul1-3*), chloramphenicol (*cmlA1*-like, *catA1*, *catIII*, *catB3*-like, *floR*), tetracyclines [*tet(A)-(G)*], β-lactams (*bla*$_{PSE}$, *bla*$_{DHA}$, *bla*$_{ACC}$, *bla*$_{MOX}$, *bla*$_{CMY}$, *bla*$_{FOX}$, *bla*$_{SHV}$, *bla*$_{LEN}$, *bla*$_{TEM}$, *bla*$_{OXA}$, *bla*$_{CTX-M}$) and integrons (*intI*1-2). The performance of the assay for 87 *Ent* was compared to the PCR analysis showing a correlation of 98.8% (Batchelor, et al., 2008). This microarray was the ancestor for other improved platforms that are provided by Identibac (http://identibac.com). The most updated assay contains probes to detect numerous virulence factors of *E. coli* and further important resistance alleles (e.g., 16S rRNA methylases, *qepA*, *bla*$_{PER}$, *bla*$_{VEB}$, *bla*$_{OXA-23/}$-40/-48/-51/-58, *bla*$_{SPM}$, *bla*$_{NDM}$, *bla*$_{IMP}$, *bla*$_{VIM}$, and *bla*$_{KPC}$). However, though the system detects an enormous number of clinically important genes (including those for non-β-lactam antibiotics), it is still unable to distinguish between ESBLs and non-ESBLs in the SHV and TEM families. Moreover, a careful validation of the assay testing large collections of well-characterized isolates is still lacking.

5.2 Clinical implementation of the microarrays

The Check KPC/ESBL was recently used by Fishbain *et al*. to detect *bla* genes in 60 positive blood culture bottles with *Ent* (n=40; of which 20 possessing *bla*$_{ESBLs}$ and/or *bla*$_{KPC}$) or non-fermenters (n=20; negative controls). Aliquots of 0.5 ml were withdrawn from the bottles and, after removing erythrocytes by centrifugation, DNA extraction was performed. The overall concordance rate between phenotypic tests and microarray was 98.8% (59/60) when the water lysis method was implemented to perform DNA extraction. This approach could reduce the notification time of ESBL and/or KPC production by 18-20 hrs (Fishbain, Sinyavskiy, Riederer, Hujer, & Bonomo, 2012). More recently, Wintermans *et al*. showed that the implementation of the first Check-Points microarray (i.e., for detecting *bla*$_{SHV}$, *bla*$_{TEM}$, and *bla*$_{CTX-M}$) instead of phenotypic tests significantly reduced the costs for unnecessary isolation for patients (i.e. those suspected to be colonized with ESBL producers) (Wintermans, Reuland, Wintermans, Bergmans, & Kluytmans, 2012). Peter *et al*. evaluated an in-house DNA microarray assay to directly detect all variants of *bla*$_{KPC}$ from simulated urine samples. The system was able to distinguish the different KPC-types with a TTR of 5-6 hrs (Peter, et al., 2012).

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5.3 Advantage and disadvantages of microarrays

Microarrays possess greater analytical capacity compared to most of the other molecular methods (e.g., those PCR-based) because they can analyze a larger number of target genes in one reaction (Cuzon, et al., 2012). Moreover, assays designed to detect different SNPs in the same genes may be able to suggest the simultaneous presence of allelic variants (e.g., $bla_{\text{SHV-11}}$ and $bla_{\text{SHV-12}}$ in *K. pneumoniae*) (Endimiani, Hujer, Hujer, Gatta, et al., 2010). This makes their implementation to screen large collections of isolates very appealing (Lascols, et al., 2012). However, the system can also find application in a clinical practice (e.g., for BSIs), especially in a context with a high prevalence of MDR-*Ent* (Fishbain, et al., 2012). The methodology is easy to perform (especially for the commercially available platforms) and to be updated. On the other hand, the TTR is moderately high (6-8 hrs including DNA extraction) and the cross reaction of the pAmpCs probes with the cAmpCs of some organisms is a problem that is difficult to solve (Batchelor, et al., 2008; Bogaerts, et al., 2011). Devices and commercial kits are also quite expensive but, due to the rapid commercialization of this promising methodology, more affordable prices are expected in the near future (Table 4).

6. MASS SPECTROMETRY-BASED METHODOLOGIES

6.1 MALDI-TOF MS

Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectroscopy (MALDI-TOF MS) allows clinical microbiologists to dissect unique protein signatures (e.g., ribosomal proteins) of bacteria in order to identify the pathogen (Wieser, Schneider, Jung, & Schubert, 2012); in addition, there is a potential for the detection of resistance mechanisms. However, the use of MALDI-TOF MS for resistance mechanisms detection is in its early evaluation state (Hrabak, Chudackova, & Walkova, 2013). Currently, multiple applications of MALDI-TOF MS are being pursued for the detection of resistance mechanisms within *Ent*. These applications include: identification of the antibiotic itself and its modified/degradated counterparts (Burckhardt & Zimmermann, 2011; Hooff, et al., 2012; Hrabak, et al., 2012; Hrabak, Walkova, Studentova, Chudackova, & Bergerova, 2011; Sparbier, Schubert, Weller, Boogen, & Kostrzewa, 2012), detection of the resistance proteins within the cell (Cai, Hu, Zhang, Zhou, & Chen, 2012; Camara & Hays, 2007; Schaumann, et al., 2012), and discovery of mutations within resistance genes through mini-sequencing (Ikryannikova, et al., 2008).

Several studies have assessed the utility of MALDI-TOF MS for the identification of β-lactams and β-lactam degradation products (Burckhardt & Zimmermann, 2011; Hooff, et al., 2012; Hrabak, et al., 2012; Hrabak, et al., 2011; Sparbier, et al., 2012). Typically, β-lactams are incubated with bacterial cultures, the cultures are centrifuged, and the supernatants are analyzed for β-lactam degradation or modified products by MALDI-TOF MS; the length of this assay (not including growth of the cells) is about 1-4 hrs. This method is effective for identifying carbapenemase-producing *Ent* (Burckhardt & Zimmermann, 2011; Hrabak, et al., 2012; Hrabak, et al., 2011). In addition, β-lactamase inhibitors can be added to the incubation to determine their efficacy against the strains being tested (Hooff, et al., 2012; Sparbier, et al., 2012). However, this method can only detect the presence of β-lactamases as

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a resistance mechanism. Other methods implementing MALTI-TOF MS are required to detect the presence of efflux pumps or changes in porins or PBPs, which also result in β-lactams resistance.

Detection of the actual proteins conferring resistance in *Ent* by MALDI-TOF MS is more problematic because the protein signatures of bacteria are very complex. Camara and Hays established a proof of principle for this method in their study in which they dissected the differences between a wild-type *E. coli* and an ampicillin-resistant strain (Camara & Hays, 2007). They identified a 29,000 atomic mass unit peak in the ampicillin-resistant *E. coli* that was not present in the wild-type strain and confirmed that this peak represented a β-lactamase. On the other hand, in the study of Schaumann *et al*., discrimination between β-lactamase producing strains and non-producing strains was found to be difficult as the accuracy rate of the method was just 70% (Schaumann, et al., 2012). Using less complex protein samples, such as OMP preparations from five carbapenem-susceptible and seven carbapenem-resistant *K. pneumoniae* isolates, MALDI-TOF MS showed loss of porin expression in several β-lactam resistant isolates (Cai, et al., 2012). Currently, MALDI-TOF MS of resistance proteins is more effective in less complex backgrounds or when using partially purified protein preparations.

Mini-sequencing using MALDI-TOF MS may detect SNPs within resistance genes. This method requires PCR of the resistance gene, extension, and purification, followed by MALDI-TOF MS analysis. As a proof of concept, Ikryannikova *et al*. focused on three nucleotide positions that correspond to the amino acid positions 104, 164, and 238, as mutations in these gene regions can confer an ESBL phenotype in TEM-type β-lactamases. The system identified polymorphisms accurately at these three sites (Ikryannikova, et al., 2008). In another investigation, Stürenburg *et al*. implemented the MALDI-TOF MS to analyze fragment masses of base-specific cleavage of bla_{SHV} PCR products after *in vitro*-generated RNA transcription. Twenty-one *Ent* possessing different SHV-types were identified unambiguously and in perfect accordance to the PCR/sequencing results (Sturenburg, et al., 2006).

6.1.1 Advantage and disadvantages of MALDI-TOF MS—The unique advantage of MALDI-TOF MS is that it is fast and inexpensive (Emonet, Shah, Cherkaoui, & Schrenzel, 2010; Ho & Reddy, 2011; Lavigne, et al., 2012). However, even if more rapid, its implementation to detect β-lactam degradation or modified products is just in addition to the second-line phenotypic tests (e.g., synergy with clavulanate or the mHT). In fact, this approach defines only if an enzyme with ESBL or carbapenemase hydrolytic spectrum is expressed by the tested pathogens (Burckhardt & Zimmermann, 2011; Hrabak, et al., 2011). We note that this information can be easily obtained implementing rapid (<2 hrs) and cost-effective phenotypic tests, such as those recently developed using phenol red in combination with different β-lactams and β-lactam/β-lactamase inhibitor combinations (Nordmann, Dortet, & Poirel, 2012; Nordmann, Poirel, & Dortet, 2012). Moreover, it is still unclear how sensitive and specific MALDI-TOF MS is for enzymes with poor affinity (K_m) and very low turnover numbers (k_{cat}). Finally, the use of MALDI TOF MS to perform mini-sequencing is, so far, too difficult and time consuming to be a candidate for replacing standard DNA sequencing methods. On the other hand, implementation of MALDI-TOF MS for detecting

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the specific atomic mass peak of β-lactamases may be a future application. However, the mass of the detected enzymes should be defined with very high resolution and accuracy guaranteeing distinction of important variants (e.g., the non-ESBL SHV-1: 28,872 *vs*. the ESBL SHV-2: 28,900). These data should also be coupled by databases able to match molecular weights with corresponding β-lactamases expressed by the tested pathogen (Table 4).

6.2 PCR/ESI MS

The PCR/Electrospray Ionization Mass Spectrometry (PCR/ESI MS) is a methodology that performs mini-sequencing of small PCR products (100-450 bp) measuring their exact molecular mass and interpreting such data with an advanced software. This system has demonstrated success in the identification of pathogens (Emonet, et al., 2010; Ho & Reddy, 2011; Lavigne, et al., 2012; Wolk, Kaleta, & Wysocki, 2012), and unlike MALDI-TOF MS has been developed into a fully automated system (PLEX-ID; Abbott Biosciences) with results available within 4-6 hrs starting from clinical samples. Like MALDI-TOF MS, the application of PCR/ESI MS for the detection of resistance mechanisms and SNPs within resistance genes in *Ent* is in its early stages (Endimiani, et al., 2011). An important study revealed the utility of PCR/ESI MS for detecting bla_{KPC} expressing *Ent*. One hundred and ten previously characterized clinical isolates of which 69 carried bla_{KPC} were used in this study. PCR/ESI MS correctly detected 100% of the bla_{KPC} producing isolates. Control strains carrying bla_{KPC-4}, bla_{KPC-5}, bla_{KPC-6}, bla_{KPC-7}, and bla_{KPC-8} were also correctly identified, however the investigators could not discriminate between bla_{KPC-6}, bla_{KPC-7}, and bla_{KPC-8} (Endimiani, Hujer, Hujer, Sampath, et al., 2010).

6.2.1. Advantage and disadvantages of PCR/ESI MS—PCR/ESI MS represents a very promising technology. The rapid and fully automated ability to detect multiple genes (not only those conferring antibiotic resistance but also certain pathogenic factors) in one reaction may represent a revolution for the diagnosis of infectious diseases (D. J. Ecker, et al., 2008). This platform also offers the ability to detect pathogens at very low copy numbers and determine genetic relatedness based upon an MLST-like approach (J. A. Ecker, et al., 2006), thus providing “real-time” data essential to prevent outbreaks and spread of hyper-epidemic clones of MDR-*Ent*. Unlike other MS type applications, the detection of DNA from organisms that are non-cultured is a major step forward in the diagnosis of “culture negative infections”. Unfortunately, PCR/ESI MS is still under development and currently only available for research applications. Moreover, due to its enormous technological complexity, this instrument and its diagnostic kits are very expensive (Table 4).

7. MISCELLANEOUS

Faria-Ramos *et al*. developed a rapid detection assay of ESBL producers based on ***flow cytometric analysis***. Bacterial cells were incubated with ceftazidime or cefotaxime for 1-2 hrs in presence or absence of clavulanate and then stained with a fluorescent dye able to diffuse across depolarized membranes. After incubating, non-ESBL isolates displayed an increased fluorescence, whereas those producing ESBLs showed this phenomenon only if incubated with clavulanate. The assay is rapid (~3 hrs) and correlates well with standard

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methods but it cannot be performed with bacteria in stationary growth phase. Moreover, it performs only a generic identification of ESBL and non-ESBL producers similarly to the phenotypic tests (Faria-Ramos, et al., 2013). Fujita *et al*. evaluated the usefulness of ***microchip gel electrophoresis (MGE)*** to analyze the results of a single PCR for $bla_{\text{CTX-M}}$ performed on positive blood cultures. All CTX-M producers tested were correctly identified, suggesting that this approach is rapid (1.5 hrs starting from DNA template) and cost-effective (US $6.50 per specimen). However, this system indicates only the presence of $bla_{\text{CTX-M}}$ (similarly to the classic single PCR) but it is unable to distinguish allelic variants (Fujita, et al., 2011). The denaturing high-performance liquid chromatography (dHPLC) is a powerful methodology that has been extensively used to detect allelic variation (Xiao & Oefner, 2001). It consists of separated multiplex PCRs, heteroduplex formation between reference and sample PCR amplicons, and analysis of heteroduplex DNA by dHPLC. Xu *et al*. implemented this technique to genotype members of all $bla_{\text{CTX-M}}$ groups. All of the 73 CTX-M-producing strains tested during the study were correctly characterized with results consistent with standard DNA sequencing (Xu, Evans, Ling, Nye, & Hawkey, 2007). Idaho Technology developed the ***FilmArray*** technique, a closed, very rapid (1 hr) and fully automated system that combines DNA extraction from clinical sample, nested multiplex PCRs, post-PCR amplicons melt curve analysis and automated readout of results (Poritz, et al., 2011). This method was recently evaluated in blood culture to rapidly identify the causative pathogens and to detect several antibiotic resistance genes, including bla_{KPC}. However, data regarding the ability to detect KPC producers was not provided (Blaschke, et al., 2012).

8. CONCLUSIONS

This review provides a summary of the existing technologies that can be applied to the molecular diagnosis of infections due to MDR-*Ent*. On the other hand, this work also shows that we are at the threshold of a “new dawn” in clinical microbiology. Each method described above promises early and more sensitive detection. This knowledge inspires us to believe that the results obtained will have an impact on clinical outcomes (reduce mortality and morbidity, minimize resistance by stewardship) and healthcare costs. Key applications here will be for the early detection of MDR organisms in severe infections (e.g., sepsis) and the recognition of colonized patients (e.g., consequences for hospital hygiene). More importantly, the methods described above have the potential to improve the sensitivity of current automated systems by detecting pathogens in settings that were previously reported to be “culture negative”. The field of clinical microbiology has long awaited this promise as the techniques we use today still reflect in large part the methods developed by Louis Pasteur. Our challenge will be to accurately and carefully use this knowledge to show that these promises can be realized. In addition, these novel molecular technologies will also lead to new insights into disease pathogenesis. It is true that we will need a considerable amount of expertise to work with these advanced approaches. However, it is also imperative for us to accept these challenges and to enact wise choices in the selection of diagnostics. We are not certain which method will best suit our needs, but, perhaps, “*chance will favor a prepared mind*”.

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Table 1

Most important antibiotic resistance traits that molecular methods should be able to detect in *Ent.* The list is based on: *i)* the frequency of observation during epidemiological studies conducted worldwide; *ii)* the clinical importance of the antimicrobials affected by the resistance mechanism(s)

Antibiotics	Mechanism of resistance	Most frequent resistance traits	Importance	Ideal level of detection/characterization
Extended-spectrum cephalosporins	Extended-spectrum β-lactamases (ESBLs)	CTX-M	++++	Generic
		TEM and SHV	++++	Distinguishing ESBLs from non-ESBLs [a]
		PER	++	Generic
		GES	++	Distinguishing ESBLs (e.g., GES-1) from carbapenemases (e.g., GES-5) [b]
		SFO	+	Generic
	Plasmid-mediated AmpCs (pAmpCs)	CMY	++++	Generic
		DHA	+++	Generic
		FOX	++	Generic
		LAT, MIR, ACT, MOX	+	Generic
	Chromosomal AmpCs (cAmpCs)	Mutations in the promoter (*E. coli*)	++	Identifying substitution (e.g., at -32/-42) and insertions (between -10 and -35)
		Mutations in *ampD* (*Enterobacter* spp.)	+	Unknown specific target
Carbapenems	Carbapenemases			
	Class A	KPC	++++	Generic
		GES	++	Distinguishing ESBLs (e.g., GES-1) from carbapenemases (e.g., GES-5) [b]
	Class B (MBL)	NDM	++++	Generic
		SME	+	Generic
		VIM	++++	Generic
		IMP	+++	Generic
		SPM and GIM	++	Generic
		AIM, SIM	+	Generic
	Class D	OXA-48	++++	Specific
		OXA-181, −162, −163	+++	Specific
	Impermeability (porins)	OmpK-35 and −36 (*K. pneumoniae*)	++	Identifying insertions and/or frame-shift mutation(s)

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Antibiotics	Mechanism of resistance	Most frequent resistance traits	Importance	Ideal level of detection/characterization
		OmpF/C (*E. coli* and *Enterobacter* spp.)	+	Unknown specific mutation/target
Quinolones	Amino acid substitutions in the QRDR	GyrA	++++	Mutation at position -248, -259/260 [c]
		ParC	++++	Mutation at position 238/239 and 250/251 [d]
	PMQR determinants	QnrA B/S/D	+	Generic
		Aac(6)-Ib-cr	++	Distinguishing the *aac(6')-Ib-cr* from the AME *aac(6')-Ib*
		QepA	++	Generic
		OqxAB	+	Generic
Aminoglycosides	AMEs	Aac(6)-I-like, Ant(3)-like, Aph(3)-like	+++	Specific
	16S rRNA methylases	ArmA, RmtA/B/C/D/E, NpmA	++++	Generic

QRDR, quinolone resistance determining region; AMEs, aminoglycoside modifying enzymes.

[a] Amino acidic substitutions E104K, R164S/H, G238S and E240K for TEM; D179N, G238S and E240K for SHV (Gniadkowski, 2008; Perilli, et al., 2008; Randegger, Keller, Irla, Wada, & Hachler, 2000);

[b] Amino acidic substitution G170A/S (Girlich, Poirel, Szczepanowski, Schluter, & Nordmann, 2012);

[c] Lead to modification of S83 and D87 (Jacoby, 2005; Qiang, et al., 2002);

[d] Lead to modification of S80 and E84 (Qiang, et al., 2002) (Jacoby, 2005)

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Table 2

Single and multiplex end-point PCRs used for the detection of the main antibiotic resistance genes in *Enterobacteriaceae*

Approach	Detected resistance target	Nr. primers sets	Cycling conditions	TTR (hrs) [b]	Comments	Reference
Single PCRs [a]	CTX-M	4	1	2	Identification of CTX-M at group level	(Pitout, Hossain, & Hanson, 2004)
	CTX-M	1	1	2	Generic detection of CTX-M	(Boyd, et al., 2004)
	TEM, SHV, PER	1, 1, 1	1, 1, 1	2	Generic detection	(De Champs, et al., 2004)
	VEB, GES, SFO	1, 1, 1	1, 1, 1	2	Generic detection	(Poirel, Le Thomas, Naas, Karim, & Nordmann, 2000)
	KPC	1	1	2	Generic detection	(Bradford, et al., 2004)
	IMP and VIM	1, 1	1, 1	2	Generic detection	(Pitout, et al., 2005)
	NDM	1	1	2	Generic detection	(Nordmann, Poirel, Carrer, Toleman, & Walsh, 2011)
	OmpK-35, OmpK-36 (*K. pneumoniae*)	2	1	2	Sequencing always required	(C. H. Lee, et al., 2007)
	OmpC (*E. cloacae*)	1	1	2	Sequencing always required	(Lou, et al., 2011)
	OmpF (*E. coli*)	1	1	2	Sequencing always required	(Kishii & Takei, 2009)
	AmpD	1	1	2	Sequencing always required	(Naas, Massuard, Garnier, & Nordmann, 2001)
	GyrA	1	1	2	Sequencing always required	(Weigel, Steward, & Tenover, 1998)
	ParC	1	1	2	Sequencing always required	(McDonald, et al., 2001)
	QnrA, QnrB	1	1	2	Generic detection	(Wang, et al., 2003)
	QnrS	1	1	2	Generic detection	(Robicsek, et al., 2006)
	Aac(6)-Ib-cr	1	1	2	Sequencing always required	(C. H. Park, et al., 2006)
	QepA	1	1	2	Specific detection	(Perichon, Courvalin, & Galimand, 2007)
	OqxAB	1	1	2	Specific detection	(Kim, et al., 2009)
	AMEs	3	1	2	Specific detection	(Miro, et al., 2012)
Multiplex PCRs	TEM, SHV, CTX-M	NA	1	2.5	Commercial kit (Hyplex)	NA
	CMY, DHA, ACC, FOX, MOX, LAT, ACT, MIR	6	1	2-4	Generic detection. DNA sequencing is required for CMY. Cross reaction with chromosomal genes	(Perez-Perez & Hanson, 2002)
	TEM, SHV, CTX-M, GES, VEB, PER KPC, IMP, VIM	21	3	3	NDM-types not included	(Dallenne, et al., 2010)

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Approach	Detected resistance target	Nr. primers sets	Cycling conditions	TTR (hrs) [b]	Comments	Reference
	CMY, DHA, ACC, FOX, MOX, LAT, ACT, MIR CMY, DHA, ACC, FOX, MOX, LAT, ACT, MIR OXA (including OXA-48-like)				Cross reaction with chromosomal genes	
	TEM, SHV, CTX-M, GES, VEB, PER KPC, SME, IMI, NMC-A, IMP, VIM, GIM, SIM, NDM, SPM CMY, DHA, MOX, ACC, ACT, MIR, FOX OXA (including OXA-48-like)	20	1	3	Partial cross reaction with chromosomal genes	(Voets, et al., 2011)
	KPC, BIC SPM, VIM, GIM, AIM, IMP, NDM, SIM, DIM OXA-48, OXA-163, OXA-181	11	1	<4	Limited to carbapenemases detection	(Poirel, et al., 2011)
	KPC, IMP, VIM, NDM, OXA-48-like	NA	1	2.5	Commercial kit (Hyplex)	(Kaase, et al., 2012)
	SME, NMC-A, KPC, GES	4	1	<3	No detection of NDM	(Hong, et al., 2012)
	KPC, NDM, VIM, IMP, OXA-48-like	5	1	<3	It uses previously reported primers	(Doyle, et al., 2012)
	QnrA, QnrB, QnrS	3	1	<3	No detection of other PMQR determinants	(Cattoir, et al., 2007)
	ArmA, RmtA, RmtB, RmtC, RmtD, NpmA	6	1	<4	Generic detection	(Bercot, et al., 2011)

Note. NA, not available

[a] Numerous single PCRs have been designed to detect the listed antibiotic resistance genes. In this table, we present the most frequently implemented PCR schemes;

[b] The TTR is calculated starting from DNA template

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Table 3

Single and multiplex real-time PCRs used for the detection of the main antibiotic resistance genes in *Enterobacteriaceae*

Approach	Detected resistance target	Nr. primers sets/probes	Cycling conditions	TTR (hrs)	Comments	Reference
Single real-time PCR	SHV (ESBLs variants)	2	1	2	SNPs are detected by the specificity of the primers	(Hammond, et al., 2005)
	TEM (ESBLs variants)	2/2	1	2	SNPs are detected by the probes	(Mroczkowska & Barlow, 2008)
	CTX-M	1/1	1	2	Specific characterization	(Oxacelay, et al., 2009)
	CTX-M	1/4	1	2	Specific detection	(Birkett, et al., 2007)
	KPC	NA	1	2	Commercial kit (EasyQ KPC test, bioMerieux)	(Spanu, et al., 2012)
	KPC	1/1	1	<4	Clinical implementation using enriched rectal swabs	(Singh, et al., 2012)
	KPC	1/SG	1	2	Used previously published primers	(Raghunathan, et al., 2011)
	NDM-1	2/3	1	3	Synthesized a positive control	(Kruttgen, et al., 2011)
	NDM-1	1/1	1	2	Good performance of the TaqMan probe for copy number determination	(Manchanda, et al., 2011)
	NDM-1	1/1	1	< 4	From spiked stools	(Naas, Ergani, et al., 2011)
	OXA-48-like	1/1	1	< 4	From spiked stools	(Naas, et al., 2013)
	CMY-2	1/SG	1	2	Quantification purpose	(Kurpiel & Hanson, 2011)
	OmpK35, OmpK36	2/2	1	2	Quantification purpose	(Landman, et al., 2009)
Multiplex real-time PCR	TEM and SHV (ESBL variants), CTX-M	NA	1	3.5	Commercial kit (CheckPoints)	(Nijhms, et al., 2012)
	CIT, MOX, FOX, DHA, ACC, EBC	6/SG	1	3	Performed with previous published primers	(Brolund, et al., 2010)
	CMY, ACT, DHA, MOX, ACC, FOX, 16S rRNA	7/7	1	3	No cross reaction with cAmpC of *E. coli*	(Geyer, et al., 2012)
	KPC, 16S rRNA	2/2	1	< 4	From over-night cultures	(Singh, et al., 2012)
	IMP, VIM	2/4	1	< 1	Specific detection	(Bisiklis, et al., 2007)
	KPC, RNaseP	2/2	1	3	Clinical implementation from blood cultures	(Hindiyeh, et al., 2011)
	KPC, TonB, GapA	4/4	1	4	Identifies peculiar *K. pneumoniae* sequencing types	(Chen, et al., 2012)

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Approach	Detected resistance target	Nr. primers sets/probes	Cycling conditions	TTR (hrs)	Comments	Reference
	GES, IMI/NMC, KPC, SME, OXA-48	5/5	1	3	It does not discriminate the carbapenemase GES variants	(Swayne, et al., 2011)
	KPC, NDM, GES, OXA-48, IMP, VIM	6/EG	1	3	Specific identification by Tm analysis	(Monteiro, et al., 2012)
	KPC, NDM, VIM, IMP, OXA-48	NA	1	3.5	Commercial kit (CheckPoints). Not yet evaluated	NA
	QnrA/B/S/C/D, QepA	6	2	< 2	Specific identification	(Guillard, et al., 2011)
	QnrA/B/ S	3	1	< 2	Coupled with High resolution melting curve analysis	(Guillard, et al., 2012)
	Aac(6)-Ib-cr	1/1	1	< 1	Coupled with high resolution melting curve analysis, specific detection	(Bell, et al., 2010)

Note. NA, not available; SB, SYBR Green; EG, EvaGreen dye

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Table 4

Main characteristics of the rapid molecular tools so far available for the detection and characterization of the antibiotic resistance genes in multidrug-resistant *Enterobacteriaceae* (MDR-*Ent*)

Molecular tool	Time	Costs [c]	Main setting(s) of application	Comments
Single PCR coupled by standard DNA sequencing	2-3 hrs (PCR) [a] 1-3 or more days (sequencing)	+ +++	Research/Epidemiology	Detect all genes but labor intensive, especially for multiple genes
Next-generation sequencing methodologies	3 hrs [a]	+++	Clinical	Much more rapid than standard sequencing, especially if coupled to real-time PCR. However, they are still unavailable in most laboratories
Multiplex end-point PCR	3-4 hrs [a]	+	Epidemiology	PCR reaction should be optimized. Moderately labor intensive
Hyplex platforms (commercial)	2.5-4 hrs [a]	+++	Clinical	Helpful for treatment and screening of carriers
Real-time PCR	2-3 hrs [a]	++	Clinical	Helpful for treatment and screening of carriers. However, only one target gene is tested
NucliSENS EasyQ KPC platform (commercial)	2 hrs [a]	+++	Clinical	It detects only bla_{KPC}
Multiplex real-time PCR	2-3 hrs [a]	++	Clinical	Reactions should be optimized. Moderately labor intensive
Check-Points (commercial)	4.5 hrs [b]	+++	Clinical	Helpful for treatment and screening of carriers of ESBL-*Ent* and/or carbapenemase producers
LAMP	1-1.5 hrs [a]	+	Clinical	Very rapid and cheap. Good for low income countries
Microarray	6-8 hrs or more [a]	++	Epidemiology	In-house platforms are difficult to be implemented for clinical applications
Check-Points (commercial kits)	5-6 hrs [a]	+++	Epidemiology/Clinical	Very versatile and easy to use
Identibac (commercial)	5-6 hrs [a]	+++	Epidemiology/Research	Capable to detect many genes (including those for non-β-lactams) but not yet validated testing large and well-characterized collections of isolates
MALDI-TOF MS (for detection of catalytic activity)	3 hrs [b]	+	Clinical/Research	It detects only the spectrum of β-lactamases without distinguishing the specific kind of enzymes
MALDI-TOF MS (for detection of atomic mass)	3 hrs [b]	+	Clinical/Research	It can detect the class of β-lactamases. The specific kind of enzymes can be distinguished if the accuracy and the databases are improved
MALDI-TOF MS (for mini-sequencing)	3 hrs [a]	++	Research	Difficult to be implemented when compared to the other easier and more rapid systems
PCR/ESI-MS (PLEX-ID, commercial)	4-6 hrs (starting from sample)	++++	(Clinical)/Research	Excellent for clinical applications. However, so far available only for research

[a] TTR calculated starting from DNA template

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[b] TTR calculated starting from colonies

[c] +, lowest cost; ++++, highest cost