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# An inventory of 44 qPCR assays using hydrolysis probes operating with a unique amplification condition for the detection and quantification of antibiotic resistance genes

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amplification condition for the detection and quantification of antibiotic resistance genes

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Early antibiotic resistance determinants (ARDs) detection in humans or animals is crucial to counteract their propagation. The ARDs quantification is fundamental to understand the perturbation caused by disruptors, such as antibiotics, during therapies.

Forty-three qPCRs on the most diffused ARDs and integrons among human and animal enterobacteria isolates, and one on the 16S rDNA for bacteria quantification, were developed.

The qPCRs, using hydrolysis probes, operated with a unique amplification condition and were tested analytically and diagnostically performing 435 reactions on five positive and negative controls for each qPCR. Diagnostic sensitivity and specificity were confirmed by PCR and genome sequencing of control isolates, demonstrating 100% performance for all qPCRs. An easy and rapid discrimination method for the epidemiologically relevant *bla*<sub>CTX-Ms</sub> is provided.

This large, non-commercial qPCRs inventory could serve for precise quantification of ARDs, but also as a rapid screening tool for surveillance purposes, providing the basis for further high-throughput developments.

Key words: TaqMan, genotyping, *bla*<sub>CTX-M</sub>, *bla*<sub>OXA</sub>, *mcr1-3*, 16S rDNA

Antimicrobial resistance (AMR) is a threat to global health for both humans and animals (Appelbaum PC 2012; O' Neill J 2016). Antibiotics are able to select and amplify antibiotic resistance determinants (ARDs) in the host, favoring their dissemination widely (Casals-Pascual C et al., 2018; Sullivan A et al., 2001). Antibiotics have different selective effects (Ianiro G et al., 2016), thus quantifying ARDs before and after antibiotic therapy usefully reveals which molecules may have limited selective impacts on the resistome. Furthermore, early detection of ARDs in hosts or the environment is crucial to temporally develop hygienic measures avoiding further dissemination, to this end rapid molecular approaches are required.

Polymerase chain reactions (PCR) remain the most propagated methodologies in clinical and research laboratories, especially in low or medium income countries. In particular, qPCR assays, based on hydrolysis probes, commercially known as TaqMan, are rapid closed-tube tools, diagnostically specific, that also allow accurate quantification of the targets of interest. Hydrolysis probes hybridize on the host complementary DNA sequence and emit fluorescence during the amplification process when the Taq polymerase removes, by hydrolysis, the fluorophore at 3'-end, which will emit a fluorescence signal once far from the quencher at the 5'-end of the probe (Lupo A et al., 2013). Quenchers with minor groove binding (MGB) motifs enhance the probe melting temperature, allowing to design short length probes while enhancing specificity. Commercially available qPCRs on ARDs exist, but they are based exclusively on ARDs of human clinical concern and, overall, few developments have included other antibiotic classes than beta-lactams.

In this study, qPCRs using hydrolysis probes for *intI1/intI2* integron genes together with 42 of the most diffused ARDs in *Enterobacterales* according to the current AMR epidemiology were designed (Poirel L et al., 2018). Integrons have been recurrently proposed as relevant indicators of multidrug resistance (Amos GC et al., 2015; Leverstein-van Hall MA et al., 2003) whereas the 42 ARDs confer resistance to beta-lactams, aminoglycosides, fluoroquinolones, colistin, folic acid

One assay was also designed to possibly quantify ARDs relatively to the bacterial abundance estimated by the amplification of a conserved region of the 16S rDNA.

## 2. Materials and Methods

### 2.1 Primers and probes design

Primers (Sigma Aldrich, France) and probes (TaqMan, Applied Biosystem, France) were designed using the Primer Express v .3.0.1 software (Applied Biosystem, USA) in order to: i) avoid primers dimers and with a melting temperature ( $T_m$ ) 10°C lower than the probe (intended at 70°C); ii) obtain the same *in silico* amplification conditions for all qPCRs; iii) allow the possibility of multiplexing; iv) discriminate the variants of family genes (e.g. *bla*<sub>CTX-M</sub> groups and variants) with genotyping assays. For the SNPs visualization, a ClustalW alignment was performed on the nucleotide sequences using BioEdit v. 7.2.5 software. The nucleotide sequences and accession number for each gene were retrieved from the Genbank database (Table 1-3).

Probes were labelled with a fluorophore (JUN, or VIC, or ABY, or FAM) at the 3'-end and a quencher (MGB or QSY) at the 5'-end.

### 2.2 Reaction conditions of qPCRs

The qPCR reactions were performed in 384-wells plates in 5µL final volume containing 2.5 µL of TaqPath ProAmp Multiplex Master Mix (Applied Biosystem, USA), 0.5µL of forward and reverse primer (1 µM), respectively, 0.25µL of TaqMan probe (50 nM), 1.25µL of sterile H<sub>2</sub>O and 1µL of DNA. For the genotyping assays, primers were used at 200µM final concentration, whereas probes (only with MGB quencher) were used at 100 µM (Malkki M et al., 2012).

For both qPCRs and genotyping assays, amplification was performed in fast mode using QuantStudio 5 Real-Time PCR System (ThermoFisher scientific, USA) (Table S1). Data were

Software v1.4.3. (ThermoFisher scientific, USA).

### 2.3 Efficiency and limit of quantification

The efficiency of qPCRs was estimated performing a calibration curve. For each gene, a PCR product was obtained using the same primers of the qPCR assays. PCR products were purified (NucleoSpin Gel and PCR Clean-up, Machery-Nagel, France) and quantified by spectrophotometer absorbance and fluorometry (NanoDrop One and Qubit 4 Fluorometer, Thermo Scientific, France) then freshly diluted (1:10 dilutions, n = 12, from 10ng/μL) and used in technical replicates. For each gene assay, three non-template controls were generated, whereas three replicates contained 10ng of DNA from ARD-free *E. coli* CIP 76.24. The mean Cq values were plotted against the respective gene copies number (Lee C et al., 2006), generating a regression curve. The efficiency (E) was calculated using the slope from the regression curve ( $E = 10^{(-1/\text{slope})} - 1$ ) and values between 90% and 110% were defined as acceptable (Bustin SA et al., 2019). The limit of quantification (LoQ) was determined according to CLSI recommendations as the Cq obtained using the highest dilution of target DNA generating values in the linear range of the reaction, with a coefficient variation < 25% (Forootan A et al., 2017).

### 2.4 Diagnostic sensibility and specificity

Diagnostic sensitivity and specificity were estimated using bacterial isolates available in the laboratory collection. The presence or absence of the target in the positive controls, at least five each except for the *rmtB* gene (n = 3) and the *bla*<sub>OXA-181</sub> gene (n = 2), was validated by PCR or next-generation sequencing (NGS) of the genome and ARDs analysis (ResFinder 3.2.0). The antibiotic susceptibility of control isolates was evaluated by disc diffusion following the veterinary CA-SFM 2019 recommendations ([https://www.sfm-microbiologie.org/wp-content/uploads/2019/07/CASFM\\_VET2019.pdf](https://www.sfm-microbiologie.org/wp-content/uploads/2019/07/CASFM_VET2019.pdf)).

### 3.1 General features of the qPCR assays

Forty-four qPCRs with common amplification conditions were developed in this study: 42 on the most common ARDs in *Enterobacterales*, two on *intI* integrons genes, and one on the 16rDNA gene for bacteria quantification. Six reactions had 100% efficiency, whereas the remaining ones had acceptable values (110% <E> 95%). LoQ varied between one and  $5,7 \times 10^3$  gene copies number (Figure 1). By testing at least five isolates carrying the intended target and five target-free isolates, all qPCRs demonstrated 100% diagnostic sensitivity and specificity considering that all positive control DNAs generated a positive result unlike negative controls, confirmed by previously developed PCR or NGS.

### 3.2 qPCR on the 16S rRNA encoding gene

Quantification of the ARDs relatively to bacterial abundance is necessary when analyzing the action of disruptors on bacterial communities, such as antibiotics on the intestinal microbiota. To do so, one qPCR targeted a conserved part of the 16S rDNA. A previously published system was adapted reducing the size of the primers to adapt the  $T_m$  (Nadkarni MA et al., 2002) (primers: F-TCCTACGGGAGGCAGCA; R-ACTACCAGGGTATCTAATCCTGTT; probe: QSY-CGTATTACCGCGGCTGCTGGCAC-JUN). The assay was optimized using a PCR product generated with the DNA of *E. coli* CIP 74.26 (Figure 1a), as representative of the most abundant cultivable species of mammalian intestinal tract (Conway T et al., 2004), and tested on further 13 species (*Acinetobacter baumannii* 32865, *Proteus mirabilis* NKU, *Enterococcus casseliflavus* CIP 102598, *Enterococcus faecalis* CIP 103214, *Enterococcus faecium* CIP 103226, *Enterococcus gallinarum* CIP 103013, *Klebsiella pneumoniae* CIP 106769, *Streptococcus agalactiae* A909, *Staphylococcus aureus* CIP 7625, *Staphylococcus epidermidis* CIP 106299, *Staphylococcus*

103219), all generating a positive amplification.

### 3.3 qPCRs on beta-lactam resistance genes

Beta-lactams are largely used in human and veterinary medicine (ECDC 2019; ESVAC 2019), because of their large spectrum of action and a favorable pharmaco-dynamics (Turck M 1982). Their enzymatic degradation by beta-lactamases has emerged and disseminated dramatically (Seiffert SN et al., 2013). Here, 17 qPCRs were designed on beta-lactams resistance genes.

Penicillins are important in veterinary medicine (Bourély C et al., 2019) but the *bla*<sub>TEM</sub> and *bla*<sub>SHV</sub> are the most worldwide diffused ARDs (Alonso CA et al., 2017; Cloeckaert A et al., 2007; Liakopoulos A et al., 2018; Partridge SR et al., 2005; Zankari E et al., 2012). The *in silico* analysis of previously published qPCRs demonstrated elevated primers T<sub>m</sub> (Alfaresi MS et al., 2010; Lachmayr KL et al., 2009; Roschanski N et al., 2014), dimerization in primers (Lachmayr KL et al., 2009; Roschanski N et al., 2014), or too many G/C in the probe's 3'-end (Alfaresi MS et al., 2010), thus qPCRs were designed *de novo* on *bla*<sub>TEM</sub> and *bla*<sub>SHV</sub> genes (Figure 1b). The *bla*<sub>TEM-52</sub> and *bla*<sub>SHV-5-12</sub> alleles encoding ESBLs (Nüesch-Inderbinen MT et al., 1997; Poyart C et al., 1998) were detected (Table 1), however penicillinase and ESBL variants could not be discriminated due to the complexity and variation of the nucleotide sequence in the region pertinent to the discrimination. A previous study discriminated two SNPs in position 164 and 240 for *bla*<sub>TEM</sub> ESBL (Mroczkowska JE et al., 2008). However, this did not include other crucial SNPs of *bla*<sub>TEM-52</sub> (Bradford PA 2001). This intrinsic limitation of the methodology could affect the epidemiological data and infection control, thus other approaches are necessary to distinguish ESBL and penicillinase encoding alleles.

The *bla*<sub>CTX-Ms</sub> are nowadays the most globally expanded genes coding for ESBLs. They include four main groups, *bla*<sub>CTX-M-1-gr</sub>, *bla*<sub>CTX-M-2gr</sub>, *bla*<sub>CTX-M-8-gr</sub>, and *bla*<sub>CTX-M-9-gr</sub>, with several alleles varying in prevalence according to the host and the geographical origin (Livermore DM et al., 2007). The association of these genes with insertion sequences and the location on a wide family of



and host distribution of *bla*<sub>CTX-Ms</sub> provides a useful epidemiological indicator to understand the evolution of ARDs dissemination.

qPCRs on *bla*<sub>CTX-Ms</sub> exist already, but in most of the cases the discrimination remained at the group level (Birkett CI et al., 2007; Mangold KA et al., 2013; Roschanski N et al., 2014; Sittová M et al., 2015) or focused on the detection of the *bla*<sub>CTX-M-1-gr</sub> members (Calero-Cáceres W et al., 2014; Quirós P et al., 2014). In this study the qPCRs allowed two levels of discrimination: four qPCRs discriminated the *bla*<sub>CTX-M</sub> groups (Table 1) all with acceptable efficiency (Figure 1, b2); seven assays discriminated the variants *bla*<sub>CTX-M-1-3-15-55</sub> of the *bla*<sub>CTX-M-1-gr</sub> and *bla*<sub>CTX-M-9-14-27</sub> of the *bla*<sub>CTX-M-9-gr</sub>. For the *bla*<sub>CTX-M-1-gr</sub> two SNPs were discriminative one at position 239 (*bla*<sub>CTX-M-1-55</sub> presented a thymine, whereas *bla*<sub>CTX-M-3-15</sub> presented a cytosine) and one at position 725 (*bla*<sub>CTX-M-1-3</sub> presented an adenine, whereas *bla*<sub>CTX-M-15-55</sub> had a guanine) (Figure S1, a). Similarly, *bla*<sub>CTX-M-9-14-27</sub> presented three discriminative residues at position 701, 702, and 725 (*bla*<sub>CTX-M-9</sub>: 701C702A, 725A; *bla*<sub>CTX-M-14</sub>: 701T702G, 725A; *bla*<sub>CTX-M-27</sub>: 701T702G, 725G) (Figure S1, b). The probe designed around the 725 residue produced amplification exclusively on the 725G variant allowing *bla*<sub>CTX-M-27</sub> discrimination. For each SNP a probe with a MGB quencher and a different fluorophore was designed and used in the same well with a unique couple of primers (Table 1). The probes enter in competition for the proper variant and the fluorophore emission corresponding to the probe with 100% nucleotide match to the target emits a stronger signal that can be interpreted with the genotyping algorithm. The assays were evaluated using the mixed probes and opportune targets (Figure 1, b3-b4) and were 100% discriminative (Figure 2), as confirmed by PCR (Lupo A et al., 2018b; Shibata N et al., 2006) or NGS on 64 positive and 41 negative controls, respectively (Table 1).

et al., 2014; Hong JS et al., 2019; Martin LC et al., 2011; Mo SS et al., 2019; Pepin-Puget L et al., 2020; van den Bunt G et al., 2020).

For the *bla*<sub>CMY-2</sub> qPCR primers previously published were adopted (Roschanski N et al., 2014). For the *bla*<sub>DHA</sub> qPCR the primers were modified from (Swayne R et al., 2013) to rise the T<sub>m</sub> and degenerating one nucleotide to amplify all variants. The probes were designed *de novo* and qPCRs validated on *bla*<sub>CMY-2</sub> and *bla*<sub>DHA-1</sub> (Figure 1, c). Specificity and sensitivity were validated by NGS (n = 16) and PCR (n = 1) (Table 1) (Carattoli A et al., 2002).

Those *bla*<sub>OXA</sub> genes encoding class D beta-lactamases with carbapenemase activity represent a great health problem due to their association with mobile elements facilitating the dissemination and for the non-susceptibility to common beta-lactamases inhibitors (Walther-Rasmussen J et al., 2006). Their detection by molecular methods is of particular interest, considering that in certain circumstances they confer low level resistance to carbapenems rendering their phenotypic detection challenging (Thomson KS 2010).

Previous qPCRs on *bla*<sub>OXA</sub> genes have been reported (Brown-Jaque M et al., 2018; Chavda KD et al., 2016; Huang X-Z et al., 2012; Swayne RL et al., 2011). Here, MGB quencher was used for the detection of *bla*<sub>OXA-48</sub> and *bla*<sub>OXA-181</sub> to achieve a better specificity allowing discrimination of *bla*<sub>OXA-48</sub> from the *bla*<sub>OXA-181</sub> variant (Table 1, Figure 1, d).

### 3.4 qPCRs on aminoglycosides resistance genes

Besides their toxicity, and the widely disseminated resistance by enzymatic modifications, aminoglycosides continue to be largely used in animals and humans. Often, a single isolate harbors several aminoglycosides resistance genes located on diverse mobile genetic elements (Bliziotis IA et al., 2005; Gow SP et al., 2008; Ho P-L et al., 2010; Ramirez MS et al., 2010; Sandvang D et al.,

al., 2019). In this study,

nine qPCRs were developed to detect aminoglycosides resistance genes (Figure 1, e1-e2; Table 2). For the *strA-strB* qPCRs the assay of Walsh *et al.* (Walsh F et al., 2011) was adopted modifying the primer sequences to avoid dimerization and using last-generation fluorophores and quenchers for the probe (Table 2), whereas the *ant(3')-Ia* gene qPCR was designed *de novo* as the previously designed probe had a too low T<sub>m</sub> (Walsh F et al., 2011). Also the *rmtB* gene qPCR was designed *de novo* to adapt the experimental conditions to the other targets (Guo X et al., 2014).

The isolates used as positive controls displayed a variable resistance phenotype depending on the presence of other ARDs validated by PCR (Fritsche TR et al., 2008; Lupo A et al., 2018b; Park CH et al., 2006) or NGS (Lupo A et al., 2017; Madec JY et al., 2012) (Table 2).

### 3.5 qPCRs on fluoroquinolones resistance genes

Three qPCRs were conceived for detecting For the *qnrS* gene the forward primer and a modified reverse primer, to obtain a proper T<sub>m</sub>, and a modified probe, in order to avoid hybridization with the primers, were adapted from Colomer-Lluch *et al.* (Colomer-Lluch M et al., 2014). For the *qnrB1* gene qPCR the assay designed by Dheilly *et al.* (Dheilly A et al., 2012) was modified to adapt the T<sub>m</sub> of the forward primer and avoid dimer formation for the reverse primer, whereas the probe was designed *de novo*. The *aac(6')-Ib-cr* gene qPCR was designed *de novo* and it amplifies the *aac(6')-Ib-cr* variant, exclusively, allowing discrimination from *aac(6')-Ib*. The presence or absence of the target was proved by PCR (Lupo A et al., 2018a; Park CH et al., 2006; Robicsek A et al., 2006) or NGS (Dahmen S et al., 2013; Lupo A et al., 2018b; Madec JY et al., 2012) (Table 3, Figure 1, f).

### 3.6 qPCRs on colistin resistance genes

et al., 2019). Here, the design from Chabou *et al.* (Chabou S et al., 2016) was modified to avoid dimers formation of the primers and using a part of the probe, avoiding the guanine at the 3'-end. A qPCR assays was developed *de novo* for the *mcr-3* allele. All the isolates confirming to harbor the gene by PCR (Yin W et al., 2017) or NGS produced a positive result (Table 3). The negative controls did not produce amplification although some harbored a *mcr* gene different from the intended target (Haenni M et al., 2018; Lupo A et al., 2018b). The qPCRs were analytically highly sensitive (Figure 1, g).

### 3.7 qPCRs on folic acid synthesis inhibitor resistance genes

For the *sul1* gene qPCR the primers designed by Brown-Jaques *et al.* (Brown-Jaque M et al., 2018) were used, whereas the probe was designed *de novo* similarly to the *sul2/3* alleles qPCRs. The reactions had similar efficiency and analytical sensitivity (Figure 1, h). All positive controls produced an amplification, unlike negative controls although they contained other *sul* alleles than the intended one (Dahmen S et al., 2013; Di Conza J et al., 2002; Lupo A et al., 2017; Nigro SJ et al., 2012) (Table 3).

Among *dfr* genes only the group *dfrA* is also detected in animal isolates (Poirel L et al., 2018) and *dfrA1/12/17* alleles are the most diffused, located on class 1 and 2 integrons (Piccirillo A et al., 2014; Sköld O 2001).

No previous qPCR assays have been designed for these genes. The novel qPCRs had variable analytical sensitivity and efficiency (Figure 1, i) and were diagnostically specific (Table 3).

### 3.8 qPCRs on tetracyclines resistance genes

Previous design on *tetA*, *tetB* and *tetM* have been published (Guarddon M et al., 2011; Peak N et al., 2007; Walsh F et al., 2011), however due to dimers formation between primers and probes three *de novo* assays were designed, here. The analytical sensitivity was high for all the genes

(Table 3).

### 3.9 qPCRs on phenicols resistance gene

The *floR* qPCR here developed (Accession no.: AF231986.2; primers: F: AGATCGGATTCAGCTTTGCC; R: CAAAGGACTTCGCGAAACG; probe: QSY-CGCCACTGTCGCGCTTGTAATGATC-JUN; amplicon size: 75 bases) was validated for analytical features (Figure 1, k) and using five positive controls (20318, Ec39, 27841 (Haenni M et al., 2018), 27845 (Haenni M et al., 2018), 32189 (Lupo A et al., 2018b)). Negative controls were the same than tetracycline resistance genes qPCRs (Table 3).

### 3.10 qPCR assays on *intI1* and *intI2* genes of class 1 and 2 integrons

Two *intI* alleles are principally reported from animal isolates, *intI1* and *intI2* determining integrons class 1 and 2 (Deng Y et al., 2015; Mazel D 2006).

For this study, primers were adapted from Barraud *et al.* (Barraud O et al., 2010) for the detection of the *intI1* and *intI2* alleles in order to satisfy the  $T_m$  requirements and last generation fluorophores and quenchers were used for the probes (Table 3). The assay remained with acceptable efficiency compared to the previous design, where both assays had a 97% efficiency. The analytical sensibility was slightly decreased for *intI1* and increased for *intI2* (Barraud O et al., 2010) (Figure 1, l).

## 4. Conclusion

This study provides the largest non-commercial inventory of qPCRs ( $n = 44$ ) based on hydrolysis probes, operating with the same amplification conditions, including the 16 rDNA, allowing ARDs quantification with accuracy, relatively to the bacterial abundance, avoiding laborious calibration curves during experiments, as required using generic dyes. To ensure the accuracy of the design, 435

alternative methods (PCR and genome sequencing). All qPCRs had 100% diagnostic specificity, confirming the main advantage for using probes. Here, the first qPCR based genotyping is proposed to easily and rapidly differentiate the main epidemiologically relevant *bla*<sub>CTX-Ms</sub> variants in both humans and animals. The turnaround time from plate preparation to analysis of results was 2hrs for seven samples, for which purified DNA is available, making the system suited to both epidemiological or research purposes. The limit for diagnostic application is linked to the articulated ARDs combination that can occur in *Enterobacteriales*, rendering difficult the phenotype prediction of pathogens. The *in silico* prediction of primers and probes performance corresponded to the real experimental conditions, suggesting that addition of other targets, following the same design, will not necessitate optimization efforts, which is the most expensive step related to qPCR based on probes. The *in silico* design took in account the possibility of multiplexing, putting the basis for further high-throughput developments.

Guidelines for reporting qPCR evaluation are available, however the analytical features of previously published qPCRs are heterogeneously reported, thus a comparison among studies is challenging.

Overall, this study with this comprehensive, evolutive and robust qPCR inventory will contribute to the fight against ARDs and to investigations on ARDs changes in bacterial communities.

**Table 1. Primers and probes (TaqMan, Applied Biosystem) sequences of 17 qPCRs on beta-lactams resistance genes and features of positive (n = 63) and negative control isolates (n = 40). Genetic content of the isolates was determined by PCR or genome sequencing (underlined isolates) and ResFinder 3.2 analysis.**

Gene, Accession number	Primer F; R; probe sequence (5'→3')	Amplicon size (bp)	Isolate containing the target (variant) and other genes relative to beta-lactams resistance	Isolates target-free and other genes relative to beta-lactams resistance <sup>a)</sup>
<i>bla</i> <sub>TEM</sub> , Y13612.1	<b>F:</b> TTGCACAACATGGGGGATC	153	<u>Ec_58</u> ( <i>bla</i> <sub>TEM-190</sub> ), <i>bla</i> <sub>CTX-M-1</sub>	<u>Ab_96:</u> <i>bla</i> <sub>SHV-182</sub> , <i>bla</i> <sub>DHA-1</sub> , <i>bla</i> <sub>OXA-1</sub>
	<b>R:</b> AGCTAGAGTAAGTAGTTCGCCAGTTAATAGTT		<u>20071</u> (Madec JY et al., 2012) ( <i>bla</i> <sub>TEM-1C</sub> ), <i>bla</i> <sub>CTX-M-15</sub> , <i>bla</i> <sub>OXA-1</sub>	<u>Kt_105:</u> <i>bla</i> <sub>SHV-11/67</sub> , <i>bla</i> <sub>DHA-1</sub> , <i>bla</i> <sub>OXA-1</sub>
	<b>QSY-AACCGGAGCTGAATGAAGCCATACCAA-ABY</b>		<u>Ec_50</u> ( <i>bla</i> <sub>TEM-1B</sub> ), <i>bla</i> <sub>CTX-M-14</sub> , <i>bla</i> <sub>OXA-1</sub> <u>Ec_41</u> ( <i>bla</i> <sub>TEM-1A</sub> ), <i>bla</i> <sub>CTX-M-14</sub> <u>Ec_60</u> ( <i>bla</i> <sub>TEM-52</sub> )	<u>Ab_90:</u> <i>bla</i> <sub>OXA-23</sub> <u>Ec_43:</u> <i>bla</i> <sub>CTX-M-55</sub>
<i>bla</i> <sub>SHV</sub> , AJ920369.1	<b>F:</b> CGATAACAGCGCCGCC <b>R:</b> TTCCCAGCGGTCAAGGC	109	<u>Ec_27</u> ( <i>bla</i> <sub>SHV-5</sub> ) <u>Kp_98</u> ( <i>bla</i> <sub>SHV-76</sub> ), <i>bla</i> <sub>TEM-1B</sub> , <i>bla</i> <sub>CTX-M-15</sub>	<u>Ec_39:</u> <i>bla</i> <sub>CTX-M-15</sub> , <i>bla</i> <sub>TEM-1B</sub> <u>Ec_50:</u> <i>bla</i> <sub>CTX-M-14</sub> , <i>bla</i> <sub>TEM-1B</sub> , <i>bla</i> <sub>OXA-1</sub>
	<b>QSY-TGACTGCCTTTTTCGCCAGATCG-JUN</b>		36250 (Diab M et al., 2017) ( <i>bla</i> <sub>SHV-12</sub> ) <u>Kp_99</u> ( <i>bla</i> <sub>SHV-182</sub> ), <i>bla</i> <sub>DHA-1</sub> , <i>bla</i> <sub>OXA-1</sub> <u>Ecl_104</u> ( <i>bla</i> <sub>SHV-12</sub> ), <i>bla</i> <sub>DHA-1</sub> , <i>bla</i> <sub>OXA-1</sub>	<u>Ec_110:</u> <i>bla</i> <sub>CTX-M-1</sub> , <i>bla</i> <sub>TEM-1A</sub> <u>Ec_04:</u> <i>bla</i> <sub>CMY-2</sub> , <i>bla</i> <sub>TEM-1B</sub>
<i>bla</i> <sub>CTX-M-1-fig.</sub> , X92506.1	<b>F-CTGGGTGTGGCATTGATTAACA</b> <sup>b)</sup> <b>R-CTCGCTGATTTAACAGATTCGGTT</b> <sup>b)</sup> <b>QSY-TGATGAGCGCTTTCGCGATGTGCA-ABY</b>	151	<u>Ec_58</u> ( <i>bla</i> <sub>CTX-M-1</sub> ), <i>bla</i> <sub>TEM-190</sub> <u>Ec_39</u> ( <i>bla</i> <sub>CTX-M-15</sub> ), <i>bla</i> <sub>TEM-1B</sub> <u>Ec_17</u> ( <i>bla</i> <sub>CTX-M-32</sub> )	<u>Ec_04:</u> <i>bla</i> <sub>CMY-2</sub> , <i>bla</i> <sub>TEM-1B</sub> <u>Ec_82:</u> <i>bla</i> <sub>CTX-M-2</sub> <u>Kp_97:</u> <i>bla</i> <sub>SHV-182</sub> , <i>bla</i> <sub>DHA-1</sub> , <i>bla</i> <sub>OXA-1</sub> <u>Ec_14:</u> <i>bla</i> <sub>CTX-M-8</sub>
	<b>MGB-ATGGCCGTGGCCG-FAM</b>		<u>27841</u> (Haenni M et al., 2018) ( <i>bla</i> <sub>CTX-M-55</sub> ), <i>bla</i> <sub>TEM-1</sub> <u>Ec_43</u> ( <i>bla</i> <sub>CTX-M-55</sub> )	
<i>bla</i> <sub>CTX-M-1-fig.</sub> , <sup>239T</sup> , <sup>b)</sup> X92506.1	<b>MGB-ATGGCCGTGGCCG-FAM</b>	151	<u>Ec_61</u> ( <i>bla</i> <sub>CTX-M-1</sub> )	<u>Ec_71:</u> <i>bla</i> <sub>CTX-M-15</sub>
			<u>27259</u> (Lupo A et al., 2018a) ( <i>bla</i> <sub>CTX-M-1</sub> )	<u>Ec_16:</u> <i>bla</i> <sub>CTX-M-15</sub>
			<u>27732</u> (Lupo A et al., 2018b) ( <i>bla</i> <sub>CTX-M-55</sub> )	<u>Ec_20:</u> <i>bla</i> <sub>CTX-M-15</sub>
			<u>32137</u> (Lupo A et al., 2018a) ( <i>bla</i> <sub>CTX-M-1</sub> )	<u>24424</u> (Dahmen S et al., 2013), <i>bla</i> <sub>CTX-M-3</sub> , <i>bla</i> <sub>TEM-1B</sub>
<i>bla</i> <sub>CTX-M-1-fig.</sub> , <sup>239C</sup> , <sup>b)</sup> Y10278.1	<b>MGB-CCAGTAAAGTGATGGCCG-VIC</b>	151	<u>Ec_71</u> ( <i>bla</i> <sub>CTX-M-15</sub> )	<u>Ec_61:</u> <i>bla</i> <sub>CTX-M-1</sub>
			<u>20071</u> (Madec JY et al., 2012) ( <i>bla</i> <sub>CTX-M-15</sub> ), <i>bla</i> <sub>TEM-1C</sub> , <i>bla</i> <sub>OXA-1</sub>	<u>27259</u> (Lupo A et al., 2018a); <i>bla</i> <sub>CTX-M-1</sub>
			<u>Ec_16</u>	M-1 <u>27732</u> (Lupo A et al., 2018b); <i>bla</i> <sub>CTX-M-55</sub>

			<u>24424</u> (Dahmen S et al., 2013) ( <i>bla</i> <sub>CTX-M-3</sub> ), <i>bla</i> <sub>TEM-1B</sub> Ec_20 ( <i>bla</i> <sub>CTX-M-15</sub> )	<u>Ec_42</u> : <i>bla</i> <sub>CTX-M-55</sub>
<i>bla</i> <sub>CTX-M-1</sub> (p-725G-AY044436.1)	<b>F:</b> AGCGAGCATTTCAGGCTGG <sup>c)</sup>	84	<u>20071</u> (Madec JY et al., 2012) ( <i>bla</i> <sub>CTX-M-15</sub> ), <i>bla</i> <sub>TEM-1C</sub> Ec_16 ( <i>bla</i> <sub>CTX-M-15</sub> )	<u>Ec_61</u> : <i>bla</i> <sub>CTX-M-1</sub>
	<b>R:</b> CAGCGGCGCACGATCT <sup>c)</sup>		27732 (Lupo A et al., 2018b) ( <i>bla</i> <sub>CTX-M-55</sub> )	<u>27259</u> (Lupo A et al., 2018a); <i>bla</i> <sub>CTX-M-1</sub> <u>32137</u> (Lupo A et al., 2018a); <i>bla</i> <sub>CTX-M-1</sub>
	<b>MGB-TGGCTATGGCACCACCA-FAM</b>		<u>Ec_42</u> ( <i>bla</i> <sub>CTX-M-55</sub> ) <u>Ec_20</u> ( <i>bla</i> <sub>CTX-M-15</sub> )	<u>24424</u> (Dahmen S et al., 2013); <i>bla</i> <sub>CTX-M-3</sub> , <i>bla</i> <sub>TEM-1B</sub>
<i>bla</i> <sub>CTX-M-1</sub> (p-725C-X92506.1)	<b>MGB-TGACTATGGCACCACCAA-VIC</b>	84	<u>24424</u> (Dahmen S et al., 2013) ( <i>bla</i> <sub>CTX-M-3</sub> ), <i>bla</i> <sub>TEM-1B</sub> Ec_61 ( <i>bla</i> <sub>CTX-M-1</sub> ) <u>27259</u> (Lupo A et al., 2018a) ( <i>bla</i> <sub>CTX-M-1</sub> ) <u>32137</u> (Lupo A et al., 2018a) ( <i>bla</i> <sub>CTX-M-1</sub> )	<u>Ec_71</u> : <i>bla</i> <sub>CTX-M-15</sub>  Ec_16: <i>bla</i> <sub>CTX-M-15</sub> <u>Ec_42</u> : <i>bla</i> <sub>CTX-M-55</sub> <u>27732</u> (Lupo A et al., 2018b); <i>bla</i> <sub>CTX-M-55</sub>
	<b>F:</b> ATCTGGGTGGTCCCGATAAAG <b>R:</b> AGCGGCGTGGTGGTATCA	130	<u>Ec_76</u> ( <i>bla</i> <sub>CTX-M-1</sub> ), <i>bla</i> <sub>CTX-M-14</sub> , <i>bla</i> <sub>TEM-1B</sub> <u>Ec_49</u> ( <i>bla</i> <sub>CTX-M-2</sub> ), <i>bla</i> <sub>TEM-1B</sub> <u>Ec_03</u> ( <i>bla</i> <sub>CTX-M-2</sub> )	<u>Ec_14</u> : <i>bla</i> <sub>CTX-M-8</sub> <u>24417</u> (Dahmen S et al., 2013); <i>bla</i> <sub>CTX-M-32</sub> Ec_61: <i>bla</i> <sub>CTX-M-1</sub> Ec_32 (Casella T et al., 2017); <i>bla</i> <sub>CMY-2</sub>
<i>bla</i> <sub>CTX-M-2</sub> -X92507.1	<b>MGB-CGCTCGTTGGGTGAT-VIC</b>		Ec_82 ( <i>bla</i> <sub>CTX-M-2</sub> ) Ec_30 ( <i>bla</i> <sub>CTX-M-2</sub> )	
	<b>F:</b> CGGCTGTGCTCAAGCAAA <b>R:</b> ATGGGATTGTAGTTAATCAGGTCT	87	<u>Es_106</u> ( <i>bla</i> <sub>CTX-M-2</sub> ), <i>bla</i> <sub>CTX-M-15</sub> , <i>bla</i> <sub>TEM-1B</sub> <u>Ec_14</u> ( <i>bla</i> <sub>CTX-M-8</sub> ) <u>Ec_48</u> ( <i>bla</i> <sub>CTX-M-8</sub> )	Ec_82: <i>bla</i> <sub>CTX-M-2</sub> <u>24417</u> (Dahmen S et al., 2013); <i>bla</i> <sub>CTX-M-32</sub> Ec_61: <i>bla</i> <sub>CTX-M-1</sub>
<i>bla</i> <sub>CTX-M-8</sub> -AFI89721.1	<b>QSY-CAACCTTCTGACTCAACACCTTCTTTTGCG-JUN</b>		Ec_57 ( <i>bla</i> <sub>CTX-M-8</sub> ) Ec_51 ( <i>bla</i> <sub>CTX-M-8</sub> ) <u>Ec_70</u> ( <i>bla</i> <sub>CTX-M-8</sub> )	<u>Kp_97</u> : <i>bla</i> <sub>SHV-182</sub> , <i>bla</i> <sub>DHA-1</sub>
	<b>F:</b> AGAGACACCACCGCCG <b>R:</b> CCAGATCACCGCAATATCATTG <b>QSY-TGGGGCAAACCCAGCGGG-ABY</b>	219	<u>Ec_09</u> ( <i>bla</i> <sub>CTX-M-9</sub> ), <i>bla</i> <sub>SHV-12</sub> <u>Ec_21</u> ( <i>bla</i> <sub>CTX-M-9</sub> ), <i>bla</i> <sub>SHV-12</sub> <u>Ec_64</u> ( <i>bla</i> <sub>CTX-M-14</sub> ), <i>bla</i> <sub>TEM-1B</sub>	<u>Ec_14</u> : <i>bla</i> <sub>CTX-M-8</sub> Ec_82: <i>bla</i> <sub>CTX-M-2</sub> <u>24417</u> (Dahmen S et al., 2013); <i>bla</i> <sub>CTX-M-32</sub> Ec_61: <i>bla</i> <sub>CTX-M-1</sub>
<i>bla</i> <sub>CTX-M-9</sub> (p-AF174129.3)	<b>F:</b> AGAGACACCACCGCCG <sup>d)</sup> <b>R:</b> CCAGATCACCGCAATATCATTG <sup>d)</sup>	209	<u>Ec_85</u> ( <i>bla</i> <sub>CTX-M-14</sub> ), <i>bla</i> <sub>TEM-1A</sub> <u>44557</u> (Melo LC et al., 2019) ( <i>bla</i> <sub>CTX-M-27</sub> ) <u>Ec_09</u> ( <i>bla</i> <sub>CTX-M-9</sub> ), <i>bla</i> <sub>SHV-12</sub> <u>Ec_21</u> ( <i>bla</i> <sub>CTX-M-9</sub> ), <i>bla</i> <sub>SHV-12</sub>	<u>Ec_74</u> : <i>bla</i> <sub>CTX-M-14b</sub> , <i>bla</i> <sub>OXA-1</sub> <u>Ec_75</u> : <i>bla</i> <sub>CTX-M-27</sub>



	MGB-TCGTGGACTGCAGGTG-VIC		<u>Ec 22</u> ( <i>bla</i> <sub>CTX-M-9</sub> )	38632 <sup>(Melo LC et al., 2019)</sup> ; <i>bla</i> <sub>CTX-M-27</sub>
			<u>Ec 88</u> ( <i>bla</i> <sub>CTX-M-9</sub> ), <i>bla</i> <sub>SHV-12</sub>	<u>Ec 11</u> : <i>bla</i> <sub>CTX-M-27</sub> , <i>bla</i> <sub>TEM-1B</sub>
			<u>Ec 66</u> ( <i>bla</i> <sub>CTX-M-9</sub> ), <i>bla</i> <sub>SHV-12</sub>	
<i>bla</i> <sub>CTX-M-9gr-70170G</sub> , AF252622,	MGB-TCGTGGACTGTGGGTG-FAM	209	<u>Ec 65</u> ( <i>bla</i> <sub>CTX-M-14</sub> ) <u>Ec 74</u> ( <i>bla</i> <sub>CTX-M-27</sub> ), <i>bla</i> <sub>OXA-1</sub> 38632 <sup>(Melo LC et al., 2019)</sup> ( <i>bla</i> <sub>CTX-M-27</sub> ) <u>Ec 41</u> ( <i>bla</i> <sub>CTX-M-14b</sub> ) <i>bla</i> <sub>TEM-1A</sub> 44557 <sup>(Melo LC et al., 2019)</sup> ( <i>bla</i> <sub>CTX-M-27</sub> )	<u>Ec 21</u> : <i>bla</i> <sub>CTX-M-9</sub> , <i>bla</i> <sub>SHV-12</sub> <u>Ec 22</u> : <i>bla</i> <sub>CTX-M-9</sub> <u>Ec 88</u> : <i>bla</i> <sub>CTX-M-9</sub> , <i>bla</i> <sub>SHV-12</sub> <u>Ec 66</u> : <i>bla</i> <sub>CTX-M-9</sub> , <i>bla</i> <sub>SHV-12</sub>
			<u>Ec 75</u> ( <i>bla</i> <sub>CTX-M-27</sub> )	<u>Ec 41</u> : <i>bla</i> <sub>CTX-M-14</sub> , <i>bla</i> <sub>TEM-1B</sub> , <i>bla</i> <sub>OXA-1</sub>
<i>bla</i> <sub>CTX-M-9gr-725G</sub> , AY156923,	MGB-TGGTGCCGTAGCCG-VIC	209	<u>Ec 86</u> ( <i>bla</i> <sub>CTX-M-27</sub> ) 38632 <sup>(Melo LC et al., 2019)</sup> ( <i>bla</i> <sub>CTX-M-27</sub> ) <u>Ec 11</u> ( <i>bla</i> <sub>CTX-M-27</sub> ), <i>bla</i> <sub>TEM-1B</sub> 44557 <sup>(Melo LC et al., 2019)</sup> ( <i>bla</i> <sub>CTX-M-27</sub> )	<u>Ec 22</u> : <i>bla</i> <sub>CTX-M-9</sub> <u>Ec 88</u> : <i>bla</i> <sub>CTX-M-9</sub> , <i>bla</i> <sub>SHV-12</sub> <u>Ec 66</u> : <i>bla</i> <sub>CTX-M-9</sub> , <i>bla</i> <sub>SHV-12</sub>
	F: GGCAAACAGTGGCAGGGTAT	101	<u>Ec 04</u> , <i>bla</i> <sub>TEM-1B</sub>	<u>Ec 50</u> : <i>bla</i> <sub>CTX-M-14</sub> , <i>bla</i> <sub>TEM-1B</sub> , <i>bla</i> <sub>OXA-1</sub>
<i>bla</i> <sub>CMY-2</sub> , X91840.1	R: AATGCGGCTTATCCCTAACG QSY-CACTTAGCCACCTATACGGCAGGCGG-ABY		<u>Ec 62</u> <u>Ec 35</u> , <i>bla</i> <sub>TEM-1B</sub> <u>Ec 23</u> , <i>bla</i> <sub>TEM-1A</sub> , <i>bla</i> <sub>OXA-1</sub> <u>Ec 36</u> , <i>bla</i> <sub>TEM-1B</sub>	<u>Ec 84</u> : <i>bla</i> <sub>CTX-M-1</sub> , <i>bla</i> <sub>TEM-1B</sub> <u>Ec 53</u> : <i>bla</i> <sub>CTX-M-15</sub> , <i>bla</i> <sub>TEM-1B</sub> <u>Ec 39</u> : <i>bla</i> <sub>CTX-M-15</sub> , <i>bla</i> <sub>TEM-1B</sub>
	F: AACTTTCACAGGTGTGCTGGG <sup>(Swayne R et al., 2013)</sup> R: GGCTGCCACTGCTGATAGAAR <sup>(Swayne R et al., 2013)</sup> MGB-AGGCGGRCTGCCGT-FAM	219	Kp_100, <i>bla</i> <sub>SHV-182</sub> , <i>bla</i> <sub>OXA-1</sub> Kp_103, <i>bla</i> <sub>SHV-182</sub> , <i>bla</i> <sub>OXA-1</sub> <u>Ec 34</u> , <i>bla</i> <sub>OXA-48</sub> , <i>bla</i> <sub>SHV-182</sub> , <i>bla</i> <sub>OXA-1</sub> <u>Ec 45</u> , <i>bla</i> <sub>OXA-48</sub> , <i>bla</i> <sub>SHV-182</sub> , <i>bla</i> <sub>OXA-1</sub> <u>Ec 87</u> , <i>bla</i> <sub>OXA-48</sub> , <i>bla</i> <sub>SHV-182</sub> , <i>bla</i> <sub>OXA-1</sub>	<u>Ec 58</u> : <i>bla</i> <sub>CTX-M-1</sub> , <i>bla</i> <sub>TEM-190</sub> <u>Ec 28</u> : <i>bla</i> <sub>CTX-M-1</sub> , <i>bla</i> <sub>TEM-1A</sub> <u>Ec 50</u> : <i>bla</i> <sub>CTX-M-14</sub> , <i>bla</i> <sub>TEM-1B</sub> , <i>bla</i> <sub>OXA-1</sub> <u>Ec 110</u> : <i>bla</i> <sub>CTX-M-1</sub> , <i>bla</i> <sub>TEM-1A</sub>
<i>bla</i> <sub>DHA-1</sub> , NC_014312.1	F: TGTTTATCAAGAATTTGCCCGC <sup>e)</sup>	232	<u>Ec 78</u> , <i>bla</i> <sub>CMY-2</sub> , <i>bla</i> <sub>TEM-1B</sub>	<u>Ec 50</u> : <i>bla</i> <sub>CTX-M-14</sub> , <i>bla</i> <sub>TEM-1B</sub> , <i>bla</i> <sub>OXA-1</sub> 33904 <sup>(Lupo A et al., 2018b)</sup> ; <i>bla</i> <sub>CTX-M-55</sub>
	R: TTCGGTCAGCATGGCTTGT <sup>e)</sup>		<u>Ec 34</u> , <i>bla</i> <sub>DHA-1</sub> , <i>bla</i> <sub>SHV-182</sub> , <i>bla</i> <sub>OXA-1</sub>	24424 <sup>(Dahmen S et al., 2013)</sup> , <i>bla</i> <sub>CTX-M-3</sub> , <i>bla</i> <sub>TEM-1B</sub>
<i>bla</i> <sub>OXA-48</sub> , AY236073.2	MGB-CGACGGTGGTATTTCGA-FAM		<u>Ec 45</u> , <i>bla</i> <sub>DHA-1</sub> , <i>bla</i> <sub>SHV-182</sub> , <i>bla</i> <sub>OXA-1</sub>	Kt_105: <i>bla</i> <sub>SHV-11</sub> , <i>bla</i> <sub>SHV-67</sub> , <i>bla</i> <sub>DHA-1</sub> , <i>bla</i> <sub>OXA-1</sub>
			<u>Ec 87</u> , <i>bla</i> <sub>DHA-1</sub> , <i>bla</i> <sub>SHV-182</sub> , <i>bla</i> <sub>OXA-1</sub> Kp_101	
<i>bla</i> <sub>OXA-181</sub> <sup>e)</sup> , JN205800,	MGB-CGATGGTGGTATTTCGATT-VIC	232	<u>Ec 02</u>	<u>Ec 34</u> : <i>bla</i> <sub>OXA-48</sub> , <i>bla</i> <sub>SHV-182</sub> <i>bla</i> <sub>DHA-1</sub> , <i>bla</i> <sub>OXA-1</sub>
			<u>Ec 15</u>	<u>Ec 45</u> : <i>bla</i> <sub>OXA-48</sub> , <i>bla</i> <sub>SHV-182</sub> <i>bla</i> <sub>DHA-1</sub> , <i>bla</i> <sub>OXA-1</sub> <u>Ec 87</u> : <i>bla</i> <sub>OXA-48</sub> , <i>bla</i> <sub>SHV-182</sub>

*bla*<sub>DHA-1</sub>, *bla*<sub>OXA-1</sub>  
Kp\_100: *bla*<sub>DHA-1</sub>, *bla*<sub>SHV-182</sub>,  
*bla*<sub>OXA-1</sub>

Note: <sup>a)</sup> All qPCRs were tested using the target-free *E. coli* CIP 74.26

<sup>b)</sup> For the *bla*<sub>CTX-M-1gr-239T</sub> and *bla*<sub>CTX-M-1gr-239C</sub> qPCR assays were used the same primers used for *bla*<sub>CTX-M-1-gr</sub>.

<sup>c)</sup> For the *bla*<sub>CTX-M-1gr-725C</sub> qPCR assay were used the same primers used for *bla*<sub>CTX-M-1gr-725G</sub>.

<sup>d)</sup> For the *bla*<sub>CTX-M-9gr-701T702G</sub> and *bla*<sub>CTX-M-9gr-725G</sub> qPCR assays were used the same primers used for *bla*<sub>CTX-M-9gr-701C702A</sub>.

<sup>e)</sup> For the *bla*<sub>OXA-181</sub> qPCR assay were used the same primers used for *bla*<sub>OXA-48-like</sub>.

Ec: *Escherichia coli*; Ab: *Acinetobacter baumannii*; Kt: *Klebsiella terrigena*; Kp: *Klebsiella pneumoniae*; EcI: *Enterobacter cloacae*; Es: *Enterobacter sakazakii*.

**Table 2. Primers and probes (TaqMan, Applied Biosystem) sequences of nine qPCRs on aminoglycosides resistance genes and features of positive (n = 32) and negative control isolates (n = 9). Genetic content of the isolates was determined by PCR or genome sequencing (underlined isolates) and analysis with ResFinder 3.2.**

Gene, Accession number	Primer F; R; probe sequence (5'→3')	Amplicon size (bp)	Isolate containing the target and other genes relative to aminoglycosides resistance	Isolates target-free and other genes relative to aminoglycosides resistance <sup>a)</sup>
<i>aac(3)-IIa</i> , AY333434.1	<b>F:</b> AGGACATCGTGACGTTCCG <b>R:</b> AACCTGAAGGCTCGCAAGAG <b>QSY</b> -CGGAGTGGTCCGAAATGCTTCTCAAG- <b>ABY</b>	105	<u>Ec_37</u> <u>32189</u> (Lupo A et al., 2018b), <i>rmtB</i> , <i>aac(6')-Ib</i> <u>Ec_44</u> , <i>strA/B</i> , <i>aph(3'')-Ia</i> , <i>ant(2'')-Ia</i> <u>Kp_98</u> , <i>strA/B</i> , <i>aac(6')-Ib-cr</i> <u>Ab_94</u> <i>ant(3'')-IIa</i> , <i>aac(6)-Ian</i>	
<i>aac(3)-IV</i> , X01385.1	<b>F:</b> ATCGGTCAGCTTCTCAACCT <b>R:</b> TCGTCCAGACCTGACCAC <b>QSY</b> -TCCACAGCTCCTCCGTAGCGTCC- <b>ABY</b>	173	<u>Ec_01</u> <u>Ec_80</u> <u>Ec_84</u> , <i>strA/B</i> , <i>aph(3'')-Ia</i> , <i>aph(4)-Ia</i> , <i>ant(3'')-Ia</i> <u>Kp_103</u> , <i>ant(3'')-Ia</i> <u>Ec_54</u> , <i>aac(3)-IIa</i> , <i>aph(4)-Ia</i> , <i>aph(3'')-Ia</i> , <i>ant(3'')-Ia</i>	<u>Ec_12</u> : none <u>Ec_13</u> : none <u>Ec_55</u> : none <u>Ec_68</u> : none
<i>aac(6)-Ib</i> , JQ808129.1	<b>F:</b> AACCTGCGAGCGATCCGA <b>R:</b> TGGCGTGTGTAACCATGTAC <b>QSY</b> -TACCTTGCTTCTCAAACCCGCTTCTC- <b>JUN</b>	101	<u>20071</u> (Madec JY et al., 2012), <i>aac(6')-Ib-cr</i> , <i>aac(3)-IIa</i> , <i>ant(3'')-Ia</i> , <i>ant(2'')-Ia</i> <u>Ec_04</u> , <i>aac(6')-Ib-cr</i> <u>Kp_102</u> , <i>strA/B</i> , <i>aac(6')-Ib-cr</i> , <i>ant(2'')-Ia</i> <u>33904</u> (Lupo A et al., 2018b), <i>aac(6')-Ib</i>	

<i>strA</i> , M95402.1	<b>F:</b> TCAATCCCGACTTCTTACCGG (Walsh F et al., 2011)	126	<u>32189</u> (Lupo A et al., 2018b), <i>aac(6')-Ib</i> , <i>rmtB</i> <u>34972</u> (Lupo A et al., 2017), <i>strA/B</i> , <i>aac(3)-IIa</i> , <i>aac(6')-Ian</i> <u>38208</u> (Lupo A et al., 2017) <i>strA/B</i> , <i>aac(3)-IIa</i> , <i>aac(6')-Ian</i> <u>Ab 107</u> (Lupo A et al., 2017)(Lupo A et al., 2017)(Lupo A et al., 2017), <i>strA/B</i> , <i>aac(3)-IIa</i> <u>Ab 109</u> (Lupo A et al., 2017)(Lupo A et al., 2017)(Lupo A et al., 2017), <i>strA/B</i> , <i>aac(3)-IIa</i> , <i>aac(6')-</i> <i>Ian</i> , <i>ant(2'')-Ia</i> <u>Ab 108</u> (Lupo A et al., 2017)(Lupo A et al., 2017)(Lupo A et al., 2017), <i>strA/B</i> , <i>aac(3)-IIa</i> , <i>aac(6')-</i> <i>Ian</i> , <i>ant(2'')-Ia</i>
	<b>R:</b> CACCATGGCAAACAACCATATC		
	<b>MGB-TGGCTCGTGTCGAAC-VIC</b>		
<i>strB</i> , M95402.1	<b>F:</b> ATCGCTTTCAGCTTTGTTTC (Walsh F et al., 2011)	112	<u>34972</u> (Lupo A et al., 2017), <i>strA/B</i> , <i>aac(3)-IIa</i> , <i>aac(6')-Ian</i> <u>38208</u> (Lupo A et al., 2017) <i>strA/B</i> , <i>aac(3)-IIa</i> , <i>aac(6')-Ian</i> <u>Ab 107</u> (Lupo A et al., 2017)(Lupo A et al., 2017)(Lupo A et al., 2017), <i>strA/B</i> , <i>aac(3)-IIa</i> <u>Ab 109</u> (Lupo A et al., 2017)(Lupo A et al., 2017)(Lupo A et al., 2017), <i>strA/B</i> , <i>aac(3)-IIa</i> , <i>aac(6')-</i> <i>Ian</i> , <i>ant(2'')-Ia</i> <u>Ab 108</u> , <i>aac(3)-IIa</i> , <i>aac(6)-Ian</i> , <i>ant(2'')-Ia</i>
	<b>R:</b> TCCGAGGCATT GCTCATCATT		
	<b>QSY-CGTCCACGCGCGATTATAGCC-JUN</b>		
<i>aph(3')-Ia</i> , V00359.1	<b>F:</b> ACCGAGGCAGTTCATAGGA	104	<u>Ab 92</u> , <i>aac(3)-Ia</i> , <i>ant(2'')-Ia</i> , <i>ant(3'')-</i> <i>Ia</i> <u>Ab 93</u> , <i>aac(3)-Ia</i> , <i>ant(3'')-Ia</i> <u>Ec 85</u> , <i>strA/B</i> , <i>ant(3'')-Ia</i> <u>Ec 87</u> , <i>aac(6')-Ib-cr</i> <u>Ec 77</u> , <i>strAB</i> , <i>aac(3)-IIa</i>
	<b>R:</b> ACCTTATTTTTGACGAGGGGAAA		
	<b>QSY-ATCCTGGTATCGGTCTGCGATTCCGA-JUN</b>		
<i>ant(2'')-Ia</i> , EU247928.1	<b>F:</b> CGGCGAGCTCGAGGC	135	<u>20071</u> (Madedec JY et al., 2012), <i>aac(3)-IIa</i> , <i>ant(3'')-Ia</i> <u>Ec 19</u> , <i>strA/B</i> , <i>aph(3')-Ia</i> , <i>ant(3'')-Ia</i> <u>Ab 109</u> , <i>strA/B</i> , <i>aac(3)-IIa</i> , <i>aac(6')-Ian</i> <u>Ec 79</u> , <i>strA/B</i> <i>ant(3'')-Ia</i> <u>Ec 89</u> , <i>strA/B</i> , <i>ant(3'')-Ia</i>
	<b>R:</b> ATTTCATACGCTTCGTCTGCC		
	<b>QSY-TCATGGAGGAGTTGGACTATGGATTCTAGCG-ABY</b>		
<i>ant(3'')-Ia</i> , AF3134 72.2	<b>F:</b> ACGTTGTCCCGCATTTGG	53	<u>Ec 17</u> , <i>aph(3')-Ia</i> <u>Ec 64</u> , <i>strA/B</i> , <i>aph(3')-Ia</i> , <i>ant(2'')-Ia</i> <u>Ec 25</u> , <i>aac(3)-IIa</i> <u>Ab 92</u> , <i>aph(3')-Ia</i> , <i>aac(3)-Ia</i>
	<b>R:</b> TCCTTCGGCGCATTTT		
	<b>MGB-ACAGCGCAGTAACC-FAM</b>		

<i>rmlB</i> , AB103506.1	<b>F:</b> CTCACCTCCATCCTGGCCT	89	<u>Ab_93</u> , <i>aph(3')-Ia</i> , <i>aac(3)-Ia</i> Ec_59	<u>Ec_50</u> : <i>strA/B</i> , <i>aac(3)-IId</i> , <i>aph(3')-Ia</i> , <i>ant(3'')-Ia</i>
	<b>R:</b> TTATGCCGCCCCCATTC		<u>32189</u> (Lupo A et al., 2018b) <i>aac(6')-Ib</i> ,	<u>Kt_105</u> : <i>strA/B</i> , <i>aac(3)-IId</i> ,
	<b>QSY-ACC</b> CGCCCTTTGCCCG- <b>JUN</b>		<u>34248</u> (Lupo A et al., 2018b) <i>aac(6')-Ib</i>	<u>Ec_43</u> : <i>strA/B</i> , <i>aac(3)-IId</i> , <i>ant(3')-Ia</i> <u>Ab_90</u> : <i>strA/B</i> , <i>aac(3)-IIa</i>

Note: <sup>a)</sup> All qPCRs were tested using the target-free *E. coli* CIP 74.26.

Ec: *Escherichia coli*; Ab: *Acinetobacter baumannii*; Kt: *Klebsiella terrigena*; Kp: *Klebsiella pneumoniae*.

**Table 3. Primes and probes (TaqMan, Applied Biosystem) sequences of qPCRs on fluoroquinolones resistance genes (*qnrB1*, *qnrS*, *aac(6)-Ib-cr*), colistin (*mcr1*, *mcr3*), inhibitors of folic acid pathways (*sul1*, *sul2*, *sul3*, *drfA1*, *drfA12*, *drfA17*), tetracyclines (*tetA*, *tetB*, *tetM*), and integrons (*intI1* and *intI2*) and features of positive and negative control isolates. Genetic content of the isolates was determined by PCR or genome sequencing (underlined isolates) and analysis with ResFinder 3.2.**

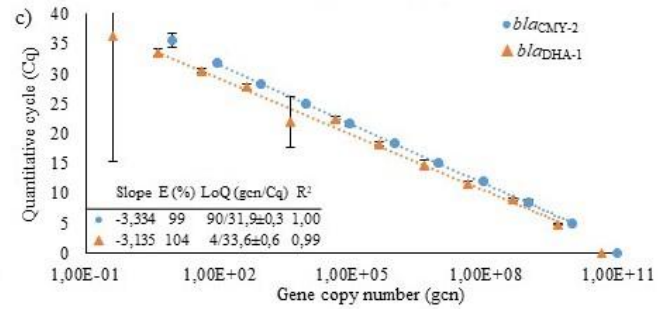
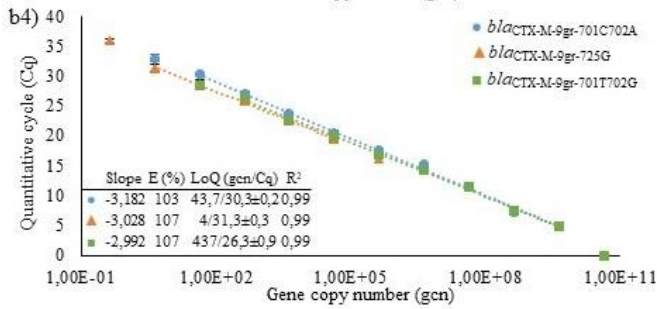
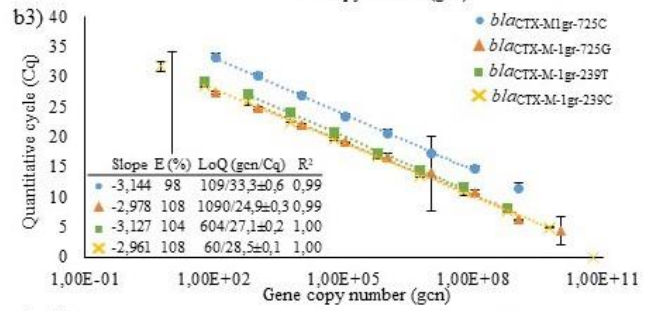
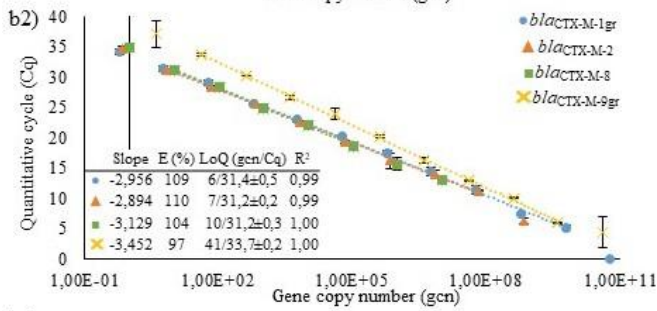
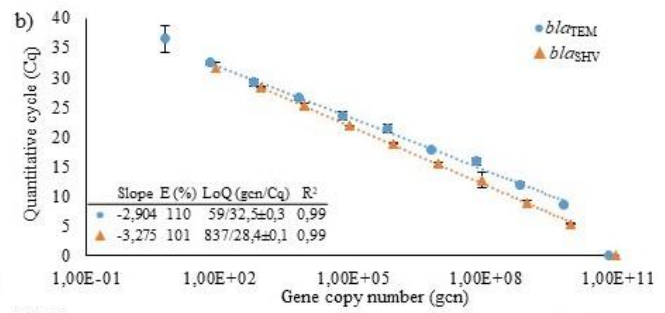
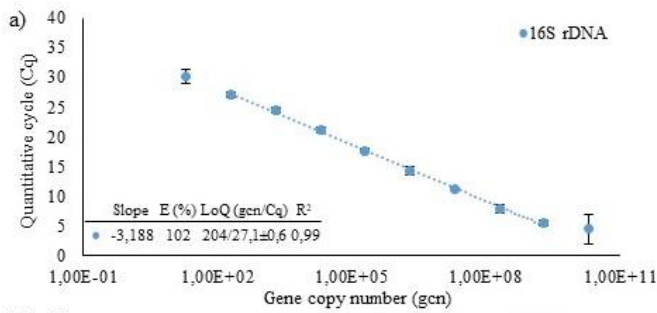
Gene, Accession number	Primer F; R; probe sequence (5'→3')	Amplicon size (bp)	Isolate containing the target and other genes relative to the resistance conferred by target	Isolates target-free and other resistance genes relative to the resistance conferred by the target <sup>a)</sup>
<i>qnrB1</i> , DQ351241.1	<b>F:</b> GTAGCGCATATATCACGAATACCAATC (Dheilly A et al., 2012) <b>R:</b> ATCTGAACCACTGAACGTCGC <b>QSY-AAGTCGTGTTGGAAAAGTGTGAGCTGTGG-JUN</b>	131	Es_106, <i>aac(6')-Ib-cr</i> Ec_75, <i>oqxAB</i> , <i>aac(6')-Ib-cr</i> Kp_98 (Lupo A et al., 2018b)(Lupo A et al., 2018b), <i>oqxAB</i> , <i>aac(6')-Ib-cr</i> Kp_104, <i>oqxAB</i> , <i>aac(6')-Ib-cr</i> Kp_102, <i>oqxAB</i> , <i>aac(6')-Ib-cr</i>	<u>Ec_12</u> : none <u>Ec_13</u> : none <u>Ec_55</u> : none <u>Ec_68</u> : none
<i>qnrS</i> , ABI187515.1	<b>F:</b> CGACGWGCTAACTTGCGTGA (Brown-Jaque M et al., 2018) <b>R:</b> GGCATTGTTGGAAACTTGCA (Brown-Jaque M et al., 2018) <b>QSY-TCATTGAACAGGGTGATATCGAAGGCTGC-JUN</b>	118	<u>Ec_84</u> <u>34228</u> (Lupo A et al., 2018b), <i>oqxA</i> , <i>qnrS</i> , <i>aac(6')-Ib-cr</i> <u>Ec_85</u> <u>Kt_105</u> , <i>oqxAB</i> , <i>qnrB</i> , <i>aac(6')-Ib-cr</i> <u>Ec_33</u>	
<i>aac(6)-Ib-cr</i> , GQ438247.1	<b>F:</b> AACTGCGAGCGATCCGA <b>R:</b> TGCGGTGTTGAACCATGTAC <b>MGB-TACGGTACCTTGCCCTCT-VIC</b>	101	Ec_26 Ec_37 (Madec JY et al., 2012) <u>20071</u> (Dahmen S et al., 2013) <u>22611</u> <u>Kp_99</u> , <i>oqxAB</i> , <i>qnrB4</i>	<u>Ec_17</u> : <i>aac(6')-Ib</i> <u>32189</u> (Lupo A et al., 2018b); <i>aac(6')-Ib</i> <u>34248</u> (Lupo A et al., 2018b); <i>aac(6')-Ib</i> <u>33058</u> (Lupo A et al., 2018b); <i>aac(6')-Ib</i>

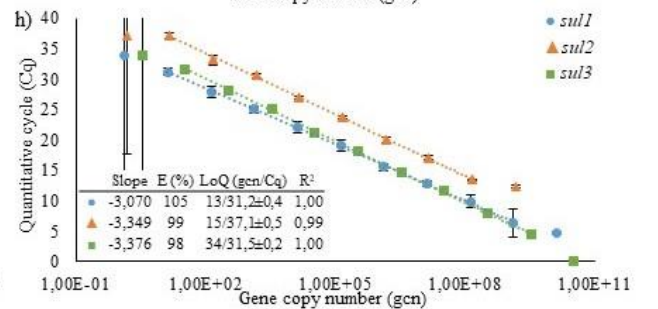
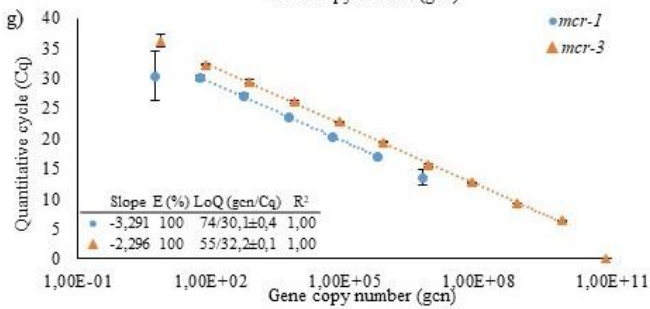
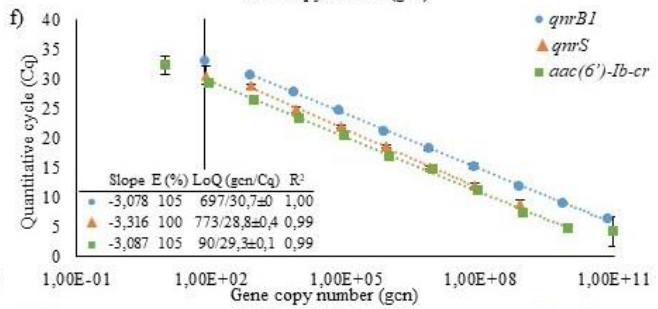
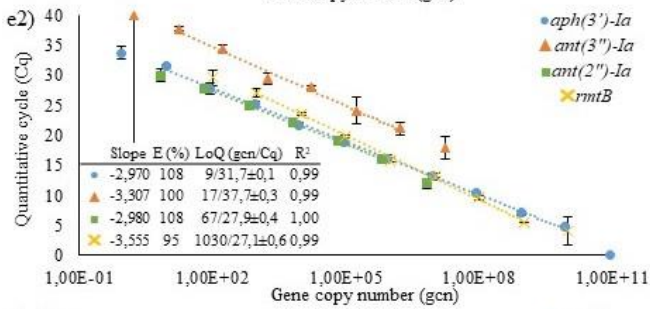
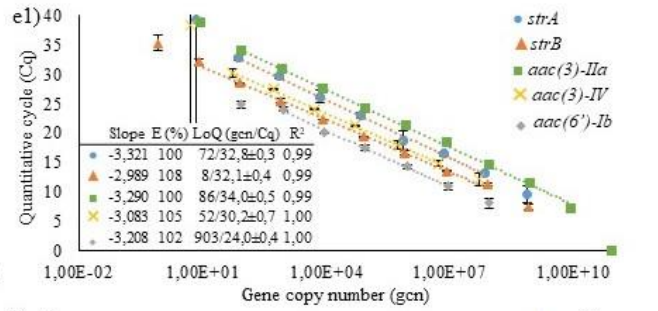
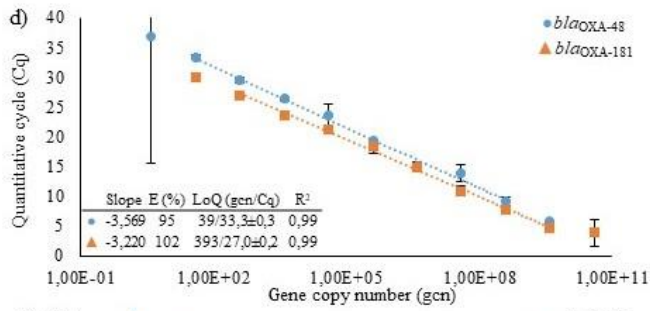
<i>mcr-1</i> , KP347127.1	<b>F:</b> TCGGTCAGTCCGTTTGTCTT <b>R:</b> TGGTGATCAGTAGCATCGCG <b>QSY-TCTTGACCGCGACCGCCAATCT-JUN</b>	166	<u>Ec_05</u> <u>Ec_10</u> 30271 (Lupo A et al., 2018b) <u>Ec_69</u> <u>Ec_43, mcr3</u>	<u>Es_106</u> 27841 (Haenni M et al., 2018); <i>mcr-3</i> 27845 (Lupo A et al., 2018b); <i>mcr-3</i> 32524 (Haenni M et al., 2018); <i>mcr-3</i>
<i>mcr-3</i> , KY924928.1	<b>F:</b> CCACGTTGATGTTTCTGGTCCG <b>R:</b> ATCATTAAAGGAGATCACGCCAC <b>QSY-TCTCGATGAATGGCTATGAGAAAGACACCA-ABY</b>	122	27841 (Haenni M et al., 2018) 32524 (Haenni M et al., 2018) 33058 (Lupo A et al., 2018b) 33904 (Lupo A et al., 2018b) <u>Ec_43, mcr1</u>	<u>Es_106</u> 32189 (Lupo A et al., 2018b) <u>Ec_07</u> <u>Ec_69: mcr-1</u>
<i>sul1</i> , X12868.1	<b>F:</b> CCGTTGGCCTTCCTGTAAAG (Czekalski N et al., 2012) <b>R:</b> TTGCCGATCGCGTGAAGT (Czekalski N et al., 2012) <b>QSY-CAGCGAGCCTTGCGGCGG-ABY</b> (Czekalski N et al., 2012)	67	<u>Ec_26, sul2</u> <u>Ec_37, sul2</u> <u>Ec_48, sul2</u> <u>Ec_43, sul3</u> <u>Ab_92</u>	<u>Ec_57: sul2</u> <u>Ec_18: sul2</u> <u>Ec_41: sul2</u> <u>Kp_105: sul2</u>
<i>sul2</i> , M36657.1	<b>F:</b> CGGCTGCGCTTCGATT (Czekalski N et al., 2012) <b>R:</b> CGCGCGCAGAAAGGATT (Czekalski N et al., 2012) <b>MGB-TGCTTCTGTCTGTTTCG-FAM</b>	60	<u>Ec_48, sul1</u> <u>Ec_51</u> <u>Ab_90</u> <u>Ec_08, sul3</u> <u>Ec_50, sul1</u>	<u>Ec_26: sul1</u> <u>Ec_04: sul1</u> <u>Ec_17: sul3</u> <u>Kp_97: sul1</u>
<i>sul3</i> , AJ459418.2	<b>F:</b> TAGGCTGCAAAGATAGGGAAAATG <b>R:</b> CAACGCCACTTCAGTTGTATC <b>QSY-TGGAGCAGATGTGATTGATTTGGGAGC-JUN</b>	268	<u>Ec_28</u> <u>Ec_39, sul1, sul2</u> <u>Ec_46, sul2, sul3</u> <u>Ec_56, sul2, sul3</u> <u>Ec_08, sul2</u>	<u>Ec_49: sul1, sul2</u> <u>Ec_72: sul2</u> <u>Ec_81: sul2</u> 22611 (Dahmen S et al., 2013); <i>sul1, sul2</i>
<i>dfrA1</i> , X00926.1	<b>F:</b> TCGAAGAATGGAGTTATCGGGA <b>R:</b> TGTCAGATGTAAACTTGAACGTGTTA <b>QSY-TGGGAGCATTACCCAACCGAAAAGTATGC-ABY</b>	184	<u>Ec_38</u> 27259 (Lupo A et al., 2018a), <i>dfrA17</i> <u>Ec_06</u> <u>Ec_63</u> <u>Ab_91</u>	<u>Ec_72: dfrA5, dfrA17</u> 22611 (Dahmen S et al., 2013); <i>dfrA12</i> <u>Kp_105</u> <u>Kp_102: dfrA14</u>
<i>dfrA12</i> , Z21672.1	<b>F:</b> TGCTGCGATGGGAGCC <b>R:</b> GACGGTTCGGTAGAGGCTTG <b>QSY-TTCGCAGACTCACTGAGGGAAAAGTCGT-ABY</b>	158	<u>Ec_17</u> <u>Ec_47</u> <u>Ec_08, dfrA17</u> <u>Ec_54</u> 45833 (Lupo A et al., 2018a)	37698 (Lupo A et al., 2018a); <i>dfrA17</i> <u>Ab_94</u> <u>Ab_91: dfrA1</u> <u>Ab_95: dfrA1</u>
<i>dfrA17</i>	<b>F:</b> GCGTAATCGGTAGTGGTCCTGA	162	<u>Ec_18, dfrA1</u>	<u>Ab_94</u>

	<b>R:</b> TTCCGTTCTTTGACACTACTGCAT <b>QSY-TTGACTCTATGGGTGTTCTTCCAATCGCA-ABY</b>		<u>27259</u> (Lupo A et al., 2018a) <u>Ec_08</u> , <u>dfrA12</u> 37698 (Lupo A et al., 2018a) <u>Ec_35</u>	45833 (Lupo A et al., 2018a); <u>dfrA12</u> <u>Ab_91</u> : <u>dfrA1</u> <u>Ab_95</u> : <u>dfrA1</u>
<i>tetA</i> , X000006.1	<b>F:</b> TCTGAGCACTGTCGCGCTC <b>R:</b> ACAGGTGCGCAGGCAAA <b>QSY-TGGTTCACCTCGAACGACGTCACCG-JUN</b>	156	<u>Ec_52</u> <u>Ec_06</u> <u>Ec_05</u> , <u>tetM</u> <u>Es_106</u> <u>Kp_105</u>	<u>Ab_94</u> : <u>tetB</u> <u>Ab_90</u> : <u>tetB</u> <u>Ec_55</u> : none <u>Ec_68</u> : none
<i>tetB</i> , EU252517.1	<b>F:</b> TGTGGCAGGAAGAATAGCCAC <b>R:</b> ATCCCTGTAAAG CACCTTGCTG <b>QSY-TTGGCTGGTGGTGGGATCGCTT-ABY</b>	221	<u>34972</u> (Lupo A et al., 2017) <u>Ab_90</u> <u>Ab_107</u> <u>Ab_92</u> , <u>tetA</u> <u>Ab_91</u>	<u>Ec_13</u> : none <u>Ec_55</u> : none <u>Ec_68</u> : none <u>Ab_92</u> : <u>tetA</u>
<i>tetM</i> , X04388.1	<b>F:</b> CGGTTTCTCTTGGATACTTAAATCAATC (Peak N et al., 2007) <b>R:</b> AGTCCGTCACATTCCAACCATAC <b>QSY-TGTTACAGCCATAGCGTATCCCCTCC-JUN</b>	102	<u>Ec_39</u> , <u>tetA/B</u> <u>Ec_51</u> , <u>tetA</u> <u>Ec_05</u> , <u>tetA</u> <u>Ec_64</u> , <u>tetB</u> <u>Ec_24</u>	<u>Ec_40</u> : <u>tetA/B</u> <u>Ec_73</u> : <u>tetB</u> <u>Ec_55</u> : none <u>Ec_68</u> : none
<i>intI1</i> , EF078697.1	<b>F-GCCTTGATGTTACCCGAGAGC</b> (Barraud O et al., 2010) <b>R-GATCGGTGCAATGCGTGTG</b> (Barraud O et al., 2010) <b>QSY-ATTCCTGGCCGTGGTCTGGGTTTT-ABY</b> (Barraud O et al., 2010)	196	<u>Ec_38</u> , <u>intI2</u>  <u>Kp_105</u>  <u>27259</u> (Lupo A et al., 2018a) <u>32137</u> (Lupo A et al., 2018a)	<u>Ec_12</u> : none <u>Ec_13</u> : none <u>Ec_55</u> : none <u>Ec_68</u> : none
<i>intI2</i> , AJ001816.1	<b>F-GACGGCTACCCTCTGTTATCTCTG</b> (Barraud O et al., 2010) <b>R-GCTTTTCCACCCTTACCGT</b> (Barraud O et al., 2010) <b>QSY-CGCTGCTGTATGGTGCAGGTTTGC-JUN</b> (Barraud O et al., 2010)	194	<u>Ec_38</u> <u>Ec_51</u> <u>Ec_18</u>  <u>Kp_105</u> <u>Ec_07</u>	<u>Ec_12</u> : none <u>Ec_13</u> : none <u>Ec_55</u> : none <u>Ec_68</u> : none

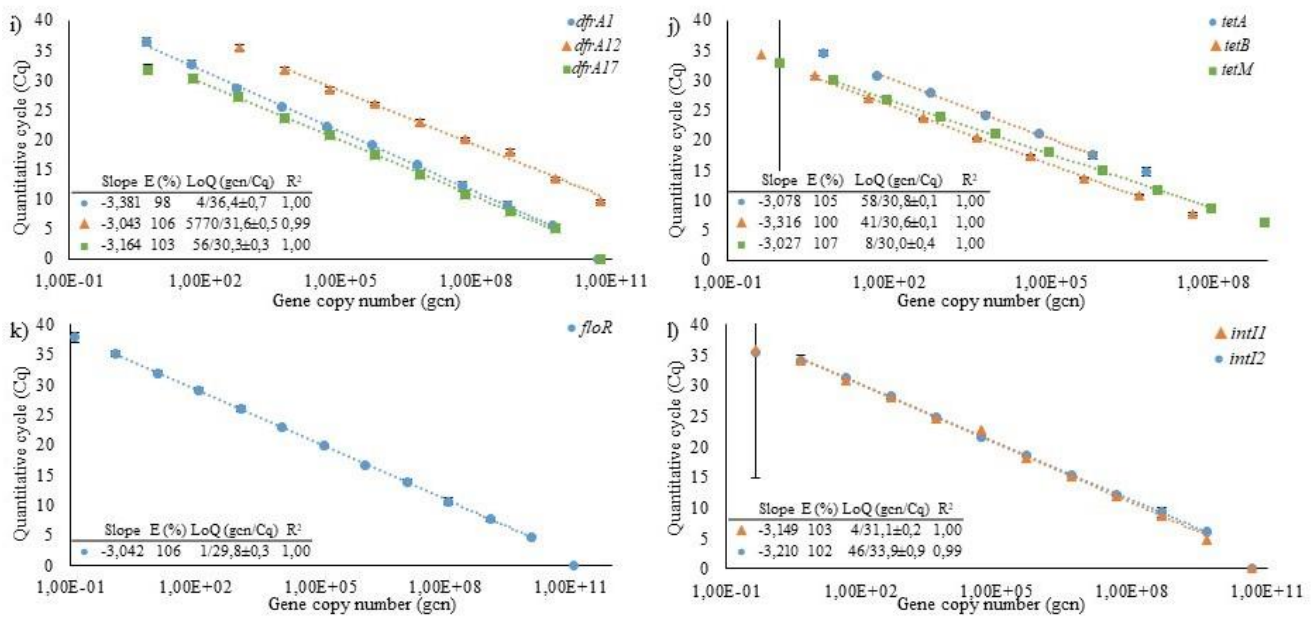
Note: <sup>a)</sup> All the qPCRs were tested using the target-free *E. coli* CIP 74.26.

Ec: *Escherichia coli*; Ab: *Acinetobacter baumannii*; Kt: *Klebsiella terrigena*; Kp: *Klebsiella pneumoniae*; Es: *Enterobacter sakazakii*.









**Figure 1.** Features of qPCRs using hydrolysis probes (TaqMan, Applied Biosystem) on the most common acquired antibiotic resistance genes in enterobacteria in Europe, 16S rDNA, and *intI1* and *intI2* genes encoding integrases of class 1 and 2 integrons. Standard curves were obtained using serial (10-fold) dilutions of purified PCR products in technical triplicates. Equations were obtained from the mean quantitative cycle (Cq) values plotted against the gene copies number (gcn). E: efficiency; LoQ: limit of quantification.

b)

**Figure 2:** Cluster plot of single nucleotide polymorphism (SNP) genotyping using hydrolysis probes (TaqMan, Applied Biosystem) on a)  $bla_{CTX-M-1}$ -group and b)  $bla_{CTX-M-9}$ -group most common variants. RFU: relative fluorescence unit.

- a) The allele 239C, whose probe is VIC labeled, is relative to  $bla_{CTX-M-3/15}$ , whereas the 239T allele, whose probe is marked with FAM, is relative to  $bla_{CTX-M-1/55}$ . The allele 725G, whose probe is labeled with FAM, is relative to  $bla_{CTX-M-15/55}$ , whereas the allele 725C, whose probe is marked with VIC, is relative to  $bla_{CTX-M-1/3}$  are in red.
- b) The allele 701C702A, whose probe is labeled with VIC, is relative to  $bla_{CTX-M-9}$ . The allele 701T702G, whose probe is labeled with FAM, is relative to  $bla_{CTX-M-14/27}$ . Only isolates harboring the 725G allele whose probe is labeled with VIC and relative to  $bla_{CTX-M-27}$  produced an amplification.

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