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Development of a set of multiplex PCR assays for the detection of genes encoding important β-lactamases in Enterobacteriaceae

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Objectives: To develop a rapid and reliable tool to detect by multiplex PCR assays the most frequently widespread β-lactamase genes encoding the OXA-1-like broad-spectrum β-lactamases, extended-spectrum β-lactamases (ESBLs), plasmid-mediated AmpC β-lactamases and class A, B and D carbapenemases.

Methods: Following the design of a specific group of primers and optimization using control strains, a set of six multiplex PCRs and one simplex PCR was created. An evaluation of the set was performed using a collection of 31 Enterobacteriaceae strains isolated from clinical specimens showing a resistance phenotype towards broad-spectrum cephalosporins and/or cephamycins and/or carbapenems. Direct sequencing from PCR products was subsequently carried out to identify β-lactamase genes.

Results: Under optimized conditions, all positive controls confirmed the specificity of group-specific PCR primers. Except for the detection of carbapenemase genes, multiplex and simplex PCR assays were carried out using the same PCR conditions, allowing assays to be performed in a single run. Out of 31 isolates selected, 22 strains produced an ESBL, mostly CTX-M-15 but also CTX-M-1 and CTX-M-9, SHV-12, SHV-5, SHV-2, TEM-21, TEM-52 and a VEB-type ESBL, 6 strains produced a plasmid-mediated AmpC β-lactamase (five DHA-1 and one CMY-2) and 3 strains produced both an ESBL (two SHV-12, one CTX-M-15) and a plasmid-mediated AmpC β-lactamase (DHA-1).

Conclusions: We report here the development of a useful method composed of a set of six multiplex PCRs and one simplex PCR for the rapid screening of the most frequently encountered β-lactamases. This method allowed direct sequencing from the PCR products.

Keywords: OXA-1-like β-lactamases, ESBLs, plasmid-mediated AmpC β-lactamases, carbapenemases, molecular epidemiology

Introduction

Resistance to β-lactam antibiotics is an increasing problem and β-lactamase production is the most common mechanism of drug resistance, especially in Gram-negative bacilli. β-Lactamases are remarkably diversified due to their continuous mutation.

Among them, extended-spectrum β-lactamases (ESBLs) are of great concern. ESBLs have been reported worldwide, most frequently in Enterobacteriaceae, but they have also been found in *Pseudomonas* spp. and *Acinetobacter baumannii*.[1,2] During the 1990s, TEM-type ESBLs and SHV-type ESBLs were dominant. During the past decade, rapid and massive spread of CTX-M-type ESBLs has been described. These enzymes are now the most prevalent ESBLs in Enterobacteriaceae in Europe and in other areas of the world.[3] Other ESBLs, less prevalent, have also been described as minor ESBLs; some of them, such as GES-type, PER-type and VEB-type β-lactamases, have now been isolated on several continents. Moreover, some GES variants also hydrolyse carbapenems.[4]

Although the plasmid-mediated AmpC β-lactamases are less common than ESBLs, they have been found in several areas of the world. Among them, the β-lactamase CMY-2 has the broadest geographical spread.[5]

Other β-lactamases conferring resistance to carbapenems, such as metallo-β-lactamases (MBLs) and KPC carbapenemases, but also some class D β-lactamases (e.g. OXA-48 in Enterobacteriaceae), have emerged more recently.[6,7]

Because many clinical isolates now harbour more than one β-lactamase, and due to the high diversity of these versatile enzymes, multiplex PCR methods are becoming widely used for their detection in epidemiological surveys.[8–10]

The purpose of the present work was to design reliable PCR assays with a maximum of consensus thermal cycling conditions for the rapid detection of frequently encountered β-lactamases by multiplexing. Six multiplex PCRs and one simplex PCR were designed, allowing direct sequencing from PCR products.

Materials and methods

Bacterial isolates

For optimization of the multiplex PCR assays, well characterized β-lactamase-producing strains were used as controls [Table S1, available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/)]. Thirty-one non-repetitive Enterobacteriaceae strains isolated in 2006 from patients hospitalized in seven Parisian university hospitals were selected by disc diffusion due to their reduced susceptibility to broad-spectrum cephalosporins (e.g. cefotaxime, ceftazidime) and/or to cephamycins (e.g. cefoxitin) and/or to carbapenems (e.g. imipenem). Biochemical identification to species level was performed using the API 20E Identification System for Enterobacteriaceae (bioMérieux SA, Marcy-l'Étoile, France). They included strains of *Proteus mirabilis* ($n=2$), *Citrobacter freundii* ($n=2$), *Enterobacter cloacae* ($n=3$), *Escherichia coli* ($n=9$) and *Klebsiella pneumoniae* ($n=15$). ESBL production was confirmed using the double-disc approximation test. AmpC β-lactamase production was evaluated by using cloxacillin (500 mg/L) as an inhibitor of AmpC enzymes. Imipenem discs supplemented with EDTA (Bio-Rad, Marnes-la-Coquette, France) were used for strains suspected of producing MBLs.

Design of group-specific primers for multiplex PCR assays

Six multiplex PCRs were designed in this study: a $bla_{TEM}/bla_{SHV}/bla_{OXA-1}$-like multiplex PCR; a bla_{CTX-M} multiplex PCR including phylogenetic groups 1, 2 and 9; a plasmid-mediated AmpC β-lactamase gene multiplex PCR including six groups based on percentage of similarity (as previously described by Pérez-Pérez and Hanson[8]); a $bla_{VEB}/bla_{GES}/bla_{PER}$ multiplex PCR; and two carbapenemase gene multiplex PCRs, one including bla_{VIM}, bla_{IMP} and bla_{KPC} genes and the other bla_{GES} and bla_{OXA-48}-like genes. One simplex PCR was also designed for $bla_{CTX-M-8/-25}$. For all of these genotypes, sequences were downloaded from the GenBank database and aligned within groups using the ClustalX software (version 2.0). Group-specific primers were designed to amplify internal fragments of several sizes (Table 1).

Multiplex PCR technique

Rapid DNA preparation was performed from one heated colony in a total volume of 100 μL of distilled water (95°C for 10 min) followed by a centrifugation step of the cell suspension. Total DNA (2 μL) was subjected to each multiplex PCR in a 50 μL reaction mixture containing 1× PCR buffer (10 mM Tris–HCl, pH 8.3/50 mM KCl/1.5 mM $MgCl_2$), 200 μM concentration of each deoxynucleotide triphosphate, a variable concentration of specific-group primers (Table 1) and 1 U of *Taq* polymerase (Sigma Aldrich, St Quentin Fallavier, France). Amplification was carried out as follows: initial denaturation at 94°C for 10 min; 30 cycles of 94°C for 40 s, 60°C for 40 s and 72°C for 1 min; and a final elongation step at 72°C for 7 min. For the carbapenemase gene multiplex PCR assays, the annealing temperature was optimal at 55°C for amplification of bla_{VIM}, bla_{IMP} and bla_{KPC} genes, and optimal at 57°C for amplification of bla_{GES} and bla_{OXA-48} genes. Amplicons were visualized after running at 100 V

Table 1. Group-specific primers used for the assays

PCR name	β-Lactamase(s) targeted	Primer name	Sequence (5′–3′)	Length (bases)	Annealing position[a]	Amplicon size (bp)	Primer concentration (pmol/μL)
Multiplex I TEM, SHV and OXA-1-like	TEM variants including TEM-1 and TEM-2	MultiTSO-T_for	CATTTCCGTGTCGCCCTTATTC	22	13–34	800	0.4
		MultiTSO-T_rev	CGTTCATCCATAGTTGCCTGAC	22	812–791		0.4
	SHV variants including SHV-1	MultiTSO-S_for	AGCCGCTTGAGCAAATTAAAC	21	71–91	713	0.4
		MultiTSO-S_rev	ATCCCGCAGATAAATCACCAC	21	783–763		0.4
	OXA-1, OXA-4 and OXA-30	MultiTSO-O_for	GGCACCAGATTCAACTTTCAAG	22	201–222	564	0.4
		MultiTSO-O_rev	GACCCCAAGTTTCCTGTAAGTG	22	764–743		0.4
Multiplex II CTX-M group 1, group 2 and group 9	variants of CTX-M group 1 including CTX-M-1, CTX-M-3 and CTX-M-15	MultiCTXMGp1_for	TTAGGAARTGTGCCGCTGYA[b]	20	61–80	688	0.4
		MultiCTXMGp1-2_rev	CGATATCGTTGGTGGTRCCAT[b]	21	748–728		0.2
	variants of CTX-M group 2 including CTX-M-2	MultiCTXMGp2_for	CGTTAACGGCACGATGAC	18	345–362	404	0.2
		MultiCTXMGp1-2_rev	CGATATCGTTGGTGGTRCCAT[b]	21	748–728		0.2
	variants of CTX-M group 9 including CTX-M-9 and CTX-M-14	MultiCTXMGp9_for	TCAAGCCTGCCGATCTGGT	19	299–317	561	0.4
		MultiCTXMGp9_rev	TGATTCTCGCCGCTGAAG	18	859–842		0.4
CTX-M group 8/25	CTX-M-8, CTX-M-25, CTX-M-26 and CTX-M-39 to CTX-M-41	CTX-Mg8/25_for	AACRCRCAGACGCTCTAC[b]	18	172–189	326	0.4
		CTX-Mg8/25_rev	TCGAGCCGGAASGTGTYAT[b]	19	497–479		0.4

Continued

Table 1. *Continued*

PCR name	β-Lactamase(s) targeted	Primer name	Sequence (5′–3′)	Length (bases)	Annealing position[a]	Amplicon size (bp)	Primer concentration (pmol/μL)
Multiplex III ACC, FOX, MOX, DHA, CIT and EBC	ACC-1 and ACC-2	MultiCaseACC_for	CACCTCCAGCGACTTGTTAC	20	744–763	346	0.2
		MultiCaseACC_rev	GTTAGCCAGCATCACGATCC	20	1089–1070		0.2
	FOX-1 to FOX-5	MultiCaseFOX_for	CTACAGTGCGGGTGGTTT	18	396–413	162	0.5
		MultiCaseFOX_rev	CTATTTGCGGCCAGGTGA	18	557–540		0.5
	MOX-1, MOX-2, CMY-1, CMY-8 to CMY-11 and CMY-19	MultiCaseMOX_for	GCAACAACGACAATCCATCCT	21	3–23	895	0.2
		MultiCaseMOX_rev	GGGATAGGCGTAACTCTCCCAA	22	900–879		0.2
	DHA-1 and DHA-2	MultiCaseDHA_for	TGATGGCACAGCAGGATATTC	21	113–133	997	0.5
		MultiCaseDHA_rev	GCTTTGACTCTTTCGGTATTCG	22	1109–1088		0.5
	LAT-1 to LAT-3, BIL-1, CMY-2 to CMY-7, CMY-12 to CMY-18 and CMY-21 to CMY-23	MultiCaseCIT_for	CGAAGAGGCAATGACCAGAC	20	570–589	538	0.2
		MultiCaseCIT_rev	ACGGACAGGGTTAGGATAGY[b]	20	1107–1088		0.2
	ACT-1 and MIR-1	MultiCaseEBC_for	CGGTAAAGCCGATGTTGCG	19	189–207	683	0.2
		MultiCaseEBC_rev	AGCCTAACCCCTGATACA	18	871–854		0.2
Multiplex IV VEB, PER and GES	GES-1 to GES-9 and GES-11	MultiGES_for	AGTCGGCTAGACCGGAAAG	19	463–481	399	0.3
		MultiGES_rev	TTTGTCCGTGCTCAGGAT	18	861–844		0.3
	PER-1 and PER-3	MultiPER_for	GCTCCGATAATGAAAGCGT	19	325–343	520	0.3
		MultiPER_rev	TTCGGCTTGACTCGGCTGA	19	844–826		0.3
	VEB-1 to VEB-6	MultiVEB_for	CATTTCCCGATGCAAAGCGT	20	187–206	648	0.3
		MultiVEB_rev	CGAAGTTTCTTTGGACTCTG	20	834–815		0.3
Multiplex V GES and OXA-48-like	GES-1 to GES-9 and GES-11	MultiGES_for	AGTCGGCTAGACCGGAAAG	19	463–481	399	0.4
		MultiGES_rev	TTTGTCCGTGCTCAGGAT	18	861–844		0.4
	OXA-48-like	MultiOXA-48_for	GCTTGATCGCCCTCGATT	18	230–247	281	0.4
		MultiOXA-48_rev	GATTTGCTCCGTGGCCGAAA	20	490-510		0.4
Multiplex VI IMP, VIM and KPC	IMP variants except IMP-9, IMP-16, IMP-18, IMP-22 and IMP-25	MultiIMP_for	TTGACACTCCATTTACDG[b]	18	194–211	139	0.5
		MultiIMP_rev	GATYGAGAATTAAGCCACYCT[b]	21	332–313		0.5
	VIM variants including VIM-1 and VIM-2	MultiVIM_for[c]	GATGGTGTTTGGTCGCATA	19	151–169	390	0.5
		MultiVIM_rev[c]	CGAATGCGCAGCACCAG	17	540–524		0.5
	KPC-1 to KPC-5	MultiKPC_for	CATTCAAGGGCTTTCTTGCTGC	22	209–230	538	0.2
		MultiKPC_rev	ACGACGGCATAGTCATTTGC	20	746–727		0.2

[a]Annealing position within the corresponding open reading frame (from the base A of start codon ATG).
[b]Y=T or C; R=A or G; S=G or C; D=A or G or T.
[c]This primer pair was previously described by Ellington *et al.*[10]

for 1 h on a 2% agarose gel containing ethidium bromide. A 100 bp DNA ladder (New England Biolabs, Ipswich, MA, USA) was used as a size marker.

Sequencing analysis of multiplex PCR products

To identify the β-lactamase genes detected in the multiplex PCR assays, DNA sequence analyses of the amplicons were performed. Amplified PCR products were purified using the ExoSap purification kit (ExoSap-it, GE Healthcare, Piscataway, NJ, USA) and bidirectional sequencing was performed. Each sequence was then compared with already known β-lactamase gene sequences by multiple-sequence alignment using the BLAST program.

Nucleotide sequence accession numbers

The GenBank nucleotide sequence accession numbers for the sequences studied in this study are detailed in Table S2 [available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/)].

Results and discussion

Analysis of the set of multiplex PCRs and simplex PCR

After optimizing the amplification conditions for each assay, amplicons of the expected sizes were obtained from all control strains used separately or mixed (Table S1) confirming the group specificity of the primers (Figure 1). For example, every template of the SHV family resulted in a 713 bp amplicon (Figure 1a).

The evaluation of chromosomal cross-hybridization was then performed with probable interfering species according to the set studied. An amplicon was observed with the species *Kluyvera ascorbata* and *Kluyvera georgiana*, respectively, when specific primers of $bla_{CTX\text{-}M}$ group 2 and groups 8/25 were used (Table S1). In the same way, amplicons of the ACC, EBC and DHA groups were observed, respectively, when the DNAs from *Hafnia alvei*, *Enterobacter asburiae* or *Morganella morganii* were used as template. No amplification product was observed from any other tested strains harbouring an AmpC gene, in contrast to the method of Pérez-Pérez and Hanson,[8] where an AmpC gene from *C. freundii* and *E. cloacae* was amplified.

Eventually, chromosomal class A genes from *K. pneumoniae* belonging to the phylogenetic group KpI were amplified with the group-specific primers of the SHV family.

Evaluation of direct sequencing from PCR products

Direct sequencing from amplicons was shown to be possible. Most amplicons contained major substitutions allowing the identification of a high number of β-lactamases. To a lesser extent, the identification of clusters of β-lactamases (each cluster containing a unique well-known enzyme) can be performed by this technique.

In the bla_{TEM}/bla_{SHV}/$bla_{OXA\text{-}1}$-like multiplex PCR, OXA-1-like β-lactamases cannot be distinguished by direct sequencing. However, due to the method, most of the TEM-type ESBL variants can be differentiated. In the same way, discrimination between ESBLs and TEM-type broad-spectrum β-lactamases became feasible, such as the distinction between SHV ESBL derivatives and SHV broad-spectrum β-lactamase variants. Another example can be found with the $bla_{CTX\text{-}M}$ multiplex PCR; the main β-lactamases belonging to phylogenetic group 1 can be discriminated. Thus, CTX-M-3 can be distinguished from CTX-M-1, and both can be differentiated from the CTX-M-15 β-lactamase. However, the common β-lactamases CTX-M-15 and CTX-M-28 cannot be separated.

For them and for other β-lactamases for whom direct sequencing is not sufficiently discriminating, further analysis may be necessary to identify the corresponding *bla* gene.

Evaluation of the six multiplex PCRs and the simplex PCR with clinical strains

In order to confirm the specificity of the set, simplex and multiplex PCR assays were performed on the 31 clinical strains selected according to their susceptibility profiles. All PCR products were submitted to direct sequencing.

ESBLs were detected in 22 isolates (71%), plasmid-mediated AmpC β-lactamases were observed in 6 isolates (19%) and 3 isolates (10%) produced both an ESBL and a plasmid-mediated AmpC β-lactamase. Isolates often carried two, occasionally three, different types of broad-spectrum β-lactamase (SHV-1, SHV-11, OXA-1 or TEM-1).

Major ESBL-producing isolates were CTX-M type, including 15 from phylogenetic group 1 (14 CTX-M-15 and 1 CTX-M-1) and 2 belonging to phylogenetic group 9 (CTX-M-9). TEM-type ESBLs (two TEM-21 and one TEM-52), SHV-type ESBLs (two SHV-12, one SHV-2 and one SHV-5) and one VEB-type ESBL were also detected. For the latter, no discrimination was possible by direct sequencing between the VEB-4, VEB-5 and VEB-6 β-lactamases.

DHA-1 enzymes were the most frequently observed plasmid-mediated AmpC β-lactamases (found in eight isolates); only one AmpC β-lactamase belonged to the CMY-2 group. Among the three isolates producing both an ESBL and a plasmid-mediated AmpC β-lactamase, two strains were found to co-produce DHA-1 and SHV-12, and the third strain was found to co-produce DHA-1 and CTX-M-15. None of the carbapenemase genes targeted was found in this study. Reduced susceptibility to imipenem found in one *K. pneumoniae* isolate was probably due to a decrease in permeability of the outer membrane. A complete description of the β-lactamases characterized in the 31 enterobacterial isolates is available in Table S3 [available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/)].

Conclusions

In summary, we report here the development of six multiplex PCRs and one simplex PCR that are able to detect prevalent β-lactamases. This PCR method is a fast, low-cost and reliable tool for the screening of frequently encountered β-lactamases. It will assist in monitoring their emergence and their spread, and it could be used in epidemiological surveys. To conclude, our study showed the high prevalence of CTX-M-type ESBLs belonging to phylogenetic group 1 among our panel of ESBL-producing Enterobacteriaceae strains, and the absence of detection of carbapenemases.

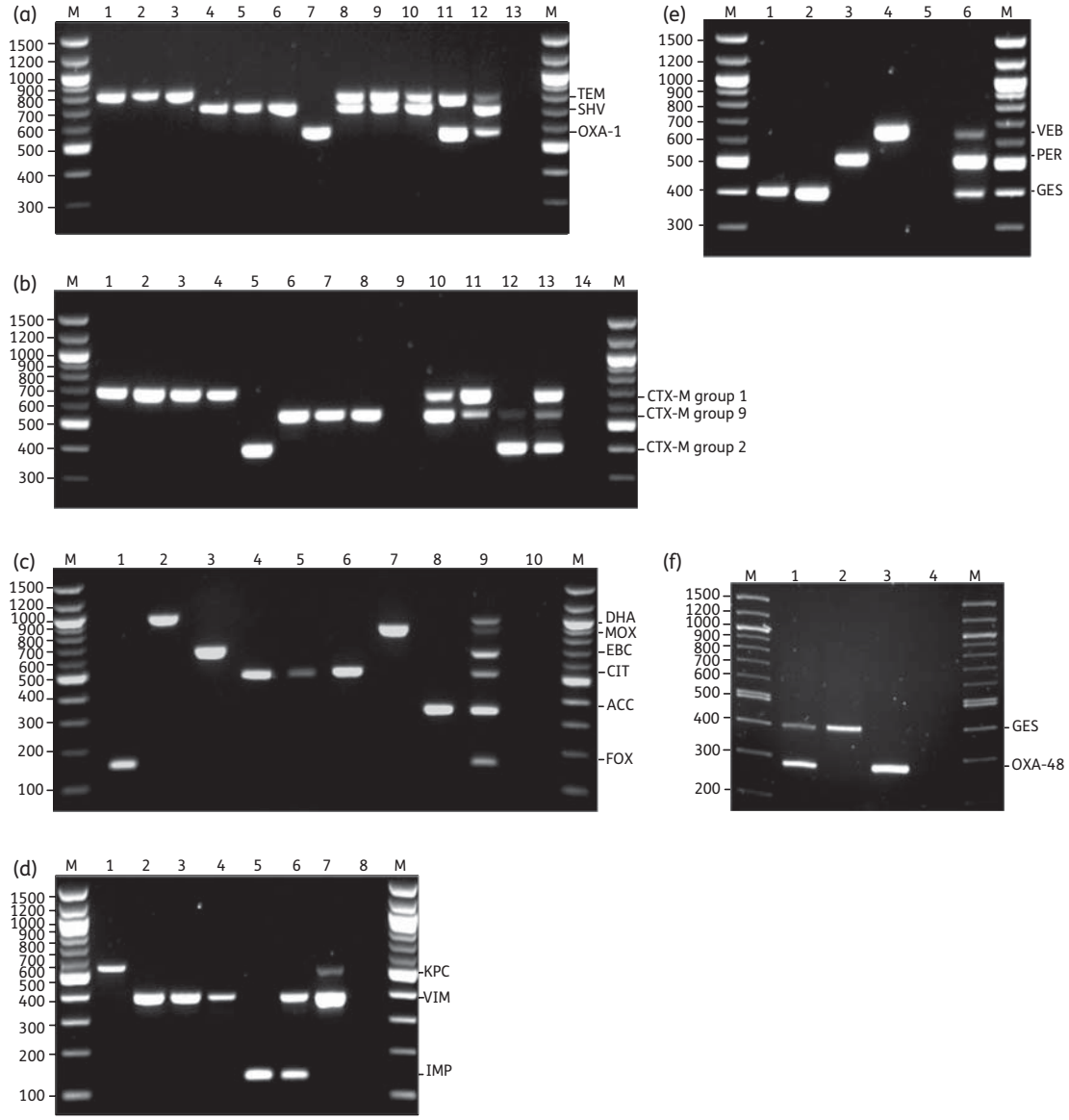

Figure 1. Multiplex PCR assays. (a) Multiplex PCR assay for the bla_{TEM}/bla_{SHV}/bla_{OXA}-like genes, amplification from control strains. Multiplex PCR products were separated in a 2% agarose gel. Lanes: 1, *E. coli* TN13; 2, *E. cloacae* 4861; 3, *K. oxytoca* 1376; 4, *K. pneumoniae* 169; 5, *K. pneumoniae* SLKM; 6, *K. pneumoniae* FLO; 7, *Salmonella* spp. RGN238; 8, *K. pneumoniae* SLK54; 9, *K. pneumoniae* BHR; 10, *K. pneumoniae* KOL; 11, *K. oxytoca* 1376+*Salmonella* spp. RGN238; 12, *K. pneumoniae* KOL+*Salmonella* spp. RGN238; 13, negative control; M, molecular size marker (in bp). (b) Multiplex PCR assay for the $bla_{CTX\text{-}M}$ genes including phylogenetic group 1, group 2 and group 9, amplification from control strains. Multiplex PCR products were separated in a 2% agarose gel. Lanes: 1, *E. coli* TN16; 2, *E. coli* TN03; 3, *K. pneumoniae* BHR; 4, *K. pneumoniae* LT; 5, *E. coli* TN19; 6, *E. coli* TN13; 7, *E. coli* TN07; 8, *E. coli* TN05; 9, *K. pneumoniae* H610; 10, *E. coli* C7; 11, *E. coli* TN05+*E. coli* TN03; 12, *E. coli* TN05+*E. coli* TN19; 13, *E. coli* TN05+*E. coli* TN03+*E. coli* TN19; 14, negative control; M, molecular size marker (in bp). (c) Multiplex PCR assay for the plasmid-mediated AmpC β-lactamase genes, amplification from control strains. Multiplex PCR products were separated in a 2% agarose gel. Lanes: 1, *K. pneumoniae* 1734; 2, *K. pneumoniae* 760; 3, *E. coli* C600 pMG231; 4, *E. coli* BS; 5, *K. pneumoniae* 169; 6, *P. mirabilis* 34955; 7, *K. pneumoniae* KOL; 8, *K. pneumoniae* SLK54; 9, *K. pneumoniae* SLK54+*K. pneumoniae* 1734+*K. pneumoniae* 760+*E. coli* C600 pMG231+*P. mirabilis* 34955+*K. pneumoniae* KOL; 10, negative control; M, molecular size marker (in bp). (d) Multiplex PCR assay for the bla_{VIM}/bla_{IMP} /bla_{KPC} genes, amplification from control strains. Multiplex PCR products were separated in a 2% agarose gel. Lanes: 1, *E. cloacae* 4861; 2, *K. pneumoniae* BHR; 3, *K. pneumoniae* FLO; 4, *P. aeruginosa* 73257; 5, *A. baumannii* 2318; 6, *K. pneumoniae* FLO+*A. baumannii* 2318; 7, *E. cloacae* 4861+*K. pneumoniae* FLO; 8, negative control; M, molecular size marker (in bp). (e) Multiplex PCR assay for the bla_{VEB}/bla_{GES}/bla_{PER} genes, amplification from control strains. Multiplex PCR products were separated in a 2% agarose gel. Lanes: 1, *K. pneumoniae* ORI-1; 2, *E. coli* 14R519(pHT9); 3, *Providencia stuartii* Ps5; 4, *E. coli* 394; 5, negative control; 6, *K. pneumoniae* ORI-1+*P. stuartii* Ps5+*E. coli* 394; M, molecular size marker (in bp). (f) Multiplex PCR assay for the bla_{GES}/$bla_{OXA\text{-}48}$-like genes, amplification from control strains. Multiplex PCR products were separated in a 2% agarose gel. Lanes: 1, *K. pneumoniae* ORI-1+*K. pneumoniae* 11978; 2, *K. pneumoniae* ORI-1; 3, *K. pneumoniae* 11978; 4, negative control; M, molecular size marker (in bp).

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Transparency declarations

None to declare.

Supplementary data

Tables S1, S2 and S3 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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