OXA-244-Producing Escherichia coli Isolates, a Challenge for Clinical Microbiology Laboratories

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OXA-244-producing *Escherichia coli* isolates: a challenge for clinical microbiology laboratories

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ABSTRACT (119)

OXA-244 is a single point mutant derivative of OXA-48 displaying reduced carbapenemase activity. Here, we report microbiological features of 7 OXA-244-producing *E. coli* isolates. Only one isolate grew on the ChromID® CARBA SMART medium (bioMérieux), but 6/7 grew on ChromID® ESBL medium (bioMérieux), as they co-produced either an Extended spectrum ß-lactamase (ESBL) and or a plasmid-encoded cephalosporinase. The presence of a carbapenemase was detected in 57.1 %, 71.4 %, 71.4 %, and 100% of the *E. coli* isolates using the Carba NP test, the RAPIDEC® CARBA NP (bioMérieux), a MALDI-TOF MS hydrolysis assay (Bruker), and OXA-48 K-SeT® (Coris bioconcept), respectively. Our results indicate that OXA-244-producing *E. coli* isolates are difficult to detect, which may lead to their silent spread.

INTRODUCTION

The emergence of carbapenemase-producing-*Enterobacteriaceae* (CPE) is becoming a major clinical issue (1). In this context, expert committees have set up guidelines to prevent the spread of CPEs (2). Thus, it is recommended to screen individuals at risk of being colonized, especially patients previously hospitalized in countries with high CPE prevalence, in-order-to isolate colonized patients as soon as possible, implement contact precautions and and "strongly" recommend cohorting with dedicated nursing staff. Screening procedures involving plating of rectal swabs on screening medium to detect all carbapenem-resistant isolates with a high sensitivity and sufficient specificity to rule out CPE carriage should be performed as soon as possible (2,3).

OXA-244, a single point mutant derivative of OXA-48 displaying reduced carbapenemase activity, was initially described in a Spainish *Klebsiella pneumoniae* isolate (4). Subsequently, it was fortuitously found in a CTX-M-producing *Escherichia coli* isolate in Germany (5), in 4 *Enterobacter aerogenes* isolates in Russia (6), in *E. coli* VAL from France (7) and in an *E. coli* isolate co-producing CTX-M14 from South East Asia (8). While in *E. coli* VAL isolate, the $bla_{\text{OXA-244}}$ gene was chromosomally-encoded it was plasmid-located in *K. pneumoniae* and *E. aerogenes* (4,6,7).

The aim of this study was to evaluate different screening approaches and confirmatory tests useful for detecting OXA-244-producing *E. coli* (OXA-244-*Ec*) isolates. In addition, we have investigated the genetic relatedness of seven OXA-244-*Ec* isolates from different geographical origins received at the French National Reference Center (F-NRC) for CPEs.

In August 2015, a 44-year-old Egyptian man was admitted to the Bicêtre Hospital (Le Kremlin-Bicêtre, France) for an episode of erysipela of his right leg, that was treated IV with amoxicillin. After two weeks, the patient was discharged with a favorable outcome. As this patient was considered at risk for MDR bacterial carriage according-to French CPE guidelines (repatriated patient), rectal swabs were plated on ChromID® CARBA SMART medium (bioMérieux, La Balme-les-Grottes, France) a selective chromogenic bi-plate for the screening of CPE and ChromID® ESBL (Biomérieux) a chromogenic plate for the screening of extended spectrum β-lactamase (ESBL)-producing Enterobacteriaceae. The ChromID® CARBA SMART medium remained sterile, while an *E. coli* 85H4 grew on the ChromID® ESBL plate (Table 1). Antimicrobial susceptibilities as determined by the disk diffusion technique on Mueller-Hinton agar (BioRad, Marnes-La-Coquette, France) and interpreted according to the EUCAST breakpoints as updated in 2016 (http://www.eucast.org), revealed that *E. coli* 85H4 isolate was highly resistant to temocillin (absence of inhibition diameter) and displayed a reduced susceptibility to ertapenem (19 mm diameter), thus requiring confirmatory testing for carbapenemase production, as recommended by EUCAST (9). The RAPIDEC® CARBA NP (bioMérieux) was positive for *E. coli* 85H4, despite that no colony grew on ChromID® CARBA SMART plate (10). In house PCR/sequencing, as previously described (11) revealed the presence of a gene that codes for OXA-244, a R214G OXA-48 variant (Table 1) (4). Since OXA-244-*Ec* 85H4 also produced an ESBL, ChromID® ESBL medium was used to screen the 34 contact-patients of the Egyptian patient. For 4 *patients* positive *E. coli* cultures on ChromID® ESBL medium were obtained. Antibiotic susceptibility testing and in house PCR was performed on five independent colonies, and none of them produced OXA-244 (data not shown).

Phenotypic characterization of OXA-244-*Ec*. The ability to reliably detect OXA-244-*Ec* using ChromID® CARBA SMART and ChromID® ESBL plates, and confirm the presence of a carbapenemase was further investigated on *E. coli* VAL (7) and 5 other OXA-244-*Ec* referred to the F-NRC for CPEs.

Antimicrobial susceptibilities of the seven OXA-244-Ec isolates to different antibiotics are shown in Table 2. For all OXA-244-*Ec* isolates, 100 µl of a 0.5 McFarland solution was plated on ChromID® ESBL and ChromID® CARBA SMART media. Only one out of the seven isolates did not grow on the ChromID® ESBL (Table 1). This isolate was susceptible to cephalosporins, explaining the absence of growth on the ChromID® ESBL medium (Table 2). At the opposite, only one isolate grew on the "OXA-48 side" of the ChromID® CARBA SMART plate (Table 1) and none on the "CARBA side". This strain displayed the highest MICs for temocillin (>1024 mg/L) and for moxalactam, a β-lactam classically used for testing impermeability problems (Table 1) (12). All the OXA-244-*Ec* isolates presented only slightly decreased susceptibility to carbapenems, thus explaining the absence of growth on the CARBA side of the ChromID® CARBA SMART plate (Table 1). The biochemical confirmation tests used for carbapenemase detection were positive for 57.1% (4/7), 71.4% (5/7) , 71.4% (5/7) and 100% (7/7) of the isolates using the Carba NP test (13), the RAPIDEC® CARBA NP (Biomérieux) (10), the MBT STAR-BL, a commercial MALDI-TOF MS-based assay (Maldi-Biotyper, Bruker, Illkirch, France), and Lateral Flow Immuno Assay (LFIA), called OXA-48 *K*-SeT assay (Coris BioConcept, Gembloux, Belgium) (14) respectively (Table 1).

Molecular detection of resistance genes. $Bla_{\text{OXA-48-like}}$ genes were detected in all 7 OXA-244-*Ec* isolates using the commercially available Xpert® Carba-R v2 assay as recommended by the manufacturer (Cepheid, Toulouse, France) (15,16).

112 Whole genome sequencing (WGS) was performed to determine the resistome
113 of these OXA-244-*Ec* isolates using the Resfinder server
114 (http://cge.cbs.dtu.dk/services/ResFinder-2.1/) (17) (Table 2). A good correlation
115 between the genetic profile and the phenotypic resistance profile for routine tested β-
116 lactams, colistin, fosfomycin, phenicol, sulphonamide/trimethoprim, and tetracycline
117 antibiotics was found (Table 2). For aminoglycosides only netilmicin, amikacin,
118 tobramycin and gentamicin were tested. However, different aminoglycoside
119 resistance genes were found in some of the isolates (eg: *aph3-1a, aph3-1b, aph6-1d,*
120 *aadA1*), which confer resistance to other aminoglycosides that were not tested, as not
121 clinically-relevant. Of note isolate OXA-244-*Ec* 86J1 was resistant to
122 fluoroquinolones due to mutations in the Quinolone Resistance Determinant Region
123 as revealed by RAST server analysis (rast.nmpdr.org) (18) and comparison with that
124 of *K. pneumoniae* ATCC 13883 (GenBank DQ673325): codons 83 (Ser83-Leu); 87
125 (Asp87-Asn) for gyrA and in codons 80 (Ser80-Ile); 84 (Glu84-Gly) for parC, which
126 are known to confer fluoroquinolone resistance.
127 Among the 7 isolates, only *E. coli* VAL had no other ß-lactam resistance gene
128 besides $bla_{\text{OXA-244}}$ gene, consequently this strain was susceptible to cephalosporins (7).
129 For the remaining 6 isolates an ESBL gene ($bla_{\text{CTX-M-14}}$, $bla_{\text{CTX-M-27}}$) or a plasmid-
130 encoded cephalosporinase gene ($bla_{\text{CMY-42}}$, $bla_{\text{CMY-2}}$) were always associated with
131 $bla_{\text{OXA-244}}$ gene (Table 2).
132 **Genetic relatedness of OXA-244-*Ec* isolates.** The seven OXA-244-*Ec*
133 isolates corresponded to four different clones as revealed by rep-PCR using the
134 DiversiLab® system (bioMérieux) following the manufacturer's recommendations.
135 Two distinct clones were identified among the Egyptian isolates (Figure 1, Table 1).
136 MLST results deduced from WGS using MultiLocus Sequence Typing server v1.8

(https://cge.cbs.dtu.dk/services/MLST/) (19) confirmed the rep-PCR results, as each rep-PCR pattern corresponded to a different Sequence Type (ST): ST-38, ST-361, ST-1722 and ST-3541 (Table 1).

Genetic environment and support of *bla*$_{\text{OXA-244}}$ genes. For three strains (78B5, 62D3, 69E6) no plasmids could be evidenced after electrophoresis of Kieser extracted DNA (11) (Supplemental Figure 1). Electroporation of the extracted plasmids as previously described (11) yielded *E. coli* TOP10 transformants only for three (86J1, 85H4, 73G4). However, only ESBL/plasmid encoded *bla*$_{\text{AMPC}}$ genes were found in these transformants. Thus, all these findings suggest a chromosomal location of the *bla*$_{\text{OXA-244}}$ gene. PCR mapping of *bla*$_{\text{OXA-244}}$ gene flanking sequences showed that all were bracketed by two IS1R copies forming an IS1R-made composite transposon, named Tn*51098* (Supplemental Figure 2A). In all isolates, even though belonging to different Rep-PCR patterns or ST-types, Tn*51098* was inserted into a gene encoding an intrinsic endonuclease from *E. coli*, as previously described (7) (Supplemental Figure 2B). Dissemination of *E. coli* isolates harboring a chromosomally-located *bla*$_{\text{OXA-48-like}}$ gene has recently been linked to one ST, namely ST38 (20,21), however in our study 4 different STs that have integrated the *bla*$_{\text{OXA-244}}$ carbapenemase gene into the chromosome were found, supporting that diffusion could be more related to the mobility of *bla*$_{\text{OXA-244}}$-carrying IS1R-made composite transposons, Tn*51098*, rather than to a clonal expansion.

Conclusion

The last years have witnessed the development of novel diagnostic tools that tremendously improved the detection or confirmation of CPEs (such as selective culture media, phenotypic testing methods, molecular techniques). Yet, the constant

161 evolution of these resistance mechanisms makes detection of CPEs a renewed
162 challenge for clinical Microbiology laboratories (22).

163 This is certainly the case for OXA-244-*Ecs*, that do not grow on ChromID®
164 CARBA SMART plates, one of the most used medium for the screening of CPEs
165 (23), likely due to weaker carbapenem and temocillin hydrolytic activities as
166 compared to OXA-48 and a chromosomal location of its gene (7,24,25). However,
167 since all but one OXA-244-*Ec* isolates also produced an ESBL, they could grow on
168 ChromID® ESBL medium. Of note, all the previous reports of OXA-244-producing
169 *Enterobacteriaceae*, revealed co-production of at least one β-lactamase hydrolyzing
170 expanded-spectrum cephalosporins (5-8). In absence of expanded-spectrum
171 hydrolyzing enzymes (such as in *E. coli* VAL), it is not possible to screen for OXA-
172 244 producers using the available screening media, and thus, OXA-244 detection
173 would rely only on molecular tests directly from rectal swabs, such as Xpert® Carba
174 v2 (15). Finally, as previously reported, the OXA-48 K-SeT® was the most reliable
175 technique to detect OXA-244-*Ec* (14).

176 In France OXA-244-producing *E. coli* are still rare (0%, 0.3% (2 isolates),
177 0.2% (2 isolates) and 0.6% (6 isolates), 0.7% (8 isolates) of OXA-48-like
178 carbapenemases in 2012, 2013, 2014 and 2015, 2016 respectively), but it is still not
179 clear whether this is a real low prevalence or simply the result of under-detection.
180
181 **Nucleotide sequence accession number.** The *E. coli* genome sequences of
182 isolates 86J1, 62D3, 69E6, 78B5, 35J9, 73G4, and 85H4 used in this study were
183 deposited under following Genbank accession numbers MKGU00000000,
184 MKGY00000000, MKGZ00000000, MKGT00000000, MKGX00000000,
185 MKGV00000000, MKGW00000000 respectively.

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TRANSPARENCY DECLARATION

LD holds an international patent for the Carba NP test that has been filed on behalf of INSERM Transfert and subsequently licenced to bioMérieux.

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297 Table 1. Clinical and phenotypic characteristics of the OXA-244-producing *E. coli* isolates and of an OXA-48 producing *E. coli* isolate.

Isolates	MLST[a]	Clones[b]	Plasmids size (c.a)	Year of isolation	Source of isolation	Origin	Susceptibility[c]					OXA-48 K-SeT®	Carba NP test[d]	RAPIDEC® Carba NP[d]	MALDI-TOF MS hydrolysis assay[d]	Chrom ID® ESBL[e]	Chrom ID® CARBA SMART[f]
							IMP (mg/L)	MEM (mg/L)	ETP (mg/L)	TEM (mg/L)	MOX (mm)						
86J1	ST-361	1	160 110 70	2015	rectal	Egypt	0.5 (S)	0.5(S)	2 (R)	>1024	7	+	+	+	+	+	+ | -
62D3	ST-1722	2	Abs	2014	urine	unknown	0.38(S)	0.38(S)	1(I)	128	21	+	+	+	+	+	- | -
69E6	ST-38	3	Abs	2014	rectal	unknown	0.25(S)	0.38(S)	3(R)	128	20	+	+/-	+	+	+	- | -
78B5	ST-38	3	Abs	2015	rectal	unknown	0.38(S)	0.5(S)	3(R)	256	21	+	+	+	+	+	- | -
VAL (4)	ST-38	3	120 60 10	2013	urine	France	0.5(S)	0.75(S)	2(R)	96	21	+	-	+/-	-	-	- | -
73G4	ST-3541	4	115	2015	unknown	Egypt	0.25(S)	0.19(S)	0.75(I)	128	20	+	+	+	+	+	- | -
85H4	ST-3541	4	115	2015	rectal	Egypt	0.38(S)	0.25(S)	2(R)	384	20	+	+/-	+/-	-	+	- | -

124G6	ST-636	NT[g]	62	NT	NT	France	0.5	0.5	2	>1024	21	+	+	+	+	-	+ | -

[a] Overview of Allele sequences and sequence types (STs) identified by MultiLocus Sequence Typing server v1.8 (25).

[b] Rep-PCR analysis by using the Diversilab technique

[c] IMP = imipenem, MEM = Meropenem, ETP = Ertapenem TEM = Temocilin, MOX = Moxalactam

[d] + = positive test, - = negative test, +/- = equivocal test

[e] + = positive culture, - = negative culture

[f] Bacterial growth was checked on both sides of the bi-plate (ChromID®OXA-48 | ChromID® CARBA), + = positive culture, - = negative culture.

[g] NT: Not tested

309 Table 2. Resistance genes and phenotypic susceptibility of the OXA-244-producing *E. coli* isolates.

Isolates	**Acquired resistance genes[a]**									**Observed Phenotype[b]**									
	β-LAC	**AMG**	**COL**	**FOS**	**C**	**FQ**	**SUL**	**TR**	**TET**	**AMX**	**AMC**	**CTX**	**AMG[c]**	**COL**	**FOS**	**C**	**FQ**	**SXT**	**TET**
86J1	*bla*$_{OXA-244}$ *bla*$_{TEM-1b}$ *bla*$_{CMY-42}$	*aph3-1b* *aph6-1d* *aadA1*	-	-	-	-	*sul1* *sul2*	*dfrA1*	*tetB*	R	R	R	S	S	S	S	R	R	R
62D3	*bla*$_{OXA-244}$ *bla*$_{CMY-2}$	-	-	-	-	-	-	-	-	R	R	R	S	S	S	S	S	S	S
69E6	*bla*$_{OXA-244}$ *bla*$_{TEM-1b}$ *bla*$_{CTX-M-14b}$	*aadA1*	-	-	*catA1*	-	-	*dfrA1*	-	R	R	R	S	S	S	R	S	R	S
78B5	*bla*$_{OXA-244}$ *bla*$_{TEM-1b}$ *bla*$_{CTX-M-14b}$	*aadA1*	-	-	*catA1*	-	-	*dfrA1*	-	R	R	R	S	S	S	R	S	R	S
VAL (4)	*bla*$_{OXA-244}$ *bla*$_{TEM-1b}$	*aph3-1b* *aph6-1d* *aadA1*	-	-	*catA1*	-	*sul2*	*dfrA1* *dfrA14*	*tetB* *tetD*	R	R	S	S	S	S	R	S	R	R
73G4	*bla*$_{OXA-244}$ *bla*$_{TEM-1b}$ *bla*$_{CTX-M-27}$	*aph3-1b* *aph6-1d* *aph3-1a*	-	-	-	-	*sul2*	*dfrA14*	*tetb*	R	R	R	S	S	S	S	S	R	R
85H4	*bla*$_{OXA-244}$ *bla*$_{TEM-1b}$ *bla*$_{CTX-M-27}$	*aph3-1b* *aph6-1d* *aph3-1a*	-	-	-	-	*sul2*	*dfrA14*	*tetb*	R	R	R	S	S	S	S	S	R	R

310

311 [a] Overview of resistance genes detected in the isolates by ResFinder (23).

312 β-lac = Beta-lactam, AMG= aminoglycoside, COL = colistin, FOS = fosfomycin, C = chloramphenicol, FQ = fluoroquinolone, SUL =

313 sulphonamide, TET = tetracycline, TR = trimethoprim.

314 [b] Antimicrobial susceptibilities were determined by the disc diffusion technique and interpreted according to the EUCAST breakpoints.

315 AMX = amoxicilin, AMC = amoxicillin/clavulanic, CTX = cefotaxime, AMG= aminoglycoside, COL = colistin, FOS = fosfomycin, CL =

316 phenicol, FQ = fluoroquinolone, SUL = sulphonamide, TET = tetracycline, TR = trimethoprim.

317 [c] aminoglycoside tested were amikacin, gentamicin, tobramycin and netilmicin

318

LEGEND OF THE FIGURES

Figure 1. Rep-PCR analysis by using the Diversilab technique. Dendrogram and computer-generated image of rep-PCR banding patterns of OXA-244-producing *E. coli* isolates and an unrelated strain of *E. coli* isolate. As recommended by the manufacturer, a cut-off for similarity of 95% defined a cluster.

Supplemental Figure 1. Plasmid analysis performed using the Kieser technique on a 0.7% agarose gel of the seven *E. coli* isolates and their transformants (if available). Lane 1, *E. coli 86J1*; Lane T1 *E. coli* TOP10 harboring plasmid 86J1 *with* $bla_{\text{CMY-42}}$ *gene*; Lane 2 *E. coli* 85H4; Lane T2 *E. coli* TOP10 harboring plasmid 85H4 with $bla_{\text{CTX-M 27}}$; Lane 3 *E. coli* 73G4; Lane T3 *E. coli* TOP10 harboring plasmid 73G4 with $bla_{\text{CTX-M 27}}$; Lane 4 *E. coli* VAL (no transformants available) (4); Lane 5 *E. coli 78B5* (no transformants available); Lane 6 *E. coli 69E6* (no transformants available); Lane 7 *E. coli* 62D3 (no transformants available); Lane M NCTC 50192.

Supplemental Figure 2 (A) Schematic map of Tn51098 structure and the surrounding sequences in the *E. coli* isolates. Open reading frames are shown as arrows or as horizontal boxes with an arrow indicating the orientation of coding sequence. Expected length (bp) between primers pairs Endonuclease (5'- GAT GAG GAT GGT AAC AAG A-3') / OXA-48b (5'- GAG CAC TTC TTT TGT GAT GGC-3') and OXA-48b (5'- GAG CAC TTC TTT TGT GAT GGC-3') / OXA-48A (5'-TTG-GTG-GCA-TCG-ATT-ATC-GG-3') are shown in squares braquets. **(B)** PCR mapping with primers pairs Endonuclease / OXA-48b (a); OXA-48A / OXA-48b (b) and negative control (c). Lane 1, *E. coli 86J1*; Lane 2 *E. coli* 85H4; Lane 3 *E. coli*

73G4; Lane 4 *E. coli 35J9*; Lane 5 *E. coli 78B5*; Lane 6 *E. coli 69E6;* Lane 7 *E. coli* 62D3; Lane M GeneRuler 1kb Plus DNA Ladder (Thermo Scientific).

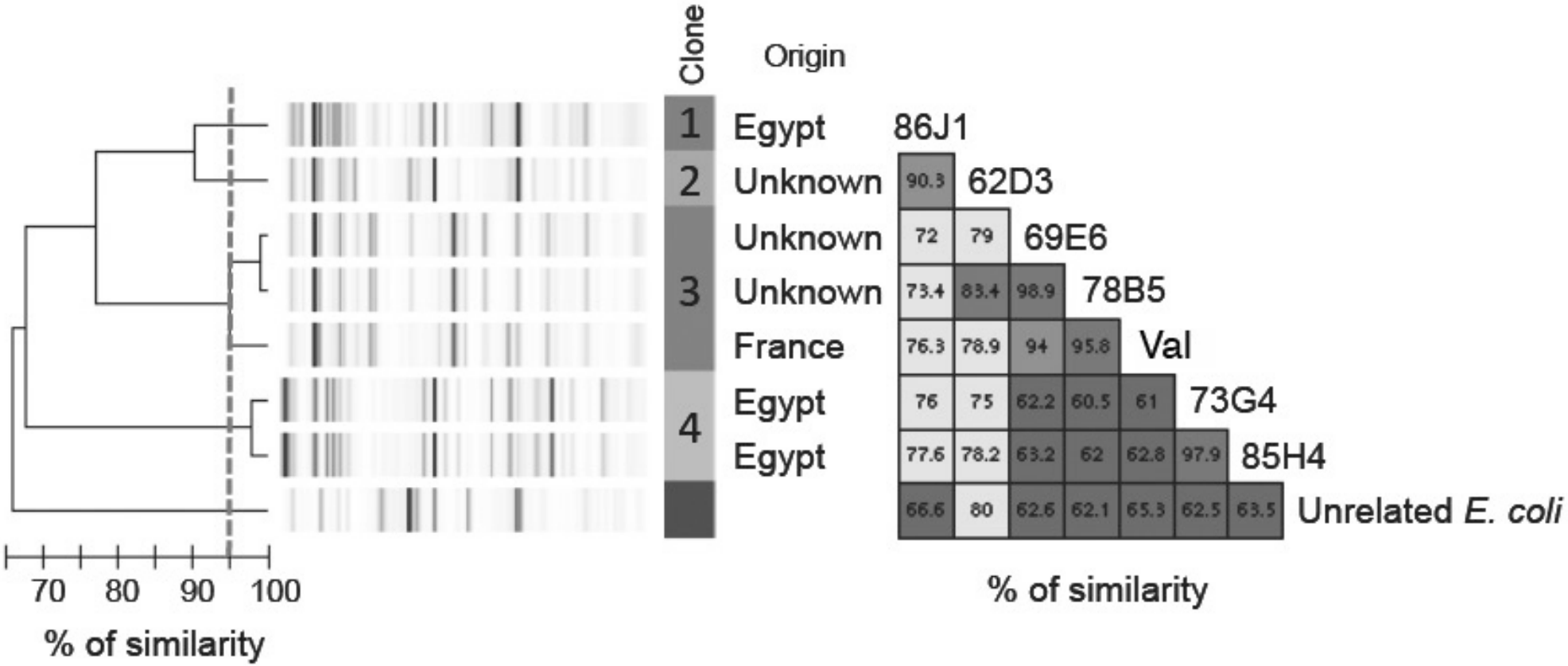
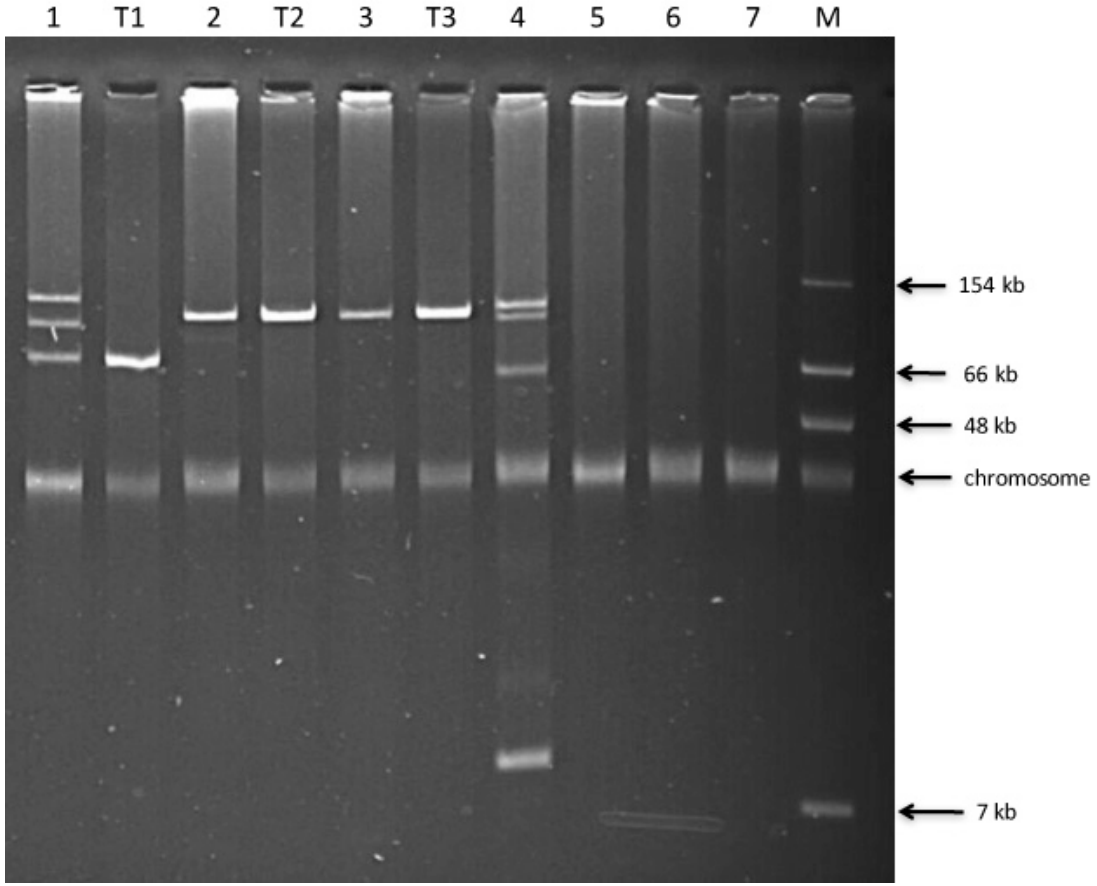
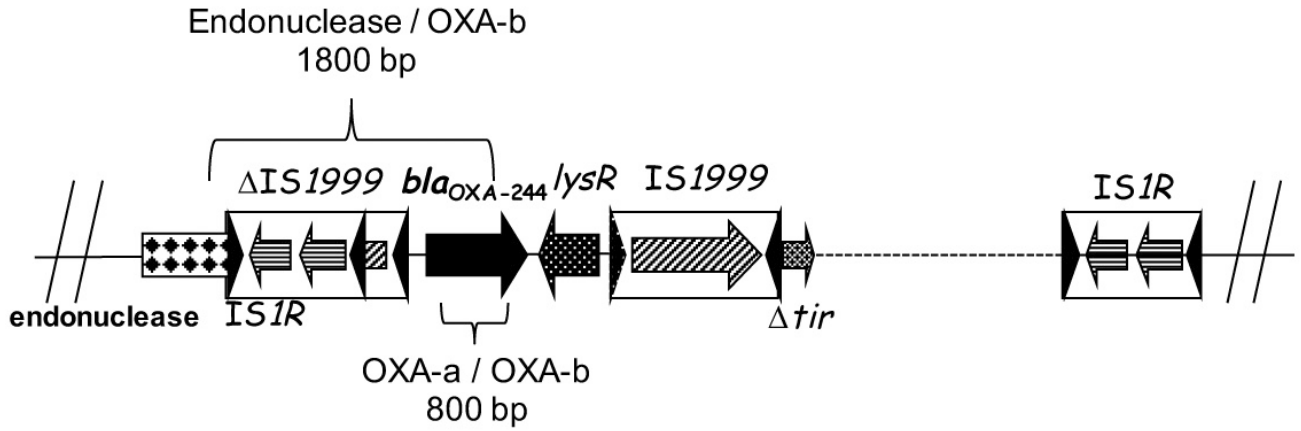

Clone
Origin
1
Egypt
86J1
2
Unknown
90.3
62D3
Unknown
72
79
69E6
3
Unknown
73.4
83.4
98.9
78B5
France
76.3
78.9
94
95.8
Val
4
Egypt
76
75
62.2
60.5
61
73G4
Egypt
77.6
78.2
63.2
62
62.8
97.9
85H4
66.6
80
62.6
62.1
65.3
62.5
63.5
Unrelated E. coli
70
80
90
100
% of similarity
% of similarity

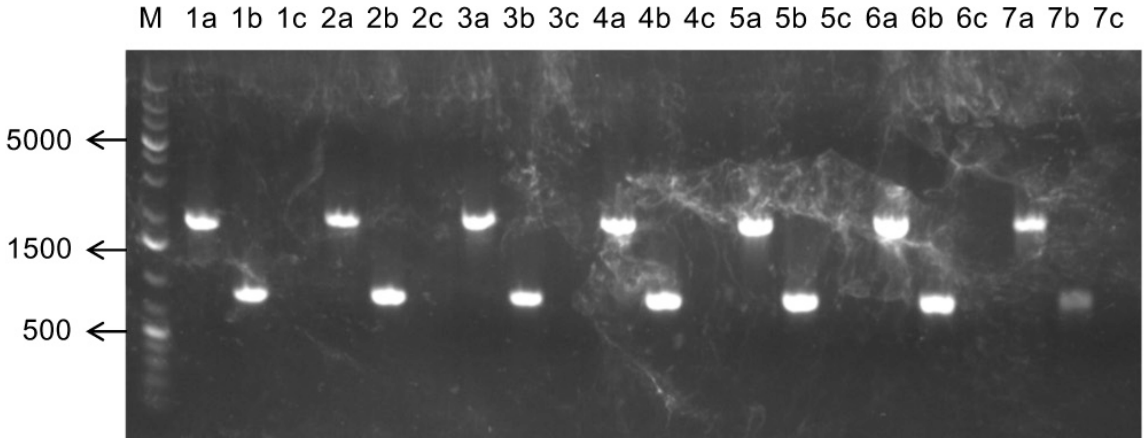
Fig. 1



Sup Fig. 1


Endonuclease / OXA-b
1800 bp
ΔIS1999
bla OXA-244
lysR
IS1999
IS1R
endonuclease
IS1R
Δtir
OXA-a / OXA-b
800 bp

(A)


M 1a 1b 1c 2a 2b 2c 3a 3b 3c 4a 4b 4c 5a 5b 5c 6a 6b 6c 7a 7b 7c
5000
1500
500

(B)

Sup. Fig. 2