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

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26 **ABSTRACT (119)**

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

38 INTRODUCTION

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

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

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

62 In August 2015, a 44-year-old Egyptian man was admitted to the Bicêtre
63 Hospital (Le Kremlin-Bicêtre, France) for an episode of erysipela of his right leg, that
64 was treated IV with amoxicillin. After two weeks, the patient was discharged with a
65 favorable outcome. As this patient was considered at risk for MDR bacterial carriage
66 according-to French CPE guidelines (repatriated patient), rectal swabs were plated on
67 ChromID[®] CARBA SMART medium (bioMérieux, La Balme-les-Grottes, France) a
68 selective chromogenic bi-plate for the screening of CPE and ChromID[®] ESBL
69 (Biomérieux) a chromogenic plate for the screening of extended spectrum β -
70 lactamase (ESBL)-producing Enterobacteriaceae. The ChromID[®] CARBA SMART
71 medium remained sterile, while an *E. coli* 85H4 grew on the ChromID[®] ESBL plate
72 (Table 1). Antimicrobial susceptibilities as determined by the disk diffusion technique
73 on Mueller-Hinton agar (BioRad, Marnes-La-Coquette, France) and interpreted
74 according to the EUCAST breakpoints as updated in 2016 (<http://www.eucast.org>),
75 revealed that *E. coli* 85H4 isolate was highly resistant to temocillin (absence of
76 inhibition diameter) and displayed a reduced susceptibility to ertapenem (19 mm
77 diameter), thus requiring confirmatory testing for carbapenemase production, as
78 recommended by EUCAST (9). The RAPIDEC[®] CARBA NP (bioMérieux) was
79 positive for *E. coli* 85H4, despite that no colony grew on ChromID[®] CARBA
80 SMART plate (10). In house PCR/sequencing, as previously described (11) revealed
81 the presence of a gene that codes for OXA-244, a R214G OXA-48 variant (Table 1)
82 (4). Since OXA-244-*Ec* 85H4 also produced an ESBL, ChromID[®] ESBL medium
83 was used to screen the 34 contact-patients of the Egyptian patient. For 4 *patients*
84 positive *E. coli* cultures on ChromID[®] ESBL medium were obtained. Antibiotic
85 susceptibility testing and in house PCR was performed on five independent colonies,
86 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*_{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).

Whole genome sequencing (WGS) was performed to determine the resistome of these OXA-244-*Ec* isolates using the Resfinder server (<http://cge.cbs.dtu.dk/services/ResFinder-2.1/>) (17) (Table 2). A good correlation between the genetic profile and the phenotypic resistance profile for routine tested β -lactams, colistin, fosfomycin, phenicol, sulphonamide/trimethoprim, and tetracycline antibiotics was found (Table 2). For aminoglycosides only netilmicin, amikacin, tobramycin and gentamicin were tested. However, different aminoglycoside resistance genes were found in some of the isolates (eg: *aph3-1a*, *aph3-1b*, *aph6-1d*, *aadA1*), which confer resistance to other aminoglycosides that were not tested, as not clinically-relevant. Of note isolate OXA-244-*Ec* 86J1 was resistant to fluoroquinolones due to mutations in the Quinolone Resistance Determinant Region as revealed by RAST server analysis (rast.nmpdr.org) (18) and comparison with that of *K. pneumoniae* ATCC 13883 (GenBank DQ673325): codons 83 (Ser83-Leu); 87 (Asp87-Asn) for *gyrA* and in codons 80 (Ser80-Ile); 84 (Glu84-Gly) for *parC*, which are known to confer fluoroquinolone resistance.

Among the 7 isolates, only *E. coli* VAL had no other β -lactam resistance gene besides *bla*_{OXA-244} gene, consequently this strain was susceptible to cephalosporins (7). For the remaining 6 isolates an ESBL gene (*bla*_{CTX-M-14}, *bla*_{CTX-M-27}) or a plasmid-encoded cephalosporinase gene (*bla*_{CMY-42}, *bla*_{CMY-2}) were always associated with *bla*_{OXA-244} gene (Table 2).

Genetic relatedness of OXA-244-*Ec* isolates. The seven OXA-244-*Ec* isolates corresponded to four different clones as revealed by rep-PCR using the DiversiLab[®] system (bioMérieux) following the manufacturer's recommendations. Two distinct clones were identified among the Egyptian isolates (Figure 1, Table 1). 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*_{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*_{AMPC} genes were found in these transformants. Thus, all these findings suggest a chromosomal location of the *bla*_{OXA-244} gene. PCR mapping of *bla*_{OXA-244} gene flanking sequences showed that all were bracketed by two IS1R copies forming an IS1R-made composite transposon, named Tn51098 (Supplemental Figure 2A). In all isolates, even though belonging to different Rep-PCR patterns or ST-types, Tn51098 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*_{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*_{OXA-244} carbapenemase gene into the chromosome were found, supporting that diffusion could be more related to the mobility of *bla*_{OXA-244}-carrying IS1R-made composite transposons, Tn51098, 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

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

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

In France OXA-244-producing *E. coli* are still rare (0%, 0.3% (2 isolates), 0.2% (2 isolates) and 0.6% (6 isolates), 0.7% (8 isolates) of OXA-48-like carbapenemases in 2012, 2013, 2014 and 2015, 2016 respectively), but it is still not clear whether this is a real low prevalence or simply the result of under-detection.

Nucleotide sequence accession number. The *E. coli* genome sequences of isolates 86J1, 62D3, 69E6, 78B5, 35J9, 73G4, and 85H4 used in this study were deposited under following Genbank accession numbers [MKGU00000000](#), [MKGY00000000](#), [MKGZ00000000](#), [MKGT00000000](#), [MKGX00000000](#), [MKGV00000000](#), [MKGW00000000](#) respectively.

186

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193

194 **TRANSPARENCY DECLARATION**

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

197

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294 Antimicrob Agents **41**:325–329.

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	+	+	+	+	-	+ -
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298

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

300 ^b Rep-PCR analysis by using the Diversilab technique

301 ^c IMP = imipenem, MEM = Meropenem, ETP = Ertapenem TEM = Temocilin, MOX = Moxalactam

302 ^d + = positive test, - = negative test, +/- = equivocal test

303 ^e + = positive culture, - = negative culture

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

306 ^g NT: Not tested

307

308

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	<i>bla</i> _{OXA-244} <i>bla</i> _{TEM-1b} <i>bla</i> _{CMY-42}	<i>aph3-1b</i> <i>aph6-1d</i> <i>aadA1</i>	-	-	-	-	<i>sul1</i> <i>sul2</i>	<i>dfrA1</i>	<i>tetB</i>	R	R	R	S	S	S	S	R	R	R
62D3	<i>bla</i> _{OXA-244} <i>bla</i> _{CMY-2}	-	-	-	-	-	-	-	-	R	R	R	S	S	S	S	S	S	S
69E6	<i>bla</i> _{OXA-244} <i>bla</i> _{TEM-1b} <i>bla</i> _{CTX-M-14b}	<i>aadA1</i>	-	-	<i>catA1</i>	-	-	<i>dfrA1</i>	-	R	R	R	S	S	S	R	S	R	S
78B5	<i>bla</i> _{OXA-244} <i>bla</i> _{TEM-1b} <i>bla</i> _{CTX-M-14b}	<i>aadA1</i>	-	-	<i>catA1</i>	-	-	<i>dfrA1</i>	-	R	R	R	S	S	S	R	S	R	S
VAL (4)	<i>bla</i> _{OXA-244} <i>bla</i> _{TEM-1b}	<i>aph3-1b</i> <i>aph6-1d</i> <i>aadA1</i>	-	-	<i>catA1</i>	-	<i>sul2</i>	<i>dfrA1</i> <i>dfrA14</i>	<i>tetB</i> <i>tetD</i>	R	R	S	S	S	S	R	S	R	R
73G4	<i>bla</i> _{OXA-244} <i>bla</i> _{TEM-1b} <i>bla</i> _{CTX-M-27}	<i>aph3-1b</i> <i>aph6-1d</i> <i>aph3-1a</i>	-	-	-	-	<i>sul2</i>	<i>dfrA14</i>	<i>tetb</i>	R	R	R	S	S	S	S	S	R	R
85H4	<i>bla</i> _{OXA-244} <i>bla</i> _{TEM-1b} <i>bla</i> _{CTX-M-27}	<i>aph3-1b</i> <i>aph6-1d</i> <i>aph3-1a</i>	-	-	-	-	<i>sul2</i>	<i>dfrA14</i>	<i>tetb</i>	R	R	R	S	S	S	S	S	R	R

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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

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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*_{CMY-42} gene; Lane 2 *E. coli* 85H4; Lane T2 *E. coli* TOP10 harboring plasmid 85H4 with *bla*_{CTX-M 27}; Lane 3 *E. coli* 73G4; Lane T3 *E. coli* TOP10 harboring plasmid 73G4 with *bla*_{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 brackets. **(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*

343 73G4; Lane 4 *E. coli* 35J9; Lane 5 *E. coli* 78B5; Lane 6 *E. coli* 69E6; Lane 7 *E. coli* 62D3;
344 Lane M GeneRuler 1kb Plus DNA Ladder (Thermo Scientific).

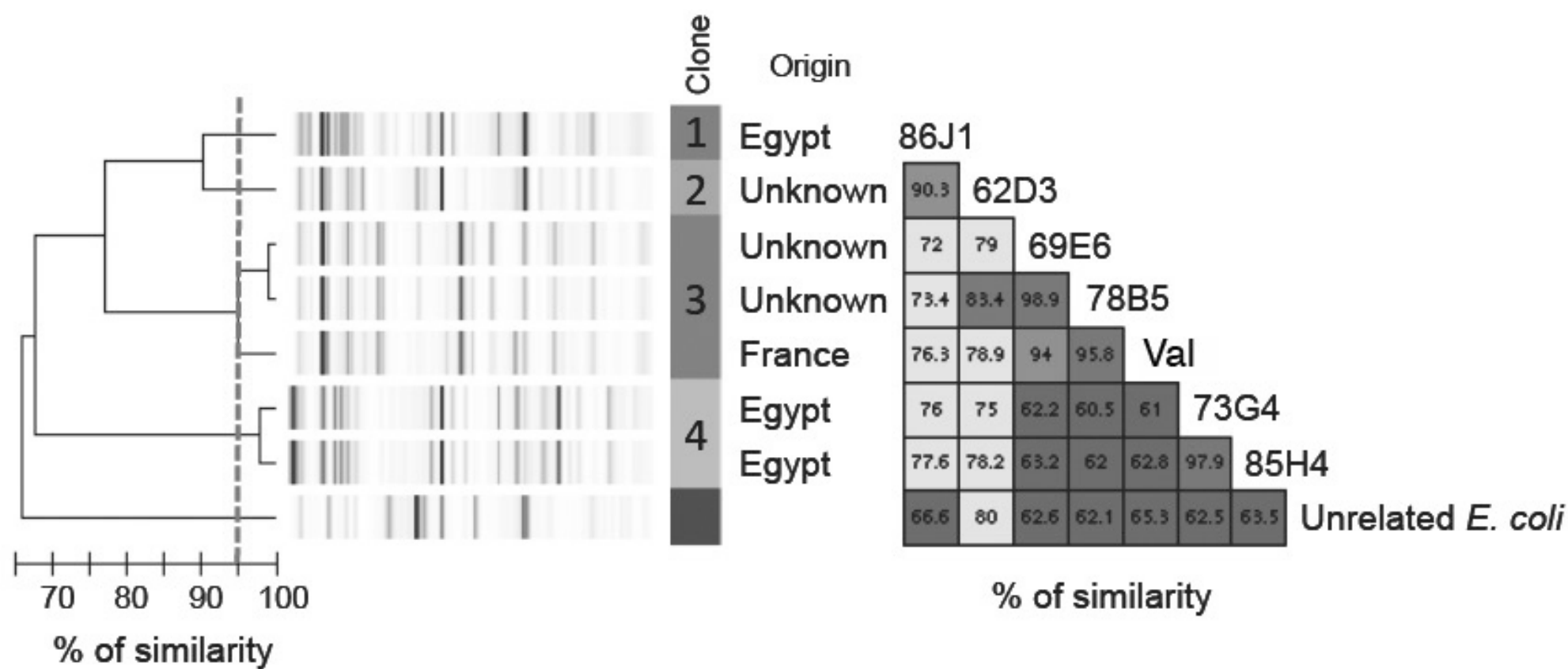
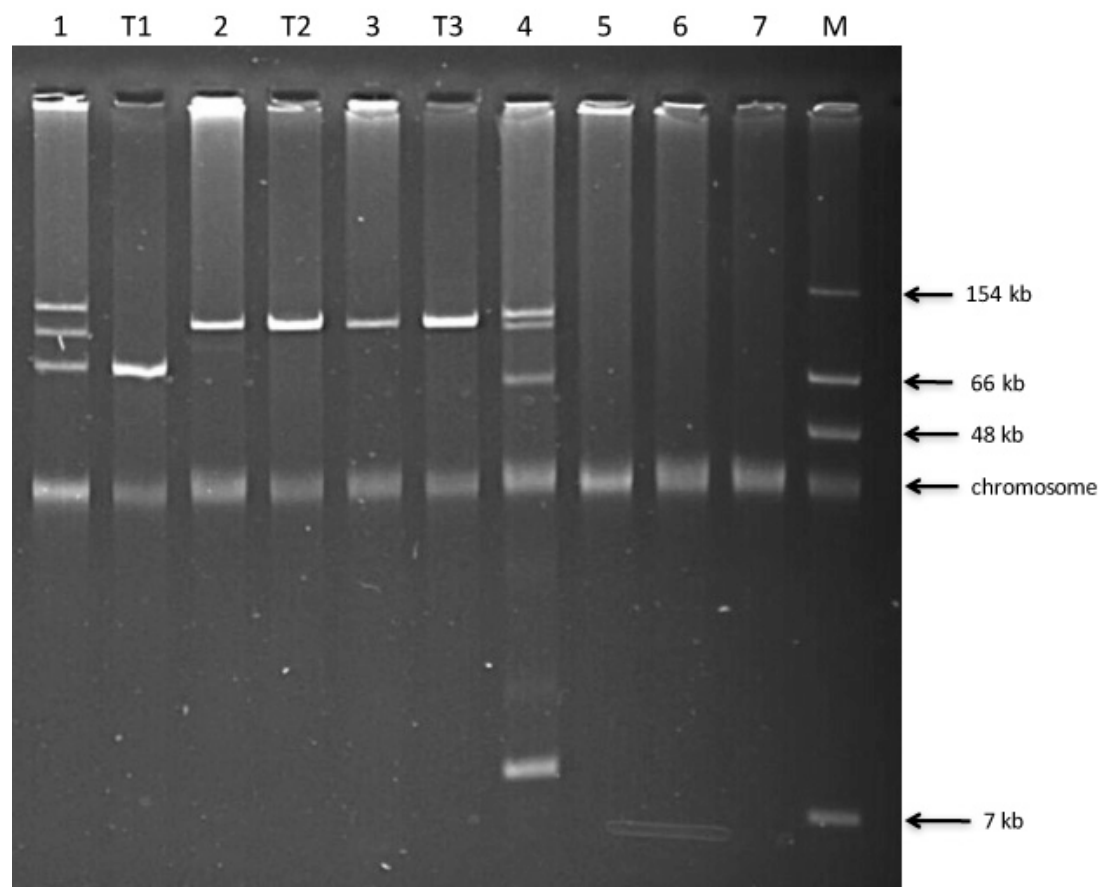
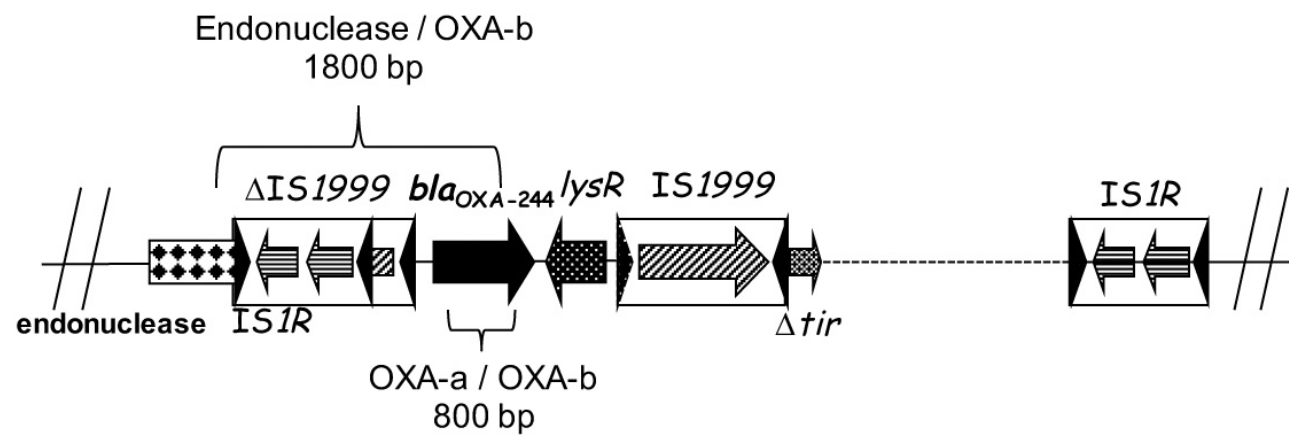


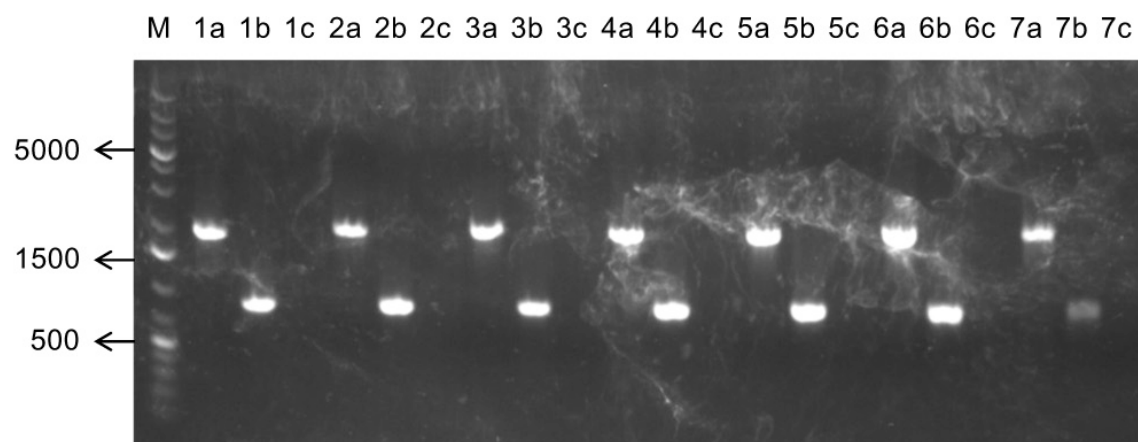
Fig. 1



Sup Fig. 1



(A)



(B)