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► To cite this version:

Thierry Naas, Chafia Bentchouala, Gaelle Cuzon, Sanàa Yaou, Abdesselam Lezzar, et al.. Outbreak of serotype Infantis producing ArmA 16S RNA methylase and CTX-M-15 extended spectrum β -lactamase in a neonatology ward in Constantine, Algeria. *International Journal of Antimicrobial Agents*, 2011, 38 (2), pp.135. 10.1016/j.ijantimicag.2011.04.012 . hal-00711302

HAL Id: hal-00711302

<https://hal.science/hal-00711302>

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PII: S0924-8579(11)00203-2
DOI: doi:10.1016/j.ijantimicag.2011.04.012
Reference: ANTAGE 3615

To appear in: *International Journal of Antimicrobial Agents*

Received date: 18-12-2010
Revised date: 11-4-2011
Accepted date: 13-4-2011

Please cite this article as: Naas T, Bentchouala C, Cuzon G, Yaou S, Lezzar A, Smati F, Nordmann P, ¹Outbreak of *Salmonella enterica* serotype Infantis producing ArmA 16S RNA methylase and CTX-M-15 extended spectrum β -lactamase in a neonatology ward in Constantine, Algeria, *International Journal of Antimicrobial Agents* (2010), doi:10.1016/j.ijantimicag.2011.04.012

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Outbreak of *Salmonella enterica* serotype Infantis producing ArmA 16S RNA methylase and CTX-M-15 extended spectrum β -lactamase in a neonatology ward in Constantine, Algeria [☆]

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ARTICLE INFO

Article history:

Received 18 December 2010

Accepted 13 April 2011

Keywords:

Methylase

Outbreak

CTX-M-15

β -Lactamase

Salmonella

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☆ This work was presented in part at the 50th Interscience Conference on

Antimicrobial Agents and Chemotherapy (ICAAC), 12–15 September 2010, Boston,

MA.

Accepted Manuscript

ABSTRACT

Plasmid-mediated 16S rRNA methylases such as ArmA, which confer high levels of resistance to aminoglycosides, are increasingly reported in Enterobacteriaceae. This study investigated the molecular mechanism of β -lactam and aminoglycoside resistance in extended-spectrum β -lactamase (ESBL)-producing *Salmonella enterica* serotype Infantis isolated at the 53-bed neonatology ward of University Hospital Benabib in Constantine, Algeria. From September 2008 to January 2009, 200 *S. enterica* isolates were obtained from 138 patients (age range 8–80 months) hospitalised in the neonatology ward. Most isolates were from stool cultures, but also from two blood cultures and one gastric fluid. The isolates were multidrug-resistant and produced TEM-1 and CTX-M-15 enzymes as well as the 16S RNA methylase ArmA. The *armA*, *bla*_{CTX-M-15} and *bla*_{TEM-1} genes were located on the same 140-kb self-transferable plasmid belonging to the IncL/M incompatibility group. All of the *S. Infantis* isolates belonged to a single clone. Increased infection control measures and thorough biodecontamination of the rooms led to control of the outbreak but did not eradicate the epidemic strain. This study further illustrates the global emergence of ArmA methylase and its frequent association with *bla*_{CTX-M} genes. Spread of 16S RNA methylase determinants at the same level as *bla*_{CTX-M} genes in Enterobacteriaceae may seriously compromise the efficacy of aminoglycosides for treating Gram-negative infections.

1. Introduction

Since 2003, high levels of resistance to aminoglycosides have been reported, conferred by plasmid-mediated 16S rRNA methylases [1]. To date, seven enzymes (ArmA, RmtA, RmtB, RmtC, RmtD, RmtE and NpmA) are known, with ArmA being the most frequently identified in Enterobacteriaceae [2]. The *armA* gene is of clinical importance since it confers high-level resistance to almost all clinically available aminoglycosides, except 4,5-disubstituted deoxystreptamines. Associations between 16S rRNA methylase- and extended-spectrum β -lactamase (ESBL)-encoding genes such as the *bla*_{CTX-M} genes have been reported [2–6]. In 2004, an outbreak of CTX-M-producing *Salmonella enterica* serotype Senftenberg isolates associated with nosocomial diarrhoea in newborns was described in the neonatology unit at University Hospital Benabib (UHB) in Constantine, Algeria [7]. These isolates remained susceptible to imipenem, ertapenem, cefoxitin, nalidixic acid, fluoroquinolones and colistin only [7]. Subsequently, a study aimed to evaluate the prevalence of 16S RNA methylase genes among 12 non-duplicate ESBL-producing *S. Senftenberg* isolates resistant to aminoglycosides recovered in the neonatology ward from January 1982 to December 2005 revealed a CTX-M-3-producing *S. Senftenberg* isolate from 1998, resistant to all aminoglycosides and found to be *armA*-positive [8]. The *bla*_{CTX-M-3} and *armA* genes were harboured on the same plasmid.

The aim of this study was to analyse the molecular mechanism of β -lactam and aminoglycoside resistance in ESBL-producing *S. enterica* serotype Infantis isolates responsible of an outbreak at UHB.

2. Materials and methods

2.1. Bacterial isolates

Salmonella enterica isolates were obtained from patients hospitalised in the 53-bed neonatology ward at UHB. Isolates collected from September 2008 to January 2009 were identified using an API 20E system (bioMérieux, Marcy-l'Étoile, France) and were serotyped on the basis of somatic O and phase 1 and phase 2 H flagellar antigens by agglutination tests with antisera (Bio-Rad, Marnes-la-Coquette, France) as specified by the White–Kauffmann–Le Minor scheme [9].

2.2. Antimicrobial agents and susceptibility testing

Routine antibiograms were determined by the disk diffusion method on Mueller–Hinton agar (Bio-Rad) and were interpreted according to Clinical and Laboratory Standards Institute (CLSI) criteria [10]. The presence of ESBLs was inferred by a synergy image using the double-disk synergy test performed with cefotaxime or ceftazidime and ticarcillin/clavulanic acid disks [11]. Minimum inhibitory concentrations (MICs) of β -lactams and aminoglycosides were determined by Etest (AB BIODISK, Solna, Sweden) performed on Mueller–Hinton agar plates (Oxoid Ltd., Basingstoke, UK) with incubation at 37 °C for 24 h as recommended by the manufacturer.

2.3. Resistance genes

Genes coding for Ambler class A β -lactamases were sought by polymerase chain reaction (PCR) using primers specific for the *bla*_{TEM}, *bla*_{SHV} and *bla*_{CTX-M} genes as

described previously [8,12]. Similarly, isolates were screened by PCR for detection of 16S rRNA methylase-encoding genes (*armA*, *rmtA*, *rmtB*, *rmtC* and *rmtD*) as described previously [2]. PCR products were purified using a QIAquick PCR Purification Kit (QIAGEN, Courtaboeuf, France) and were sequenced on both strands with an ABI PRISM[®] 3100 Automated Sequencer (Applied Biosystems, Foster City, CA). The nucleotide and deduced amino acid sequences were analysed using software available on the Internet (<http://www.ncbi.nlm.nih.gov/>).

2.4. Pulse-field gel electrophoresis (PFGE)

PFGE was performed with *Xba*I-restricted whole-cell DNA embedded in 1% agarose plugs and separated in a 1% pulsed field-certified agarose gel using a CHEF DRII System (Bio-Rad) as described previously [13,14]. The banding pattern was acquired using the Gel Doc[™] System (Bio-Rad) and pulsotypes were assigned by eye, especially since isolates displayed all the same banding patterns.

2.5. Plasmid analysis and mating out experiments

Mating experiments were performed using azide-resistant *Escherichia coli* J53 recipient strains as previously described [8]. Transconjugants were selected on trypticase soy (Bio-Rad) agar plates containing sodium azide (100 mg/L) and cefotaxime (1 mg/L) and on trypticase soy agar plates containing sodium azide (100 mg/L) and amoxicillin (100 mg/L).

Natural plasmids were extracted using the Kieser extraction method [15]. Plasmid extracts were subsequently analysed by electrophoresis on a 0.7% agarose gel. *Escherichia coli* 50192 was used as a reference strain for plasmid extraction [16].

2.6. Replicon typing and genetic environment of resistance genes

PCR-based replicon typing of the main plasmid incompatibility groups reported in Enterobacteriaceae was performed as described previously [17]. The *armA* genetic environment was investigated by PCR as previously described [3,6,8].

3. Results

3.1. Clinical data

Between September 2008 and January 2009, 200 *S. enterica* isolates were obtained from 138 patients hospitalised in the 53-bed neonatology ward at UHB (Fig.1).

All of the isolates belonged to serotype Infantis and no other serotype was isolated during the study period in the neonatology ward. Patient age ranged from 8 months to 80 months (mean 54 months). Most of isolates were found in stool cultures, but two newborns had positive blood cultures and another had positive gastric fluid. Patients with diarrhoea without signs of severity of infection received oral colistin therapy, whereas the two septic patients received imipenem and amikacin treatment. None of the patients died of their infection.

3.2. Antibiotic susceptibility

Disk diffusion antibiograms revealed that all but two of the *S. Infantis* isolates were resistant to all β -lactams (except cephamycins and carbapenems), kanamycin, netilmicin, tobramycin, amikacin, gentamicin, rifampicin and trimethoprim/sulfamethoxazole. The isolates remained susceptible to nalidixic acid, all the fluoroquinolones, tetracycline, chloramphenicol, colistin and fosfomycin. Presence of an ESBL was suspected by a synergy image using the double-disk synergy test performed with cefotaxime or ceftazidime and ticarcillin/clavulanic acid disks. MICs of β -lactams confirmed the presence of an ESBL with high-level resistance to ceftazidime and cefotaxime and revealed a high level resistance to aminoglycosides.

3.3. Resistance genes

For further molecular analysis, 16 *S. Infantis* isolates were randomly chosen throughout the study period along with one *S. Infantis* isolate susceptible to all β -lactams (*S. Infantis* 1). *Salmonella* Senftenberg 1998, isolated in the same hospital in 1998 and known to be CTX-M-3- and ArmA-positive, was used as a control (Table 1) [8]. The *S. Infantis* isolates were positive for *armA* 16S rRNA methylase gene, *bla*_{CTX-M-like} and *bla*_{TEM-like} genes. Sequencing of the amplified fragments confirmed the presence of *bla*_{CTX-M-15} and *bla*_{TEM-1} genes. Isoelectric focusing performed as described previously confirmed that in addition to CTX-M-15 [isoelectric point (pI) of 8.6], TEM-1 (pI 5.4) was also expressed (data not shown). PCR mapping of the region containing the *bla*_{CTX-M-15} gene showed its association with insertion sequence *ISEcp1* as previously described (Fig. 2A) [18]. Sequencing of the 16S rRNA methylase-encoding genes showed identity with previously reported *armA* genes.

PCR mapping of the region containing the *armA* gene showed its association with ISCR1 inside a *sul1*-type integron structure, with the same configuration observed in Tn 1548 (Fig. 2B) [1,6]. Tn 1548 typically contains additional genes conferring resistance to azithromycin, streptomycin–spectinomycin, sulfonamides and trimethoprim [6].

3.4. Genetic support

Plasmid extraction and conjugation assays with an azide-resistant recipient *E. coli* J53 strain performed as described previously revealed a 140-kb self-transferable plasmid in all strains (frequency of transfer 1×10^{-6}). The 16S rRNA methylase and ESBL determinants were co-transferred by conjugation [8]. The 16S rRNA methylase-positive transformants and transconjugants displayed the same antimicrobial resistance profile and expressed high-level resistance to aminoglycosides (Fig. 3; Table 2). The *armA*, *bla*_{TEM-1} and *bla*_{CTX-M-15} genes were located on the same plasmid belonging to the IncL/M incompatibility group, as observed for plasmid pCTX-M-3 [8]. No additional resistance marker was identified according to results of routine antibiograms performed with the transconjugants and testing the same antibiotics as for the parental strains.

3.5. Genetic relatedness

One *S. Infantis* isolate per patient was typed by PFGE. All of the isolates, including those displaying a multisusceptible phenotype, were genetically related as revealed by similar PFGE patterns (data not shown). *Salmonella* Infantis 1, which was one of the two susceptible isolates, was ESBL- and ArmA-negative by PCR and lacked the

plasmid, suggesting that *S. Infantis* isolates may have acquired the plasmid or that *S. Infantis* 1 may have lost it. *Salmonella* Senftenberg isolates could be the source of this plasmid, since similar plasmids have been found in that species, with the exception of CTX-M-3, whilst *S. Infantis* expressed CTX-M-15.

4. Discussion

Implementing and strengthening standard hygiene procedures, infection control measures (isolation precautions, chlorhexidine hand-washing and barrier protections) and thorough biodecontamination of rooms of colonised patients led to control of the outbreak but did not eradicate the epidemic strain, as revealed by sporadic cases since January 2009 (Fig. 1). Persistence of this strain is probably a consequence of re-hospitalisation of colonised patients in the ward, but since staff members were not screened for colonisation during that period they might also be a possible source of re-introduction of the strain into the ward.

This is the first report of an outbreak involving Arm-type 16S rRNA methylase enzymes in Algeria and from *S. Infantis*. CTX-M-producing enterobacterial isolates are increasingly documented worldwide as well as in Algeria [7,8,18–22]. In Algeria, ESBL-producing *Salmonella* isolates have also been described [7,8,22]. However, this is the first description of an outbreak involving *S. Infantis* producing CTX-M-15 and ArmA. ArmA has recently been identified in non-Typhi *Salmonella* isolates from the USA and from a patient directly transferred from India and from Bulgaria [5,6]. In the latter case, the *armA* gene was associated with a broad-host-range IncL/M conjugative plasmid and was linked to *bla*_{TEM-1} and *bla*_{CTX-M-3}, which confer resistance to all β -lactams with the exception of carbapenems, and also to the *aac3*, *ant3*"9,

sul1 and *dfxII* genes [6]. Similarly, in Algeria ArmA was identified in a *S.* Senftenberg isolate from the hospital of Constantine in 1998, located on an IncL/M conjugative plasmid also linked to *bla*_{TEM-1} and *bla*_{CTX-M-3} [8]. Here, 12 years later, a similar plasmid is still present in the neonatology ward, but in another *Salmonella* serovar, and the CTX-M-gene has evolved into a CTX-M-15 variant, which also includes ceftazidime in its substrate profile. This shift from CTX-M-3 to CTX-M-15, a more potent enzyme, has been observed on several occasions and illustrates the trend towards an even broader resistance profile [18].

This study underlines the association on the same plasmid of 16S rRNA methylase- and CTX-M-15-encoding genes. Their localisation on the same conjugative plasmid is worrying since it has the potential to further expand the threat of multidrug-resistant *Salmonella*. Furthermore, this plasmid could be present in the neonatology ward for more than 12 years, in different *Salmonella* serovars, suggesting exchange of this plasmid between different serovars and likely between species. Fine mapping or complete sequencing of the plasmid could confirm this hypothesis. A spread of these aminoglycoside resistance determinants at the same level as *bla*_{CTX-M} genes in Enterobacteriaceae may seriously compromise the efficacy of aminoglycosides for treating Gram-negative infections. The prevalence of 16S rRNA methylase determinants among ESBL-producers is still low, ranging from 0.03% in Japan to 3% in South Korea, but may be as high as 10% in China [2,4,23,24].

Finally, this work further underlines that *Salmonella* spp. may be a reservoir for ESBL genes and for 16S rRNA methylase genes in the community and that these strains may then be responsible for hospital-acquired infections.

Funding

This work was funded by INSERM (France), by a grant from the Ministère de l'Éducation Nationale et de la Recherche (UPRES-EA3539), Université Paris XI (Paris, France), by the Assistance Publique-Hôpitaux de Paris (France) and by the European Community's Seventh Framework Programme FP7/2007-2013 under grant agreement no. 241742.

Competing interests

None declared.

Ethical approval

Not required.

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Fig. 1. Epidemic curve of the outbreak between September 2008 and January 2009.

Fig. 2. Schematic representation of the genetic environments of (A) *bla*_{CTX-M-15} and (B) *armA* genes. Genes and insertion sequences are indicated as boxes with an arrow indicating the translational orientation. *tnpU* and *tnpD* are putative transposase genes. Small arrows indicate the primers used for polymerase chain reaction (PCR) mapping.

Fig. 3. Plasmid analysis and Southern hybridisation using an *arm* probe: (A) plasmid extraction by the Kieser method [15]; and (B) Southern hybridisation carried out with an internal probe for *armA*. Lanes 1–16, *Salmonella enterica* serotype Infantis as in Table 1; lane 17, *Escherichia coli* 50192 harbouring four plasmids (7, 48, 66 and 154 kb); lane 18, *Salmonella enterica* serotype Senftenberg [8].

Table 1*Salmonella* isolates used in this study

No.	Bacteria	Date of isolation	Sample	Reference
1	S. Infantis 1	18 Sept. 2008	Stool	This study
2	S. Infantis 2	18 Sept. 2008	Blood culture	This study
3	S. Infantis 3	18 Sept. 2008	Gastric fluid	This study
4	S. Infantis 4	28 Sept. 2008	Stool	This study
5	S. Infantis 5	30 Sept. 2008	Stool	This study
6	S. Infantis 6	8 Oct. 2008	Stool	This study
7	S. Infantis 7	14 Oct. 2008	Stool	This study
8	S. Infantis 8	14 Oct. 2008	Stool	This study
9	S. Infantis 9	22 Oct. 2008	Stool	This study
10	S. Infantis 10	23 Oct. 2008	Stool	This study
11	S. Infantis 11	28 Oct. 2008	Stool	This study
12	S. Infantis 12	14 Nov. 2008	Stool	This study
13	S. Infantis 13	19 Nov. 2008	Stool	This study
14	S. Infantis 14	25 Nov. 2008	Stool	This study
15	S. Infantis 15	5 Dec. 2008	Stool	This study
16	S. Infantis 16	9 Jan. 2009	Stool	This study
17	S. Senftenberg 1998	1998	Stool	[8]

Table 2

Minimum inhibitory concentrations (MICs) of antibiotics for *Salmonella* clinical isolates producing 16S rRNA methylases, its corresponding *armA*-positive transformant, and wild-type *Escherichia coli*^a

Bacterial isolates	MIC (mg/L)							
	AMK	NET	GEN	TOB	CTX	CAZ	IPM	CIP
<i>S. Senftenberg</i> 1998 ^b	>256	>256	>256	>256	>32	3	0.19	0.003
<i>E. coli</i> (pS98) ^c	>256	>256	>256	>256	16	1	0.19	0.002
<i>S. Infantis</i> 3 ^d	>256	>256	>256	>256	>32	>32	0.19	0.003
<i>E. coli</i> (p3) ^e	>256	>256	>256	>256	>32	>32	0.19	0.002
<i>E. coli</i> TOP10 ^f	1	0.25	0.25	0.38	0.05	0.38	0.19	0.002

AMK, amikacin; NET, netilmicin; GEN, gentamicin; TOB, tobramycin; CTX, cefotaxime; CAZ, ceftazidime; IPM, imipenem; CIP, ciprofloxacin.

^a MICs of antibiotics were determined by Etest according to the manufacturer's recommendations.

^b The clinical isolate was also resistant to sulfonamides and trimethoprim [8].

^c MICs for *E. coli* TOP10 transformants harbouring pS98 expressing ArmA and CTX-M-3 [8].

^d Clinical isolates were also resistant to sulfonamides and trimethoprim.

^e MICs for *E. coli* TOP10 transformants harbouring p5086 expressing ArmA and CTX-M-15.

^f MICs for *E. coli* TOP10.

Figure 1

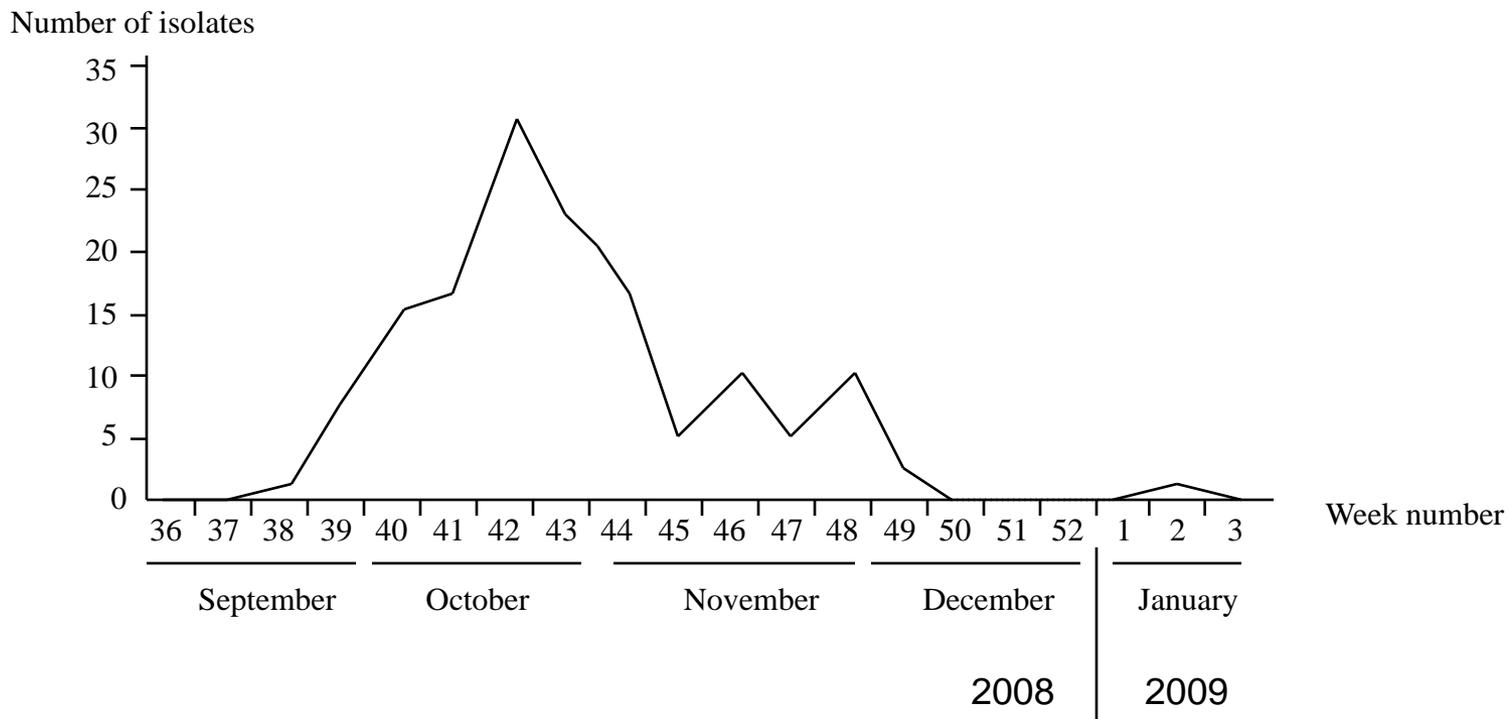


Figure 2

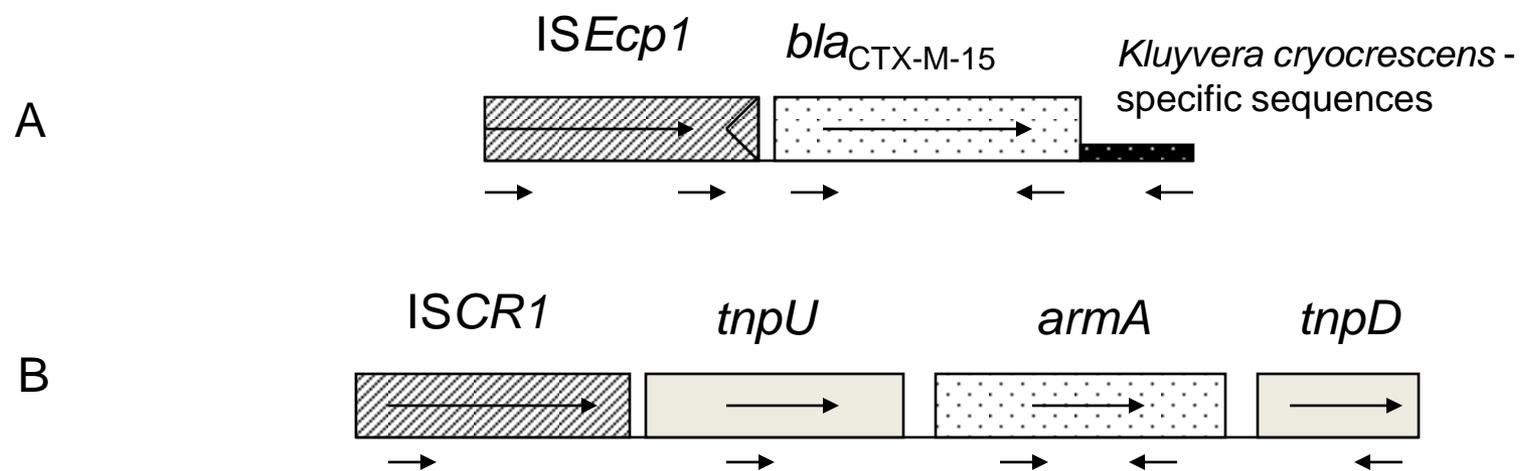
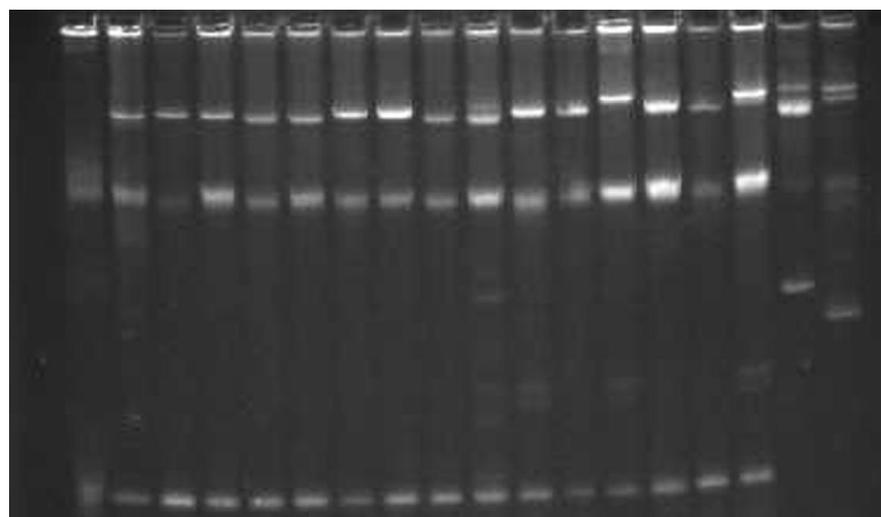


Figure 3

A

1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8
1 1 1 1 1 1 1 1 1



← 154 →
← 66 →
← 48 →
← Chr →
← 7 →

B

1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8
1 1 1 1 1 1 1 1

