Virulence-resistance plasmids (pUO-StVR2-like) in meat isolates of *Salmonella enterica* serovar Typhimurium

Ignacio Montero[a], Ana Herrero, M. Carmen Mendoza[a], Rosaura Rodicio[b], M. Rosario Rodicio[a]*

[a]*Department of Functional Biology, Laboratory of Microbiology, University of Oviedo, Oviedo, Spain*

[b]*Department of Biochemistry and Molecular Biology, University of Oviedo, Oviedo, Spain*

* *Author for correspondence*

Mailing address: Área de Microbiología, Facultad de Medicina, Universidad de Oviedo, Julián Clavería 6, 33006-Oviedo, Spain

Phone. +34985103562

Fax: +34985103148

e-mail: rrodicio@fq.uniovi.es

ABSTRACT

During a screening of *Salmonella enterica* in foods of animal origin four isolates of serovar Typhimurium carrying hybrid virulence-resistance plasmids were detected. Three of them, one from pork and two from chicken meat, contained pUO-StVR2, a derivative of the pSLT virulence plasmid with the *bla*$_{\text{OXA-1}}$, *catA1*, *aadA1*, *sulI* and *tet*(B) genes, which confer resistance to ampicillin, chloramphenicol, streptomycin-spectinomycin, sulfonamides and tetracycline, respectively. The fourth isolate harboured a pUO-StVR2 variant (termed ν8) with an additional *dfrA10* gene responsible for resistance to trimethoprim. This gene is part of the *orf513-dfrA10-qacEΔ1-sul1* element characteristically found in complex class 1 integrons. Pulsed-field gel electrophoresis identified two XbaI-BlnI combined profiles: X2-B2 generated from the three pUO-StVR2 isolates, and X12-B17 shown by the pUO-StVR2-ν8 isolate. The same profiles have also been found in clinical and ill pig isolates, supporting chicken and pork meat as vehicles for transmission of Typhimurium carrying pUO-StVR2-like plasmids.

Keywords: virulence-resistance plasmid, pSLT, resistance island, multidrug resistance, complex class 1 integron, plasmid evolution.

1. Introduction

Non-typhoid serovars of *Salmonella enterica* subspecies *enterica* are one of the leading causes of food-borne diseases worldwide. In humans they mainly cause a common gastroenteritis, which usually presents as a localized and self-limited infection, associated with intestinal inflammation and diarrhoea. Nevertheless, the bacteria can also spread beyond the intestine, leading to bacteraemia and focal extraintestinal infections, particularly in immuno-compromised hosts. Although usually not required, treatment with antimicrobials is essential for invasive salmonellosis, and for patients at risk of extraintestinal disease (Parry & Threlfall, 2008).

The emergence and spread of multidrug-resistant strains of *S. enterica* is threatening the application of reliable therapies (Parry & Threlfall, 2008). Many of the resistance genes are associated with mobile genetic elements, including the integron-gene cassette system, transposons and plasmids (Carattoli, 2003; Miriagou, Carattoli & Fanning, 2006). Of particular interest are the hybrid virulence-resistance plasmids, generated by combination of a serovar-specific virulence plasmid with antimicrobial resistance determinants (Chu *et al*., 2001; Guerra, Soto, Helmuth & Mendoza, 2002; Herrero, Rodicio, Echeita & Mendoza, 2008; Izumiya *et al*., 2011; Kingsley *et al*., 2009; Rodriguez, Guerra, Mendoza & Rodicio, 2011; Villa & Carattoli, 2005). One of these plasmids, designated pUO-StVR2, has been widely characterized in our laboratory. pUO-StVR2 (132 kb) is a conjugative derivative of pSLT, the virulence plasmid specific of *S. enterica* serovar Typhimurium. It differs from pSLT by the presence of a 47.6 kb fragment inserted between the *ccdAB* genes (toxin-antitoxin system) and the *pefI* gene (plasmid encoded fimbriae) of the virulence plasmid, replacing a 12 kb fragment of DNA located in between (Fig. 1; Herrero, Mendoza, Rodicio & Rodicio, 2008). The missing DNA comprises the IncFIB/*repA2* replicon, including the overlapping *rsk* locus (resistance to serum killing), and most of the *pef* operon. The

acquired DNA consists of a central antimicrobial resistance island of 28.8 kb, which confers resistance to ampicillin, chloramphenicol, streptomycin-spectinomycin, sulfonamides and tetracycline, encoded by *bla*$_{OXA-1}$, *catA1*, *aadA1*, *sul1* and *tet*(B), respectively. These genes are supplied by a class 1 integron, termed InH (*bla*$_{OXA-1}$, *aadA1*, *sul1*), carried by a Tn*21*-like transposon inserted within Tn*9* (*catA1*). The resulting composite transposon is in turn associated with a defective Tn*10* [*tet*(B)]. Flanking the resistance island, the conventional left (7.1 kb) and right (11.7 kb) regions provide a second toxin-antitoxin system (*vagCD*) and a putative iron uptake system, respectively, which could further increase the virulence of the strains harbouring this hybrid plasmid (Fig. 1; Herrero, Mendoza, Rodicio & Rodicio, 2008).

pUO-StVR2 has been found in clinical isolates of Typhimurium which are widespread in Spain and were also detected in the United Kingdom (Bances, Herrero, Gonzalez, Rodicio & Gonzalez-Hevia, 2007; Guerra, Soto, Helmuth & Mendoza, 2002; Herrero, Mendoza, Threlfall & Rodicio, 2009; Herrero, Rodicio, Echeita & Mendoza, 2008; Herrero, Rodicio, Gonzalez-Hevia & Mendoza, 2006). In the present study we report on the detection of pUO-StVR2-like plasmids in Typhimurium isolates from chicken and pork meat, and demonstrate the close relationship existing between these isolates and others found to be involved in human salmonellosis.

2. Materials and methods

2.1. Isolation of Typhimurium carrying pUO-StVR2-like plasmids

The Typhimurium pUO-StVR2-like isolates analyzed in this work (Table 1) were recovered during surveys of *S. enterica* in foods of animal origin for human consumption performed in Spain at the Laboratory of Microbiology of Oviedo University (LMO). Identification of the isolates used standard methods and serotyping

was done at the National Center of Microbiology (Madrid), acting as Spanish Reference Laboratory for *Salmonella* and *Shigella*. Typhimurium LT2 (pSLT; Liu, Hessel & Sanderson, 1993), and LSP 146/02 (pUO-StVR2; Herrero, Mendoza, Rodicio & Rodicio, 2008) were included as controls in different experiments.

2.2. Antimicrobial susceptibility

The isolates were tested for antimicrobial susceptibility by a disk diffusion technique, using commercially available disks (Oxoid, Madrid, Spain). The antimicrobials tested were ampicillin, chloramphenicol, ciprofloxacin, kanamycin, gentamicin, nalidixic acid, spectinomycin, streptomycin, sulfonamides, tetracycline, and trimethoprim. The method and interpretative criteria were according to CLSI (Clinical and Laboratory Standards Institute, 2009a, 2009b).

2.3. Plasmid analysis, Southern hybridization and conjugation

Plasmid DNA was extracted from Typhimurium strains by an alkaline lysis method, particularly suitable for visualization of large undigested plasmids (Kado & Liu, 1981), and sequentially hybridized with *spvC* and *bla*$_{\text{OXA-1}}$ probes, used as pUO-StVR2 virulence and resistance markers, respectively (Herrero, Rodicio, Echeita & Mendoza, 2008; Sambrook & Russell, 2001). The probes were obtained from LT2 (*spvC*) and LSP 146/02 (*bla*$_{\text{OXA-1}}$) by PCR amplification with the primers reported by Herrero, Rodicio, Echeita & Mendoza (2008), and the PCR DIG labelling mix (Roche Diagnostics, Barcelona, Spain), which includes digoxigenin-labelled dUTP in addition to the four dNTPs. The generated amplicons were purified with the GFX$^{\text{TM}}$ DNA and Gel Band Purification Kit (VWR, Barcelona, Spain), and hybridizing fragments were detected with the "DIG Nucleic Acid Detection Kit" (Roche). Plasmid DNA was also extracted

with the HiPure plasmid purification kit (Invitrogen, Barcelona, Spain), digested with different restriction enzymes, including SphI, and hybridized with the *bla*$_{OXA-1}$ probe. Conjugation experiments using each of the Typhimurium isolates carrying pUO-StVR2-like plasmids as donors and *Escherichia coli* K12 J53 (rifampicin-resistant) as recipient were performed as reported (Herrero, Rodicio, Echeita & Mendoza, 2008).

2.4. PCR procedures, cloning and sequencing

Resistance genes and pSLT-virulence genes were screened by PCR amplification using *E. coli* transconjugants as the source of the template DNA, and previously reported primers and conditions (Herrero, Rodicio, Echeita & Mendoza, 2008). PCR mapping of the DNA gained by pSLT in pUO-StVR2-like plasmids was done as in Herrero, Mendoza, Rodicio & Rodicio (2008). Further characterization of a pUO-StVR2 variant detected in this work was achieved by cloning of an about 10.5 kb SphI fragment carrying the *bla*$_{OXA-1}$ resistance gene. Fragments generated by SphI digestion of the plasmid variant were ligated with pUK1921, a pUC19-derivative which contains kanamycin instead of ampicillin as selectable marker (Heinisch, 1993). The ligation mixture was introduced into chemically-competent cells of *Escherichia coli* DH5α (Invitrogen), and selection of the desired fragment was achieved by inoculation of the transformants in LB (Luria-Bertani broth) medium (Sambrook & Russell, 2001) containing kanamycin (25 mg L^{-1}) plus ampicillin (100 mg L^{-1}). Partial nucleotide sequencing of the cloned DNA was performed at Secugen (Madrid, Spain).

2.5. Pulsed-field gel electrophoresis (PFGE) analysis

Total DNA from food isolates carrying pUO-StVR2-like plasmids and from the two control strains was independently digested with XbaI and BlnI, as previously described

(Herrero, Rodicio, Echeita & Mendoza, 2008). The generated fragments were separated by PFGE, performed in a CHEF-DR III System (Bio-Rad Laboratories) under standardized conditions (Peters *et al*., 2003).

3. Results and Discussion

3.1. Detection and genetic structure of pUO-StVR2-like plasmids in food isolates of Typhimurium

Four Typhimurium isolates (LMO 11/04, LMO 12/04, LMO 34/06 and LMO 23/04, recovered from pork and chicken meat during surveys of *S. enterica* in food products, lacked the virulence plasmid pSLT (94 kb) but contained plasmids larger than 100 kb (Fig. 2a). These plasmids were putatively ascribed to the Typhimurium pUO-StVR2-like group (here represented by LSP 146/02) on the basis of the resistance properties conferred to their hosts (Table 1). The first three isolates showed the penta-resistance profile (phenotype and responsible genes) associated with pUO-StVR2 (see Introduction). The remaining isolate, LMO 23/04, displayed additional resistance to trimethoprim, encoded by the *dfrA10* gene. The presence of hybrid plasmids in the four isolates was confirmed by Southern hybridization with probes specific for *spvC* and $bla_{\text{OXA-1}}$ (Fig. 2b and 2c). As expected, only *spvC* hybridized with pSLT extracted from Typhimurium LT2, while the two probes mapped on the large plasmids detected in the meat isolates and in the control LSP 146/02. Small, high copy number plasmids of unknown function were also observed in the food isolates (Figs. 2 and 3).

Like pUO-StVR2, the newly detected plasmids were conjugative and conferred to the recipient *E. coli* strain all resistance properties shown by the original hosts. In addition, they were PCR-positive for *spvC, rck*, IncFIIA/*repA*, *parAB, traT* and *traX*, and negative for *pefABCD*, *rsk* and IncFIB/*repA2*, all carried by pSLT. The genetic

structure of the DNA inserted within pSLT was then determined by PCR mapping. This revealed that the conventional left region of the hybrid plasmids from meat isolates, and its insertion site adjacent to the *ccdAB* genes of pSLT, matched those of pUO-StVR2 (Fig. 1). The same was true for the central resistance island, the right region and the pSLT right junction in the plasmids carried by LMO 11/04, LMO 12/04 and LMO 34/06. In contrast, the insertion in the plasmid from LMO 23/04 was somewhat different, and therefore this plasmid was considered as a pUO-StVR2 variant termed pUO-StVR2-v8. Six other pUO-StVR2 variants (differing in size, restriction profile and/or resistance pattern) have been previously found in clinical isolates (Herrero, Mendoza, Threlfall & Rodicio, 2009; Herrero, Rodicio, Echeita & Mendoza, 2008).

With regard to the structure of VR2-v8, PCR mapping demonstrated the absence of the entire right region, the *pefI* gene and part of *orf7*, all present in pUO-StVR2 (Fig. 1). Moreover, hybridization of the $bla_{\text{OXA-1}}$ probe with plasmid DNA digested with SphI revealed an insertion of about 4 kb in the central resistance island of the new variant. The probe mapped on a SphI fragment of about 10.5 kb in VR2-v8, instead of the 6.3 kb SphI fragment observed in the original plasmid (Fig. 3). Cloning of the 10.5 kb fragment of pUO-StVR2-v8, which conferred ampicillin resistance to the *E. coli* strain used as host, followed by partial nucleotide sequencing, demonstrated the insertion of an *orf513-dfrA10-qacEΔ1-sul1* element between *orf5* and *sul1* of the InH integron (Fig. 1). *orf513* is part of a 2,154 bp segment, known as common region (CR1) to distinguish it from the 3' conserved segment (CS) of conventional class 1 integrons (Parsons, Hall & Stokes, 1991; Partridge & Hall, 2003). *orf513*/CR1 shows characteristics of IS*91*, an unusual insertion sequence that uses rolling-circle replication for transposition (Toleman, Bennett, & Walsh, 2006). It is typically found in complex class 1 integrons, and has also been detected in several variants of SGI1 (*Salmonella* Genomic Island)

carried by serotypes Typhimurium, Agona, Kiambu and Infantis (Mulvey, Boyd, Olson, Doublet & Cloeckaert, 2006; Nielsen, Torpdahl, Ethelberg & Hammerum, 2009).

3.2. Genomic typing

To determine the genomic relationship between the meat isolates, they were analysed by PFGE with the restriction enzymes XbaI and BlnI (Fig. 4, Table 1). LMO 11/04, LMO 12/04 and LMO 34/06, as well as the control LSP 146/02 showed the X2-B2 combined profile, which has been previously detected in clinical isolates from Spain and the United Kingdom (Herrero, Mendoza, Threlfall & Rodicio, 2009; Herrero, Rodicio, Echeita & Mendoza, 2008; Herrero, Rodicio, Gonzalez-Hevia & Mendoza, 2006). LMO 23/04 generated X12 and B17 patterns, showing four and five mismatching fragments, some of plasmid origin, when compared with X2 and B2, respectively. The X12-B17 profile has recently been found in two clinical isolates of Typhimurium, as well as in one isolate recovered from an ill pig. Interestingly, these isolates also carried the pUO-StVR2-v8 variant (A. Herrero and M. R. Rodicio, unpublished), which appears as a single band of about 124 kb in the B17 profile (Fig. 4).

4. Conclusions

Results of the present study support food-producing animals as reservoir of Typhimurium carrying pUO-StVR2-like plasmids, and chicken and pork meat as vehicles for their transmission. They also reveal that both Typhimurium and the hybrid plasmid are evolving over time. While the consequences of bacterial evolution remain unknown, the emergence of the new plasmid variant has led to additional resistance to trimethoprim. The occurrence of resistance and virulence genes in conjugative plasmids

allows co-selection and simultaneous spread of both properties, thereby representing a hazard to animal and human health.

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Table 1

Origin and characteristics of the *Salmonella enterica* serovar Typhimurium isolates used in this work

Isolate Source	Transferable resistance pattern phenotype/genotype	V or VR-plasmid (size in kb)	XbaI-BlnI PFGE-profile
LT2	-	pSLT (94)	X0-B0
LSP 146/02 Clinical	A,C,S,Su,T/*bla*$_{OXA-1}$,*catA1*,*aadA1*,*sul1*,*tet*(B)	pUO-StVR2 (132)	X2-B2
LMO 11/04 Chicken	A,C,S,Su,T/*bla*$_{OXA-1}$,*catA1*,*aadA1*,*sul1*,*tet*(B)	pUO-StVR2 (132)	X2-B2
LMO 12/04 Chicken	A,C,S,Su,T/*bla*$_{OXA-1}$,*catA1*,*aadA1*,*sul1*,*tet*(B)	pUO-StVR2 (132)	X2-B2
LMO 34/06 Pork	A,C,S,Su,T/*bla*$_{OXA-1}$,*catA1*,*aadA1*,*sul1*,*tet*(B)	pUO-StVR2 (132)	X2-B2
LMO 23/04 Pork	A,C,S,Su,T,Tp/*bla*$_{OXA-1}$,*catA1*,*aadA1*,*sul1*,*tet*(B),*dfrA10*	pUO-StVR2-v8 (125)	X12-B17

LSP-Laboratory of Public Health of Asturias (Spain), LMO-Laboratory of Microbiology of the Oviedo University, A-ampicillin, C-choramphenicol, S-streptomycin, S-sulfonamides, T-tetracycline, Tp-trimethoprim. Three resistance genes, *bla*$_{OXA-1}$-*aadA1* and *sul1*, are carried by a class 1 integron termed InH.

337 **Figure legends**

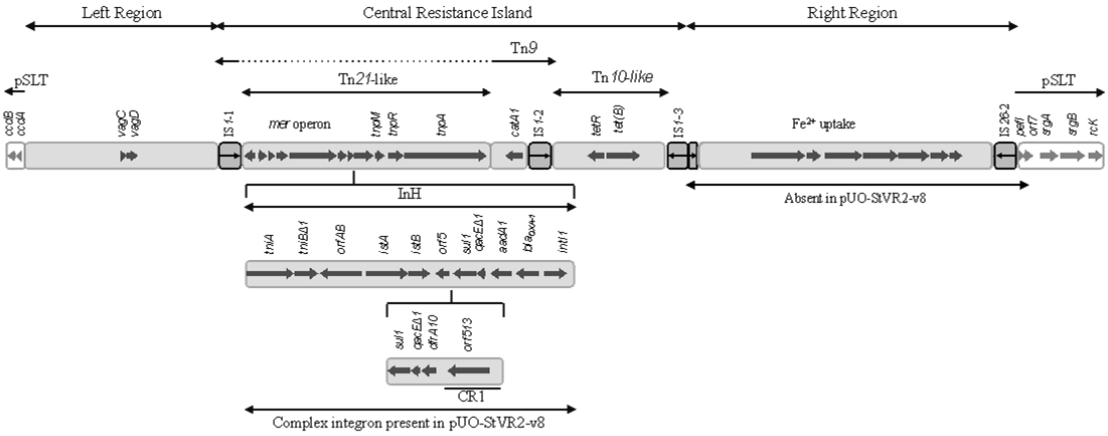

Figure 1

338

339 **Fig. 1.** Structure of the DNA acquired by pSLT in pUO-StVR2-like plasmids from meat
340 isolates. pUO-StVR2 was carried by LSP 146/02 (control strain), LMO 11/04, LMO
341 12/04 and LMO 34/06. pUO-StVR2-v8 was detected in LMO 23/04. CR1, conserved
342 region of complex class 1 integrons carrying *orf513*; InH, class 1 integron with the
343 ~2000 bp/bla_{OXA-1}-*aadA1* variable region, characteristically found in pUO-StVR2-like
344 plasmids.

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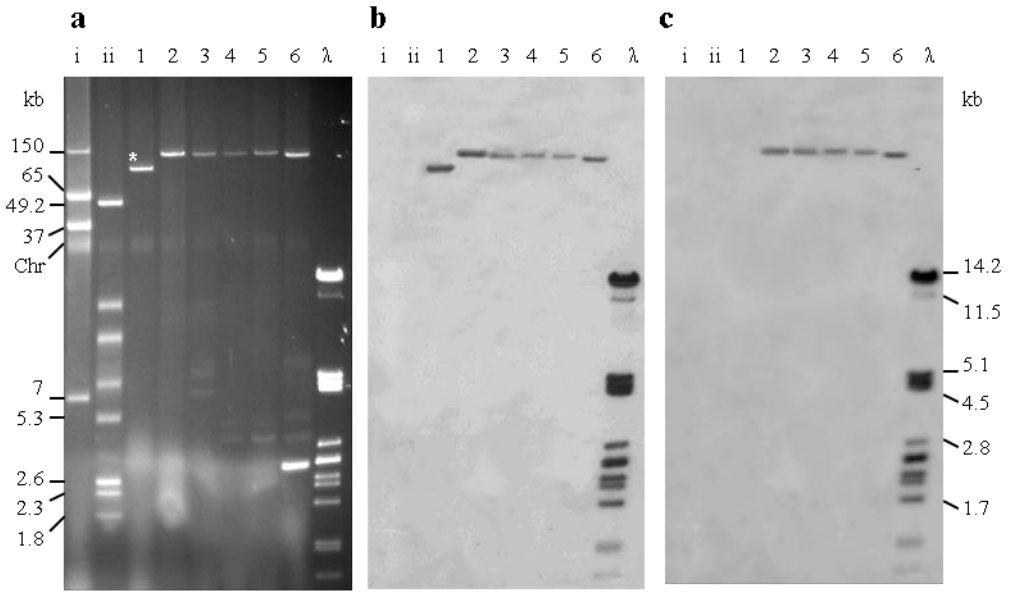

Figure 2

Fig. 2. Plasmid profiles of *Salmonella enterica* serovar Typhimurium isolates from meat samples. Undigested plasmids (a) were hybridized with *spvC* (b) and $bla_{\text{OXA-1}}$ (c) probes. i and ii, plasmids obtained from *Escherichia coli* V517 (NCTC 50193) and *E. coli* 39R861 (NCTC 50192) used as molecular size standards for uncut DNA; lanes 1 to 6, plasmids from LT2, LSP 146/02, LMO 34/06, LMO 11/04, LMO 12/04 and LMO 23/04; λ, DNA of phage lambda digested with PstI. Chr, chromosomal DNA.

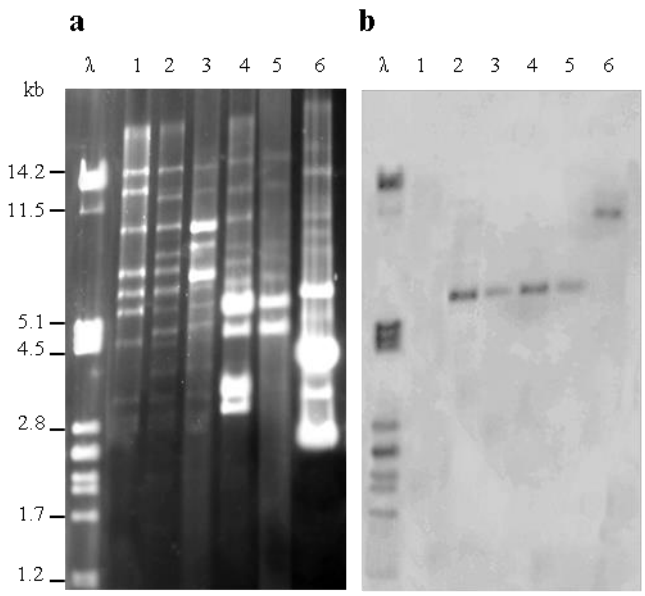

Figure 3

Fig. 3. Detection of the DNA inserted within the resistance island of pUO-StVR2-v8. Plasmids from meat isolates were digested with SphI (a) and hybridized with the *bla*$_{\text{OXA-1}}$ probe (b). λ, DNA of phage lambda digested with PstI (size standard); lanes 1 to 6, plasmid DNA from LT2, LSP 146/02, LMO 34/06, LMO 11/04, LMO 12/04 and LMO 23/04, digested with SphI. Strong bands in lanes 3 to 6 correspond to small, high copy number plasmids, also seen in Fig. 2.

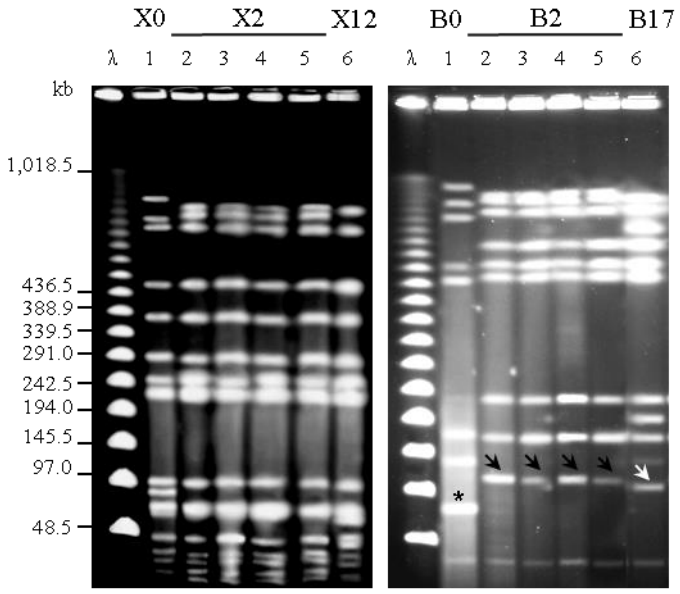

Figure 4

Fig. 4. XbaI (a) and BlnI (b) macrorestriction-PFGE analysis of *Salmonella enterica* serovar Typhimurium isolates from food samples. λ, lambda Ladder PFG Marker (New England BioLabs) used as size standard; lanes 1 to 6, LT2, LSP 146/02, LMO 34/06, LMO 11/04, LMO 12/04 and LMO 23/04. pSLT, pUO-StVR2 and pUO-StVR2-v8, each having a single recognition site for BlnI, are marked with an asterisk, and with black and white arrows in the respective B0, B2 and B17 profiles.