1 **Molecular epidemiology of emergent multidrug-resistant *Salmonella enterica* serotype**
2 **Typhimurium strains carrying the virulence resistance plasmid pUO-StVR2**

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Abstract:

Objectives: To evaluate the incidence of a distinct multidrug-resistant (MDR) grouping of *Salmonella* serotype Typhimurium strains carrying the hybrid virulence resistance plasmid pUO-StVR2, and its possible evolution in the region where it was first detected [Principality of Asturias (PA), Spain].

Methods: pUO-StVR2-containing isolates were tentatively identified by two genetic markers: the $bla_{\mathrm{OXA\text{-}30}}$ gene and the class 1 integron InH:2000 bp/$bla_{OXA\text{-}30}$-*aadA1a*. Positive isolates were examined for resistance profile (RP), plasmid content, virulence profile (VP) and genomic polymorphisms using macrorestriction–PFGE.

Results: A total of 182 out of 248 Typhimurium clinical isolates recorded in the PA over 2001–02 were ampicillin-resistant and could be distributed into several MDR groupings. A MDR grouping carrying pUOStVR2, with a defined RP (AMP/$bla_{\mathrm{OXA\text{-}30}}$, CHL/*catA1*, [STR-SPT]/[*strA/B,aadA1a*], SUL/[*sul1,sul2*], TET/*tet*(B), *qacEΔ1*, *merA*, ±TMP/*dfrA12*, and containing InH), was represented by 49 isolates. The VPs of these isolates (24 genes screened) differed from that of the type strain LT2 by the absence of the *sopE1* and *pef* genes. Macroestriction analysis established six combined *XbaI/BlnI* PFGE profiles, and supported a clonal relationship among most of the isolates.

Conclusions: During 2001–02, the isolates carrying pUO-StVR2 constituted the second most frequent *S.* Typhimurium MDR grouping recorded in the PA, preceded only by the pandemic pentaresistant DT104. Polymorphisms on the genomic DNA, different phage types, different plasmid profiles and the detection of trimethoprim resistance in one isolate encoded by an additional plasmid, were consistent with both intra-cluster evolution and horizontal transfer of the hybrid plasmid.

Keywords: multidrug resistance, hybrid plasmid, PFGE, virulence genotype.

45 **Introduction**

46 *Salmonella enterica* serotype Typhimurium (*S.* Typhimurium) is one of the most common
47 bacteria causing food-borne disease in developed countries[1]. As with many other non-typhoid
48 serotypes, infection usually results in self-limited gastroenteritis, which does not require
49 antimicrobial therapy. However, bacteria can occasionally be invasive, and even fatal,
50 particularly for patients with underlying risk factors, such as immunosuppression. In these
51 cases, effective antimicrobial therapy is necessary. A wide diversity of virulence (V) factors
52 have been described for *Salmonella*, and they can be located on the bacterial chromosome,
53 frequently as part of pathogenicity islands, on plasmids and prophages[2–4]. The V plasmid of *S.*
54 Typhimurium (pSLT) has a size of 94 kb, belongs to the incompatibility group IncFII and
55 contains the *spvABCDR* locus (responsible for an increase in the bacterial growth rate in mice
56 during the systemic phase of disease), the *pefBACDI* operon (for biosynthesis of fimbriae
57 involved in adherence to the intestinal epithelium) and the *rck* and *rsk* genes (resistance to
58 complement killing), in addition to genes encoding for essential plasmid functions (such as
59 plasmid replication and maintenance)[4,5].

60 *S.* Typhimurium LT2 (the type strain of Typhimurium) is susceptible to antimicrobial drugs, as
61 were the majority of *Salmonella* strains circulating until the 1980s. Since then, multidrug-
62 resistant (MDR) groupings have emerged and, nowadays, isolates pertaining to them are far
63 more frequent than drug susceptible isolates[1,6]. A MDR clone, defined as *S.* Typhimurium
64 DT104, with resistance (R) to ampicillin, chloramphenicol–florfenicol, streptomycin–
65 spectinomycin, sulphonamides and tetracyclines (AMP-CHL-STR-SUL-TET phenotype), was
66 initially detected in animals and later in humans. Today it is widely spread and could be
67 considered as pandemic[1,6,7]. In this MDR clone, the genes encoding the five antimicrobial-R
68 [$bla_{\text{PSE-1}}$- *floR-aadA2-sul1-tet*(G)] are located within a chromosomal island (SGI1), with
69 different variants reported over time, which include two class 1 integrons (designated as

InC:1200 bp/*bla*$_{\text{PSE-1}}$ and InD:1000 bp/*aadA2*) described as In4-type integrons[6,8]. In 1997, a new MDR grouping that is monophasic [4,(5),12:i:-] appeared in Spain[9]. In general, it displays gentamicin-R and trimethoprim-R, in addition to the DT104 pentaresistant phenotype, with the R genes located on large plasmids carrying a class 1 integron (InI:1900 bp/*dfrA12-aadA2*). In 2002, our laboratory characterized 12 MDR clinical *S.* Typhimurium isolates collected during 1993–2000 in the Principality of Asturias (PA), Spain[10]. They had the same R phenotype as the pentaresistant DT104 associated to a different R genotype [*bla*$_{\text{OXA-1}}$-*catA1-[strA/B-aadA1a]-[sul1-sul2]-tet*(B)], with two of the genes pertaining to a distinctive integron (InH:2000 bp/*bla*$_{\text{OXA-1}}$-*aadA1a*). The R genotype was conferred by pUO-StVR2, a hybrid self-transferable plasmid, apparently derived from pSLT. These isolates generated a common and distinctive *XbaI* macrorestriction profile and could be considered as clonally related. Recently, it was established that the *bla*$_{\text{OXA}}$ gene present in pUO-StVR2 was in fact *bla*$_{\text{OXA-30}}$ that differs from *bla*$_{\text{OXA-1}}$ by only 1 nt (GenBank accession number AY534545).

When pUO-StVR2 was first described[10], it was already highlighted that the coexistence of virulence and resistance genes in the same extrachromosomal element represents a serious threat for human health that deserves epidemiological surveillance. In the present work we aimed to follow the impact on human health of the *S.* Typhimurium clone carrying pUO-StVR2 during 2001–02, and to ascertain its possible evolution in the region where this hybrid plasmid was first detected.

Materials and methods

Bacterial isolates

A total of 182 *S.* Typhimurium ampicillin-R isolates (97 collected in 2001 and 85 in 2002, with 8 and 3 being assigned to the [4,(5),12:i:-] variant, each year) were analysed in this study (Table 1). They represented 73.4% of the total *S.* Typhimurium clinical isolates recorded at the 'Laboratorio de Salud Pública' (LSP, acting as *Salmonella* Reference Centre for the PA) during those 2 years. *S.* Typhimurium LT2 (pSLT), LSP 31/93 (pUO-StVR2), LSP 14/92 (bla_{PSE}, InC and InD positive) and LSP 389/97 (bla^{TEM}, InI and In0 positive) strains[4,10,11] were used as controls in different experiments. Phage typing of the isolates and control strains was performed at the 'Centro Nacional de Microbiología' (Madrid, Spain).

Antimicrobial susceptibility

Antimicrobial susceptibility was tested according to NCCLS guidelines[12] by a disc-diffusion technique using commercial discs (bioMérieux and Oxoid). The antimicrobials and quantities in micrograms tested were: ampicillin (AMP), 10; aztreonam (ATM), 30; cefotaxime (CTX), 30; ceftazidime (CAZ), 30; chloramphenicol (CHL), 30; co-amoxiclav (AMC), 30; gentamicin (GEN), 10; nalidixic acid (NAL), 30; spectinomycin (SPT), 10; streptomycin (STR), 10; sulfadiazine (SUL), 300; tetracycline (TET), 30; and trimethoprim (TMP), 5.

PCR procedures and DNA sequencing

Single and multiple PCRs, using specific primers and conditions previously described,[10,13–16] were performed for detection of V-pSLT genes (*spvC, rck, pefA* and *pefC*), V chromosomal genes (*iroB, phoP/Q, agfA, stn, slyA, invE/A, sopE1* and *sefD*), R genes [bla_{OXA}, bla_{PSE}, bla_{TEM}, *catA1, strA/B, aadA1a, sul1, sul2, tet*(B), *dfrA1* and *dfrA12*] and Class 1 integrons. Primers for other pSLT and chromosomal V genes were designed for the present work: *pefB* (GGCACTCAGGGACTACCTTG/TGATGCGTGACAGGCGGTTC),

118 *pefD* (CTTTAAGGTCAGGCCCAAGG/TCCGTTCAGCGACAGTTTCC), *parA*
119 (GAAGTACGCGATGACGATTC/GGTGCTTCCATCATAGGTTG), *parB*
120 (GAGATGACTGACACCCAAAG/GAGCTATCAATGCCTGAGAG), *sodCI*
121 (CCAGTGGAGCAGGTTTATCG/GGTGCGCTCATCAGTTGTTC), *orgA* (GATAAGGCGAAATCGTCAA
122 ATG/GTAAGGCCAGTAGCAAAATTG), *ttrC* (GTGGGCGGTACAATATTTCTTTT/TCACGAATAATAAT
123 CAGTAGCGC), *ssaQ* (GAATAGCGAATGAAGAGCGTCC/CATCGTGTTATCCTCTGTCAGC), *mgtC*
124 (TGACTATCAATGCTCCAGTGAAT/ATTTACTGGCCGCTATGCTGTTG), *misL* (GACGTTGATAGTCT
125 GCCATCCAG/CAATGCCGCCAGTCTCCGTGC), *spi4R* (GATATTTATCAGTCTATAACAGC/ATTCTC
126 ATCCAGATTTGATGTTG), *spi4D* (GAATAGAAGACAAAGCGATCATC/GCTTTGTCCACGCCTTTCA
127 TC), *sopB* (GATGTGATTAATGAAGAAATGCC/GCAAACCATAAAAACTACACTCA) and *pipA*
128 (CTCTTGGATGATTTTCTTCTTTA/CTTATCTCAGGCGCGGGTGG).
129 For a correct description of the type of bla_{OXA} gene carried by pUOStVR2/InH, the 708 bp
130 amplicons obtained with the bla_{OXA} primers[13] from LSP 31/93 and LSP 153/02 were sequenced.
131 In both cases the gene was identified as bla_{OXA-30}. Confirmation of the insertion of bla_{OXA-30}
132 and *aadA1a* into the 2000 bp amplicons generated with the 50CS/30CS primers was achieved
133 by nested-PCR, using the 2000 bp amplicon as the template DNA, and primers specific for the
134 bla_{OXA} and *aadA1a* genes[17].

135 *Plasmid analysis and Southern hybridization*

136 Plasmid DNA was routinely purified by the method of Kado and Liu[18]. However, for a better
137 resolution of large plasmids with a similar size, plasmid extraction from selected isolates was
138 also performed by S1-PFGE[19]. Plasmids ranging in size from 7 to 150 kb extracted from
139 *Escherichia coli* 39R861 (NCTC 50192) and λ Ladder PFG Marker (New England BioLabs)
140 were used as molecular size standards for undigested and S1-digested DNA, respectively.
141 Selected plasmid profiles were sequentially hybridized with probes specific for *spvC*,

142 bla_{OXA-30} and *dfrA12*. The probes were obtained from LT2 (*spvC*), LSP 31/93 (bla_{OXA-30}) and
143 LSP 174/01 (*dfrA12*) by PCR amplification using DIG-labelled dNTPs (PCR DIG labelling
144 mix; Roche Applied Science), followed by gel extraction and purification with the GFXTM
145 DNA and Gel Band Purification Kit (Amersham Biosciences).

146 *Genomic macrorestriction–PFGE analysis*

147 *S.* Typhimurium isolates were analysed by macrorestriction and PFGE. Slices of the DNA-
148 containing plugs were subjected to *XbaI* (Takara Biomedicals, 30 U; 4 h at 37ºC) and *BlnI*
149 (Takara Biomedicals, 20 U; overnight at 37ºC) digestion. A CHEF-DRIII system (Bio-Rad
150 Laboratories) was used to separate the fragments under the standardized conditions
151 recommended by 'The Salm-gene project'[20]. The LT2 isolate was used as PFGE marker[21].
152 Similarity between *XbaI–BlnI* profiles was evaluated by the Jaccard's coefficient (S), and
153 cluster analysis was performed by the unweighted pair group method with arithmetic averages,
154 using the software program MVSP (Multivariate Statistics Package for PCs, RockWare Inc.).
155 Strains showing profiles with similarity coefficients of ≥0.70 were considered members of the
156 same cluster.

157

158 **Results**

159 *S. Typhimurium ampicillin resistance in the Principality of Asturias*

160 A total of 1427 *S. enterica* isolates causing human disease were recorded at the LSP over the
161 period 2001–02 (702 in 2001 and 725 in 2002), corresponding to rates of 66.2 and 68.4 per 105
162 inhabitants/year, during 2001 and 2002, respectively. Of them, 248 belonged to *S.*
163 Typhimurium [rates of 10.2 and 13.2 per 105 inhabitants/year, during 2001 (108 isolates) and
164 2002 (140 isolates), respectively]. Regarding the antigenic formula the *S.* Typhimurium isolates

165 could be subdivided into typical or biphasic [4,(5),12:i:1,2] and atypical or monophasic
166 [4,(5),12:i:-] variants, with 231 and 17 isolates, respectively.
167 Regarding ampicillin, 182 (73.4%) out of the 248 *S.* Typhimurium isolates were resistant. All
168 of them were tested for the presence of three *bla* genes and for In profile by PCR procedures.
169 Results showed that 91, 37 and 49 isolates generated the expected amplicons for bla_{PSE} (419
170 bp), bla_{TEM} (503 bp) and bla_{OXA} (708 bp), respectively, while in the remaining five, the
171 ampicillin-R determinants were not identified. In addition, 151 isolates were In-positive, and
172 could be differentiated into four In profiles, In-P1 (1200 + 1000 bp); In-P2 (1900 ± 150 bp);
173 In-P3 (2000 bp); and In-P4 (1600 bp), with 91, 10, 49 and 1 isolate(s), respectively. Both traits,
174 *bla* gene and In profile, together with the antigenic formula, were used to discriminate the
175 isolates into three major groupings: G1 (bla_{PSE}, In-P1), G2 (bla_{TEM}, In-P2) and G3 (bla_{OXA}, In-
176 P3), apart from other, as yet undefined, groupings (Table 1). The presence of bla_{OXA} and the
177 *aadA1a* gene within the 2000 bp amplicon characteristic of In-P3 (InH) was confirmed by
178 nested-PCR amplification using specific primers for each gene (not shown).

179 *Characterization of G3 isolates*

180 The 49 isolates belonging to G3 and the control strains LT2 and LSP 31/93 were tested for the
181 presence of pSLT determinants, plasmid profile, resistance profile (RP) and virulence profile
182 (VP). All these, as well as the control LSP 31/93 strain, were *spvC-, rck-* and *parA/B*-positive
183 and *pefABCD*-negative, and carried a plasmid of the size expected for pUO-StVR2 (~140 kb;
184 readily visualized after extraction by the Kado and Liu method). In addition, some G3 isolates
185 contained one or more plasmids of smaller size and unknown function (data not shown). As
186 expected, G3 isolates showed the RP conferred by pUO-StVR2, but one of them (LSP 174/01)
187 was also trimethoprim-R. PCR amplification revealed that *dfrA12* was the gene responsible for
188 such resistance (not shown). Plasmid extraction from this and several other isolates (LSP 31/93,
189 LSP 238/02 and LT2) by S1-PFGE, followed by Southern hybridizations, demonstrated that,

190 together with pUO-StVR2, a plasmid of ~110 kb (labelled pUO-StR12) was present in LSP
191 174/01, and the *dfrA12* mapped on it (Figure 1a and d). In contrast, the bla_{OXA} and *spvC* probes
192 mapped on the 140 kb plasmid from the G3 isolates (LSP 31/93 and LSP 238/02) and the
193 second also on the 94 kb plasmid from LT2 (Figure 1b and c).

194 To further characterize the isolates belonging to G3, they were analysed for VP. Results
195 revealed that all of them, as well as the control strains, were positive for the genes used as
196 indicators of five *Salmonella* pathogenicity islands (*invE/A* and *orgA*, SPI1; *ttrC* and *ssaQ,*
197 SPI2; *mgtC* and *misL*, SPI3; *spi4R* and *spi4D*, SPI4; and *sopB* and *pipA*, SPI5). In addition,
198 they were also positive for seven out of nine other V genes tested (*phoP/Q, agfA, slyA, stn, iroB*
199 and *sodCI*). However, all were negative for *sefD* and all except LT2 were also negative for
200 *sopE1.*

201 *Dispersion of pUO-StVR2 between genomic types of S. Typhimurium*

202 By *XbaI*-macrorestriction PFGE analysis the clinical isolates could be discriminated into five
203 *XbaI* profiles (X1–X5), with most isolates (85.7%) belonging to X1 and X2. LSP 31/93, the
204 G3 strain from the previous period, generated the X1 profile while LT2 gave a distinct profile,
205 here termed X0 (Table 2 and Figure 2a). Total DNA from representative isolates of the different
206 *XbaI* profiles (6, 6, 1, 1 and 2 belonging to X1–X5, respectively; Table 2), and the controls LSP
207 31/93 and LT2, were also analysed by *BlnI* macrorestriction (Figure 2b). In general, results
208 obtained with the second endonuclease matched those obtained with *XbaI*. In fact, all except
209 one of the isolates tested with *BlnI* could be assigned to the B0–B5 profiles, which
210 corresponded to the X0–X5 profiles. The exception was LSP 174/01 (*dfrA12*-positive) that
211 generated the X2 profile but a distinct *BlnI* profile, termed B6 (Table 2 and Figure 2b), which
212 differed from B2 by the presence of an additional ~110 kb fragment (probably corresponding
213 to pUO-StR12). The *XbaI* and *BlnI* profiles were combined, and a dendrogram of similarity

214 was constructed (Figure 2c). At a cut-off point of *S* = 0.73 all profiles from pUO-StVR2 isolates
215 fall into a single cluster, which was related to LT2 at *S* = 0.54. It is interesting to note that the
216 newly described profiles differed from the earliest X1–B1 by 2–6 bands. Moreover, the two
217 combined profiles that included most of the analysed isolates (X1–B1 and X2–B2) differed
218 only by three bands. Accordingly, the G3 grouping appears to be highly clonal[22].

219

220 **Discussion**

221 Using two genetic markers, type of *bla* gene and In profile, three well-defined MDR groupings
222 (G1–G3), together with other not well-defined groupings of *S.* Typhimurium could be
223 distinguished as a cause of human salmonellosis in the PA over the period 2001–02. As in other
224 countries[23], the pentaresistant DT104 clone (here defined as G1: bla_{PSE}, InC:1200 bp and
225 InD:1000 bp), was the most frequent. It was involved in at least 89 salmonellosis episodes
226 associated with 91 clinical isolates [89 collected from faeces and two from patients suffering
227 from bacteraemia secondary to gastroenteritis (with positive blood and faeces cultures)]. They
228 represented 6.4 and 36.7% of the *S. enterica* and *S.* Typhimurium clinical isolates recorded in
229 the LSP, respectively. The second most frequent grouping was G3, identified as positive for
230 bla_{OXA}, InH:2000 bp (containing the bla_{OXA}-*aadA1a* gene cassettes) and pUO-StVR2. It was
231 implicated in at least 44 sporadic episodes including 47 isolates, and one family outbreak (here
232 represented by two isolates with the same macrorestriction profiles). Apart from the outbreak,
233 38 sporadic episodes were of gastroenteritis (two isolates were collected at different days from
234 the same patient, and showed different phage type: DT104 and DT193). The remaining four
235 corresponded to bacteraemia, and isolates with identical traits were recovered from both blood
236 and ascitic fluid in one patient, and from blood and faeces in the other three. The 49 G3-isolates
237 represented 3.4 and 19.7% of *S. enterica* and *S.* Typhimurium, respectively, recorded at the

LSP during 2001–02. A third MDR grouping, G2, corresponding to the [4,(5),12:i:-] variant (here identified as positive for *bla*$_{TEM}$ and InI:1900 bp – In0:150 bp) was related to 10 sporadic episodes of gastroenteritis, representing 0.7 and 4.0% of *S. enterica* and serotype Typhimurium, respectively. It should be kept in mind that the actual frequency of salmonellosis and of the three major ampicillin-R groupings in the PA could be much higher, since in intestinal disease episodes, only some people require hospital attention, and, usually, *Salmonella* organisms are only collected from them. In addition, not all the isolates collected in the PA Microbiology Laboratories are forwarded to the LSP.

It is of note that direct information about the distribution of G3 in places other than the PA is not available. However, recent studies from different Spanish laboratories have demonstrated the presence of InH:2000 bp/*bla*$_{OXA}$-*aadA1a* integrons in isolates of S. Typhimurium[24–26], although its relation with hybrid plasmids has not been investigated, or could not be demonstrated. Noteworthy, one of the analysed isolates yielded a PFGE profile similar to X1[25]. In studies from four other European countries (Albania, Italy, Portugal and Norway), MDR plasmids carrying InH-type integrons were reported in *S.* Typhimurium. The Albanian isolates, which were associated with sporadic cases of acute gastroenteritis in children, carried self-transferable R plasmids of ~140 kb, belonging to the incompatibility group IncFI, and conferring the AMP-CHL-KAN-STR-SUL-TMP-TET MDR phenotype[27]. More recently, a *bla*$_{OXA}$-containing integron was found in the virulence plasmid of an MDR *S.* Typhimurium strain collected in Italy[28]. This plasmid, apparently non-conjugative, was of ~110 kb in size, conferred the AMP-GEN-KAN-STR-SPT-SUL-TMP phenotype, and belonged to the incompatibility group IncFII. The Portuguese isolates[29], recovered from humans and pork, carried InH-type integrons on self-transferable plasmids (reported as >70 kb and showing different restriction profiles), which encoded the AMP-STR-SUL-TET-CHL R phenotype. These isolates generated *XbaI* profiles similar to X1 and X2 found in the PA isolates. Finally,

263 *bla*$_{OXA}$-*aadA1* integrons were also detected in four AMP-CHL-STR-SUL-TET strains from
264 patients hospitalized in Norway[30]. Interestingly, although one of the strains was reported as
265 domestically acquired, the remaining three were acquired in Spain.

266 With respect to virulence, V determinants common to LT2 and G3 included 10 chromosomal
267 genes representing the 5 SPIs as well as 6 non-SPI located genes (*phoP/Q, agfA, slyA, stn* and
268 *iroB*)[31–35] and the bacteriophage-associated *sodCI* gene[36]. However, the *sopE1* gene was
269 present in LT2 but not in pUO-StVR2-positive isolates. This gene, located on a temperate
270 bacteriophage, encodes an effector protein of the SPI1 type III secretion system, which
271 contributes to host cell invasion and intestinal inflammation in animal models[37]. As expected,
272 LT2 and G3 isolates were negative for *sefD*, a gene of the *sef* operon, which encodes the SEF18
273 fimbriae in *Salmonella* Enteritidis[16,32]. With respect to plasmid genes, all G3 isolates contained
274 *spvC, rck, parA* and *parB* but lacked the *pef* operon, while LT2 was positive for all these genes.
275 According to this, deletions in the *pef* operon could have occurred when the region was inserted
276 into pSLT to originate the hybrid plasmid. Such a possibility is currently being investigated. It
277 is interesting to note that 12 G3 isolates, collected during 1993–2000, and previously subjected
278 to PFGE analysis, showed a single *XbaI* profile (X1)[10]. In contrast, the 49 isolates recovered
279 over 2001–02 generated five *XbaI* profiles (X1–X5), although >85% belonged to X1 and X2.
280 Comparisons of these, and the corresponding *BlnI* profiles, supported that most G3 isolates are
281 clonally related. Accordingly, vertical transmission could be the main way for the spread of
282 pUO-StRV2, although horizontal transfer of the plasmid cannot be ruled out. In fact, the
283 observed polymorphisms on the genomic DNA, together with the different phage types, the
284 different plasmid profiles and the acquisition of a new trimethoprim-R determinant (*dfrA12*),
285 are compatible not only with the evolution of the progeny of an original G3 strain, but also
286 with the transfer of the plasmid to relatively related *S.* Typhimurium strains. Further

epidemiological surveillance will be required to determine the potential of a wider distribution of the emergent MDR cluster carrying pUO-StRV2.

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Tables

Table 1. Features of ampicillin-resistant Salmonella serotype Typhimurium isolates and control strains.

Grouping (no.)	Antigenic formula	*bla* gene	In profile[a]	Phage type (no.)	Origin[b]		
					B	F	AF
G1 (91)	[4,(5),12:i:1,2]	*bla*$_{PSE}$	In-P1:1200 + 1000[c]	DT104 (41)	1	40	–
				U302 (36)	1	35	–
				NT (7)	–	7	–
				RDNC (4)	–	4	–
				others (3)[d]	–	3	–
G2 (10)	[4,(5),12:i:-]	*bla*$_{TEM}$	In-P2:1900 ± 150[e]	DT 193 (5)	–	5	–
				DT U302 (4)	–	4	–
				NT (1)	–	1	–
G3 (49)	[4,(5),12:i:1,2]	*bla*$_{OXA}$	In-P3:2000[f]	RDNC (26)	1	24	1
				NT (9)	–	9	–
				DT104 (4)	1	3	–
				others (10)[g]	2	8	–
Others (32)	[4,(5),12:i:1,2]	*bla*$_{TEM}$	In-P4:1600	U302 (1)	–	1	–
	[4,(5),12:i:1,2]	*bla*$_{TEM}$	–	DT193 (13)	–	13	–
				NT (5)	–	5	–
				others (7)[h]	–	7	–
	[4,(5),12:i:1,2]	NI		RDNC (4)	–	4	–
				DT208 (1)	–	1	–
	[4,(5),12:i:-]	*bla*$_{TEM}$	–	PT195 (1)	–	1	–
Controls							
LT2	[4,(5),12:i:1,2]	–	–	RDNC			
LSP 14/92 (G1)	[4,(5),12:i:1,2]	*bla*$_{PSE}$	In-P1:1000 + 1200[c]	DT104			
LSP 389/97 (G2)	[4,(5),12:i:-]	*bla*$_{TEM}$	In-P2:1900 + 150[e]	DTU302			
LSP 31/93 (G3)	[4,(5)12:i:1,2]	*bla*$_{OXA}$	In-P3:2000[f]	DT104b			

No., number of isolates; NI, not identified; NT, non-typeable; RDNC, reaction does not conform.
[a]Size in bp of the amplicon generated by 5′CS/3′CS primers.
[b]Number of isolates from blood (B), faeces (F) and ascitic fluid (AF).
[c]Associated with InC and InD.[10]
[d]NI (1), DT312 (1) and DT193 (1).
[e]Associated with InI and In0.[10]
[f]Associated with InH.[10]
[g]DT104b (2), DT193 (2), DT208 (2), DT27 (1), DT160 (1), DT 41 (1) and DTU302 (1).
[h]DT208 (2), U302 (2), DT104 (2) and NI (1).

Table 2. Differential traits of G3 isolates.

Representative strain—phage type	R profile[a]	V profile[b]	PFGE profiles	
			*Xba*I (no.)	*Bln*I (no.)[c]
LT2—RDNC	S	VP1	X0	B0
LSP 31/93—DT104b	RP1	VP2	X1 (15)	B1 (7)
LSP 137/01—RDNC	RP1	VP2	X2 (28)	B2 (5)
LSP 353/01—DT41	RP1	VP2	X3 (1)	B3 (1)
LSP 509/01—DT104b	RP1	VP2	X4 (2)	B4 (1)
LSP 200/02—DT208	RP1	VP2	X5 (3)	B5 (2)
LSP 174/01—DT27	RP2	VP2	X2 (1)	B6 (1)

No., number of isolates; RDNC, reaction does not conform.
[a]The 49 clinical isolates and the control LSP 31/93 were positive for the R genes *bla*$_{OXA}$, *catA1*, *strA/B*, *aadA1a*, *sul1/I2* and *tet*(B). S, susceptible to antimicrobials; RP1, all pUO-StVR2 resistance determinants; RP2, RP1 and *dfrA12* positive.
[b]All were positive for the V genes *spvC*, *rck*, *iroB*, *phoP/Q*, *agfA*, *stn*, *slyA*, *sodCI*, *invE/A*, *orgA*, *ttrC*, *ssaQ*, *mgtC*, *misL*, *spi4R*, *spi4D*, *sopB*, *pipA* and *parA/B*. VP1, all V-pSLT and V chromosome genes tested except *sefD*; VP2, all V genes tested except *pefABCD*, *sefD* and *sopE1*.
[c]Only 16 out of the 49 G3 isolates, representing the five *Xba*I profiles, were tested by *Bln*I macrorestriction.

422 **Captions to Figures**

423 **Figure 1.** Plasmid analysis by S1-PFGE and hybridizations. (a) Plasmid profiles obtained by
424 S1-PFGE. Lanes 1–4: LT2 (pSLT), LSP 31/93 (pUOStVR2), LSP 238/02 and LSP 174/01,
425 respectively. M, l ladder PFGE marker. (b–d) Hybridization of (a) with *spvC*, *bla*$_{OXA-30}$ and
426 *dfrA12* probes, mapping on fragments of (140 and 94 kb), (140 kb) and (110 kb),
427 respectively.

428 **Figure 2**. Macrorestriction–PFGE analysis of representative *S.* Typhimurium pUO-StVR2
429 isolates. (a) *XbaI* profiles: lanes 1–6, profiles showed by LSPstrains 31/93, 60/01, 174/01,
430 353/01, 509/01 and 238/02, respectively. L, X0-profile generated by LT2. The arrow indicates
431 the fragment corresponding to pSLT. (b) *BlnI* profiles: lanes 1–7, profiles shown by LSP
432 strains 31/93, 60/01, 137/01, 353/01, 509/01, 238/01 and 174/01, respectively. L, B0-profile
433 generated by LT2. The arrow indicates the fragment corresponding to pSLT. (c) Dendogram
434 of similarity of *XbaI*–*BlnI* profiles corresponding to G3 isolates. At *S* = 0.73, the six profiles
435 generated by G3 isolates were clustered, while the LT2 profile remains out of the group (*S* =
436 0.54). Asterisk indicates the branch in which the G3 prototype strain (LSP 31/93) was also
437 included.

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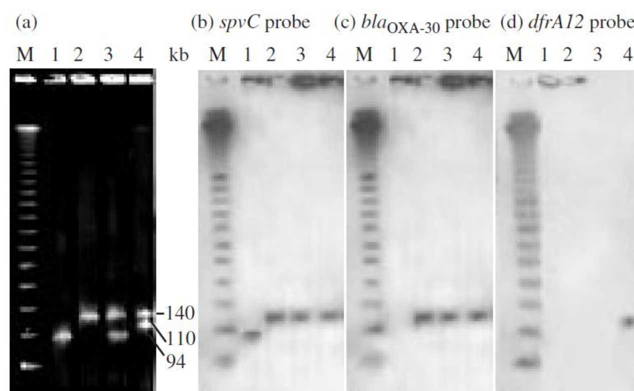

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440 **Figure 1.**

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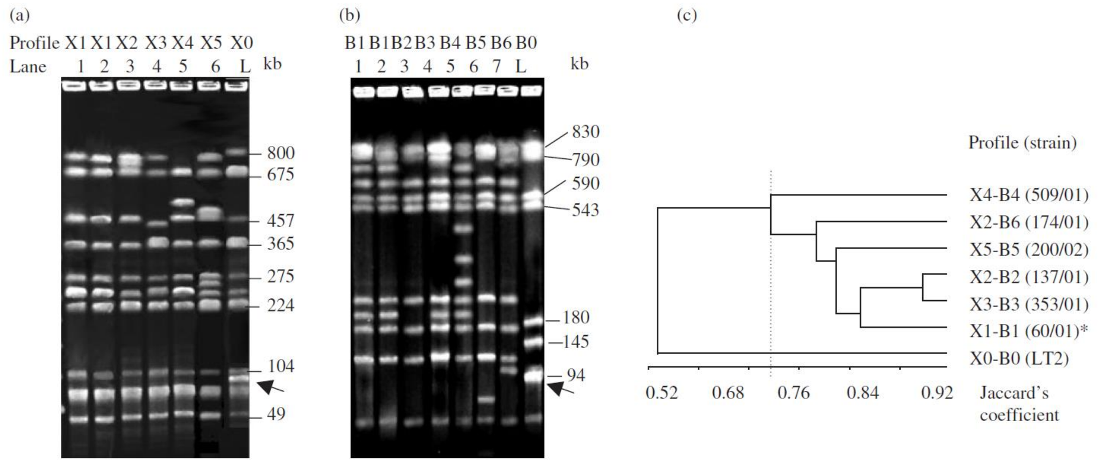

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443 **Figure 2.**

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