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Dissemination of Sulfonamide Resistance Genes (*sul1*, *sul2*, and *sul3*) in Portuguese *Salmonella enterica* Strains and Relation with Integrons

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In 200 sulfonamide-resistant Portuguese *Salmonella* isolates, 152 *sul1*, 74 *sul2*, and 14 *sul3* genes were detected. Class 1 integrons were always associated with *sul* genes, including *sul3* alone in some isolates. The *sul3* gene has been identified in isolates from different sources and serotypes, which also carried a class 1 integron with *aadA* and *dfrA* gene cassettes.

Salmonella enterica is a zoonotic pathogen transmitted through the food chain to humans, with contaminated foods of animal origin being important sources of infection. In particular, *S. enterica* serotype Typhimurium definitive phage type 104 (DT104), with resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracyclines (R-type ACSSuT), has emerged as a global health problem. The use of antimicrobial agents in food animals has been a major factor in the emergence of *Salmonella* with decreased susceptibility to antibiotics (19). A basic role in the spread of antimicrobial resistance in *Salmonella* has been attributed to class 1 (6, 17) and class 2 (4, 13) integrons. Sulfonamide resistance in gram-negative bacilli generally arises from the acquisition of either of the two genes *sul1* and *sul2*, encoding forms of dihydropteroate synthase that are not inhibited by the drug (2). The *sul1* gene is normally found linked to other resistance genes in class 1 integrons, while *sul2* is usually located on small nonconjugative plasmids (18) or large transmissible multiresistance plasmids (2). Recently, a new plasmid-borne sulfonamide resistance gene called *sul3* has been discovered (15). The objective of this study was to evaluate the incidence of sulfonamide resistance genes and class 1 and class 2 integrons among sulfamethoxazole-resistant nontyphoidal *Salmonella* isolates from different sources in Portugal.

For this study all the sulfonamide-resistant isolates (200) from a total of 1,183 Portuguese *Salmonella* isolates apparently epidemiologically unrelated collected during 2002 and 2003 were selected. The strain collection was obtained from the National Center of *Salmonella* (Lisbon, Portugal) and from clinical and food microbiology laboratories dispersed in our country. The MIC of sulfamethoxazole was determined by the agar dilution method, according to the NCCLS (12), with Mueller-Hinton agar 2 (bioMérieux, Marcy-l'Etoile, France). The breakpoint used was the one defined by the NCCLS (12) for the family *Enterobacteriaceae* (≥512 μg/ml). These 200 isolates were recovered from human clinical sources (120), food products (73), the environment (five), and unknown sources (two) and belong to several serogroups, principally B, C, and D. By a PCR assay performed according to the work of Pritchett et al. (16), all the *Salmonella* isolates contained the *invA* gene and 66 were identified as belonging to the DT104 phage type. Clonality among the isolates was assessed by pulsed-field gel electrophoresis (PFGE) following XbaI digestion of genomic DNA according to the standard 1-day protocol set by the Centers for Disease Control and Prevention (CDC) (1). Isolates with electrophoretic patterns that differed by three bands at most were assigned to the same clone.

All the sulfonamide-resistant isolates were screened by PCRs, which were performed with primers specific for *sul1* (9), *sul2* (9), and *sul3* (15) genes. In 200 sulfonamide-resistant isolates, 152 (76%) *sul1*, 74 (37%) *sul2*, and 14 (7%) *sul3* genes were detected. In 34 isolates, more than one gene coding for sulfonamide resistance was present: *sul1* and *sul2* in 24; *sul1* and *sul3* in four; and *sul1*, *sul2*, and *sul3* in six (Table 1). The sequencing data from one of the PCR products obtained with primers for the *sul3* gene showed identity with the newly described *sul3* gene (15). The 14 *sul3*-positive *Salmonella* isolates were from human clinical samples (six), from foods of animal origin (six), and from environmental sources (two), obtained from geographically dispersed regions in Portugal and belonging to three *Salmonella* serotypes. The PFGE profiles showed one clone which includes the four *S. enterica* serotype Rissen isolates, all from swine end products, and three clones were identified among the nine serotype Typhimurium isolates, with one of them carrying *sul1*, *sul2*, and *sul3* genes isolated from swine end products, humans, and the environment (Table 2; Fig. 1).

The presence of class 1 integrons was tested by PCR, with the primers 5′CS-3′CS (10) and the specific primer *int1* (9) in all the sulfamethoxazole-resistant isolates. To characterize the conserved segment 3′CS, the presence of *sul1* and *qacE*Δ1 genes was determined in all the isolates by PCR, with specific primers (17). To determine the content of the variable regions of the integrons, sequencing and PCR with the 5′CS primer in combination with reverse primers for several genes were performed. Class 2 integrons were detected by PCR with primers specific for *int2* (11). PCR analysis revealed that, of 200 sulfonamide-resistant isolates, 149 (75%) contained class 1 inte-

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TABLE 1. Distribution of sulfonamide resistance genes in *Salmonella* isolates and relation to class 1 and class 2 integrons

Strain characteristic (*n*)	No. of isolates with gene(s):					
	sul1	*sul2*	*sul3*	*sul1*, *sul2*	*sul1*, *sul3*	*sul1*, *sul2*, *sul3*
Sulfonamide resistance (200)	118	44	4	24	4	6
Class 1 integron (149)	112	1	4	22	4	6
Class 1 and 2 integrons (5)	4	0	0	1	0	0

grons and 5 (3%) contained class 1 and class 2 integrons (Table 1). Five of the sulfamethoxazole-resistant strains carried class 1 integrons, which lacked the *qacE*Δ1 and *sul1* genes at the 3′CS.

Among the 154 isolates carrying class 1 integrons, 149 presented the *sul1* gene, found alone (116 isolates) or simultaneously with *sul2* (23 isolates) or *sul3* (four isolates) and with *sul2* and *sul3* (six isolates) (Table 1). In the five strains with class 1 integrons, which lacked the *qacE*Δ1 and *sul1* genes, four carried a *sul3* gene and the other one carried a *sul2* gene. The *sul1* gene is frequently located on class 1 integrons, which could be confirmed by the fact that, of the 152 *sul1*-positive isolates, 149 (98%) harbored class 1 integrons. Interestingly, the *sul3* gene occurs in *Salmonella* isolates carrying class 1 integrons, with identical cassette genes (*dfrA12* and *aadA2*) in all but one isolate (*dfrA1* and *aadA1*), which confer resistance to the same antimicrobial agents (Table 2).

Conjugation assays on an agar plate were carried out with the recipient strain *Escherichia coli* K802N. Transconjugants were selected on Mueller-Hinton agar 2 (bioMérieux) containing sulfamethoxazole (256 μg/ml) plus nalidixic acid (64 μg/ml). Sulfonamide resistance was transferred from 6 out of the 14 *sul3*-positive isolates; transconjugants were confirmed by PCR specific for *sul3*. Resistance to other antimicrobial agents and class 1 integrons was also cotransferred in those isolates (Table 2).

Class 1 integrons and sulfonamide resistance genes are disseminated among *Salmonella* in contrast with class 2 integrons. A significant proportion (77%) of isolates resistant to sulfonamides carried class 1 integrons; in nearly all cases (98%) the *sul1* gene was a consistent marker for the presence of this class of integrons. The presence in all isolates with class 1 integrons of at least one of the *sul* genes provides, when bacteria are submitted to selective pressure by sulfonamides, a useful tool for the maintenance and further extension of resistance to other antimicrobial agents.

In our *Salmonella* isolates the *sul1* gene was the most frequent mechanism of resistance to sulfonamides. In contrast, recently the spread of *sul2* seems to have increased in other European countries (2, 9), as the gene was reported to be more widespread among clinical isolates of *E. coli* than the *sul1* gene.

A new sulfonamide resistance gene, named *sul3*, has been detected in *E. coli* isolates from pigs in Switzerland (15) and also among German *E. coli* isolates from various animals and foods (7) and *Salmonella* isolates (8). The *sul3* gene was also identified in two different human clinical isolates of *E. coli* in Sweden (5). In our study, the newly described *sul3* gene has now been identified in 14 *Salmonella* isolates from three serotypes collected from human and nonhuman sources in Portugal, being mainly observed in isolates from swine food products. The consumption of sulfonamides for veterinary use is generally widespread in Portugal, particularly for swine production. So, the appearance of a newly described gene and the simultaneous presence of several *sul* genes may reflect its high usage in food-producing animals, as verified in our country by Pena et al. (14). The association of *sul3* genes with conjugative plasmids in the food-borne isolates of the serotype Rissen clone and serotype Typhimurium and in one human *Salmo-*

TABLE 2. Characterization of *Salmonella* isolates with the *sul3* gene

Serotype and isolate (phage type)	PFGE type[a]	Isolation date/area of Portugal	Source	Class 1 integron genes[b]	*sul* gene(s)[b]
Rissen					
SFCL-5	A	July 2002/north	Swine product	***int1*, *dfrA12*, *aadA2*, *qacE*Δ1, *sul1***	***sul1*, *sul3***
BF033	A	October 2002/north	Swine product	***int1*, *dfrA12*, *aadA2*, *qacE*Δ1, *sul1***	***sul1*, *sul3***
SFS-2	A2	2002/north	Sausage	***int1*, *dfrA12*, *aadA2***	***sul3***
BF128	A2	August 2003/north	Swine product	***int1*, *dfrA12*, *aadA2*, *qacE*Δ1, *sul1***	***sul1*, *sul3***
Typhimurium					
IH459/02 (DT104)	B	August 2002/south	Human (hospital 1)	*int1*, *dfrA12*, *aadA2*, *qacE*Δ1, *sul1*	*sul1*, *sul2*, *sul3*
AF30 (DT104)	B3	May 2003/south	Swine product	*int1*, *dfrA12*, *aadA2*, *qacE*Δ1, *sul1*	*sul1*, *sul2*, *sul3*
IE352/03 (DT104)	B3	June 2003/north	Seawater (beach 1)	*int1*, *dfrA12*, *aadA2*, *qacE*Δ1, *sul1*	*sul1*, *sul2*, *sul3*
IE357/03 (DT104)	B3	June 2003/north	Seawater (beach 2)	*int1*, *dfrA12*, *aadA2*, *qacE*Δ1, *sul1*	*sul1*, *sul2*, *sul3*
IH752/03 (DT104)	B3′	2003/south	Human (hospital 2)	*int1*, *dfrA12*, *aadA2*, *qacE*Δ1, *sul1*	*sul1*, *sul2*, *sul3*
IH779/03 (DT104)	B3″	2003/north	Human (hospital 3)	*int1*, *dfrA12*, *aadA2*, *qacE*Δ1, *sul1*	*sul1*, *sul2*, *sul3*
IH184/03	C	April 2003/north	Human (hospital 4)	*int1*, *dfrA12*, *aadA2*	*sul3*
BH212	C	2003/north	Human (hospital 5)	*int1*, *dfrA12*, *aadA2*	*sul3*
AF29	D	May 2003/south	Swine product	***int1*, *dfrA1*, *aadA1*, *qacE*Δ1, *sul1***	***sul1*, *sul3***
IIIb:65:lv:enxz15					
IH842/03	E	2003/south	Human (hospital 6)	***int1*, *dfrA12*, *aadA2***	***sul3***

[a] Clones are designated by capital letters, and subtypes are defined by a subindex that indicates the number of bands that differ from the strain considered to be the initial PFGE type.

[b] Resistance genes produced in transconjugants appear in boldface.

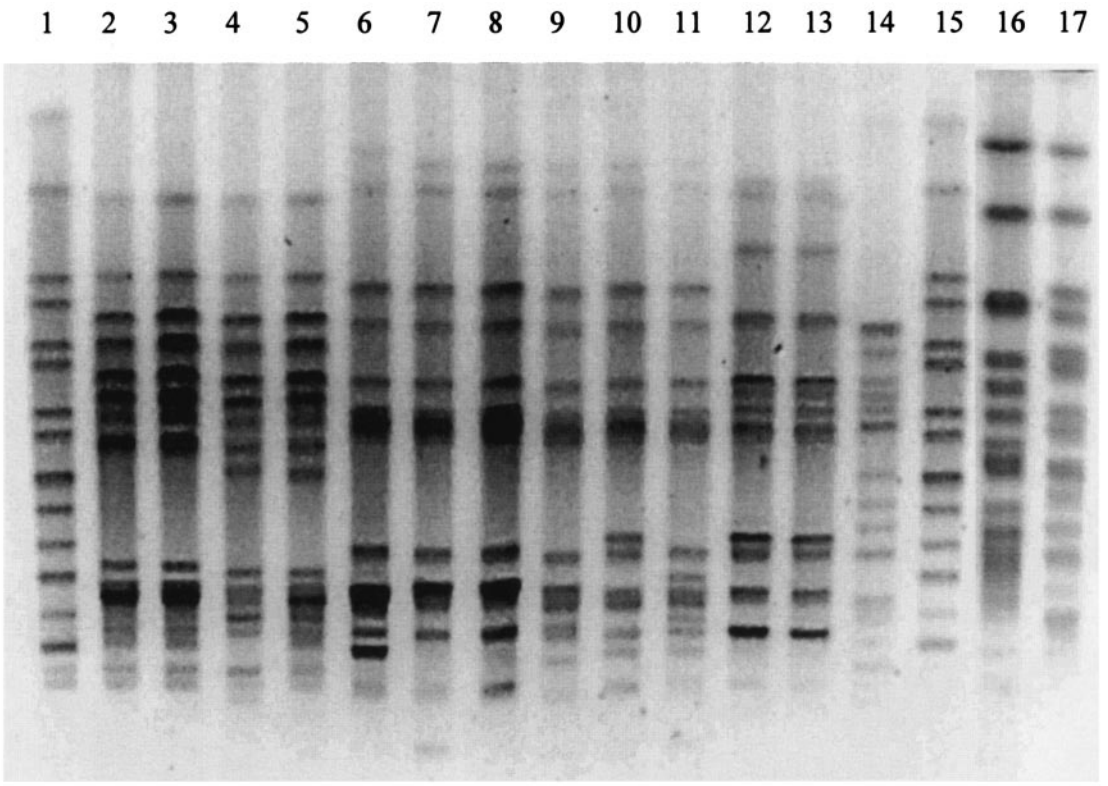

FIG. 1. PFGE patterns of *sul3*-carrying *Salmonella* isolates. Lanes: 1, 15, and 17, *Salmonella* serotype Braenderup H9812 (CDC); 2, SFCL-5 (type A); 3, BF033 (type A); 4, SFS-2 (subtype A2); 5, BF128 (subtype A2); 6, IH459/02 (type B); 7, AF30 (subtype B3); 8, IE352/03 (subtype B3); 9, IE357/03 (subtype B3); 10, IH752/03 (subtype B3′); 11, IH779/03 (subtype B3″); 12, IH184/03 (type C); 13, BH212 (type C); 14, AF29 (type D); 16, IH842/03 (type E).

nella isolate of another serotype could facilitate the further spread of this gene to other bacteria.

Interestingly, the *sul3* gene occurs in *Salmonella* carrying class 1 integrons with *aadA* and *dfrA* gene cassettes, which allows isolates to survive exposure to sulfamethoxazole and trimethoprim, a combination frequently used in therapeutics. It is of note that serotype Typhimurium was the main serotype carrying the *sul3* gene and the only serotype associated with the three *sul* genes. The persistence of several sulfonamide resistance genes may be the result of the successive pressure exerted by sulfonamides and other antimicrobial agents that are also commonly used and may be mitigated by the fact that not all sulfonamide-resistant determinants exert a fitness cost, as described for the *sul2*-encoding plasmid (3).

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REFERENCES

1. **Centers for Disease Control and Prevention.** 2002. One-day (24-48h) standardized laboratory protocol for molecular subtyping of *Escherichia coli* O157:H7, non-typhoidal *Salmonella* serotypes, and *Shigella sonnei* by pulsed field gel electrophoresis (PFGE). PulseNet PFGE manual. Centers for Disease Control and Prevention, Atlanta, Ga.
2. **Enne, V. I., D. M. Livermore, P. Stephens, and L. M. C. Hall.** 2001. Persistence of sulfonamide resistance in *Escherichia coli* in the UK despite national prescribing restriction. Lancet **357:**1325–1328.
3. **Enne, V. I., P. M. Bennett, D. M. Livermore, and L. M. C. Hall.** 2004. Enhancement of host fitness by the *sul2*-coding plasmid p913 in the absence of selective pressure. J. Antimicrob. Chemother. **53:**958–963.
4. **Goldstein, C., M. D. Lee, S. Sanchez, C. Hudson, B. Phillips, B. Register, M. Grady, C. Liebert, A. O. Summers, D. G. White, and J. Maurer.** 2001. Incidence of class 1 and 2 integrases in clinical and commensal bacteria from livestock, companion animals, and exotics. Antimicrob. Agents Chemother. **45:**723–726.
5. **Grape, M., L. Sundstrom, and G. Kronvall.** 2003. Sulfonamide resistance gene *sul3* found in *Escherichia coli* isolates from human sources. J. Antimicrob. Chemother. **52:**1022–1024.
6. **Guerra, B., S. Soto, S. Cal, and M. C. Mendoza.** 2000. Antimicrobial resistance and spread of class I integrons among *Salmonella* serotypes. Antimicrob. Agents Chemother. **44:**2166–2169.
7. **Guerra, B., E. Junker, A. Schroeter, B. Malorny, S. Lehmann, and R. Helmuth.** 2003. Phenotypic and genotypic characterization of antimicrobial resistance in German *Escherichia coli* isolates from cattle, swine and poultry. J. Antimicrob. Chemother. **52:**489–492.
8. **Guerra, B., E. Junker, and R. Helmuth.** 2004. Incidence of the recently described sulfonamide resistance gene *sul3* among German *Salmonella enterica* strains isolated from livestock and food. Antimicrob. Agents Chemother. **48:**2712–2715.
9. **Kerrn, M. B., T. Klemmensen, N. Frimodt-Moller, and F. Espersen.** 2002. Susceptibility of Danish *Escherichia coli* strains isolated from urinary tract infections and bacteraemia, and distribution of *sul* genes conferring sulfonamide resistance. J. Antimicrob. Chemother. **50:**513–516.
10. **Lévesque, C., L. Piché, C. Larose, and P. H. Roy.** 1995. PCR mapping of integrons reveals several novel combinations of resistance genes. Antimicrob. Agents Chemother. **39:**185–191.
11. **Mazel, D., B. Dychinco, V. A. Webb, and J. Davies.** 2000. Antibiotic resistance in the ECOR collection: integrons and identification of a novel *aad* gene. Antimicrob. Agents Chemother. **44:**1568–1574.
12. **National Committee for Clinical Laboratory Standards.** 2003. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 6th ed. Approved standard M7-A6. National Committee for Clinical Laboratory Standards, Wayne, Pa.

13. **Orman, B. E., S. A. Pineiro, S. Arduino, M. Galas, R. Melano, M. I. Caffer, D. O. Sordelli, and D. Centrón.** 2002. Evolution of multiresistance in nontyphoid *Salmonella* serovars from 1984 to 1998 in Argentina. Antimicrob. Agents Chemother. **46:**3963–3970.
14. **Pena, A., C. Serrano, C. Réu, L. Baeta, V. Calderón, I. Silveira, J. C. Sousa, and L. Peixe.** 2004. Antibiotic residues in edible tissues and antibiotic resistance of faecal *Escherichia coli* in pigs from Portugal. Food Addit. Contam. **21:**749–755.
15. **Perreten, V., and P. Boerlin.** 2003. A new sulfonamide resistance gene (*sul3*) in *Escherichia coli* is widespread in the pig population of Switzerland. Antimicrob. Agents Chemother. **47:**1169–1172.
16. **Pritchett, L. C., M. E. Konkel, J. M. Gay, and T. E. Besser.** 2000. Identification of DT104 and U302 phage types among *Salmonella enterica* serotype Typhimurium isolates by PCR. J. Clin. Microbiol. **38:**3484–3488.
17. **Sandvang, D., F. M. Aarestrup, and L. B. Jensen.** 1998. Characterisation of integrons and antibiotic resistance genes in Danish multiresistant *Salmonella enterica* Typhimurium DT104. FEMS Microbiol. Lett. **160:**37–41.
18. **Skold, O.** 2000. Sulfonamide resistance: mechanisms and trends. Drug Resist. Updates **3:**155–160.
19. **Threlfall, E. J.** 2002. Antimicrobial drug resistance in *Salmonella*: problems and perspectives in food- and water-borne infections. FEMS Microbiol. Rev. **26:**141–148.