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Antimicrobial resistance and class I integrons in *Salmonella enterica* isolates from wild boars and Bísaro pigs

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Summary. The antibiotic resistance phenotype and genotype and the integron type were characterized in 58 *Salmonella enterica* isolates recovered from Bísaro pigs and wild boars (20 *S.* Typhimurium, 17 *S.* Rissen, 14 *S.* Enteritidis and 7 *S.* Havana). Most *S.* Typhimurium isolates (15/20 of Bísaro pigs and wild boars) showed ampicillin, chloramphenicol, streptomycin, tetracycline, sulfonamide, and amoxicillin-clavulanic acid resistances. Of the 17 *S.* Rissen isolates of both origins, 13 were resistant to ampicillin, tetracycline and trimethoprim-sulfamethoxazole. Among the *S.* Enteritidis isolates of Bísaro pigs, eight were nalidixic acid-resistant and three were sulfonamide-resistant. The *tet*(A) or *tet*(G) genes were detected in most tetracycline-resistant isolates. The *intI1* gene was identified in 72.5% of *S. enterica* isolates in which the conserved region 3′ of class 1 integrons (*qacE*Δ1+*sul1*) was also amplified, whereas none had the *intI2* gene. The *dfrA12+orfF+aadA2* gene cassette arrangement was found in the variable region of class 1 integrons in 14 *S.* Rissen isolates. Fifteen *S.* Typhimurium isolates had two integrons with variable regions of 1000 and 1200 bp that harbored the *aadA2* and bla_{PSE-1} gene cassettes, respectively. In these isolates the *floR* and *tet*(G) genes were also amplified, indicative of the genomic island 1 (SGI1). *Salmonella* Typhimurium and *S.* Rissen of animal origin frequently show a multi-antimicrobial resistant phenotype, which may have implications in public health. [**Int Microbiol** 2011; 14(1):19-24]

Keywords: *Salmonella* spp. · antibiotic resistance · wild boars · Bísaro pigs

Introduction

Bacterial antibiotic resistance has become a worldwide public health problem with direct impact on food safety. The monitoring of food-borne pathogens that have important animal reservoirs, such as *Salmonella*, is of outmost importance, as highlighted in the European Union's proposed legislation [Directive 2003/99/EC of the European Parliament and of the Council of 17 November 2003 on the monitoring of zoonoses and zoonotic agents, amending Council Decision 90/424/EEC and repealing Council Directive 92/117/EEC. Official Journal L 325, 2003, pp 31–40]. *Salmonella* spp. are among of the most frequently reported causes of food poisoning in the world [31]. A study devel-

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oped in Portugal revealed that *Salmonella* was responsible for 41.8% food-poisoning-related outbreaks in the period 1987–1991 in that country [Araújo A (1996) Segurança alimentar. Meribérica/Liber Eds., Lisboa]. Salmonellosis is a major public health problem in most industrialized countries [6]; the primary reservoir for *Salmonella* sp. is the intestinal tract of animals, and colonization is favored by intensive animal production [2]. Although *Salmonella enterica* serovars are some of the best studied bacterial pathogens, much remains to be learned about them, especially taking into account that they cause significant morbidity and mortality worldwide, have broad host ranges, are able to establish persistent infections acting as reservoirs for transmission/shedding, and are increasingly resistant to many antibiotics [6]. The prevalence of resistance among *Salmonella* to several antibiotics, including ampicillin and trimethoprim-sulfamethoxazole, has increased in recent decades [Centers for Disease Control and Prevention (2007) National Antimicrobial Resistance Monitoring System for Enteric Bacteria, 2004. Human Isolates Annual report. U.S. Dept. of Health and Human Services, CDC, Atlanta, GA, USA].

The location of specific antibiotic-resistance genes on mobile genetic elements (such as plasmids and transposons) allows the transmission of resistance among bacteria, even among different species [8]. Furthermore, single genetic elements, such as integrons, may contain several genes involved in the resistance to different families of antibiotics, thus making the bacteria multi-resistant to different antibiotics [16]. The widespread use of antibiotics in food-animal production has contributed to the occurrence of *Salmonella* with decreased susceptibility to antibiotics. These strains can, in turn, be transmitted to humans through food products, particularly those of animal origin [25]. To our knowledge, in Portugal, there has been only one previous study, performed by our research group in which the prevalence of *Salmonella* in wild animals was determined, although antibiotic resistance was not evaluated in that study [33]. The aim of the present work was to evaluate antibiotic resistance phenotypes and the implicated mechanisms of resistance in *Salmonella* sp. from wild boars and Bísaro pigs in Portugal and to characterize the integrons in these isolates.

Materials and methods

Sampling and bacteria. Fecal samples of 35 Bísaro swine (endemic breeding) were recovered from a Bísaro pig farm located in Northern Portugal during December 2007. In this farm system of pig production, grower pigs are housed indoors in group-housing or straw-lined sheds, whilst pregnant sows are confined in sow stalls (gestation crates) and give birth in farrowing crates. Each fecal sample corresponded to one batch pen (with 10–15 animals). The age of the animals ranged from five weeks to adult age and none of them had received antibiotics in the previous four months.

In addition, 22 *Salmonella enterica* isolates (16 *S.* Typhymurium and 6 *S.* Rissen) recovered from fecal samples of wild boars (*Sus scrofa*), previously obtained and serotyped [33], were included in this study.

Microbiological culture method. The fecal samples of the Bísaro pigs were analyzed by means of standard culture methods, according to ISO norm 6579:2002-07 [Microbiology of food and animal feeding stuffs - Horizontal method for the detection of *Salmonella* spp.]. Briefly, 10 g of feces were suspended in buffered peptone water (BPW Merck, Darmstadt, Germany) (1:10). The suspension was homogenized in a Stomacher (90 s), incubated at 37°C for 18 ± 2 h, after which 0.1 ml and 1.0 ml were, respectively, inoculated in Rappaport-Vassiliadis medium containing Soya peptone (RVS broth, Oxoid, Cambridge, UK) and in Muller-Kauffmann tetrathionate/novobiocin broth (MKTTn broth, Merck, Darmstadt, Germany). The RVS broth was incubated at 41.5 ± 1°C for 24 h ± 3 h, and the MKTTn broth at 37ºC ± 1°C for 24 h ± 3 h. In a second stage, one loop of each selective enrichment broth was streaked onto the surface of two selective solid media: Hektoen and xylose-lysine-deoxycholate (XLD) agar (Oxoid, Cambridge, UK). Finally, isolates of presumptive *Salmonella* (1 or 2 colonies from each sample) were confirmed by means of biochemical tests [oxidase reaction, triple sugar iron agar (Oxoid, Cambridge, UK), urea broth (Merck, Darmstadt, Germany), L-lysine descarboxylation medium (Oxoid, Cambridge, UK)], and serological agglutination with Poly A-I & Vi antiserum (Difco, Lawrence, Kansas, USA).

***Salmonella* serotyping.** *Salmonella* isolates from Bísaro pigs were serotyped from each positive sample according to the Kauffmann-White scheme [Popoff MY (2001) Antigenic formulas of the *Salmonella* serovars. 8th rev. Institute Pasteur, WHO Collaborating Centre for Reference and Research on *Salmonella*] in the LNIV-National Reference Laboratory for *Salmonella.*

Antibiotic susceptibility testing. Antibiotic susceptibility testing to 17 antibiotics was performed in all 58 *Salmonella* isolates of Bísaro pigs and wild boars by a disk diffusion method [Clinical and Laboratory Standards Institute (CLSI) (2008) Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals. Approved standard. CLSI document M31-A3. CLSI, Wayne, PA, USA]. The antibiotics tested were (μg/disc): ampicillin (10), amikacin (30), amoxicillin-clavulanic acid (10), cefotaxime (30), ceftazidime (30), aztreonam (30), cefoxitin (30), gentamicin (10), tobramycin (10), streptomycin (10), sulfonamides (200), tetracycline (30), trimethoprim-sulfamethoxazole (1.25 + 23.75), nalidixic acid (30), ciprofloxacin (5), chloramphenicol (30), and imipenem (10). AmpC phenotype was studied by comparing disk diffusion susceptibility to cefoxitin (30 μg) with and without cloxacillin (200 μg) [28], and the ESBL-positive phenotype was checked as previously recommended [Ref. CLSI, 2008].

Antibiotic resistance genes. The presence of genes encoding SHV, TEM, OXA, and PSE-1 type β-lactamases was studied by specific PCRs [7,10], and the obtained amplicons were sequenced. DNA sequences were compared with those included in the GenBank database as well as with those deposited at the website [http:// www.lahey.org/Studies/], in order to determine the specific type of β-lactamase gene. The following resistance genes were also studied by PCR [15,26]: *tet*(A) and *tet*(B) (in tetracycline-resistant isolates), *aadA* (in streptomycin-resistant isolates), *cmlA* (in chloramphenicol-resistant isolates), *sul1* (in SXT-resistant isolates) and *tet*(G) and *floR* genes (in bla_{PSE-1} positive isolates). The *gyrA* and *parC* genes were amplified

by PCR and sequenced in nalidixic-acid-resistant isolates [15]. Class 1 and 2 integrases were analyzed by PCR, as was the 3′-conserved region (3′-CS) of class 1 integrons, *qacE*Δ1+*sul1*. The variable region of class 1 integrons were amplified by PCR and sequenced to determine their gene cassette composition [15].

Results

Of the 35 fecal samples of Bísaro pigs analyzed in this study, 30 were positive for *Salmonella* detection (86%), and 36 isolates were recovered (one per positive sample, and two in the case of different profile). The serotypes detected among these isolates were (number of isolates): *S.* Enteritidis (14), *S.* Rissen (11), *S.* Havana (7) and *S.* Typhimurium (4). We selected 22 *Salmonella* isolates previously recovered from wild boars [33] for the characterization of antibiotic-resistance phenotypes and genotypes in the present study (16 *S.* Typhimurium and 6 *S.* Rissen). The number of *Salmonella* isolates of wild boars and Bísaro pigs resistant to the tested antibiotics is shown in Table 1. Regarding the isolates of wild boars, 13 of 16 *S.* Typhimurium isolates showed ampicillin and chloramphenicol resistance, and most of them also had streptomycin, tetracycline, sulfonamide and amoxicillin-clavulanic acid resistance. In addition, five different phenotypes of antibiotic resistance were identified among the *S.* Rissen isolates, three of them conferring resistance to at least four different families of antibiotics (Table 2).

Regarding the isolates of Bísaro pigs, five different phenotypes of resistance were detected (Table 2). The AmpC phenotype was studied in all ampicillin-resistant isolates of wild boars and Bísaro pigs but negative results were obtained in all cases. Table 2 shows the antibiotic resistance genes detected according to the specific antibiotic resistance phenotype in 40 *Salmonella* isolates that were resistant to at least one antibiotic. None of the isolates had a positive ESBL-phenotype. The bla_{PSE-1} gene was identified in 15 out of the 29 ampicillin-resistant *Salmonella* isolates of this study (all of them of the serotype Thyphimurim), but the bla_{TEM}, bla_{SHV} or bla_{OXA} genes were not found among ampicillin-resistant isolates. The *tet*(A) or *tet*(G) genes were detected in eight *S.* Ris-

Table 1. Number of ntibiotic-resistant *Salmonella* isolates of the different serovars and origins

Antibiotic	Wild boars (n = 22)		Bísaro pigs (n = 36)			
	Salmonella Typhimurium (n = 16)	*Salmonella* Rissen (n = 6)	*Salmonella* Typhimurium (n = 4)	*Salmonella* Rissen (n = 11)	*Salmonella* Enteritidis (n = 14)	*Salmonella* Havana (n = 7)
AMP	13	3	3	10	0	0
AMC	12	3	3	4	0	0
CTX	0	0	0	0	0	0
CAZ	0	0	0	0	0	0
AZT	0	0	0	0	0	0
FOX	0	0	0	0	0	0
IMP	0	0	0	0	0	0
GEN	0	0	0	0	0	0
TOB	0	0	0	0	0	0
STR	12	3	3	3	0	0
AK	0	0	0	0	0	0
TET	12	5	3	10	0	0
SUL	12	5	3	10	3	0
SXT	0	4	0	10	0	0
NAL	0	0	0	0	8	0
CIP	0	0	0	0	0	0
CHL	13	0	3	0	0	0

AMP, ampicillin; AMC, amoxicillin-clavulanic acid; CTX, cefotaxime; CAZ, ceftazidime; AZM, aztreonam; FOX, cefoxitin; IMP, imipenem; GEN, gentamicin; TOB, tobramycin; STR, streptomycin; AK, amikacin; TET, tetracycline; SUL, sulfonamides; SXT, trimethoprim-sulfamethoxazole; NAL, nalidixic acid; CIP, ciprofloxacin; CHL, chloramphenicol.

Table 2. Antibiotic resistance phenotypes and genes detected in 40 resistant (to at least one antibiotic) *Salmonella* isolates from wild boars and Bísaro pigs

				Class 1 integrons		
Serotype (no. of isolates)	Animal origin (no. of isolates)	Antibiotic-resistance phenotype[a]	Resistance genes[b]	*intI1*	*qacE*Δ1 +*sul1*	Variable region
S. Rissen (16)	Wild boar (1)	TET	*tet*(A)	–	–	Negative
	Wild boar (1)	STR, SUL	–	–	–	Negative
	Wild boar (1)	TET, SUL, SXT	–	+	+	2000 bp (*dfrA12* + *orfF* + *aadA2)*
	Wild boar (1)	AMP, AMC, TET, SUL, SXT	*tet*(A)	+	+	2000 bp (*dfrA12* + *orfF* + *aadA2)*
	Wild boar (1)	AMP, AMC, STR, TET, SUL, SXT	*tet*(A)	+	+	2000 bp (*dfrA12* + *orfF* + *aadA2)*
	Wild boar (1)	AMP, AMC, STR, TET, SUL, SXT	–	+	+	2000 bp (*dfrA12* + *orfF* + *aadA2)*
	Bísaro pig (4)	AMP, TET, SUL, SXT	*tet*(A)	+	+	2000 bp (*dfrA12* + *orfF* + *aadA2*)
	Bísaro pig (2)	AMP, TET, SUL, SXT	–	+	+	2000 bp (*dfrA12* + *orfF* + *aadA2*)
	Bísaro pig (1)	AMP, AMC, STR, TET, SUL, SXT	*tet*(A)	+	+	2000 bp (*dfrA12* + *orfF* + *aadA2*)
	Bísaro pig (3)	AMP, AMC, STR, TET, SUL, SXT	–	+	+	2000 bp (*dfrA12* + *orfF* + *aadA2*)
S. Typhimurium (16)	Wild boar (1[c])	AMP, CHL	ND	ND	ND	ND
	Wild boar (12)	AMP, AMC, STR, TET, SUL, CHL	*floR, tet*(G)	+	+	1000 / 1200 bp (*aadA*2 / bla_{PSE-1})
	Bísaro pig (3)	AMP, AMC, STR, TET, SUL, CHL	*floR, tet*(G)	+	+	1000 / 1200 bp (*aadA*2 / bla_{PSE-1})
S. Enteritidis (8)	Bísaro pig (5)	NAL[d]	–	–	–	Negative
	Bísaro pig (3)	NAL[d], SUL	–	–	–	Negative

[a]Antibiotics: AMP, ampicillin; AMC, amoxicillin-clavulanic acid; STR, streptomycin; TET, tetracycline; SUL, sulfonamides; SXT, trimethoprim-sulfamethoxazole; NAL, nalidixic acid; CHL, chloramphenicol.
[b]Detected outside integron variable regions.
[c]This strain was lost and could not be further characterized.
[d]Asp87Tyr amino acid change detected in GyrA protein. No mutations were detected in the parC gene.
ND: Not determined.

sen and 15 *S.* Typhimurium isolates, respectively, among the 30 tetracycline-resistant isolates (15 *S.* Typhimurium and 15 *S.* Rissen).

The *intI1* gene, encoding the integrase of class 1 integrons, was identified in 29 isolates (72.5%) in which the conserved region 3′ (*qacE*Δ1+*sul1*) of this type of integron was also amplified. All isolates were negative for the *intI2* gene. The *dfrA12*+*orfF*+*aadA2* gene cassette arrangement was found in the 14 integron-positive *S.* Rissen isolates. Fifteen *S.* Typhimurium isolates showed two integrons with variable regions of 1000 and 1200 bp that harbored the *aadA2* and bla_{PSE-1} gene cassettes, respectively. These 15 bla_{PSE-1}-positive isolates also amplified the *floR* and *tet*(G) genes, encoding resistance to chloramphenicol and tetracycline, respectively, and indicative of *Salmonella* genomic island 1.

Discussion

An emerging problem related to food-borne diseases that represent a threat to public health has been described by different authors [18,29]. In this context, *Salmonella* sp. has assumed a prominent role, due to the important increase in human salmonellosis that has occurred in recent decades [Araújo A (1996) Segurança alimentar. Meribérica/Liber Eds., Lisboa]. In this study and in a previous one [33], *Salmonella* was isolated from fecal samples of Bísaro pigs and wild boars (86% and 22%, respectively). The percentages of *Salmonella* strains isolated from animals intended for human consumption were higher than those reported in other studies [3,4]. High percentages were also found in similar

studies conducted in slaughtered pigs (61% of samples) and in chickens (above 50%), such that these animals were considered unsuitable for consumption [9,30]. In a previous study performed in Portugal, *Salmonella* sp. was recovered from 27.7% of pigs slaughtered for consumption [32], with similar results obtained in other countries [Proceedings of the 4th International Symposium on the Epidemiology and Control of *Salmonella* and other foodborne pathogens in pork, Leipzig, Germany, 2001. Davies R et al., pp 162-173; Sorensen O et al., pp 183-185].

In 2000, the two most common *S. enterica* serotypes isolated from human sources were Typhimurium and Enteritidis [Centers for Disease Control and Prevention (2001) *Salmonella* surveillance: Annual summary, 2000. U.S. Department of Health and Human Services, CDC, Atlanta, GA, USA].

In our study, the serotypes detected in isolates from Bísaro pigs were *S.* Typhimurium, *S.* Rissen, *S.* Enteritidis, and *S.* Havana. In the study developed by Vieira-Pinto et al. [32] in pigs, eight different serotypes were identified and the most prevalent serotype was *S.* Typhimurium. This serotype should be given special attention because of its virulence for humans and animals [5] and its high resistance rates to antibiotics [Proceedings of the 3rd International symposium on the Epidemiology and Control of *Salmonella* in Pork. Washington DC, USA (1999) Nielsen B et al., pp 261-263]. On the other hand, *S.* Enteritidis was the most frequent serotype among Bísaro pig isolates in our study. Among clinical and environmental *Salmonella* isolates, there is a strong predominance of the *S.* Typhimurium serotype (61%), followed by the *S.* Enteritidis serotype and, at a lower percentage, the *S*. Rissen serotype (9%) [2].

In previous research conducted in Portugal, most of the clinical and environmental *S. enterica* isolates studied were found to be resistant to tetracycline, streptomycin, ampicillin, and sulfonamides [2]. In our study, 73% and 36% of *Salmonella* isolates from wild boars and Bísaro pigs, respectively, were ampicillin resistant, and 77% and 36% were tetracycline resistant. Conventional antimicrobial agents, such as ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole, were the drugs of choice in the treatment of salmonellosis before the 1980s. However, multidrug resistance, with resistance rates to these antimicrobial agents of more than 50%, has been reported in many areas of the world [10,14,27]. We found that 18% and 28% of the isolates from wild boars and Bísaro pigs, respectively, were resistant to trimethoprim-sulfamethoxazole.

The presence of *tet* genes has been reported as a prevailing mechanism for tetracycline resistance in *E. coli* isolates from pet animals [11] and wild animals [12,23]. In our study, the *tet*(A) gene was only detected in isolates of the S. Rissen serotype, and *tet*(G) in isolates of the *S.* Typhimurium serotype.

In our study, 15 out of 16 chloramphenicol-resistant *S.* Typhimurium isolates amplified the *floR* gene, as previously reported [20]. In addition, these 15 isolates carried the *tet*(G) gene and two integrons containing bla_{PSE-1} and *aadA2* as gene cassettes within their variable regions, which is characteristic of the *Salmonella* genomic island type 1 (SGI1) [2,13,17].

Integrons provide a great selective advantage to the bacteria that carry them. The high percentage of isolates in our study that contained class 1 integrons is indicative of their high rate of occurrence in *Salmonella* strains. As also described previously [17,21,22], our results demonstrated a strong association of class 1 integrons with the identified resistance to specific antibiotics, attributed in part to the presence of resistance gene cassettes within these integrons.

In accordance with previous investigations, we confirmed a predominance of gene cassettes conferring resistance to β-lactam (bla_{PSE-1}), streptomycin (*aadA*), and trimethoprim (*dfrA*), and of *aadA* genes carried by all integron-containing *Salmonella* serotypes [2]. The persistence of these genes, which have been reported worldwide in isolates from different origins [1,19], might be associated with the extensive use of streptomycin, sulfonamides, and other antibiotics in food-producing animals.

The results presented in this study highlight the importance of antibiotic resistance among *Salmonella* isolates from food-producing and wild animals, that can constitute a serious public health problem. The high frequencies of multiantibiotic resistant isolates of the serovars *S*. Typhymurim and *S*. Rissen in fecal samples from these animals is of special interest for human health. Most of these resistant isolates carry integrons containing some of the resistant genes. Thus, it is crucial to track the evolution of multiresistant *S. enterica* isolates in different types of animals and to analyze the implications for human health and the potential transmission of these bacteria through the food chain [24].

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Competing interests. None declared.

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