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Pheno and genotyping of *Salmonella* from slaughtered pigs in a Portuguese abattoir reveal differential persistence ability

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ABSTRACT

Pork is one of the most common vehicles of non-typhoid foodborne *Salmonella*, with the slaughterhouse representing a key point for the infection of pigs and carcass contamination. By comparing matching samples taken from animals at the dirty (skin) and clean (inner and outer carcass surface) areas of the slaughterline, this study aimed to assess potential *Salmonella* contamination routes of pig carcasses within a Portuguese abattoir. Forty-four *Salmonella* isolates were retrieved from 120 pigs, and further characterized through pheno and genotypical methods. Most frequent serotypes found were *Salmonella* 4, [5],12:i:- (47.7%), *Salmonella* Rissen (40.9%) and *Salmonella* Derby (11.4%). Isolates were most commonly collected from the skin of pigs sampled at the dirty area (59.1%), followed by the inner (38.1%) and outer (9.1%) carcass surface sampled at the clean area. Most isolates (79.5%) were considered to be multidrug resistant and all harbored the virulence associated genes *invA*, *invH*, *sopB*, *stn*, *slyA*, *phoP*, *phoQ* and *agfA*. PFGE analysis revealed that most bacterial isolates belonging to the same serotype, recovered from animals from different farms, and slaughtered at separate days were genetically undistinguishable. Furthermore, our findings suggest that *Salmonella* Rissen might have an increased ability to endure on the slaughterhouse environment when compared with the other serotypes.

Concluding, this study shows that the slaughterhouse may be a key point for the dissemination of resistant and virulent *Salmonella* strains, which stresses the importance of the implementation of good hygiene practices at the slaughterhouse and of the application of corrective measures to avoid cross-contamination.

1. Introduction

Salmonella enterica is a major cause of food-borne illness in the European Union (EU), with a total 91,662 confirmed human salmonellosis cases in 2017, accountable for 24.4% of all food-borne outbreaks (EFSA and ECDC, 2018). Human salmonellosis due to non-typhoidal salmonellae is commonly characterized by gastroenteritis, but in specific risk groups the gastrointestinal illness can lead to serious and potentially fatal complications, such as bacteremia or endocarditis (Acheson and Hohmann, 2001). It has been estimated that 10–20% of all human salmonellosis cases in the EU are associated with pigs and pork, though these values vary among different member states (EFSA and ECDC, 2010). In 2017, pig meat and related products were considered to be the food vehicle implicated in 4.5% of all food-borne outbreaks caused by *Salmonella* in the EU (EFSA and ECDC, 2018).

Transmission of *Salmonella* occurs mainly through a fecal-oral route. Infected pigs frequently become asymptomatic carriers, meaning that this pathogen can be isolated from tonsils, intestines and associated lymph nodes of apparently healthy animals (Fedorka-Cray et al., 2000). *Salmonella*-free pigs can be colonized at different steps along the pork production chain, ranging from breeding to slaughter, each step presenting diverse associated risk factors (Bonardi, 2017). In fact, the slaughterhouse is considered a key point for pig infection and carcass contamination (De Busser et al., 2011; Gomes-Neves et al., 2012). The most recent data on *Salmonella* monitoring in animals in the EU point out for a prevalence of 14.2% in pigs at slaughter (EFSA and ECDC, 2018). Despite the role of positive slaughtered pigs in *Salmonella* dissemination (Vieira-Pinto et al., 2005, 2006), the possibility of carcass contamination due to the presence of endemic microbiota present on the slaughterhouse environment should also be taken into account (van Hoek et al., 2012; Arguello et al., 2013a).

Different studies have sought to understand the origin of carcass

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contamination by tracking *Salmonella* isolates at multiple points or operations at slaughterhouses of multiple European countries (Vieira-Pinto et al., 2006; De Busser et al., 2011; Gomes-Neves et al., 2012; van Hoek et al., 2012; Arguello et al., 2013b; Bonardi et al., 2016; Pesciaroli et al., 2017), but the Portuguese scenario is still crudely understood.

The aim of this study was to investigate the aspects of *Salmonella* carcass contamination within a slaughterhouse, through a pheno and genotypical characterization of the bacterial isolates recovered at two different points of the slaughter line, namely at the clean and dirty areas.

2. Materials and methods

2.1. Salmonella isolates

A total of 44 *Salmonella enterica* subsp. *enterica* isolates were included in this study. The isolates were obtained from 360 samples collected between January and April 2014 on a multispecies slaughterhouse located on the Northern region of Portugal, with a throughput of 90 pigs/hour and a daily processing average of 250 pigs. A non-destructive sampling method was performed using sterile sponges soaked with 10 ml of Tryptone-salt broth (*Biokar Diagnostics*® – BK014HA). Briefly, for each carcass three sponges were used, one to swab 1000 cm^2 of skin after stunning and bleeding (dirty area) and the remaining sponges to swab 1000 cm^2 of the outer and of the inner carcass surfaces before cooling (clean area). This procedure was performed on 120 carcasses originating from 11 different farms (Table 1). The isolation of *Salmonella* present on the tested skin and carcass surfaces was achieved according to ISO norm 6579:2202, and all isolates were serotyped according to the Kauffman-White scheme at the Instituto Nacional de Investigação Agrária e Veterinária (INIAV) that is the National Reference Laboratory for *Salmonella* identification in food samples. Isolates belonged to three serotypes: *Salmonella* 4, [5],12:i:- (n = 21; 47.7%) *Salmonella* Rissen (n = 18; 40.9%) and *Salmonella* Derby (n = 5; 11.4%).

2.2. Antimicrobial susceptibility testing

Antimicrobial susceptibility was tested by the disc diffusion method according to the CLSI guidelines for veterinary antimicrobial susceptibility testing (CLSI, 2012), using amoxicillin/clavulanic acid (AMC, 30 μg), ampicillin (AMP, 30 μg), chloramphenicol (C, 30 μg), cephalexin (CL, 30 μg), gentamicin (CN, 10 μg), cefotaxime (CTX, 30 μg), enrofloxacin (ENR, 5 μg) nalidixic acid (NA, 30 μg), penicillin (P, 10 U), streptomycin (S, 25 μg), sulfamethoxazole/trimethoprim (SXT, 10 μg) and tetracyclin (TE, 30 μg). *E. coli* ATCC 25922 was used as the control strain for test performance. Multidrug resistance (MDR) phenotype was considered to be present whenever an isolate revealed resistance to three or more antimicrobial compounds belonging to different classes (Magiorakos et al., 2012).

2.3. Detection of virulence associated genes

Detection of virulence genes associated with plasmid (*spvC*), invasion (*invA*, *invH*, *sopB*), enterotoxin (*stn*), cytolysin (*slyA*), survival within macrophages (*phoP*, *phoQ*) and fimbriae formation (*agfA*, *pefA*, *safC*) was carried out by PCR as previously described (Huehn et al., 2009). In order to evaluate the method's reproducibility, a randomly selected sample representing 10% of all isolates was also tested. *Salmonella enterica* subsp. *enterica* CECT 443 and CECT 722 were used as positive controls.

2.4. Genomic typing

In order to assess the relatedness between isolates genomic fingerprinting was performed by Pulsed Field Gel Electrophoresis (PFGE) using a CHEF-DRIII apparatus (Bio-Rad Laboratories, San Diego, USA) after DNA digestion with *XbaI* (Nzytech, Portugal), according to the PulseNet protocol (Centers for Disease Control and Prevention, 2017). Genomic DNA from *Salmonella* Braenderup H9812 was also subjected to restriction with *XbaI* and used as a standard.

Each PFGE pattern was analyzed both visually and by computer-assisted cluster analysis using BioNumerics® version 6.6 software (Applied Maths, SintMartens-Lantem, Belgium).

The reproducibility was evaluated with 10% of isolates randomly selected. The similarity between each pair of the duplicates was obtained from the analysis based on a dendrogram computed with Dice coefficient and the unweighted pair group method with arithmetic average (UPGMA) as the agglomerative clustering. Also used for band matching a value of 1.25% of tolerance. The reproducibility value was determined as the average value (90%) for all pairs of duplicates. Patterns with levels of similarity above the reproducibility value were considered undistinguishable and assigned to a same PFGE type.

2.5. Quantification of biofilm production by optical density determination

The quantification of biofilm formation was performed as already described (Christensen et al., 1985). Isolates were inoculated in Tryptic Soya Broth (TSB) (Oxoid, CM0129B) and incubated for 18 h at 37 °C.

Table 1
Distribution of *Salmonella* positive samples, and related information, by sample collection chronological order.

Date of collection	Pig	Farm	Serotype	Biological sample	Sample code
06/01/2014	1	A	Rissen	Skin	p1
06/01/2014	3	A	Rissen	Skin	p3
06/01/2014	4	A	Rissen	Skin	p4
06/01/2014	5	A	Rissen	Skin	p5
03/02/2014	21	C	Rissen	Carcass Ext.	ce21
	21	C	Rissen	Carcass Int.	ci21
03/02/2014	25	C	Rissen	Skin	p25
17/02/2014	31	D	Rissen	Skin	p31
17/02/2014	37	A	Rissen	Carcass Ext.	ce37
17/02/2014	38	A	Rissen	Carcass Int.	ci38
24/02/2014	44	E	Rissen	Carcass Ext.	ce44
24/02/2014	55	F	4,[5],12:i:-	Skin	p55
	55	F	Rissen	Carcass Int.	ci55
24/02/2014	56	F	4,[5],12:i:-	Skin	p56
24/02/2014	57	F	Rissen	Carcass Int.	ci57
24/02/2014	58	F	Rissen	Skin	p58
03/03/2014	61	G	Rissen	Skin	p61
03/03/2014	62	G	Rissen	Skin	p62
03/03/2014	64	G	Rissen	Skin	p64
03/03/2014	66	G	Derby	Skin	p66
03/03/2014	67	G	Derby	Skin	p67
03/03/2014	68	G	Derby	Skin	p68
	68	G	Derby	Carcass Int.	ci68
03/03/2014	70	G	Derby	Carcass Ext.	ce70
31/03/2014	96	J	4,[5],12:i:-	Skin	p96
07/04/2014	104	K	4,[5],12:i:-	Skin	p104
	104	K	4,[5],12:i:-	Carcass Int.	ci104
07/04/2014	105	K	4,[5],12:i:-	Carcass Int.	ci105
07/04/2014	106	K	4,[5],12:i:-	Skin	p106
07/04/2014	107	K	4,[5],12:i:-	Skin	p107
07/04/2014	108	K	4,[5],12:i:-	Carcass. Int.	ci108
07/04/2014	109	K	4,[5],12:i:-	Skin	p109
	109	K	4,[5],12:i:-	Carcass Int.	ci109
07/04/2014	110	K	4,[5],12:i:-	Skin	p110
	110	K	4,[5],12:i:-	Carcass Int.	ci110
07/04/2014	111	K	4,[5],12:i:-	Carcass Int.	ci111
07/04/2014	112	K	4,[5],12:i:-	Skin	p112
07/04/2014	114	K	4,[5],12:i:-	Skin	p114
07/04/2014	115	K	4,[5],12:i:-	Skin	p115
	115	K	4,[5],12:i:-	Carcass Int.	ci115
07/04/2014	116	K	Rissen	Skin	p116
	116	K	4,[5],12:i:-	Carcass Int.	ci116
07/04/2014	117	K	4,[5],12:i:-	Carcass Int.	ci117
07/04/2014	118	K	4,[5],12:i:-	Skin	p118

After a 1:40 dilution in TSB supplemented with 0.25% glucose, 200 ml of each dilution were distributed in 96-well plates (Oxvital, 167008). Negative control wells contained only the glucose supplemented TSB. The plates were incubated for 18 h at 37 °C, washed three times with PBS, pH 7.0, air-dried for 1 h at 60 °C, and stained with 0.25% crystal violet (Benton-Dickinson, 212525) for 1 min. After washing, the OD570 nm was determined. The *Salmonella* isolates were classified as non-adherent, weakly, moderately or strongly adherent according to previously specified parameters (Stepanović et al., 2000).

All tests were performed in triplicate.

3. Results

3.1. Distribution of Salmonella isolates and serotypes

The samples from which each *Salmonella* isolate was retrieved from are displayed on Table 1. Overall, the skin was the most frequent source of the isolates found (n = 26; 59.1%), followed by the internal (n = 14; 31.8%) and the external surface (n = 4; 9.1%) of the carcasses. In eight cases it was possible to isolate *Salmonella* from two different biological samples obtained from the same pig/carcass, commonly from the skin and the internal surface of the carcass.

Regarding the serotypes, *Salmonella* 4,[5],12:i:- (n = 12; 27.3%), *Salmonella* Rissen (n = 11; 25.0%) and *Salmonella* Derby (n = 3; 6.8%) were recurrently recovered from the skin at the dirty area. At the clean area, *Salmonella* 4,[5],12:i:- (n = 9; 20.4%), *Salmonella* Rissen (n = 4; 9.1%) and *Salmonella* Derby (n = 1; 2.3%) were isolated from the samples taken from the inner surface of the carcasses, and only *Salmonella* Rissen (n = 3; 6.8%) and *Salmonella* Derby (n = 1; 2.3%) were found to be present at outer surface of the carcasses.

3.2. Antimicrobial susceptibility phenotypes

The studied isolates were subjected to antimicrobial susceptibility testing regarding a panel of 12 antimicrobial agents (Table 2). High levels of resistance were observed to compounds of diverse antimicrobial groups, namely to P (n = 44; 100%), TE (n = 36; 81.8%), S (n = 34; 77.3%) and AMP (n = 26; 59.1%). Resistance to ENR (n = 11; 25%), C (n = 10; 22.7%), NA (n = 4; 9.1%), AMC (n = 2; 4.5%) and CN (n = 1; 2.3%) were also registered at overall lower levels. None of the isolates exhibited resistance to CL nor CTX.

Furthermore, 35 (79.5%) isolates were classified as MDR, displaying resistance to three or more antimicrobial classes (Magiorakos et al., 2012). Among these MDR isolates, diverse resistance phenotypes were observed in several strains within the same serotype, and frequently a combination of five or more antimicrobials. The most frequent phenotypes were AMP/P/S/SXT/TE, presented by eight *Salmonella* 4,[5],12:i:- isolates (22.8%), AMP/ENR/P/S/SXT/TE, presented by six *Salmonella* 4,[5],12:i:- isolates (17.1%), AMP/C/P/S/SXT/TE, presented by five *Salmonella* Rissen isolates (14.3%), P/S/TE, presented by five *Salmonella* Derby isolates (14.3%), followed by a C/P/S pattern presented by three *Salmonella* Rissen isolates (8.6%) and a AMP/ENR/NA/P/S/SXT/TE pattern presented by three *Salmonella* 4,[5],12:i:- isolates (8.6%). Only one *Salmonella* 4,[5],12:i:- isolate (2.9%) presented resistance to eight compounds, namely AMC/AMP/ENR/NA/P/S/SXT/TE. Other unique MDR resistance profiles were also recorded.

On the other hand, the *Salmonella* isolates which were not considered MDR (n = 9; 20.5%) belonged all to the Rissen serotype, only having in common a P resistance phenotype.

3.3. Virulence associated genes

Almost all isolates revealed a common genetic profile regarding the virulence genes studied (Table 3). The *invA*, *invH*, *sopB*, *stn*, *slyA*, *phoP*, *phoQ*, *agfA* genes were present in 100% of the isolates, contrasting with absence of *spvC* and *pefA* genes in all isolates. The variability on the genetic profile observed was associated with the *safC* gene, which was absent in 18 isolates (40.9%), corresponding to *Salmonella* Rissen.

Table 2
Phenotypic antimicrobial resistance of the *Salmonella* isolates.

Sample code	AMC	AMP	C	CL	CN	CTX	ENR	NA	P	S	SXT	TE
p1	S	S	S	S	S	S	S	S	R	I	S	R
p3	S	S	S	S	S	S	S	S	R	S	S	S
p4	S	S	S	S	S	S	S	S	R	S	S	S
p5	S	S	S	S	S	S	S	S	R	I	S	S
ce21	S	R	R	S	S	S	S	S	R	R	R	R
ci21	S	R	R	S	S	S	S	S	R	R	R	R
p25	S	S	R	S	S	S	S	S	R	I	I	S
p31	S	S	R	S	S	S	S	S	R	R	S	S
ce37	S	R	R	S	S	S	S	S	R	R	R	R
ci38	S	S	R	S	S	S	S	S	R	R	I	R
ce44	S	S	R	S	S	S	S	S	R	R	S	S
p55	S	R	S	S	R	S	S	S	R	I	S	R
ci55	S	S	S	S	S	S	S	S	R	I	S	S
p56	S	R	I	S	S	S	S	S	R	R	S	R
ci57	S	S	S	S	S	S	S	S	R	I	S	R
p58	S	S	S	S	S	S	S	S	R	I	S	R
p61	S	R	R	S	S	S	S	S	R	R	R	R
p62	S	S	R	S	S	S	S	S	R	R	I	S
p64	S	R	R	S	S	S	S	S	R	R	R	R
p66	S	S	S	S	S	S	S	S	R	R	S	R
p67	S	S	S	S	S	S	S	S	R	R	I	R
p68	S	S	S	S	S	S	S	S	R	R	S	R
ci68	S	S	S	S	S	S	S	S	R	R	I	R
ce70	S	S	S	S	S	S	S	S	R	R	S	R
p96	S	R	S	S	S	S	R	R	R	R	R	R
p104	S	R	S	S	S	S	I	S	R	R	R	R
ci104	S	R	S	S	S	S	I	I	R	R	R	R
ci105	S	R	S	S	S	S	R	I	R	R	R	R
p106	S	R	S	S	S	S	R	R	R	R	R	R
p107	S	R	S	S	S	S	I	I	R	R	R	R
ci108	S	R	S	S	S	S	I	I	R	R	R	R
p109	I	R	S	S	S	S	R	I	R	R	R	R
ci109	S	R	S	S	S	S	R	I	R	R	R	R
p110	I	R	S	S	S	S	I	I	R	R	R	R
ci110	S	R	S	S	S	S	I	I	R	R	R	R
ci111	S	R	S	S	S	S	R	I	R	R	R	R
p112	I	R	S	S	S	S	R	R	R	R	R	R
p114	R	R	S	S	S	S	R	R	R	R	R	R
p115	R	R	S	S	S	S	R	I	R	R	R	R
ci115	I	R	S	S	S	S	R	I	R	R	R	R
p116	S	S	S	S	S	S	S	S	R	S	S	R
ci116	S	R	S	S	S	S	I	I	R	R	R	R
ci117	S	R	S	S	S	S	I	I	R	R	R	R
p118	I	R	S	S	S	S	R	I	R	R	R	R

AMC, amoxicillin/clavulanic acid, 30 µg; AMP, ampicillin, 30 µg; C, chloramphenicol, 30 µg; CL, cephalexin, 30 µg; CN, gentamicin, 10 µg; CTX, cefotaxime, 30 µg; ENR, enrofloxacin, 5 µg; NA, nalidixic acid, 30 µg; P, penicillin, 10 U; S, streptomycin, 25 µg; SXT, sulfamethoxazole/trimethoprim, 10 µg; TE, tetracyclin, 30 µg.
S, sensible; I, intermediate; R, resistant.

3.4. PFGE analysis

The PFGE typing of the 44 isolates resulted in 7 different PFGE types, according to the criteria previously mentioned (Fig. 1).

The vast majority (n = 17; 94.4%) of *Salmonella* Rissen isolates, except ci38, were grouped in a cluster with a common genetic similarity of 85.3%, which was subdivided into two smaller groups (IA and IB) with values of similarity higher than 90%, and so each representing one same type. The seven isolates belonging to group IA derived from animals originating from three different farms (A, F and K) slaughtered within a time gap of three months. The 10 isolates from group IB were retrieved from swabs performed on pigs from six different farms slaughtered during a one month period. These isolates were found to be present on the skin and in the internal and external surfaces of the carcasses.

Table 3
Detection of virulence genes by PCR in the *Salmonella* isolates.

Sample code	*spvC*	*invA*	*invH*	*sopB*	*stn*	*slyA*	*phoP*	*phoQ*	*agfA*	*pefA*	*safC*
p1	–	+	+	+	+	+	+	+	+	–	–
p3	–	+	+	+	+	+	+	+	+	–	–
p4	–	+	+	+	+	+	+	+	+	–	–
p5	–	+	+	+	+	+	+	+	+	–	–
ce21	–	+	+	+	+	+	+	+	+	–	–
ci21	–	+	+	+	+	+	+	+	+	–	–
p25	–	+	+	+	+	+	+	+	+	–	–
p31	–	+	+	+	+	+	+	+	+	–	–
ce37	–	+	+	+	+	+	+	+	+	–	–
ci38	–	+	+	+	+	+	+	+	+	–	–
ce44	–	+	+	+	+	+	+	+	+	–	–
p55	–	+	+	+	+	+	+	+	+	–	+
ci55	–	+	+	+	+	+	+	+	+	–	–
p56	–	+	+	+	+	+	+	+	+	–	+
ci57	–	+	+	+	+	+	+	+	+	–	–
p58	–	+	+	+	+	+	+	+	+	–	–
p61	–	+	+	+	+	+	+	+	+	–	–
p62	–	+	+	+	+	+	+	+	+	–	–
p64	–	+	+	+	+	+	+	+	+	–	–
p66	–	+	+	+	+	+	+	+	+	–	+
p67	–	+	+	+	+	+	+	+	+	–	+
p68	–	+	+	+	+	+	+	+	+	–	+
ci68	–	+	+	+	+	+	+	+	+	–	+
ce70	–	+	+	+	+	+	+	+	+	–	+
p96	–	+	+	+	+	+	+	+	+	–	+
p104	–	+	+	+	+	+	+	+	+	–	+
ci104	–	+	+	+	+	+	+	+	+	–	+
ci105	–	+	+	+	+	+	+	+	+	–	+
p106	–	+	+	+	+	+	+	+	+	–	+
p107	–	+	+	+	+	+	+	+	+	–	+
ci108	–	+	+	+	+	+	+	+	+	–	+
p109	–	+	+	+	+	+	+	+	+	–	+
ci109	–	+	+	+	+	+	+	+	+	–	+
p110	–	+	+	+	+	+	+	+	+	–	+
ci110	–	+	+	+	+	+	+	+	+	–	+
ci111	–	+	+	+	+	+	+	+	+	–	+
p112	–	+	+	+	+	+	+	+	+	–	+
p114	–	+	+	+	+	+	+	+	+	–	+
p115	–	+	+	+	+	+	+	+	+	–	+
ci115	–	+	+	+	+	+	+	+	+	–	+
p116	–	+	+	+	+	+	+	+	+	–	–
ci116	–	+	+	+	+	+	+	+	+	–	+
ci117	–	+	+	+	+	+	+	+	+	–	+
p118	–	+	+	+	+	+	+	+	+	–	+

All *Salmonella* 4,[5],12:i:- isolates clustered together, with a pattern similarity level of 83.0%. Isolates p55 and p56, deriving from animals of farm F represent a subcluster (IIA) at 86.7% similarity and the other *Salmonella* 4,[5],12:i:- isolates have a pattern similarity level of 93.5%. Apart from p96, all isolates were recovered from the skin or the internal surface of carcasses of animals from farm K, and slaughtered at the same day.

Finally, the five *Salmonella* Derby isolates where clustered together at a 91.0% similarity level, being all recovered from animals from farm G slaughtered at the same day.

3.5. Biofilm production

Of all the *Salmonella* isolates, only three (9.1%) were considered capable of producing biofilm, and were all classified as weakly adherent. Three isolates belonged to the Rissen serotype (p3, p31 and ci57) and the remaining one to the 4,[5],12:i:- serotype (p110).

4. Discussion

The implementation of good hygiene practices at the slaughterhouse and slaughtering processes is of paramount importance to control *Salmonella* contamination of pork and the possibility of human exposure. It has been suggested that specific interventions carried out at slaughterhouses can produce a greater decrease in human Salmonellosis cases in the EU, in a short-term period, when comparing with interventions at the farm level (Hill et al., 2016).

In the present study, we report data regarding the contamination of pig carcasses by *Salmonella*, comparing two distinct areas of a Portuguese slaughterhouse. A clear predominance of *Salmonella* 4,[5],12:i:- and Rissen serotypes among the isolates recovered was found. On the other hand, *Salmonella* Derby accounted for only one-ninth of the serotypes identified. Other studies have also reported the isolation of the mentioned serotypes, though with differences regarding the frequencies (van Hoek et al., 2012; Bonardi et al., 2013, 2016, 2018; Pesciaroli et al., 2017; Van Damme et al., 2018). Previous reports on *Salmonella* isolation from pig slaughterhouses in Portugal are limited (Vieira-Pinto et al., 2005; Gomes-Neves et al., 2012). Vieira-Pinto et al. (2005) have reported a clear predominance in *Salmonella* Typhimurium, followed by *Salmonella* Rissen serotype, but only a 4.4% frequency of *Salmonella* 4,[5],12:i:-. Likewise, Gomes-Neves and collegues (2012) did not find *Salmonella* 4,[5],12:i:- in any of the carcass samples on their study, but only on the lymph nodes, even though they tested a bigger sample of animals. Inversely with these two previous reports, in our study none of the isolates corresponded to *Salmonella* Typhimurium. The same scenario was reported to occur in recent studies carried out in Italy, where only the monophasic variant of *Salmonella* Typhimurium had been found (Bonardi et al., 2016; Pesciaroli et al.,

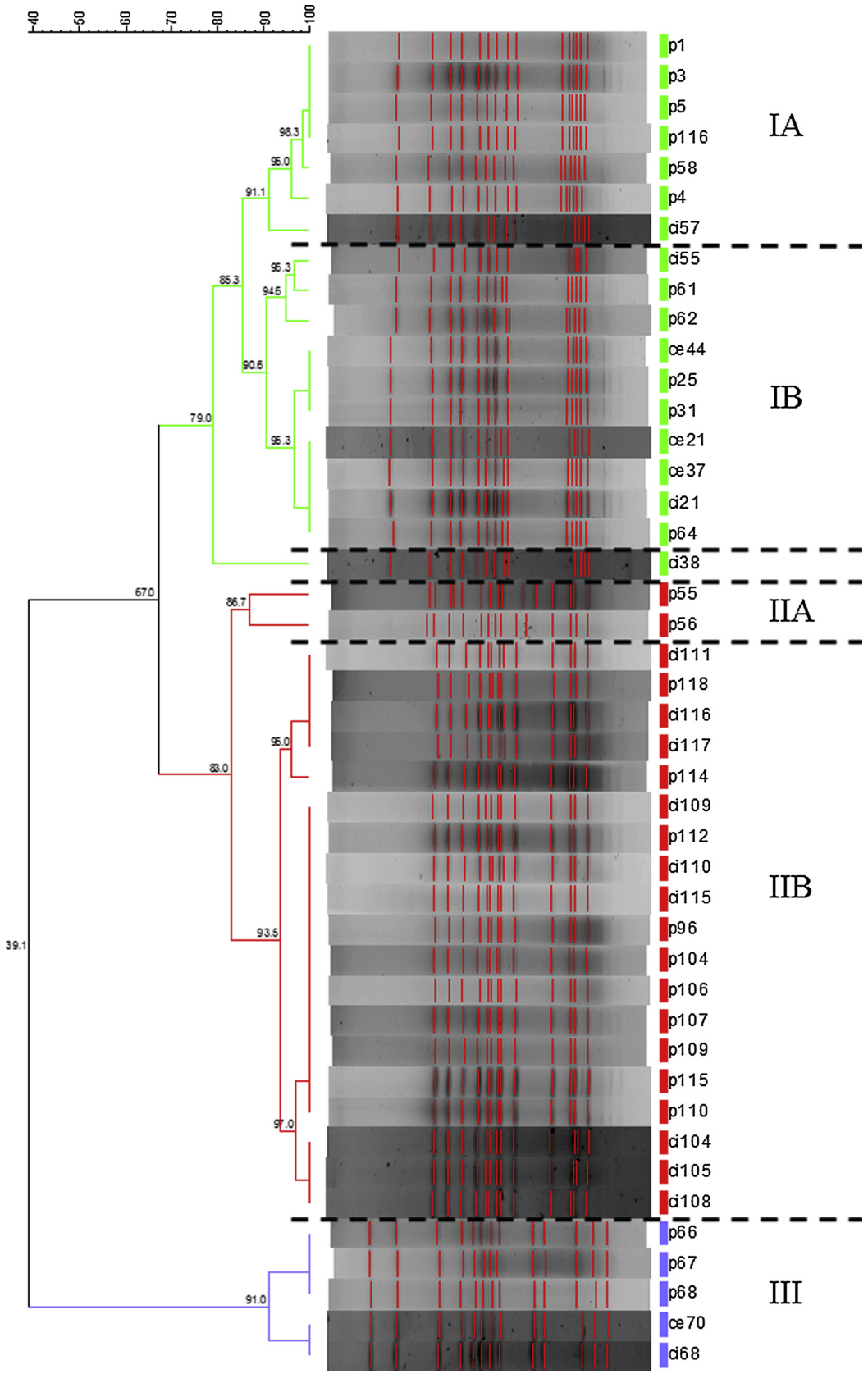

Fig. 1. Dendrogram based on *XbaI* – PFGE patterns of the *Salmonella enterica* subsp. *enterica* isolates recovered from skin and internal and external surfaces of pig carcasses.

2017). Our results are in accordance with the opinion that *Salmonella* Typhimurium is gradually being substituted by the monophasic variant 4,[5],12:i:- in swine population (Bonardi et al., 2016). Also, it is not surprising to find that the *Salmonella* Rissen serotype was very frequent among all isolates studied, since Portugal, along with Spain, was pointed out as a country where *Salmonella* Rissen is frequently isolated from pig lymph nodes (EFSA, 2008). Furthermore, *Salmonella* 4,[5],12:i:- and *Salmonella* Rissen, which are commonly found in pig carcasses (Bonardi, 2017), were some of the most frequently associated serotypes in human salmonellosis cases in Portugal over the past few years (Silveira et al., 2018).

The antimicrobial resistance levels found were worrisome. Most isolates (79.5%) were resistant to three or more of the antimicrobial agents tested. All the *Salmonella* 4,[5],12:i:- isolates were MDR, exceeding the 77.2% reported in the latest EU report on antimicrobial resistance in zoonotic bacteria (EFSA and ECDC, 2019). The most common pattern of resistance found in MDR *Salmonella* Rissen isolates was AMP/C/P/S/SXT/TE, which is in accordance with the results from the above-mentioned report. Similar to the monophasic *Salmonella* Typhimurium isolates studied, *Salmonella* Derby isolates were all MDR, presenting a P/S/TE resistance pattern. These findings are of great concern, particularly in the Portuguese scenario, since pork is the most common type of meat consumed in the country, along with the impact of *Salmonella* 4,[5],12:i:- and *Salmonella* Rissen in human salmonellosis cases (Silveira et al., 2018). Furthermore, the high levels of resistance to AMP and SXT reduce antimicrobial treatment options whenever severe infections occur, since these are some of the first-line antibiotics recommended for treatment of non-typhoidal human salmonellosis cases (Shane et al., 2017).

Additionally to the high levels of antimicrobial resistance, all isolates harboured several virulence associated genes, namely *invA*, *invH*, *sopB*, *stn*, *slyA*, *phoP*, *phoQ* and *afgA*. The only variation on gene virulence repertoire was the absence of the *safC* gene in *Salmonella* Rissen isolates. It appears that *Salmonella* Rissen might not express any of the *saf* gene cluster products, namely *safA*, *safB*, *safC*, since none of these genes were found in a previous report comparing several *Salmonella* serovars responsible for human disease (Folkesson et al., 1999) The biological impact of the absence of this gene is pigs is unknown.

The genomic relatedness between isolates was assessed by PFGE. Isolates sharing high levels of genetic similarity were isolated from animals of different farms and slaughtered at distinct dates. The most striking case was the *Salmonella* Rissen isolate p116, which had an indistinguishable PFGE profile from those of isolates p1, p3 and p5, although all recovered from the skin of animals from a distinct farm slaughtered three months earlier. The surface where each isolates was retrieved from must be taken into account when trying to assess the possibility of cross-contamination. In fact, sampling the outer surface of the carcass should reveal contamination before slaughter or cross-contamination and samples of the inner carcass surface point out to contamination with intestinal contents (Arguello et al., 2013a). Likewise, samples taken from the unprocessed skin of pigs after stunning and exsanguination should reveal contamination originating from the farm, transport or lairage stages. Thus, this result might be associated with the maintenance of *Salmonella* Rissen at the lairage area, since skin contamination has been demonstrated to be associated with contamination of the area where animals rest before being slaughtered (Rossel et al., 2009). In another study, *Salmonella* Rissen was repeatedly reported as being present in the slaughterhouse equipment, thus contributing for the contamination found at the end of the slaughter line (van Hoek et al., 2012). Furthermore, isolates p3 and p31 were capable of producing biofilm, though the capability of adherence was considered to be weak. On the other hand, the fact that some of the *Salmonella* Rissen isolates were obtained from the external surface of the carcasses can be indicative of cross-contamination; however, the source of this contamination was not assessed. Further studies must be undertaken in order to confirm the capability of *Salmonella* Rissen to persist in the slaughterhouse environment, namely at lairage. This is especially important considering the dissemination of this serotype in 7 of the 8 positive farms, which reflects the EFSA report on the distribution of *Salmonella* serotypes among EU countries, on which Portugal was found to be part of the cluster where *Salmonella* Rissen could most likely be isolated from pig carcass lymph nodes (EFSA, 2008).

A similar result was observed regarding the *Salmonella* 4,[5],12:i:- isolate p96 and several others recovered from the skin and internal surface of carcasses belonging to animals of a different farm, slaughtered one week later. However, a different pattern was observed, since though isolated from the skin of the animals sampled at the dirty area, *Salmonella* 4,[5],12:i:- was never recovered from the external surface of carcasses before chilling, but strictly from the internal surface. As mentioned before, this can point towards a scenario where these animals were already colonized before entering the slaughterhouse, being the skin contamination a result of faecal contamination. Again, these results show that skin contamination can be indicative of final carcass contamination, as previously revealed (Rossel et al., 2009).

Regarding *Salmonella* Derby, since all isolates were recovered from animals slaughtered at the same day and in sequence, the results seem to indicate that cross-contamination could have occurred.

Overall, these results confirm that implementing good hygiene practices at the slaughterhouse is crucial in order to prevent *Salmonella* contamination of pork and to promote the safeguarding of food safety. A high prevalence of MDR isolates harbouring several virulence genes was detected not only on the skin, but also on carcasses at the end of the slaughter line. Furthermore, our findings draw attention to a possible differential persistence of *Salmonella* in the slaughterhouse that can be related with serotype. Further studies are required, encompassing the three stages (pre-harvest, harvest and post-harvest), to better understand the situation of *Salmonella* in pigs and pork in Portugal.

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