Salmonella and *Yersinia enterocolitica* through the pig meat chain in Sardinia: occurrence, antimicrobial resistance and genetic insight

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Abstract

This study aimed to characterize *Salmonella* and *Yersinia enterocolitica* detected in fattening pigs in Sardinia, examining genetic similarity and antimicrobial resistance of isolates from farms and slaughterhouses and evaluating carcass hygiene. Environmental samples were collected from six pig farms, and the same pigs were also sampled at the slaughterhouses. Palatine tonsils, mesenteric lymph nodes, colon content, and carcass surface samples were collected and tested for *Salmonella* and *Y. enterocolitica*. Antimicrobial resistance testing and whole genome sequencing were performed on all isolates. Carcass surface samples were tested for total aerobic colony count (ACC) and *Enterobacteriaceae* count (EBC). *Y. enterocolitica* was found in two farms (33%), while *Salmonella* was absent in environmental farm samples. At slaughterhouses, 13.1% of pigs were found positive for *Salmonella* (lymph nodes, colon content, and palatine tonsils samples), but *Y. enterocolitica* was not detected. *Salmonella* isolates were typed as monophasic *S.* Typhimurium ST34 and *S.* Goldcoast ST358, with few allelic differences among isolates of the same ST. *Salmonella* ST34 showed resistance towards ampicillin, streptomycin, sulfonamide, and tetracycline (*blaTEM-1B, aph(3)-Ib, sul2* and *tetB* genes, R-type ASSuT). *Y. enterocolitica* isolates (biotype 2, ST853, and ST859) showed resistance to ampicillin and amoxicillin-clavulanic acid (*blaA* gene). Process hygiene criteria were generally met, with mean (log_{10} CFU/cm^2 ± standard deviation) values for ACC and EBC being 2.23±0.74 and 0.75±0.81. Pigs of Sardinia are confirmed carriers of *Salmonella* and *Y. enterocolitica*, but overall hygienic status in farms and slaughterhouses in Sardinia is acceptable. Monophasic *S.* Typhimurium and *Y. enterocolitica* isolates showed typical resistance patterns. Monophasic *S.* Typhimurium ST34 isolates with R-type ASSuT are confirmed as epidemic clones.

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Introduction

Pork is frequently associated with foodborne outbreaks, particularly involving *Salmonella* and *Yersinia enterocolitica* (YE). Pigs can harbor numerous *Salmonella* and YE serovars, including those with significant public health implications, as the monophasic variants of *S.* Typhimurium (MST) (Fedorka-Cray *et al.,* 2000). Infections often occur at the farm level, starting from the early stages of life and persisting asymptomatically in palatine tonsils and gut-associated lymphoid tissue. Asymptomatic pigs cannot be identified through routine inspections, therefore, causing the entrance of pathogens into the slaughter line (EFSA, 2011). Monitoring zoonotic pathogens like *Salmonella* and *Yersinia* in the meat production process is crucial for ensuring food safety. Farms and slaughterhouses serve as pivotal points for epidemiological research.

Sardinia (Italy), with 162,731 pigs registered as of 3rd October 2024, has 2.02% of the Italian pig population. Productions of pigs in Sardinia are mainly suckling pigs (Ministero della Salute, 2024). The number of farms is under 11,000 and over 88% of these have fewer than 15 adult pigs, with most farms dedicated to self-consumption and only 5% commercial (Mur *et al*., 2016), with

approximately fifty farrow-to-finish farms overall (Ministero della Salute, 2024).

The occurrence and antimicrobial resistance of microorganisms can differ considerably between different geographical areas and production systems. Therefore, studies at the regional level are necessary to acquire information on the epidemiological status of the local area. In this framework, we aimed to characterize *Salmonella* and YE detected at farms and slaughterhouses in fattening pigs in Sardinia.

Materials and Methods

Sampling at farms

Environmental sampling was conducted on six pig farms (A, B, C, D, E, and F) across Sardinia. "Medium-to-big sized", commercially oriented farms were selected to consider those with a more relevant impact on food safety. They were selected based on comparable procedures, with a minimum population of 150 pigs, including at least 50 fattening pigs each. Farms A and B were in the northern area (Sassari province), farms C and D in the central area (Oristano province), and farms E and F in the southern area (Medio Campidano province). On each farm, finisher pigs over 16 weeks old, housed together and scheduled for slaughter within the next 14 days, were selected for sampling. Sampling was conducted using sterile sock kits (Technical Service Consultants Ltd, Heywood, United Kingdom). Each pen, housing 8-12 pigs, was sampled by walking over the floor, covering at least 50% of the area, including corners, water supply, and troughs, to collect fecal material. Farmers were interviewed regarding farm production, management, and biosecurity procedures, covering aspects such as building and equipment characteristics, production processes, feed management, hygiene procedures, pathologies, and antibiotic usage.

Sampling at slaughterhouse

The farms did not send pigs regularly to the slaughterhouse; therefore, within 14 days of collecting environmental samples on the farms, pigs were sampled at the slaughterhouse. Five slaughterhouses (S1, S2, S3, S4, and S5) were selected based on the farms they serviced. Two farms (A and B) sent pigs to the same slaughterhouse (S1), which was thus sampled twice. Only pigs that had been in the sampled pens were selected for sampling at the slaughterhouse, and pigs from the same farm and slaughtered on the same day were considered as a batch. A total of 38 animals were sampled: 6 from farm A, 3 from farm B, 10 from farm C, 3 from farm D, 10 from farm E, and 6 from farm F. Samples from each pig included palatine tonsils, mesenteric lymph nodes, colon content, and carcass surface, collected aseptically immediately post-evisceration, as previously described (Piras *et al*., 2011). Carcass surface samples were obtained using a sterile sponge kit (3M Health Care, Milan, Italy) and, following the ISO norm (ISO, 2015) the sampling was carried out using the same sponge for four points in the carcass, namely ham, loins, abdomen and throat with a sterile 10×10 cm^2 delimiter (Copan, Brescia, Italy), from the least contaminated point (ham) to the most contaminated (throat). Environmental samples at the slaughterhouse were also taken post-slaughter and pre-cleaning, using a sponge kit and a sterile delimiter (10×10 cm^2). The surface samples were food contact surfaces (FCS) (cutting equipment and bristles removal equipment), non-food contact surfaces (NFCS) (walls near the stunning and killing area, walls and drain surface of the pre-chilling area), scalding water (SW) (approximately 100 mL). Overall, 36 environmental samples were collected: 12 FCS samples, 18 NFCS samples, and 6 SW samples.

Microbiological analysis

All samples were tested for *Salmonella* and YE. For the detection of *Salmonella*, the ISO norm was used (ISO, 2020). Species were confirmed by polymerase chain reaction (PCR) (Kiskároly *et al.,* 2017). Phenotypic serotyping of *Salmonella* isolates was conducted by slide agglutination (ISO, 2020). For the detection of YE, the ISO norm was used (ISO, 2017a). Species identification was performed by PCR (Garzetti *et al.,* 2014). YE isolates were biotyped through biochemical reaction and serotyped using the slide agglutination test with somatic antigens (ISO 10273:2017).

To evaluate carcass hygiene, total aerobic colony count (ACC) and *Enterobacteriaceae* count (EBC) were conducted according to ISO norms (ISO, 2013 and 2017b). The results were compared with the process hygiene criteria set in Regulation (EC) No. 2073/2005 for pig carcasses (European Commission, 2005). Since non-destructive sampling was used, thresholds were adjusted as per national guidelines (Conferenza Stato-Regioni, 2016) and reduced by 20%. Also, the environmental surface samples were tested for ACC and EBC counts to assess contamination levels at slaughterhouses, excluding drain surface samples from the counts to avoid data imbalance, with only pathogen detection conducted in those samples.

Antimicrobial susceptibility testing

The isolates were tested for antimicrobial susceptibility following the guidelines provided by the EUCAST (2024). For *Salmonella* isolates, antimicrobial susceptibility was tested by broth microdilution (Sensititre automated system, Thermo-Fisher Scientific, Monza, Italy) using the EUVSEC2 AST and EUVSEC3 AST Plates (Thermo-Fisher Scientific, Monza, Italy). Since streptomycin was not included in the plates, its resistance was tested with the disc-diffusion method with commercial antimicrobial susceptibility discs (S10, 10 µg; Thermo-Fisher Scientific, Monza, Italy) and Mueller-Hinton agar (Microbiol, Cagliari, Italy).

For YE isolates, the disc-diffusion method was applied by using commercial antimicrobial susceptibility discs (ThermoFisher Scientific, Monza, Italy) and Mueller-Hinton agar (Microbiol, Cagliari, Italy). The isolates were tested for amikacin (30 µg), ampicillin (10 µg), amoxicillin/clavulanic acid (20 µg /10 µg), azithromycin (15 µg) cephazolin (30 µg), ceftriaxone (30 µg), cefotaxime (30 µg), ceftazidime (5 µg), ciprofloxacin (5 µg), imipenem (10 µg), kanamycin (30 µg), levofloxacin (5 µg), meropenem (10 µg), nalidixic acid (30 µg), streptomycin (10 µg), sulphonamide (300 µg), tetracycline (30 µg), trimethoprim/sulfamethoxazole (1:19, 25 µg).

Whole genome sequencing

Whole genome sequencing was carried out on *Salmonella* and YE isolates on the NextSeq 550 (Illumina, San Diego, USA) platform using the Nextera XT 300-cycle Kit (Illumina, San Diego, USA). Quality control of the obtained sequencing data was conducted using Bifrost software (Bifrost Inc, Westerly, Rhode Island, US) as previously described (Siddi *et al.*, 2024). Raw sequence FASTQ data for this study are available at NCBI, under Bioproject Bioproject PRJNA1120634.

Results

Characteristics of farms

Management characteristics of the farms are summarized in Table 1. The median number of fattening pigs per farm was 170, ranging from 80 to 700. The fattening period lasted around 170 days, with pigs reaching about 120 kg. Most farms practiced semi-intensive farming with a closed breeding system, except farm E, which occasionally purchased piglets from outside Sardinia. Daily feces removal and the all-in-all-out technique were common, except in farms C and F where cleaning frequency varied. Dry pellet feed was standard, with occasional whey supplementation in farms B and D. Three farms did not use antibiotics, while others used amoxicillin, trimethoprim-sulfamethoxazole, and oxytetracycline for sick animals or mixed with feed (farm B). All pigs were transported within 100 km to slaughterhouses and slaughtered after 15-18 hours of fasting.

Microbiological analysis

YE was detected in 2/6 farms (B and C) with a prevalence of 33.4%. No farm tested positive for *Salmonella*.

Salmonella was detected in 5/38 (13.1%) pigs at slaughter, all originating from farm E, with an overall prevalence of 4.6% in all samples and the highest prevalence in lymph nodes (13.1%), as reported in Table 2. Among *Salmonella*-positive pigs, one tested simultaneously positive in lymph nodes and colon content and one tested simultaneously positive in lymph nodes and tonsils. No YE was found in pig samples, though one pig tested positive for *Y. frederiksenii* (colon content of pigs coming from farm D) and two pigs for *Y. aleksiciae* (colon content of pigs coming from farms B and E). The prevalence of *Salmonella* and *Yersinia* in pig samples is reported in Table 2. No *Salmonella* or YE isolates were found in environmental samples collected from the slaughterhouses.

Salmonella characterization

Two out of 7 *Salmonellae* (28.6%) were typed as monophasic S. Typhimurium (MST) (serotype 1,4,[5],12:i- and ST34) and five (71.4%) were typed as *S.* Goldcoast (ST358). The genetic characterization of the isolates is shown in Figure 1. The MST isolates were collected from lymph nodes and tonsils of two different animals and were closely related with 8 allelic differences (AD). The five *S.* Goldcoast, coming from four different animals, were highly related to 0-1 AD between isolates.

Genotypically, all MST detected in the present study possessed the *blaTEM-1B*, *sul2*, *aph(3'')-Ib,* and *tet(B)* antimicrobial resistance (AMR) genes. In *S.* Goldcoast isolates no resistance genes were detected.

Table 1. Management characteristics of the farms and detection of *Salmonella* and *Yersinia enterocolitica* in environmental samples.

Farm	Herd size (n)	Fattening pigs (n)	Fattening period (days)	Floor of the fattening pen	Cleaning of the fattening pen	Feed	Water	Pest control	Antibiotic compounds used	*Salmonella* detection	*Y. enterocolitica* detection
A	300	220	Approx. 150	S	Daily, AIAO	CP	Well	Rodents	Aug, Sxt	-	-
B	180	130	Approx. 180	NS, external paddock	Daily	CP + whey	Mains	Rodents	-	-	+
C	150	80	Approx. 210	S, external paddock	Twice a day	CP	Mains + well	Rodents	Aug, Ox	-	+
D	150	80	Approx. 170	NS	AIAO	CP + whey	Well	Rodents	Aug	-	-
E	5000	500	Approx. 120	NS	Daily, AIAO	CP	Well	Rodents	-	-	-
F	1400	700	Approx. 120	S	Daily, AIAO	CP	Mains	Rodents	-	-	-

S, slatted; NS, not slatted; AIAO, all-in/all-out; CP, commercial pellet; Aug, amoxicillin-clavulanic acid; Sxt, trimethoprim-sulfamethoxazole; Ox, oxytetracycline.

Table 2. Prevalence of *Salmonella* and *Yersinia enterocolitica* in slaughtered pigs including samples from palatine tonsils, mesenteric lymph nodes, colon content and carcass surface (positive/total, prevalence %).

Pathogen	Tested animals	Positive animals	Positive samples				
			Palatine tonsils	Mesenteric lymph nodes	Colon content	Carcass surface	Total
Salmonella	38	5	1/38 (2.6)	5/38 (13.1)	1/38 (2.6)	0/38	7/152 (4.6)
Y. enterocolitica	38	0	0/38	0/38	0/38	0/38	0/152

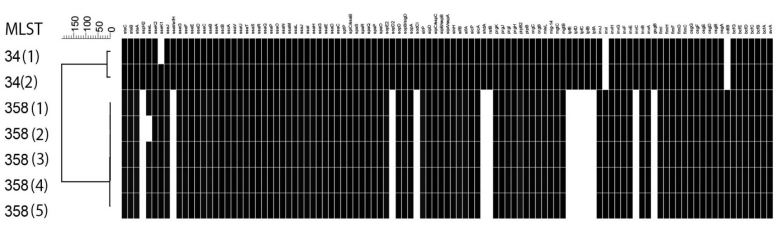

Figure 1. Core genome multilocus sequence typing single linkage clustering tree showing virulence genes of *Salmonella* isolates. Branch length is the number of allelic differences (AD) and the tree is shown with a branch length cut off at 200 AD.

The two MST showed resistance towards ampicillin, streptomycin, sulphonamide, and tetracycline. *S.* Goldcoast isolates were susceptible to all antimicrobials tested.

Yersinia spp. characterization

The two YE isolates were identified as biotype 2. One isolate was identified as serotype O:3 and for the other isolate, it was not possible to identify the serotype phenotypically. The isolates were typed as ST853 (farm C) and ST859 (farm B). Regarding virulence genes, the virulence profile of the isolates is shown in Figure 2. The AMR *blaA* gene was detected in both YE isolates, which showed resistance to amoxicillin-clavulanic acid and ampicillin (AugAmp).

Contamination of carcasses and slaughterhouse surfaces

Table 3 shows the results of ACC and EBC counts in carcass surface samples collected at slaughterhouses. Concerning compliance with the process hygiene criteria, the mean ($\log_{10}$ CFU/cm^2 ± standard deviation) values for ACC and EBC were overall 2.23±0.74 and 0.75±0.81 respectively. More specifically, 34/38 (89.5%) of the samples taken from the carcass surfaces showed ACC values <3.2 $\log_{10}$ CFU/cm^2 and were considered satisfactory. For the EBC, 32/38 (84.2%) samples showed values <1.6 $\log_{10}$ CFU/cm^2 and were considered satisfactory.

Regarding environmental contamination, FCS had a median value ($\log_{10}$ CFU/ cm^2) of 2.24 for ACC and 0.67 for *Enterobacteriaceae*. NFCS showed lower values, with ACC median value of 0.82 and EBC median value below the detection limit.

Discussion

In the present investigation, *Salmonella* was not detected in environmental samples from the pig farms, while YE was found in 33.4% of them. The two YE isolates belonged to biotype 2, which has been previously isolated in pigs at slaughterhouses (Fredriksson-Ahomaa *et al.,* 2007) and in our previous study in Sardinia (Fois *et al*., 2017). The surveyed farms reported implementing biosecurity measures, including the "all-in/all-out" system in most cases (4/6), aiding in minimizing cross-contamination between production cycles and reducing pathogen exposure. It is noteworthy that YE was detected in farms where cleaning was not carried out with the "all-in-all-out" technique (farms B and C). The YE environmental contamination poses a potential infection route for pigs, highlighting the importance of proper disinfection protocols (Andres *et al.,* 2015).

Salmonella was detected in 13.1% of pigs at one slaughterhouse. Regarding this, studies conducted in Sardinia between 2011 and 2020 on the occurrence of *Salmonella* in "local" pigs (born and raised in Sardinia or raised on the island starting from weaning) show a noteworthy reduction over the years: from 35.5% in 2011 (Piras *et al*., 2011), to 8.1% in 2014 (Fois *et al.,* 2017), and finally to 7.5% in 2020 (Siddi *et al*., 2021). In the present investigation, the occurrence level was slightly higher, but the pathogen was detected in one single farm. Also, another study on YE conducted in 2014 reported an occurrence of 18.6% in local pigs (Fois *et al*., 2018), while YE was never detected in pig samples in the present study. We can hypothesize that this decline in pathogenic bacteria could be attributed to the implementation of the African Swine Fever (ASF) Eradication Plan of 2015-2018 (Regione Sardegna, 2014) and the following plans, which led to significant advancements in biosecurity measures across pig farms in Sardinia. Biosecurity regulations, farmer education, and stricter controls were enforced as part of the ASF Eradication Plan, which successfully improved farm hygiene. These measures were critical in eradicating ASF in Sardinia, as recognized by the European Commission in 2024 (European Commission, 2024). The reduced prevalence of *Salmonella* and YE, particularly after 2016, is likely due to the application of biosecurity protocols.

Despite samples being collected from different animals, *Salmonella* isolates were closely related with less than 10 AD, suggesting epidemiological links and circulation of the same cluster. The occurrence of *Salmonella* on farm E may have been caused by the incoming of new animals. In the past, the imports of live animals were more frequent (Piras *et al.,* 2011); now the progressive shift to importing already slaughtered meat, in Sardinia and at the

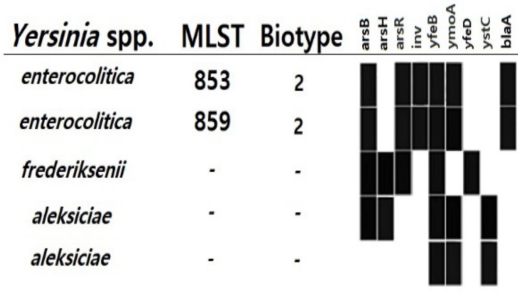

Figure 2. Core genome multilocus sequence typing, biotype, virulence genes and antimicrobial resistance genes of *Yersinia* isolates.

Table 3. Carcass contamination during the sampling days (mean $\log_{10}$ CFU/cm^2) in slaughterhouses.

	Slaughterhouses	1	1	2	3	4	5
	Farm of origin	A	B	C	D	E	F
	Number of samples	6	3	10	3	10	6
ACC	Mean	1.29	2.47	2.33	3.03	1.99	3.06
	Median	1.48	2.03	2.20	3.10	1.99	2.84
	Minimum value	0.52	1.86	1.95	2.68	1.51	2.49
	Maximum value	1.94	3.53	3.00	3.32	2.50	4.26
EBC	Mean	0.18	0.71	0.60	0.50	0.36	2.33
	Median	0.01	1.02	0.70	0.51	0.34	2.38
	Minimum value	BDL	0.02	BDL	BDL	BDL	1.79
	Maximum value	0.84	1.08	1.22	1.00	0.94	3.13

ACC, aerobic colony count; EBC, *Enterobacteriaceae* counts; BDL, below detection limit.

national level (ANAS, 2022), has also reduced the risk of pathogen introduction, further improving animal health and biosecurity in Sardinian farms. The fact that the *Salmonella*-positive pigs all came from the same farm that imports live piglets is a risk factor that supports the hypothesis of the benefits brought by the ASF Eradication Plan. The non-detectability of *Salmonella* in environmental samples from farm E is likely because *Salmonella* infection typically occurs during weaning, with pigs developing immunity after the infectious phase, resulting in fewer pigs excreting *Salmonella* at the slaughter age (Fedorka-Cray *et al*., 2000). The farm's "all-in/all-out" cleaning approach likely limited pathogen spread. Additionally, the carrier pigs detected at slaughter validate the existing knowledge that pigs become more susceptible to *Salmonella* due to stressful factors, such as during transport and pre-slaughter phases, increasing the likelihood of them becoming carriers and spreaders from farm to slaughterhouse (Primavilla *et al.,* 2021). Monophasic MST and *S.* Goldcoast were identified. MST poses a serious concern to public health, being among the most prevalent identified serovar from human cases of salmonellosis and the most common serotype from pig sources in Europe (EFSA and ECDC, 2023). Conversely, *S.* Goldcoast is uncommon in human infections, however, outbreaks related to fermented sausages and salami consumption have been reported (Bremer *et al.,* 2004).

Y. frederiksenii and *Y. aleksiciae* were detected from the colon content of three pigs. These strains are YE-like species, which lack typical YE virulence genes. There is an ongoing debate about whether they are true or opportunistic pathogens (Falcão *et al.,* 2014). However, both *Y. aleksiciae* isolates harbored the *ystC* gene, which encodes for the production of the YE heat-stable enterotoxin YST (Platt-Samoraj, 2022).

The MST isolates possessed the *blaTEM-1B*, *aph(3)-Ib*, *sul2,* and *tet(B)* genes, confirmed by phenotypic resistance towards ampicillin, streptomycin, sulphonamide, and tetracycline. This resistance pattern is known as R-type "ASSuT" and the clonal line ST34 with this R-type is considered an epidemic and known as the "European clone", detected in both food-borne infections and pig meat in Europe and USA (Hopkins *et al.,* 2010).

The *blaA* gene detected in all YE isolates was expected, as the *blaA* gene and an intrinsic resistance to -lactams, even in association with clavulanic acid, is commonly present in all YE biotypes (EUCAST, 2024).

The resistance patterns detected in our isolates were common and frequently observed among isolates of the same species and ST (Hopkins *et al.,* 2010; EUCAST, 2024). This allows us to speculate that the genes were likely not acquired from the farm environment, suggesting that the selective AMR pressure was low in the farms and that pathogen entrance is a source of AMR genes. This is further supported by the absence of AMR in the other *Salmonella* and *Yersinia* isolates.

Regarding environmental contamination, the highest levels were detected on meat-contact surfaces, highlighting the need for stringent hygiene measures for tools and surfaces to prevent carcass and meat contamination.

Conclusions

Implementing focused cleaning practices like the "all-in/all-out" system and minimizing the introduction of animals from other farms are critical for biosecurity. According to the results of this research, the "farrow-to-finish" system reduces the risk of introducing diseases and AMR genes in the farm and appears to be the most recommended from a food safety point of view. The data in this study show an overall good carcass hygiene status, with generally acceptable contamination levels. However, the presence of pigs carrying *Salmonella* and *Yersinia* spp. represents a risk for the consumer and confirms the crucial importance of the application of hygiene measures at the farms and at the slaughterhouse. MST ST34 with R-type ASSuT is confirmed as an epidemic isolate. Low AMR pressure, indicated by the identification of isolates with resistance patterns common to the species, is a positive result but the need for continuous monitoring and control remains crucial.

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