Epidemiological survey on the prevalence of *Salmonella* spp. in the Sardinian pig production chain, using real-time PCR screening method

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Abstract

The aim of this study was to evaluate the prevalence of *Salmonella* spp. in the Sardinian pig production chain in order to establish the incidence of monophasic serovariant of *Salmonella* Typhimurium on isolates with molecular methods (real-time PCR and multiplex PCR). Samples were collected in three EC slaughterhouses, four small slaughterhouses annexed to farmhouses, one meat distribution center, four meat cutting laboratories and four sausage processing plants. A total of 166 samples were collected and analyzed: 46 environmental samples, 48 finishing pigs, 16 piglets, 24 samples of non-processed meat, 28 meat preparations and 4 meat products. All samples were processed with an initial screening using the real-time PCR *MicroSEQ® Salmonella spp detection Kit* (*Applied biosystems, life technologies*) and with the TaqMan® Real-time PCR to confirm the kit results. Samples that tested positive for *Salmonella* spp were confirmed with cultural method using the standard ISO 6579. Positive samples were submitted to phenotypic identification. One colony from each positive sample was serotyped with multiplex PCR method. *Salmonella* spp was isolated in 7 on 166 samples (4.22 %). Among the positive samples, two came from finishing pigs, two belonged to the category meat preparations, two to meat products, one was an environmental sample. Multiplex PCR confirmed that the collected strains belonged to the species *Salmonella* Typhimurium (1), *Salmonella derby* (3) and monophasic serovariant of *Salmonella* Typhimurium (3).

Introduction

Salmonella spp is the second zoonotic agent responsible in Europe of about 95.000 confirmed cases of foodborne salmonellosis each year (EFSA and ECDC, 2017). Data related to pig meat in Europe shows a prevalence of 2.38% of *Salmonella* spp in fresh meat and 1.93% in ready to eat food minced meat, meat preparations and meat products (EFSA and ECDC, 2017). *S.* Enteritidis, *S.* Typhimurium and monophasic serovariant of *Salmonella* Typhimurium are the main serovariants involved in cases of human salmonellosis (EFSA and ECDC 2017).

The monophasic serovariant of *Salmonella* Typhimurium 4,5,12:i:- appears to be genetically related to *Salmonella* Typhimurium (which has the antigenic formula 4,5,12:i:1,2) but lacks in expression of the second phase flagellar antigen, which is 1,2 in *Salmonella* Typhimurium (Soyer *et al*., 2009; Ido *et al.,* 2014). The Center for Disease Control and Prevention (CDC) reported that the prevalence of monophasic serovariant of *Salmonella* Typhimurium has increased considerably in many Countries in the world over the last 10 years. The diffusion of this *Salmonella* serotype was responsible for many human salmonellosis outbreaks, including the ones in Spain in 1998, in the United States in 2004 and 2007, and in Luxemburg in 2006 (Agasan *et al.,* 2002; Mossong *et al.,* 2007). The monophasic serovariant of *Salmonella* Typhimurium is frequently isolated from a large number of different foods and animals.

Regarding the transmission methods of the microorganism, it has been demonstrated that the contaminations of pork products by *Salmonella* spp depend on two main factors: the origin of the animals and the application of good hygienic and manufacturing practices during slaughtering (Fois *et al*., 2017). EFSA reported a *Salmonella* prevalence of 6.7% at herd level and of 3.5% at animal level.

Salmonella prevalence at the farm level depend on various factor, as the origin and type of the feed, the management procedures, different types of herds like farrow to finish herds or fattening herds (Bonardi, 2017).

Pigs are healthy carriers of *Salmonella* spp, they can excrete the microorganism with feces or host it in tissues, particularly in the large intestine and ileum, in the lymph nodes or in the tonsils (García-Feliz *et al.,* 2009). Some studies show that healthy carriers may shed *Salmonella* if subjected to stressful factors, such as the transfer to the slaughterhouse (Hurd *et al.,* 2002; Berardi, 2017). If animal welfare procedures and good manufacturing practices are not applied during slaughtering, Salmonella is disseminated over the carcasses or on the surfaces and equipment of the processing environments.

In Sardinia the pork industry is an important resource for the agricultural economy. There are approximately 16.000 small farms and there is an annual consumption of 50.000 quintals of pork meat, of which 35% is represented by pork products (Fois *et al.,* 2017). However, local production accounts for less than 50% of the regional needs, making it necessary to import pigs from other European countries. Often in Sardinia were slaughtered pigs imported from different European countries.

A survey, including 19,071 pigs from 24 European countries, (EFSA, 2008) found an overall bacteriological prevalence in the mesenteric lymph nodes and feces of slaughtered pigs. This prevalence varied widely among participating countries (Davies, 2011). At mesenteric lymph nodes level, the prevalence of *Salmonella* ranges from 7,4% to 26% while the prevalence reported in faecal content is about 20-30% (Bonardi, 2017).

Contaminated carcasses are the main vehicle of *Salmonella* spp in the pork meat processing industry, as well as in distribution centers, meat cutting laboratories and sausage factories. Different levels of prevalence have been detected in pig carcasses in EU countries, ranging from

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0,35% to 17,41% (EFSA; ECDC).

Once inside the environment, if good hygiene practices are not applied, *Salmonella* can persist within niches of the processing environment, protected by a structured biofilm ecosystem. Starting from these niches the microorganisms find their way to contaminate exposed food through direct contact, aerosol, dripping or water splashes and by means of operators (Simoes *et al.,* 2009). Data provided by EFSA, reported an overall *Salmonella* prevalence of 0,5% in fresh pork and 0,7% in ready-to-eat minced meat, meat preparations and meat products (Bonardi, 2017).

The aim of this study is to investigate the situation of Sardinian pig meat production in relationship with the contamination by *Salmonella* spp and to determine the prevalence of monophasic serovariant of *Salmonella* Typhimurium.

Materials and Methods

Between July 2017 and April 2018, an epidemiological survey was carried out over a total of sixteen structures between pork meat processing plants and slaughterhouses in Sardinia. In detail, environmental and products samples were collected in three EC slaughterhouses, four small slaughterhouses annexed to farmhouses, one meat distribution center, four meat cutting laboratories and four sausage factories. A single sampling day was conducted at each plant.

Samples collection

All samples were collected in 15 different food plants located in Sardinia. Meat industries and laboratories included in this study were: EC slaughterhouses (3); slaughterhouses annexed to a farmhouse (4); meat distribution center (1); meat cutting laboratories (4); sausage factories (4). The different number of samples collected from each food plant was proportional to its production capacity. A total of 166 samples were collected and analyzed: 46 environmental samples, 48 finishing pigs, 16 piglets, 24 samples of non processed meat (fresh meat before processing, fresh bacon), 28 meat preparations (minced meat, fresh sausage) and 4 meat products (fermented sausage). By way of illustration, the environmental samples included 20 surfaces not in contact with meat and 26 surfaces in contact with meat. Surfaces not in contact with meat, like walls, floors and floor drains were sampled in each visited plant (slaughterhouses, meat distribution center, meat cutting laboratories and sausage factories). Surfaces in contact with meat like mincing machine, mixing machine, sausage stuffers and trolleys were sampled at sausage factories; while cutting tables, saw machine and knives were sampled from each structure. The pig scalding dehairing machines was sampled at slaughterhouse. Environmental samples were collected using a commercial sponge sampling kit (3M, St. Paul, Minnesota, USA). Each kit contained a sterile sponge moistened with 10 mL of buffer peptone water (BPW), sterile gloves and sterile bag to transport the sponge. Sponges were scrubbed on the selected site, bounded with a sterile plastic delimiter, in order to cover an area of about 0.3 m^2 (Carpentier & Barre, 2012). Sponges were also used to sample the surfaces of finishing pigs and piglets carcasses, scrubbing the sponge on an area of 0.4 m^2. From each carcass four point were swabbed: ham, back, belly and jowl. After collection, each sponge was placed into its sterile bag. Samples of about 100 grams were collected from non-processed meat, meat preparations and meat products and placed in a sterile bag. All samples were transported in coolers with ice packs (3±1°C), received and processed at the Food Hygiene Department of Institute for Experimental Veterinary Medicine of Sardinia within 24 hours after collection.

Screening with real-time PCR

Detection of *Salmonella* in both environmental samples and product samples was performed using real-time PCR followed by microbiological confirmation (Bonardi *et al.,* 2017). A pre-enrichment broth was prepared suspending the sponge in 90 mL of Buffered Peptone Water BPW (Oxoid, Basingstoke, UK), 25 g of non-processed meat and meat products in 225 mL of BPW and 10 g of meat preparations in 90 mL of BPW. All samples were placed into Paddle Blender Bagfilter® and homogenate in a stomacher blander. After 24±2 h at 37±1°C DNA was extracted from 750 µL of the pre-enrichment culture using the PrepSEQ™ Rapid Spin Sample Preparation Kit *Salmonella* spp (Applied biosystems, life technologies). 30 µL of the bead-free supernatant from the DNA preparation step was then transferred into the tubes of MicroSEQ® *Salmonella* spp Detection Kit containing lyophilized PCR reagents. Real-time PCR reactions were run on the Applied Biosystems 7500 Fast Real-Time PCR System with the RapidFinder™ Express Software. The thermal profile used was: 95°C for 2 minutes, followed by 40 cycles at 95°C for 3 seconds and 60°C for 30 seconds. All samples were also processed with the TaqMan® Real-time PCR to confirm the kit results (Cremonesi *et al.*, 2014). Using the DNA obtained with PrepSEQ™ Preparation Kit, the PCR reactions were performed in a final volume of 20 mL containing 2 mL of template genomic DNA, 10 mL of TaqMan Universal PCR Master Mix, 20 mM for each primer, 5 mM for probe, the TaqMan Exogenous Internal Positive Control (IPC) Reagents VIC-labeled (2 mL of the Exo IPC Mix) (primers/probe) and 0.5 mL of the Exo IPC DNA (target DNA) and 4 mL of molecular grade water. A DNA from target and non-target reference strains correlating the designed assays was used. The reactions were carried out in 96-well plates sealed with heat bonding film. Amplification was achieved using an Applied Biosystems 7500 Fast Real-time PCR System (Life Technologies Inc, Italy). Each sample was tested in duplicate and the thermal profile used was: 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 60 seconds. The samples with a cycle threshold (CT) lower than 40 were considered positive. The samples above this CT were considered negative for *Salmonella* spp.

Microbiological analysis

All real-time PCR positive samples were processed using conventional culture-based methods according to the International Organization for Standardization [ISO] (2017) protocol ISO 6579:2017. Starting from the same pre-arrichment PBW (24±2 h at 37±1°C) used for biomolecular screening, from stomacher bag 100 µL and 1 mL of samples were taken and mixed with 10 mL of Rappaport Vassialidis soya RVS broth (Oxoid) and Muller Kauffmann tetrathionate-novobiocin MKTTn broth (Microbiol & C. s.n.c., Italy), respectively. Cultures were incubated overnight at 37±2°C for MKTTn broth and at 42±2°C for RSV broth. After the selective enrichment step, a loopful of each sample was streaked on selective and differential medium Xylose Lysine Desoxyscholate XLD (Microbiol) and ChromID™ Salmonella Agar SM2 (Biomerieux SA, France). Presumptive *Salmonella* colonies isolated on plating media were inoculated in Triple Sugar Iron semi-solid agar TSI (Oxoid) and incubated for 24±2 h at 37±1°C. Cultures giving typical reactions for *Salmonella* were submitted for biochemical identification test. Biochemical tests on suspected colonies were carried out using a miniaturized kit for rapid biochemical characterization of *Salmonella* Vitek 2 compact (Biomerieux).

Typing of isolates

Serotyping of isolates was performed following the White-Kauffmann-Le Minor

scheme by slide agglutination with specific sera for O and H antigen. The phenotypically discrimination of monophasic variant from *Salmonella* Typhimurium was done by repeating phase inversion at least three times without getting expression of the phase-2 flagellar antigens. Subsequently the genotypical discrimination was carried out by multiplex PCR protocol previously described by Alvarez *et al.* (2004), by Tennant *et al.* (2010) and then partly modified by Barco *et al.* (2011). This method allows a simultaneous amplification of a fragment between the genes *flj*B and *flj*A and the phase-2 flagellar gene (*flj*B). As described by Barco *et al.* (2011), template DNA was obtained by boiling of pure bacterial culture for 10 minutes. The PCR assay was performed in a total volume of 30 µL containing 2.5 mM $MgCl_2$, 0.6 mM of dNTPs, 1X Buffer-Taq, 1U of AmpliTaq GoldTM DNA Polymerase (Applied Biosystems, Roche), 0.1 mM of primers specific for fliB-fliA intergenic region, 1 mM of primers specific for *flj*B gene, and 5 µL of template DNA. The amplification profile was denaturation (95°C for 2 minutes), amplification (30 cycles: 95°C for 30 seconds, 64°C for 30 seconds, 72°C for 90 seconds), and final extension (72°C for 10 minutes).

Results

Detection of *Salmonella spp*

The CT values of both real-time PCR methods used in the study were lower than 40 in 13 out of 166 samples (7.83%). Of the 13 positive samples, 2 were surfaces in contact with meat, 3 were carcasses of finishing pigs, 5 belonged to the category of non-processed meat, 2 were meat preparations and 1 was a fermented pork sausage. The prevalence calculated from the real-time PCR results is showed in Table 1.

Salmonella spp was isolated from 7 out 13 (53.85%) of the PCR-positive samples, therefore the prevalence of *Salmonella* spp in all tested samples was 4.22% (7/166).

Salmonella spp was isolated from 1 out of 3 EC slaughterhouses (33.3%) and 3 out of 4 meat cutting laboratories (75%).

The prevalence of *Salmonella* spp in the environmental samples was 2.17% (1/46). In particular the prevalence in samples of surfaces in contact with meat was 3.85% (1/26). The microorganism was isolated from pig scalding dehairing machine in 1 out of 3 EC slaughterhouses visited. *Salmonella spp* was not isolated in the environmental samples not in contact with meat (walls, floors, floor drains). Results are showed in Table 2.

In regards to the carcass samples, a prevalence of 3.13% (2/64) was found. Positive samples belonged to the category of finishing pigs with a prevalence of 4.16% (2/48) from 1 EC slaughterhouse out of 16 plants visited. *Salmonella* spp was not isolated in any piglet sample. Results are showed in Table 3.

Salmonella spp was isolated in 4 out of 56 food products, showing a prevalence of 7,14 %. Positive samples belonged to the categories "non-processed meat" (2 samples of pork bacon) and "meat preparations" (2 samples of fresh sausage). Results are showed in Table 4.

The serotyping of strains isolated in this study showed that the 7 *Salmonella* spp belonged to *Salmonella* Typhimurium (1/7), *Salmonella* derby (3/7) and monophasic serovariant of *Salmonella* Typhimurium (3/7), with a prevalence of respectively 14.3%, 42.9% and 14.3%.

Table 1. Prevalence of *Salmonella* spp with real-time PCR screening method.

Samples	Plants				
	EC slaughterhouses (3)	Small slaughterhouses (4)	Meat cutting laboratory (4)	Sausage factory (4)	Meat distribution center (1)
Environmental					
SCM	16.7%(1/6)	ne	- (0/4)	6.25%(1/16)	ne
SNCM	ne	-(0/7)	- (0/1)	- (0/10)	- (0/2)
Total	6	7	5	26	2
Carcasses					
Finishing Pigs	9.1%(2/22)	-(0/6)	16.7%(1/6)	-(0/4)	-(0/10)
Piglets	-	-(0/12)	-(0/3)	-	-(0/1)
Total	22	18	9	4	11
Product					
Non-Processed Meat	ne	ne	33.33%(4/12)	8.33%(1/12)	ne
Meat Preparations	ne	ne	7.7%(2/26)	- (0/2)	ne
Fermented pork sausage	ne	ne	ne	25% (1/4)	ne
Total			38	18	

SCM, surfaces in contact with meat; SNCM, surfaces not in contact with meat. The number of positive samples out of the total is reported in brackets; -, not detected; ne, not evaluated.

Table 2. Prevalence of *Salmonella* spp in environmental samples in relationship with plants and sample categories.

Samples	Plants				
	EC slaughterhouses (3)	Small slaughterhouses (4)	Meat cutting laboratory (4)	Sausage factory (4)	Meat distribution center (1)
SCM	16.7%(1/6)	ne	- (0/4)	- (0/16)	ne
SNCM	ne	-(0/7)	- (0/1)	- (0/10)	- (0/2)
Total	6	7	5	26	2

SCM, surfaces in contact with meat; SNCM, surfaces not in contact with meat. The number of positive samples out of the total is reported in brackets; -, not detected; ne, not evaluated.

Table 3. Prevalence of *Salmonella* spp in carcasses of finishing pigs and piglets in relationship with plants.

Samples	Plants				
	EC slaughterhouses (3)	Small slaughterhouses (4)	Meat cutting laboratory (4)	Sausage factory (4)	Meat distribution center (1)
Finishing Pigs	9.1%(2/22)	-(0/6)	-(0/6)	-(0/4)	-(0/10)
Piglets	ne	-(0/12)	-(0/3)	ne	-(0/1)
Total	2218	18	9	4	11

The number of positive samples out of the total is reported in brackets; -, not detected; ne, not evaluated.

The multiplex PCR used to distinguish *Salmonella* Typhimurium from the monophasic serovariant of *Salmonella* Typhimurium confirmed that among the 4 strains of *Salmonella* Typhimurium, 3 lacked in expression of the second phase flagellar antigen.

Table 4. Prevalence of *Salmonella* spp in products in relationship with plants.

Samples	Plants	
	Meat cutting laboratory (4)	Sausage factory (4)
Non-Processed Meat	16.67%(2/12)	-(0/12)
Meat Preparations	7.7%(2/26)	- (0/2)
Fermented pork sausage	ne	- (0/4)
Total	38	18

The number of positive samples out of the total is reported in brackets; -, not detected; ne, not evaluated.

Discussion

Data obtained by the initial screening using the real-time PCR method, showed a higher prevalence of *Salmonella* spp if compared with the results of microbiological analysis. One possible explanation could be the higher sensitivity of real-time PCR. This aspect has been demonstrated by several studies. In 2014 Rodriguez-Lazaro *et al.* observed that a real-time PCR protocol was able to detect down to 2-4 *Salmonella* CFU in 25 g of different samples, including raw pork. Other authors demonstrated that limit of detection of the ISO 6579:2002 is down to 10 CFU per 25 g, therefore the real-time PCR can represent an excellent alternative (Delibato *et al*., 2014). Furthermore, another possible explanation could be the ability of real-time PCR to amplify DNA also from dead microorganisms (Barbau-Piednoir *et al.,* 2014), as probably happened in the fermented sausage sample and in the environmental sample.

This study showed a large variability in *Salmonella* prevalence among different plants. The obtained results confirmed what previously demonstrated by a study conducted by Piras *et al*., in 2014 on Sardinian abattoirs, affirming that contaminations depend on the slaughterhouse procedures (hygienic parameters and qualification of personnel), on the sampling day and on the origin and the number of infected pigs delivered during the same day. The positive carcasses to *Salmonella,* in one of three visited slaughterhouses, came from a European country (Spain), confirming that subclinical infected pigs may start excreting *Salmonella* when they are exposed to stress for example during transfer to the slaughterhouse (Rostagno, 2009). In fact, in the present study *Salmonella* spp was never isolated in small abattoirs annexed to farmhouses, where only pigs born in the farm are slaughtered. The isolation of *Salmonella* from equipment, as the scalding dehairing machine, confirms the ability of the microorganism to adhere to steel surfaces and to produce biofilm, as demonstrated by Piras *et al*. in 2015. This aspect can increase the risk of cross-contamination for carcasses during the dehairing phases and the subsequent contamination of offal and meat. The non confirmation of positivity at the microbiological analysis in the environmental samples not in contact with meat, that were positives at real-time PCR, shows the effectiveness of good hygiene practices during pre and post operative cleaning phases. Overall, data on the prevalence of *Salmonella* spp concerning environments, products and Salmonella typing obtained in this work confirm the ones reported in literature (D'ostuni *et al.,* 2016; Terentjeva *et al.*, 2017). As demonstrated by several research, our study demonstrated that the most common *Salmonella* serovariant isolated from pig in the EU are *Salmonella* Typhimurium, *Salmonella* Derby and monophasic serovariant of *Salmonella* Typhimurium (Bonardi, 2017; Barilli *et al.,* 2018; EFSA and ECDC, 2016).

Conclusions

The swine meat supply chain represents a potential alternative to the local agriculture as well as a strategic resource for the growth of the Sardinian economy, especially following the positive achievements obtained over the last years on animal health and breeding. Therefore, it becomes necessary to collect epidemiological data related to one of the most important anthropozoonotic agents carried by pigs in the industrial food chain.

Although in low prevalence, *Salmonella* appears to be present in slaughterhouses and products.

Considering the risk associated to *Salmonella* spp contamination in pig meat, the followings should be adopted as guideline: periodical monitoring of the meat supply chain (over all the steps of the process); enforcement of good hygiene practices and, whereas possible, restrict raw material selection to qualified farms.

The high sensitivity of real-time PCR, makes this method a complimentary alternative to the conventional microbiological techniques, especially for the initial sample screening that, once confirmed, are then processed according to the ISO. In light of the above, the real-time PRC allows to save time and improve response time efficiency mainly for the laboratories that perform a large number of checks.

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