



Occurrence of *Salmonella* spp. in samples from pigs slaughtered for consumption: A comparison between ISO 6579:2002 and 23S rRNA Fluorescent *In Situ* Hybridization method

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ABSTRACT

Contamination of pork products during slaughter represents an important vehicle for *Salmonella* spp. dissemination to humans. Salmonellosis poses an important risk for public health and presents an important economic issue to pork producers. This study aimed to evaluate the occurrence of this foodborne pathogen in pork carcasses and risk tissues (ileum, ileocolic and mandibular lymph nodes and tonsils) by two methods: the reference culture method (ISO 6579:2002) and a rapid Fluorescent *In Situ* Hybridization (FISH) method. The culture method identified the presence of *Salmonella* spp. in 13.7% of the samples, while the FISH technique revealed that 38.2% of the samples were positive. From these FISH positive samples, only 58 were concordant to the positive results obtained by the culture method.

These results confirm the potential risk that pork represents in salmonellosis transmission, suggesting that additional measures should be taken during evisceration practices and extraction of tonsils and mandibular lymph nodes after slaughter, in order to achieve a better control of *Salmonella* contamination during slaughter. The FISH method showed to be a rapid screening tool for *Salmonella* spp. detection in pork samples.

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1. Introduction

Salmonella spp. is the most frequently reported cause of food poisoning worldwide (Arnold, Scholz, Marg, Rosler, & Hensel, 2004). Salmonellosis poses a high risk for public health, being responsible for important economic losses for pork producers (Feder et al., 2001; Fedorka-Cray, Kelley, Stabel, Gray, & Laufer, 1995). Epidemiological studies in several European countries confirmed the importance of pork as a source of *Salmonella* infection to humans (Bone et al., 2010; Bruun et al., 2009; Ethelberg et al., 2008). Approximately 5 to 30% of the foodborne salmonellosis cases in industrialized countries are caused by the ingestion of pork and pork products (Araújo, 1996; Blaha, 2001; Hald, Wingstrand, Swanenburg, Von Altröck, & Thorberg, 2003; Käsbohrer et al., 2000). Pigs may be asymptomatic carriers of *Salmonella*, which may be found in the animals' digestive system, associated lymphatic tissues (tonsils and mesenteric lymph nodes), stomach content, lungs, skin and oral cavity (Hald et al., 2003; Jung, Lee, Lee, Kim, & Kim, 2001; Käsbohrer et al., 2000; Metcalf et al., 2000).

Pork slaughtering process includes several steps where carcass contamination with *Salmonella* may occur, through the oro-faryngic route, stomach content and feces (Borch, Nesbakken, & Christensen, 1996; Hald et al., 2003; Olsen, Jensen, Dahl, & Christensen, 2001). The product processing, storage and food preparation that occur after slaughter may also contribute to the multiplication of these contaminating bacterial agents (Bailey et al., 2003).

The European Food Safety Authority (EFSA) has published in 2008 a survey on *Salmonella* levels detected in slaughtered pigs across the European Union in 2006–2007. *Salmonella* was estimated to be present in one in ten pigs slaughtered for human consumption (10.3%). Levels for *Salmonella* detected in pigs ranged from 0% to 29% between Member States. *Salmonella* types most frequently identified were *Salmonella* Typhimurium and *Salmonella* Derby, two common types found in human infections, detected in 4.7% and 2.1% of pigs slaughtered for human consumption, respectively.

Rapid analytical techniques, able to provide data on *Salmonella* spp. contamination of fresh pork meat within few hours, are fundamental to the development of control and surveillance measures, which may allow for the implementation of corrective measures in time to avoid *Salmonella* transmission to consumers. Current rapid methods for *Salmonella* spp. detection are mainly based on immunological reactions (Kich et al., 2007; Valdivieso-Garcia,

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Desruisseau, Riche, Fukuda, & Tatsumi, 2003; Yeh, Tsai, Chen, & Liao, 2002) and PCR detection (Arnold et al., 2004; Hyeon et al., 2010; Lee et al., 2009; Löfstrom, Knutsson, Axelsson, & Radstrom, 2004).

The objective of this study was to evaluate the occurrence of *Salmonella* spp. in pork carcasses and tissues of known risk (ileum, ileocolic and mandibular lymph nodes and tonsils) that can be involved in pork product contamination during slaughter, using two methods: the reference culture method based in the ISO 6579:2002 Norm and the Fluorescent *In Situ* Hybridization (FISH) method as a rapid screening tool.

FISH was chosen as an alternative method since results from previous studies related to the application of FISH using Sal3 showed its potential as a sensitive, specific and rapid method for *Salmonella* detection in food samples (Fang, Brockmann, Botzenhart, & Wiedemann, 2003; Oliveira, Blasco, Ferrer, & Bernardo, 2005). Besides, FISH protocols can be optimized for specific detection of bacterial genera. Ribosomal genes contain highly conserved regions as well as variable regions that are transcribed into a high number of ribosomes (10^3 to 10^5 ribosomes/cell) during bacterial growth (Amann, Ludwig, & Shleifer, 1995). Oligonucleotide probes can be targeted to signature sites of the rRNA molecules that are specific for some microorganisms, allowing their detection through FISH (Moter & Göbel, 2000). Due to the abundance of rRNA in cells, the binding of fluorescent probes to individual cells is easily visualized by FISH (De Long, Wickham, & Pace, 1989). Finally, another reason for the choice of FISH is the fact that this technique is a low-cost technique (Oliveira et al., 2005).

2. Materials and methods

2.1. Sampling procedure

From June 2003 to September 2004, ten visits were made to an abattoir located in the North of Portugal. In each visit, approximately ten pigs were randomly selected, corresponding to one in every 20 slaughtered pigs. A total of 101 pigs were sampled.

From each pig, samples were collected from the ileum (25 g), ileocolic lymph nodes (25 g), mandibular lymph nodes (10 g) and tonsils (10 g). An internal surface swab was performed in the corresponding half carcass, using cotton sterilized gauze (hydrated in 25 ml of Buffered Peptone Water with 0.1% Tween) (Vieira-Pinto, Temudo, & Martins, 2005). A total of 505 samples were analyzed.

2.2. Sample preparation

Samples were processed up to 4 h after collection. All samples, with the exception of the carcass swab, were submerged in boiling water for 10 s for surface decontamination (Jung et al., 2001). Samples were then suspended in Buffered Peptone Water (BPW, Merk®, 1.07228) (1:10) and homogenized for 90 s in a Stomacher (IUL Instruments, Barcelona, Spain).

2.3. Microbiological culture method

Salmonella spp. detection was performed using the conventional microbiological protocol recommended by the ISO 6579:2002 standard, as already described by Vieira-Pinto, Temudo, et al. (2005).

2.4. Fluorescent *In Situ* Hybridization (FISH)

For the rapid detection of *Salmonella* spp., a specific FISH protocol was applied, using a 23S rRNA oligonucleotide probe, Sal3 (5'-AATCACTTACCTACGTG-3'; *E. coli* 1713→1730; Nordentoft, Christensen, & Wegener, 1997; Vieira-Pinto, Oliveira, Bernardo, & Martins, 2005). The probe was synthesized and labeled with fluorescein at the 5'-end (MWG-Biotech, Ebersberg, Germany).

The cells from 1 ml of the BPW pre-enrichment suspensions performed for conventional microbiological detection were harvested by centrifugation (19,500 g, 10 min; HERMLE Z233M-2, HERMLE AG, Gosheim, Germany) and fixed in 500 μ l of a 4% paraformaldehyde (w/v) solution in PBS for 4 h.

Ten microliters of the fixed suspensions were placed on the wells of Teflon slides (Heinz Herenz, Hamburg, Germany), air dried and dehydrated with 50%, 80% and 96% ethanol for 3 min at each concentration. After air drying, 10 μ l of hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl, pH 7.2, 0.01% SDS) containing 5 ng/ μ l of the Sal3 probe was added. Slides were incubated in a humid chamber at 45 °C for 3 h and washed in a buffer solution (0.9 M NaCl, 20 mM Tris-HCl, pH 7.2, 0.1% SDS) at 45 °C for 15 min.

Finally, slides were mounted in Vectashield® Mounting Medium (Vector Laboratories, H1000) and immediately visualized by fluorescent microscopy at $\times 1000$ (objective HCX PLAN APD) in a Leica DMR microscope (Leica Microsystemas Lda., Lisbon, Portugal).

2.5. Statistical analysis

Results obtained by both methods were compared according to the ISO/FDIS 16140:2000(E) standard – “Microbiology of food and animal feeding stuffs – protocol for the validation of alternative method”. The agreement between positive and negative results obtained by both methods was determined, as well as the positive (false-positive results) and negative deviations (false-negative results) (Table 2). Relative accuracy, sensitivity and specificity of the FISH method were also determined according to the ISO/FDIS, and a chi-squared analysis was applied for evaluating the difference between methods (Table 3).

3. Results

Results concerning the occurrence of *Salmonella* spp. in 505 pork samples as determined by the two methods are summarized in Table 1.

Ileocolic lymph nodes were found to be the most contaminated tissue from which *Salmonella* was isolated through the culture method. It was observed that 18.8% of the ileocolic lymph nodes samples were *Salmonella*-positive, followed by ileum (13.9%), mandibular lymph nodes (12.9%), carcasses (12.9%) and tonsil (9.9%) samples. *Salmonella* serovars identified from positive samples were previously described by Vieira-Pinto, Oliveira, et al. (2005) and Vieira-Pinto, Temudo, et al. (2005). It was observed that the most prevalent serotype was Typhimurium (47.8%), followed by Rissen (27.5%). Less prevalent serotypes included Tennessee (7.2%), Enteritidis (5.8%), 4, [5],12:i:- (4.4%), Anatum (2.9%), Give (2.9%) and Derby (1.5%).

According to FISH, the ileocolic lymph nodes (46.5%), carcasses (40.6%), mandibular lymph nodes (39.6%), ileum (38.6%) and tonsil (25.7%) samples revealed to be positive for *Salmonella* spp.

The agreement between the positive and negative results obtained by both methods was determined according to the ISO/FDIS 16140:2000(E) standard, as well as the positive (false-positive results) and negative deviations (false-negative results), and are presented in Table 2. Statistical analysis of FISH results obtained for all samples revealed that FISH has a relative accuracy of 71.1%, a relative specificity of 69.0% and a relative sensitivity of 84.1%. Determination of the agreement between methods ($\alpha < 0.05$) showed that they are different (Table 3).

4. Discussion

Salmonella spp. have been shown to persist for long periods in swine, which may carry this foodborne pathogen to the slaughterhouse (Gray, Fedorka-Cray, Stabel, & Ackermann, 1995). As long as animal carriers enter the slaughterhouse, *Salmonella* spp. may be transmitted to the consumers, even if slaughter is performed

Table 1
Identification of positive pork samples by conventional bacteriology (ISO 6579:2002) and the FISH method.

Samples	Carcass (n = 101)	Ileocolic lymph nodes (n = 101)	Ileum (n = 101)	Mandibular lymph nodes (n = 101)	Tonsils (n = 101)	Total (n = 505)
ISO 6579:2002 method	13	19	14	13	10	69
FISH method	41	47	39	40	26	193

according to stringent codes of Good Manufacturing Practices (GMP) (Berends, Van Knapen, Snijders, & Mossel, 1997).

Salmonella spp. presence in different tissues emphasizes the importance of pork sanitary status in bacterial transmission during slaughtering and its role as potential vehicles to the consumers. The ileocolic lymph nodes presented the highest *Salmonella* spp. occurrence. This result also alerts for the potential risk that mesenterium and mesenteric lymph nodes, which are sold in Portugal for making traditional gastronomic meals, can assume in *Salmonella* spp. cross-contamination.

Salmonella spp. presence in the ileocolic lymph nodes may point to the potential presence of this microorganism in the gut, via lymphatic drainage (Fedorka-Cray et al., 1999). Therefore, an evisceration process should be performed in order to minimize *Salmonella* spp. dissemination.

Since January 2006, the Regulation (EC) No. 2073/2005, defines the compulsory control of *Salmonella* spp. in pork carcasses by the ISO 6579:2002 standard. Nevertheless, this regulation allows the use of rapid alternative methods. Our study suggests that the FISH technique has a great potential for the rapid detection of *Salmonella* in pork carcasses. As a rapid screening tool, this technique would reduce the high volume of negative samples that are routinely analyzed, and expedite the detection of presumptive positive samples in time to protect consumers at risk.

Fluorescent *In Situ* Hybridization also showed that *Salmonella* spp. occurrence in mandibular lymph nodes was higher than in tonsils, although these tissues were consistently normal in appearance. Tonsils constitute an important contamination source to the carcass and other edible products (Castagna, Schwartz, Canal, & Cardoso, 2004), and according to the Regulation EC No. 853/2004, they are compulsorily removed during pork slaughter procedure. Our results suggest that the compulsory tonsil removal should be followed by the extraction of the mandibular lymph nodes. *Salmonella* spp. presence in the mandibular lymph nodes, usually with normal appearance, may constitute a risk to the consumer, since they can remain in the head carcass (Castagna et al., 2004; Wood, Pospischil, & Rose, 1989) and are routinely incised during sanitary meat inspection, leading to cross-contamination (Vieira-Pinto & Martins, 2003). Therefore, tonsil and mandibular incision extractions should be performed according to GMP practices.

Fluorescent *In Situ* Hybridization results are in agreement with conventional microbiological detection. From the 505 samples

analyzed, more samples (193 samples) were found to be positive by this method, being 58 of the FISH positive samples concordant with the ISO 6579:2002 results, while 135 were considered positive deviation. Result discrepancy and the high percentage of FISH false-positive results (26.7%) may be due to the detection of injured or non viable cells that still produce enough rRNA to be visualized by FISH and by the presence of inhibitory factors in the bacteriological culture media (Fang et al., 2003). FISH was also responsible for false-negative results (2.2%), which is in accordance with the work by Fang et al. (2003). These results could be due to the presence of small numbers of the target molecules (Fang et al., 2003; Stender et al., 2001) or to the insufficient accessibility of the target molecules (Amann et al., 1995). Although some authors suggest that a pre-enrichment step should be included to improve method sensitivity (Blasco, Ferrer, & Pardo, 2003; Fang et al., 2003; Moreno et al., 2003), in our study this step didn't eliminate the false-positive results. The authors believe that the lower number of *Salmonella* spp. presented in the original sample as well as the presence of debris in the suspension were responsible for the occurrence of false-negative results.

Although the FISH method generated a substantial number of false-positive and false-negative results, it showed a high relative accuracy (71.1%), a high relative specificity (69.0%) and a high relative sensitivity (84.1%). Nevertheless, it should be noted that the determination of the agreement between methods showed that the FISH protocol is not equivalent to the conventional bacteriological method.

In conclusion, Fluorescent *In Situ* Hybridization results suggest that this method should be considered as an important rapid screening tool for *Salmonella* spp. detection in pork samples, indicating that deeper studies should be performed for evaluating the origin of the high number of positive results and to eliminate the false-negative results.

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Table 2
Comparison between the results obtained in the detection of *Salmonella* spp. in pork samples by conventional bacteriology and the FISH method.

Samples	Carcass (n = 101)	Ileocolic lymph nodes (n = 101)	Ileum (n = 101)	Mandibular lymph nodes (n = 101)	Tonsils (n = 101)	Total (n = 505)
Positive agreement (M ^a + ^b and F ^c +))	11 (10.9%)	16 (15.8%)	12 (11.9%)	11 (10.9%)	8 (7.9%)	58 (11.5%)
Negative deviation (M+ and F- ^d)	2 (2.0%)	3 (3.0%)	2 (2.0%)	2 (2.0%)	2 (2.0%)	11 (2.2%)
Positive deviation (M- and F+)	30 (29.7%)	31 (30.7%)	27 (26.7%)	29 (28.7%)	18 (17.8%)	135 (26.7%)
Negative agreement (M- and F-)	58 (57.4%)	51 (50.5%)	60 (59.4%)	59 (58.4%)	73 (72.3%)	301 (59.6%)

^a M – Microbiological result.

^b F – FISH result.

^c + – positive result.

^d – – negative result.

Table 3

Statistical evaluation of FISH results for all samples according to the ISO/FDIS 16140:2000(E) standard – “Microbiology of food and animal feeding stuffs – protocol for the validation of alternative method”.

Parameter	Formula	Results
Number of samples (N)	$NA^a + PA^b + PD^c + ND^d$	505
Number of negative results (N-)	$NA + PD$	436
Number of positive results (N+)	$PA + ND$	69
Relative accuracy (AC)	$[(PA + NA)/N] \times 100\%$	71.1%
Relative specificity (SP)	$(NA/N-) \times 100\%$	69.0%
Relative sensitivity (SE)	$(PA/N+) \times 100\%$	84.1%
Methods agreement ($\alpha < 0.05$)	$Y = PD + ND$ If $Y > 22$, chi-squared = D^2/Y , where $D = PD - ND$ If chi-squared > 3.841 , the two methods are different	$Y = 146$ Chi-squared = 105.3, therefore the two methods are different

^a NA – negative agreement.

^b PA – positive agreement.

^c PD – positive deviation.

^d ND – negative deviation.

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