Application of a 23S rRNA Fluorescent In Situ Hybridization method for rapid detection of *Salmonella* sp. in slaughtered pigs

Vieira-Pinto, M. M.¹; Oliveira, M.²; Martins, C.¹ and Bernardo, F.²

²CIISA/Laboratório de Inspeção Sanitária, Faculdade de Medicina Veterinária, Lisboa, Portugal.

*corresponding author: mmvpinto@utad.pt*

**Abstract**

Pork products contamination during the slaughter process represents an important vehicle of *Salmonella* dissemination to humans. It’s urgent to develop rapid, sensitive and accurate methods that allow the detection of a large number of *Salmonella*-positive samples, in order to control these risks efficiently and in a practical time. This study evaluates the suitability of Fluorescent In Situ Hybridization (FISH) method as a rapid screening tool for *Salmonella* sp. detection in pork carcasses, as well in some risk tissues (ileum, ileocolic and mandibular lymph nodes and tonsils), which could be involved in *Salmonella* contamination during slaughter process. For that, FISH was comparatively analysed with the labour intensive reference microbiological culture method (ISO 6579:2002) whose results were previously published by the same authors. From the 69 (13.7 %) positive samples identified by the culture method, 58 were also identified by FISH, 7 hours after the pre-enrichment step. Additionally, FISH has detected *Salmonella* sp. in more 135 samples (26.7%), being this difference highly significant (p-value<0.001). These results indicate FISH as a promising tool for rapid *Salmonella* sp. detection in pork samples, which could be considered an important ally concerning to the actual European Regulation in matter of *Salmonella* sp. control in slaughtered pigs. Nevertheless, this study must be considered as a base line work that must be improved with additional studies in order to evaluate the origin of the higher number of positive results.

**Introduction**

Programs to reduce *Salmonella* sp. transmission to meat in the slaughterhouse should be implemented in order to promote food safety. To guarantee the efficacy of those programs is fundamental the use of sensitive methods that provide results more rapidly without a prohibitive cost. Only in this way it is possible the implementation of corrective measures in due time avoiding additional costs associated to storage of materials and products prior to use and distribution. According to Fang et al., (2003), FISH is a potential method that could be successfully implemented in food microbiology due to its favorable characteristic, such as: less prone to inhibitory substances, rapid availability of quantitative results, identification of genetic markers (greater sensitivity and specificity), the possibility of simultaneous identification of different species in the same sample and the relatively low cost per experiment. In 1997, studies developed by Nordentoft et al. (1997) revealed that FISH using Sal3, a fluorescence-labelled oligonucleotide probe, combined with a simple hybridization protocol, could be used to rapidly and accurately detect *S. enterica* serovars. Subsequently, other studies, related to the application of FISH with Sal3, indicated its potential as a sensitive and specific rapid method for *Salmonella* detection in food samples (Oliveira and Bernardo; 2002, Fang et al., 2003; Vieira-Pinto et al., 2005a). The objective of this study was to evaluate the efficacy of a 23S rRNA Fluorescent In Situ Hybridization (FISH) method, using Sal3, as a rapid screening tool for *Salmonella* sp. detection in pork carcasses and in some risk tissues (e.g. ileum, ileocolic and mandibular lymph nodes and tonsils) that can be involved in *Salmonella* sp. contamination during slaughter. The use of FISH as rapid method for *Salmonella* sp. detection at the slaughter level could constitute an important ally concerned to the actual European Regulation (Regulation (EC) N°. 2160/2003 and Regulation (EC) N°. 2073/2005) in matter of *Salmonella* sp. control in slaughter pigs, as well as an important tool to be use within Hazard Analysis Critical Control Point (HACCP) programmes.
Material and methods

From June 2003 to September 2004, a randomly selected group of 101 pigs was sampled in an abattoir in the North of Portugal during the slaughter procedure. In each pig, samples of the ileum (25g), ileocolic lymph nodes (25g), mandibular lymph nodes (10g) and tonsils (10g) were collected. In the corresponding half carcass, an internal surface swab was performed, with a cotton sterilised gauze (hydrated in 25 ml of Buffered Peptone Water with 0.1% Tween) (Vieira-Pinto et al., 2005a).

At the laboratory, four hours after sample collection, samples were suspended in Buffered Peptone Water (BPW, Merk®, 1.07228) (1:10) and homogenised during 90 seconds in the Stomacher. The isolation of Salmonella was performed according to ISO 6579:2002 Norm, as already described by Vieira-Pinto et al. (2005a).

For the rapid Salmonella detection, a specific 23S rRNA oligonucleotide probe was used, Sal3 (5'-AATCACTTCACCTACGTG-3'; E. coli 1713→1730; Nordentoft et al., 1997; Vieira-Pinto et al., 2005b). The probe was synthesized and labeled with fluorescein in the 5'-end (MWG-Biotech, Ebersberg, Germany). The cells from 1 ml of the suspensions of each dilution in BPW were recovered by centrifugation (14000 rpm, 10 min; HERMLE Z233M-2, HERMLE AG, Gosheim, Germany) and fixed with a 4% paraformaldehyde (w/v) solution in PBS for four hours.

Ten µl of the fixed suspensions were placed on the wells of teflon slides (Heinz Herzen, Hamburg, Germany) and air dried, after which were dehydrated with ethanol at 50%, 80% and 96%, during 3 minutes at each concentration. After drying, 10 µl of hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl, pH 7.2, 0.01% SDS) containing 5 ng/µl of the Sal3 probe were added. The slides were incubated in a humid chamber at 45 °C during 3 hours. After incubation, the slides were washed in a buffer solution (0.9 M NaCl, 20 mM Tris-HCl, pH 7.2, 0.1% SDS) at 45 °C during 15 min. The slides were mounted in Vectashield® Mounting Medium (Vector Laboratories, H1000) and immediately visualized by fluorescent microscopy at x1000 (objective HCX PLAN APD) in a Leica DMR microscope (Leica Microsystems Ltda., Portugal).

Positive deviations (false-positive results), negative deviations (false-negative results), sensitivity and specificity of FISH comparatively to ISO 6579:2002 method, were determined according to ISO/FDIS 16140:2000(E) Norm which specify the protocol validation of alternative microbiological methods for food and animal feeding stuffs.

A McNemar test (using Chi-square approach with Yates correction) for matched samples (D'Hainaut, 1992) was applied in order to determine the significance level of the difference between the results achieved using FISH and ISO method, with respect to the ratio of positive to negative results. Differences were considered significant at P<0.05.

Results

The results concerned to the detection of Salmonella sp. in the 505 pork samples determined by both methods and the statistical analysis are summarized in Table 1.

Table 1 - Results and statistical analysis concerned to the detection of Salmonella sp. determined by the reference microbiological method and FISH.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Carcass</th>
<th>Ile. Ln.</th>
<th>Ileum</th>
<th>Mand. Ln</th>
<th>Tonsils</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Method</td>
<td>n=101</td>
<td>n=101</td>
<td>n=101</td>
<td>n=101</td>
<td>n=101</td>
<td>n=505</td>
</tr>
<tr>
<td>Microbiological</td>
<td>13 (12.9%)</td>
<td>19 (18.8%)</td>
<td>14 (13.9%)</td>
<td>13 (12.9%)</td>
<td>10 (9.9%)</td>
<td>69 (13.7%)</td>
</tr>
<tr>
<td>FISH</td>
<td>41 (40.6%)</td>
<td>47 (46.6%)</td>
<td>39 (38.6%)</td>
<td>40 (39.6%)</td>
<td>26 (25.7%)</td>
<td>193 (38.2%)</td>
</tr>
<tr>
<td>Posit. deviation</td>
<td>30 (29.7%)</td>
<td>31 (30.7%)</td>
<td>27 (26.7%)</td>
<td>29 (28.7%)</td>
<td>18 (17.8%)</td>
<td>135 (26.7%)</td>
</tr>
<tr>
<td>Neg. deviation</td>
<td>2 (2.0%)</td>
<td>3 (3.0%)</td>
<td>2 (2.0%)</td>
<td>2 (2.0%)</td>
<td>2 (2.0%)</td>
<td>11 (2.2%)</td>
</tr>
</tbody>
</table>

**Il. - ileocolic; Ln. - Lymph nodes; Mand. - Mandibular; Posit. - Positive; Neg. - Negative; *** - p-value < 0.001 (Highly significant difference)
According to the results presented in Table 1, the difference among the results from the two methods were highly significant (p-value<0.001). Sensitivity and specificity of FISH comparatively to ISO 6579:2002 method determined according to ISO/FDIS 16140:2000(E) Norm, using the results presented in Table 1, was 84% and 69%, respectively.

Discussion
FISH allowed *Salmonella* sp. detection in approximately 7 hours (necessary time for fixation, hybridization and observation of the samples), with a consumable cost of 0.45 to 3.0 euros per sample, which is in accordance with previous results reported by Oliveira and Bernardo (2002) and Blasco et al. (2003).

Concerned to the differences in results obtained by FISH, comparatively to those achieved by the culture method, it was observed that, from the 69 samples where *Salmonella* sp. was isolated by the culture method, FISH detected 58 failing in detection of 11, corresponding these ones to the false-negatives or negative deviations. The number of negative results are inversely related with the sensitivity value of FISH, that in our study was 84%. Fang et al. (2003) also found false-negative results using the FISH method for detection of *Salmonella* in food. False negatives or weak signals can be obtained when small numbers of the target molecules are present, since the reduced number of bacteria in the sample can limit the FISH applicability because only a small part of the specimen is viewed within a reasonable time, or when the accessibility of the target molecules is insufficient (Stender et al. 2001; Fang et al. 2003). In our case we believe that the lower number of *Salmonella* sp. presented in the original sample as well as the presence of debris in the suspension observed trough FISH, were the main responsible for the occurrence of false-negative results.

Additionally to the 58 positive samples detected simultaneously by FISH and by the culture method, the Hybridization method has detected more 135 positive samples (26.7%) where no *Salmonella* was isolated by the cultural method, corresponding these cases to the false-positive samples or positive deviation. Several authors previously reported higher number of positive results by hybridization comparatively to the standard culture method (Bottari et al., 1995 and Eckner et al., 1994). Particularly to the application of FISH to food samples, Fang et al. (2003) previously reported that in 56 positive samples screened by FISH (using Sal3), *Salmonella* was not recovered in 28 samples of them by the conventional cultural method.

The large number of positive deviations achieved in this study, leaded to a low value of FISH specificity (69%). In view of these reduced value, two important questions must be prompt: Could these results be related to the FISH detection of other bacteria rather than *Salmonella* sp. or could these bacteria be dead? or this result reflects a ISO method limitation concerned to *Salmonella* sp. isolation in these kind of samples due to the presence of injured cells (viable but not cultivable) or a high competitive flora?

With regard to the large number of false-positive results verified, it is necessary to understand its origin and, most of all, to investigate if the noncultivable cells detected by FISH are viable. The presence of viable bacterial cells in food is of extreme importance to the food industry and to the consumers. To correspond to these expectations the authors suggests an optimization of the FISH method, in order to allow a reliable detection of viable cells, based on the work of Buchrieser and Kaspar (1993). These authors showed that the previous sample incubation with inhibitory antibiotics of the DNA gyrase, generated a significative cellular elongation in the physiologic active cells and increased the fluorescent intensity of the hybridized viable cells through the increase of the intracellular rRNA.

Conclusions
The FISH results, previously described, corroborated and increased the culture results, allowing the quick (7 hours) observation of individual cells by epifluorescent microscopy.
From the 505 samples analyzed, FISH allowed the detection of a superior number of positive samples (193 samples) comparatively to ISO method. The larger number of positive results detected by FISH that should be confirmed by the cultural method should not be an imposing limitation, since the negative results are most often encountered in food analysis. In addition, the use of FISH as a screening method would avoid an unnecessary waist of time and material in the
analyses of these negative samples, as well as expedite the results in a proper time to allow adequate consumers protection from the microbiological risk. The results from this study suggests that FISH could be considered an important rapid screening tool for *Salmonella* sp. detection in pork samples, constituting an important ally concerned to the actual European Regulation (Regulation (EC) N°. 2160/2003 and Regulation (EC) N°.2073/2005) in matter of *Salmonella* sp. control in slaughter pigs, as well as an important tool to be use within Hazard Analysis Critical Control Point (HACCP) programs. Nevertheless, additional studies should be performed in order to evaluate the origin of the high number of the positive results and to eliminate the false-negative results.

**References**


OLIVEIRA, M. e BERNARDO, F. 2002. "Fluorescent *In Situ* Hybridization" aplicado à detecção rápida de *Salmonella* de origem alimentar e ambiental. Revista Portuguesa de Ciências Veterinárias. 97, 81-85
