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Rapid detection of *Salmonella* sp. in pork samples using fluorescent *in situ* hybridization: a comparison with VIDAS®-SLM system and ISO 6579 cultural method

[*Detecção rápida de Salmonella sp. em amostras de suinos por hibridação in situ fluorescente: comparação com o sistema VIDAS®-SLM e com o método de cultura ISO 6579*]

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ABSTRACT

This study reports the use of the fluorescent *in situ* hybridization (FISH) with Sal3 probe for *Salmonella* detection in swine carcasses inner surface (swab); and in the correspondent samples of ileum, ileocolic, and mandibular lymph nodes; and tonsils, after dilution (1:10) in buffered peptone water and a pre-enrichment step (37^0C, 18h). In order to evaluate the efficiency of FISH, 235 naturally contaminated samples were simultaneously tested by the cultural method (ISO 6579) and by the Vitek Immuno Diagnostic Assay System (VIDAS®) - *Salmonella* (SLM) system. The cultural method identified 39 positive samples. From these, VIDAS®- SLM only detected 23. FISH identified 115 positive samples. This difference was highly significant ($P<0.001$). From positive samples, 32 were also confirmed by the cultural method. The results indicate FISH as a promising tool for rapid *Salmonella* detection in samples of pork and swine carcasses.

Keywords: swine, *Salmonella*, FISH, ELFA

RESUMO

Descreve-se a utilização da técnica de hibridação in situ *fluorescente (FISH), utilizando a sonda Sal3, para detecção de* Salmonella *na superfície interna de carcaças de suínos (zaragatoa), em amostras correspondentes de íleo, linfonodos ileocólicos, linfonodos mandibulares e amígdalas, após terem sido diluídas (1:10) e submetidas a uma fase de pré-enriquecimento em água peptonada tamponada (a 37ºC, 18h). Para avaliar a eficácia do método FISH, analisaram-se 235 amostras naturalmente contaminadas, usando o método de cultura ISO 6579 e o sistema* Vitek Immuno Diagnostic Assay System *(VIDAS®)-* Salmonella *(SLM), simultaneamente. O método de cultura identificou 39 amostras positivas, das quais o método VIDAS®-SLM detectou apenas 23. O método FISH identificou 115 amostras positivas. A diferença entre os métodos foi altamente significativa ($P<0.001$). Das amostras positivas, 32 foram confirmadas pelo método de cultura. Os resultados indicam que a FISH constitui uma promissora técnica de detecção rápida de* Salmonella *em amostras de suínos abatidos para consumo.*

Palavras-chave: suíno, *Salmonella*, FISH, ELFA

INTRODUCTION

Salmonella sp. is the most frequently reported cause of food poisoning in the world and an increase in the number of human cases of salmonellosis has been revealed in the last decade (Botteldoorn et al., 2001; Pless et al., 2001).

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In recent years, pork has gained increasing attention as a source of human salmonellosis (Jacob et al., 2001; Castagna et al., 2004). *Salmonella* species have been shown to persist for long periods in swine (Silva et al., 2006), resulting in contamination of the slaughterhouse (Gray et al., 1995). Swine can carry *Salmonella* in several tissues, especially those of the digestive tract and the associated lymph nodes, thus representing a potential risk for consumers which must be properly identified and controlled (Jung et al., 2001; Castagna et al., 2004; Silva et al., 2006).

Cultural method for *Salmonella* detection in foods are labor-intensive and time-consuming (requiring between four to six working days), which does not allow result provision in time to avoid possible distribution of the contaminated meat. Culture methods are also not convenient for routine monitoring of a large number of samples (De Medici et al., 1998). To control these risks efficiently and in due time, it is urgent to develop rapid, sensitive and accurate methods that allow the screening of a large number of *Salmonella*-suspected samples (Fang et al., 2003).

Studies developed by Nordentoft et al. (1997) revealed that the fluorescent *in situ* hybridization (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. The target sequence of Sal3 is located at the helix 63 of *Salmonella* 23SrRNA, and this study showed to be accessible for *in situ* hybridization.

FISH with rRNA-targeted oligonucleotide probes has been developed for the *in situ* identification of individual microbial cells and now is a well-established technique (Amann et al., 2001). Due to the abundance of rRNA in cells, the binding of the fluorescent probes to individual cells is easily visualized by FISH (DeLong et al., 1989). According to Amann et al. (1995), the 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. Oligonucleotide probes can be targeted to signature sites of the rRNA molecules specific for some microorganims, allowing detection through FISH (Moter and Gobel, 2000).

Results from previous studies related to the application of FISH with Sal3 indicated its potential as a sensitive, specific and rapid method for *Salmonella* detection in food samples (Oliveira and Bernardo, 2002; Fang et al., 2003; Vieira-Pinto et al., 2005).

This study was carried out to investigate the use of FISH with Sal3 as an accurate and rapid method to detect *Salmonella* in swine carcasses; ileum, ileocolic and mandibular lymph nodes; and tonsils. For that reason, FISH was compared with the labour intensive conventional cultural method (ISO 6579:2002) and Vitek Immuno Diagnostic Assay System (VIDAS)®-SLM[1], a fully automated enzyme–linked fluorescent immunoassay (ELFA) validated by the AFNOR, which is a rapid screening method used in several food analyses for the presumptive detection of foodborne pathogens.

MATERIALS AND METHODS

During two consecutive months (September and October 2003), 47 animals were randomly sampled in an abattoir. From each selected pig, a portion of the ileum (I=25g), ileocolic lymph nodes (IL=25g), and mandibular lymph nodes (ML=10g); and tonsils (T=10g) were collected. In the corresponding half carcass (C), an internal surface swab was performed, with a cotton sterilised gauze, hydrated in 25ml of BPW[2] with 0.1% Tween. A total of 235 samples were analysed. All the samples were individually packed in a sterile labelled container and transported to the laboratory under refrigerated conditions.

All the samples, except the carcass swabs, were heated in boiling water for 10sec in order to decontaminate the external surface (Jung et al., 2001). After that, samples were diluted in BPW, (1:10), homogenised in the Stomacher (90sec) and incubated at 37^0C for 18h. Afterwards, FISH, VIDAS and the cultural methods were performed.

Cultural method was performed according to ISO 6579:2002 applied to *Salmonella* detection in food and animal feeding stuffs. Presumptive

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colonies of *Salmonella* were confirmed by biochemistry: oxidase reaction, triple sugar iron agar[3], urea broth[4], L-Lysine descarboxylase broth[5]. Serological agglutination with Poly A-I & Vi antisera[6] was also analysed. *Salmonella* isolates from each positive sample were serotyped according to Kauffmann-White scheme (Popoff, 2001) in the Laboratório de Investigação Veterinária – Portuguese National Reference Laboratory for *Salmonella*.

VIDAS®-SLM protocol was carried out according to the procedures recommended by the manufacturer. For FISH protocol, ten-well Teflon-coated slides[7] were used after treatment with a 2% 3-Trimethoxysilylpropylamine in acetone solution[8].

After 18 hours incubated in BPW at 37ºC, 2ml of each sample were fixed using the protocol adapted from Hodson et al. (1995). Briefly, the cells were recovered by centrifugation (14000rpm, 10min), washed twice with 1ml of PBS and fixed with a 4% paraformaldehyde (w/v) solution in PBS for two hours. Afterwards, they were washed twice in PBS, re-suspended in 5ml ethanol 50% in PBS, and stored at –20ºC.

From the fixed samples, 10µl were collected and placed into the slide wells and the hybridization protocol described by Blasco et al. (2003) was performed, using a specific probe for the 23S rRNA of *Salmonella*, Sal3 (5'-AATCACTTCACCTACGTG-3'; *E. coli* 1713→1730), labeled with fluorescein[9] at the 5'-end (Nordentoft et al., 1997; Oliveira and Bernardo, 2002; Vieira-Pinto et al., 2005).

The slides were visualized by fluorescent microscopy at 1000x amplification[10] in a Leica DMR microscope equipped with a 100V mercury lamp and an I3 filter[11]. In the first well of each slide, the reference strain *S.* Enteritidis CECT4300 was used as positive control.

[3]Oxoid®– CM277 - Basingstoke, Hampshire, England
[4]Merk® – 1.08483 - Darmstadt, Germany
[5]Oxoid® – CM308S - Basingstoke, Hampshire, England
[6]Difco® - 222641, Beckton Dickinson - Franklin Lakes, NJ, USA
[7]Heinz Herenz - Hamburg, Germany
[8]Merk Sharp and Dohme - Lisbon, Portugal
[9]MWG-Biotech - Ebersberg, Germany
[10]Objective HCX PLAN APD
[11]Leica Microsistemas Ltda. - Lisbon, Portugal

A McNemar test (D'Hainaut, 1992) was applied in order to determine the significance of the difference between the results achieved using FISH, VIDAS, and ISO culture method.

RESULTS AND DISCUSSION

In order to assess the accuracy of FISH, 235 naturally contaminated samples were simultaneously tested by the ISO 6579 cultural method and VIDAS®-SLM. Results from these analysis are summarised in the Tables 1 and 2.

VIDAS®-SLM only detected 23 of the 39 positive samples identified by the cultural method, failing in the detection of 16, which can be described as false negatives. This difference is highly significant ($P<0.001$). The reason for samples tested negative with VIDAS®-SLM but positive with cultural method is unknown. These results are not according to those achieved by De Medici et al. (1998) and Yeh et al. (2002), who use, respectively, VIDAS-ICS and VIDAS-SLM for *Salmonella* sp. detection in food samples (poultry meat and swine carcass sponge samples) naturally contaminated, and found no significant difference between these methods and the cultural. Comparatively analysing these two studies with the one described in this manuscript, no particular reason was found to explain the high level of false negative results obtained by VIDAS®-SLM in this study. Further studies with artificial contaminated samples should be developed in order to comprehend the origin of these results.

FISH allowed positive detection of *Salmonella* in approximately seven hours (time necessary for fixation, hybridization and observation of the samples), which is according to previous results reported by Oliveira and Bernardo (2002) and Blasco et al. (2003).

From the 39 samples, in which *Salmonella* was isolated by the cultural method, FISH detected 32 of the confirmed positive. Additionally, FISH detected 83 further positive samples, in which no *Salmonella* was recovered by the cultural method.

Table 1. Comparison between results obtained using the cultural method VIDAS®-SLM in naturally contaminated carcasses (swab); ileum, ileocolic, and mandibular lymph nodes; and tonsils of slaughtered swine

		Cultural method		
		Positive	Negative	Total
VIDAS®-SLM	Positive	23	0	23
	Negative	16(FN)	196	212
	Total	39	196	235

VIDAS®: Vitek Immuno Diagnostic Assay System. FN: false negative. McNemar's test for paired samples (P<0.05)

Table 2. Comparison between results obtained using the cultural method and FISH in naturally contaminated carcasses (swab); ileum, ileocolic, and mandibular lymph nodes, and tonsils of slaughtered swine

		Cultural method		
		Positive results	Negative results	Total
FISH	Positive results	32	83	115
	Negative results	7(FN)	113	120
	Total	39	196	235

FISH: fluorescent *in situ* hybridization. FN: false negative.
McNemar's test for paired samples (P<0.05)

Again, this difference was highly significant (P<0.001). These results are according to those obtained by Fang et al. (2003), who showed that in 56 positive samples screened by FISH (using Sal3), *Salmonella* was not recovered in 28 by the conventional cultural method. The occurrence of a large number of positive results, comparatively to the culture method, can be partially explained by the presence of injured cells or inhibitory factors, which can leave the cells viable (and visible by microscopy), but not culturable (Fang et al., 2003). According to these authors, FISH efficiency seems to be less influenced by different physical and chemical properties of preserved foods (temperature, salt concentration and pH) which might stress *Salmonella* cells that become not culturable. In fact, these particular properties are two of the main advantages of FISH for food analyses when compared with the cultural method (Blasco et al., 2003). Otherwise, the higher number of FISH positive results should not be attributed to the detection of dead cells be low, since pre enrichment step at 37°C allows the rapid RNA degradation of inactivated cells (Moreno et al., 2001; Fang et al., 2003). Nevertheless, since the identification of the viability state of the detected cells is an issue of major interest to the industry, further detailed investigation should be performed to understand if the non-culturable cells detected by FISH are viable, and represent a potential risk to the consumers. For that, parallel viability tests such as the LIVE/DEAD Bac Light viability kit[12] (Auty et al., 2001) and the direct viable count, described by Villarino et al. (2000) should be performed in order to account for non-culturable FISH positive samples.

Another reason for the high number of FISH positive results, comparatively to the cultural method, can be related to the presence of competing bacteria that may interfere with *Salmonella* isolation (Cudjoe and Krona, 1997; De Medici et al., 1998), but do not interfere with its detection by FISH.

FISH failed to detect seven positive samples (false negative) identified by the cultural method. Fang et al. (2003) also obtained similar results. In the present study, background fluorescence was observed in several samples, which might influence the *Salmonella* visualization, especially if the microorganims are present in low number. For this reason, it is suggested for future studies the inclusion of a slight centrifugation (Cui et al., 2003) or a filtration step (Rijpens et al., 1999)

[12]Molecular Probes In. - Eugene

before the sample fixation, in order to eliminate only the major particles that could interfere with the fluorescence background. The comparison between the results obtained by VIDAS®-SLM and FISH was also established. The results are presented in Table 3.

Table 3. Comparison between results obtained using the cultural method, FISH, and VIDAS®-SLM in naturally contaminated carcasses (swab); ileum, ileocolic, and mandibular lymph nodes; and tonsils from slaughtered swine

	Cultural method		
	Positive	Negative	Total
FISH+/ VIDAS®-SLM +	23	0	23
FISH+/ VIDAS®-SLM -	9	83	92
FISH-/ VIDAS®-SLM +	0	0	0
FISH-/ VIDAS®-SLM -	7	113	120
Total	39	196	235

VIDAS®: Vitek Immuno Diagnostic Assay System. FISH: fluorescent *in situ* hybridization.
+: positive; -: negative
McNemar's test for paired samples ($P<0.05$)

All the presumptive positive samples detected by VIDAS® were also detected by FISH. Furthermore, FISH detected other nine presumptive positive samples which were not detected by VIDAS®-SLM but were identified as positive by the cultural method. Thus, FISH was more effective for *Salmonella* detection than VIDAS®–SLM, and this difference was highly significant ($P<0.001$).

Considering the increasing importance of pork as a *Salmonella* carrier, it is imperative to implement in the slaughterhouse a rapid and accurate screening method for an adequate risk assessment, promoting food safety, increasing consumer confidence and improving products marketing. This fact has particular relevance in the EU, since the *Salmonella* control in swine carcasses in the slaughterhouse is compulsory, but the carcasses decontamination at the end of the slaughter is not allowed. In this context, FISH has a particular interest. Comparatively to the conventional cultural method and to VIDAS®–SLM, FISH detected more presumptive positive samples. In addition, this method has the advantages to allow enumeration and simultaneously detect several microorganims in the same microscopic field, as well as being faster and less expensive than cultural methods.

The large number of positive results detected by FISH, which should be confirmed by the cultural method, should not be an imposing limitation, since the negative results are most often encountered in food analysis. 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 it would allow the indication of presumptive positive results in a proper time to guaranteeing an adequate protection to consumers from the microbiological risk. The use of FISH could be particularly advantageous in the application of hazard analysis and critical control points.

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