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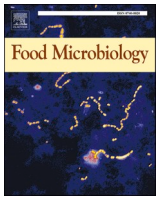

ELIME-IMS hybrid assay for *Salmonella* detection in swine mesenteric lymph nodes at slaughterhouse

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ABSTRACT

Salmonella contamination in pig slaughterhouses is linked to infection rate on farms. Accurate diagnosis in heavy pigs relies on isolating pathogens from the gut wall or lymph nodes. A key technique is Immunocapture using Magnetic Beads (IMS), which purifies target bacteria from *Salmonella* enrichment broths. This is followed by an Enzyme-Linked Immunomagnetic Electrochemical (ELIME) assay for rapid detection. In our study, we developed an ELIME-IMS hybrid assay to detect *Salmonella* in swine mesenteric lymph nodes (MNL), involving a clean-up with N-acetylcysteine and centrifugation. Detection limits for *S.* Typhimurium and *S.* Derby were estimated at 2.80 and 3.52 Log CFU/ml, respectively. We analysed 103 MNL samples from a northern Italy slaughterhouse. Additionally, we examined 15 carcass swabs. Both the ELIME assay and the IMS-based culture method showed strong agreement with the ISO 6579–1:2017 method, especially after 20 h of enrichment (89.47% concordance). The clean-up step significantly influenced the results, as samples processed without it showed higher variability. A logistic regression model indicated high classification accuracy for negative samples using ELIME values. The ELIME-IMS assay facilitates rapid *Salmonella* screening and isolation in swine mesenteric lymph nodes.

1. Introduction

Salmonellosis is a major foodborne disease in the European Union (EU), with 65,208 confirmed cases in 2022, a hospitalization rate of 38.9%, and a fatality rate of 0.22% (EFSA and ECDC, 2023). It spreads through contaminated food and can cause gastrointestinal symptoms, with some cases leading to bacteraemia and long-term effects like reactive arthritis (Ajene et al., 2013). *Salmonella* infections are common in swine, and contaminated pork products can infect humans (Bonardi, 2017). While fattening pigs are usually asymptomatic, host-adapted strains can persist in macrophages of mesenteric lymph nodes (MLNs), allowing *Salmonella* to survive silently for months. Infected herds show chronic infections with low *Salmonella* excretion (Kempf et al., 2022; Lahodny et al., 2017), but stress during transportation can increase shedding, thus causing contamination of the slaughter environment (Henry et al., 2018; Massacci et al., 2020; Possebon et al., 2020) and carcasses (Zeng et al., 2021).

The burden of *Salmonella* entering pig slaughterhouses is linked to the prevalence of infection at farm level (Martínez et al., 2020; Roasto et al., 2023; Snary et al., 2016). Reliable infection diagnostics involve pathogen isolation from the gut wall or gut-associated lymph nodes (Bessire et al., 2018; Deane et al., 2022; EFSA 2008). In some EU countries, serological diagnosis is an alternative for *Salmonella* control in fattening pigs (EFSA, 2006), although it cannot identify currently infected or shedding pigs. For this purpose, antibody titres scoring system (using ELISA cut-off values) were developed to assess *Salmonella* risk on farms (EFSA 2010; Szabó et al., 2008). Serology is often preferred for pig herds surveillance due to its cost-effectiveness and ease of sampling through blood or muscle juice collection at the slaughterhouse (Correia-Gomes et al., 2021; De Lucia et al., 2020; Nielsen et al., 1998).

In 2011, the European Food Safety Authority (EFSA) proposed harmonized epidemiological indicators (HEIs) for pigs as part of risk-based meat inspection, but their application remains unregulated (EFSA, 2011; Ferri et al., 2023; Li et al., 2023). *Salmonella* control measures in the EU are governed by several directives and regulations, including Directive 2003/99/EC, Regulation (EC) No 2160/2003,

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Regulation (EC) No 2073/2005, and Regulation (EU) No 217/2014, which mandate national control programs to reduce significant serotype prevalence in target animals. While poultry species have established prevalence targets, pigs lack defined requirements for sampling, testing and reporting (EFSA and ECDC, 2023). As a result, not all Member States (MS) have implemented *Salmonella* monitoring programmes (SMPs) for pigs, leading to a lack of harmonization (Correia-Gomes et al., 2021). Food Business Operators (FBOs) could benefit from classification of the incoming batches of pigs in order to properly manage the risk of carcasses contamination before and after chilling (Li et al., 2023; Roasto et al., 2023).

Sensitive and rapid detection of pathogens in lymph nodes and carcasses is essential. Electrochemical biosensors using antibodies effectively reduce time and costs of *Salmonella* detection (Nastasijevic et al., 2021). These biosensors use electrodes for transduction, with antibodies serving as receptors for bacteria antigens (Cesewski and Johnson, 2020). Magnetic beads coated with anti-*Salmonella* antibodies are often employed in techniques like immunomagnetic separation (IMS) to capture *Salmonella* from samples. This is followed by coupling secondary anti-*Salmonella* enzyme-linked antibodies in a sandwich assay format, known as Enzyme-Linked Immunomagnetic Electrochemical (ELIME) (Awang et al., 2021; Delibato et al., 2009; Volpe et al., 2016). Various research groups have developed new immunological and molecular methods for rapid *Salmonella* detection, and several assays are commercially available (Awang et al., 2021; Silva et al., 2018). However, these methods do not integrate the screening efficiency of biosensor-based techniques with *Salmonella* isolation, crucial for isolate characterization and contamination source tracing. This study aims to develop a time-effective assay that detects *Salmonella enterica* in swine mesenteric lymph nodes and carcasses within 24 h, and to compare its performance with the ISO 6579–1:2017, 2017 method. The procedure should also assess the viability of target bacteria, thanks to *Salmonella* culturing on suitable media, and enable isolate typing.

2. Materials and methods

2.1. Preparations of control strains and standardization of cultures

Salmonella enterica strains, including *S*. Typhimurium, *S*. Typhimurium monophasic variant 1, 4, [5], 12:i: , *S*. Derby, *S*. Rissen, *S*. Infantis, *S*. Enteritidis, *S*. Napoli and *S*. Thompson, as well as isolates belonging to other species (*Pseudomonas aeruginosa*, *Escherichia coli*, *Pantoea* (*Enterobacter*) *agglomerans, Yersinia enterocolitica* O:8) were provided by the Unit of Inspection of Food of animal origin, University of Parma. The selection of bacteria other than *Salmonella* was based on their close relation to *Salmonella* or their common occurrence in the same environment under similar growth conditions. In our study, the bacteria not belonging to *Salmonella* genus are considered interferents. For selectivity tests, *Salmonella* or non-*Salmonella* isolates were cultured aerobically with gentle shaking at 37 °C for 18–20 h in Buffered Peptone Water (BPW, ThermoFisher Scientific, Rodano, Italy). The enriched cultures were then diluted in BPW to achieve a turbidity (transmittance) of 20–40% at 560 nm. Serial 1:10 dilution with Phosphate Buffered Saline pH 7.4 (PBS; Merk Life Science, Milano, Italy) were made to obtain concentrations of 3–6 $\log_{10}$ CFU/ml. Calibration curves for *S*. Typhimurium and *S*. Derby were generated through the dilution of standardized cultures in PBS, as well as in the sample matrix, which consisted of lymph node enrichment broths that tested negative for *Salmonella*.

2.2. MLN and carcass swab preparation

Between May 2022 and February 2024, 103 mesenteric lymph node (MLN) samples were collected during 20 sampling sessions from 42 batches of pigs at slaughterhouse located in northern Italy. The samples were sealed in sterile bags and transported under refrigeration (+4 °C) to the University of Parma laboratory (PR-Lab) within 2 h from collection. Additionally, from May to July 2023, 15 carcass samples were collected using a sterile sponge moistened with 10 mL of BPW swabbing a 400 cm^2 area following the ISO 17604:2015 method. Both MLNs and carcass swabs were analysed using the ISO 6579–1:2017, 2017 method for *Salmonella* detection by PR-Lab. Upon arrival, MLNs were aseptically cleaned from fat tissue, flame-sterilized to eliminate any surface contamination, weighted up to 25 g, and diluted 1:10 in 225 mL of BPW. They were homogenized in a Stomacher blender for 2 min and incubated at 34 °C–38 °C. Carcass sponges were similarly homogenized in 90 mL of BPW and incubated under the same conditions.

After 8 h of incubation, two 1 mL aliquots of the enrichment broths were refrigerated at 4 °C and transported to the University of Bologna diagnostic laboratory (BO-Lab) for testing using an ELIME assay with the Dynabeads® *Salmonella* detection System (ThermoFisher Scientific), following a modified manufacturer protocol (Fig. 1). One mL aliquots were taken after 20 h of incubation to detect Salmonella using the ISO method. Starting from sample No. 49 (January 2023), an additional 2 mL aliquot was refrigerated, transported to BO-Lab, and analysed, thus enhancing the IMS-based method sensitivity. *Salmonella* spp. isolates detected at PR- and BO-Labs were sub-cultured on Tryptic Soy Agar (TSA, Oxoid, ThermoFisher Scientific) and serotyped according to ISO/TR 6579–3:2014.

2.3. Artificially contaminated samples

To validate the ELIME assay, a blind test was conducted on 10 MLN samples and one carcass swab sample. At the PR-Lab, the BPW pre-enrichment broths were artificially contaminated with 10–15 CFU of *S*. Typhimurium (ATCC 14028). The contaminated *Salmonella*-samples (pre-enrichment spiked samples; pre-ESS) were made indistinguishable from the other samples. These were incubated for 8 and 20 h at 34 °C–38 °C, processed as described in the previous paragraph, and transported to the BO-Lab for blind proficiency testing.

2.4. Secondary and labelled antibodies

The antibody solution was prepared using mouse monoclonal antibodies (K26D, *AbSal*) targeting the common LPS core of all O-serogroups of *Salmonella*, along with affinity purified goat anti-mouse IgG (H&L) F (ab)'2 fragment cross-adsorbed horseradish peroxidase (HRP) conjugate. Both antibodies were at a concentration of 1 μg/mL in a 0.1x casein blocker from ThermoFisher Scientific. The tubes were incubated at 37 °C for 30 min in a hybridization incubator with slow rotation (2 rpm). The anti-mouse IgG conjugated with HRP served as the detector antibodies in a sandwich ELIME assay.

2.5. Calibration curves, blocking and determination of the limit of detection

Calibration curves were created to establish the relationship between concentration of *Salmonella* and instrumental response. Standard solutions of either *S*. Typhimurium or *S*. Derby were prepared in 1-ml PBS and captured using 20 μL of Dynabeads® anti-*Salmonella* suspension (ThermoFisher Scientific). The resulting signal was measured via amperometry, as detailed in paragraph 2.6. The calibration curves were fitted using a nonlinear regression with a four-parameter dose-response equation (4PL) in GraphPad Prism (Motulsky, 2016):

$$y = \mathrm{d} + \frac{x^b \bullet (a-d)}{x^b + c^b} \qquad \text{equation 1}$$

where: y is the ELIME response signal (μA); x is the *Salmonella* concentration (CFU/mL), a and d represent the maximum and minimum signal response of the calibration curves, respectively; b is the Hill coefficient which represent the slope-like parameter; and c is the *Salmonella*

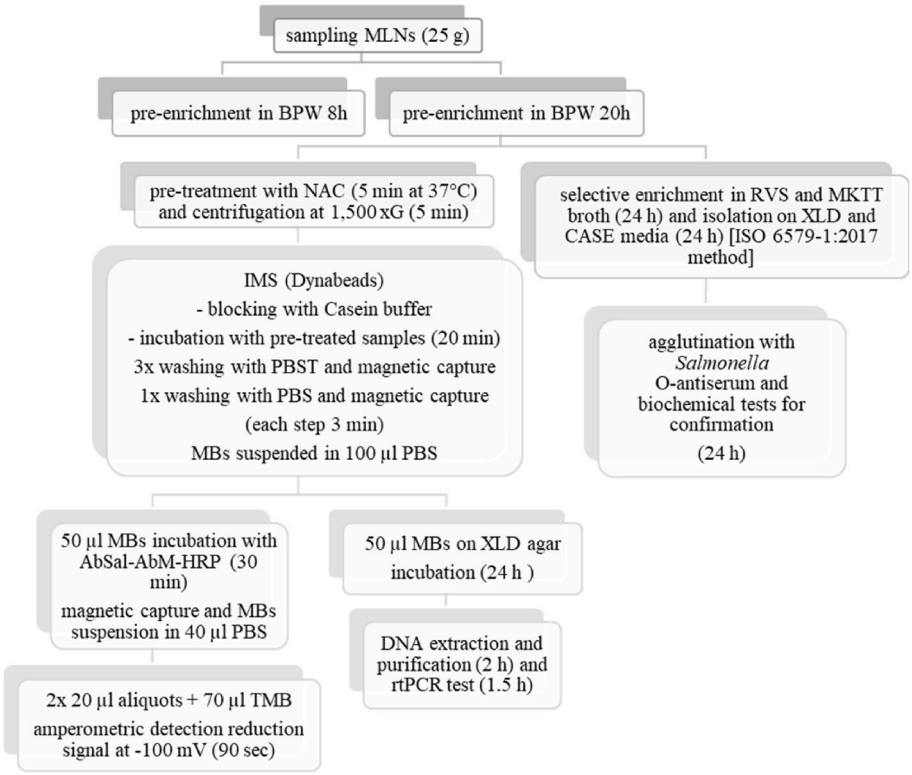

Fig. 1. Protocol of the culture-based methods for detection of Salmonella and the ELIME assay.

concentration producing a 50% signal response. The limit of detection (LOD) was calculated based on the value of three times the standard deviation of the blank values (NTC, not template controls). These values were then interpolated from the sigmoidal dose-response 4-PL equation, and the corresponding concentration of *Salmonella* value was then calculated including the confidence interval (Masdor, 2017; Masdor et al., 2019).

2.6. Blocking of the magnetic beads

The magnetic beads were blocked by coating the Dynabeads® anti-*Salmonella* suspension with a casein blocker to prevent non-specific antibody binding and minimize cross-reactivity with goat proteins that might interfere with polyclonal anti-mouse antibodies. The treatment involved the following steps: 1. washing the Dynabeads with phosphate-buffered saline (PBS); 2. incubating the beads in a 0.5x casein blocker solution for 30 min at room temperature to saturate unoccupied binding sites; 3. treating the beads with a 0.1x casein blocker for 5 min. The pre-treated magnetic beads were then ready for the ELIME assay.

2.7. ELIME assay

The aliquots from BPW enriched samples at 8 and 20 h, along with the artificially contaminated samples (pre-ESS), were centrifuged at 5,000xG for 5 min. The pellet was resuspended in 0.01 M Phosphate buffered saline pH 7.4 (PBS, Merk Life Science). Each assay included the addition of 1 mL of PBS and 1 mL of *S.* Typhimurium ATCC 14028 solution (approximately 4 Log CFU/mL) as negative (NTC) and positive control, respectively. Twenty µL of Dynabeads® anti-*Salmonella* suspension (ThermoFisher Scientific) were added to each sample and control to capture the target bacteria, followed by incubation at room temperature with slow rotation for 20 min using a Dynabeads® MX1 Mixer (ThermoFisher Scientific).

To minimize interferences from non-target bacteria, three washing steps with a 0.05% Tween solution in PBS (20x Tween-20, ThermoFisher Scientific) and one washing step with PBS were performed. A sample clean-up step was introduced due to the presence of viscous, gel-like material in many MLN samples that could interfere with IMS (Fig. S1). This step began with sample No 79 in May 2023. In the modified procedure, 1 mL of the samples was treated with 100 µL of N-acetylcysteine (100 µg/mL) (Fluimucil®, Zambon, Vicenza, Italy) known for its mucolytic properties (Aldini et al., 2018; Balsamo et al., 2010). The N-acetylcysteine (NAC) treatment needed incubation of the sample at 37 °C for 5 min to effectively lyse the slime material, followed by centrifugation at 1,500xG for 5 min to remove the debris. Positive controls (*Salmonella* suspensions in PBS) were treated similarly to verify that the NAC treatment did not affect the number or viability of the target bacteria. The supernatant from the clean-up step was used for *Salmonella* detection.

The Dynabeads®-captured bacteria were suspended in 100 µL of PBS; a 50 µL-aliquot was seeded on Xylose-Lysine-Desoxycholate Agar plates (XLD; Oxoid, ThermoFisher Scientific) and incubated at 34–38 °C for 24h; the remaining 50 µL-aliquot was suspended in 500 µL of antibodies solution and incubated at 37 °C for 30 min in the Amersham RPN2510E Hybridization oven/shaker (VWR International, Milano, Italy) with slow rotation. The immunomagnetic Dynabeads®-*Salmonella* complexes were cleaned from non-conjugated antibodies through two washes in 0.05% Tween solution and one wash in PBS. The IMBs were then captured using magnets and resuspended in 40 µL PBS. From these, 20 µL aliquots were applied to unmodified disposable screen-printed carbon electrodes (Dropsens DRP-150, Metrohm, Origgio, Italy). A neodymium magnet positioned beneath the electrode facilitated IMBs capture, after which 70 µL of the ready-to-use 3, 3′, 5, 5’ Tetramethylbenzidine (TMB; Merk Life Science, Milano, Italy) substrate was added. Chronoamperometry was employed to measure the current flowing through the working electrode over time using a potentiostat (Eco Chemie Autolab PGSTAT12), connected to a DRP-DSC cell (Metrohm-DropSens) controlled by Nova 2.1 software. A constant potential of −100 mV was set, with a recording time of 90 s and a pre-step delay time of 60 s for bulk solution equilibrium. Background current from NTC

samples in each batch was subtracted to account for pipetting errors in antibodies preparation.

2.8. Real Time-PCR

DNA from suspect *Salmonella* colonies on XLD was extracted and purified using the QIAamp® UCP Pathogen Mini Kit (QIAGEN, Milano, Italy). For real time PCR assay the *Salmonella*-specific oligonucleotide primers (ttr-6 and ttr-4) and the target probe (ttr-5) were designed by Malorny et al. (2004) based on conserved sequences in the tetrathionate resistance (ttr RSBCA) locus. The reaction conditions, as per the TaqPath™ BactoPure™ Microbial detection PCR master mix guidelines (Applied Biosystems, Monza, Italy), included an initial denaturation step at 95 °C for 2 min, followed by 45 cycles of denaturation at 95 °C for 10 s and annealing at 65 °C for 30 s, with a final elongation at 60 °C for 60 s. The baseline subtraction option was always selected, and the threshold line for calculating the threshold cycle number (C_T) was set manually to a fluorescence value of 0.06 for standardization. The DNA samples were analysed without internal amplification control.

2.9. Enriched sample spiked aliquots

To determine the minimum number of CFUs required for a positive result in the ELIME assay, eight trials were conducted using a pool of known negative samples (identified as negative by culture method and ELIME results below 0.1 μA, with PBS-blank values subtracted). Each 0.9-mL aliquot was then spiked with 100 μL of standardized dilutions of *S.* Derby or *S.* Virchow containing 3.1–5.9 log10 CFUs. These spiked samples are referred to as post-ESS, and the ELIME test was repeated following the above-described procedure.

2.10. Statistics

Descriptive statistics tools in Microsoft Excel (Office 365) were used to produce boxplot and calibration plots. GraphPad Prism (version 10.2.3 for macOS) was used for nonlinear regression (4 PL dose-response curves and logistic regression) and other statistical analyses (normality test, Kruskall-Wallis and Kolmogorov-Smirnov test) to evaluate differences in ELIME current values between samples testing positive or negative with the culture-based *Salmonella* detection methods. The Cohen's Kappa test was then used to measure the consistency between the IMS-based and ISO 6579–1:2017, 2017 methods for *Salmonella* detection.

3. Results

3.1. Calibration curves

The calibration curves shown in Fig. 2 illustrate the relationship between the detected ELIME signal and the concentration of a reference standard. Sigmoidal, 4 PL (four-parameter logistic) regression curves were derived from data relative to *S.* Typhimurium and *S.* Derby (four assay repetitions using ten-fold dilutions of the bacteria in PBS). The graph also shows incremental signals from negative MLN pools after being spiked with *Salmonella*. A right shift in the calibration curves was observed, with *S.* Typhimurium exhibiting a more pronounced left shift compared to *S.* Derby. LogEC50 values (c coefficients of 4 PL dose-response equations) for *S.* Typhimurium and *S.* Derby were 5.34 and 5.71, respectively, with current values exceeding 1 μA observed at concentrations above 3.04 and 3.5 Log CFU/ml, respectively. The reduction in current values (μA) at the working electrode analysing NTC (no template control samples) was -0.46 ± 0.12 μA, based on 29 assays with various lots of antibodies and magnetic beads. The current (μA) for different concentrations (x) of *S.* Typhimurium and *S.* Derby in PBS can be estimated with equations (1a) and (1b), respectively:

$$y = -21.89 + \frac{x^{-0.67} \bullet (-21.89 + 0.40)}{x^{-0.67} + 216,941^{-0.67}} \quad \text{equation 1a}$$

$$y = -16.86 + \frac{x^{-0.71} \bullet (-16.90 + 0.37)}{x^{-0.71} + 512,314^{-0.71}} \quad \text{equation 1b}$$

The estimated LODs for *S.* Typhimurium and *S.* Derby were 2.80 (95%UCL 3.24) and 3.52 (95%UCL 4.03) Log CFU/ml, respectively.

3.2. Inclusivity and exclusivity tests

The inclusivity test (Fig. 3a) demonstrated that the method effectively detects various *Salmonella* serotypes, with signal increasing (5-7x) at higher concentrations. The reduction current values (μA) at the working electrode, due to HRP (anti-mouse IgG HRP conjugate), were notably high (over 4 μA) at *Salmonella* concentrations of 4–5 Log CFU/ml. In the exclusivity test, lower current values were recorded for non-target bacteria, with *E. coli* reaching a maximum of 0.4 μA at 4.8 Log CFU/ml (Fig. 3b).

3.3. Analysis of MLNs with culture tests

The IMS-based culture detection method for *Salmonella* correctly identified all pre-ESS contaminated with 10–15 CFU of *S.* Typhimurium. Comparative analyses of culture tests with the non-spiked samples,

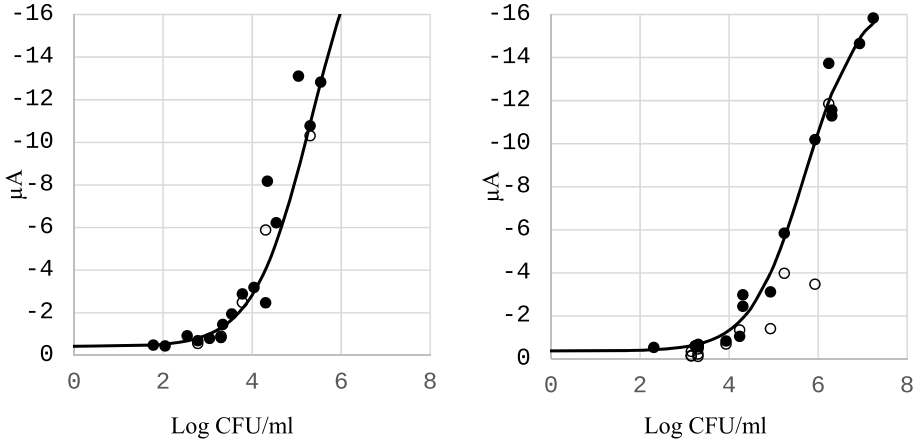

Fig. 2. Concentration-response curve of S. Typhimurium (left) and S. Derby (right) in PBS.
Legend: full and empty circles represent PBS concentration standards and ESS data.

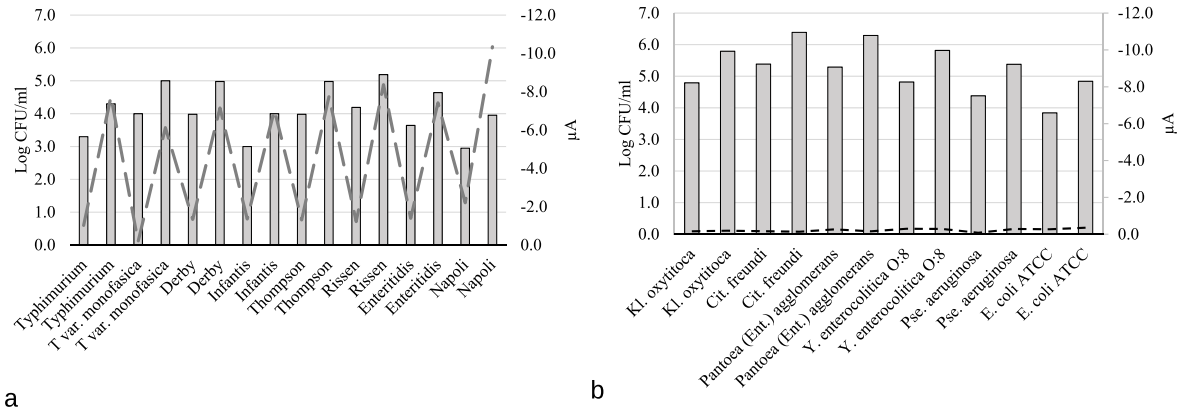

Fig. 3. Inclusivity and exclusivity tests for various *Salmonella* serovars and non-target bacteria.

reported in Table 1, showed 'strong' agreement (McHugh, 2012) after 20 h of enrichment (89.48% of the observations) and similar results for 8-h enriched samples using the IMS-based method (87.38% of the observations). The Kappa values for these agreements were 0.716 and 0.553, respectively, indicating reliability. The false negative rate for the 57 samples analysed at 20 h with both methods was 17.65%, while for samples analysed after 8 h with the IMS method and 20 h with ISO method the rates were 33.33% and 20.83%, respectively (see Table 2).

Overall, *Salmonella* spp. isolates were detected in 24 out of 103 samples (23.3%). Eight serotypes were identified, with *S.* Derby, *S.* Typhimurium 4,[5],12:i: and *S.* Bredeney being found in 45.8%, 16.7% and 12.5% of the positive samples (Table S1). Occurrence of two different serovars were observed in two samples when analysed in the two laboratories, demonstrating possible superinfections in the swine herds (Table 3).

The results of the ELIME assay for MLN samples are shown in Fig. 4 and Table S2. The clean-up step had a discernible impact on the outcomes. Samples analysed after 8 or 20 h of enrichment that tested negative for *Salmonella* using culture-based methods, exhibited significantly higher signals and greater variability when analysed without the clean-up step. The median values were as follows: without the clean-up step, −0.2540 μA and −0.2445 μA after 8 and 20 h of enrichment, respectively; with the clean-up step, −0.005 μA and 0.050 μA after 8 and 20 h, respectively. The Kolmogorov-Smirnov test revealed significant differences between these groups with P-value of 0.0004 for the 8-h samples and 0.0015 for the 20-h samples (see Table S2).

The differences in ELIME signals between *Salmonella*-positive and negative samples were statistically significant for those analysed after the clean-up step, but for the uncleaned samples (Table S2). The likelihood of obtaining positive results using culture-based methods can be estimated through logistic regression. Specifically, this involves fitting a logistic regression model to the log odds:

$$LN\frac{p}{1-p} = -4.236 + \mu A \bullet (-7.853) \quad \text{(equation 3)}$$

where −4.236 and −7.853 are β_0 and β_1 in the Logit equation, specifically the y-intercept and the slope of the log-odds as a function of the ELIME signal. The above equation can be rewritten as:

$$p(x) = \frac{1}{1 + e^{(\beta_0 + \beta_1 \bullet x)}} \quad \text{(equation 4)}$$

where x is the ELIME signal (μA) and p is the probability of positive culture for *Salmonella*. The equation parameters were estimated using Maximum Likelihood Estimation (MLE) based on experimental data. The dataset consisted of 40 samples: 8 tested positives using the ISO and/or IMS-based method, while 32 tested negatives. The standard errors (SE) and associated probabilities (p-values) for the parameters are as follows.

- For the intercept (β_0): SE = 1.528; p = 0.0056
- For the slope (β_1): SE = 3.289; p = 0.017

Fig. 5b illustrates the predicted probabilities of positive and negative culture results compared to the observed outcomes in 20-h enriched samples. With a classification cutoff of −0.5 μA, 93.75% of negatives and 75% of positives were correctly classified. Raising the cutoff to −0.8 μA achieved 100% accuracy for negatives while maintaining the 75% for positives. Two samples were incorrectly classified as negatives, likely due to the low numbers of *Salmonella* in the enriched sample aliquots.

Fitting the ELIME signals at 8 h yielded a β_1 coefficient of −2.984 (SE 1.939; p = 0.1239). Since the p-value exceeded the 0.05 significance level of, we failed to reject the null hypothesis that β_1 is equal to zero, indicating that the ELIME signals at 8 h do not significantly distinguish positive *Salmonella* samples (Fig. 5a).

3.4. Analysis of carcasses

Only one out of 15 carcasses (6.7%) tested positive for *Salmonella* (*S.* Derby), and this result was obtained solely through the IMS-based method, classifying the culture-based pairs as 'discordant.' Fig. 6 illustrates the results from the naturally contaminated carcass swab, along with pre-ESS and post-ESS samples, allowing for comparisons across data sets. The Kolmogorov-Smirnov test found no significant differences between culture-negative samples and those spiked with 3.1–3.9 and 4.2–4.9 Log CFU/ml (p-values equal to 0.0645 and 0.0571).

Table 1
– Comparative results of analysis made in 8 h (a) and 20 h (b) enriched cultures.

		ISO		
		positive	negative	sum
IMS-based	positive	11 (10.68%)	5 (4.85%)	16 (15.53%)
	negative	8 (7.77%)	79 (76.70%)	87 (84.47%)
	sum	19 (18.45%)	84 (81.55%)	103 (100%)

		ISO		
		positive	negative	sum
IMS-based	positive	11 (19.30%)	3 (5.26%)	14 (24.56%)
	negative	3 (5.26%)	40 (70.18%)	43 (75.44%)
	sum	14 (24.56%)	43 (75.44%)	57 (100%)

Table 2
Antigenic characteristics of isolates detected in MLN by ISO and IMS-based methods.

Sample ID	ISO culture	IMS-based detection			Serotype isolate detected by method	
		culture	hours*	Clean-up	ISO	IMS-based
BS 41	positive	positive	8–20	NO	*S.* Derby	*S.* Derby
BS 50	positive	positive	8–20	NO	*S.* Derby	*S.* Derby
BS 73	positive	positive	8–20	NO	S. Derby	*S.* Derby
BS 58	positive	positive	8–20	NO	*S.* Derby	*S.* Derby
BS 62	positive	positive	20	NO	*S.* Derby	*S.* Derby
BS 66	positive	positive	8–20	NO	S. Derby	*S.* Derby
BS 78	positive	negative		NO	*S.* Derby	*na*
BS 17	positive	*negative*		NO	*S.* Derby	*na*
BS 48	*negative*	positive	8-*na*	NO	*na*	*S.* Derby
BS 74	*negative*	positive	8–20	NO	*na*	*S.* Derby
BS 87	positive	positive	8–20	YES	*S.* Derby	*S.* Rissen
BS 52	positive	positive	8–20	NO	*S.* Enteritidis	*S.* Derby
BS 67	positive	positive	8–20	NO	MVST	MVST
BS 69	positive	*negative*		NO	MVST	*na*
BS 118	positive	*negative*		YES	MVST	*na*
BS 45	positive	*negative*		NO	MVST	*na*
BS 103	positive	positive	8–20	YES	*S.* Bredeney	*S.* Bredeney
BS 105	positive	positive	8–20	YES	*S.* Bredeney	*S.* Bredeney
BS 107	positive	positive	8–20	YES	*S.* Typhimurium	*S.* Typhimurium
BS 19	positive	*negative*		NO	*S.* Infantis	*na*
BS 39	*negative*	positive	8-*na*	NO	*na*	*S.* Bredeney
BS 89	*negative*	positive	8–20	YES	*na*	*S.* Rissen
BS 119	*negative*	positive	8–20	YES	*na*	*S.* Nottingham
BS 129	positive	positive	20	YES	*S.* Choleraesuis	*S.* Choleraesuis

Legend: *enrichment time; in italic discordant results; *na* = not analysed/detected; MVST. *S.* Typhimurium 4.[5]0.12:i:

Table 3
descriptive statistics and results of Kruskal-Wallis test (P-values) for ELIME assay results in MNL enriched samples pre-treated or not with the cleanup procedure.

	24h				8h			
	positive	discordant	negative	P-value	positive	discordant	negative	P-value
a. with samples cleanup								
Number of values	5	3	32		5	3	32	
Minimum	−12.000	−0.780	−0.570		−5.100	−0.470	−0.670	
25% Percentile	−11.000	−0.780	−0.010		−5.000	−0.470	−0.060	
Median	**−7.900**	**−0.510**	**0.050**	**0.3731**	**−3.100**	**−0.040**	**0.005**	**0.3615**
75% Percentile	−4.700	−0.290	0.080		−0.310	0.040	0.038	
Maximum	−1.600	−0.290	0.150		−0.010	0.040	0.150	
Range	11.000	0.490	0.720		5.100	0.510	0.820	
Mean	−8.000	−0.530	−0.010		−2.700	−0.160	−0.059	
Std. Deviation	4.100	0.250	0.170		2.400	0.270	0.180	
Std. Error of Mean	1.800	0.140	0.030		1.100	0.160	0.032	
	24h				8h			
	positive	discordant	negative	P-value	positive	discordant	negative	P-value
b. without sample cleanup								
Number of values	6	3	8		8	8	47	
Minimum	−4.903	−0.949	−0.693		−5.531	−3.056	−4.262	
25% Percentile	−4.692	−0.949	−0.540		−1.253	−0.892	−0.480	
Median	**−1.311[a]**	**−0.713[b]**	**−0.245[c]**	**0.0001**	**−0.343[a]**	**−0.310[ab]**	**−0.254[b]**	**0.0128**
75% Percentile	−0.183	0.042	−0.136		−0.161	−0.054	0.009	
Maximum	−0.138	0.042	0.012		−0.132	−0.024	0.164	
Range	4.765	0.991	0.705		5.399	3.032	4.426	
Mean	−2.080	−0.540	−0.315		−1.085	−0.674	−0.587	
Std. Deviation	2.234	0.518	0.240		1.852	1.016	1.065	
Std. Error of Mean	0.912	0.299	0.085		0.655	0.359	0.155	

Differences between groups with different superscript letters were significant (Kolmogorov-Smirnov test).

4. Discussion

4.1. Sample pre-treatment

ELIME assays use magnetic beads as a support for the immunological reaction and electrochemical readout to measure signals. The assay's specificity and sensitivity can be affected by the number and affinity of binding sites and interferences from unwanted molecules (Haukanes and Kvam, 1993). To address matrix interferences, blocking agents and clean-up techniques are necessary (Paniel and Noguer, 2019). Interfering substances like glycoproteins, lipids, and other molecules from culture media, can hinder the bacteria-antibodies interaction. At the BO-lab, we observed slime material on the walls of tubes containing the refrigerated enriched gut bacteria cultures and abundant sediment. Exopolysaccharides (EPSs)-producing bacteria, such as *Enterobacter*, *Enterococcus*, and *Klebsiella* species, are common in the swine gut and can grow in culture media used to detect *Salmonella,* producing EPSs (Netrusov et al., 2023). These EPSs may interfere with the binding ability of magnetic beads coated with antibodies in the ELIME assay and IMS-based culture method. Biofilm formation is crucial for many enteric

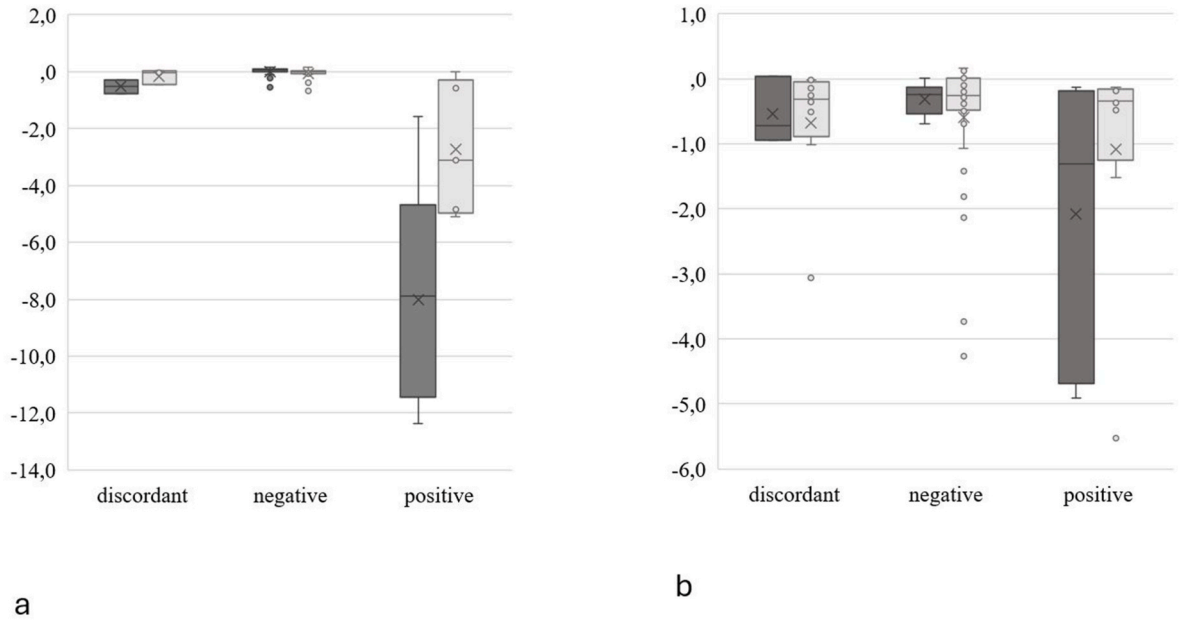

Fig. 4. ELIME signals in the groups of MLN samples classified according to the results of the culture tests and use of cleanup step.
Legend: a = clean-up; b = no clean-up; dark filling 20h; clear filling 8h; discordant = different results with sample aliquots analysed using ISO and IMS-based detection method.

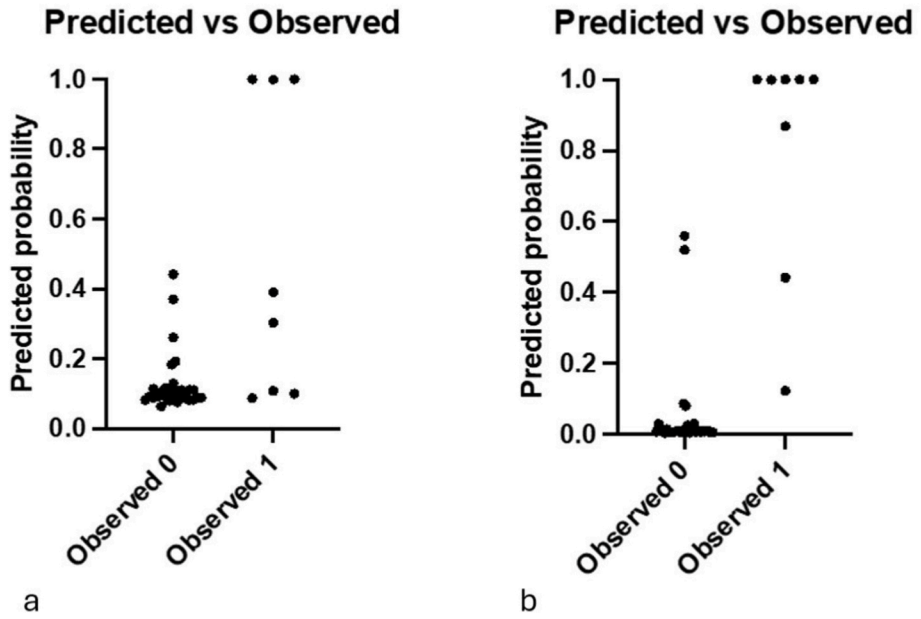

Fig. 5. Predicted probability for positive and negative samples and observed results.
Legend: a = 8h; b = 20h.

bacteria, including *Salmonella*, allowing them to persist outside hosts and colonize multiple hosts. In lymph nodes, EPSs can affect the immune response and contribute to the pathogenesis of chronic infections (Dsouza et al., 2024; Harrell et al., 2021; Perry and Tan, 2023; Schiopu et al., 2023).

It is unclear whether EPSs production occurs during incubation at 37 °C or while enriched samples are refrigerated. However, to enhance the sensitivity of the ELIME assay, we introduced a clean-up step before Immunomagnetic Separation using N-acetylcysteine (NAC) as a mucolytic agent and low-speed centrifugation to separate particulate material without affecting planktonic bacteria. NAC, often used in medical treatments, is effective for disrupting microbial biofilms in enriched samples (Blasi et al., 2016; Dinicola et al., 2014; Kregiel et al., 2019). By disrupting disulphide bonds in EPSs, NAC reduces nonspecific bacterial adhesion to magnetic beads. These pre-treatments significantly improved the detection rates compared to the ISO 6579–1:2017, 2017 method since May 2023, demonstrating the superior performance of the ELIME assay.

4.2. The effect of incubation time on the detection of Salmonella isolates in MLN' enrichments

IMS-based detection methods are not culture-independent and the number of *Salmonella* in samples and their growth in enrichment

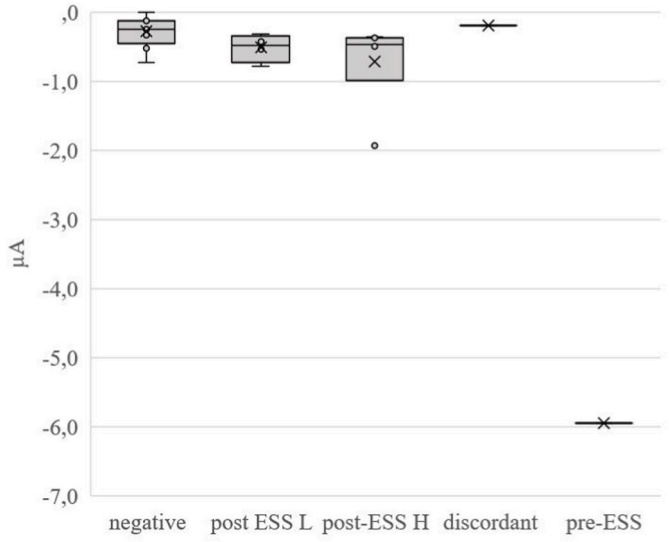

Fig. 6. ELIME signals in the groups of carcass swab samples classified according to the results of the culture tests and artificial samples inoculated with salmonella.
Legend: post-EES L and post-ESS H = pool of enriched samples inoculated with *S.* Derby at concentration in the ranges: Low, 3.10–3.8 Log (CFU/ml); High 4.2–4.9 Log (CFU/ml).
pre-ESS swab homogenate inoculated with *S.* Typhimurium.

cultures significantly influence the outcomes. This explains the differences observed between the samples incubated for 8 and 20 h. The detection rate depends on the initial population size in 25 g samples and specific growth parameters, such as growth rate (μ) at 37 °C, maximum growth rate (Nmax), and lag phase (λ) (Baranyi and Roberts, 1994; Oksuz and Buzrul, 2020). Although studies on the physiological state of *Salmonellae* in lymph nodes are limited, these organs can serve as niches for bacterial growth due to the bacteria's ability to survive there (Martins et al., 2013).

In pigs at slaughter weight, *Salmonella* spp. is primarily found in the digestive tract, its contents, and associated lymph nodes, which act as immune inductive sites. Following infection with *S.* Typhimurium and its monophasic variant, most pigs recover, but some become *Salmonella* carriers, intermittently shedding the pathogen for up to 5 months (Griffith et al., 2019). Approximately 5–30% of these animals may continue to excrete *Salmonella* spp. at the end of the finishing period, and this percentage can double during transport and lairage (Berends et al., 1996). The farm-phase is central to these issues, with reports indicating that *S.* Typhimurium can be detected in MLNs of infected pigs from 2 h to 6 weeks after oral inoculation (Rostagno et al., 2011).

In pig faecal samples with low numbers of *Salmonella*, the ISO method demonstrated a sensitivity of 91.5% for *S.* Typhimurium and 88.5% for *S.* Derby (Mooijman et al., 2019), while sensitivity for MLNs was close to 87% (Mainar-Jaime et al., 2013). These variations can significantly affect *Salmonella* detection in MLNs and should be considered when using culture-dependent assays to assess the *Salmonella* status of farms supplying pigs to slaughterhouses. Bacteria in lymph nodes face stressful conditions that can influence the lag phase duration during enrichment, unlike artificially introduced bacteria. In our experiments, we detected *Salmonella* using the IMS-based method in samples artificially contaminated with 10–15 colony-forming units (CFU) after an 8-h enrichment period. Facultative intracellular lymph-tropic bacterial pathogens like *Salmonella* can exist in higher numbers free in lymph nodes than cell-associated, with significant differences in infection among strains of the same species (Siggins and Sriskandan, 2022).

4.3. Comparison between results of IMS-based culture method, ELIME assay and ISO method

Previous studies indicated that the IMS detection method had a 90% concordance with the ISO 6579 method in various food matrices inoculated with low levels of *Salmonella* (Cudjoe and Krona, 1997). Additionally, combining IMS with immunological methods like ELISA has proven successful (Bai et al., 2023; Wang et al., 2020). In our study on swine MLNs, the two culture-based methods exhibited substantial agreement. After 20 h of enrichment, the results were consistent in 100% of the cases, and after just 8-h consistency was observed in 87.38% of the cases. Concerning the ELIME assay, the signals from samples enriched for 20 h correlated with the likelihood of positive and negative results from culture-based methods. However, three samples (%) exhibited inconsistent results between IMS-based and ISO culture methods, with two having ELIME signals falling between positive and negative categories, likely due to low *Salmonella* load.

5. Conclusion

The primary objective of our study was to demonstrate the ELIME assay utility as a rapid screening test for detecting *Salmonella* in swine mesenteric lymph nodes. The results show that the ELIME assay, applied to samples enriched for 20 h, effectively assesses the likelihood of MLN samples being positive or negative for *Salmonella*, as confirmed by using culture-based methods. The ELIME-IMS Hybrid Assay also shows substantial agreement with the ISO 6579–1:2017, 2017 method, highlighting its potential for detecting *Salmonella* in swine gut tissues, aligning well with established standards.

CRediT authorship contribution statement

M. Trevisani: Writing – review & editing, Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **M. Conter:** Writing – review & editing, Validation, Investigation, Formal analysis, Data curation. **M. Cecchini:** Writing – review & editing, Investigation, Formal analysis, Data curation. **L. Lamperti:** Writing – review & editing, Investigation, Formal analysis, Data curation. **L. Andriani:** Writing – review & editing, Investigation, Formal analysis, Data curation. **M. Rega:** Writing – review & editing, Formal analysis, Data curation. **C. Bacci:** Writing – review & editing, Formal analysis, Data curation. **M. Perri:** Writing – review & editing, Formal analysis, Data curation. **S. Bonardi:** Writing – review & editing, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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