Detection of *Salmonella* spp. in meat products: A 48 hr solution from enrichment to confirmation using qPCR automation and chromogenic media.

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Introduction

The detection of foodborne pathogens typically involves a multi-stage workflow consisting of enrichment, screening, and confirmation. Traditional methods such as ISO 6579-1:2017[1] for *Salmonella* detection can be time consuming (up to seven days) and require multiple culture media, decreasing laboratory productivity and increasing costs. In contrast, alternative methods that streamline this process and provide timely results with enhanced sensitivity and selectivity are pivotal for industrial laboratory routines. This study demonstrates a complete AFNOR validated solution for detection and confirmation of *Salmonella* spp. within 48 hr, using an automated real-time PCR system followed by plating in selective chromogenic media and latex agglutination test for confirmation (Figure 1). Furthermore, the study explores the method's robustness by testing its efficacy with meat and bone meal, a challenging matrix not tested in the original validation, known for its high levels of qPCR inhibitors and background flora that may hinder pathogen recovery.

Methods

Bacterial Inoculation and Sample Enrichment Process

Bacterial strains were pre-cultured in Brain Heart Infusion (BHI) at 37 ± 1 ºC for 18-24 hours, then diluted in 1% saline solution to reach the required concentrations.

Sixty-three meat and bone meal samples (25 g), previously confirmed as *Salmonella*-negative, were artificially inoculated with *Escherichia coli* ATCC 25922 (1000 CFU/sample) to serve as an interferent. Then, thirty samples were also co-inoculated with *Salmonella* Typhimurium ATCC 14028 (<10 CFU/sample) to mimic real contamination conditions.

After inoculation, samples were diluted ten-fold in Buffered Peptone Water (BPW) and enriched at 37 ± 1 ºC for 22 ± 2 hours.

Screening protocol using the automated qPCR method.

Aliquots of 700 µL of each enriched sample were transferred to an iQ-Check Deep Well Microplate (Bio-Rad Laboratories, Catalog #3594900) and placed in the iQ-Check Prep Automation System (Bio-Rad Laboratories, Catalog #3594911). The iQ-Check Prep carried out an enzymatic treatment to remove free DNA from samples, using the iQ-Check Free DNA Removal Solution (Bio-Rad Laboratories, Catalog #3594970). Subsequently, bacterial DNA extraction was carried out (Easy I protocol) and qPCR plates were set-up without human manipulation, using the iQ-Check *Salmonella* II Kit (Bio-Rad Laboratories, Catalog #3578123). Real-time PCR reactions were performed by the CFX96 Touch Deep Well System (Bio-Rad Laboratories, Catalog #3600037) using the Fast APF. Results were analyzed automatically by the CFX Manager Industrial Diagnostic Edition Software (Bio-Rad Laboratories, Catalog #3593893).

Confirmation protocol using RAPID'*Salmonella* chromogenic media and Latex test.

Ten positive and ten negative samples were randomly chosen and plated (10 µl) on the surface of RAPID'*Salmonella* (Bio-Rad Laboratories, Catalog #3564705), directly (Short protocol) and after secondary enrichment in RVS broth (41,5 ± 1°C for 22 hr; Double Enrichment protocol), as described in figure 1 (Step 4). Additionally, samples were also plated on XLD for media comparison, integrating the ISO 6579-1:2017 method. All plates were incubated at 37 ± 1°C for 24 ± 2 hr. Typical colonies were picked from each media and confirmed using *Salmonella* latex test (Bio-Rad Laboratories, Catalog #3556710).

Results were analyzed considering sensitivity, specificity, precision, false positive and false negative rate, according to DOC-CGCRE-089 (2017)[2] from the INMETRO General Accreditation Coordination.

Results

The combined solution of qPCR and chromogenic media proved capable of detecting the pathogen in all inoculated samples, bringing a time to results saving of at least 2 and 3 days for negative and positive samples, respectively, in comparison to the ISO 6579-1:2017 reference method. The results obtained with the alternative method demonstrated that, even analyzing a challenging matrix as meat and bone meal, no qPCR or bacteria growth inhibitions were found, and the tests showed sensitivity, specificity, and precision of 100%. Additionally, no false positive or false negative results were identified with the proposed solution. Further, the statistical entity Kappa shows that there is a excellent agreement between the results obtained by the alternative method and the reference method (Table 1).

Table 1. Results of the sensitivity study between the reference and each step of the alternative method tested.

Isolation step	PA	NA	PD	ND	Kappa	SE_{ref}	SE_{alt}	SP_{alt}	PR_{alt}
RAPID'*Salmonella* - Short protocol	100%	100%	0%	0%	1.0	100%	100%	100%	100%
RAPID'*Salmonella* - Double enrichment protocol	100%	100%	0%	0%	1.0	100%	100%	100%	100%

PA	NA	PD	ND	Kappa	SE_{ref}	SE_{alt}	SP_{alt}	PR_{alt}
Positive agreement	Negative agreement	Positive deviation	Negative deviation	Degree of agreement between the alternative method and the reference method.	Sensitivity for the reference method	Sensitivity for the alternative method (Considering screening, isolation and confirmation steps)	Specificity for the alternative method (Considering screening, isolation and confirmation steps)	Precision for the alternative method (Considering screening, isolation and confirmation steps)

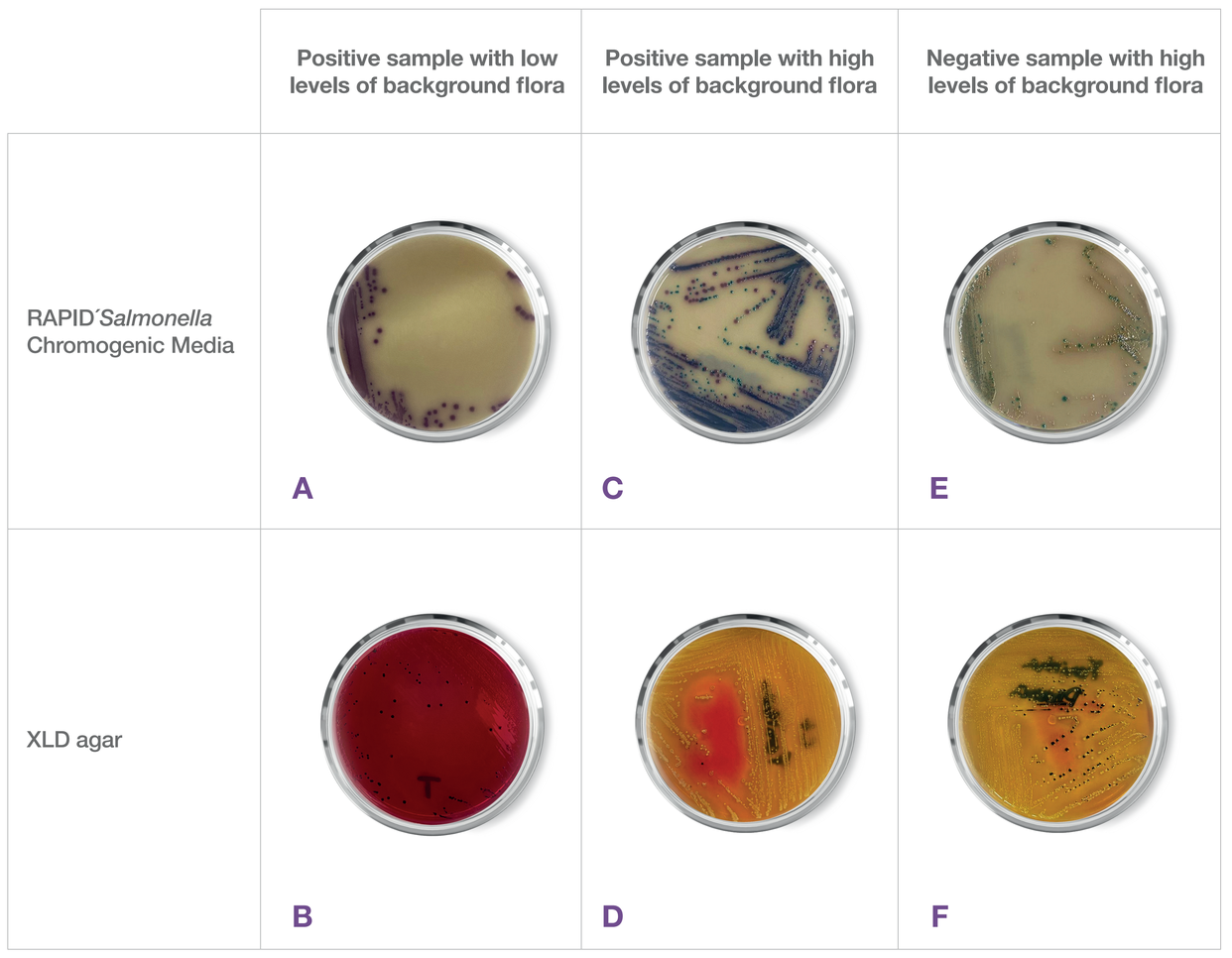
The iQ-Check PREP System performed all steps of PCR plate preparation without human intervention, representing a traceability and cross-contamination prevention tool.

Both isolation protocols, Short and Double enrichment, tested with the RAPID'*Salmonella* chromogenic medium allowed the target recovery in all inoculated samples. On RAPID'*Salmonella* chromogenic medium, *Salmonella* spp. forms distinct, easily identifiable magenta colonies, attributed to the activity of a C8 esterase enzyme. Both media tested, traditional and chromogenic, demonstrated satisfactory results for pathogen recovery in positive samples with low levels of background flora (Figures 2A and 2B). However, *Salmonella* recovery in positive samples with high background flora populations was higher on RAPID'*Salmonella* compared to XLD agar (Figures 2C and 2D). Additionally, XLD agar presented a presumptive positive result confirmed as negative by ISO 6579-1 method and *Salmonella* latex test (Figures 2E and 2F). This may be attributed to other *Enterobacteriaceae* producing H_2S, resulting in black colonies that resemble *Salmonella* on XLD agar.

Salmonella latex test has proven to be a fast, easy-to-use and effective alternative to biochemical and serological tests for confirming *Salmonella* of groups B to E and G, providing results in up to 1 min.

Figure 1. Comprehensive workflow for detection and confirmation of *Salmonella* spp., comprising enrichment in BPW (Step 1), automated qPCR method for screening (Steps 2 and 3), bacteria isolation using RAPID'*Salmonella* Chromogenic Media (Step 4) and *Salmonella* Latex test for confirmation (Step 5).

Figure 2. Comparison between the RAPID'*Salmonella* and XLD agar on the *Salmonella* Typhimurium ATCC 14028 recovery rates from different samples.



Conclusions

This study demonstrated the effectiveness of the complete solution for *Salmonella* detection in less than 48 hr. The power of automation associated with the sensitivity of qPCR and the selectivity of the chromogenic media proves to be a potent tool for industrial laboratories, shortening time to results and operational steps, and improving the laboratory profitability as well as analysis reliability.

References

[1]ISO 6579-1:2017 Microbiology of the food chain – Horizontal method for the detection, enumeration and serotyping of *Salmonella* – Pat 1: Detection of *Salmonella* spp.

[2]DOQ-CGCRE-089:2017 INMETRO General Accreditation Coordination. Guidelines on evaluation of analytical methods performance – Microbiology.

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