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Comparison of ISO 6579–1, VIDAS Easy SLM, and SureFast® Salmonella ONE Real-time PCR, for *Salmonella* Detection in Different Groups of Foodstuffs

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Abstract
In the European Union (EU), *Salmonella* was the causative agent responsible for almost one in three (30.7%) of all foodborne outbreaks reported by member states during 2018, causing 11,581 cases of illness, which represented an increase of 20.6% compared to 2017. Considering the importance of this foodborne zoonotic bacterium in food safety and human health, several strategies for the control and consequent detection of *Salmonella* in foodstuffs are continuously being developed. In this study, we have tested 137 food samples (78 potentially naturally contaminated, 21 artificially contaminated with high levels of *Salmonella*, and 38 artificially contaminated with low levels of *Salmonella*) in order to compare the results and performance of three *Salmonella* detection methods: standard conventional culture (ISO 6579–1), SureFast® Salmonella ONE real-time PCR, and VIDAS® (Vitek Immunodiagnostic Assay System) Easy SLM, an Enzyme Linked Fluorescent Assay (ELFA). Although SureFast® Salmonella ONE real-time PCR was the fastest, it showed more inconclusive results, due to PCR inhibition and false positive results. ISO and VIDAS® protocols gave identical results and proved to be more robust than SureFast® Salmonella ONE real-time PCR when testing different food matrices, despite its longer response times. SureFast® Salmonella ONE real-time PCR may be appropriate to be used when the objective is to test food matrices that are known not to interfere with PCR and expected to be negative for *Salmonella*. All the analytical tested methods have advantages and limitations and thus, depending on the situation, may be used as the elected method for *Salmonella* detection in foodstuffs in accordance with the purpose of the laboratorial analysis.

Keywords Foodstuffs · *Salmonella* detection · Conventional and alternative methods · Food safety

Introduction

Salmonella is a flagellated, rod-shaped, Gram-negative, facultative anaerobe, belonging to the *Enterobacteriaceae* family, which can be harbored by different animal species, and also by humans. *Salmonella* genus includes one of the most common zoonotic foodborne pathogens causing morbidity, mortality, and burden of disease in all regions of the world (Jajere 2019; Newell et al. 2010; Eguale 2018; Zishiri et al. 2016; Ed-dra et al. 2017; Gal-Mor 2019).

The presence, in foodstuffs, of pathogenic bacteria capable of causing food-borne diseases remains a significant public health concern for consumers worldwide, with economic consequences for the producers and the industry, challenging a multisectorial One Health approach for the timely implementation of preventive measures (Hussain and Dawson 2013).

In the EU, salmonellosis was the second most common zoonotic disease, after campylobacteriosis, being *Salmonella* a common etiologic agent in foodborne disease outbreaks. In 2018, 91,857 human salmonellosis confirmed cases were reported by EU member states resulting in a notification rate of 20.1 cases per 100,000 population.

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The highest notification rates were reported by the Czech Republic and Slovakia (> 50 cases/100,000 population), while the lowest rates were found in Greece, Italy, Portugal, Netherlands, Ireland, Bulgaria, and Romania (< 10.0 cases per 100,000 population) (EFSA 2019). In fact, in 2018, *Salmonella* was the causative agent identified in a total of 1581 food-borne outbreaks (FBO) affecting 11,581 people in 24 member states. Most of the *Salmonella* outbreaks were caused by *S. enteritidis*, frequently associated to the consumption of "Eggs and egg products" followed by "Bakery products" and "Mixed food" (EFSA 2019).

The gold standard strategy for *Salmonella* detection in foods still relies in the use of conventional microbiological culturing techniques that employ the use of sequential enrichments with increasing selectivity, ending in the isolation of *Salmonella* on selective-differential agar plates. Confirmation is usually based on biochemical testing of sugar and other nutrient utilization or performed by mass spectrometry (Bell et al. 2016). Although this method is sensitive and inexpensive, it is labor intensive, making it unsuitable for multi-sample analysis, and the time to result may be too long, especially for fresh food (Bell et al. 2016).

To overcome these weaknesses, research is focusing on the development of rapid, sensitive, and specific user-friendly methodologies, appropriate for multi-sample analysis.

Several immunological methods, which rely on antigen–antibody binding, have been developed for the detection of *Salmonella* (Valdivieso-Garcia et al. 2003, Schneid et al. 2006, Magliulo et al. 2007, Cho et al. 2014, Wang et al. 2015). The targets of these assays are either the whole bacterial cells or specific cellular components, like lipopolysaccharides, or other biomolecules of bacterial outer membrane. Despite a shorter assay time compared to traditional culture techniques, antibody-based detection still potentially presents some problems like low sensitivity, low affinity of the antibody to the pathogen, and potential interference of contaminants (Velusamy et al. 2010, Paniel and Noguer 2019).

Furthermore, advances in biotechnology have led to the development of diverse nucleic acid-based methods for *Salmonella* detection, such as polymerase chain reaction (PCR) (Heymans et al. 2018, Barrere et al. 2020, Sahu et al. 2019). In PCR-based approaches, detection depends on the amplification of a target sequence that can be amplified 1 million-fold in less than an hour. PCR-based methods have shown better specificity, higher sensitivity, shorter analysis time, and better accuracy. PCR has been the base for pathogen detection systems using nucleic acids and is often utilized to improve the sensitivity of nucleic acid-based assays.

More recently, new technologies like biosensors have also been developed as alternatives for routine laboratories (Cinti et al. 2017, Velusamy et al. 2010, Paniel and Noguer 2019).

In this study, we compared the performance of the conventional microbiological culturing techniques according to International standard ISO 6579–1: 2017 with two other methodologies used for the detection of *Salmonella* spp.: a real-time PCR-based method (SureFast® Salmonella ONE real-time PCR) and an automated system for the detection of *Salmonella* antigens, based on the ELFA technology (VIDAS® Easy SLM System) (Crowley et al. 2011).

Materials and Methods

Sample Description

One hundred and thirty-seven (137) food samples, divided into seven different categories (raw meats, ready-to-eat foods, seafood, spices, cocoa and derivatives, egg products, and dairy products) were selected and analyzed. Convenience sampling was performed. Samples were purchased in different local supermarkets or collected in canteens as part of the official control carried out by the laboratory. Samples were transported to, and stored in, the laboratory, at room temperature, or refrigerated when adequate and analyzed within a maximum of 24 h after collection.

From the 137 food samples, 78 were tested as potentially naturally contaminated samples, and 59 were artificially contaminated with *Salmonella*—21 at a high level (> 10^7 CFU (colony-forming units)/25 g of sample) and 38 at a low level (< 120 CFU/25 g of sample). Several *Salmonella* serovars (*S.* Nottingham, *S.* Typhimurium, *S.* Abony, *S.* Enteritidis, *S.* Essen and *S.* Goldcoast) preserved as ISO 17,034 certified Lenticules® (PHE, London, UK) or reference stock cultures (on Tryptic Soy Broth (TSB-Biokar Diagnostics, Allonne, France) supplemented with 20% volume fraction glycerol and prepared in accordance with ISO 11,133:2014) were used for artificial contamination of the samples.

As positive controls, three samples from the External Quality Assessment (EQA—PHE, London, UK) containing *S.* Essen, *S. Typhimurium*, and *S. Enteritidis*, respectively, and two samples of buffered peptone water (BPW—Oxoid Ltd., Cheshire, UK), one contaminated with *S.* Goldcoast (13 CFU) and the other with *S. Typhimurium* (30–120 CFU) certified Lenticules®, were used. Sterile BPW was used as negative control.

Preparation of Food Samples and Non-selective Pre-enrichment Procedure

Sample preparation and non-selective pre-enrichment were performed as described in ISO 6579–1: 2017 protocol: 225 mL of BPW in a sterile plastic bag was homogenized with 25 g of food sample and stomached for 1 min (Lab Blender, model 4001, Seward Medical, London, UK). The

mixture was incubated at 37 ± 1 ºC, for 18 ± 2 h. These steps were common to all tested methods.

In the case of dehydrated and low a_w products like oregano, cinnamon, cocoa, and clove, samples were also prepared in accordance with ISO 6887–4: 2017. Thus, specific and suitable dilution ratios were made: for oregano and cinnamon 1:100 (w/v) on 0.1% sterile BPW; for clove 1:100 and 1:1000 (w/v) on 0.1% sterile BPW and regarding cocoa, the pre-enrichment solution was performed with milk (1:10 w/v), instead of BPW.

For high contaminated samples, one *Salmonella* pure colony (obtained by streaking an aliquot of a reference stock culture of *Salmonella* on tryptic soy agar (TSA—Biokar Diagnostics, Allonne, France) and incubating at 37 ± 1 ºC, overnight) or 0.1 ml of a brain heart infusion (BHI—Biokar Diagnostics, Allonne, France) culture previously inoculated with one *Salmonella* isolated colony and incubated at 37 ± 1 ºC for 24 ± 2 h, was added at this stage (> 10^7 CFU/25 g of sample).

For low contaminated samples, one *Salmonella* Lenticule® *disc* or 0.1 ml of a 10^{-7} or 10^{-8} dilution of a BHI culture previously inoculated with one *Salmonella* isolated colony (and incubated at 37 ± 1 ºC for 24 ± 2 h) was added at this step. The contamination level of low-level samples was between < 5 and 120 CFU (Table 2; Supplementary Table 1). The number of CFU was evaluated by counting the number of isolated colonies after platting serial dilutions of *Salmonella* BHI culture on tryptic soy agar (TSA) and incubating at 37 ± 1 ºC for 24 ± 2 h. The reported CFU for each of the artificial contaminations was the ones added to the 25 g of sample, before the non-selective pre-enrichment.

EQA freeze-dried samples were reconstituted by following the manufacturer's instructions: 200 mL of BPW (prewarmed to 30 ± 2 ºC) was used to hydrate these samples, and 25 mL of the reconstituted samples was used, as described above for 25 g of sample.

The obtained mixtures were homogenized in a Stomacher for 1 min. All samples (potentially naturally contaminated, artificially high contaminated, artificially low contaminated, and controls) were incubated at 37 ± 1 ºC, for 18 ± 2 h.

Tested Methods

After the pre-enrichment step, *Salmonella* detection was evaluated by the three different methods described below. Figure 1 presents a comparative diagram of the tested methods.

SureFast® Salmonella ONE Real-Time PCR

Detection of *Salmonella* spp. using SureFast Salmonella® ONE kit (R-Biopharm AG, Darmstadt, Germany) was carried out in accordance with the manufacturer's manual. This method is validated by MICROVAL (License nº 2014LR43) and by the AOAC Research Institute (certificate nº 081,803), against the reference method ISO 6579.

DNA preparation: 500 µl of the upper third of the non-selective pre-enrichment cultures, or of incubated sterile BPW (extraction control), was transferred to 2 ml tubes and 500 µl of the kit lysis buffer was added. After vortexing, the tubes were incubated at 95 ± 1 ºC, for 10 min, in a heating block (bioSan- T5-100C, bioSan, Riga, Latvia). After 1 min at room temperature, 5 µl of the upper part of each lysate was used as DNA template on the PCR reactions.

Preparation of the real-time PCR mix: Each PCR tube contained 19.3 µl of the kit reaction mix plus 0.7 µl of Taq DNA polymerase (Master-mix) plus 5 µl of DNA template. In addition to the extraction control, a real-time PCR negative control (where 5 µl of nuclease free, molecular grade water, was added to the Master-mix) and a positive control (where 5 µl of the kit positive control was added to the Master-mix) were used. The Master mix included an internal amplification control for each reaction, to check for potential PCR inhibition.

Reaction mixtures were subjected to amplification in a RIDA® CYCLER multiplex real-time PCR thermocycler (R- Biopharm AG, Darmstadt, Germany), starting with an initial denaturation step of 1 min, at 95 ºC, followed by 45 cycles, each involving a denaturation step of 15 s, at 95 ºC, and one annealing/extension step of 30 s, at 60 ºC.

Interpretation of results: A sample was stated *Salmonella* positive when showing an amplification in the detection system (FAM channel). A sample was stated negative when showing no amplification in the detection system and presenting an amplification of the internal control (VIC/HEX channel) with a shift in Cq value ≤ 2 compared with the negative control. A result was stated as inconclusive when showing no amplification in the detection system and presenting no amplification of the internal control (or presenting an amplification of the internal control with a shift in Cq value > 2 compared with the negative control).

VIDAS® Easy SLM System

VIDAS Easy SLM (bioMérieux, Marcy L'Etoile, France) is an AFNOR validated method (BIO-12/16–09/05) and is performed after a non-selective pre-enrichment step. From each BPW suspension, 100 µl was transferred into 10 ml of Salmonella Xpress 2 (SX2) broth (bioMérieux, Marcy L'Etoile, France) and incubated at 41.5 ± 1 ºC, for 24 ± 2 h. After incubation, 0.5 ml of the SX2 broth was transferred into the sample well of the VIDAS SLM reagent strip, heated for 15 min, at 95–100 ºC, and after 10 min at room temperature analyzed according to the manufacturer's instructions. A Test Value Range VT ≥ 0.23 was considered a presumptive positive result. The results were automatically obtained

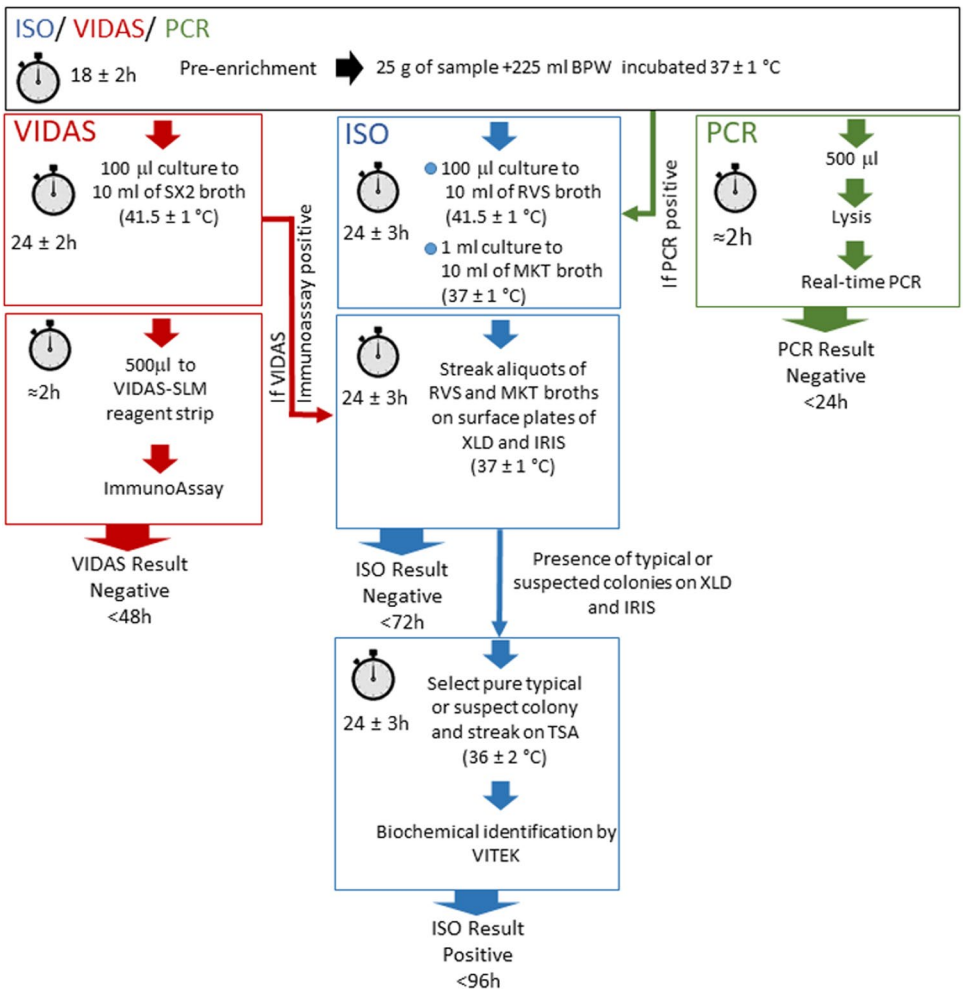

Fig. 1 Procedure for *Salmonella* detection — comparative diagram of the tested methods. IRIS - IRIS Salmonella agar, MKT - Muller-Kauffmann tetrathionate, RVS - Rappaport–Vassiliadis, SX2 - Salmonella Xpress 2, TSA - Tryptic soy agar, XLD - Xylose lysine deoxycholate agar

after 45 min and expressed as presence (positive) or absence (negative) of *Salmonella* in 25 g of sample.

Furthermore, when samples were positive in VIDAS (test value ≥ 0.23), a drop of SX2 broth was streaked on xylose lysine deoxycholate (XLD) agar (bioMérieux, Marcy L'Etoile, France) and another one on IRIS Salmonella agar (BIOKAR Diagnostics, Allonne, France) and incubated at 37 ± 1 ºC, for 24 ± 3 h. Both media are selective and differential for the isolation of *Salmonella* spp.

In XLD agar, typical *Salmonella* strains are lactose and sucrose negative, and lysine decarboxylase positive, producing red colonies with or without black centers. IRIS Salmonella agar shows a high specificity for the detection of *Salmonella* including atypical species and serovars, ensuring reliable detection of lactose- and sucrose-positive strains. A contrast between clear agar and pink-magenta *Salmonella* colonies and the secondary flora, which appears in blue to dark blue, is also evinced. When presenting *Salmonella* characteristics, these colonies were confirmed by biochemical identification on VITEK 2 compact system (bioMérieux, Marcy L'Etoile, France).

ISO 6579–1: 2017

After the incubation of non-selective pre-enrichment, 0.1 ml and 1 ml of the BPW suspension were transferred and mixed with 10 ml of Rappaport–Vassiliadis medium with Soya (RVS) broth (BIOKAR Diagnostics, Allonne, France), and 10 ml of Muller-Kauffmann tetrathionate (MKT) broth (BIOKAR Diagnostics, Allonne, France), respectively. Cultures were incubated during 24 ± 3 h, at 41.5 ± 1 ºC for RVS broth and at 37 ± 1 ºC for MKT broth.

Finally, after incubation of the selective enrichment broths, one drop of each enriched samples was streaked on XLD agar and on IRIS Salmonella agar and incubated at 37 ± 1 ºC, for 24 ± 3 h. The obtained *Salmonella* culture suspected colonies were isolated on tryptic soy agar (TSA), incubated at 36 ± 2 ºC, for 24 ± 3 h, and, when presenting *Salmonella* characteristics, were confirmed by biochemical identification on VITEK 2 compact system.

Results and Discussion

Comparison of the Methods Results

In this work, the results obtained for *Salmonella* spp. detection, by both VIDAS® Easy-SLM assay test and conventional culture using ISO 6579–1, were in agreement, for the total analyzed samples—potentially naturally contaminated and artificially contaminated (with low level and high level of *Salmonella*) (Table 1).

Comparison of VIDAS® Easy SLM and ISO 6579–1 outcomes, when analyzing the results obtained for potentially contaminated samples, shows that 3 out of the 78 tested samples (3.8%) gave positive results, and the remaining tested negative.

On the other hand, when analyzing the results obtained for artificially contaminated samples, shows that, except in the case of Cinnamon, where bacterial growth inhibition has occurred (samples 104 and 105, Table 2; Supplementary Table 1), all the other samples have tested positive (36/38 (94.7%) of low contaminated samples and 21/21 (100%) of high contaminated samples), even when the contamination level corresponded only to < 5 CFU (samples 106, 112 and 113; Table 2; Supplementary Table 1). The use of different *Salmonella* serovars for artificial contamination of samples did not affect the expected results (Supplementary Table1), proving that these methods are not serovar-specific.

Cinnamon bacterial growth inhibition is a known phenomenon already reported by others (Hong et al. 2013; Nabavi et al. 2015). Moreover, following the procedures specified in ISO 6887–4: 2017 (preparation of the initial suspension of cinnamon using a 1: 100 (w/v) cinnamon/buffered peptone water (BPW) ratio on non-selective pre-enrichment) allowed to overcome the bacterial growth inhibition effect (Sample 109; Table 2; Supplementary Table 1). However, it is important to remember that a high dilution ratio, at this stage, can eventually debilitate *Salmonella* detection limit and eventually result in a false negative result.

In addition, considering conventional microbiological culturing techniques, according to International standard ISO 6579–1: 2017, as the reference method, none of the two other methodologies used in this study gave false-negative results. Regarding false-positive and inconclusive results, methodologies showed different results for the food samples tested. While, when using VIDAS® Easy SLM, neither false-positive nor inconclusive results were observed; looking at the outcomes of SureFast® Salmonella ONE real-time PCR method, we can see two types of potential problems: false positive and real-time PCR inhibition.

In fact, when looking at PCR outcomes, three out of the 78 potentially naturally contaminated samples (3.8%) gave false-positive results (samples 12, 28 and 29; Table 2; Supplementary Table 1). This may be related with the potential existence of non-viable or viable but non-culturable (VBNC) *Salmonella* cells in the samples, which did not grow during the enrichment step but could be detected by PCR. This hypothesis could have been confirmed by performing the PCR assay before and after the enrichment incubation step. However, the realization of this assay for all the tested samples would involve higher financial costs and would not be an advantage because, like in the case of the VIDAS method, all positive PCR results must always be confirmed by the conventional ISO method. It is important to notice that, contrarily to non-viable *Salmonella* cells, VBNC *Salmonella* cells may retain their pathogenicity, and the resuscitation of these pathogenic cells has become an important field of investigation as a result of the public health implications for both humans and animals (Dong et al. 2020).

Table 1 Comparison of methods results (ISO 6579–1, VIDAS® Easy-SLM and SureFast® Salmonella ONE real-time PCR)

		Salmonella spp. detection results			
Type of samples	**Sample no**		**SureFast® Salmonella ONE (%)**	**VIDAS® Easy-SLM (%)**	**ISO 6579–1 (%)**
Potentially naturally contaminated	78	+	6/78 (7.7)*	3/78 (3.8)	3/78 (3.8)
		–	59/78 (75.6)	75/78 (96.2)	75/78 (96.2)
		?	13/78 (16.7)*	0/78 (0)	0/78 (0)
Low level	38	+	33/38 (86.8)	36/38 (94.7)	36/38 (94.7)
		–	2/38 (5.3)	2/38 (5.3)	2/38 (5.3)
		?	3/38 (7.9)*	0/38 (0)	0/38 (0)
High level	21	+	21/21 (100)	21/21 (100)	21/21 (100)
		–	0/21 (0)	0/21 (0)	0/21 (0)
		?	0/21 (0)	0/21 (0)	0/21 (0)

+positive results, – negative results, ? inconclusive results

*Real-time PCR results were not in accordance with the ones obtained by ISO and VIDAS® methods.

Table 2 Detection of *Salmonella* spp. with different methodologies—results comparison

Identification No	Food products	Matrices	Pre-enrich. ratio	Sample type	Results		
					PCR	**VIDAS**	**ISO**
1–7	Gizzards	Raw meat	1:10	PNC	−	−	−
8–10	Gizzards	Raw meat	1:10	PNC	+	+	+
11, 13–22	Raw vegetable salads	Ready-to-eat	1:10	PNC	−	−	−
12	Raw vegetable salad (tomato, onion, carrot, lettuce, beet)	Ready-to-eat	1:10	PNC	+ (FP)	−	−
23–27, 30–34	Mixed foods (cooked)	Ready-to-eat	1:10	PNC	−	−	−
28/29	Cooked tuna salad/Valencian rice	Ready-to-eat	1:10	PNC	+ (FP)	−	−
35, 36, 41, 42/37–40	Clams/shrimp	Seafood	1:10	PNC	−	−	−
43/44/45/46/48, 49/50/51/54	Red chilies/paprika/cinnamon/mustard/pepper/fennel/nutmeg/curry	Spices	1:10	PNC	−	−	−
47/52/53	Clove/oregano/piri-piri	Spices	1:10	PNC	? (PCR Inh.)	−	−
47A	Clove	Spices	1:10	PCR dil. (1/10)	? (PCR Inh.)	na	na
53A	Piri-piri	Spices	1:10	PCR dil. (1/10)	−	na	na
55	Oregano	Spices	1:100	PNC	? (PCR Inh)	−	−
56	Clove	Spices	1:100	PNC	? (PCR Inh)	−	−
57	Clove	Spices	1:1000	PNC	−	−	−
58	Cinnamon	Spices	1:100	PNC	−	−	−
59, 61	Low-fat cocoa	Cocoa and deriv	1:10	PNC	? (PCR Inh.)	−	−
59A	Low-fat cocoa	Cocoa and deriv	1:10	PCR dil. (1/2)	−	na	na
60	Chocolate powder	Cocoa and deriv	1:10	PNC	−	−	−
62	Low-fat cocoa	Cocoa and deriv	1:10 (in UHT milk)	PNC	? (PCR Inh.)	−	−
63–67	Eggs	Egg products	1:10	PNC	? (PCR Inh.)	−	−
63A–67A	Eggs	Egg products	1:10	PCR dil.(1/2)	? (PCR Inh.)	na	na
68,70	Egg white	Egg products	1:10	PNC	−	−	−
69, 71	Egg yolk	Egg products	1:10	PNC	? (PCR Inh.)	−	−
69A	Egg yolk	Egg products	1:10	PCR dil. (1/2)	? (PCR Inh.)	na	na
72, 73, 76/75/77/74,78	Cow's milk/chocolate milk/goat's milk/cheese	Dairy products	1:10	PNC	−	−	−
79–88	Gizzards	Raw meat	1:10	LOW (30–120 CFU)	+	+	+
89–93/94–99	Raw vegetable salads/mixed foods (cooked)	Ready-to-eat	1:10	LOW (2–99 CFU)	+	+	+
100, 101/102	Clams/shrimps	Seafood	1:10	LOW (9–13 CFU)	+	+	+
103	Shrimps	Seafood	1:10	LOW (99 CFU)	+ (Neg. IC)	+	+
103A	Shrimps	Seafood	1:10	PCR dilution (1/2)	+	na	na
104	Cinnamon	Spices	1:10	LOW (13 CFU)	? (PCR Inh.)	−	−
104A	Cinnamon	Spices		PCR dilution (1/2)	−	−	−
105	Cinnamon	Spices	1:10	LOW (13 CFU)	−	−	−
106	Oregano	Spices	1:100	LOW (< 5 CFU)	? (PCR Inh.)	+	+
107	Clove	Spices	1:100	LOW (7 CFU)	? (PCR Inh.)	+	+
108	Clove	Spices	1:1000	LOW (7 CFU)	+	+	+
109	Cinnamon	Spices	1:100	LOW (7 CFU)	+	+	+
110	Low-fat cocoa	Cocoa and deriv	1:10 (in UHT milk)	LOW (7 CFU)	? (PCR Inh.)	+	+
111	Eggs	Egg products	1:10	LOW (10 CFU)	+ (Neg. IC)	+	+
112	Egg white	Egg products	1:10	LOW (< 5 CFU)	+	+	+
113	Egg yolk	Egg products	1:10	LOW (< 5 CFU)	+ (Neg. IC)	+	+

Table 2 (continued)

Identification No	Food products	Matrices	Pre-enrich. ratio	Sample type	Results		
					PCR	**VIDAS**	**ISO**
114/115/116	Chocolate milk/cow's milk/goat's milk	Dairy products	1:10	LOW (13 CFU)	+	+	+
117–126	Gizzards	Raw meat	1:10	HIGH	+	+	+
127–131/132–134	Raw vegetable salads/ mixed foods (cooked)	Ready-to-eat	1:10	HIGH	+	+	+
135, 136	Clams	Seafood	1:10	HIGH	+	+	+
137	Cinnamon	Spices	1:10	HIGH	+	+	+
138–140	EQA	Standard	na	EQC	+	+	+
141, 142	Lenticule on BPW	Standard	na	Lenticule (13–120 CFU)	+	+	+
	BPW	Control	na	Water	−	−	−
	PCR negative controls	Control	na	Neg. Control	−	na	na
	PCR positive controls	Control	na	Pos. Control	+	na	na

+positive result, −negative result, ? inconclusive result, *na* non-applicable, *FP* false positive, *PCR Inh* PCR inhibition, EQA External Quality Assessment Control, *Cocoa and deriv.* Cocoa and derivatives, *BPW* buffered peptone water, *Neg IC* negative internal control, *PNC* potentially naturally contaminated.

In addition to the occurrence of false-positive PCR results, certain food matrices may contain PCR interferences that potentially affect different steps of the PCR analysis (Schrader et al. 2012). In the present study, this type of interferences and/or inhibitory effects occurred when testing foodstuffs like clove, oregano, piri-piri, low-fat cocoa, eggs, egg yolk, Vietnam shrimp, and cinnamon. In order to try to solve this problem, a dilution of the extracted nucleic acids was made, a widely applied strategy that is known to automatically result in a dilution of PCR inhibitors (Schrader et al. 2012; Scipioni et al. 2008; Widjojoatmodjo et al. 1992). However, for applications involving heavily degraded or low-copy templates, this strategy may be undesirable due to the further reduction of template amounts. In these cases, a more efficient DNA-extraction and purification method may be required. Moreover, different results might have been obtained if we had used different PCR kits (Ali et al. 2017).

In this study, inhibition of PCR was overcome by diluting the lysis extract, in the case of piri-piri (1:10 dilution, sample 53A), low-fat cocoa (1:2 dilution, sample 59A), Vietnam shrimp (1:2 dilution, sample 103A) and cinnamon (1:2 dilution, sample 104A) samples (Table 2; Supplementary Table 1). In some cases, PCR inhibition was also solved following the procedures specified in ISO 6887–4: 2017 for the pre-enrichment initial suspension during preparation step. Regarding clove, for instance, neither the dilution of the lysis extract (1:10) before PCR reaction (sample 47A) nor the use of a 1:100 (w/v) sample/BPW ratio on pre-enrichment step (samples 56 and 107) solved PCR inhibition; however, when using a 1:1000 dilution ratio on pre-enrichment (samples 57 and 108), this interference was removed (Table 2; Supplementary Table 1). On the other hand, in the case of low-fat cocoa, the use of 1:10 (w/v) sample/UHT milk ratio on pre-enrichment did not solve the inhibition problem because both internal standard and *Salmonella* PCR reactions were inhibited (samples 62 and 110). In this study, PCR inhibition could not be solved for eggs (samples 63, 64, 65, 66, 67, 111) and oregano (samples 52, 55, 106) (Table 2; Supplementary Table 1).

Taking into account that egg yolk and the egg white differ significantly in composition, and eggs are the most important food vehicle involved in EU *Salmonella* foodborne outbreaks (FBO), we have decided to evaluate the inhibitory effect of egg white and egg yolk on PCR amplification, concluding that egg yolk matrix (samples 69, 71, 113) contain more PCR inhibitors than egg white (samples 68, 70, 112) (Table 2; Supplementary Table 1). These results are in accordance with those previously obtained by Xiaohua He et al. (2007).

It is important to notice that, although internal standard PCR inhibition occurred in shrimp and egg products, when artificially contaminated with *Salmonella*, the presence of this microorganism was detected by the specific PCR reaction (samples 103, 111, 113; Table 2; Supplementary Table 1).

Comparative Evaluation of the Methods

In the last decade, the growing concern both by the food industry and the consumers about food safety and the economic impact of foodborne diseases has driven the urgent development of new alternative methodologies that are more rapid, sensitive, and user-friendly, especially for the detection of pathogens like *Salmonella* in foodstuffs.

In this study, the methods VIDAS® Easy-SLM and SureFast Salmonella One real-time PCR used in parallel with the reference ISO method were tested as alternative technologies.

Figure 1 shows the comparison between the times required to produce a result (positive for *Salmonella* spp. — detected or negative for *Salmonella* spp. — not detected) for the 3 different methods used in this study.

The results obtained in our study showed that if the sample to be tested is negative for *Salmonella*, SureFast Salmonella One real-time PCR is the fastest method, with a shorter detection time < 24 h, followed by VIDAS method < 48 h and ISO 6579–1 with a total elapsed time < 72 h. However, because the gold standard strategy for the detection of *Salmonella* spp. still recommends following ISO methodology, when a positive result is obtained by PCR or VIDAS methods, confirmation is imperative and only occurs on the fourth day (< 96 h).

Although among the three tested *Salmonella* detection methodologies, ISO standard is the most laborious and time consuming, it was also the less expensive since VIDAS and SureFast Salmonella One real-time PCR require specific highly expensive equipment and consumables.

Moreover, SureFast Salmonella One real-time PCR is highly susceptible to contamination. Considering that several food samples may be tested at the same time and on the same plate, if a contaminant is introduced in an earlier step, the error may be amplified and lead to false or inaccurate results. In order to avoid PCR contamination, it is necessary to create a proper laboratory environment with separated rooms for each PCR reaction step (or with PCR hoods to create the needed separation), to establish a unidirectional workflow and to use proper materials specific to sensitive PCR applications.

The cost calculation regarding the different methods is highly dependent on the laboratory setups and sample numbers, and, especially, if considering other factors, like hands-on time and storage, this calculation might change. Also, particularly in the case of PCR approaches, successful cost-saving pooling strategies have been implemented in industries where prevalence of positive samples is low (Witte et al. 2020).

Conclusions

Regarding the diversity of alternative methods for *Salmonella* detection developed and improved in the last 20 years, the election of a specific methodology will be dependent on the specific situation and aim of the microbiological analysis by the foods testing laboratories. Considering the context of this specific study, we thus suggest that:

- SureFast Salmonella One real-time PCR may be an appropriate methodology for situations where the tested matrices are from the same food category, are known not to interfere with the PCR reaction (or when methods for the removal of PCR inhibitors, or for the reduction of their effects, are well known), and the majority of the samples is expected to test negative for *Salmonella*. This is the case of screening the presence of *Salmonella* in food industries that produce always the same type of food products
- VIDAS™ may be adequate to test a large diversity of foods and when is expected that most of the results be negative. This may be the case, for instance, in a food safety laboratory that examines ready-to-eat foods
- ISO 6579 standard must be used: (i) when requested by the entity that requires the food analysis; (ii) for the food categories included in the European Commission (EC) Regulation No 2073/2005 *on microbiological criteria for foodstuffs* as amended, whenever is not implemented an equivalent validated method; or (iii) as the confirmation method for the presumptive positive results obtained by any of the other elected alternative methodologies

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Author Contribution Furtado R, Coelho A, Morais M, and Batista R designed the project, performed most of the experimental work and data analyses; Batista R wrote the manuscript; Leitão AL, Saraiva M, and Correia CB had an advisory role in the project design and helped with the data analysis and manuscript writing. All the authors have read and contributed to the manuscript.

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Code Availability Not applicable.

Declarations

Ethics Approval Not applicable.

Consent to Participate All authors have agreed to participate in this study.

Consent for Publication All authors have read and agreed with the final version of the manuscript.

Conflict of Interest Rosália Furtado declares that she has no conflict of interest. Anabela Coelho declares that she has no conflict of interest.

Marisa Morais declares that she has no conflict of interest. Ana Lúcia Leitão declares that she has no conflict of interest. Margarida Saraiva declares that she has no conflict of interest. Cristina Belo Correia declares that she has no conflict of interest. Rita Batista declares that she has no conflict of interest.

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