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Short communication

Fast and discriminative CoSYPS detection system of viable *Salmonella* spp. and *Listeria* spp. in carcass swab samples

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ABSTRACT

In this study, the complete CoSYPS Path Food workflow including all steps, namely swab sample enrichment, SYBR®Green qPCR detection of *Salmonella* spp. and *Listeria* spp., isolation and confirmation of the detected strain, was validated on beef carcass swabs. To perform the validation, the results of the complete workflow were compared, according to the ISO 16140:2003, with the ISO reference methods for detection, isolation and confirmation of *Listeria monocytogenes* and *Salmonella* spp. The results showed that the relative level of detection and the limit of detection of the complete workflow and ISO reference methods are in a range from 2 to 16 CFU/swab for both bacteria. The relative specificity, sensitivity and accuracy identified during this validation were all 100% since the results obtained with the complete CoSYPS Path Food workflow and the ISO reference methods were identical (Cohen's kappa index = 1.00). In addition the complete CoSYPS Path Food workflow is able to provide detection results (negative or presumptive positive) in half the time needed as for the ISO reference methods. These results demonstrate that the performance of the complete CoSYPS Path Food workflow is not only comparable to the ISO reference methods but also provides a faster response for the verification of beef carcasses before commercial distribution.

1. Introduction

Worldwide there are annually 1.3 billion cases of human gastroenteritis due to *Salmonella* spp. (Bhunia, 2008b). In European Union (EU), *Salmonella* is the first notification cause of microbial foodborne contamination (Commission of the European Union, 2012), and the main reported causative agent in foodborne outbreaks (EFSA and ECDC, 2014). The reservoirs are mainly poultry, but also cattle, swine and sheep (Pui et al., 2011). Human salmonellosis is mainly caused by contaminated food consumption (EFSA and ECDC, 2014).

Listeria monocytogenes has a low annual incidence worldwide. About 1500 and 2500 cases per year are recorded in EU and in the USA, respectively (Bhunia, 2008a; EFSA and ECDC, 2014). However, because of its high mortality rate (between 20 and 30%), listeriosis ranks among the most frequent human death causes due to foodborne illnesses in the USA and EU (Barton et al., 2011; EFSA and ECDC, 2014). *Listeria* spp. principal reservoirs are soil, forage, water and farm animals' intestinal tract (cattle, sheep, goats, etc.) (Bhunia, 2008a). The main transmission route to humans is contaminated food consumption (EFSA and ECDC, 2014).

As foodborne pathogen reservoirs are mainly farm animals, foodstuffs from animals are controlled according to the regulation (EC) No. 854/2004 (Commission of the European Union, 2004). Meat is one of the most important foodborne pathogen vehicles (Commission of the European Union, 2005). Meat is usually contaminated on the surface during the slaughter process by faecal contamination during evisceration (FSA, 2002). The meat contamination by foodborne pathogens is assessed by carcasses monitoring at slaughterhouse. Carcasses sampling can be performed by destructive (excision or drilling) or by non-destructive methods (swabbing). The latter presents the advantages to be non-destructive and causes no damage to the carcasses (no commercial impact), and it allows the sampling of a large surface (up to 1600 cm^2/carcass (EFSA and ECDC, 2014)). This large surface sampling is an important advantage for detection of low contamination levels (Lindblad, 2007).

The regulation (EC) No. 2073/2005 (EFSA and ECDC, 2014) indicates the official analytical reference methods to detect foodborne pathogens and the microbiological acceptance limits for each food category. For *Salmonella* spp. and *L. monocytogenes* detection, the reference methods are culture-based (ISO, 1996, 2002). Although efficient, these methods

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are time-consuming and labour-intensive, i.e. each target bacterium requires its own protocol and up to 7 days are needed to confirm their presence. This hampers a rapid answer in case of outbreaks where a swift (re-)action is required.

Molecular methods are increasingly accepted as good alternatives since they are fast, sensitive and specific. Up to now, several real-time PCR (qPCR) assays have been developed for detection of *Salmonella* spp. (e.g. Hein et al., 2006; Josefsen et al., 2007; Liming and Bhagwat, 2004; Malorny et al., 2004, 2007; Pasquali et al., 2013; Perelle et al., 2004; Seo et al., 2004; Wang and Mustapha, 2010) and *L. monocytogenes* (e.g. Berrada et al., 2006; Hough et al., 2002; Nogva et al., 2000; O'Grady et al., 2008, 2009; Oravcova et al., 2007; Rossmanith et al., 2006; Rudi et al., 2005) in food products. These systems provide single-genus or single-species detection systems for *Salmonella* spp. and *L. monocytogenes*, respectively. Moreover, they target a single-gene with a single-assay. This could lead to false negative results in case of targeted gene mutation or deletion (Barbau-Piednoir et al., 2013b; Hu et al., 2008). To mitigate these inconveniences, approaches targeting two genes for *Salmonella* spp. detection (Gonzalez-Escalona et al., 2012) or targeting several bacteria at the same time (Garrido et al., 2012a, 2012b; Ma et al., 2014; Köppel et al., 2013; Singh et al., 2012) have been developed. Recently, this strategy was further improved with the Combinatory SYBR®Green qPCR Screening system for pathogen detection in food samples (CoSYPS Path Food), able to detect in a single-step both *Salmonella* spp. and *Listeria* spp., and to give information about species and subspecies detected (Barbau-Piednoir et al., 2013a, 2013b). This system contains several target genes per bacterium to create a multi-level detection system. All SYBR®Green qPCR assays of this CoSYPS Path Food system have been validated (Barbau-Piednoir et al., 2013a, 2013b) and can be used together as a single-plate detection system. This detection system is part of the complete CoSYPS Path Food workflow, studied in the present paper, which includes all steps from swab sample enrichment, DNA extraction, *Salmonella* spp. and *Listeria* spp. qPCR detection, isolation and confirmation of the detected strains.

In order to validate this complete CoSYPS Path Food workflow as an alternative method to the reference ISO methods, a final confirmation is required. The ISO 16140:2003 (ISO, 2003) describes the process to validate an alternative method by comparing it with a reference method. The complete validation is composed of two steps: i) a comparison study of the alternative method versus the reference method carried out in the organizing laboratory, and ii) an inter-laboratory study performed with both methods in parallel. The first part allows alternative method to be used in the laboratory under accreditation. The second part is performed for commercialisation purposes.

In this paper, the first part of the ISO 16140:2003-validation was performed. The results of *Salmonella* spp. and *Listeria* spp. detection in carcass swab samples obtained by the complete CoSYPS Path Food workflow were compared to those obtained with the reference methods: ISO 6579:2002/Cor 1:2004 and ISO 11290-1:1996/Amd.1:2005 (ISO, 1996, 2002, 2004a, 2005). The limits of detection of each method were determined and compared. Different validation criteria such as relative detection level, relative sensitivity, relative specificity, relative accuracy, Cohen kappa index and practicability of both detection methods were investigated. Finally the advantages of using the complete CoSYPS Path Food workflow were discussed.

2. Materials and methods

2.1. Bacterial strains and spiked preparation

Salmonella enterica subsp. *enterica* Enteritidis (H,VI,6,32 from Belgian *Salmonella* NRC) and *L. monocytogenes* serotype 1/2a (ATCC 51772) were used to artificially contaminate the swab samples. A single colony was inoculated in 10 ml of Brain Heart Infusion (BHI) broth and cultured at 37 °C without shaking for 16–18 h. This culture was diluted in sterile BHI broth to get an $OD_{600\ nm}$ at 1 (around 5.10^8 CFU/ml). This dilution called D0 was used as starter in a 10-fold serial dilution until D-9 in buffered peptone water (BPW). To perform the enumeration of D-6 to D-9, 100 µl of these dilutions was plated in triplicate on nutrient agar plates and incubated for 18 ± 2 h at 37 °C. These four dilutions were used to spike the swab samples.

2.2. Spiked carcass swab samples

To create artificial beef carcass swab samples containing the same resident microflora as the genuine beef carcass swab samples, 25 g of minced meat (100% beef) (free of *Salmonella* spp. and *Listeria* spp.) from a retail shop was stomached in 225 ml of BPW medium in a filter stomacher bag giving a "minced meat juice". A BPW-hydrated sponge (swab) introduced into a new filter stomacher bag was soaked with 10 ml of this "minced meat juice". To spike these swabs, 100 µl of D-6 to D-9 was added onto the swab. The spiked swabs were stored for 18 ± 2 h at fridge temperature to mimic the storage and transport steps that are undergone by real swab samples between sampling and analysis (ISO, 2004b).

2.3. Listeria spp. and Salmonella spp. detection, isolation and confirmation methods

The workflow of the procedure followed by all the detection methods is shown in detail in Fig. 1.

2.3.1. Common pre-enrichment step

Spiked swab samples were pre-enriched in 90 ml BPW for 24 ± 2 h at 37 °C without shaking. As requested by ISO16140:2003, the same sample was used for the analysis with the ISO reference methods as well as with the complete CoSYPS Path Food workflow.

2.3.2. ISO reference methods

The reference method used to detect *L. monocytogenes* was the ISO 11290-1:1996 amended by ISO11290-1/A1:2005 (ISO, 1996, 2005). A variation from this protocol was performed. BPW was used instead of Half-Fraser for the pre-enrichment to be able to perform *Salmonella* and *Listeria* detection at the same time. The choice was made to use a single swap sample instead of using two swap samples (respectively enriched by BPW and Half-Fraser) that would introduce a bias in the contamination level. The reference method used for *Salmonella* spp. detection was ISO 6579:2002.

2.3.2.1. Enrichment step. The BPW pre-enrichment broth, after incubation, was used to inoculate a selective Fraser enrichment broth, for *Listeria* detection and Rappaport-Vassiliadis Soja (RVS) and Müller-Kauffmann tetrathionate-novobiocin (MKTTn) selective broths for *Salmonella* detection.

2.3.2.2. Isolation. The isolation was performed by plating out the selective enrichment broth on selective solid media. For *Listeria* spp., the isolation was performed on Agar *Listeria* according to Ottaviani and Agosti (ALOA) and Rapid L'Mono (RLM) agar, while for *Salmonella* spp., the isolation media were Xylose-Lysine-Deoxycholate (XLD) agar and ChromID™ *Salmonella* (SMID) agar.

2.3.2.3. Confirmation. For *Listeria* spp., no confirmation was performed. Indeed, the presence of typical colonies on selective plates was enough to get a positive result as they were spiked samples.

For *Salmonella* spp., one typical colony on each of the selective plates, i.e. two typical colonies were selected. These colonies were biochemically confirmed. In this validation, the expected feature on Hajna-Kligler iron agar (= Triple sugar iron agar (TSI)) was considered as a positive result. As the samples were *Salmonella*-spiked, neither Rapid ID32E nor serological confirmation was performed.

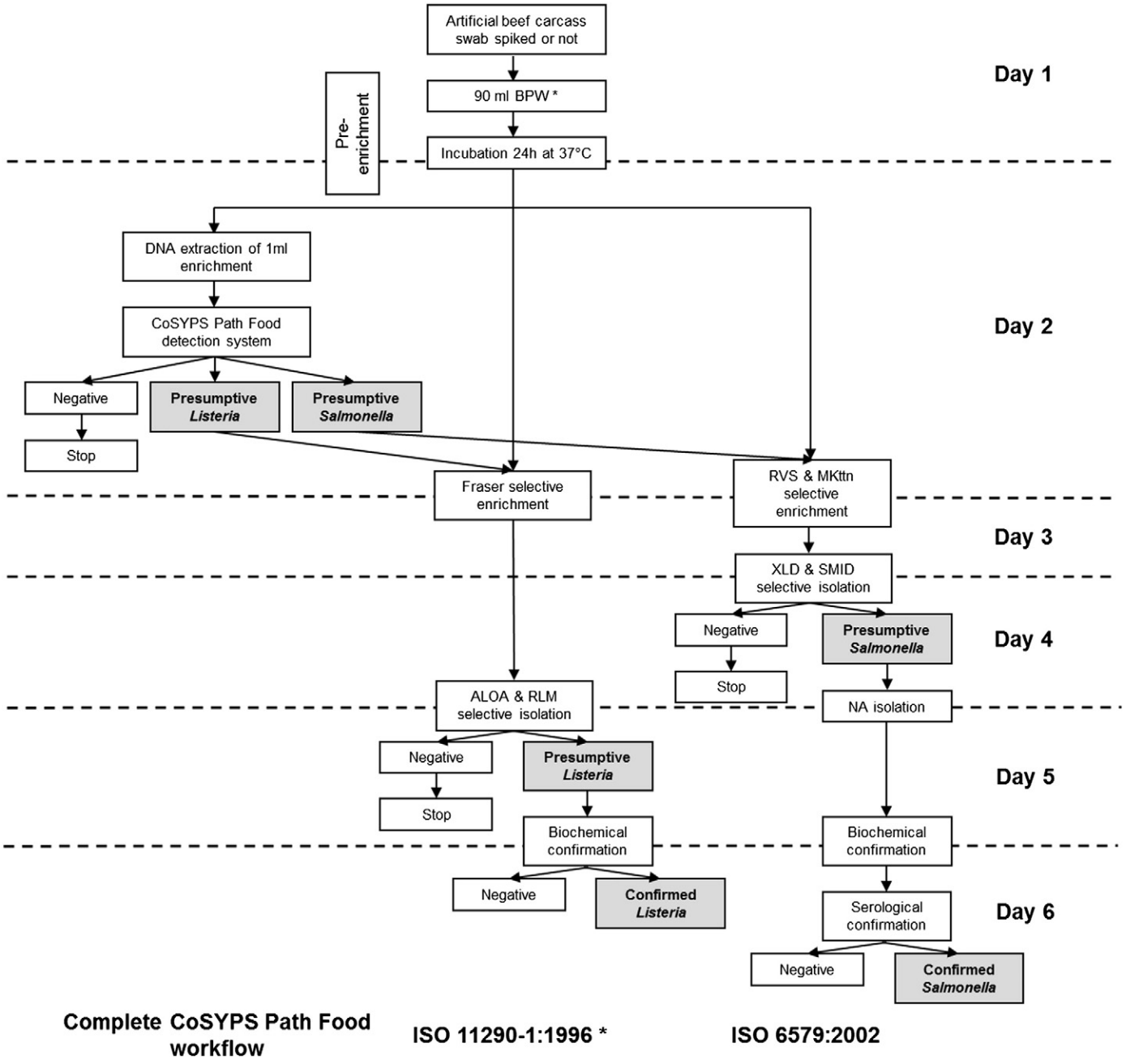

Fig. 1. Flow chart illustrating the different steps of the entire CoSYPS Path Food workflow, the modified (*) ISO 11290-1:1996 and ISO 6579:2002 on beef carcass swab samples. * Half-Frazer pre-enrichment has been replaced by BPW pre-enrichment. Abbreviations are as follows: BPW: Buffer peptone water, RVS: Rappaport-Vassiliadis Soja, MKttn: Müller-Kauffmann tetrationate-novobiocine, XLD: Xylose-Lysine-Deoxycholate, SMID: ChromID™ *Salmonella*, NA: Nutrient Agar, ALOA: Agar *Listeria* according to Ottaviani and Agosti and RLM: Rapid L'Mono.

2.3.3. The complete CoSYPS Path Food workflow

2.3.3.1. DNA extraction. After 24 h of pre-enrichment, 1 ml of the pre-enrichment broth was transferred into a 1.5 ml micro-centrifuge tube, centrifuged for 10 min at 6000 ×*g* at room temperature and the supernatant was discarded. The pellet was extracted with the Nucleospin food kit (Macherey–Nagel®) according to the manufacturer's recommendations.

2.3.3.2. CoSYPS analysis. The *Salmonella* and *Listeria* spp. detection were performed using the CoSYPS Path Food detection system. This system is composed of respectively seven and four SYBR®Green qPCR assays, for the detection of *Salmonella* spp. and *Listeria* spp. and their discrimination at species and sub-species levels (Barbau-Piednoir et al., 2013a, 2013b). These eleven qPCR assays can be run separately or together on the same plate (as eleven singleplex). All qPCR assays were performed on an iCycler iQ™5 Real-Time PCR Detection System (Bio-rad) with iCycler iQ™ PCR plates, 96 wells (Bio-rad) closed with the PCR Sealers Microseal B films (Bio-rad). All qPCR assay reactions were performed according to the same protocol: the reactions were performed in a final volume of 25 µl containing 5 µl of the diluted DNA extract (1/2 for *Listeria* qPCR assays and 1/1000 for *Salmonella* qPCR assays), 1X SYBR®Green PCR Mastermix (DMSG-2X-A300, Diagenode), and the appropriate concentration of each primer (Barbau-Piednoir et al., 2013a, 2013b).

Primers were purchased from Eurogentec (Belgium). The following thermal programme was applied: a single cycle of DNA polymerase activation for 10 min at 95 °C followed by 40 amplification cycles of

15 s at 95 °C (denaturing step) and 1 min at 60 °C (annealing–extension step). Subsequently, melting temperature analysis of the amplification products was performed by gradually increasing the temperature from 60 °C to 95 °C over 20 min (±0.6 °C/20 s). The fluorescent reporter signal was normalized against the internal reference dye (ROX) signal and the threshold limit was set manually at the beginning of the exponential amplification phase. "No Template" Controls (NTC) using DNase and RNase free water were included in each reaction to assess primer dimer formation or non-specific amplification. A positive control using 10^4 copies of gDNA of *L. monocytogenes* 1/2a strain ATCC 51772 or *S. enterica* subsp. *enterica* Enteritidis (Belgian CNR *Salmonella* ref H.V.6.32) from pure strains extracted with the DNeasy® Blood and tissue Extraction kit (Qiagen) was included in each qPCR reaction.

For the interpretation of a SYBR®Green qPCR assay, two criteria were analysed: the quantification cycle (Cq) value, and the melting temperature of the amplicon (Tm). The Cq-value represents the fractional cycle at which the PCR amplification reaches the threshold level for the reaction (Bustin, 2000). Since it is a screening assay, only a qualitative response is required. To be considered as positive, a signal generated in the CoSYPS Path Food detection system should display an (exponential) amplification above the limit of detection of each qPCR determined previously, with the expected Tm-value (Barbau-Piednoir et al., 2013a, 2013b). The combination of positive assays generates the list of bacteria possibly present into the sample (presumptive positive) according to the decision tree presented in Fig. 2.

2.3.3.3. Selective enrichment, isolation and confirmation. The selective enrichment, isolation and the confirmation were performed only if a presumptive positive result was obtained. All these steps were performed as previously described in the ISO reference methods section.

2.4. Validation of the complete CoSYPS Path Food workflow

The complete CoSYPS Path Food workflow was validated for the enrichment, detection, isolation and confirmation of the presence of *Listeria* spp. and *Salmonella* spp. in beef carcass swab samples. The samples were analysed in parallel by the reference ISO methods and the complete CoSYPS Path Food workflow as depicted in Fig. 1. The results obtained with each method were compared using the criteria described in the ISO 16140:2003, the NordVal guidelines and the AFNOR technical board listed thirteen practicability criteria (AFNOR, 2013; ISO, 2003; NordVal, 2009).

According to ISO 16140:2003 at least three levels of contamination should be tested. In this study, four levels were analysed (D-6 to D-9). They were spiked on four different swabs. Four independent analyses of these four swabs were performed for each tested bacteria. As the ISO 16140:2003 requires at least six repetitions, the dilution identified as relative detection level with each individual detection method was re-analysed with six swabs.

This ISO also requires the use of twenty samples to validate a system on a food category, with 50% positive and 50% negative samples. The same samples should be analysed by both the alternative and the reference methods. In this study, twenty samples were prepared presenting different spike concentrations and samples were inoculated with one or both targets (*Salmonella* spp. and *Listeria* spp.) of the complete CoSYPS Path Food workflow (Table 2): i) five samples containing none of the targets were used as negative samples; ii) six samples contained only one of the targets at the LOD, ten times or hundred times above the LOD; iii) one sample contained both targets at the LOD; iv) four samples contained one target at the LOD and the other target ten or hundred times above the LOD; v) two samples contained one target ten times above the LOD and the other target hundred times above the LOD; vi) one sample contained both targets ten times above the LOD and vii) one sample contained both targets hundred times above the LOD.

2.5. Validation criteria

2.5.1. Limit of detection and relative detection level (LOD)

The LOD of each method is defined as the lowest number of microorganisms per assay that is positive in 95% of the occasions (ISO, 2011).

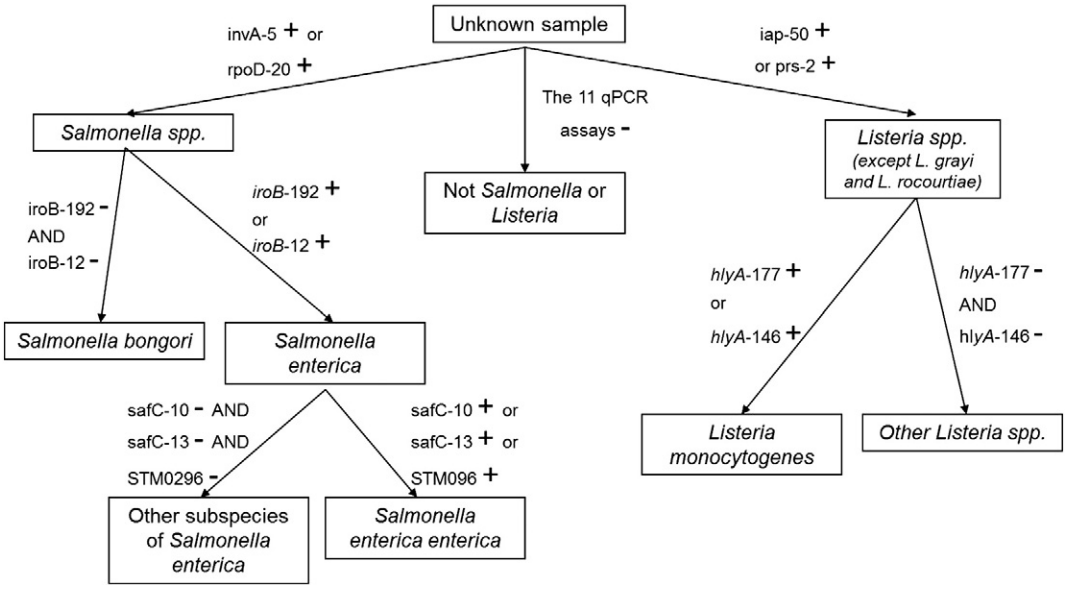

Fig. 2. Decision tree of the entire CoSYPS Path Food workflow. The CoSYPS Path Food detection system is multi-genera (*Listeria* and *Salmonella*) and multi-level (Genus, species and subspecies) SYBR®Green qPCR system. Each level of detection is performed by two qPCR assays: *iap*-50 and *prs*-2 for *Listeria* spp. (except *L. grayi*); *hlyA*-177 and *hlyA*-146 from *L. monocytogenes*; *invA*-5 and *rpoD*-20 for *Salmonella* spp.; *iroB*-192 and *iroB*-12 for *Salmonella enterica*; and *safC*-10, *safC*-13 and STM096 for *Salmonella enterica enterica*. Regarding the positive (+) or negative (−) signal observed with each assay, the presumptively present strain is identified. More details in (Barbau-Piednoir et al., 2013a,b). Abbreviations for the genes are as follows: *iap*: invasion-associated protein, *prs*: phosphoribosylpyrophosphate synthetase, *hlyA*: Listeriolysin O, *invA*: invasine gene, *rpoD*: RNA-polymerase sigma factor, *iroB*: C-glycosyltransferase, *safC*: *Salmonella* atypical fimbriae outer membrane usher protein and STM0296: putative cytoplasmic protein AAL19253.

The relative detection level is the smallest number of culturable micro-organisms that can be detected in the sample in 50% of the occasions by the alternative and reference methods.

2.5.2. Relative sensitivity, relative specificity and relative accuracy

The relative specificity (SP) is the ability of the alternative method to not detect the analyte when it is not detected by the reference method (ISO, 2003). The relative sensitivity (SE) is the ability of the alternative method to detect the analyte when it is detected by the reference method (ISO, 2003). The relative accuracy (AC) is the degree of correspondence between the response obtained by the reference method and the one obtained by the alternative method on identical samples (ISO, 2003). They were determined by comparing results obtained by analysing 20 samples of spiked artificial swabs with the ISO methods (reference methods) and with the complete CoSYPS Path Food workflow (alternative method).

$$SE = \frac{PA \times 100}{PA + ND}$$

$$SP = \frac{NA \times 100}{NA + PD}$$

$$AC = \frac{(PA + NA) \times 100}{PA + NA + PD + ND}.$$

PA is the positive agreement: number of samples that are positive by both reference and alternative methods. NA is the negative agreement: number of samples that are negative by both reference and alternative methods. PD is the positive deviation: number of samples that are negative with the reference method and positive with the alternative method. ND is the negative deviation: number of samples that are positive with the reference method and negative with the alternative results.

2.5.3. Cohen kappa index

The Cohen kappa index (κ), expressing the degree of acceptance between two methods was calculated according to the following formula (Cohen, 1960):

$$\kappa = \frac{p_o - p_c}{1 - p_c}$$

where

$$p_o = \frac{PA + NA}{PA + NA + PD + ND}$$

$$p_c = \frac{[(PA + ND) \times (NA + PD)] + [(PA + PD) \times (NA + ND)]}{(PA + NA + PD + ND)^2}.$$

Cohen kappa values are categorised as follow: ≤0.20 poor agreement, between 0.21 and 0.40 fair agreement, between 0.41 and 0.60 moderate agreement, between 0.61 and 0.80 good agreement, and ≥0.81 very good agreement (Landis and Koch, 1977; NordVal, 2009).

2.5.4. Practicability

AFNOR technical board listed thirteen practicability criteria (AFNOR, 2013). Some of these criteria, judged as relevant by the authors, were evaluated in the present study: the training of the operator, the lab equipment and the time required to get results.

3. Results and discussion

3.1. LOD and the relative level of detection determination

Beef carcass swab samples spiked with different *L. monocytogenes* or *S. enterica* subsp. *enterica* concentrations were analysed in parallel with the ISO reference methods and the complete CoSYPS Path Food workflow. The swabs spiked with the dilutions D-6 and D-7 were positive in the four independent repeats with the complete CoSYPS Path Food workflow as well as with the ISO reference methods. The D-8 gave 50% (2/4 repeats) of positive with ISO 11290-1:1996 and 25% of positive with the ISO 6579:2002 and the complete CoSYPS Path Food workflow. The D-9 gives 25% of positives with all the tested methods. Considering these results, the limit of detection (LOD) of both conventional and CoSYPS methods as well as the relative detection level (RDL) are at dilution −7 (D-7), i.e. between 4 and 16 CFU/swab for *L. monocytogenes* detection and between 2 and 11 CFU/swab for *S. enterica* subsp. *enterica* detection. To confirm these LOD and RDL, six additional swabs spiked with D-7 were analysed. These six additional repeats gave all positive results, confirming both criteria (Table 1). The study demonstrated that the complete CoSYPS Path Food workflow is as efficient as the reference ISO methods to detect low concentration of targets.

3.2. Relative specificity, relative specificity and relative accuracy and Cohen's kappa index determination

Twenty beef carcass swab samples spiked with different *L. monocytogenes* and/or *S. enterica* subsp. *enterica* concentrations were analysed in parallel with the ISO reference methods and the complete CoSYPS Path Food workflow. Each of the swabs spiked with the different concentrations of bacteria gave the expected positive signal (12/12) with both approaches, whereas the non-spiked swabs gave all a negative signal (8/8) (Table 2). This demonstrates that the complete CoSYPS Path Food workflow was able to detect both target bacteria in a tested sample, even when one target was present at very low concentration. This is particularly important for samples containing low *Listeria* spp. numbers. Indeed, *Listeria* spp. grow slower than *Enterobacteriaceae* and its growth could be inhibited by the beef carcass co-resident microflora, and by *Salmonella* spp. in case of double contamination and the pre-enrichment in BPW instead of Half-Fraser is less optimal for *Listeria* spp. growth. However, these results demonstrated that BPW is efficient enough for *Listeria* detection as low as 4–16 cfu/swab with an overnight storage of the swab samples at fridge temperature.

From these results, the positive and negative agreements (PA and NA), the positive and negative deviations (PD and ND) were assessed. For both targets, the PA and the NA result were 12 and 8 respectively while the ND and PD were 0. These values allowed the calculation of a 100% relative sensitivity (SE), 100% relative specificity (SP) and 100% relative accuracy (AC) and a Cohen's kappa index of 1.00 of the results obtained with the complete CoSYPS Path Food workflow compared with the results obtained with the ISO methods (Table 2). These values demonstrated that the complete CoSYPS Path Food workflow is as efficient as the reference methods in detecting *Salmonella* spp. and *L. monocytogenes* in beef carcass swab samples.

3.3. Practicability

To perform the ISO reference methods, as well as the complete CoSYPS Path Food workflow, classical L2 laboratory microbiological equipments are required. In addition, the complete CoSYPS Path Food workflow required qPCR well-trained personnel operating a properly maintained qPCR apparatus.

The ISO 11290-1:1996 and ISO 6579:2002 comprise a pre-enrichment step, a selective enrichment step and isolation on

Table 1
Comparative results of swab spiked with a 10-fold serial dilution of *S.* Enteritidis or *L. monocytogenes* tested in parallel with the ISO reference methods or the CoSYPS Path Food workflow.

		ISO	CoSYPS	ISO	CoSYPS	ISO	CoSYPS	ISO	CoSYPS	ISO	CoSYPS
Listeria detection	Spike: CFU/swab (dilution)	77–150 (D-6)		4–16 (D-7)		0–2 (D-8)		0–1 (D-9)		0 (C-)	
	Repeat 1	+	+	+	+	−	−	−	−	−	−
	Repeat 2	+	+	+	+	+	+	−	−	−	−
	Repeat 3	+	+	+	+	−	−	+	+	−	−
	Repeat 4	+	+	+	+	+	−	−	−	−	−
	Repeat 5	nt	nt	+	+	nt	nt	nt	nt	−	−
	Repeat 6	nt	nt	+	+	nt	nt	nt	nt	−	−
	Repeat 7	nt	nt	+	+	nt	nt	nt	nt	−	−
	Repeat 8	nt	nt	+	+	nt	nt	nt	nt	−	−
	Repeat 9	nt	nt	+	+	nt	nt	nt	nt	−	−
	Repeat 10	nt	nt	+	+	nt	nt	nt	nt	−	−
	Percentage of positive	100	100	100	100	50	25	25	25	0	0
Salmonella detection	Spike: CFU/swab (dilution)	30–60 (D-6)		2–11 (D-7)		0–1 (D-8)		0 (D-9)		0 (C-)	
	Repeat 1	+	+	+	+	−	−	−	−	−	−
	Repeat 2	+	+	+	+	+	+	+[a]	+[a]	−	−
	Repeat 3	+	+	+	+	−	−	−	−	−	−
	Repeat 4	+	+	+	+	−	−	−	−	−	−
	Repeat 5	nt	nt	+	+	nt	nt	nt	nt	−	−
	Repeat 6	nt	nt	+	+	nt	nt	nt	nt	−	−
	Repeat 7	nt	nt	+	+	nt	nt	nt	nt	−	−
	Repeat 8	nt	nt	+	+	nt	nt	nt	nt	−	−
	Repeat 9	nt	nt	+	+	nt	nt	nt	nt	−	−
	Repeat 10	nt	nt	+	+	nt	nt	nt	nt	−	−
	Percentage of positive	100	100	100	100	25	25	25	25	0	0

ISO methods are ISO11290-1** and ISO 6579, for *Listeria* and *Salmonella* detection, respectively.
CoSYPS: complete CoSYPS Path Food workflow.
[a] This positive is not a false positive, it is due to the bias in the spike enumeration, i.e. the spike is evaluated by enumeration of 3 repetitions of the spike (an enumeration of the spike itself is not possible).
** Slightly modified as enrichment is done with BPW instead of Half-Frazer.

selective plates (Fig. 1). These different steps need four and five days for *Salmonella* spp. and *L. monocytogenes* detection, respectively, since each culture step requires an 18 to 24 h of incubation (48 h for Fraser selective enrichment). If no typical colonies are observed on selective plates, the sample is concluded as containing no *Salmonella* spp. or *L. monocytogenes* and the analysis is stopped. If typical colonies are

Table 2
Comparative results of the culture-based reference methods with the CoSYPS *Salmonella* and *Listeria* on the detection in spiked artificial swabs of beef meat.

	Listeria detection comparison				*Salmonella* detection comparison			
	Spiked CFU *Listeria*/swab	CoSYPS *Listeria*	ISO 11290	Comparison	Spiked CFU *Salmonella*/swab	CoSYPS *Salmonella*	ISO 6579	Comparison
Sample 1	0	−	−	=	0	−	−	=
Sample 2	10–19	+	+	=	8–10	+	+	=
Sample 3	121–131	+	+	=	56–74	+	+	=
Sample 4	1105–1555	+	+	=	760–786	+	+	=
Sample 5	1105–1555	+	+	=	0	−	−	=
Sample 6	121–131	+	+	=	8–10	+	+	=
Sample 7	10–19	+	+	=	56–74	+	+	=
Sample 8	0	−	−	=	760–786	+	+	=
Sample 9	121–131	+	+	=	0	−	−	=
Sample 10	1105–1555	+	+	=	8–10	+	+	=
Sample 11	0	−[a]	−	=	56–74	+	+	=
Sample 12	10–19	+	+	=	760–786	+	+	=
Sample 13	10–19	+	+	=	0	−	−	=
Sample 14	0	−	−	=	8–10	+	+	=
Sample 15	1105–1555	+	+	=	56–74	+	+	=
Sample 16	121–131	+	+	=	760–786	+	+	=
Sample 17	0	−	−	=	0	−	−	=
Sample 18	0	−	−	=	0	−	−	=
Sample 19	0	−	−	=	0	−	−	=
Sample 20	0	−	−	=	0	−	−	=
PA	12				12			
NA	8				8			
ND	0				0			
PD	0				0			
SE (%)	100				100			
SP (%)	100				100			
AC (%)	100				100			
Cohen's kappa index	1.00				1.00			
Cohen's kappa conclusion	Very good agreement				Very good agreement			

[a] This sample have to be reextracted since in first extract a false positive was observed; +: positive result; −: negative result; PA: positive agreement; NA: negative agreement; ND: negative deviation; PD: positive deviation; SE: relative sensitivity; SP: relative specificity; and AC: relative accuracy.

observed on the selective plates, it is a presumptive positive result, and further biochemical confirmations are performed, which takes an additional day (Fig. 1). The complete CoSYPS Path Food workflow comprises a pre-enrichment step, followed by a DNA extraction and a CoSYPS detection system (qPCR analysis). These steps can be completed within two days (including an overnight enrichment) as DNA extraction and CoSYPS analysis are easily performed within a single day. Indeed, DNA extraction requires maximum 3 h and the CoSYPS analysis needs around 4 h (preparation, running and result interpretation). If the CoSYPS analysis result is negative, the sample is concluded as containing neither *Salmonella* spp. nor *Listeria* spp. and the analysis is stopped. If the CoSYPS analysis result is positive, it is a presumptive positive. Thus, the CoSYPS Path Food workflow provides a negative or presumptive positive result in half the time needed for the ISO detection methods (two days instead of four/five days). This reduced time is an important advantage, especially in case of outbreaks and for self-control of short-life products. Moreover, for a food business operator a presumptive positive is enough to take action. To be confirmed, a presumptive positive sample must continue the complete workflow with the selective enrichment, isolation on selective plate and confirmation of the isolated strain which require four additional days (Fig. 1). Thus, a confirmed positive result requires the same number of days, i.e., 6 days for *Salmonella* spp. and *Listeria* spp. analysis.

4. Conclusion

This validation study confirms that the complete CoSYPS Path Food workflow is as efficient as the reference methods in detecting *Salmonella* spp. and *L. monocytogenes* in beef carcass swab samples. Thus, it is a valuable alternative to the ISO reference methods for beef carcasses control before commercial distribution.

This validation was performed on artificially contaminated swab samples. Although this validation replies to the ISO 16140 requirements, for the full implementation of the developed workflow in a laboratory, the authors recommend analyzing real-swab samples in parallel with the ISO reference methods. This would confirm its reliability and consequently, then, the current ISO methods could be replaced by the complete CoSYPS Path Food workflow.

The complete CoSYPS Path Food workflow presents several advantages. Firstly, as a multi-genus system, this workflow is able to detect the presence of both pathogens in a single plate and from a single sample. Secondly, as a multi-level system, it has the advantage over other previously developed qPCR-based detection systems to provide information about detected strain species and/or subspecies. Thirdly, it gives negative or presumptive positive results in two days whereas four and five days are required for ISO 6579:2002 and ISO 11290-1:1996, respectively. Finally, it presents an additional advantage of great flexibility over other available qPCR-based detection systems. The CoSYPS Path Food qPCR detection step is indeed adaptable to the sample requirements: i) the tested target list can be adapted to the analysis purpose and ii) new foodborne pathogens can be added into the qPCR detection system as long as new qPCR assays are developed to be run in the already used PCR conditions (Barbau-Piednoir et al., 2013a, 2013b). The selective enrichment, isolation and confirmation steps would have to be specific to the added foodborne pathogen, using the protocols provided into the respective ISO reference methods (when available). Thus, this workflow could be upgraded with additional foodborne pathogen targets with a limited amount of work.

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