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METHODS AND PROTOCOLS

SYBR®Green qPCR *Salmonella* detection system allowing discrimination at the genus, species and subspecies levels

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Abstract In this work, a three-level *Salmonella* detection system based on a combination of seven SYBR®Green qPCR was developed. This detection system discriminates *Salmonella* at the genus, species and subspecies levels using a single 96-well plate. The SYBR®Green qPCR assays target the *invA*, *rpoD*, *iroB* and *safC* genes, as well as the STM0296 locus, putatively coding for a cytoplasmic protein. This study includes the design of primer pairs, in silico and in situ selectivity, sensitivity, repeatability and reproducibility evaluations of the seven SYBR®Green qPCR assays. Each detection level displayed a selectivity of 100 %. This combinatory SYBR®Green qPCR system was also compared with three commercially available *Salmonella* qPCR detection kits. This comparison highlighted the importance of using a multi-gene detection system to be able to detect every target strain, even those with deletion or mutation of important genes.

Keywords Real-Time PCR · SYBR®Green · Foodborne pathogens · Detection · *Salmonella*

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Introduction

The food safety is an important concern worldwide. Foodborne diseases cause not only an enormous economic burden due to sick leaves, treatments, hospitalisations and mortality (Scharff 2012) but also economical losses of farmers and industry. The recent outbreak of the O104:H4 serotype of *Escherichia coli* in Germany was a dramatic illustration of all the consequences of a severe outbreak (European Commission 2011). Currently, the official methods for the detection of pathogenic bacteria in food samples are norms from the International Organization for Standardization (ISO). These norms are culture-based methods. They are reliable but focus on individual pathogenic species, are time consuming and labour intensive (e.g. at least 5 days for *Salmonella* detection (ISO 6579 norm 2002)). Therefore, developing fast and simple foodborne pathogens detection tools, able to simultaneously detect several pathogens, remains a not only necessity but also a real challenge.

Real-time PCR (qPCR) assays have gained confidence as faster and reliable alternatives (Postollec et al. 2011), and several qPCR assays have already been developed (e.g. Beutin et al. 2009; Botteldoorn et al. 2003; Fukushima et al. 2009; Garrido et al. 2012b; Kim and Cho 2010; Löfström et al. 2010; Singh et al. 2012). However, their use all together into the same detection system is hampered by several drawbacks: they are using different technologies (e.g. TaqMan® and SYBR®Green), they are using different protocols and they were developed for different purposes and validated following different ways. Moreover, current detection assays are mostly based on the detection of a single virulence gene (e.g. *invA* for *Salmonella* or *hlyA* for *Listeria* detection). Yet, it has now been documented that *Salmonella* can lose an entire Pathogenicity island while remaining infectious (Hu et al. 2008). Single-gene target detection systems could, in this case, lead to a false negative signal.

In Europe, *Salmonella* is the first cause of notification of foodborne contamination due to microorganisms into the Rapid

Alert System for Food and Feed (European Commission 2012) and the first detected causative agent in foodborne outbreaks reported, representing *ca.* 30 % of the total number of reported outbreaks in Europe in 2011 (European Food Safety Authority, European Centre for Disease Prevention and Control 2013). Salmonellosis is caused by the consumption of contaminated food (Gomez-Aldapa et al. 2012) (e.g. meat, raw eggs, cheese, fruit or vegetables) and, to a lesser extent, person-to-person transmission (Medus et al. 2006), waterborne transmission and environmental or animal exposure. The reservoirs are mainly poultry but also involve cattle, swine, sheep, birds, rats and reptiles (Bertrand et al. 2008; Pui et al. 2011). In developing countries, some *Salmonella* serovars can provoke typhoid and paratyphoid fevers. These serovars are not transmitted by food but mainly by drinking water spoiled by urine and faeces of infected individuals.

The *Salmonella* genus includes two species: *Salmonella enterica* and *Salmonella bongori*, both pathogenic for humans (Guibourdenche et al. 2010). The *S. enterica* species is itself divided into six subspecies: *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae* and *indica* (Baumler 1997). The subspecies *enterica* is the most frequently found in food samples and is therefore the most relevant for human illnesses (Bertrand 2010). *S. bongori* and the other subspecies of *S. enterica* are rarely found in salmonellosis cases since they are associated with cold-blood invertebrates (Bertrand et al. 2008).

This paper reports on the development and validation of a *Salmonella* sp. detection system based on three detection levels. The first level detects all *Salmonella* spp., the second level detects all *S. enterica* and the third level detects all the *Salmonella enterica enterica.* Each level is composed of two to three SYBR®Green qPCR assays. The seven qPCR assays forming this detection system were developed to be run simultaneously on a single 96-well plate. Finally, this Combinatory SYBR®Green qPCR System (CoSYPS) is compared with commercial single-gene target *Salmonella* detection kits.

Material and methods

Bacterial strains

The bacterial strains used in this study are listed in Table 1. A panel of 111 bacterial strains (72 *Salmonella* strains and 39 other genera), two mould strains, two yeast strains and two viruses have been tested. These strains were obtained from National Reference Centres and Laboratories.

Bacterial growth conditions, DNA extraction and DNA quantification

Overnight cultures of each bacterial strain were grown in Brain-Heart Infusion (BHI) broth or Bolton broth (for *Campylobacter*) at the adequate temperature and oxygen condition. The total DNA from bacterial strains was extracted using the DNeasy Blood and Tissue Kit (Qiagen). Genomic DNA (gDNA) from yeast was extracted using the Invisorb Spin Plant Mini Kit (STRATEC Molecular GmbH). Fungal gDNA was extracted with ZR Fungal/Bacterial gDNA extraction (Zymo Research). Viral RNA was extracted using the RNeasy mini kit (Qiagen), and cDNA was obtained by reverse transcription using the Transcriptor high fidelity cDNA synthesis kit (Roche). The cDNA amplificability was checked with a specific PCR amplification. All kits were used according to the manufacturer's recommendations. The DNA quality was controlled on agarose gel, and the DNA concentration was measured using a Nanodrop® 2000 device according to the manufacturer's recommendations.

Development and in silico assessment of primer pairs

A uniform primer design approach was applied in the development of all primer pairs. The first step consisted of identifying genes of interest, either genus or species specific, by means of a bibliographic study (Chen et al. 1997; Galan et al. 1992; Gonzalez-Escalona et al. 2009; Parkhill et al. 2001; Porwollik et al. 2002; Soyer et al. 2009). The second step included the collection of DNA sequences relevant for the chosen targets retrieved from the NCBI public database (http://www.ncbi.nlm.nih.gov/sites/entrez). The primer pairs were designed, preferentially within conserved regions, using the "Primer 3" program (http://frodo.wi.mit.edu/primer3/ (Rozen and Skaletsky 2000) with the "product size range" specification set at "60 to 120 bp" and "primer size" optimal set at "22 bases". An in silico test of the primer pairs was then performed. It consisted in a bioinformatic analysis carried out with the "wprimersearch" software available on wEMBOSS Open Source Software package (https://wemboss.uio.no/wEMBOSS/) (Rice et al. 2000; Sarachu and Colet 2005). This software mimics the PCR amplification of the tested primers on a database of bacterial genomes sequences from NCBI of 217 bacteria, representing 103 species belonging to 61 genera. When mismatches between primer and one of its targets were observed, degenerate nucleotides were introduced into the primer sequence. However, primer pairs with no degenerate nucleotides were always preferred. Only primer pairs that gave the expected in silico amplification were retained for the following steps.

Qualitative SYBR®Green qPCR assay and optimal primer concentration

All qPCR assay reactions were performed according to the protocol described in Barbau-Piednoir et al. (2013). For the interpretation of a SYBR®Green qPCR assay, two criteria were taken into consideration: the quantification cycle (Cq)

Table.1 Specificity assessment of the seven SYBR®Green qPCR methods: "*invA*-5", "*rpoD*-20", "*iroB*-192", "*iroB*-12", "*safC*-10","*safC*-13" and "STM0296"

Genus	Species	Subspecies	Serovar	Serogroup	GRAM	Origin	Reference	*invA*-5	*rpoD*-20	*iroB*-192	*iroB*-12	*safC*-10	*safC*-13	STM0296
Salmonella	*enterica*	*enterica* (I)	Paratyphi A	O:2 (A)	-	1	10-03665	+	+	+	+	+	+	+
Salmonella	*enterica*	*enterica* (I)	Agona	O:4 (B)	-	1	NH.II.18.12	+	+	+	+	+	+	+
Salmonella	*enterica*	*enterica* (I)	Brandendurg	O:4 (B)	-	1	H.V.62.54	+	+	+	+	+	+	+
Salmonella	*enterica*	*enterica* (I)	Derby	O:4 (B)	-	1	H.II.34.34	+	+	+	+	+	+	+
Salmonella	*enterica*	*enterica* (I)	Heidelberg	O:4 (B)	-	1	NH.III.67.26	+	+	+	+	+	+	-
Salmonella	*enterica*	*enterica* (I)	Paratyphi B	O:4 (B)	-	1	H.VII.64.65	+	+	+	+	+	+	+
Salmonella	*enterica*	*enterica* (I)	Thyphimurium var. Copenhagen	O:4 (B)	-	1	H.II.13.13	+	+	+	+	+	+	+
Salmonella	*enterica*	*enterica* (I)	Thypimurium	O:4 (B)	-	1	H.II.32.32	+	+	+	+	+	+	+
Salmonella	*enterica*	*enterica* (I)	Infantis	O:7 (C1)	-	1	NH.III.66.30	+	+	+	+	+	+	+
Salmonella	*enterica*	*enterica* (I)	Ohio	O:7 (C1)	-	1	H.III.52.53	+	+	+	+	+	+	+
Salmonella	*enterica*	*enterica* (I)	Paratyphi C	O:7 (C1)	-	1	H.I.21.21	+	+	+	+	+	+	+
Salmonella	*enterica*	*enterica* (I)	Virchow	O:7 (C1)	-	1	H.V.42.56	+	+	+	+	+	+	-
Salmonella	*enterica*	*enterica* (I)	Montevideo	O:7 (C1)	-	1	H.V.33.50	+	+	+	+	+	+	+
Salmonella	*enterica*	*enterica* (I)	Kentucky	O:8 (C2-C3)	-	1	H.I.70.26	+	+	+	+	+	+	+
Salmonella	*enterica*	*enterica* (I)	Muenchen	O:8 (C2-C3)	-	1	H.I.79.35	+	+	+	+	+	+	+
Salmonella	*enterica*	*enterica* (I)	Newport	O:8 (C2-C3)	-	1	H.V.70.42	+	+	+	+	+	+	+
Salmonella	*enterica*	*enterica* (I)	Enteritidis	O:9 (D1)	-	1	H.VI.6.32	+	+	+	+	+	+	+
Salmonella	*enterica*	*enterica* (I)	Typhi	O:9 (D1)	-	1	H.III.28.24	+	+	+	+	+	+	+
Salmonella	*enterica*	*enterica* (I)	Baildon	O:9,46 (D2)	-	1	H.III.30.26	+	+	+	+	+	+	+
Salmonella	*enterica*	*enterica* (I)	Anatum	O:3,10 (E1)	-	1	H.II.76.36	+	+	+	+	+	+	+
Salmonella	*enterica*	*enterica* (I)	Give	O:3,10 (E1)	-	1	H.V.52.50	+	+	+	+	+	+	+
Salmonella	*enterica*	*enterica* (I)	London	O:3,10 (E1)	-	1	H.II.60.20	+	+	+	+	+	+	+
Salmonella	*enterica*	*enterica* (I)	Gnesta	O:1,3,19 (E4)	-	1	H.II.47.7	+	+	+	+	+	+	+
Salmonella	*enterica*	*enterica* (I)	Kouka	O:1,3,19 (E4)	-	1	H.II.41.1	+	+	+	+	+	+	+
Salmonella	*enterica*	*enterica* (I)	Krefeld	O:1,3,19 (E4)	-	1	NH.I.50.10	+	+	+	+	-	-	+
Salmonella	*enterica*	*enterica* (I)	Liverpool	O:1,3,19 (E4)	-	1	H.II.52.12	+	+	+	+	+	+	+
Salmonella	*enterica*	*enterica* (I)	Senftenberg	O:1,3,19 (E4)	-	1	H.II.50.10	+	+	+	+	-	-	+
Salmonella	*enterica*	*enterica* (I)	Senftenberg (lactose +)	O:1,3,19 (E4)	-	1	H.II.48.8	+	+	+	+	-	-	+
Salmonella	*enterica*	*enterica* (I)	Vilvoorde	O:1,3,19 (E4)	-	1	H.VI.10.55	+	+	+	+	+	+	+
Salmonella	*enterica*	*enterica* (I)	Marseille	O:11 (F)	-	1	H.III.55.3	+	+	+	+	+	+	+
Salmonella	*enterica*	*enterica* (I)	Tours	O:11 (F)	-	1	H.VI.76.12	+	+	+	+	+	+	+
Salmonella	*enterica*	*enterica* (I)	Poona	O:13 (G)	-	1	NH.II.32.2	+	+	+	+	+	+	+
Salmonella	*enterica*	*enterica* (I)	Florida	O:6,14 (H)	-	1	H.IV.29.8	+	+	+	+	+	+	+
Salmonella	*enterica*	*enterica* (I)	Gaminara	O:16 (I)	-	1	H.III.74.12	+	+	+	+	+	+	+
Salmonella	*enterica*	*enterica* (I)	Carmel	O:17 (J)	-	1	H.III.79.3	+	+	+	+	+	+	+
Salmonella	*enterica*	*enterica* (I)	Cerro	O:18 (K)	-	1	H.IV.18.1	+	+	+	+	+	+	+
Salmonella	*enterica*	*enterica* (I)	Minnesota	O:21 (L)	-	1	H.IV.20.1	+	+	+	+	+	+	+
Salmonella	*enterica*	*enterica* (I)	Cotham	O:28 (M)	-	1	H.VII.63.23	+	+	+	+	+	+	+
Salmonella	*enterica*	*enterica* (I)	Pomona	O:28 (M)	-	1	H.VIII.24.27	+	+	+	+	+	+	+
Salmonella	*enterica*	*enterica* (I)	Urbana	O:30 (N)	-	1	H.IV.52.1	+	+	+	+	+	+	+
Salmonella	*enterica*	*enterica* (I)	Adelaïde	O:35 (O)	-	1	H.IV.35.1	+	+	+	+	+	+	+
Salmonella	*enterica*	*enterica* (I)	Monschaui	O:35 (O)	-	1	NH.II.6.2	+	+	+	+	+	+	+
Salmonella	*enterica*	*enterica* (I)	Inverness	O:38 (P)	-	1	H.VII.23.12	+	+	+	+	+	+	+
Salmonella	*enterica*	*enterica* (I)	Perth	O:38 (P)	-	1	H.VI.24.6	+	+	+	+	+	+	-
Salmonella	*enterica*	*enterica* (I)	Anfo	O:39 (Q)	-	1	NH.III.58.2	+	+	+	+	+	+	+
Salmonella	*enterica*	*enterica* (I)	Champaign	O:39 (Q)	-	1	H.IV.62.2	+	+	+	+	+	+	+
Salmonella	*enterica*	*enterica* (I)	Hofit	O:39 (Q)	-	1	H.IV.63.3	+	+	+	+	+	+	-
Salmonella	*enterica*	*enterica* (I)	Kokomlemle	O:39 (Q)	-	1	H.II.47.8	+	+	+	+	+	+	+
Salmonella	*enterica*	*enterica* (I)	Namur	O:39 (Q)	-	1	H.VII.35.5	+	+	+	+	-	-	+
Salmonella	*enterica*	*enterica* (I)	Wandsworth	O:39 (Q)	-	1	H.IV.36.1	+	+	+	+	+	+	+
Salmonella	*enterica*	*enterica* (I)	Johannesburg	O:40 (R)	-	1	H.VII.29.6	+	+	+	+	+	+	+
Salmonella	*enterica*	*enterica* (I)	Waycross	0:41(S)	-	1	H.VI.39.7	+	+	+	+	+	+	+
Salmonella	*enterica*	*enterica* (I)	Frederiksberg	O:42 (T)	-	1	H.VII.65.5	+	+	+	+	+	+	+

Table.1 (continued)

Genus	Species	Subspecies	Serovar	Serogroup	GRAM	Origin	Reference	*invA*-5	*rpoD*-20	*iroB*-192	*iroB*-12	*safC*-10	*safC*-13	STM0296
Salmonella	*enterica*	*enterica* (I)	Berkeley	O:43 (U)	-	1	H.IV.73.4	+	+	+	+	+	+	+
Salmonella	*enterica*	*enterica* (I)	Lawra	O:44 (V)	-	1	H.IV.55.3	+	+	+	+	+	+	+
Salmonella	*enterica*	*enterica* (I)	Tornow	O:45 (W)	-	1	NH.III.15.2	+	+	+	+	+	+	+
Salmonella	*enterica*	*enterica* (I)	Djakarta	O:48 (Y)	-	1	H.IV.45,1	+	+	+	+	+	+	-
Salmonella	*enterica*	*arizonae* (IIIa)	51:24z23:-	O:51	-	1	H.VI.61.2	+	+	+	+	-	-	-
Salmonella	*enterica*	*diarizonae* (IIIb)	47:lv:z35	O:47 (X)	-	1	NH.III.6.6	+	+	+	+	-	-	-
Salmonella	*enterica*	*diarizonae* (IIIb)	53:z10:z35	O:53	-	1	H.VII.42.1	+	+	+	+	-	-	-
Salmonella	*enterica*	*diarizonae* (IIIb)	57:-:-	O:57	-	1	NH.IV.4.1	+	+	+	+	-	-	-
Salmonella	*enterica*	*diarizonae* (IIIb)	59: Z52:Z53	O:59	-	1	H.V.27.1	+	+	+	+	-	-	-
Salmonella	*enterica*	*diarizonae* (IIIb)	60:r:z	O:60	-	1	H.V.10.2	+	+	+	+	-	-	-
Salmonella	*enterica*	*diarizonae* (IIIb)	61:lv:1,5,7	O:61	-	1	H.IV.77.1	+	+	+	+	-	-	-
Salmonella	*enterica*	*diarizonae* (IIIb)	65:(k):35	O:65	-	1	H.V.13.3	+	+	+	+	-	-	-
Salmonella	*enterica*	*houtenae* (IV)	44:z4z23:-	O:44 (V)	-	1	H.IV.40.1	+	+	+	+	-	-	-
Salmonella	*enterica*	*houtenae* (IV)	50:Z4:Z32	O:50 (Z)	-	1	H.V.9.5	+	+	+	+	-	-	-
Salmonella	*enterica*	*houtenae* (IV)	50:z4z23:-	O:50 (Z)	-	1	H.V.7.3	+	+	+	+	-	-	-
Salmonella	*enterica*	*indica* (VI)	1,6,14,25:a:enx	O:6,14 (H)	-	1	1240 K	+	+	+	+	-	-	-
Salmonella	*enterica*	*salamae* (II)	48:z:1,5	O:48 (Y)	-	1	H.VII.48.10	+	+	+	+	-	-	-
Salmonella	*enterica*	*salamae* (II)	58:c:z6	O:58	-	1	H.VII.72.10	+	+	+	+	-	-	-
Salmonella	*bongori*	NA	66:z41:-	O:66	-	2	1224/72	+	+	-	-	-	-	-
Pseudomonas	*aeruginosa*	NA	NA	NA	-	3	LMG 6395	-	-	-	-	-	-	-
Vibrio	*parahaemolyticus*	NA	NA	NA	-	3	TIAC 610	-	-	-	-	-	-	-
Aeromonas	*hydrophila*	NA	NA	NA	-	4	M/2862	-	-	-	-	-	-	-
Citrobacter	*freundi*	NA	NA	NA	-	3	TIAC 554	-	-	-	-	-	-	-
Citrobacter	*diversus*	NA	NA	NA	-	4	M/849	-	-	-	-	-	-	-
Enterobacter	*cloacae*	NA	NA	NA	-	3	TIAC 445	-	-	-	-	-	-	-
Enterobacter	*gergoviae*	NA	NA	NA	-	4	5502	-	-	-	-	-	-	-
Enterobacter	*aerogenes*	NA	NA	NA	-	4	M/3785	-	-	-	-	-	-	-
Escherichia	*coli*	NA	NA	NA	-	3	ATCC 25922	-	-	-	-	-	-	-
Escherichia	*coli*	NA	NA	O157:H7	-	5	EH 630	-	-	-	-	-	-	-
Escherichia	*fergusonii*	NA	NA	NA	-	5	95/394	-	-	-	-	-	-	-
Escherichia	*hermanii*	NA	NA	NA	-	5	Div 2663	-	-	-	-	-	-	-
Escherichia	*vulneris*	NA	NA	NA	-	5	Seq048	-	-	-	-	-	-	-
Hafnia	*alvei*	NA	NA	NA	-	4	7186	-	-	-	-	-	-	-
Klebsiella	*oxytoca*	NA	NA	NA	-	3	TIAC 695	-	-	-	-	-	-	-
Klebsiella	*pneumoniae*	NA	NA	NA	-	3	TIAC 446	-	-	-	-	-	-	-
Morganella	*morganii*	NA	NA	NA	-	4	M/100	-	-	-	-	-	-	-
Proteus	*mirabilis*	NA	NA	NA	-	3	TIAC 726	-	-	-	-	-	-	-
Proteus	*vulgaris*	NA	NA	NA	-	4	M/654	-	-	-	-	-	-	-
Providencia	*rettgeri*	NA	NA	NA	-	4	M/831	-	-	-	-	-	-	-
Serratia	*foncticola*	NA	NA	NA	-	4	3429	-	-	-	-	-	-	-
Serratia	*marcescens*	NA	NA	NA	-	4	7015	-	-	-	-	-	-	-
Serratia	*odorifera*	NA	NA	NA	-	4	3436	-	-	-	-	-	-	-
Shigella	*boydii*	NA	NA	15	-	1	10-02874	-	-	-	-	-	-	-
Shigella	*dysenteriae*	NA	NA	6	-	1	10-01857	-	-	-	-	-	-	-
Shigella	*flexneri*	NA	NA	2a	-	1	10-03891	-	-	-	-	-	-	-
Shigella	*sonneï*	NA	NA	NA	-	1	10-03865	-	-	-	-	-	-	-
Yersinia	*enterocolitica*	NA	NA	NA	-	3	LMG 15558	-	-	-	-	-	-	-
Campylobacter	*coli*	NA	NA	NA	-	3	TIAC 539	-	-	-	-	-	-	-
Campylobacter	*jejuni*	NA	NA	NA	-	3	ATCC 33291	-	-	-	-	-	-	-
Campylobacter	*lari*	NA	NA	NA	-	3	TIAC 544	-	-	-	-	-	-	-
Clostridium	*perfringens*	NA	NA	NA	+	3	ATCC 13124 T	-	-	-	-	-	-	-
Bacillus	cereus	NA	NA	NA	+	3	ATCC 14579	-	-	-	-	-	-	-
Brochotrix	*thermosphacta*	NA	NA	NA	+	3	TIAC 400	-	-	-	-	-	-	-
Listeria	*innocua*	NA	NA	NA	+	6	CIP 8011	-	-	-	-	-	-	-
Listeria	*ivanovii*	NA	NA	NA	+	6	CIP 7842	-	-	-	-	-	-	-

Table.1 (continued)

Genus	Species	Subspecies	Serovar	Serogroup	GRAM	Origin	Reference	*invA*-5	*rpoD*-20	*iroB*-192	*iroB*-12	*safC*-10	*safC*-13	STM0296
Listeria	*monocytogenes*	NA	NA	1/2a	+	6	ATCC 51772	-	-	-	-	-	-	-
Staphylococcus	*aureus*	NA	NA	NA	+	3	ATCC 25923	-	-	-	-	-	-	-
Lactobacillus	*acidophillus*	NA	NA	NA	+	3	TIAC 642	-	-	-	-	-	-	-
Aspergillus	*fumigatus*	NA	NA	NA	NA	7	BCCM/IHEM 19436	-	-	-	-	-	-	-
Cladosporium	*sphaerospermum*	NA	NA	NA	NA	7	BCCM/IHEM 24474	-	-	-	-	-	-	-
Saccharomyces	*cerevisiae*	NA	NA	NA	NA	7	BCCM/IHEM 3961	-	-	-	-	-	-	-
Candida	*parapsilosis*	NA	NA	NA	NA	7	BCCM/IHEM 6478	-	-	-	-	-	-	-
Hepatitis A Virus			NA	NA	NA	3	27 (WZ)	-	-	-	-	-	-	-
Norovirus GI			NA	NA	NA	3	2593	-	-	-	-	-	-	-
No template control					NA	NA	NA	-	-	-	-	-	-	-

+ There is an amplification and a Tm value similar for all corresponding strains; - No amplification; *1*: Scientific Institute of Public Health, *Salmonella* and *Shigella* National Reference Centre, rue Juliette Wytsmanstraat 14, 1050 Brussels, Belgium; *2* French National Reference Center Salmonella, Institut Pasteur, 25-28 rue du Dr. Roux, 75724 Paris Cedex 15, France; *3* Scientific Institute of Public Health, Food pathogens laboratory, rue Juliette Wytsmanstraat 14, 1050 Brussels, Belgium; *4* Scientific Institute of Public Health, Quality of medical Laboratories, rue Juliette Wytsmanstraat 14, 1050 Brussels, Belgium; *5* Department Microbiology and Infection Control, VTEC National Reference Centre, Free University of Brussels, Laarbeeklaan 101-1090 Brussel; *6* Scientific Institute of Public Health, *Listeria* National Reference Centre, rue Juliette Wytsmanstraat 14, 1050 Brussels, Belgium; *7* Mycology & Aerobiology, Scientific Institute of Public Health, rue Juliette Wytsmanstraat 14, 1050 Brussels, Belgium; *NA* not applicable

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, a qualitative respond is required. To be considered as positive, a signal generated in SYBR®Green qPCR analysis should display an (exponential) amplification above the threshold level, with a single peak upon melting analysis giving a unique Tm value. A signal was considered as negative when no Cq value was obtained. The optimal concentration of the selected primer pairs was determined by testing different concentrations of each primer, between 250 and 1,000 nM. The concentrations giving the lowest Cq value without formation of a high level of primer dimer were selected. When using a positive sample at a concentration around the limit of detection (LOD), the primer dimer dissociation peak should not be higher than the positive sample dissociation peak. The primer pairs and their concentrations used in this study are presented in Table 2.

Selectivity test and accuracy calculation

Primer pairs selected from the in silico evaluation were tested in situ. In brief, the selectivity of the SYBR®Green qPCR assay is tested according to the ISO 22118 norm (2011) on target and non-target strains. The selectivity of an assay is assessed by inclusivity (target strains giving a positive signal) and exclusivity (non-target strains not giving a positive signal). The selectivity test is fully described in Barbau-Piednoir et al. (2013). From the selectivity test, the accuracy can be calculated. The accuracy represents the closeness of agreement between a test result and the accepted reference value (ISO 3534–1 1993). Its formula is found in ISO 16140 norm (2003).

Amplicon cloning and sequencing

In order to check the correctness of the amplicon obtained with each developed primer pair, the amplicons obtained by the amplification of *S. enterica enterica* Enteritidis (H.V.6.32) were cloned, sequenced and verified using the same approach as recently described by Barbau-Piednoir et al. (2013). Sequencing reactions were run on an ABI 3130xl at the Platform Biotechnology and Molecular Biology at the Scientific Institute of Public Health.

Full genome sequencing

The total DNA from *S. enterica enterica* serovar Namur was extracted with Blood & Cell Culture DNA Midi kit (Qiagen). The quality and quantity of the DNA were controlled with the 260/280-nm ratio and by spectrophotometer measurement, respectively. This DNA was sent to Baseclear for sequencing using the Illumina technology.

Dynamic range and calculation of the PCR efficiency

The dynamic range of a qPCR assay is the range of concentrations where it performs in a linear manner. It was assessed by the analysis in duplicate of a serial dilution in a carrier DNA (4 ng/μL Calf Thymus DNA (CTD) (Invitrogen) of pure strain DNA (1,000 to 0.01 theoretical genomic copies) of *S. enterica enterica* Enteritidis (H.V.6.32) and *S. enterica*

Table 2 Primer pairs and amplicon size for each SYBR®Green qPCR assay

Targeted genus	Targeted species	Targeted subspecies	Targeted sequence	qPCR assay name	Prime name	Primer sequence (5' ->3')	Position on the *S.* Enteritidis genome (AM933172)	Primer concentration (nM)	Product Size (bp)	Tm amplicon	References
Salmonella	All	All	Sigma factor *(rpoD)*	rpoD-20	rpoD-20-F	ACATGGGTATTCAGGTAATGGAAGA	3,264,501–3,264,525	250	75	77–78 °C	Adapted from Botteldoorn et al. 2006
					rpoD-20-R	C**R**GTGCTGGTGGTATTTTCA	3,264,574–3,264,555	1000			
			Invasine gene *(invA)*	invA-5	invA-5-deg-F	GAT**Y**TGAA**R**GCCGGTATTATTG	2,924,646–2,924,625	250	107	80–82 °C	This study
					invA-5-R	ATAAACTTCATCGCACCGTCA	2,924,540–2,924,560	250			
	enterica	All	C-glycosyltransferase *(iro-B)*	iroB-192	iroB-192-F	CCAAGAGATCTGGCGTGGATAG	2,801,780–2,801,101	250	86	78 °C (*S. enterica salamae*-79 °C *(S. enterica enterica* and *S. enterica arizonae*) - 80 °C (*S. enterica doutenae* and *S. enterica diarizonae*)	Adapted from Murphy et al. 2007
					iroB-192-R	TATTGCATGGAGATAACCGGCT	2,801,865–2,801,844	250			
				iroB-12	iroB-12-deg-F	GG**R**ACAAAAATGGGG**M**ACTTCT	2,801,531–2,801,552	500	105	80.5–83 °C	This study
					iroB-12-R	AAGGGGAGG**R**TAGACGATGA	2,801,635–2,801,616	500			
		enterica	Outer membrane usher protein (*safC)*	safC-10	safC-10-F	CACTAAAAAGTCGTCTGACTCTGG	323,795–323,818	250	72	75–76 °C	This study
					safC-10-R	CGGTAAATGGCACACTATCAAAT	323,866–323,844	250			
				safC-13	safC-13-F	ATGATGACAGCTACGGACTGAGTT	324,665–324,688	250	76	77–78 °C	This study
					safC-13-R	CTGTTTTGATTCCAGTTCAATGAC	324,740–324,717	250			
			STM0296 (Acc n: AAL19253)	STM0296	STM0296-F	A**M**GCCCATCCCTTCAATACAG	320,053–320,073	500	56	76–78 °C	This study
					STM0296-R	GTGAT**Y**ACGCCGCAGCATACCC	320,108–320,087	500			

Degenerated nucleotides: R=A or G, Y=C or T, M=A or C

enterica Typhimurium (H.II.32.32). The carrier DNA avoids the improper dilution due to low concentration of gDNA. This analysis allows the assessment of the coefficient of determination (R^2) and the PCR efficiency (E) for each SYBR®Green qPCR assays. The coefficient of determination (R^2) is an indicator of the correlation of data regarding the linear regression curve. The R^2 of the dynamic range curve should be above 0.98 (European Network of GMO Laboratories 2008). The PCR efficiency (E) should be between 89.6 and 110.2 % and can be calculated according to the formula reported by Rutledge and Cote (2003).

Sensitivity test

Primer pairs passing the selectivity test were subsequently examined for their sensitivity. Using serial dilution, the LOD of each SYBR®Green qPCR assays was evaluated. The LOD is defined as the lowest concentration of an analyte giving a positive result with a probability of 95 % (European Network of GMO Laboratories 2008). The strains used were *S. enterica enterica* Enteritidis (H.V.6.32) and *S. enterica enterica* Typhimurium (H.II.32.32). The calculation of the target genomic copy numbers for each dilution point was performed according to Barbau-Piednoir et al. (2013), considering the gDNA size of *S. enterica enterica* Enteritidis and Typhimurium as 4,685,848 and 4,857,432 bp (Acc. Num.: AM933172 and AE006468, respectively). To determine the LOD, a range of copy number between 10 and 0.1 theoretical copies was tested (i.e. 10, 5, 2, 1, 0.5, 0.2 and 0.1). Each dilution was tested in six replicates per plate, for both strains. Moreover, the analysis was performed three independent times, under repeatable conditions, resulting in 36 repeats for each dilution point. It has to be noticed that the dilution points beyond the theoretical single copy were carried out to assess the dilution series correctness. Indeed, as it is statistically impossible to get amplification in all replicates with the dilution points beyond one theoretical copy, none of these dilution points should give 100 % of positive signals.

Repeatability calculation

To evaluate the repeatability of the assay, independent tests were performed with the same protocol, with the same samples, by the same operator using the same apparatus within a short interval of time (ISO 3534–1 norm 1993). The repeatability limit (r) and the relative standard deviation of repeatability (RSD_r) were calculated according to ISO 16140 norm (2003). RSD_r should be ≤ 25 % for all the dilution range above the LOD (European Network of GMO Laboratories 2008). The RSD_r and r values of the Cq values were calculated at each dilution point while the RSD_r and r values of the Tm values were calculated with all the Tm values coupled with amplification (Cq <40).

Reproducibility study and calculation

To evaluate the reproducibility of the assays (ISO 3534–1 norm 1993), independent tests were performed with the same protocol, using the same samples, in two different laboratories, by two different operators using different apparatus (Bio-Rad iQ5 and ABI 7300). The nine tested samples were all gDNA from *S. enterica enterica* strains at different concentrations between 20 and 100 genomic copies per reaction. Each sample was analyzed in duplicate by each operator.

Two reproducibility measures can be calculated from these results: the relative standard deviation of reproducibility (RSD_R) and the expanded uncertainty (U). RSD_R represents the absolute value of the coefficient of variation. It is expressed in percent and is obtained by multiplying the reproducibility standard deviation by 100 and dividing this product by the reproducibility median (ISO 16140 2003). The RSD_R should be ≤35 % for all the tested samples (European Network of GMO Laboratories 2008). The RSD_R of the Cq values are calculated for the nine tested samples. The RSD_R of the Tm values are calculated with all the Tm values coupled with an amplification (Cq <40).

The uncertainty is "the parameter associated with the result of a measurement that characterizes the dispersion of the values that could reasonably be attributed to the measurand". The uncertainty can be expressed by the expanded uncertainty (U) which is the quantity defining "an interval about the result of a measurement that may be expected to encompass a large fraction of the distribution of values that could reasonably be attributed to the measurand". The expanded uncertainty (U) is obtained by multiplying the combined standard uncertainty by a coverage factor (Joint Committee for Guides in Metrology 2008).

CoSYPS *Salmonella*

The seven qPCR SYBR®Green assays have been run, on the same plate with the same PCR program (Barbau-Piednoir et al. 2013) using the appropriate concentration of each primer (Table 2) on 10^4 copies of five gDNA extraction from pure culture of *S. enterica enterica* Enteritidis (H.VI.6.32), *Salmonella enterica salamae* (H.VII.72.10), *Salmonella enterica arizonae* (H.VI.61.2), *Salmonella enterica diarizonae* (NH.IV.4.1) and *S. bongori* (1224/72).

Comparison of CoSYPS *Salmonella* with other commercial kits

The CoSYPS *Salmonella*, composed of the seven SYBR®Green qPCR assays as detection/identification system, was compared with three *Salmonella* detection kits: iQ-Check™ *Salmonella* II from Bio-Rad, foodproof® *Salmonella* Detection Kit from Biotecon and GeneDisc® *Salmonella* spp. from Pall® Life

Sciences (according to manufacturer's protocol). Eleven gDNA *Salmonella* samples from pure culture at 10^4 copies per assay have been tested in duplicate with the three detection systems.

Results

In silico selection of the primer pairs and optimisation of primer concentration

As a first step, specific genes of *Salmonella* genus, *S. enterica* species and *S. enterica enterica* subspecies were identified. For the specific detection of the *Salmonella* genus, *invA* and *rpoD* were retained. The *rpoD* gene encodes the RNA polymerase sigma 70 factor (Bai et al. 2012), while *invA* plays an important role during invasion of the intestinal cells (Galan et al. 1992). *InvA* is located in the *Salmonella* Pathogenicity Island I (SPI-I) (Baumler et al. 1997) shown to be present in all phylogenetic lineages of the *Salmonella* genus (Li et al. 1995). For the specific detection of the *S. enterica*, the chosen gene was *iroB*, which belongs to the Fur regulon that mediates gene regulation in response to iron availability (Ernst et al. 1978). This gene is not only present in *S. enterica* but also in all its subspecies (Porwollik et al. 2002). Finally, for the specific detection of *S. enterica* subsp. *enterica*, the *safC* and *ratB* genes were selected (Porwollik et al. 2002). *SafC* belongs to the *safABCD* operon (Folkesson et al. 2002) located in the *Salmonella* Pathogenicity Island 6 (SPI-6) (Parkhill et al. 2001). This operon encodes the *Salmonella* atypical fimbriae (Saf) pilus (Salih et al. 2008). *RatB* encodes an outer membrane protein essential in the colonization of the caecum by *Salmonella*.

During in silico evaluation of these primer pairs, some nucleotides were degenerated when necessary. Twenty primer pairs were first tested in situ with the "light" selectivity test (data not shown). From these, seven primer pairs were retained: *invA*-5 and *rpoD*-20 for the *Salmonella* spp. specific detection, *iroB-192*, *iroB*-2 and *iroB-12* for the *S. enterica* specific detection and *safC-1* and *safC-13* for the *S. enterica enterica* detection. The optimal concentration of the seven primer was also evaluated (Table 2). These assays were then evaluated in vivo for their selectivity.

In situ optimisation of primer pair and targets

During in situ selectivity test, the primer pairs *invA*-5 and *iroB*-12 were found to less efficiently (latter Cq value) amplify *S. bongori* and *Salmonella enterica houtenae,* respectively, while strains *S. enterica enterica* Senftenberg, Namur and Krefeld were not amplified by the *safC* assays.

Optimisation of invA-5 and iroB-12 primer pairs

The locus containing the primer pair *invA*-5 in *S. bongori* and *iroB*-12 in *S. enterica houtenae* was sequenced (data not shown) and aligned with the published sequences in order to identify mismatches. The new degenerated primers *invA-5-deg-F* and *iroB-12-deg-F* are displayed in Table 2. The new assays performed with these degenerated primers amplified *S. bongori* and *S. enterica houtenae*, respectively, as efficiently (giving a comparable Cq value at the same concentration) as the other positive strains.

Optimisation of S. enterica enterica target

SafC could not be found in the genomes of both *S. enterica enterica* Senftenberg (CAGQ00000000.1 and AFCU 00000000.1) and Namur (AWGG00000000) (see below). However, a short region (56 bp) into the STM0296 gene of *S. enterica enterica* Typhimurium (AE006468), upstream of the *saf* operon, was found to be highly conserved in *S. enterica* subsp. *enterica* including Senftenberg and Namur. Therefore, a new primer pair, named STM0296, was designed to amplify this 56-bp sequence (Table 2).

Determination of SYBR®Green qPCR assay selectivity

The primer pairs *invA*-5 and *rpoD*-20 amplified 100 % (72/72) of the *Salmonella* spp. tested strains, none (0/45) of the non-target strains nor the no template control (NTC) (Table 1). The *iroB*-192 and *iroB*-12 assays for *S. enterica* detection gave a specific amplification with 100 % (71/71) of *S. enterica* tested strains and 0 % (0/46) of the non-target strains and NTC (Table 1). Thus, these four assays were considered 100 % accurate for their target (*S. enterica*) giving 0 % of false positive and 0 % of false negative.

The four *safC* assays amplified 94.6 % (52/55) of the tested serovars and 0 % (0/61) of the non-target strains and NTC (Table 1). Primer pairs *safC*-10 and *safC*-13 were selected among four candidates since they amplified different loci of *safC* and gave the smallest amplicon size with two distinct Tm values (Table 2). These two assays give 0 % of false positive and 5.45 % (3/55) of false negative (serovars Senftenberg, Namur and Krefeld). The accuracy of these two assays is 97.4 % for detection of *S. enterica enterica*

The STM0296 assay amplifies 90.9 % (50/55) of the tested serovars and 0 % (0/61) of non-target strains and NTC (Table 1). This assay gives 0 % of false positive and 9.1 % (5/55) of false negative (serovars Heidelberg, Virchow, Perth, Hofit and Djakarta. The accuracy of this assay is 95.7 % for detection of *S. enterica enterica.* However, 100 % (55/55) of the tested serovars were amplified by at least one *S. enterica enterica* assay. Then, the combination of *safC* and STM0296 assays forming the level detection system was 100 % accurate for the specific detection of *S. enterica enterica.*

Table 3 Standard curve, amplification efficiency, coefficient of correlation, LOD, repeatability and reproducibility of the seven *Salmonella* assays

			Accepted range	*rpoD*-20	*invA*-5	*iroB*-192	*iro*B-12	*safC*-10	*safC*-13	STM0296
		Standard curve equation	−3,1<slope<−3,6	−3.2399 (logX) +34.657	−3.3546 (logX) +35.473	−3.3136 (logX) +34.290	−3.3477 (logX) +35.651	−3.3765 (logX) +34.951	−3.2543 (logX) +35.771	−3.1876 (logX) +33.795
		Amplification efficiency (*E*)	89,6–110.2 %	104 %	99 %	100 %	99 %	98 %	103 %	106 %
		Coefficient of correlation (R^2)	>0.98	0.997	0.982	0.985	0.996	0.988	0.999	0.998
		LOD	<10 copies	2–5 copies	2–5 copies	2–5 copies	2–5 copies	5–10 copies	2–5 copies	1–2 copies
Repeatability	*Salmonella enterica enterica* Enteritidis	Cq RSDr (%)	≤25 %	2.2	5.1	3.2	2	3.7	3.1	2.9
		Cq r	na	2	4.7	2.9	1.9	3.5	2.9	2.8
		Tm value±SD (°C)	na	77.5±0.2	80.3±0.4	78.7±0.3	81.1±0.3	75.0±0.4	77.6±0.2	76.1±0.22
		Tm RSDr (%)	≤25 %	0.2	0.4	0.4	0.3	0.5	0.2	0.3
		Tm r	na	0.5	1	0.9	0.8	1	0.5	0.6
Reproducibility	*Salmonella enterica enterica* Typhimurium	Cq RSDr (%)	≤25 %	2.2	3.5	2	2.9	2.5	2.2	1.7
		Cq r	na	2	3.1	1.9	2.7	2.2	2	1.5
		Tm value±SD (°C)	na	77.2±0.3	80.6±0.4	78.6±0.3	81.2±0.3	75.0±0.3	76.9±0.3	77.7±0.29
		Tm RSD_r (%)	≤25 %	0.3	0.4	0.3	0.4	0.4	0.4	0.4
		Tm *r*	na	0.7	1	0.7	0.8	0.9	0.9	0.8
	RSD_R (%) (min - max)	Cq	≤35 %	1.64–4.79	0.73–4.12	1.35–3.11	2.57–5.05	3.62–7.34	3.11–8.25	0–1.95
		Tm	≤35 %	0.36–0.96	0.13–0.66	0.27–0.63	0.35–0.78	0–0.84	0.05–1.01	0–0.37
	Uncertainty at 99 %	Cq	na	2.41	2.68	1.8	3.27	4.38	4.78	0.92
		Tm	na	1.32	0.94	1.17	1.24	1.14	1.47	0.39

Standard curves, *E* and R^2 were obtained from two replicates for each concentration (from 1,000 to 1 copies) from two different strains; LOD is based on 36 repetitions with the two genomic targets as DNA template; repeatability is based on 18 repetitions with the genomic target as DNA template; *X* copy number; *LOD* limit of detection, *na* not applicable

These seven assays tested on *S. enterica enterica* Enteritidis showed a unique band at the expected size upon agarose gel analysis (data not shown). The sequence of each amplicon was determined and shown to correspond to the sequence of *S. enterica enterica* Enteritidis (data not shown). In addition, the seven detection assays gave a unique melting peak with a specific melting temperature for each assay (Table 2).

SafC presence in *S. enterica enterica* Namur, Krefeld and Senftenberg

As indicated above, the genome of *S. enterica enterica* Namur was fully sequenced (data not shown). The presence of the *Salmonella* atypical fimbrial (*saf*) operon was neither been found in the Senftenberg strain whole genome shotgun contigs (CAGQ00000000.1 and AFCU00000000.1), nor in the Namur strain sequence (AWGG00000000). The *S. enterica enterica* Senftenberg, Namur and Krefeld strains were also PCR-tested using primers flanking, or located inside, the *saf* operon. None of them was positive confirming the absence of this operon in these three strains (data not shown). The lack of this *saf* operon is in accordance with the phylogenetic study of den Bakker et al. (2011) demonstrating the diversification of virulence factors content, mainly fimbrial operons, between two clades of *S. enterica enterica*.

Determination of SYBR®Green qPCR assay dynamic range and PCR efficiency

The seven developed assays performed in a linear manner between 1 and 1,000 copies as their R^2 values were between 0.982 and 0.999, above the required 0.98 (ENGL 2008) (Table 3). From the dynamic range analyses, the PCR efficiency (E) of each assay was calculated. The seven assays displayed PCR efficiencies ranging between 98 and 106 %, falling into the accepted limits of 89.6–110.2 % (ENGL 2008) (Table 3).

Determination of SYBR®Green qPCR assay sensitivity and repeatability

The LOD of the seven assays fell between two and ten copies (Table S1) complying with the requirement "between 1 and 10 CFU" (ISO 22118 norm 2011). The r values at the LOD of the Cq values ranged between 1.9 and 4.7 Cq and those of the Tm values ranged between 0.5 and 1 °C (Table 3). The RSD_r values at LOD of the Ct values of the seven assays were between 1.7 and 5.1 % while those of the Tm values ranged between 0.2 and 0.5 % (Table 3). All these RSD_r values were below ≤25 % as requested by the ENGL guidelines (ENGL 2008).

Determination of SYBR®Green qPCR assay reproducibility

For all the developed SYBR®Green qPCR assays, the RSD_R values were below ≤35 % as requested by the ENGL guideline (ENGL 2008): between 0 and 1.01 % for the Tm values and between 0.00 and 7.34 % for the Ct values (Table S2). The expanded uncertainty at 99 % of confidence was also calculated from the reproducibility data from the eight samples. U was ranging between 0.39 and 1.47 °C for the Tm values and between 0.92 and 4.78 for the Cq values (Table S2).

CoSYPS Salmonella

The seven SYBR®Green qPCR assays were run together on the same plate. *S. enterica enterica* Enteritidis was amplified by the seven assays, whereas *S. enterica arizonae, S. enterica salamae* and *S. enterica diarizonae* subspecies were amplified by the four assays amplifying all the *S. enterica* subspecies. The *S. bongori* species was only amplified by the two assays that are amplifying all the species of the *Salmonella* genus (Table 4).

Table 4 Matrix of amplified targets with the different SYBR®Green qPCR assays

Genus	Species	Subspecies	Serovars	*rpoD*-20	*invA*-5	*iroB*-192	*iroB*-12	*safC*-10	*safC*-13	STM0296
Salmonella	*enterica*	*enterica* (I)	Senftenberg, Namur and Krefeld	+	+	+	+	-	-	+
		enterica (I)	Djakarta, Heidelberg, Hofit, Perth, Virchow	+	+	+	+	+	+	-
		enterica (I)	Others	+	+	+	+	+	+	+
		salamae (II)	na	+	+	+	+	-	-	-
		arizonae (IIIa)	na	+	+	+	+	-	-	-
		diarizonae (IIIb)	na	+	+	+	+	-	-	-
		houtenae (IV)	na	+	+	+	+	-	-	-
		indica (VI)	na	+	+	+	+	-	-	-
	bongori (V)	na	na	+	+	-	-	-	-	-

na not applicable

Table 5 Comparison CoSYPS *Salmonella* with other commercial qPCR *Salmonella* detection kits

	CoSYPS *Salmonella*	FoodProof® *Salmonella*	GeneDisc® *Salmonella* spp.	iQ-Check *Salmonella* II
Control positif kit	+	+	+	+
No template Control	-	-	-	-
S. bongori	+	+	-	+
S. enterica indica	+	+	+	+
S. enterica houtenae	+	+	+	+
S. enterica arizonae	+	+	+	+
S. enterica diarizonae	+	+	+	+
S. enterica salamae	+	+	+	+
S. enterica enterica Typhimurium	+	+	+	+
S. enterica enterica Senftenberg	+	+	+	+
S. enterica enterica Senftenberg (-SPI-1)	+	+	+	-
S. enterica enterica Enteritidis	+	+	+	+
S. enterica enterica Namur	+	+	+	+

Comparison with other *Salmonella* qPCR detection kits

The CoSYPS Path Food detection/identification system based on seven qPCR assays allowed the detection of the eleven tested samples. The iQ-Check™ *Salmonella* II did not detect *S. enterica enterica* Senftenberg, isolated from a Chinese outbreaks that do not contain the SPI-1 (Hu et al. 2008) and the GeneDisc® *Salmonella* spp. did not detect *S. bongori.* The foodproof® of Biotecon detects all tested strains (Table 5).

Discussion

In order to significantly reduce the incidence of foodborne outbreaks, food chain surveillance is necessary (Official journal of the European Union 2004). Although standard protocols based on microbiology to detect foodborne pathogens are available (ISO 11290–1 norm 1996; ISO 16654 norm 2001; ISO 6579 norm 2002; ISO 10272 norm 2006), there are time consuming, labour intensive and target only single pathogenic bacteria. On the contrary, real-time PCR is much more rapid to detect foodborne pathogens (Postollec et al. 2011), and several assays have already been developed and published (e.g. Amagliani et al. 2010; Chen et al. 2000; Fukushima et al. 2009; Garrido et al. 2012a; Hein et al. 2006; Lund et al. 2004; McCabe et al. 2011; Perelle et al. 2004; Sharma 2002; Sharma and an-Nystrom 2003). Unfortunately, these assays are not harmonized: they are using different chemistry (SYBR®green or TaqMan®), different protocols and different validation procedures, hampering their use as coherent detection system. Moreover, these assays target a single DNA sequence, often virulence or associated virulence genes, potentially leading to false negative signal when the target is mutated or absent. Such drawbacks could be avoided in this study. For instance, the primers for *invA* and *iroB* were successfully degenerated to better amplify *S. bongori* and *S. enterica houtenae*, respectively, illustrating the variability of target genes. Also, comparison of different *Salmonella* qPCR detection kits demonstrated that single target kits were unable to detect *S. bongori* or an atypical *S. enterica enterica* Senftenberg lacking the entire Pathogenicity Island I (Hu et al. 2008) (Table 5). Therefore, strategy targeting several genes is highly advisable for the appropriate and efficient detection of pathogenic bacteria.

In this study, seven simplex qualitative detection SYBR® Green qPCR assays, targeting all *Salmonella* spp., one of the

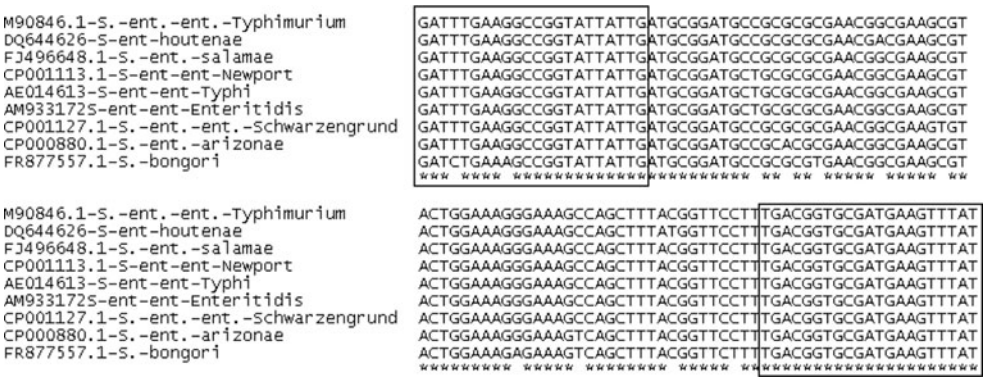

Fig. 1 Alignment of *invA* amplicon from different *Salmonella* species and subspecies

most important foodborne pathogen (European Food Safety Authority, European Centre for Disease Prevention and Control 2013), were successfully developed and in-house validated. Considering that all *Salmonella* spp. are pathogenic for human, these assays were developed targeting five distinct loci (*invA*, *rpoD*, *iroB*, safC and STM0296) for a full coverage of all the *Salmonella* species, subspecies and serovars. These assays were developed and validated, to be run simultaneously with the same PCR program, according a harmonized standard procedure described in Barbau-Piednoir et al. (2013).

The seven assays allowed a three-level detection system for the discrimination between the different species and subspecies of S*almonella* (Table 4). Two assays allow the detection of the *Salmonella* genus bacteria based on *invA* and *rpoD* detection. Two assays permit the specific identification of the species *S. enterica* based on *iroB* detection. A third level is composed of two assays based on *safC* and an additional one targeting the STM0296 sequence. The last assay was developed to detect *S. enterica enterica* serovars presenting a deletion of *safC*. This deletion seems to be relatively frequent: present in three serovars (Senftenberg, Krefeld and Namur) among the 55 tested. This type of operon deletion was also reported in den Bakker et al. (2011) and hypothetically explained by a selective pressure. The three assays (*safC*-10, *safC*-13 and STM0296) specifically and exhaustively detected *S. enterica* subsp. *enterica* (Table 4), amplifying all the serovars tested in this study (Table 1).

The SYBR®Green chemistry was preferred to the commonly used TaqMan® chemistry since SYBR®Green assays present the advantage of allowing the use of only two primers in the conserved region surrounding the more polymorphic sequence. As shown in Fig. 1, the design of a TaqMan® probe between *invA-5* primers would have been impossible as polymorphisms are observed between all the *Salmonella* species and subspecies. In addition, SYBR®Green assays are cheaper and permit post-amplification verification of the amplicon specificity by melting temperature analysis (Postollec et al. 2011).

The seven SYBR®Green qPCR assays for *Salmonella* detection developed and validated in this paper could be combined with the four assays for *Listeria* detection previously described (Barbau-Piednoir et al. 2013) and run all together on a single plate. Since this approach is modular, detection assays can be added to detect a wider range of foodborne pathogens or emerging pathogens. The only requirement is to develop an assay able to be run with the same conditions, allowing its use in high throughput on the same 96-well plate.

Combined with a decision support system (Van den Bulcke et al. 2008; 2009), such detection method represents a very useful approach in managing the experimental analysis of samples for a high-quality screening system and a remarkable food surveillance tool which can be modulated in response to the laboratory needs. It will also considerably reduce the time and the cost of a sample analysis. Such simultaneous detection may be extremely useful when a global screening and rapid identification of the foodborne pathogens is requested, as in the case of a bio-emergency or outbreaks of unknown origin.

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