Development of a Real-Time PCR Assay to Specifically Detect *Salmonella* Typhimurium

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Introduction

Salmonella is a leading cause of foodborne illness worldwide, with poultry and pork products being a primary source of infection to humans. *Salmonella enterica* subsp. *enterica* is the most clinically significant subspecies, causing 99% of *Salmonella* infections. This subspecies is further sub-divided into more than 2,500 serovars among which Typhimurium and Enteritidis are the most frequently associated with foodborne outbreaks. In 2011, a mandatory testing was implemented in the EU, requesting that all fresh poultry must be examined for *S.* Typhimurium adulteration. The most widely-used method used to characterize *Salmonella* is Kauffman-White serotyping, requiring up to 5 days according to the ISO 6579 method. qPCR is a fast, sensitive and specific alternative to conventional serotyping. However, due to their close genetic relatedness, finding a signature unambiguously characterizing a small subset of serovars is a challenging task. Here we describe a novel PCR assay relying on a unique sequence identified by genome-wide comparison enabling the specific detection of *Salmonella* Typhimurium, including monophasic variants.

Methods

Design of the assay

We aimed at developing a novel qPCR assay for the detection of *Salmonella* Typhimurium in food samples. Several genes such as FliC (Hadjinicolaou et al., 2009), STM4492 (McCarthy et al., 2009), STM4497 or STM2755 (Shanmugasundaram et al., 2009) were previously proposed as suitable targets to identify Typhimurium, however, none of the published assays yielded satisfactory results under our qPCR conditions. Therefore, we used a comparative genomics method based on 8 full Typhimurium target genomes and 21 non Typhimurium non-target genomes to identify a unique signature of target (Ho et al., 2012). Primers and probes were designed according to iQ-Check® PCR assays specifications and validated against Genbank database. An internal control was designed consisting in an heterologous target sequence amplified with primer identical to the ones of the *S.* Typhimurium assay.

Specificity

The specificity of the qPCR assay was validated on a comprehensive panel of inclusivity and exclusivity strains. Briefly, strains were isolated on TCS or BGA media and one colony was suspended in 500 µl of sterile water. Five µl of this suspension were tested using the novel *Salmonella* Typhimurium qPCR assay. All *Salmonella* strains were also tested using the iQ-Check *Salmonella* II kit which was previously developed for *Salmonella* screening in food and environmental samples.

Sensitivity

The sensitivity of a qualitative qPCR method can be expressed as the limit of detection (LOD_{PCR}) which can be approximated by the lowest amount of genome units among a series of dilutions yielding a positive signal with a 90% threshold for 10 replicates (Broeders et al., 2014). One colony of *Salmonella* Typhimurium RDCM 118 strain was grown at 37°C in TCS and serial dilutions were prepared. Ten DNA extractions were performed for each dilution using Easy I iQ-Check protocol and amplified with both iQ-Check *Salmonella* II® kit and the new *Salmonella* Typhimurium assay.

Performance on food samples

Pork samples were prepared using the same protocol as for testing with iQ-Check *Salmonella* 2 kit. Briefly, 25 g samples were contaminated using 100 µl of a *Salmonella* Typhimurium (ATCC 14028) suspension which concentration was estimated at 100 CFU/ml. Samples were enriched for 20 hr at 37°C in 225 ml of Buffered Peptoned Water (BPW). Bacteria DNA was subsequently extracted from a 100 µl aliquot of enriched media using iQ-Check *Salmonella* II kit Easy protocol. 5 µl of the DNA extract were tested with the novel *S.* Typhimurium assay. All Real-Time PCR assays were run on the CFX96 Touch™ Deep Well instrument.

Results

In silico comparison of target and non-target genomes yielded a unique 1388bp sequence that presented a perfect identity with all *S.* Typhimurium genomes. Importantly the 4,[5],12:i:- genome corresponding to monophasic *S.* Typhimurium variant was adequately detected. It is noteworthy that although serovars Aqua, Derby, Give, SaintPaul, Serenga, Tallahasse, Uganda and Wandsworth shared partial identity, no serovars known to be highly prevalent in food were identified as putative cross-reaction. No non-*Salmonella* strains returned significant identity.

Table 1. Comparison of PCR detection of relevant *Salmonella* serovars with both *Salmonella* spp. PCR and simplex *Salmonella* Typhimurium PCR assays.

	iQ-Check *Salmonella* spp. II assay			iQ-Check *Salmonella* Typhimurium assay		
Serovars	Target Cq	Internal control Cq	Result	Target Cq	Internal control Cq	Result
Negative ctrl	**N/A**	32.84	Negative	N/A	32.49	Negative
Positive ctrl	31.72	32.24	Positive	31.95	31.78	Positive
Typhimurium	**19.69**	**34.60**	**Positive**	**19.36**	**33.43**	**Positive**
Monophasic Typhimurium	**18.35**	**N/A**	**Positive**	**18.08**	**34.20**	**Positive**
Enteritidis	20.34	32.72	Positive	N/A	32.15	Negative
Infantis	20.86	32.40	Positive	N/A	31.78	Negative
Virchow	19.71	33.28	Positive	N/A	32.00	Negative
Hadar	21.20	32.29	Positive	N/A	31.89	Negative
Paratyphi B Java	20.24	33.27	Positive	N/A	31.91	Negative
Livingstone	20.84	33.21	Positive	N/A	32.38	Negative
Kentucky	18.67	34.26	Positive	N/A	32.17	Negative
Dublin	21.24	32.68	Positive	N/A	31.99	Negative
Newport	20.23	32.67	Positive	N/A	31.98	Negative

A 122 bp amplicon encompassing part of the unique signature, with minimum coverage of the partial identity observed for non *S.* Typhimurium serovars was selected and adequate primer/probes were designed. A series of *Salmonella* serovars, including Typhimurium as well as some of the most relevant serovars for the food industry according to data recorded from recent outbreaks in the US were tested (Jackson et al., 2013). As a control, all serovars were tested in parallel with the iQ-Check *Salmonella* spp. II assay. As shown in Table 1, all serovars tested yielded positive results with the *Salmonella* species assay. In contrast, only *Salmonella* Typhimurium was adequately detected with the new qPCR assay. Furthermore, monophasic *S.* Typhimurium, a growing threat to the food industry, was also adequately detected. Direct comparison of the Cq obtained with both assays demonstrated that the sensitivity of the *S.* Typhimurium assay was comparable to the one of the *Salmonella* spp. assay, thereby suggesting that new *Salmonella* Typhimurium assay is well suited for either confirmatory or direct screening on samples.

Table 2. *Salmonella* enterica subspecies and serovars tested with the simplex *Salmonella* Typhimurium PCR assay.

Abaetetuba	Bredeney	Fischerkietz	Indiana	Mbandaka	Paratyphi B java	Taksony
Aberdeen	Budapest	Ferruch	indica	Muenchen	Postdam	Tallahassee
Adelaïde	California	Give	Inverness	Montevideo	Poona	Tournai
Agama	Cerro	Gaminara	Johannesburq	Moscow	Puttin	Tenessee
Albany	Carrau	Gallinarum	Infantis	Napoli	Quentin	Thompson
Anatum	Canoga	Glostrup	Kentucky	Nienstedten	Rostock	Treforest
arizonae	Crossness	Grumpensis	Kirkee	Naestved	Salamae	Tranoroa
Bambylor	Cubana	Grabow	Kottbus	Newport	Rubislaw	Utrecht
Bareilly	Choleraesuis	Goldgoast	Kedougou	Nottingham	Senftenberg	Virchow
Berta	diarizonae	Havana	Lomita	Oranienburg	Saint Paul	Zuerich
Betioky	Dalhem	Hadar	Livingstone	Ouakam	Schwarzengrund	Yoruba
Blegdam	Derby	Guinea	Manica	Okatie	Singapore	Wayne
Blockley	Dublin	Havanna	London	Ohio	Sheffield	Worthington
bongori	Emek	houtenae	Miami	Phoenix	Sundsvall	
Braenderup	Duisberg	Illinois	Minnesota	Panama	Springs	
Brandenburg	Enteritidis	Heidelberg	Maregrosso	**Paratyphi B***	Strasbourg	

A study of the assay specificity was performed on a series of *Salmonella* serovars. Results are shown in Table 2. All serovars tested yielded negative results, except one *S.* Parathyphi B which cross-reacted. Nevertheless, the *Salmonella* Parathyphi B variant Java, of peculiar relevance to food samples, was not detected, thus strengthening the conclusion that the assay displays a very good specificity. A series of non *Salmonella* bacteria was also tested with the qPCR assay. As shown in Table 3, none of the 39 species were detected, thereby confirming the remarkable selectivity for a simplex assay.

Table 3. Exclusivity study with non-salmonella bacteria. All species yielded negative results.

Acinetobacter baumanii	*Campylobacter upsaliensis*	*Enterobacter amnigenus*	*Listeria monocytogenes*	*Shigella flexneri*
Aeromonas hydrophila	*Citrobacter freundii*	*Enterobacter cowanii*	*Micrococcus luteus*	*Shigella sonnei*
Aeromonas hydrophila/caviae	*Cronobacter sakazakii*	*Enterococcus faecium*	*Pantoea agglomerans*	*Staphylococcus aureus*
Bacillus licheniformis	*Enterobacter cloacae*	*Escherichia coli*	*Proteus mirabilis*	*Staphylococcus*
Bacillus cereus	*Enterobacter pyrinus*	*Escherichia hermanii*	*Pseudomonas fluorescens*	*internmedius*
Campylobacter jejuni	*Enterobacter sakazakii*	*Hafnia alvei*	*Pseudomonas aeruginosa*	*Staphylococcus xylosus*
Campylobacter coli	*Enterobacter aerogenes*	*Klebsiella oxytoca*	*Raoultella terrigena*	*Staphylococcus epidermidis*
Campylobacter lari	*Enterobacter asburiae*	*Klebsiella pneumoniae*	*Serratia marcescens*	*Yersinia enterocoloitica*

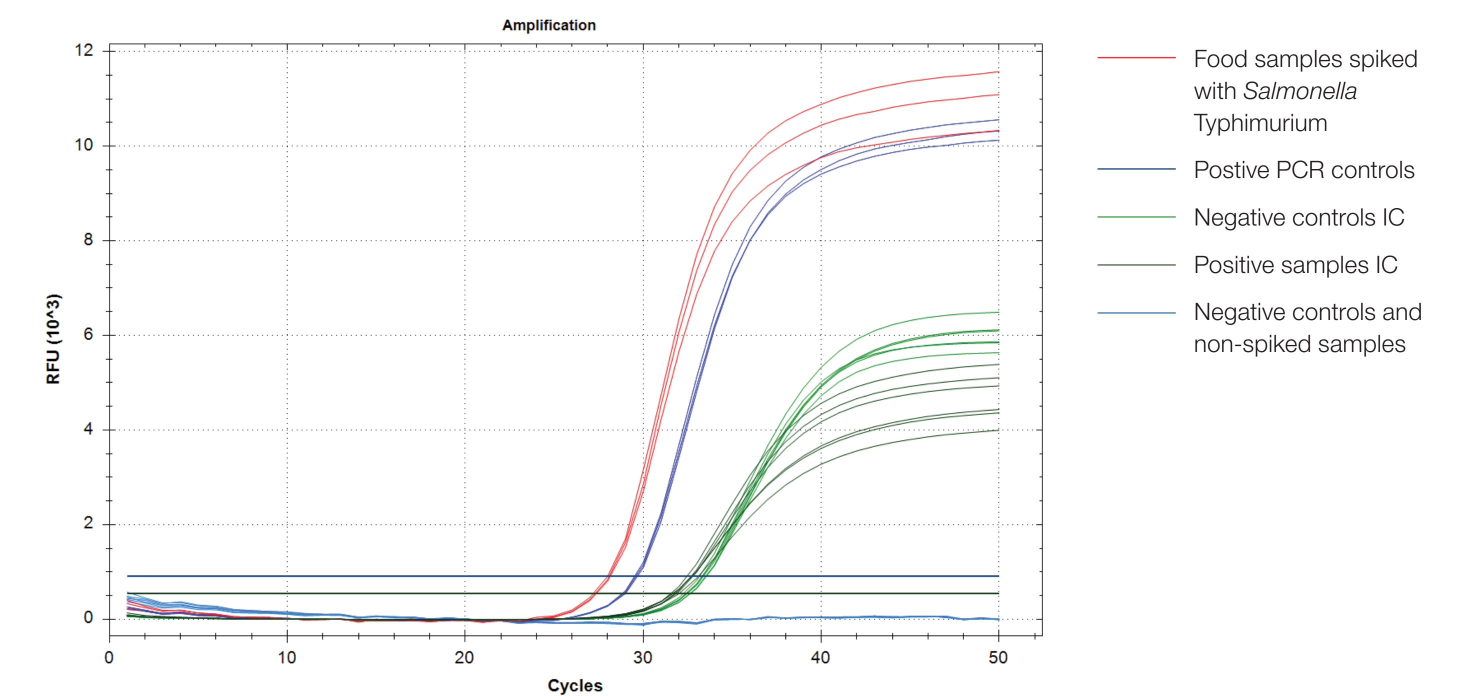
The LOD_{PCR} of the *Salmonella* Typhimurium assay was compared to the one of the iQ-Check *Salmonella* II PCR screening assay by testing 10 independent replicates of a series of dilutions of a culture of *Salmonella* Typhimurium. As shown on Table 4, 100% detection was repeatedly obtained for 10^3 to 10^7 fold dilutions from initial bacterial culture. The limit of detection of the test as determined by the 90% threshold was similar for both assays. This result is consistent with an LOD close to 100CFU/mL.

Table 4. Comparison of iQ-Check *Salmonella* II and *Salmonella* Typhimurium PCR assays LODs

	Number of positives	
-Fold dilutions from initial culture	*Salmonella* Typhimurium assay	*Salmonella* spp. assay
1.00E+08	9	9
1.00E+07	10	10
1.00E+06	10	10
1.00E+05	10	10
1.00E+04	10	10
1.00E+03	10	10

Finally, a full assay was designed which contains a synthetic positive control and an internal control to rule out the possibility of false negative results due to inhibitory effect of the food matrix. The novel qPCR assay was tested on food sample consisting in 25 gr pork meat portion spiked with approximately 10CFU of *Salmonella* Typhimurium (ATCC 14028). Results of three independent food samples are shown in Figure 1. Both positive controls and spiked samples were adequately detected. Internal controls were correctly amplified indicating that there was no inhibition due to the food matrix. Negative controls and non-spiked samples did not show any amplification. Note that preliminary data also indicate compatibility with the iQ-Check *Salmonella* protocol for environmental primary production samples testing (data not shown).

Figure 1. Detection of *Salmonella* Typhimurium in Food samples with the simplex PCR assay.



Conclusions

We had previously developed a real-time PCR method allowing specific detection of *Salmonella* Typhimurium that can be used either as a rapid typing step after the detection of *Salmonella* spp or as a direct combined screening & typing method. This assay was based on a duplex PCR system, resulting in 100% inclusivity on 84 *Salmonella* Typhimurium strains, including monophasic variants, and 93% exclusivity on 214 *Salmonella* non-Typhimurium strains. We have now developed a novel version of the test that relies on the detection of a unique target, which was identified using a strategy of whole genome alignment and subtraction of regions of similarity. This simplified qPCR assay displays 100% inclusivity on all *S.* Typhimurium strains tested including monophasic variant and >99% on 254 non Typhimurium *Salmonella* strains. First results indicated that the simplex assay was capable of detecting specifically *Salmonella* Typhimurium in food samples with sensitivity comparable to the one of the *Salmonella* spp. assay. Additional field evaluations are currently ongoing, especially in the poultry industry.

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