A Novel Simplex Real-Time PCR Assay for Rapid Molecular Detection and Typing of *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 serotypes defined by somatic and flagellar antigens. *Salmonella* enterica subsp. enterica Typhimurium and *Salmonella* enterica subsp. enterica Enteritidis are the most frequently reported serotypes associated with human cases of *Salmonella* infection from foodborne outbreaks. In 2011, mandatory testing was implemented in the EU, requiring that all fresh poultry must be examined for *S.* Typhimurium adulteration. The most widely-used method used to characterise *Salmonella* serotypes is the Kauffman-White serotyping system, based on the variability of the O, H and Vi antigens. The current method for isolation and characterization of *Salmonella* from food samples takes up to 5 days according to the ISO 6579. Conversely, qPCR is a fast, sensitive and specific alternative to conventional serotype identification. However, due to their close genetic relatedness, finding a signature unambiguously characterizing a small subset of serotypes is a challenging task, justifying that serotype-specific assays most often resort to multiplex PCR. Here we describe a novel PCR assay relying on a unique sequence identified by genome-wide comparison method enabling the specific detection of *Salmonella* Typhimurium, including monophasic variants.

Methods

Identification of a novel marker

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, our first goal was to identify a novel genetic marker which would enable specific and reliable detection of *Salmonella* Typhimurium. Eight full Typhimurium genomes were selected as targets and 21 non Typhimurium genomes were selected as non-target sequences. We then used a comparative genomics method (Ho et al., 2012) to identify a set of unique signatures of target genomes.

Inclusivity / exclusivity

Primers and probes were designed according to iQ-Check® PCR assay specifications and validated against Genbank database. An internal control was designed consisting of a heterologous target sequence amplified with primer identical to the ones of the *S.* Typhimurium assay. The specificity of the qPCR assay was validated on a comprehensive panel of inclusivity and exclusivity strains. *Salmonella* strains were obtained from the field and from Bio-Rad's internal strain collections. Non-*Salmonella* strains were from Bio-Rad's internal collection. Briefly, strains were isolated on TCS or BGA media and one colony was then suspended in 500 µl of sterile water. Five µl of this suspension were then tested using the novel *Salmonella* Typhimurium qPCR assay consisting in a mix of 40 µl of a dedicated amplification solution with 5 µl of primer/probe solution. All *Salmonella* strains were also tested using the iQ-Check *Salmonella* II kit which was previously developed for *Salmonella* spp. screening in food and environmental samples.

Salmonella Typhimurium qPCR assay on food samples

Pork samples were prepared using the same protocol as for testing with iQ-Check *Salmonella* II kit. Briefly, 25g samples were contaminated using 100 µl of a *Salmonella* Typhimurium (ATCC 14028) suspension which concentration was estimated at 100 CFU / mL by turbidimetry and confirmed by plating on TCS medium. Samples were enriched for 20 h at 37°C in 225 mL of Buffered Peptone 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.

Results

Identification of a novel marker

In silico comparison of target and non-target genomes yielded a set of unique sequences specific of the *S.* Typhimurium targets. All sequences identified were aligned against both the whole Nr/rt and *Salmonella* WGS Genbank databases to identify sequences showing perfect identity shared among all target genomes. One 1490 bp sequence was eventually selected, that presented a perfect identity with all *S.* Typhimurium genomes while serotypes Aqua, Derby, Give, SaintPaul, Serenga, Tallahasse, Uganda and Wandsworth shared partial identity. No other *Salmonella* or non-*Salmonella* strains returned significant identity. This result was of particular interest as the number of putative cross-reactions was limited as compared to the number of known serotypes. Furthermore, no serotypes known to be highly prevalent in food environment in particular serotypes of high concern such as S. Livingstone, Enteritidis, Infantis, Hadar, Virshow, Dublin were identified as putative cross-reaction. It was also important to note that the 4,[5],12:i:- genome corresponding to monophasic *S.* Typhimurium variant was adequately detected. We performed a round of primer/probe optimization to select an adequate amplicon of 122 bp encompassing part of the unique signature, with minimum coverage of the partial identity observed for non *S.* Typhimurium serotypes. Primer/probes physicochemical parameters were chosen to match the thermo-profile used for the commercial iQ-Check *Salmonella* spp. assay.

We first tested a series of *Salmonella* serotypes, including Typhimurium as well as some of the most relevant serotypes for the food industry according to data recorded from recent outbreaks in the US (Jackson et al., 2013). As a control, all serotypes were tested in parallel with the iQ-Check *Salmonella* spp. assay, dedicated to the *Salmonella* spp. screening of food and environmental samples. As shown in Table 1, all serotypes tested yielded positive results with the *Salmonella* species assay. In sharp contrast, only *Salmonella* Typhimurium was adequately detected with the new simplex 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 of samples.

Table 1

	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**	**34,2**	**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	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

Table 1. Comparison of PCR detection of relevant *Salmonella* serotypes with both *Salmonella* spp. PCR and simplex *Salmonella* Typhimurium PCR assays. Only Typhimurium serovars were adequately detected. Cq obtained with both assays were similar, thus demonstrating that the Typhimurium assay achieved a comparable sensitivity to the one of the *Salmonella* spp. assay.

Table 2

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

A comprehensive study of the assay specificity was performed on *Salmonella* serotypes consisting in strains from Bio-Rad library and field strains. Results are shown in Table 2. All serovars tested yielded negative results, except one S. Parathyphi B which cross-reacted. Nevertheless, the S. Parathyphi B variant Java, of peculiar relevance to food samples, was not detected. Moreover, the serotypes such as S. Give, Talahasse and SaintPaul who showed partial identity to the signature, hinting to possibly cross-reactions, yielded negative results, thus reinforcing the conclusion that the assay was very specific.

Table 2. *Salmonella* enterica subspecies and serotypes tested with the simplex *Salmonella* Typhimurium PCR assay. *Salmonella* enterica subspecies (italics) *Salmonella* enterica subsp. enterica serotypes (plain text) yielded negative results, excepted one strain of Paratyphi B.

Table 3

Acinetobacter baumanii	*Enterobacter sakazakii*
Aeromonas hydrophila	*Enterobacter aerogenes*
Aeromonas hydrophila/caviae	*Enterobacter asburiae*
Bacillus licheniformis	*Enterobacter amnigenus*
Bacillus cereus	*Enterobacter cowanii*
Campylobacter jejuni	*Enterococcus faecium*
Campylobacter coli	*Escherichia coli*
Campylobacter lari	*Escherichia hermanii*
Campylobacter upsaliensis	*Hafnia alvei*
Citrobacter freundii	*Klebsiella oxytoca*
Cronobacter sakazakii	*Klebsiella pneumoniae*
Enterobacter cloacae	*Listeria monocytogenes*
Enterobacter pyrinus	*Micrococcus luteus*

A series of non-*Salmonella* bacteria was also tested with the qPCR assay. As shown on Table 3, none of the 39 species were detected, thus demonstrating a remarkable selectivity for a simplex assay.

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

Finally, a full assay was designed which contains 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 samples consisting of 25 g pork meat portion spiked with approximately 10 CFU of *Salmonella* Typhimurium (ATCC 14028). A positive and a negative control were run in parallel. Results of three independent 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

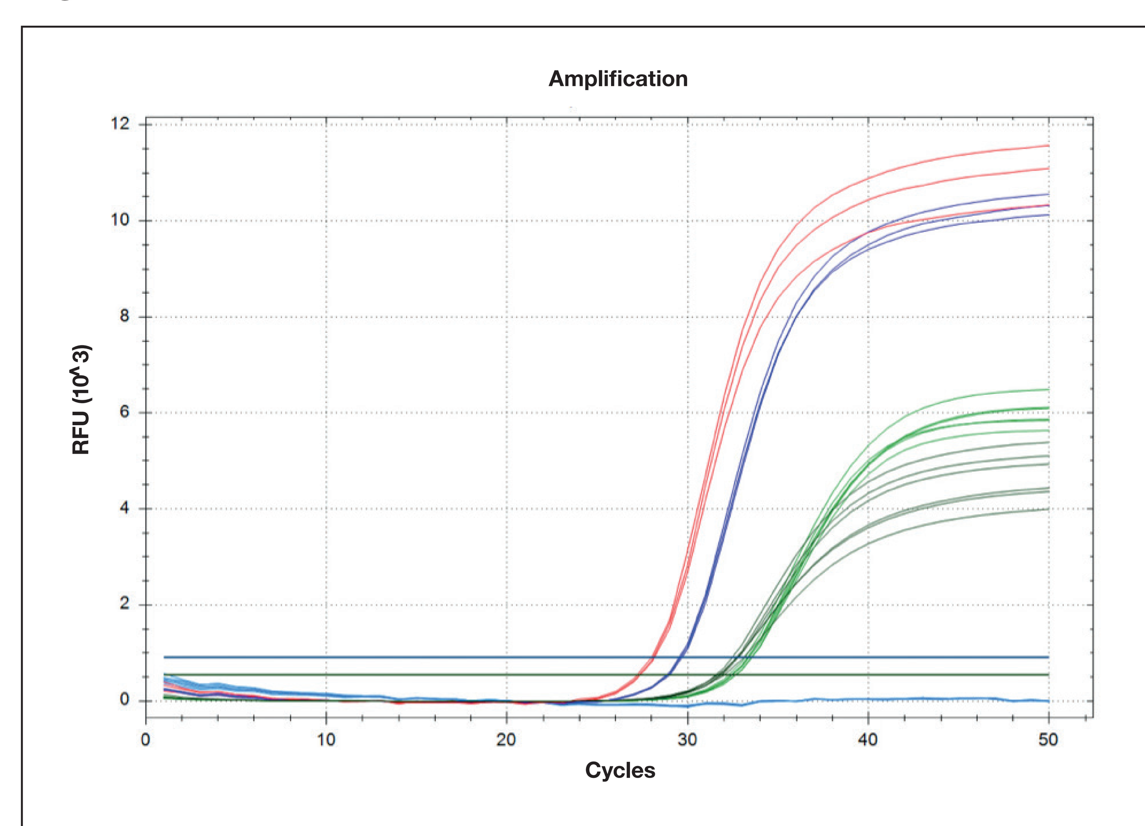

Figure 1. Detection of *Salmonella* Typhimurium in Food samples with the simplex PCR assay.
Food samples spiked with *Salmonella* Typhimurium (Red); Positive PCR controls (dark blue); Negative controls IC (light green); Positive samples IC (dark green); negative controls and non-spiked samples (light blue).

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. A time-to-result shorter than the one of standard method is therefore expected, fostering a faster release of products free of contamination. Additional field evaluations are currently ongoing, especially in the poultry industry. Altogether, the performance of qPCR assay should be a key attractive criterion for food chain operators for contributing to the control of this major public health concern.

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