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Molecular detection assay of five Salmonella serotypes of public interest: Typhimurium, Enteritidis, Newport, Heidelberg, and Hadar

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ABSTRACT

Foodborne illnesses due to Salmonella represent an important public-health concern worldwide. In the United States, a majority of Salmonella infections are associated with a small number of serotypes. Furthermore, some serotypes that are overrepresented among human disease are also associated with multi-drug resistance phenotypes. Rapid detection of serotypes of public-health concern might help reduce the burden of salmonellosis cases and limit exposure to multi-drug resistant Salmonella.

We developed a two-step real-time PCR-based rapid method for the identification and detection of five Salmonella serotypes that are either overrepresented in human disease or frequently associated with multi-drug resistance, including serotypes Enteritidis, Typhimurium, Newport, Hadar, and Heidelberg. Two sets of four markers were developed to detect and differentiate the five serotypes. The first set of markers was developed as a screening step to detect the five serotypes; whereas, the second set was used to further distinguish serotypes Heidelberg, Newport and Hadar. The utilization of these markers on a two-step investigation strategy provides a diagnostic specificity of 97% for the detection of Typhimurium, Enteritidis, Heidelberg, Infantis, Newport and Hadar. The diagnostic sensitivity of the detection makers is >96%. The availability of this two-step rapid method will facilitate specific detection of Salmonella serotypes that contribute to a significant proportion of human disease and carry antimicrobial resistance.

1. Introduction

Foodborne diseases in general, and salmonellosis in particular, result in an important economic burden worldwide due to employee absenteeism, treatment, hospitalization, and mortality, with *Salmonella* reported to cause the largest burden on public health in the United States (Batz et al., 2012; Scallan et al., 2011). Because of its zoonotic nature, *Salmonella* also results in substantial economical and animal losses for farmers (Scallan et al., 2011; Scharff, 2012). Further, the ability of *Salmonella* to proliferate in a large variety of conditions makes it able to contaminate a wide variety of food products contributing to its importance in public health.

The United States Department of Agriculture-Food Safety and Inspection Service (USDA-FSIS) dictates that *Salmonella* is an adulterant in ready-to-eat meat and poultry products and therefore has established a required testing policy regarding this pathogen (FDA, 2011a, 2011b, 2014a). Meat and poultry are subject to a zero tolerance policy, in regard to visible fecal contamination on carcass surfaces during the slaughtering process (USDA:FSIS, 1998). *Salmonella* is not normally considered an adulterant, but rather, its presence in various raw products is used as a performance standard in order to evaluate the effectiveness of sanitary slaughter procedures (USDA:FSIS, 2011). Furthermore, as part of the national school lunch program (NSLP), the agricultural marketing service (AMS) of the USDA requires ground beef suppliers to adhere to strict tolerances for *Salmonella*, that is used as measurement of food safety. Regarding meat products supposed to enter the NSLP, *Salmonella* is considered as an adulterant in raw ground beef, and trim (Ollinger et al., 2014). Performance of raw chicken suppliers for the NSLP has also improved regarding *Salmonella* contamination (Ollinger et al., 2015).

Salmonella is a highly diverse genus, which is divided into two species: *S. enterica* and *S. bongori*. *S. enterica* is the most diverse, frequently encountered, and is subdivided into 6 subspecies: *enterica* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV), and *indica* (VI), with the majority of *Salmonella* serotypes belonging to *S. enterica* subsp. *enterica* (>1500) (Grimont and Weill, 2007). Because of its status of adulterant in ready-to-eat meat and poultry products, multiple molecular assays have been developed to detect the presence of *Salmonella* genus strains on food samples. Such molecular assays are often based on the detection of conserved genes, such as *invA* or *ttrC* (Bugarel et al., 2011; Chiu and Ou, 1996; Soto et al., 2006; Wang et al., 1997). However, despite this large diversity, only a small number of serotypes are

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overrepresented in human clinical cases. The national surveillance annual summary, published by the Centers for Disease Control and Prevention (CDC), identified the three serotypes: Enteritidis, Typhimurium and Newport, to be the most frequently encountered and to cause the greatest disease burden on consumers in the United States between 1999 and 2009. Together, these 3 serotypes represented 41.8% and 44% of the clinical isolates reported by the CDC in 2009 (CDC, 2011) and by the CDC-FoodNet in 2013 (CDC-FoodNet, 2013), respectively. Furthermore, the CDC reported that the four most threatening serotypes of *Salmonella enterica* to public health are Typhimurium, Newport, Hadar and Heidelberg, because of their association with multidrug resistance (MDR) (CDC, 2011). The contamination of food products with MDR pathogenic bacterial strains represents an important public health concern. Antibiotic resistant foodborne pathogens can be associated with an increased risk of hospitalization for infected people. Furthermore, a large proportion of resistance mechanisms are encoded on genetic mobile elements, which can be transmitted horizontally to other bacteria potentially present in the intestinal tract. More specifically recent reports show that a high proportion of *Salmonella* strains belonging to Heidelberg, Newport and Typhimurium serotypes from retail meat and poultry products are multi-drug resistant (CDC, 2014b; FDA, 2014a, 2014b).

The CDC considers MDR *Salmonella* a serious threat that requires rapid and efficient actions to avoid aggravation of the situation (CDC, 2014a, 2014b). In response, a petition declared specific MDR strains of *Salmonella* to be considered adulterants in raw ground meat and poultry products, including *S.* Heidelberg, *S.* Hadar, *S.* Newport and *S.* Typhimurium (Citizen-Petition, 2011; DeLauro and Slaughter, 2014). Raw meat products are highly perishable and a decision on how to handle the product has to be made based on a rapid method to detect the presence or absence of molecular markers by screening a sample directly after selective enrichment. Assays have been already developed for the overall detection of *Salmonella* genus. However, there is no existing rapid method to screen sample enrichments to detect and identify these *Salmonella* serotypes. Between March 2013 and July 2014, a large outbreak involving 634 persons from 29 different states and Puerto Rico was reported. This major outbreak involving seven strains of MDR *S.* Heidelberg was linked to the consumption of poultry products (CDC, 2014a, 2014b). This outbreak highlighted the need for specific and rapid methods to detect MDR *Salmonella* serotypes associated with a significant public health burden to facilitate outbreak detection and microbial source tracking to shortly identify the food vehicle responsible for disease and mitigate the dissemination of such threatening pathogens.

Multiple molecular assays to determine the serotype of *Salmonella* isolates have already been developed (Fitzgerald et al., 2007; Franklin et al., 2011; Herrera-Leon et al., 2007; Luk et al., 1993; McQuiston et al., 2004; Mortimer et al., 2004; Yoshida et al., 2016). However, these assays are based on the identification of the somatic and flagellar antigens, which determine antigenic formula thus following the Kauffmann-White-Le Minor scheme (KWM). The KWM scheme summarizes *Salmonella* nomenclature and all the *Salmonella* antigenic formulae encountered to date, encompassing >2600 serotypes (Grimont and Weill, 2007).

Currently, 46 somatic and 114 flagellar antigens have been identified in *Salmonella* genus. Genes involved in the expression of somatic antigens are grouped into a large regulon called the *rfb* cluster. In particular, this cluster contains *wzx* and *wzy* genes encoding for O-antigen flippase and polymerase, respectively. These genes are frequently targeted for the design of serogrouping markers in molecular assays mimicking the KWM scheme (Fitzgerald et al., 2007; Herrera-Leon et al., 2007; Luk et al., 1993). Genes encoding for the flagellin structural proteins are *fliC* (phase 1 flagellin) and *fljB* (phase 2 flagellin). These genes are also frequent targets for the identification of the flagellar variants in molecular serotyping protocols following the KWM scheme (Franklin et al., 2011; McQuiston et al., 2004; Mortimer et al., 2004). Major drawbacks of this kind of molecular serotype determination approaches are linked to their complexity as they are following the KWM workflow. Furthermore, given that they require the utilization of several markers for the identification of a serotype they have to be used on isolated strains and not directly on complex matrices, such as food products or human stools.

In this study, we focused on the development of single markers to detect and identify five important *Salmonella* serotypes chosen based on their association with human disease, their potential role in spreading antimicrobial resistance, and their potential future involvement in regulation.

We developed a two-step rapid detection and identification method for *Salmonella* serotypes: Typhimurium, Heidelberg, Newport and Hadar as a tool to address the petition in the event it spurs regulatory action. In addition, we included *S.* Enteritidis in our method, although primarily characterized as pan-susceptible to tested antimicrobials, this serotype alone accounts for 17.5% of human cases and it is frequently associated with large multi-state outbreaks of foodborne illness attributed to poultry products (CDC, 2011). The present study focusses on the evaluation of these newly developed markers as direct detection assay of these five important *Salmonella* serotypes on complex matrix such as ground beef samples.

2. Material and methods

2.1. Strain collection

A total of 447 strains belonging to 149 serotypes were investigated in this study. The characteristics of these strains are presented on Supplemental Table 1. Of these, 205 were from the Food Microbe Tracker strain collection at Cornell University (Ithaca, New York), 82 were supplied by the French Agency for Food, Environmental and Occupational Health & Safety (Anses, at Maisons-Alfort, France) and had been isolated from food products in France, and the remaining were available to us in the isolate collection of the International Center for Food Industry Excellence (ICFIE) laboratory in the department of Animal and Food Sciences at Texas Tech University (Lubbock, Texas).

All isolates used in this study had been previously serotyped using the conventional approach in France (Anses, Maisons-Alfort) or at Cornell University (Ithaca, NY) or using a hybrid method combining the phenotypic identification of somatic antigens and the molecular identification of flagellar antigens (USDA, Nebraska).

The investigated collection was composed of an inclusivity panel grouping together a minimum of 25 strains belonging to the five serotypes of interest: Enteritidis (n = 26), Typhimurium (n = 25), Newport (n = 30), Hadar (n = 25), and Heidelberg (n = 27), and an exclusivity panel containing 121 serotypes from *S. enterica* subsp. *enterica* together with few strains of the 5 other *Salmonella* subspecies. The exclusivity panel counted up to 5 strains for each of the 30 most prevalent serotypes in human salmonellosis according to the CDC report (CDC, 2011). Furthermore, we also included outside genus isolates from the *Enterobacteriaceae* family as well as similar organisms to *Salmonella enterica*. A total of 13 different non-*Salmonella* genera were included on this study.

2.2. DNA extraction methods

DNA extractions used in this study were either provided by the French Food Safety Agency or were performed either by the boiling extraction protocol or following the manufacturer's instructions of the GenElute Bacterial Genomic DNA kit (Sigma-Aldrich, St. Louis, MO). Genomic DNA extractions were quantified using the Nanodrop 2000c Spectrophotometer (ThermoScientific, Waltham, MA).

2.3. Primer and probe designs

Genome comparisons were performed in silico using BLAST with sequences available on NCBI GenBank in order to identify serotype-specific regions. Primers and hydrolysis probes were designed by hand on chromosome sequences, and as much as possible on annotated open reading frames on NCBI GenBank available sequences. Forward and reverse primers were designed with a Tm of 60 °C and probes with a Tm of 70 °C. Properties of designed primers (temperature, and secondary structures) were investigated using online software (https://www.idtdna.com/calc/analyzer, http://biotools.nubic.northwestern.edu/OligoCalc.html). Probes were labeled in 5′ with the HEX, Cy5 or FAM fluorophores and at their 3′ end with one of the following quenchers, as appropriate: BHQ1, BHQ2 or Iowa BRQ (Integrated DNA Technologies, Coralville, IA). The sequences and labels of the markers are listed in Table 1.

2.4. RNA extraction

Total RNA was extracted according to a modified protocol of Roberts and collaborators (Roberts et al., 2009). Briefly, overnight cultures of *Salmonella* were passaged by two 1% inoculum transfers and grown in flasks containing 50 mL of Brain-Heart Infusion (BHI) (Becton Dickinson, Franklin Lakes, NJ) broth to stationary phase (10 h post-inoculation) at 37 °C with shaking. Total RNA was isolated using RNA Protect (Qiagen, Venlo, The Netherlands), followed by a treatment with TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH), and then bromochloropropane (Sigma, St Louis, MO). Finally, the pellets of RNA were washed with a 75% ethanol solution, dried, treated with DNAse (Promega, Madison, WI), quantified using the Nanodrop 2000c Spectrophotometer (ThermoScientific, Waltham, MA), and stored at −80 °C.

2.5. Real-time PCR and reverse transcription real-time PCR conditions

Real-Time PCRs were performed in simplex using the following conditions: 1X Brilliant II QPCR low ROX master mix (Agilent Technologies, Santa Clara, CA), primers at a final concentration of 0.3 μM, hydrolysis probes at a final concentration of 0.6 μM, and 2.5 μL of the template DNA, for a total reaction volume of 25 μL. The reactions were carried out on a Stratagene Mx3005P thermocycler (Agilent Technologies, Santa Clara, CA), that was programmed as follows: an initial denaturation step at 95 °C for 10 min, then 35 cycles each of 30 s at 95 °C and 1 min at 61 °C. Reverse Transcriptase real-time PCR used 25 ng of total RNA extraction per reaction. The reactions were performed using the Brilliant II QRT-PCR low ROX 1-step master mix (Agilent Technologies, Santa Clara, CA) with the same conditions described for the Real-Time PCR and the following conditions of amplification: an initial reverse transcription step at 50 °C for 30 min, a denaturation step at 95 °C for 10 min, and then 40 cycles each of 30 s at 95 °C and 1 min at 61 °C.

Table 1
Sequences and efficiency of the screening and discrimination markers.

Primer and probe IDs	Sequence (5′… 3′)
Ent6-F	TCGTACCTGCTGATGCTGGG
Ent6-P	HEX-TATGCGCTGGTTCCGTTCCGTTTTCTGG-BHQ2
Ent6-R	AGGATGAAGACGGGTAATGTCC
Newp2-F	AATGGCTGGTAGCCTGTTCG
Newp2-P	Cy5-TCATGCTATGCACTGGGAACAATTTCTGGC-Iowa BRQ
Newp2-R	AGGGAAAGCAAGGAACAGTAG
STM2-F	AGATATTCCGTAGCAATTGAGTTG
STM2-P	FAM-TGTGTTCAAGCAATGGTGAACAAACATAATCCC-BHQ2
STM2-R	AATAGCTAAAAATGACTGGGACTC
Heid2-F	CCTGCAGAAAGATATGTTTGGC
Heid2-P	HEX-TTAATCTGTGCGACGAATTGGGCAGCC-BHQ2
Heid2-R	TGCGATGAAGATTGATGATGCC
Heidspe-F3	TCATGATTATGCCGAAATTATGCG
Heidspe-P3	HEX-AATGGTGGTTGGGATTGGGATAGGTCTC-BHQ2
Heidspe-R3	ATCATAAGAGACTAATTGCCCATC
Infspe-F2	AAGTTGCCCCCTTGAGTTCC
Infspe-P2	FAM-TGTTGCCAGCGGCATGAAGAAAATCCC-BHQ1
Infspe-R2	AATGTGCGCACGTTTGGTGG
Hadspe-F1	GAATCAAACCGCTTTATGGTGC
Hadspe-P1	FAM-ATCCGGCGCTGCATGGGTATATGACC-BHQ1
Hadspe-R1	TGCTCTTGAGCATCCCGTTG
Newpspe-F1	TGATGCTTTCTTATTATGAACAAGG
Newpspe-P1	HEX-ATTATTCTGAGCTAACGCCATCGCAGAGG-BHQ2
Newpspe-R1	TCTATAGGCATATGAATACTCGC

2.6. Efficiencies of the real-time PCR markers

Marker efficiency represents the rate at which a PCR amplicon is generated. To determine these efficiencies, standard curves using various concentrations of gDNA (10^7, 10^6, 10^5, 10^4, 10^3 and 10^2 copies of gDNA per reaction) from a set of positive control strains belonging to serotypes Newport, Enteritidis, Typhimurium, Hadar, Infantis, and Heidelberg, were performed. The number of gDNA copies was determined using the online program "Copy number calculator for real-time PCR" (http://scienceprimer.com/copy-number-calculator-for-realtime-pcr). This calculation is based on the amount of DNA added to the reaction and an average estimate of *Salmonella* genome length (4.5 Mb). PCR efficiency (E) is estimated using the following eq. $E = 10^{-(1/\text{slope})} - 1$. An efficiency of 100% represents is reached if an amplicon doubles in quantity during the reaction of amplification. This value is used to estimate the concentration of input target DNA before amplification using real-time PCR. The efficiency value represents an estimate of the overall performance of the real-time PCR assay.

2.7. Food sample detection

Twenty-five grams of ground beef were added to 225 mL of modified tryptone soya broth (mTSB) (USDA:FSIS, 2013) and inoculated with two concentrations of each reference strains belonging to the targeted serotypes: 1.02 and 0.1 CFU/g of ground beef for *S.* Typhimurium, 1.55 and 0.15 CFU/g of ground beef for *S.* Enteritidis, 1.82 and 0.18 CFU/g for *S.* Hadar, 2.52 and 0.25 CFU/g for *S.* Newport, and 1.04 and 0.1 CFU/g for *S.* Heidelberg. These concentrations were estimated by plating dilutions of the overnight culture of the reference strains used to inoculate the ground beef sample. Then, the enrichment bags were homogenized 2 min by hand and incubated at 42 ± 1 °C for 24 h. Three samples of 500 μL, 100 μL and 50 μL were used to extract DNA with a heat treatment at 97 °C for 15 min. Then real-time PCR was performed using 2.5 μL of these DNA extractions as described above.

2.8. Assay performance

In order to characterize the performances of the developed assays, we determined the diagnostic sensitivity and diagnostic specificity for each of them. The diagnostic sensitivity represents the ratio of positive samples that are identified as positive by the assay. The diagnostic specificity represents the ratio of negative samples that are identified as negative by the assay (Saah and Hoover, 1997).

The inclusivity panel size (number of strains belonging to the targeted serotype of each assay) is used as denominator for the calculation of the diagnostic sensitivity. The exclusivity panel size is used as denominator for the calculation of the diagnostic specificity. The exclusivity panel is composed of our entire DNA collection (n = 447) except the strains included in the inclusivity panel, which represent 403 strains for the STM2 marker, 421 strains for the Ent6 marker, 420 strains for the Heid2 marker and 392 for the Newp2 marker. The performance results are described in Tables 2 and 3.

Table 2
Summary of the performances of the screening markers.

Marker	Targeted serotypes	Efficiencies	Inclusivity panel	Exclusivity panel size[a]	Cross-reacting serotypes	Cross-reactivity[b] (%)	Diagnostic sensitivity[c]	Diagnostic specificity[d]
STM2	Typhimurium and its monophasic variants	86.7%	44/44	403		0%	1	1
Ent6	Enteritidis	82.5%	24/26	421		0%	0.96	1
Heid2	Heidelberg	62.2%	26/27	420	Infantis (11/11), Kintambo (1/1), *S*.I 4,5,12:r:- (1/1)	1.53%	0.96	0.97
Newp2	Newport and Hadar	89.8%	30/30 for Newport + 23/25 for Hadar	392	Bardo[e] (1/1), Istanbul[f] (1/1), Blockley (1/1), Glostrup (1/1), Bovismorbificans (1/1), Kottbus (1/1), Litchfield (1/1), Muenchen (3/9), Manhattan (2/2), Virchow (1/10)	3.58%	0.96	0.97

[a] Exclusivity panel size is determined by the number total of tested strains (n = 447) minus the strains of the inclusivity panel.
[b] At the serotype level, in human cases in the US between 1999 and 2009 according to the CDC report (CDC, 2011).
[c] Diagnostic sensitivity: number of tested positive strains/number of true positive strains (Saah and Hoover, 1997). Calculation based on the inclusivity panel.
[d] Diagnostic specificity: number of tested negative strains/number of true negative strains (Saah and Hoover, 1997). Calculation based on the effective of the exclusivity panel, represented by the total panel of strains (n = 447) – inclusivity panel. Size of each panel described in material and methods, paragraph 2.8.
[e] Not distinguishable from Newport according to Mikoleit et al. (2012).
[f] Not distinguishable from Hadar according to Mikoleit et al. (2012).

3. Results and discussion

3.1. Detection markers for the five serotypes of interest

We designed a set of four markers for the identification of isolates belonging to the serotypes Enteritidis, Typhimurium, Newport, Hadar, and Heidelberg. To do so, we targeted serotype-specific chromosomal sequences. We purposely avoided plasmid sequences in order to use stable target sequences.

The STM2 marker designed for the detection of *Salmonella* Typhimurium yielded positive results for all of the 25 investigated *S.* Typhimurium strains. This marker also detects all the strains of both *S.* Typhimurium flagellar variants: 1,4,[5],12:i:- strains (n = 17), and 1,4,[5],12:-:1,2 (n = 1) (Table 2).

In Europe, the regulation acknowledges that these monophasic variants of *S.* Typhimurium should be considered and therefore handled as *S.* Typhimurium (EFSA, 2010). Multiple studies have been performed to characterize these new *S.* Typhimurium variants that emerged about 10 years ago (Amavisit et al., 2005; Bugarel et al., 2012; Garaizar et al., 2002; Henry et al., 2015; Hopkins et al., 2010; Ido et al., 2011; Moreno Switt et al., 2009; Tavechio et al., 2009). All these studies agreed that these serotypes, and more specifically the serotype 1,4,[5],12:i:-, represent a public health threat similar to that posed by *S.* Typhimurium (EFSA, 2010). Additionally, this STM2 marker is also present in one strain belonging to the O:5-negative variant of *S.* Typhimurium, *S.* Typhimurium variant Copenhagen. This variant is mainly isolated from cattle, swine and other animals (Frech et al., 2003; Hedge et al., 2005).

On another hand, this marker cross-reacts with a single *S.* Heidelberg isolate (Table 2). That particular *S.* Heidelberg isolate is the only isolate belonging to the Heidelberg serotype that is not detected by the Heidelberg marker. The antigenic formulae of *S.* Typhimurium (1,4,[5],12:i:1,2) and Heidelberg (1,4,[5],12:r:1,2) are very close, differing only by their first flagellar phase. Furthermore, based on both pulsed-field gel electrophoresis (PFGE) and clustered regularly interspaced short palindromic repeats (CRISPR) subtyping, *S.* Typhimurium and Heidelberg are closely related (Fricke et al., 2011; Kerouanton et al., 2007). It is likely this *S.* Heidelberg strain was misclassified by conventional serotyping and actually belongs to the serotype Typhimurium.

The Heidelberg marker, Heid2, detected 26 of the 27 strains tested (Table 2). This marker cross-reacted with all the *S.* Infantis strains tested (n = 12). *S.* Infantis, however, only accounted for about 1.6% of all human clinical isolates reported to the CDC in 2009 (CDC, 2011). This marker also presents cross-reactions with two strains belonging to the serotypes *S.* Kintambo and *S*.I 4,[5],12:r:- (Table 2). However, these two serotypes combined represent about 0.04% of all the human isolates reported to CDC between 1999 and 2009 (CDC, 2011).

As assay optimization and inclusivity panels were performed, the marker designed for *S.* Newport, Newp2, amplified all the 30 investigated strains belonging to the targeted serotype. *S.* Newport ranked as the third serotype isolated from human sources in 2009 (CDC, 2011). *S.* Newport displays important genomic diversity, and has been characterized as polyphyletic based on various subtyping methods, including multilocus enzyme electrophoresis, PFGE and multilocus sequence typing (Cao et al., 2013; Harbottle et al., 2006; Sangal et al., 2010; Sukhnanand et al., 2005; Torpdahl et al., 2005). Various subdivisions of this serotype have been proposed (Cao et al., 2013; Harbottle et al., 2006; Sangal et al., 2010; Sukhnanand et al., 2005; Torpdahl et al., 2005). A previous study identified a large gap between two lineages within the *Salmonella enterica* species (den Bakker et al., 2011). Most of the serotypes fall into one or the other evolutionary lineage, however some serotypes such as *S.* Newport occur in few unrelated phylogenetic lineages, named clade A and B (den Bakker et al., 2011). These two lineages specially differ in genes involved in ecology and transmission characteristics, such as utilization of carbon and nitrogen sources, or host and tissue tropism (den Bakker et al., 2011). Within the set of strains investigated in the validation of the *S.* Newport marker, we included strains belonging to both lineages; 4 strains belonged to clade A and 5 to clade B.

In order to cover *S.* Newport diversity, the marker had to be wide-range, which lead to the detection of some non-targeted strains. The Newp2 marker also detected 19 out of the 20 *S.* Hadar tested strains. *S.* Hadar has been ranked as the 20th most frequent serotype isolated from human sources reported to the CDC in 2009 (CDC, 2011). *S.* Hadar only represents 0.8% of laboratory-confirmed cases (CDC, 2011), however this serotype has been recently involved in multiple large multi-state outbreaks of foodborne illness. This serotype is mainly linked to poultry-associated infections (Basler et al., 2015; Jackson et al., 2013). According to Jackson and collaborators, >80% of outbreaks

Table 3
Summary of the performances of the discrimination markers.

Markers	Efficiencies	Inclusivity	Exclusivity
Heidspe3	86.2%	Heidelberg: detection of 23/23	Infantis: detection of 0/11
Infspe2	93.4%	Infantis: detection of 11/11	Heidelberg: detection of 0/23
Hadspe1	87.4%	Hadar: detection of 22/22	Newport: detection of 0/28
Newspe1	45.3%	Newport: detection of 28/28	Hadar: detection of 0/22

caused by *S.* Hadar are attributed to eggs or poultry products (Jackson et al., 2013). For this reason, *S.* Hadar is regulated in poultry in Europe (Regulation (EC) No 2160/2003) (EFSA, 2009).

In addition the Newp2 marker detected 2 strains belonging to the serotypes Bardo and Istanbul. Newport (6,8,20:e,h:1,2) and Hadar (6,8:z_{10}:e,n,x) harbor very close antigenic formulae with Bardo (8:e,h:1,2) and Istanbul (8:z_{10}:e,n,x), respectively. These four serotypes belong to the C2 serogroup. This *Salmonella* serogroup is defined by one major antigen (O:8) and two minor antigens O:6 and O:20 (CDC, 2011). There are currently 39 serotype pairs in the KWM scheme, which differ only by the presence or absence of O:6, with Newport/Bardo and Hadar/Istanbul being two of these C2 serogroup pairs. However the O:6 antigen is variably expressed in many of the commonly encountered C2 serotypes (Mikoleit et al., 2012). For this reason, Mikoleit and collaborators suggested that both serotypes within a pair are not distinct serotypes (Mikoleit et al., 2012).

Based on this observation, it is not surprising that the Newp2 marker displays cross-reactions with the Bardo and Istanbul serotypes.

In addition, cross-reactions for the Newp2 marker also occur with other strains belonging to the C2 serogroup: Bovismorbificans (1/1), Blockley (1/1), Kottbus (1/1), Litchfield (1/1), Glostrup (1/1), Manhattan (2/2), and Muenchen (3/9). However, despite the important cross-reactivity of the Newp2 marker with strains belonging to the C2 serogroup, additional tested strains belonging to other serotypes of this serogroup (Kentucky, Corvallis and Albany) do not react with the Newp2 marker. In addition, the Newp2 marker presents a cross-reaction with 1 out of 10 tested strains of *S.* Virchow (Table 2). Despite all these cross-reactions, all together these non-targeted serotypes only represent about 3.58% and 2.5% of the human isolates collected by the CDC between 1999 and 2009 and of the isolates from nonhuman sources reported to the National Veterinary Services Laboratories (NVSL) in 2009, respectively (CDC, 2011).

Finally, *S.* Enteritidis is the most common *Salmonella* serotype implicated in human salmonellosis cases in the United States. *S.* Enteritidis represented up to 17.5% of all the *Salmonella* strains collected by the CDC in 2009, about 16.3% of the human isolates reported to the CDC between 1999 and 2009, and 14.6% of the non-clinical nonhuman isolates reported to the NVSL in 2009 (CDC, 2011). Strains belonging to this serotype are usually pan-susceptible; an average of 89.2% of strains isolated from human sources were characterized as pan-susceptible between 2000 and 2009 according to the National Antimicrobial Resistance Monitoring System for enteric bacteria (NARMS) (CDC, 2010). However, this serotype represents a very common threat to consumers, especially in the consumption of chicken products as about 50% of *S.* Enteritidis from non-clinical non-human sources were isolated from chicken (CDC, 2011). Also prior to 2009, in the European Union, *S.* Enteritidis has been associated with 52.3% of all the confirmed cases of human salmonellosis (EFSA and ECDC, 2011). Furthermore, this serotype has been regulated in poultry products in Europe for >10 years (European Regulation (EC) no. 2160/2003, completed by the European regulations (EC) no. 1168/2006 and 646/2007). In order to identify strains belonging to the *S.* Enteritidis serotype we designed the Ent6 marker, which is able to properly identify 24 out of the 26 investigated *S.* Enteritidis strains. This marker doesn't cross-react with any of the 421 investigated strains of the exclusivity panel, representing 148 non-targeted *Salmonella* serotypes (Table 2).

3.2. Markers for the discrimination of Infantis/Heidelberg and Newport/Hadar

In order to clarify the identity of the detected strains with Heid2 and Newp2 markers, we designed a second set of 4 additional markers.

These 4 markers were only investigated against strains from the serotypes to be distinguished and the cross-reacting serotypes. Reactivity with other serotypes has not been investigated. This set of primers is meant to be used in a second assay after a positive screening result is detected with Heid2 or Newp2 markers.

In order to distinguish *S.* Heidelberg from *S.* Infantis, the main cross-reaction of the Heid2 marker, we designed the Heidspe3 and Infspe2 markers. The Heidspe3 marker shows positive results with the 23 *S.* Heidelberg investigated strains and negative results with all of the 11 tested strains of *S.* Infantis (Table 3). At the opposite, the Infspe2 marker detects all the *S.* Infantis strains without any cross-reaction with any *S.* Heidelberg strains (Table 3). In addition, only the marker Heidspe3 still presents a cross-reaction with the serotype *S.*I 4,5,12:r:-. The strain from the serotype Kintambo, that displayed a cross-reaction with the Heid2 marker, does not react with any of Heidspe3 and Infspe2 markers.

Regarding the discrimination between *S.* Newport and *S.* Hadar, both detected by the Newp2 marker, we designed the Hadspe1 and Newspe1 markers. The Hadspe1 marker positively reacts with all the *S.* Hadar strains investigated (n = 22) and negatively with all the *S.* Newport strains tested (n = 28).

On the other hand, the Newspe1 marker showed a positive result for each of the 28 tested strains belonging to the Newport serotype and negative results for the 22 tested Hadar strains (Table 3). Regarding the rare cross-reacting serotypes, only Blockey still shows a cross-reaction with Newpspe1, and Istanbul with Hadspe1.

Most of the rare serotypes cross-reacting with the detection markers Heid2 and Newp2 can be distinguished from the serotypes of interest, thanks to this set of discrimination markers.

3.3. Expression of marker targets

In order to provide an even more powerful detection assay for the most relevant *Salmonella* serotypes, we explored the possibility to use these markers to only detect viable cells by detecting the biosynthesized mRNA of these marker targets. We first evaluated the PCR efficiencies of the different markers. Real-time PCRs were performed on serial dilutions of a genomic DNA extraction of one positive-control strain from each serotype. Two out of the eight designed markers show a low PCR efficiency around 50%. Newpspe1 and Heid2 have an average PCR efficiency of 45.3% and 62.2%, respectively (Tables 2 and 3). On another hand, the 6 remaining markers show very good efficiencies, between 82.5% and 93.4%. Ent6, Heidspe3, STM2, Hadspe1, Newp2 and Infspe2 markers show an average PCR efficiency of 82.5%, 86.2%, 86.7%, 87.4%, 89.8%, and 93.4%, respectively (Tables 2 and 3).

Furthermore, based on the prediction of open reading frames from GenBank annotated genomes, only the Ent6 marker doesn't target an encoding sequence. Reverse transcription real-time PCRs were performed on 25 ng of total RNA of each reference strain using developed markers in order to determine the presence of mRNA for each targeted sequences. We detected mRNA expression for the targeted sequences of the STM2, Heid2, Newp2, Infspe2, and Heidspe3 markers. Only Ent6, Hadspe1, and Newspe1 markers do not target expressed sequences on investigated conditions. Based on these results, it is feasible to use screening markers to detect viable *Salmonella* strains belonging to serotype Typhimurium, Heidelberg, Infantis, Hadar, and Newport directly into complex matrices.

3.4. Ground beef sample testing

Artificial contamination of ground beef samples was performed using a representative strain of each serotype at two different ranges of concentration (average of 1.59 and 0.156 CFU/g of ground beef).

Ground beef samples of 25 g were spiked with a single strain or a cocktail composed of the five representative strains. After enrichment, three different samples were collected from the enrichment bags (500 μL, 100 μL, and 50 μL) to perform DNA extractions to be tested on real-time PCR. The markers specifically detected the spiked strains in all the investigated conditions (Table 4), even with the lowest spiked bacterial concentration and the smallest testing sample.

Table 4
Summary of the results of the direct testing on enriched ground beef.

Inoculation conditions	Extraction conditions	Markers			
		STM2	Newp2	Heid2	Ent6
Typhimurium at 1.02 CFU/g	500 μL	+	−	−	−
	100 μL	+	−	−	−
	50 μL	+	−	−	−
Typhimurium at 0.1 CFU/g	500 μL	+	−	−	−
	100 μL	+	−	−	−
	50 μL	+	−	−	−
Enteritidis at 1.82 CFU/g	500 μL	−	−	−	+
	100 μL	−	−	−	+
	50 μL	−	−	−	+
Enteritidis at 0.18 CFU/g	500 μL	−	−	−	+
	100 μL	−	−	−	+
	50 μL	−	−	−	+
Hadar at 2.52 CFU/g	500 μL	−	+	−	−
	100 μL	−	+	−	−
	50 μL	−	+	−	−
Hadar at 0.25 CFU/g	500 μL	−	+	−	−
	100 μL	−	+	−	−
	50 μL	−	+	−	−
Newport at 1.04 CFU/g	500 μL	−	+	−	−
	100 μL	−	+	−	−
	50 μL	−	+	−	−
Newport at 0.1 CFU/g	500 μL	−	+	−	−
	100 μL	−	+	−	−
	50 μL	−	+	−	−
Heidelberg at 1.04 CFU/g	500 μL	−	−	+	−
	100 μL	−	−	+	−
	50 μL	−	−	+	−
Heidelberg at 0.1 CFU/g	500 μL	−	−	+	−
	100 μL	−	−	+	−
	50 μL	−	−	+	−
All the strains at higher concentrations	500 μL	+	+	+	+
	100 μL	+	+	+	+
	50 μL	+	+	+	+
All the strains at lower concentrations	500 μL	+	+	+	+
	100 μL	+	+	+	+
	50 μL	+	+	+	+

4. Conclusions

As previously highlighted, there is a definite need for rapid and easy-to-use methods for the detection of some of the most prevalent and threatening serotypes of *Salmonella enterica* for public health.

The molecular markers developed during this study show high diagnostic specificity (above 97%) and sensitivity (above 96%) (Table 2) (Saah and Hoover, 1997). Furthermore, the utilization of the detection markers was investigated directly on ground beef samples. This investigation shows that the four developed markers specifically detected pre-enrichment bacterial concentration as low as 0.156 CFU/g of ground beef. These results allow good confidence in the ability of these markers to specifically detect the appropriate targets.

The markers developed and described in this study should be utilized in two successive steps: (i) detection of the 5 serotypes of interest, Enteritidis, Typhimurium, Newport, Hadar, and Heidelberg, using the markers Ent6, STM2, Newp2, and Heid2, and (ii) if the markers Heid2 and Newp2 are positive, the identity of the strain can be refined using the second set of markers composed by Heidspe3/Infspe2 or Hadspe1/Newpspe1. Using real-time PCR, this entire two-step investigation can be processed in about 4 h. In addition to being even faster, the screening step of this procedure may have the potential to be performed without an isolation step. Further studies are needed to continue to evaluate the ability of the two-step real-time PCR developed here to detect the presence of these five *Salmonella* serotypes directly in food sample enrichments, other than ground beef, including a variety of matrices and enrichment procedures. Finally, the last advantage of this assay is that the presence and identification of the serotypes of public health interest associated to their potential multidrug resistance abilities (Typhimurium, Newport, Hadar and Heidelberg) could be investigated only for live bacteria by using a reverse transcription real-time PCR on expressed mRNAs of the biomarker targets. The availability of these new markers will provide a detection system for specific *Salmonella* serotypes in the event these serotypes do become targeted for regulation and as a result facilitate improvement of consumer safety by reducing the number of salmonellosis cases and by mitigating the propagation of multidrug resistant strains.

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