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A multiplex oligonucleotide ligation-PCR method for the genoserotyping of common *Salmonella* using a liquid bead suspension assay

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ABSTRACT

Salmonella is a major pathogen having a public health and economic impact in both humans and animals. Six serotypes of the *Salmonella* genus are mentioned in the Belgian and European regulation as to be rapidly excluded from the food chain (EU regulation N°2160/2003, Belgian royal decree 27/04/2017). The reference method for *Salmonella* serotyping, including slide-agglutination and biochemical tests, is time-consuming, expensive, not always objective, and therefore does not match the fast identification criteria required by the legislation. In this study, a molecular method, using genetic markers detected by Multiplex Oligonucleotide Ligation – PCR and Luminex technology, was developed for the identification of the 6 *Salmonella* serotypes and their variants subjected to an official control. The resulting method was validated with the analysis of 971 *Salmonella* isolated from different matrixes (human, animal, food or environment) and 33 non-*Salmonella* strains. The results were compared with the reference identifications, achieving an accuracy of 99.7%. The cost-effective high-throughput genoserotyping assay is performed in 1 day and generates objective results, thanks to the automatic interpretation of raw data using a barcode system. In conclusion, it is fully adapted to the implementation in first line laboratories and meets the requirements of the regulation.

1. Introduction

In 2017, human salmonellosis was still the second bacterial zoonosis reported in Europe after *Campylobacter* infections (EFSA, 2018; Eng et al., 2015). *Salmonella*, the causing agent of salmonellosis, can infect both humans and animals which leads to public health issues and economical loss. This pathogen can contaminate a large variety of food products from vegetables to products of animal origins including eggs, dairy products and meat. The genus *Salmonella* is divided according to a complex classification system including 2 species, 6 subspecies and more than 2500 serotypes. The species and subspecies are characterized by biochemical tests and the serotypes are determined by slide-agglutination, following the Kaufman-White-Le Minor (KWL) scheme (Grimont and Weill, 2007; Ryan et al., 2017). The most common serotypes belong to the 1500 *Salmonella enterica* subsp. *enterica* and include Enteritidis, Hadar, Infantis, Virchow, Typhimurium including its monophasic variant 1,4,[5],12:i:- and Paratyphi B including its variant which has the ability to ferment the dextrorotatory [(L(+)]-tartrate also named Java or dT+. These 6 serotypes and their variant must be monitored because they are the most frequently isolated in Belgium and mentioned in the European and Belgian legislations as to be rapidly excluded from the food chain (EU regulation N°2160/2003, Belgian royal decree 27/04/2017 and Belgian FASFC note BP-FDS/LABO/1470050 v7). Indeed, in Belgium, if one of these *Salmonella* serotypes is detected in poultry farms, the flocks must be eliminated by logistic slaughtering and a thermic treatment must be performed on the eggs

Abbreviation: AC, Allele call; CTRL_+ and CTRL_-, positive and negative controls; eBG, eBurst group; GPP, Gödel Prime Product; KWL, Kauffman-White-Le Minor; MFI, Medium Fluorescence Intensity; MLST, MultiLocus Sequence Typing; MOL-PCR, Multiplex Oligonucleotide Ligation – Polymerase Chain Reaction; SNR, Signal to Noise Ratio; ST, Sequence Type

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before use in the food industry. Additionally, the farm must be completely decontaminated and absence of these serotypes must be proven before arrival of a new poultry batch. Regarding this situation, a rapid and accurate identification of these *Salmonella* serotypes is required to avoid economical loss for the breeders and to be in agreement with the regulation.

The classical methods for *Salmonella* serotyping consist of biochemical tests and slide-agglutination which are very complex, subjective, expensive and time-consuming. Therefore, most of the *Salmonella* isolates must be sent by the first line laboratories to National Reference Centers (NRCs) where the reference techniques are fully mastered and the complete serotype identification can be obtained. This additional step has the effect to increase the analysis time and cost. Additionally, only these NRCs hold the complete collection of the expensive antisera needed for the slide-agglutination, contrarily to the first line laboratories which usually are a smaller structure with limited resources. Luckily these last years, molecular techniques based on the detection of molecular markers, specific to the targeted serotypes, have proven to be a better alternative as they yield more objective and accurate results (Wattiau et al., 2011). In addition these new techniques are sometimes cheaper or faster and consequently more adapted to the rapid exclusion of serotypes demanded by the legislation and can be done directly at the first line level. Most of the time, these genoserotyping methods are PCR based, therefore requiring a detection by electrophoresis on agarose gel, or real-time PCR based, and they target only one or few serotypes at the same time, or several assays are needed for complete identification (Fitzgerald et al., 2007; Franklin et al., 2011; Gand et al., 2019; Malorny et al., 2003; Maurischat et al., 2015; Rajtak et al., 2011; Wattiau et al., 2011). To avoid the use of multiple assays, a better option is the Multiplex Oligonucleotide Ligation – PCR (MOL-PCR), using a liquid bead suspension assay (Luminex xTAG technology), which allows a high level of multiplexing (Wuyts et al., 2015a; Yoshida et al., 2014). The MOL-PCR consists of the detection of molecular markers through a ligation-dependent amplification reaction, in combination with the xTAG technology. The latter is based on color-coded microspheres, divided into distinct (color) sets. Each bead set is coated with an oligonucleotide allowing the specific capture of MOL-PCR fragments after hybridization, and detection via fluorescence measurements on a Luminex instrument. A commercial Luminex kit already exists for the identification of the 100 most common *Salmonella* serotypes (kit: xMap Salmonella Serotyping Assay Kit). Nevertheless, this kit is too expensive, not modular nor adjustable because commercial without detailed probe information, and is too labor-intensive (3 multiplex assays and using the Lx200 apparatus) for the first line laboratories which aim to detect in priority the 6 *Salmonella* serotypes and their variants mentioned in the legislation elaborated above. Data interpretation of the results generated by this kit is not automated and can be complex. Consequently, there is a need for a simpler method, focused on the identification of mandatory *Salmonella* serotypes subjected to an official control, that can be adapted following the evolution of the legislation criteria or serotype prevalence.

In this study, a MOL-PCR assay using the Luminex technology was developed for a fast, accurate and cheap detection of *Salmonella* isolates previously isolated from human, animal, food or environmental samples and belonging to the serotypes, and their variants, to be combatted as outlined by the European and Belgian regulations. The molecular markers targeted by the method were selected from the MultiLocus Sequence Typing (MLST) database named EnteroBase or from the scientific literature. The detection of the MOL-PCR fragments is performed on a Luminex platform called the MagPix which allows the simultaneous detection of up to 50 molecular markers. Compared to the Lx200, the MagPix apparatus is smaller, less expensive and therefore more suitable and cost-effective for implementation in first line laboratories. Moreover, the results' interpretation is facilitated by a barcode system using the Gödel Prime Product (GPP) (Van den Bulcke et al., 2008; Van Den Bulcke et al., 2010) and ensuring an objective conversion of the fluorescence data into serotype identifications. The developed method was validated with the analysis of 1004 bacterial isolates composed of 971 *Salmonella* strains from 114 different serotypes and 33 non-*Salmonella* strains.

2. Materials and methods

2.1. Bacterial strains

The strains used in this study are reference isolates coming from the collection of the Belgian National Reference Center (NRC) for *Salmonella* and *Shigella*. The *Salmonella* strains were previously isolated by the first line laboratories between 2005 and 2018 from food, animal or human matrixes, or from environment. These isolates were sent to the NRC for further characterization, including serotyping by slide-agglutination following the KWL scheme, after confirmation of *Salmonella* genus identification by selective media (XLD agar) or MALDI-TOF method if needed. All isolates are available upon request. The *Salmonella* strains and strains from other bacterial genus were cultured on Nutrient agar (Neogen® Culture Media, Lansing, USA).

2.2. DNA extraction

For MOL-PCR, the bacterial DNA was extracted by heat lysis (as described by Wuyts et al., 2015a) from an overnight (14–20 h) culture at 37 °C. Briefly, a single colony was sampled in 60 µl sterile de-ionised water and incubated at 95 °C in a heating block for 10 min. After cooling for minimum 20 min at 4 °C (in the fridge) and centrifugation for 10 min at 11,000 × *g*, the supernatant was used immediately or stored at −20 °C for further analysis. Positive controls were made by mixing in one tube a single colony of specific strains in function of their targets characteristics (Table 1). The DNA of the strain's mix is extracted by heat lysis as previously described.

For Whole Genome Sequencing (WGS), genomic DNA was extracted with the GenElute Bacterial Genomic DNA kit (Sigma-Aldrich, Saint-Louis, USA) according to the manufacturer's instructions.

2.3. Selection of molecular markers from EnteroBase and NCBI database

The MLST database, EnteroBase (Achtman et al., 2012; Alikhan et al., 2018; https://enterobase.warwick.ac.uk), was screened to select conserved housekeeping alleles among the genetically close related strains of a targeted serotype which are clustered together in Sequence Types (STs) or eBurst Groups (eBGs). In order to find a Single Nucleotide Polymorphism (SNP) that can discriminate this allele, and hence the genoserotype to which it belongs, genetic alignments were made using MUSCLE (Edgar, 2004) in the MEGA6 software (Tamura et al., 2013) with these conserved alleles against all the alleles present in EnteroBase for a given housekeeping gene (a total average of 500 alleles per housekeeping gene, in early 2016). The specificity of the selected MLST SNP markers were therefore confirmed *in silico* on the whole MLST Database composed of 31,848 entries, after in-house curation, in early 2016 (Table 2).

Other markers selected from the scientific literature are based on a SNP or on the presence or absence of a complete sequence (Abs/Pres) specific to a serotype. The specificity of these markers was checked *in silico* by BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi) using the NCBI Database (Table 3).

2.4. Design of ligation probes

The first step of the MOL-PCR consists in the adjacent annealing of the upstream and downstream ligation probes, to the genomic DNA, for a specific detection of the selected molecular marker. These ligation probes were designed using Visual OMP (version 7.6.58.0; DNA Software) according to the guidelines of Wuyts et al. (2015a) (Tables 2

Table 1
Negative and positive controls composition.

Mix Number	CTRL name	Targeted characteristics	Strains ID	Probes controlled for positive signal[a]
1	CTRL_- Vibrio	*Vibrio alginolyticus*	M/5035	NA
	CTRL_+ H	*S.* Hadar	S17BD01821	STID3, STID334_WT, STID34_SNP, STID4_SNP, STID491_WT, STID5, STID71_WT, STID191
2	CTRL_+ PB	*S.* Paratyphi B dT-	II-37-NH	invA, SAL-73, STID16, STID334_SNP, STID34_WT, STID4_WT, STID71_SNP
3	CTRL_+ 12	*S.* Anatum	S16BD07249	STID171, STID2, STID31, STID491_SNP
		S. Enteritidis	S17BD07653	
		S. Enteritidis ST183	S16BD09144	
4	CTRL_+ 24	*S.* Infantis	S17BD01991	rpoB, STID50, STID6
		S. Indiana	S17BD06592	
5	CTRL_+ 34	*S.* Minnesota	S17BD02503	STID13, STID15, STID18, STID35
		S. Virchow	S17BD00950	

dT-: non fermenting D-tartrate strain.
CTRL_-: negative control.
CTRL_+: positive control.
NA: Not Applicable.
[a] Probes for which a positive signal is expected with the associated CTRL_+.

and 3). For markers coming from the scientific literature, ligation probes are based on existing probes, primers or specific amplified sequences. For some SNP markers, for which an interpretation using allelic discrimination is needed, a probe with the wild-type (WT) allele was also designed. All probes and primers were ordered from Integrated DNA Technologies (IDT, Leuven, Belgium) with a standard desalted purification.

2.5. MOL-PCR protocol and Luminex read-out

The MOL-PCR assay, the hybridization to MagPlex-TAG microspheres (Luminex, Austin, USA) and the staining reaction using streptavidin-R-phycoerythrin (SAPE) (Thermofischer Scientific, Waltham, USA) were performed following a modified version of the protocol described by (Wuyts et al., 2015a). Briefly, all the probes (Tables 2 and 3), except invA-U and invA-D, were mixed together at a final concentration of 50 nM and stored at −20 °C in single use aliquots. This probe mix was added to the ligation mix like a single probe at a final concentration of 2 nM. Probes invA-U and invA-D were identically diluted and stored but added separately to the ligation mix to avoid high background noise. DNA from *Vibrio alginolyticus* strain M/5035 was extracted and used in the assay, like described for other samples, as a negative control (CTRL_-) for all probes. Identically, five mixes of *Salmonella* DNA (see composition of the mix in Table 1) were prepared as described in section 2.2 and used in the assay as positive controls (CTRL_+), in a way that each marker targeted by the method was present in one of these DNA mixes. The ligation and PCR reactions were performed in a thermal cycler SimpliAmp (Applied Biosystems, Foster City, USA).

For the Luminex read-out, a volume of 100 μL of MOL-PCR amplicons hybridized to Luminex beads was analyzed by a MagPix device (Luminex, Austin, USA) at 37 °C with a minimum beads count of 50 and a sample wash, according to the manufacturer's instructions.

2.6. Data processing and interpretation

For each sample, the MagPix gives the Median Fluorescence Intensity (MFI) corresponding to each marker. For a Signal-to-Noise Ratio (SNR) interpretation, the signal to noise ratio of the marker "a" was calculated by dividing the MFI of the sample "x" by the corresponding MFI of the CTRL_- (Equ (1)):

$$\mathrm{SNR}_{\,sample\ x\ marker\ a} = \frac{\mathrm{MFI}_{Sample\ x_{marker\ a}}}{\mathrm{MFI}_{CTRL\_-marker\ a}} \tag{1}$$

For some SNP markers which need an allelic discrimination interpretation, a SNP Allele Call (AC) was calculated by dividing the SNR of the SNP probe by the sum of the SNR of the SNP probe and the SNR of its corresponding WT probe (Equ (2)):

$$\mathrm{AC\_SNP}_{sample\ x\ SNP\ a} = \frac{\mathrm{SNR}_{\,Sample\ x\ SNP\ a}}{\mathrm{SNR}_{\,Sample\ x\ SNP\ a} + \mathrm{SNR}_{\,Sample\ x\ WT\ a}} \tag{2}$$

It is recommended to everyone who wants to implement this method, to perform a small validation procedure, including at least 25 *Salmonella* strains per targeted serotypes, 75 untargeted *Salmonella* strains and 25 bacterial strains belonging to another genus, in order to determine the proper cut-off values which can vary a bit between laboratories. Indeed, Wuyts et al. (2015b), showed that the intensity of the fluorescence signals generated by the assay can depend on some intrinsic factors like for example the heating and cooling rates of the thermocycler used during the MOL-PCR step. Here, the cut-off values were calculated for each marker from the MFI generated during the validation procedure and by taking into account the variability obtained with the background noise (MFI signal of CTRL_-). Briefly, for a SNR interpretation, the maximum MFI of the negative samples (increased by 10% when lower than 200) and the minimum MFI of the positive samples (decreased by 10% when higher than 400) were determined, and respectively divided by the weakest MFI signal encountered in CTRL_- and the highest MFI value allowed in CTRL_-. The cut-off values were calculated as the average of these 2 values. A marker is present in the genome of the *Salmonella* isolate, when the SNR of its corresponding probe is above or equal to its determined cut-off value. For an AC interpretation, the cut-off values were determined as the average of the maximum AC value of negative samples and the minimum AC value of positive samples, encountered during the validation process. If the SNP allele call is higher than the cut-off value, then the SNP allele is assigned to the sample.

A barcode system using the GPP (Van den Bulcke et al., 2008; Van Den Bulcke et al., 2010) was used to identify if a combination of molecular markers detected in the analyzed *Salmonella* sample is specific to a serotype (Table 4). Similarly as previously described by Wuyts et al. (2015a), a prime number was allocated to each marker. For each sample, the prime numbers of all detected markers were all multiplied to give a product which was subsequently compared to the expected GPPs listed in Table 4, which are associated to serotype identifications. In case of an unknown GPP was obtained, the product was divided by each prime number of the corresponding serogrouping probes: i.e. STID16, STID18, STID31, STID35, STID171 and STID191. If the result of one of these divisions is an integer, the molecular marker linked to the probe was present in the genome of the *Salmonella* isolate, and consequently, the serogroup could be determined (Table 3).

All the processing of the data (SNR/AC calculation, GPP attribution, CTRL check and final identification results displaying) was automatically performed using a programmed Excel workbook compatible with the Comma Separated Value (CSV) files generated by the MagPix

Table 2
Ligation probes designed from molecular marker selection using EnteroBase.

Target	Probe	DNA sequence	Beads region[b]	Interpretation[c]	MLST gene	Allele number	SNP position[d]
S. Enteritidis ST183	STID491-U_SNP	*TAATACGACTCACTATAGG*gtgattgaatagtagattgtttaaCAGCTTCGCCGAAACGGCGGA**G**	46	AC	*purE*	60	132
	STID491-U_WT	*TAATACGACTCACTATAGG*gttatgatatagtgagttgttattCAGCTTCGCCGAAACGGCGGAA	77				
	STID491-D	P-GAGAACGGATATCAAGTGATTATTGC*TCCCTTTAGTGAGGGTTAAT*					
S. Hadar	STID3-U_SNP	*TAATACGACTCACTATAGGG*agtagaaagttgaaattgattatgCTTCTCGCTGTCCACGCT**T**	12	SNR	*dnaN*	5	183
	STID3-D	P-CCTGCCGCCGATTTCCCGA*TCCCTTTAGTGAGGGTTAAT*					
	STID4-U_SNP[a]	*TAATACGACTCACTATAGG*gattgatatttgaatgtttgtttgCACCACCGAGATCCCGGC**A**	22	AC	*thrA*	12	423
	STID4-U_WT[a]	*TAATACGACTCACTATAGG*gtatgttgtaatgtattaagaaagCACCACCGAGATCCCGGCG	25				
	STID4-D[a]	P-CGAGACATGGCGGCGAAAAC*TCCCTTTAGTGAGGGTTAAT*					
	STID5-U_SNP	*TAATACGACTCACTATAGG*gatagatttagaatgaattaagtgACGCCTGATGCTGACATTGCC**T**	28	SNR	*hisD*	7	385
	STID5-D	P-GCAAGGTGGCGGAGGCGGTAGA*TCCCTTTAGTGAGGGTTAAT*					
S. Indiana	STID50-U_SNP	*TAATACGACTCACTATAGGGa*gtgaatgtaagattatgtatttgTGAATGTTATCAACGATGTG**A**	13	SNR	*hemD*	11	351
	STID50-D	P-GAAACATTACGATGGCGCGG*TCCCTTTAGTGAGGGTTAAT*					
S. Infantis	STID6-U_SNP	*TAATACGACTCACTATAGGG*aattagaagtaagtagagtttaagGCGCCAAATTCCCGGG**G**	56	SNR	*sucA*	21	69
	STID6-D	P-GCGAAACGTTTCTCGCTCGA*TCCCTTTAGTGAGGGTTAAT*					
	STID71-U_SNP	*TAATACGACTCACTATAGGG*attgtgaaagaaagagaagaaattGTCGAAACTGGCCTGTG**G**	14	AC	*hemD*	22	99
	STID71-U_WT	*TAATACGACTCACTATAGG*gtgttatagaagttaaatgttaagTCGAAACTGGCCTGTGT	30				
	STID71-D	P-CGCCGCGCTATTTCGCG*TCCCTTTAGTGAGGGTTAAT*					
S. Virchow	STID13-U_SNP[a]	*TAATACGACTCACTATAGGG*aataagagaattgatatgaagatgGGCCATCGAAAACTGGGTCG**A**	35	SNR	*dnaN*	7	271
	STID13-D[a]	P-TTCAATCAGGCGCTTCATCGTGGC*TCCCTTTAGTGAGGGTTAAT*					
	STID15-U_SNP[a]	*TAATACGACTCACTATAGG*gtaagattagaagttaatgaagaaCGCTAAACATCGCCATGTT**A**	52	SNR	*thrA*	14	339
	STID15-D[a]	P-TTAAGGTTAGAGATCCCTTTAACCGGC*TCCCTTTAGTGAGGGTTAAT*					

P-: Phosphate.
Primer (T7 and T3), anti-TAG, target-specific sequences and SNP positions are indicated by italic, lower-case, underlined and bold sequences, respectively.
[a] The probes anneal on the reverse complement strand of the MLST gene.
[b] Correspond to the specific color and TAG sequence of the bead.
[c] The presence of the molecular marker is determined by the calculation of the Signal to Noise Ratio (SNR) or Allele Call (AC).
[d] Correspond to the location of the SNP in multiple alignments of all alleles related to the considered MLST gene.

Table 3
Ligation probes designed from molecular markers selected in the scientific literature.

Target	Probe	DNA sequence	Beads region[a]	Type[b]	Interpretation[c]	Marker	Based on	Source
S. Enteritidis	STID2-U	*TAATACGACTCACTATAGGG*tattagagtttgagaataagtagtCGGCGCATTCCTCCGTTT	33	Abs/Pres	SNR	*sdr*	Primer sdr-R	Rajtak et al. (2011)
	STID2-D	P-TTTCGTCGTGGGCGTCAGTA*TCCCTTTAGTGAGGGTTAAT*						
S. Paratyphi B (including var. Java)	STID334-U_SNP	*TAATACGACTCACTATAGG*gttgtaaattgtagtaaagaagtaGCGTAAACTTCATCGGCATAGT**T**	15	SNP	AC	*SPAB_04460*	TaqMan probes ParaB_SNP and ParaB_WT	Gand et al. (2019)
	STID334-U_WT	*TAATACGACTCACTATAGG*gttagttatgatgaatattgtgtaGCGTAAACTTCATCGGCATAGTC	45					
	STID334-D	P-AGATCTTTGCCTTTGCTACCCA*TCCCTTTAGTGAGGGTTAAT*						
	STID34-U_SNPdT-	*TAATACGACTCACTATAGGG*tgtatatgttaatgagatgttgtaTCATATATCATTGATTGGATAATTATAAATAT**A**	29	SNP	AC	*STM3356*	Primer #166	Malorny et al. (2003)
	STID34-U_WTdT +	*TAATACGACTCACTATAGGG*tttaagtgagttatagaagtagtaTCATATATCATTGATTGGATAATTATAAATATG	37					
	STID34-D	P-GAACCCATTACCCTTACCTTATGTT*CCCTTTAGTGAGGGTTAAT*						
Salmonella	invA-U	*TAATACGACTCACTATAGGG*gataagaaagtgaaatgtaaattgATAAACTTCATCGCACCGTCA	51	Abs/Pres	SNR	*invA*	Ligation probes invA-U and invA-D	Wuyts et al. (2015a)
	invA-D	P-AAGGAACCGTAAAGCTGGCTT*TCCCTTTAGTGAGGGTTAAT*						
Serogroup O:4	STID16-U	*TAATACGACTCACTATAGGG*tttgatttaagagtgttgaatgtaTCAAGTTGGAACTGGTGCT	26	Abs/Pres	SNR	*rfbJ*	Sequence amplified by B_rfbJ_F and B_rfbJ_R	Franklin et al. (2011)
	STID16-D	P-GGGGTAAGTTTGAAAGATTTTCTGG*TCCCTTTAGTGAGGGTTAAT*						
Serogroup O:9	STID171-U_SNP	*TAATACGACTCACTATAGGG*aattgagaaagagataaatgatagCATATACTAAACAAAAAGCAAATGAA**C**	72	SNP	SNR	*prt*	Serogroup D capture probe	Fitzgerald et al. (2007)
	STID171-D	P-TCGCCGCCGCCATTATAGAT*CCCTTTAGTGAGGGTTAAT*						
Serogroup O:6,7	STID18-U	*TAATACGACTCACTATAGG*gtaagagtattgaaattagtaagaCGTTGGCAGACTGGTACTGATTG	66	Abs/Pres	SNR	*wbaA*	Primer C1_wbaA_F3	Franklin et al. (2011)
	STID18-D	P-GCTCCCCTATTACGATGATTTC*TCCCTTTAGTGAGGGTTAAT*						
Serogroup O:8	STID191-U	*TAATACGACTCACTATAGGG*aaataagaatagagagagaaagttTTATAAATTTACGTTTAGAACATGTTTAC	43	Abs/Pres	SNR	*rfbJ*	Sequence amplified by C2_rfbJ_F and C2_rfbJ_R	Franklin et al. (2011)
	STID191-D	P-GGTGAGAGGGATAAAGCAGGTAAAAT*CCCTTTAGTGAGGGTTAAT*						
Serogroup O:10	STID31-U	*TAATACGACTCACTATAGGG*tgatatagtagtgaagaaataagtTCTCTACGCAGACAATTATGTCA	34	Abs/Pres	SNR	*wzx*	Primer E_wzx_F	Franklin et al. (2011)
	STID31-D	TGGAGTTATTATCCGGATGGGT*CCCTTTAGTGAGGGTTAAT*						
Serogroup O:21	STID35-U	*TAATACGACTCACTATAGG*gttgagaattagaatttgataaagCCACTGTCATTGGTGGTTATGAG	73	Abs/Pres	SNR	*wzx*	Primer L_wzx_F2	Franklin et al. (2011)
	STID35-D	P-TATGAATGGCTGGTATACGACATC*TCCCTTTAGTGAGGGTTAAT*						

(*continued on next page*)

Table 3 (*continued*)

Target	Probe	DNA sequence	Beads region[a]	Type[b]	Interpretation[c]	Marker	Based on	Source
S. Typhimurium (including var. monophasic)	rpoB-U	*TAATACGACTCACTATAGGG*gtaattgaattgaaagataagtgt TTTCTCAGCTGCACCGTAGC	18	SNP	SNR	*rpoB*	Ligation probes rpoB-U and rpoB-D	Wuyts et al. (2015a)
	rpoB-D	P-CCTGGCGTCTTCTTTGACTCGTCCCTTTAGTGAGGG *TTAAT*						
	SAL-73-U	*TAATACGACTCACTATAGGG*tgaaatgtgtattgtatgtttag CCAGCCGCAAGGGTTACTGTAC	62	Abs/Pres	SNR	*fljB*[d]	Ligation probes SAL-73-U and SAL-73-D	Wuyts et al. (2015a)
	SAL-73-D	P-CGTCAGTAGCAAGGTTAACTTCATAATCCCTTTAGTGAGG *GTTAAT*						

P-: Phosphate.
Primer (T7 and T3), anti-TAG, target-specific sequences and SNP positions are indicated by italic, lower-case, underlined and bold sequences, respectively.
[a] Correspond to the specific color and TAG sequence of the bead.
[b] Single Polymorphism Nucleotide (SNP) or presence or absence of a sequence (Abs/Pres).
[c] The presence of the molecular marker is determined by the calculation of the Signal to Noise Ratio (SNR) or Allele Call (AC).
[d] Targeting fljB sequence coding for flagellar antigen H:1,2 but also positive for some *Salmonella* isolates belonging to H:1,5 H:1,6 and H:1,7.

and containing all the MFI values for each assay. This Excel template is available upon request.

2.7. Comparison study

A total of 1004 bacterial isolates, including 971 *Salmonella* and 33 non-*Salmonella*, were analyzed with the developed genoserotyping test. From this total, 690 *Salmonella* isolates belonged to the serotypes targeted by the method including 134 *S.* 1,4,[5],12:i:- , 106 *S.* Enteritidis, 31 *S.* Hadar, 154 *S.* Infantis, 13 *S.* Paratyphi B, 84 *S.* Paratyphi B var. Java, 123 *S.* Typhimurium and 45 *S.* Virchow. The remaining strains, which are not targeted by the method, were composed of 281 *Salmonella* isolates from 108 other serotypes, 1 *S. bongori* and 33 non-*Salmonella* isolates (Table 5). Prior to use, the serotype identification of each *Salmonella* isolate was confirmed by the reference methods including slide-agglutination and biochemical tests following the KWL scheme, from the same culture used for DNA extraction. If discordant identifications were obtained between the 2 methods, 2 repetitions of the slide-agglutination analysis were performed, i.e. one blind test and one performed by a different technician, to confirm the results. The accuracy of the method, which means the closeness of agreement between the test result and the reference identification, was determined from inclusivity (sensitivity) and exclusivity (specificity) tests as previously described by (Gand et al., 2019).

2.8. Whole genome sequencing

The whole genome of 3 *S.* Virchow (S16BD00604, S17BD03634 and S17BD08736) was obtained with an Illumina MiSeq (2 × 300 bp, Nextera XT libraries) (Illumina, San Diego, United States). FASTQ reads from all sequences were deposited at the Sciensano-*Salmonella* BioProject at NCBI (PRJNA509747).

In CLC Genomics Workbench 8.0 (Qiagen, Hilden, Germany), the raw FASTQ reads were first trimmed to quality score limit 0.05 with maximum 2 ambiguous nucleotides and reads with length below 30 nucleotides were discarded. These trimmed reads were then *de novo* assembled with automatic bubble and word size, in mapping mode "map reads back to contigs" with scaffolding and a minimum contig length of 1000 nucleotides. For each isolate, the serotype and the 7 gene MLST ST were characterized using SISTR (Yoshida et al., 2016) with genome assemblies (FASTA format) as input.

3. Results

3.1. Selection of molecular markers

In this study, molecular markers were selected for the specific detection of the 6 *Salmonella* serotypes and their variants frequently isolated in Belgium and mentioned in the regulation as to combat (EU regulation N°2160/2003, Belgian royal decree 27/04/2017 and Belgian FASFC note BP-FDS/LABO/1470050 v7). A total of 12 molecular markers was inspired from the literature for the specific detection of *S.* Enteritidis, *S.* Paratyphi B including the Java variant, *S.* Typhimurium including its monophasic variant and for sample serogrouping (Table 3). These markers are based on a SNP or on the presence or absence of a complete sequence. Their specificity was checked successfully *in silico* using BLASTn on the NCBI Database (data not shown).

The rest of the molecular markers (9) was selected from the MLST database (EnteroBase) for the specific identification of *S.* Hadar, *S.* Infantis, *S.* Virchow, a specific ST of *S.* Enteritidis (ST183) and the exclusion of *S.* Indiana, which can be mistaken with a low percentage of *S.* Typhimurium isolates (Table 2). First, specific SNPs were screened among the allele sequences of the 7 housekeeping genes (which constitute the MLST scheme: *aroC*, *dnaN*, *hemD*, *hisD*, *purE*, *sucA*, and *thrA*) that are conserved in a serotype population. After an *in silico* verification on the whole EnteroBase database, it became clear that used

Table 4
Genotype profiles which can be detected by the method.

Probe ID:	invA	rpoB	SAL-73	STID2	STID3	STID4	STID5	STID6	STID13	STID15	STID16	STID18
Targets:	*Salmonella*	Typhimurium*	*fljB*	Enteritidis	Hadar	Hadar	Hadar	Infantis	Virchow	Virchow	O:4**	O:7**
Probe prime numbers:	3	23	5	67	41	31	37	47	19	59	7	43
	+	+	–	–	–	–	–	–	+[1]	–	+	–
	+	+	–	–	–	–	–	–	+[2]	–	+	–
	+	+	–	–	–	–	–	–	–	–	+	–
	+	+	–	–	–	–	–	–	–	–	+	–
	+	–	–	+	–	–	+	–	–	–	–	–
	+	–	–	–	–	–	+	–	–	–	–	–
	+	–	–	–	+	+	+[5]	–	–	–	–	–
	+	–	–	–	+	+	–	–	–	–	–	–
	+	–	+	–	–	–	–	+	–	–	–	+
	+	–	+	–	–	–	–	–	–	–	+	–
	+	–	+	–	–	–	–	–	–	–	+	–
	+	–	+	–	–	–	–	–	–	–	+	–
	+	–	+	–	–	–	–	–	–	–	+	–
	+	+	+	–	–	–	–	–	+[1]	–	+	–
	+	+	+	–	–	–	–	–	+[2]	–	+	–
	+	+	+	–	–	–	–	–	–	–	+	–
	+	+	+	–	–	–	–	–	–	–	+	–
	+	–	+	–	–	–	–	–	+	+	–	+

Probe ID:	STID31	STID34	STID35	STID50	STID71	STID171	STID191	STID334	STID491	GPP	Associated serotyping result
Targets:	O:10**	dT- var.	O:21**	Indiana	Infantis	O:9**	O:8**	Paratpyhi B*	Enteritidis		
Probe prime numbers:	73	17	79	61	11	53	29	13	71		
	–	–	–	–	+[1]	–	–	–	–	100947	Monophasic Typhimurium
	–	–	–	–	–	–	–	–	–	9177	Monophasic Typhimurium
	–	–	–	–	+[3]	–	–	–	–	5313	Monophasic Typhimurium
	–	–	–	–	–	–	–	–	–	483	Monophasic Typhimurium
	–	–	–	–	–	+	–	–	–	394161	Enteritidis
	–	–	–	–	–	+	–	–	+	417693	Enteritidis (ST183[4])
	–	–	–	–	–	–	+	–	–	4091349	Hadar
	–	–	–	–	–	–	+	–	–	110577	Probably Hadar*** (ST473)
	–	–	–	–	+	–	–	–	–	333465	Infantis
	–	+	–	–	+[6]	–	–	+	–	255255	Paratyphi B (dT-)
	–	+	–	–	–	–	–	+	–	23205	Paratyphi B (dT-)
	–	–	–	–	+[6]	–	–	+	–	15015	Paratyphi B var. Java (dT+)
	–	–	–	–	–	–	–	+	–	1365	Paratyphi B var. Java (dT+)
	–	–	–	–	+[1]	–	–	–	–	504735	Typhimurium
	–	–	–	–	–	–	–	–	–	45885	Typhimurium
	–	–	–	–	+[3]	–	–	–	–	26565	Typhimurium
	–	–	–	–	–	–	–	–	–	2415	Typhimurium
	–	–	–	–	–	–	–	–	–	723045	Virchow

According to the in silico analysis (using EnteroBase, 2016) performed during marker selection:
[1]The SNP #99 of the allele *hemD* and the SNP #271 of the gene *dnaN* are present together in 94% of the Typhimurium population.
2: The SNP #271 of the gene *dnaN* is present in 0.7% of the Typhimurium population.
[3]The SNP #99 of the gene *hemD* is present in 4% of the Typhimurium population.
[4]The ST183 represents 1.7% of the Enteritidis population.
[5]The SNP #385 of the gene *hisD* is present in 94% of the Hadar population.
[6]The SNP #99 of the gene *hemD* is present in 54% of the Paratyphi B (dT-/dT+) population.
*: Including variants.
**: Serogroup tageted by the associated probe.
***: To be confirmed by classical method (i.e. slide-agglutination).
Monophasic Typhimurium: 1,4,[5],12:i:-.
dT-: non d-tartrate fermenting strains.

separately, no single SNP candidate was sufficient for the specific detection of the targeted serotypes. Consequently, the choice was made to use a combination of SNP markers selected among at least 2 conserved alleles. Used together with the serogroup markers selected from the literature (Table 3), these combinations of MLST markers (Table 4) gave a false positive rate of 0% for the concerned serotypes, after an *in silico* verification on EnteroBase (2016). Few strains of the targeted serotypes, belonging to rare STs, were reported as false negative: i.e. less than 6% for Hadar serotype and less than 3% for the 4 other serotypes mentioned earlier in this paragraph. These false negative rates were investigated during the validation process (section 3.3).

3.2. MOL-PCR development

From the selection of molecular markers, a total of 26 upstream and 21 downstream probes were designed (Tables 2 and 3). In the programmed Excel workbook, a serotype identification is automatically assigned to a sample when a specific combination of positive probes is obtained and converted into a known GPP. If no positive signal is obtained for the marker *invA* targeting all *Salmonella* species, no GPP interpretation is performed. If the obtained GPP is unknown, the serogroup of the sample is determined, if possible, as described in materials and methods. The expected combinations of molecular

Table 5
Determination of the sensitivity, specificity and accuracy of the developed method.

	Reference identification[a]		Nbr of isolates	Expected result with the Luminex assay			Obtained results with the Luminex assay			Comparison results[c]
	Serotype	Serogroup		GPP	Identification	Serogroup[b]	GPP	Identification	Serogroup[b]	
Targeted isolates	*S.* 1,4,[5],12:i:-	O:4	133	100947, 9177, 5313 or 483	*S.* Typhimurium var. monophasic	NA	100947	*S.* Typhimurium var. monophasic	NA	133 TP
	S. 1,4,[5],12:i:-	O:4	1	100947, 9177, 5313 or 483	*S.* Typhimurium var. monophasic	NA	9177	*S.* Typhimurium var. monophasic	NA	1 TP
	S. Enteritidis	O:9	99	394161 or 417693	*S.* Enteritidis	NA	394161	*S.* Enteritidis	NA	99 TP
	S. Enteritidis	O:9	7	394161 or 417693	*S.* Enteritidis (ST184)	NA	417693	*S.* Enteritidis (ST184)	NA	7 TP
	S. Hadar	O:8	29	110577 or 4091349	*S.* Hadar	NA	4091349	*S.* Hadar	NA	29 TP
	S. Hadar	O:8	2	110577 or 4091349	Probably *S.* Hadar (ST474)[d]	O:8	110577	Probably *S.* Hadar (ST474)[d]	O:8	2 TP
	S. Infantis	O:7	154	333465	*S.* Infantis	NA	333465	*S.* Infantis	NA	154 TP
	S. Paratyphi B (dT-)	O:4	13	23205 or 255255	*S.* Paratyphi B (dT-)	NA	255255	*S.* Paratyphi B (dT-)	NA	13 TP
	S. Paratyphi B var. Java (dT+)	O:4	83	1365 or 15015	*S.* Paratyphi B var. Java (dT+)	NA	15015	*S.* Paratyphi B var. Java (dT+)	NA	83 TP
	S. Paratyphi B Var. Java (dT+)	O:4	1	1365 or 15015	*S.* Paratyphi B var. Java (dT+)	NA	1365	*S.* Paratyphi B var. Java (dT+)	NA	1 TP
	S. Typhimurium	O:4	122	504735, 45885, 26565 or 2415	*S.* Typhimurium	NA	504735	*S.* Typhimurium	NA	122 TP
	S. Typhimurium	O:4	1	504735, 45885, 26565 or 2415	*S.* Typhimurium	NA	26565	*S.* Typhimurium	NA	1 TP
	S. Virchow	O:7	42	723045	*S.* Virchow	NA	723045	*S.* Virchow	NA	42 TP
	S. Virchow	O:7	2	723045	*S.* Virchow	NA	38055	*Salmonella*	O:7	2 FN
	S. Virchow	O:7	1	723045	*S.* Virchow	NA	645	*Salmonella*	O:7	1 FN
Untargeted isolates	*Acinetobacter baumanii*	NA	1	–	Non-*Salmonella*	NA	–	Non-*Salmonella*	NA	1 TN
	Bacillus cereus	NA	1	–	Non-*Salmonella*	NA	–	Non-*Salmonella*	NA	1 TN
	Citrobacter koseri	NA	1	–	Non-*Salmonella*	NA	–	Non-*Salmonella*	NA	1 TN
	Enterobacter aerogenes	NA	1	–	Non-*Salmonella*	NA	–	Non-*Salmonella*	NA	1 TN
	Enterococcus faecium	NA	1	–	Non-*Salmonella*	NA	–	Non-*Salmonella*	NA	1 TN
	Escherichia coli	NA	2	–	Non-*Salmonella*	NA	–	Non-*Salmonella*	NA	2 TN
	Klebsiella oxytoca	NA	1	–	Non-*Salmonella*	NA	–	Non-*Salmonella*	NA	1 TN
	Klebsiella pneumoniae	NA	1	–	Non-*Salmonella*	NA	–	Non-*Salmonella*	NA	1 TN
	Listeria monocytogenes	NA	5	–	Non-*Salmonella*	NA	–	Non-*Salmonella*	NA	5 TN
	Morganella morganii	NA	1	–	Non-*Salmonella*	NA	–	Non-*Salmonella*	NA	1 TN
	Neisseria meningitidis	NA	4	–	Non-*Salmonella*	NA	–	Non-*Salmonella*	NA	4 TN
	S. Abony	O:4	1	Unknown GPP	*Salmonella*	O:4	1239	*Salmonella*	O:4	1 TN
	S. Agama	O:4	1	Unknown GPP	*Salmonella*	O:4	105	*Salmonella*	O:4	1 TN
	S. Ago	O:30	1	Unknown GPP	*Salmonella*	Unknown	3	*Salmonella*	Unknown	1 TN
	S. Agona	O:4	11	Unknown GPP	*Salmonella*	O:4	21	*Salmonella*	O:4	11 TN
	S. Agoueve	O:13	1	Unknown GPP	*Salmonella*	Unknown	33	*Salmonella*	Unknown	1 TN
	S. Ajiobo	O:13	4	Unknown GPP	*Salmonella*	Unknown	39	*Salmonella*	Unknown	4 TN
	S. Albany	O:8	2	Unknown GPP	*Salmonella*	O:8	87	*Salmonella*	O:8	2 TN
	S. Albany	O:8	1	Unknown GPP	*Salmonella*	O:8	957	*Salmonella*	O:8	1 TN
	S. Altona	O:8	1	Unknown GPP	*Salmonella*	O:8	435	*Salmonella*	O:8	1 TN
	S. Anatum	O:3,10	3	Unknown GPP	*Salmonella*	O:10	12045	*Salmonella*	O:10	3 TN
	S. Anecho	O:35	1	Unknown GPP	*Salmonella*	Unknown	3	*Salmonella*	Unknown	1 TN
	S. Bardo	O:8	2	Unknown GPP	*Salmonella*	O:8	17166405	Probably *S.* Bardo/Newport[e]	O:8	2 TN
	S. Bardo	O:8	1	Unknown GPP	*Salmonella*	O:8	148335	Probably *S.* Bardo/Newport/Blockley[d]	O:8	1 TN
	S. Bardo	O:8	1	Unknown GPP	*Salmonella*	O:8	435	*Salmonella*	O:8	1 TN
	S. Bareilly	O:7	2	Unknown GPP	*Salmonella*	O:7	645	*Salmonella*	O:7	2 TN
	S. Berta	O:9	1	Unknown GPP	*Salmonella*	O:9	2067	*Salmonella*	O:9	1 TN
	S. Blockley	O:8	2	Unknown GPP	*Salmonella*	O:8	148335	Probably *S.* Bardo/Newport/Blockley[d]	O:8	2 TN

(*continued on next page*)

Table 5 (*continued*)

Reference identification[a]		Nbr of isolates	Expected result with the Luminex assay			Obtained results with the Luminex assay			Comparison results[c]
Serotype	Serogroup		GPP	Identification	Serogroup[b]	GPP	Identification	Serogroup[b]	
S. bongori	NA	1	Unknown GPP	*Salmonella*	Unknown	3	*Salmonella*	Unknown	1 TN
S. Bovismorbificans	O:8	2	Unknown GPP	*Salmonella*	O:8	2697	*Salmonella*	O:8	2 TN
S. Braenderup	O:7	3	Unknown GPP	*Salmonella*	O:7	1419	*Salmonella*	O:7	3 TN
S. Brancaster	O:4	1	Unknown GPP	*Salmonella*	O:4	21	*Salmonella*	O:4	1 TN
S. Brandenburg	O:4	1	Unknown GPP	*Salmonella*	O:4	21	*Salmonella*	O:4	1 TN
S. Bredeney	O:4	3	Unknown GPP	*Salmonella*	O:4	105	*Salmonella*	O:4	3 TN
S. Brive	O:42	1	Unknown GPP	*Salmonella*	Unknown	3	*Salmonella*	Unknown	1 TN
S. Butantan	O:3,10	1	Unknown GPP	*Salmonella*	O:10	219	*Salmonella*	O:10	1 TN
S. Carmel	O:17	1	Unknown GPP	*Salmonella*	Unknown	3	*Salmonella*	Unknown	1 TN
S. Cero	O:18	2	Unknown GPP	*Salmonella*	Unknown	3	*Salmonella*	Unknown	2 TN
S. Cero	O:18	3	Unknown GPP	*Salmonella*	Unknown	33	*Salmonella*	Unknown	3 TN
S. Cero	O:18	3	Unknown GPP	*Salmonella*	Unknown	15	*Salmonella*	Unknown	3 TN
S. Chester	O:4	3	Unknown GPP	*Salmonella*	O:4	21	*Salmonella*	O:4	3 TN
S. Choleraesuis	O:7	1	Unknown GPP	*Salmonella*	O:7	645	*Salmonella*	O:7	1 TN
S. Coeln	O:4	2	Unknown GPP	*Salmonella*	O:4	3255	*Salmonella*	O:4	2 TN
S. Colindale	O:7	1	Unknown GPP	*Salmonella*	O:7	939765	Probably *S.* Colindale (ST584)[d]	O:7	1 TN
S. Corvallis	O:8	1	Unknown GPP	*Salmonella*	O:8	87	*Salmonella*	O:8	1 TN
S. Cotham	O:28	2	Unknown GPP	*Salmonella*	Unknown	3	*Salmonella*	Unknown	2 TN
S. Derby	O:4	11	Unknown GPP	*Salmonella*	O:4	21	*Salmonella*	O:4	11 TN
S. Derby	O:4	6	Unknown GPP	*Salmonella*	O:4	231	*Salmonella*	O:4	6 TN
S. Dublin	O:9	5	Unknown GPP	*Salmonella*	O:9	159	*Salmonella*	O:9	5 TN
S. Dugbe	O:45	1	Unknown GPP	*Salmonella*	Unknown	3	*Salmonella*	Unknown	1 TN
S. Durban	O:9	1	Unknown GPP	*Salmonella*	O:9	159	*Salmonella*	O:9	1 TN
S. Durham	O:13	1	Unknown GPP	*Salmonella*	Unknown	141	*Salmonella*	Unknown	1 TN
S. Eastbourne	O:9	1	Unknown GPP	*Salmonella*	O:9	159	*Salmonella*	O:9	1 TN
S. Ebrie	O:35	1	Unknown GPP	*Salmonella*	Unknown	33	*Salmonella*	Unknown	1 TN
S. Escanaba	O:7	1	Unknown GPP	*Salmonella*	O:7	1419	*Salmonella*	O:7	1 TN
S. Gallinarum var Gallinarum	O:9	1	Unknown GPP	*Salmonella*	O:9	5883	Probably *S.* Gallinarum[d]	O:9	1 TN
S. Gallinarum var Pullorum	O:9	1	Unknown GPP	*Salmonella*	O:9	5883	Probably *S.* Gallinarum[d]	O:9	1 TN
S. Gaminara	O:16	1	Unknown GPP	*Salmonella*	Unknown	15	*Salmonella*	Unknown	1 TN
S. Give	O:3,10	4	Unknown GPP	*Salmonella*	O:10	1095	*Salmonella*	O:10	4 TN
S. Glostrup	O:8	1	Unknown GPP	*Salmonella*	O:8	94047	*Salmonella*	O:8	1 TN
S. Goldcoast	O:8	1	Unknown GPP	*Salmonella*	O:8	87	*Salmonella*	O:8	1 TN
S. Grumpensis	O:13	1	Unknown GPP	*Salmonella*	Unknown	15	*Salmonella*	Unknown	1 TN
S. Havana	O:13	2	Unknown GPP	*Salmonella*	Unknown	3	*Salmonella*	Unknown	2 TN
S. Heidelberg	O:4	2	Unknown GPP	*Salmonella*	O:4	61845	Probably *S.* Kisangani/Heidelberg/Saintpaul/Stanleyville[d]	O:4	2 TN
S. Hvittingfoss	O:16	1	Unknown GPP	*Salmonella*	Unknown	627	Probably *S.* Hvittingfoss[d]	Unknown	1 TN
S. Ibadan	O:13	1	Unknown GPP	*Salmonella*	Unknown	17205	*Salmonella*	Unknown	1 TN
S. Idikan	O:13	2	Unknown GPP	*Salmonella*	Unknown	15	*Salmonella*	Unknown	2 TN
S. Indiana	O:4	5	Unknown GPP	*Salmonella*	O:4	147315	Probably *S.* Indiana[d]	O:4	5 TN
S. Isangi	O:7	1	Unknown GPP	*Salmonella*	O:7	1419	*Salmonella*	O:7	1 TN
S. Ituri	O:4	1	Unknown GPP	*Salmonella*	O:4	21	*Salmonella*	O:4	1 TN
S. Jalisco	O:11	1	Unknown GPP	*Salmonella*	Unknown	15	*Salmonella*	Unknown	1 TN
S. Javiana	O:9	1	Unknown GPP	*Salmonella*	O:9	159	*Salmonella*	O:9	1 TN
S. Jerusalem	O:7	1	Unknown GPP	*Salmonella*	O:7	129	*Salmonella*	O:7	1 TN
S. Kapemba	O:9	1	Unknown GPP	*Salmonella*	O:9	795	*Salmonella*	O:9	1 TN
S. Kasenyi	O:38	2	Unknown GPP	*Salmonella*	Unknown	42009	*Salmonella*	Unknown	2 TN
S. Kedougou	O:13	3	Unknown GPP	*Salmonella*	Unknown	33	*Salmonella*	Unknown	3 TN
S. Kentucky	O:8	10	Unknown GPP	*Salmonella*	O:8	87	*Salmonella*	O:8	10 TN
S. Kisarawe	O:11	1	Unknown GPP	*Salmonella*	Unknown	3	*Salmonella*	Unknown	1 TN

(*continued on next page*)

Table 5 (*continued*)

Reference identification[a]		Nbr of isolates	Expected result with the Luminex assay			Obtained results with the Luminex assay			Comparison results[c]
Serotype	Serogroup		GPP	Identification	Serogroup[b]	GPP	Identification	Serogroup[b]	
S. Kottbus	O:8	2	Unknown GPP	*Salmonella*	O:8	87	*Salmonella*	O:8	2 TN
S. Lagos	O:4	1	Unknown GPP	*Salmonella*	O:4	21	*Salmonella*	O:4	1 TN
S. Limete	O:4	2	Unknown GPP	*Salmonella*	O:4	4935	*Salmonella*	O:4	2 TN
S. Litchfield	O:8	3	Unknown GPP	*Salmonella*	O:8	17835	*Salmonella*	O:8	3 TN
S. Livingstone	O:7	14	Unknown GPP	*Salmonella*	O:7	129	*Salmonella*	O:7	14 TN
S. Livingstone	O:7	3	Unknown GPP	*Salmonella*	O:7	1677	*Salmonella*	O:7	3 TN
S. London	O:3,10	1	Unknown GPP	*Salmonella*	O:10	1095	*Salmonella*	O:10	1 TN
S. Manhattan	O:8	1	Unknown GPP	*Salmonella*	O:8	87	*Salmonella*	O:8	1 TN
S. Mbandaka	O:7	8	Unknown GPP	*Salmonella*	O:7	1419	*Salmonella*	O:7	8 TN
S. Meleagridis	O:3,10	1	Unknown GPP	*Salmonella*	O:10	2847	*Salmonella*	O:10	1 TN
S. Mgulani	O:38	1	Unknown GPP	*Salmonella*	Unknown	6105	*Salmonella*	Unknown	1 TN
S. Miami	O:9	1	Unknown GPP	*Salmonella*	O:9	159	*Salmonella*	O:9	1 TN
S. Mikawasima	O:7	1	Unknown GPP	*Salmonella*	O:7	129	*Salmonella*	O:7	1 TN
S. Minnesota	O:21	16	Unknown GPP	*Salmonella*	O:21	237	Probably *S.* Minnesota[e]	O:21	16 TN
S. Monschaui	O:35	1	Unknown GPP	*Salmonella*	Unknown	3	*Salmonella*	Unknown	1 TN
S. Montevideo	O:7	1	Unknown GPP	*Salmonella*	O:7	129	*Salmonella*	O:7	1 TN
S. Muenchen	O:8	3	Unknown GPP	*Salmonella*	O:8	4785	*Salmonella*	O:8	3 TN
S. Muenster	O:3,10	9	Unknown GPP	*Salmonella*	O:10	219	*Salmonella*	O:10	9 TN
S. Napoli	O:9	1	Unknown GPP	*Salmonella*	O:9	159	*Salmonella*	O:9	1 TN
S. Newport	O:8	2	Unknown GPP	*Salmonella*	O:8	256215	Probably *S.* Bardo/ Newport[d]	O:8	2 TN
S. Newport	O:8	4	Unknown GPP	*Salmonella*	O:8	148335	Probably *S.* Bardo/ Newport/Blockley[d]	O:8	4 TN
S. Newport	O:8	1	Unknown GPP	*Salmonella*	O:8	25665	*Salmonella*	O:8	1 TN
S. Newport	O:8	5	Unknown GPP	*Salmonella*	O:8	435	*Salmonella*	O:8	5 TN
S. Norwich	O:7	2	Unknown GPP	*Salmonella*	O:7	1419	*Salmonella*	O:7	2 TN
S. Nyborg	O:3,10	1	Unknown GPP	*Salmonella*	O:10	1095	*Salmonella*	O:10	1 TN
S. Ohio	O:7	2	Unknown GPP	*Salmonella*	O:7	129	*Salmonella*	O:7	2 TN
S. Oranienburg	O:7	2	Unknown GPP	*Salmonella*	O:7	129	*Salmonella*	O:7	2 TN
S. Panama	O:9	1	Unknown GPP	*Salmonella*	O:9	795	*Salmonella*	O:9	1 TN
S. Paratyphi A	O:2	3	Unknown GPP	*Salmonella*	Unknown	3	*Salmonella*	Unknown	3 TN
S. Pomona	O:28	1	Unknown GPP	*Salmonella*	Unknown	15	*Salmonella*	Unknown	1 TN
S. Poona	O:13	1	Unknown GPP	*Salmonella*	Unknown	15	*Salmonella*	Unknown	1 TN
S. Potsdam	O:7	1	Unknown GPP	*Salmonella*	O:7	4773	*Salmonella*	O:7	1 TN
S. Rissen	O:7	6	Unknown GPP	*Salmonella*	O:7	129	*Salmonella*	O:7	6 TN
S. Rissen	O:7	1	Unknown GPP	*Salmonella*	O:7	7869	Probably *S.* Rissen (ST1846)[d]	O:7	1 TN
S. Rubislaw	O:11	1	Unknown GPP	*Salmonella*	Unknown	3	*Salmonella*	Unknown	1 TN
S. Saintpaul	O:4	4	Unknown GPP	*Salmonella*	O:4	105	*Salmonella*	O:4	4 TN
S. Sandiego	O:4	1	Unknown GPP	*Salmonella*	O:4	21	*Salmonella*	O:4	1 TN
S. Schwarzengrund	O:4	1	Unknown GPP	*Salmonella*	O:4	105	*Salmonella*	O:4	1 TN
S. Senftenberg	O:1,3,19	6	Unknown GPP	*Salmonella*	Unknown	3	*Salmonella*	Unknown	6 TN
S. Singapore	O:7	1	Unknown GPP	*Salmonella*	O:7	1677	*Salmonella*	O:7	1 TN
S. Soerenga	O:30	1	Unknown GPP	*Salmonella*	Unknown	141	*Salmonella*	Unknown	1 TN
S. Stanley	O:4	1	Unknown GPP	*Salmonella*	O:4	105	*Salmonella*	O:4	1 TN
S. Stanleyville	O:4	1	Unknown GPP	*Salmonella*	O:4	273	*Salmonella*	O:4	1 TN
S. Stanleyville	O:4	2	Unknown GPP	*Salmonella*	O:4	21	*Salmonella*	O:4	2 TN
S. Stanleyville	O:4	1	Unknown GPP	*Salmonella*	O:4	23541	Probably *S.* Stanleyville[d]	O:4	1 TN
S. Takoradi	O:8	1	Unknown GPP	*Salmonella*	O:8	87	*Salmonella*	O:8	1 TN
S. Tees	O:16	1	Unknown GPP	*Salmonella*	Unknown	20163	*Salmonella*	Unknown	1 TN
S. Telelkebir	O:13	1	Unknown GPP	*Salmonella*	Unknown	3	*Salmonella*	Unknown	1 TN
S. Tennessee	O:7	5	Unknown GPP	*Salmonella*	O:7	129	*Salmonella*	O:7	5 TN
S. Thompson	O:7	1	Unknown GPP	*Salmonella*	O:7	129	*Salmonella*	O:7	1 TN

(*continued on next page*)

Table 5 (*continued*)

Reference identification[a]		Nbr of isolates	Expected result with the Luminex assay			Obtained results with the Luminex assay			Comparison results[c]
Serotype	Serogroup		GPP	Identification	Serogroup[b]	GPP	Identification	Serogroup[b]	
S. Typhi	O:9	1	Unknown GPP	*Salmonella*	O:9	159	*Salmonella*	O:9	1 TN
S. Uganda	O:3,10	2	Unknown GPP	*Salmonella*	O:10	2409	*Salmonella*	O:10	2 TN
S. Umbilo	O:28	1	Unknown GPP	*Salmonella*	Unknown	93	*Salmonella*	Unknown	1 TN
S. Urbana	O:30	1	Unknown GPP	*Salmonella*	Unknown	3	*Salmonella*	Unknown	1 TN
S. Wandsworth	O:39	1	Unknown GPP	*Salmonella*	Unknown	2145	*Salmonella*	Unknown	1 TN
S. Weltevreden	O:3,10	2	Unknown GPP	*Salmonella*	O:10	219	*Salmonella*	O:10	2 TN
Serratia odorifera	NA	1	–	Non-*Salmonella*	NA	–	Non-*Salmonella*	NA	1 TN
Shigella boydii	NA	1	–	Non-*Salmonella*	NA	–	Non-*Salmonella*	NA	1 TN
Shigella flexneri	NA	1	–	Non-*Salmonella*	NA	–	Non-*Salmonella*	NA	1 TN
Shigella sonei	NA	1	–	Non-*Salmonella*	NA	–	Non-*Salmonella*	NA	1 TN
Staphylococcus aureus	NA	1	–	Non-*Salmonella*	NA	–	Non-*Salmonella*	NA	1 TN
Staphylococcus epidermidis	NA	1	–	Non-*Salmonella*	NA	–	Non-*Salmonella*	NA	1 TN
Staphylococcus mileri	NA	1	–	Non-*Salmonella*	NA	–	Non-*Salmonella*	NA	1 TN
Staphylococcus saprophyticus	NA	1	–	Non-*Salmonella*	NA	–	Non-*Salmonella*	NA	1 TN
Streptococcus agalactiae	NA	1	–	Non-*Salmonella*	NA	–	Non-*Salmonella*	NA	1 TN
Streptococcus bovis	NA	1	–	Non-*Salmonella*	NA	–	Non-*Salmonella*	NA	1 TN
Streptococcus dysgalactiae	NA	1	–	Non-*Salmonella*	NA	–	Non-*Salmonella*	NA	1 TN
Streptococcus pyogenes	NA	1	–	Non-*Salmonella*	NA	–	Non-*Salmonella*	NA	1 TN
Vibrio alginolyticus	NA	1	–	Non-*Salmonella*	NA	–	Non-*Salmonella*	NA	1 TN
Yersinia enterocolitica	NA	1	–	Non-*Salmonella*	NA	–	Non-*Salmonella*	NA	1 TN
Total number of samples		1004					Total TP		687
							Total TN		314
							Total FP		0
							Total FN		3
							Inclusivity: sensitivity		99,6%
							Exclusivity: specificity		100%
							Accuracy		99,7%

1,4,[5],12:i:- : Typhimurium monophasic.
Paratyphi B (dT-): non D-tartrate fermenting Paratyphi B isolates (dT-).
Paratyphi B var. Java (dT+): D-tartrate fermenting Paratyphi B isolates (dT+).
NA: not applicable.
TP: True Positive.
TN: True Negative.
FP: False Positive.
FN: False Negative.

[a] Determined by classical methods i.e., slide-agglutination and biochemical tests.
[b] Determination of the serogroup (if possible), only mentioned when the sample does not belong to one of the targeted serotypes.
[c] Expected serotype identification vs. obtained serotype identification.
[d] Probability determined according to the present results and the *in silico* analysis on Enterobase.
[e] Probability determined according to the present results.

markers, the GPPs and the associated serotyping results are listed in Table 4. In case of unknown or partial identification, the sample must be serotyped by the classical method.

Based on preliminary results (data not shown), an allelic discrimination, i.e. AC interpretation including the use of a WT probe, was needed to properly detect some SNP markers and to improve the fluorescence signal of probes STID4, STID34, STID334, STID71 and STID491, allowing the characterization of *S.* Hadar, *S.* ParatyphiB, *S.* Infantis and *S.* Enteritidis. All other probes are interpreted using SNR calculation, as elaborated in material and methods.

The probe STID50 targeting *S.* Indiana was introduced in the multiplex to exclude this serotype from a relatively infrequent population of *S.* Typhimurium isolates identified by GPP 2415 and representing less than 1% of the *S.* Typhimurium population, according to an *in silico* analysis performed on EnteroBase (2019). Additionally, STID491 was added to the assay for the identification of *S.* Enteritidis isolates belonging to ST183, representing only 1.6% of the *S.* Enteritidis isolates on EnteroBase (2019), but circulating in Belgium (Supplementary Table S1), and not detected by STID2 which is targeting the other *S.* Enteritidis isolates. Finally, the marker targeted by STID5 is not able to detect a small part of the *S.* Hadar population belonging to ST473 with a frequency expected to be 6% like previously anticipated during the marker selection from EnteroBase (2016). Consequently, when the GPP 110577 will be obtained, the Hadar genoserotype result will have to be confirmed by slide-agglutination because false positive results can be retrieved when obtaining a positive result for the probe combination including invA, STID191, STID3, STID4 and negative for STID5.

3.3. Comparison between the reference and the developed molecular method

With the aim to check the specificity of the developed genoserotyping test compared to the reference methods, i.e. serotyping by slide-agglutination and biochemical tests, 690 *Salmonella* isolates belonging to the targeted serotypes, 281 *Salmonella* isolates belonging to untargeted serotypes and 33 non-*Salmonella* isolates were analyzed with both methods. When an unknown GPP was retrieved, the presence of a serogroup marker was screened among the signals of the serogrouping probes. The obtained results are listed in Table 5 and a detailed version including the probe combinations is available in Supplementary Table S1. According to the results, except for 3/45 *S.* Virchow isolates which gave false negative results (with ID numbers S16BD00604, S17BD03634 and S17BD08736), all the other 687 targeted isolates were correctly identified. For the 314 untargeted isolates, no false positive results were obtained.

To investigate why the 3 *S.* Virchow isolates (S16BD00604, S17BD03634 and S17BD08736) were not detected by STID13 and/or STID15, the identification was confirmed by 2 repetitions of the slide-agglutination and their full genome was sequenced. The upload of the corresponding genome assemblies to the SISTR tool also confirmed the *S.* Virchow identification. Additionally, the MLST function (7 genes MLST) of SISTR clustered these 3 isolates into a rare ST (ST 2563 for S16BD00604 and S17BD08736, representing only 0.7% of *S.* Virchow strains in EnteroBase (2019)) or into an unknown ST (for S17BD03634) which are not targeted by the SNP markers of STID13 and/or STID15.

Also, of the 97 *S.* Paratyphi B (dT-/dT+) tested, only one isolate yielded the GPP 1365 and no isolate resulted in the theoretically possible GPP 23205 (both lacking marker of STID71) demonstrating that these two populations of *S.* Paratyphi B are not frequently distributed in Belgium while their expected frequency was near 46% according to the percentage obtained during the selection of molecular markers from EnteroBase (2016) (Tables 4 and 5).

From the 30 *S.* Hadar isolates that were analyzed during this comparison study, 2 (6.6%) were identified as belonging to the ST473 (combination of 4 markers leading to GPP 110577), which is close to the expected frequency determined during the *in silico* analysis using EnteroBase (2016) (Tables 4 and 5). Additionally, for all other *Salmonella* isolates tested, no other genoserotyping results linked to GPP 110577 were obtained. This could lead to the hypothesis that an as good specificity for *S.* Hadar detection can be obtained with GPP 110577 and 4 markers as with GPP 4091349 and 5 markers.

Interestingly, some molecular markers combinations, other than those foreseen for the targeted serotypes (Table 4), seem to be specific for certain other serotypes (Table 5). When possible, for MLST markers in association with serogroup and/or *fljB* markers, the specificity of these combinations was checked *in silico* on EnteroBase (2019) and allowed the prediction of "probable serotype" identification: i.e. *S.* Bardo, *S.* Blockley, *S.* Colindale ST584, *S.* Gallinarum, *S.* Heidelberg, *S.* Hvittingfoss, *S.* Indiana, *S.* Kisangani, *S.* Newport, *S.* Rissen ST1836, *S.* Saintpaul and *S.* Stanleyville eBG79 (Supplementary Table S1). Additionally, considering the few serotypes present in the serogroup O:21 (Grimont and Weill, 2007) and the detection frequency of *S.* Minnesota in Belgium (personal communication, NRC), it is likely that samples resulting in the GPP 237 belong to Minnesota serotype. All these "probable serotype" predictions were in agreement with the results obtained during the comparison study with the reference methods (Table 5). Consequently, the GPPs linked to these predictions were added to the list of possible identifications (Supplementary Table S1) which can be interpreted by the automated Excel file.

According to the inclusivity tests, i.e. identification results of targeted *Salmonella* strains, and exclusivity tests i.e. identification results of untargeted strains, the sensitivity and specificity were calculated to be 99.6% and 100%, respectively. Based on these results, the accuracy of the developed method was determined to be 99.7% (Table 5). During this comparison study, one technician was able to serotype a maximum of 25 samples per day by slide-agglutination. This did not include the determination of the second antigenic phase or the performing of biochemical tests for variant discrimination, when needed, which required at least 1 to 8 additional days. In comparison, using the developed genoserotyping method for the targeted serotypes, the complete identification could be obtained for 90 samples in only one working day, in a single 96-well plate. The price of a *Salmonella* serotype identification by classical and new molecular methods was estimated using to the data of the Belgian NRC. This estimation included the current cost of the consumables, the reagents, the technicians, the purchase of a MagPix apparatus and its maintenance (personal communication, NRC). Already with the analysis of only one sample, the developed genoserotyping assay is 1.7 times cheaper than the classical method. If 25 samples are considered, the new method is 3.5 times less expensive. Finally, if a full 96-well plate is analyzed, which can be performed in one day by one technician, the cost per sample is 7.5 times cheaper.

4. Discussion

In this study, a *Salmonella* genoserotyping tool, based on genetic markers (selected from EnteroBase and scientific literature) and using the Luminex technology, was developed. This tool is able to identify the most common serotypes in Belgium, i.e. Enteritidis, Hadar, Infantis, Virchow, Paratyphi B including its variant Java and Typhimurium including its monophasic variant, that are subjected to an official control (EU regulation N°2160/2003, Belgian royal decree 27/04/2017 and Belgian FASFC note BP-FDS/LABO/1470050 v7). To check the specificity of this new method, 1004 isolates including 971 *Salmonella* and 33 non-*Salmonella* isolates were analyzed. Resulting genoserotyping profiles were compared with serotyping identifications obtained using the reference methods, i.e. slide-agglutination and biochemical tests, and lead to an accuracy of 99.7%. Only 3 *S.* Virchow isolates of 45 tested, representing 6.8% of the tested *S.* Virchow isolates and belonging to rare STs, were not correctly identified by the molecular test. Consequently, when these genoserotypes will be analyzed by the developed method, they will be reported as *Salmonella* belonging to the serogroup O:7 and the Virchow serotype identification will have to be obtained by slide-agglutination.

Some differences were observed concerning the genoserotype distribution between the percentages estimated during the *in silico* analysis and the results obtained with the comparison study. This can be explained by the genoserotype distribution present in EnteroBase which was not always representative of the *Salmonella* population circulating in Belgium between 2005 and 2018, and used during the comparison study. This was especially the case for *S.* Paratyphi B (dT-/dT+).

Interestingly, the developed method was able to make genoserotype predictions, based on specific marker combinations other than those used for the targeted serotypes. Although only a few *Salmonella* isolates were tested to check these predictions, the specificity of the associated marker combinations was validated *in silico* in EnteroBase. In addition to the clustering made by the serogrouping probes, these predictions are an added value to the method, as they can give some clues about the identification of untargeted serotypes and contribute to the confirmation by classical methods. For example, one of these predictions concerns the serotype Newport which is present in the top 5 of the most common serotypes in Europe, reported by EFSA (2018) for the year 2017, and the closely related serotype Bardo. These 2 serotypes differ by only one somatic antigen (O:6 vs. O:6,8) in their antigenic formula (Grimont and Weill, 2007), which explains that they are hardly discriminated with molecular methods. Consequently, when the result "Probably *S.* Bardo/Newport" is retrieved by the GPP automatic interpretation, slide-agglutination of antigens H1:e,h for exclusion of other serotypes and O:6 for Bardo/Newport discrimination, can be performed, in the same day to confirm the identification. Identically, the slide-agglutination of targeted antigens, following the KWL scheme, can be done for the verification of the other genoserotyping predictions obtained during the comparison study.

As the developed test uses a barcode system (GPP) and an automated Excel file to process Luminex data generated by the MagPix, the results are more accurate and objective compared to the classical method, for which trained technicians are required to properly interpret subjective slide-agglutinations and biochemical tests. However, this genoserotyping system follows the KWL classification and is, therefore, fully compatible with the regulation and the serotypes mentioned in it (EU regulation N°2160/2003 Belgian royal decree 27/04/2017 and Belgian FASFC note BP-FDS/LABO/1470050 v7). Moreover, the complete genoserotyping analysis, from DNA extraction to data interpretation, takes only one working day compared to the classical method where at least 2–9 days are sometimes required to have a complete identification, including variant determination like for example *S.* Paratyphi B var. Java for which a complex and time-consuming biochemical test is needed (Alfredsson et al., 1972; Barker, 1985). The method is also cost-effective as it is performed in 96-well plates and only one well is required per sample, making it 7.5 times less expensive compared to the classical serotyping. To be able to perform this genoserotyping assay, commonly used equipment such as a PCR instrument and a centrifuge are needed, in addition to the purchase of a MagPix instrument.

Retrospectively considering the serotyping analyses performed by the Belgian NRC during the last 10 years using classical methods, the developed genoserotyping method could have identified more than 77% of the *Salmonella* isolates sent to the center, and could have given serogroup orientation or probable serotype prediction for even more (annual reports and personal communication, NRC). In conclusion, although the developed *Salmonella* genoserotyping method can detect less serotypes than other methods (Wattiau et al., 2011; Yoshida et al., 2016) or the commercially available *Salmonella* identification kit, it is perfectly adapted to first line laboratories for which a fast, accurate and cost-effective tool is needed, avoiding the sending of most *Salmonella* samples to the NRC. Like this, the results are rapidly transmitted to the professionals of the food sector who then know if they are in agreement with the criteria of the regulation and they can sell properly their food products as soon as possible. Furthermore, other MOL-PCR assays compatible with the MagPix apparatus exist for pathogen diagnostics (Wessels et al., 2014), subtyping (Ventola et al., 2019; Wuyts et al., 2015a) or antimicrobial resistance screening (Ceyssens et al., 2016). All these compatible tests can be performed with the MagPix in the same run using the multi-batch function (with a limit of 96 reactions in total) thereby lowering the analysis price per sample. Moreover, the present method is modular and the target composition can easily be adapted following the evolution of the most common *Salmonella* serotypes or the required law adaptations. In the future, additional MOL-PCR assays could be developed to detect other *Salmonella* serotypes commonly encountered in the Belgian food sector.

Declaration of competing interest

All authors declare that they have no conflict of interest

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2019.103394.

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