One-Step Triplex High-Resolution Melting Analysis for Rapid Identification and Simultaneous Subtyping of Frequently Isolated *Salmonella* Serovars

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Salmonellosis is one of the most important food-borne diseases worldwide. For outbreak investigation and infection control, accurate and fast subtyping methods are essential. A triplex gene-scanning assay was developed and evaluated for serotype-specific subtyping of *Salmonella enterica* isolates based on specific single-nucleotide polymorphisms in fragments of *fljB*, *gyrB*, and *ycfQ*. Simultaneous gene scanning of *fljB*, *gyrB*, and *ycfQ* by high-resolution melting-curve analysis of 417 *Salmonella* isolates comprising 46 different serotypes allowed the unequivocal, simple, and fast identification of 37 serotypes. Identical melting-curve profiles were obtained in some cases from *Salmonella enterica* serotype Enteritidis and *Salmonella enterica* serotype Dublin, in all cases from *Salmonella enterica* serotype Ohio and *Salmonella enterica* serotype Rissen, from *Salmonella enterica* serotype Mbandaka and *Salmonella enterica* serotype Kentucky, and from *Salmonella enterica* serotype Bredeney, *Salmonella enterica* serotype Give, and *Salmonella enterica* serotype Schwarzengrund. To differentiate the most frequent *Salmonella* serotype, Enteritidis, from some *S. Dublin* isolates, an additional single PCR assay was developed for specific identification of *S. Enteritidis*. The closed-tube triplex high-resolution melting-curve assay developed, in combination with an *S. Enteritidis*-specific PCR, represents an improved protocol for accurate, cost-effective, simple, and fast subtyping of 39 *Salmonella* serotypes. These 39 serotypes represent more than 94% of all human and more than 85% of all nonhuman *Salmonella* isolates (including isolates from veterinary, food, and environmental samples) obtained in the years 2008 and 2009 in Austria.

Salmonella enterica is one of the most important global food-borne pathogens. Every year, millions of human cases of salmonellosis are reported all over the world, resulting in thousands of deaths (28). In Austria, the incidence of salmonellosis was about 38 cases per 100,000 inhabitants in 2008 and 34 cases per 100,000 inhabitants in 2009 (9, 10). The gold standard for *Salmonella* subtyping is based on the scheme developed by Kauffmann, White, and Le Minor, using the serologic identification of O (somatic) and H (flagellar) antigens (4). Presently, more than 2,500 different *Salmonella* serotypes (or serovars) have been defined. Despite the usefulness of serotyping, testing with a complete set of antisera is time-consuming and requires a well-trained technician (5). Furthermore, serotyping does not reveal the genetic relatedness of different isolates (25). Thus, many molecular-based subtyping methods, i.e., pulsed-field gel electrophoresis (PFGE), amplified fragment length polymorphism (AFLP) (14), multilocus sequence subtyping (MLST), multilocus enzyme electrophoresis (MLEE) (25), multiple-locus variable-number tandem-repeat analysis (MLVA) (11), and microarray techniques (15), are routinely used for *Salmonella* subtyping. The advantage of sequence-based methods, like MLST or single-nucleotide polymorphism (SNP), over DNA band pattern-based methods, like PFGE or AFLP, is better and easier comparability of data (1, 6, 16).

For fast identification and subtyping of isolates, especially for outbreak situations and routine diagnostics, a PCR-based subtyping method is preferable in terms of cost, simplicity, turnaround time, and potential for standardization, since MLST and detection of multiple SNPs still represent time-consuming and cost-intensive approaches.

High-resolution melting (HRM) analysis represents a relatively new method using single-nucleotide polymorphisms for strain differentiation. The principle of HRM analysis is based on the generation of different melting-curve profiles due to sequence variations in double-stranded DNA. Single-nucleotide changes represent the smallest genetic changes and are divided into four classes distinguished by different melting-temperature (T_m) shifts (13, 27). SNP class 1 involves C/T and G/A, and SNP class 2 involves C/A and G/T, base exchanges that can easily be genotyped by HRM due to T_m differences of more than 0.5°C (13). In contrast, in SNP class 3, only C/G base exchange occurs, and SNP class 4 is described by A/T base exchange, producing very small T_m differences (<0.4°C for SNP class 3 and <0.2°C for SNP class 4) (21).

In general, HRM analysis is a simple, inexpensive, and rapid scanning method for known and unknown mutations (21, 22, 29) and can dramatically reduce the turnaround time for mutation screening and testing. Here, we describe the development and evaluation of a triplex HRM assay based on sequence variations in three genes (*fljB*, *gyrB*, and *ycfQ*), in combination with a *Salmo-*

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nella enterica serotype Enteritidis-specific real-time PCR assay, for the fast identification of *Salmonella* serotypes.

MATERIALS AND METHODS

Microorganisms. A set of 33 *Salmonella* isolates representing the 14 most frequent *Salmonella enterica* serotypes found in 2008 in Austria (*S. Enteritidis* [$n = 4$], *Salmonella enterica* serotype Typhimurium biphasic variant [$n = 3$, including the reference strain, ATCC 14028], *S. Typhimurium* monophasic variant [$n = 2$], *Salmonella enterica* serotype Infantis [$n = 2$], *Salmonella enterica* serotype Saintpaul [$n = 2$], *Salmonella enterica* serotype Hadar [$n = 2$], *Salmonella enterica* serotype Agona [$n = 2$], *Salmonella enterica* serotype Newport [$n = 2$], *Salmonella enterica* serotype Thompson [$n = 2$], *Salmonella enterica* serotype Abony [$n = 2$], *Salmonella enterica* serotype Montevideo [$n = 2$], *Salmonella enterica* serotype Senftenberg [$n = 2$], *Salmonella enterica* serotype Dublin [$n = 2$], and *Salmonella enterica* serotype Tennessee [$n = 2$]) and one relatively rare serotype (*Salmonella enterica* serotype Indiana [$n = 2$]) was initially used to develop a triplex high-resolution melting-curve assay. To evaluate the performance of the assay, an additional collection of 385 *Salmonella* isolates comprising 46 serotypes was analyzed. Ten *Salmonella enterica* serotype Gallinarum and 10 *Salmonella enterica* serotype Choleraesuis (including nine *S. Choleraesuis* var. *kunzendorf*) isolates closely related to *S. Enteritidis* were analyzed by the specific *S. Enteritidis* *edrI* assay.

All 438 *Salmonella* isolates were provided by the Austrian National Reference Centre for *Salmonella* and serotyped according to the White-Kauffmann-Le Minor scheme.

Bacteria were grown overnight at 37°C on BD BBL XLD agar (Becton, Dickinson and Company, Franklin Lakes, NJ). Genomic DNA (gDNA) was isolated with the Genelute Bacterial Genomic DNA kit (Sigma, St. Louis, MO) according to the instructions of the manufacturer. The concentration and quality of the purified gDNA were determined by UV spectrophotometry at 260 and 280 nm and agarose gel (1.5% [wt/vol]) electrophoresis.

Detection of serotype-specific SNPs. Serotype-specific SNPs and fragments were identified through extensive sequence analysis of the published sequences of *atpD*, *fljB*, and *gyrB*, as well as through isolation, reamplification, and sequencing of AFLP fragments (23). Finally, loci within *fljB*, *gyrB*, and *ycfQ* that allowed the unequivocal differentiation of the initial set of isolates were chosen for amplification and subsequent HRM analysis. The Primer-BLAST online tool from the National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov>) was used for specific HRM primer design.

High-resolution melting-curve PCR analysis. HRM analysis was performed by specific coamplification of a 170-bp fragment of *fljB* using the primers *fljB*-forward (5'-GTGAAAGATACAGCAGTAACAACG-3') and *fljB*-reverse (5'-ACAAAGTACTTGTATTATCTGCG-3'), a 171-bp fragment of *gyrB* using the primers *gyrB*-forward (5'-AAACGCCGATCCACCCGA-3') and *gyrB*-reverse (5'-TCATCGCCGCACGGAAG-3'), and a 241-bp fragment of *ycfQ* using the primers *ycfQ*-forward (5'-GCCTACTCTATGCGGAATTAC-3') and *ycfQ*-reverse (5'-GATATCGCGCAGGAGGCG-3'). All the primers had the respective M13 sequence attached to the 5' end of the gene-specific priming sequence for subsequent sequencing. PCR and HRM analysis were performed on the LightCycler LC480 (Roche Diagnostics, Penzberg, Germany). In a final volume of 10 μ l, the HRM PCR mixture contained 2 ng of gDNA, 0.1 pmol of each *gyrB* primer, 0.075 pmol of each *fljB* primer, 0.075 pmol of each *ycfQ* primer, and 3 mM MgCl₂ in the LightCycler 480 High Resolution Melting Master Mixture containing ResoLight dye (Roche Diagnostics). HRM PCR included an activation step at 95°C for 10 min, followed by 45 cycles of 95°C for 10 s, 60°C for 10 s, and 72°C for 10 s. Prior to HRM analysis, the amplification products were heated to 95°C for 1 min and then cooled to 40°C for 1 min. HRM analysis was performed from 60°C to 95°C, rising at 1°C/s, with 25 acquisitions per degree.

We used the LC 480 gene-scanning software version 1.5 with manual settings for sensitivity at 0.30 and for temperature shift at threshold 5, a

premelt normalization range from 80.87 to 81.51, and a postmelt normalization range from 89.17 to 89.92 for HRM analysis. After normalizing and temperature shifting of the melting curves, difference plots were generated by selecting HRM profile 6, representing *S. Thompson*, as the baseline. Only amplification products reaching the plateau phase were analyzed. For optimal performance (i.e., correct assignment of melting-curve profiles to a known sequence) of HRM experiments, each run must contain well-characterized standards (i.e., strains with known sequences) because melting-curve profiles obtained from different runs cannot be compared directly.

Sequencing of HRM products. For sequencing of HRM products, a singleplex reaction (20- μ l final volume) was applied with the respective primer pair (10 pmol of each primer), 2 ng of gDNA, and the RedTaq Ready Mix (Sigma). The amplification reaction was performed in a Master Cycler Eppgradient S (Eppendorf, Hamburg, Germany) programmed as follows: an initial step of 30 s at 95°C and 35 cycles of 30 s at 95°C, 30 s at 55°C, and 1 min at 72°C. After amplification, 5 μ l of each PCR product was analyzed on a 1.5% (wt/vol) agarose gel. PCR products were purified using the QIAquick PCR purification kit (Qiagen, Inc., Chatsworth, CA). Sequence analysis was performed using a SequiTerm Excel II Cycle Sequencing Kit (Epicentre, Madison, WI) with fluorescence-labeled primers M13 universal (5'-TGTAACGACGCGCCAGT-3') and M13 reverse (5'-CAGGAACAGCTATGACC-3') (MWG-Biotech, Ebersberg, Germany) in a Licor 4300 automated DNA sequencer (Li-Cor Bioscience, Lincoln, NE) according to the manufacturer's instructions. All sequences obtained were assembled, edited, and compared to determine sequence variations.

Specific detection of *S. enterica* serovar Enteritidis. In previous experiments, AFLP analysis resulted in the identification of an *S. Enteritidis*-specific fragment (23) identical to *Salmonella enterica* subsp. *enterica* serovar Enteritidis difference region I (AF370716) (2). For specific detection of *S. Enteritidis*, a 156-bp fragment of *S. Enteritidis* difference region I was amplified using the primers *EntI*-forward (5'-GACGAGCTCTTACAC TCCATCAGTT-3') and *EntI*-reverse (5'-GAAAGTGTTCAGAACTC TTGTTGCAT-3'). PCR was performed on a LightCycler LC480 (Roche Diagnostics). The specificity of the assay was evaluated using 86 *S. Enteritidis* and 352 non-*S. Enteritidis* isolates (including the closely related *S. Dublin*, *S. Gallinarum*, and *S. Choleraesuis*) as described in "Microorganisms" above.

The reaction mixture contained 2 ng of gDNA, 0.25 pmol of each primer, and 3 mM MgCl₂ in the LightCycler 480 SYBR green I Master Mix (Roche Diagnostics) with PCR grade water adjusted to a final volume of 10 μ l. The reaction conditions included an initial denaturation step at 95°C for 10 min, followed by 35 cycles of 95°C for 10 s, 60°C for 10 s, and 72°C for 40 s.

Multilocus sequence subtyping. MLST analysis was performed on 84 isolates, representing the serotypes *S. Enteritidis* ($n = 21$), *S. Dublin* ($n = 13$), *S. Montevideo* ($n = 19$), *S. Saintpaul* ($n = 13$), *S. Newport* ($n = 13$), and *Salmonella enterica* serotype Paratyphi B var. *d-tartrate*⁺ ($n = 5$), by determining the sequences of seven housekeeping genes (*aroC*, *dnaN*, *hemD*, *hisD*, *purE*, *sucA*, and *thrA*), as described previously (8). Amplification was performed in a 20- μ l reaction mixture using RedTaq Ready Mix (Sigma) and 10 pmol of each primer. The amplification conditions were as follows: an initial step of 30 s at 95°C and 45 cycles of 30 s at 95°C, 30 s at 53°C, and 1 min at 72°C. After amplification, 5 μ l of each PCR product was analyzed on a 1.5% (wt/vol) agarose gel. Prior to sequencing, the amplification products were purified with Exo Sap-It (GE Healthcare, Buckinghamshire, United Kingdom). Two microliters of purified amplification product was used for subsequent sequencing with the BigDye Terminator v3.1 sequencing kit (Applied Biosystems, Carlsbad, CA). The products were analyzed on an ABI Genetic Analyzer 3500Dx (Applied Biosystems). The sequences obtained were compared to the reference sequences available at the *Salmonella* MLST database (<http://mlst.ucc.ie/mlst/dbs/Senterica>) for allele identification. Novel alleles and sequence types (STs) were submitted for allele, ST, and clonal complex (CC) des-

FIG 1 Difference plot of melting-curve profiles of normalized and temperature (Temp)-shifted amplification products of the genes *fljB*, *gyrB*, and *ycfQ* from the 15 different *Salmonella* serotypes evaluated in the initial set of isolates showing HRM-CPs 1 to 16.

ignations. CLUSTAL W was used for sequence comparison and determination of the genetic relatedness of the different MLSTs (<http://www.clustal.org/>) (12).

Nucleotide sequence accession numbers. The sequences of the eight different *fljB* sequence types were deposited in GenBank under accession numbers JQ514786 and JQ629418 to JQ629424, the 21 different *gyrB* sequence types under GenBank accession numbers JQ514787 and JQ629443 to JQ629462, and the 19 different *ycfQ* sequence types under GenBank accession numbers JQ595558 and JQ629425 to JQ629442.

RESULTS

High-resolution melting-curve PCR analysis. We performed parallel gene scanning of three amplification products of *gyrB*, *fljB*, and *ycfQ* on the initial set of 33 *Salmonella* isolates, comprising 15 Austrian *S. enterica* serotypes (the 14 most common serotypes and one rare serotype, accounting for 85% of all tested *Salmonella* isolates in 2008 in Austria). They yielded 16 different melting-curve profiles (HRM-CP 1 to 16), as shown in Fig. 1. Most *S. enterica* serotypes had one specific melting-curve profile, except *S. Newport*, which had two specific HRM profiles (HRM-CP 3 and HRM-CP 4) (Fig. 1 and Table 1). The triplex HRM assay was blindly tested on an arbitrary collection of 385 *Salmonella* isolates. Two hundred sixty-eight (69.6%) of these 385 additional isolates could be assigned to the initial 16 HRM-CPs. The remaining 117 isolates yielded 39 new melting-curve profiles derived from 36 serotypes (curves not shown). Classical serotyping revealed that the isolate collection (all 418 isolates) contained 46 different serotypes that yielded 55 distinct melting-curve profiles (Table 1).

Serotypes characterized by single, unique melting-curve profiles are shown in Table 1. In addition, subtyping within a serotype was possible for *S. Saintpaul*, *S. Thompson*, *S. Senftenberg*, *S. Montevideo*, *Salmonella enterica* serotype Kedougou, *Salmonella enterica* serotype Derby, and *Salmonella enterica* serotype Livingstone, each characterized by two distinct melting-curve profiles; *S. Paratyphi* B var. d-tartrate⁺ yielded three unique melting-curve profiles, and *S. Newport* showed five characteristic melting-curve profiles (Table 1). All 86 investigated *S. Enteritidis* isolates and 7

out of 13 *S. Dublin* isolates shared HRM-CP 14; all 10 *Salmonella enterica* serotype Mbandaka and all three *Salmonella enterica* serotype Kentucky isolates shared HRM-CP 42; the single *Salmonella enterica* serotype Ohio isolate and all four *Salmonella enterica* serotype Rissen isolates shared HRM-CP 46; all five *Salmonella enterica* serotype Bredeney, all three *Salmonella enterica* serotype Schwarzengrund, and all three *Salmonella enterica* serotype Give isolates shared HRM-CP 33 (Table 1).

Sequencing of the three amplification products of *fljB*, *gyrB*, and *ycfQ* detected eight different *fljB* sequence types, 21 different *gyrB* sequence types, and 19 different *ycfQ* sequence types (Tables 2 to 4).

Amplification of the *fljB* fragment was observed in 22 serotypes (Table 1).

Amplification of the *gyrB* and the *ycfQ* fragment was possible with all *Salmonella* isolates. Based on the combination of the sequences of the amplified fragments, 57 different HRM sequence types (HRM-STs) could be defined, resulting in HRM-CP 1 to 55 (Table 1).

For accurate differentiation of *S. Enteritidis* from *S. Dublin* isolates showing HRM-CP 14, a PCR assay specific for the *S. Enteritidis* *edrI* region was developed. The specific *edrI* fragment could be amplified from all 86 *S. Enteritidis* isolates tested, but not from the 352 non-*S. Enteritidis* isolates (Fig. 2).

Multilocus sequence typing analysis. To assess the HRM results obtained for *S. Enteritidis* and *S. Dublin*, as well as serotypes displaying multiple HRM profiles, a set of arbitrarily chosen isolates was analyzed by multilocus sequence typing. MLST of 21 *S. Enteritidis* isolates and all seven *S. Dublin* isolates showing HRM-CP 14 revealed that all 21 *S. Enteritidis* isolates belonged to MLST 11 (ST-11) and the seven *S. Dublin* isolates to ST-1494. ST-11 and ST-1494 could be distinguished due to sequence differences in *hisD* (13 SNPs), *purE* (2 SNPs), *sucA* (8 SNPs), and *thrA* (4 SNPs) (Table 5). HRM-CP 15, characteristic of the remaining six *S. Dublin* isolates, belonged to ST-10 (5 isolates) and ST-1487 (1 isolate). Neighbor-joining placed ST-10, ST-1487, and ST-1494

TABLE 1 Sequence types and triplex HRM profiles of *fljB*, *gyrB*, and *ycfQ*^a

Serotype (no. of strains)	Curve profile/sequence type				
	HRM-CP	HRM-ST	<i>fljB</i> -ST	<i>gyrB</i> -ST	<i>ycfQ</i> -ST
<i>S. Abony</i> (21)	9	9		5	4
<i>S. Agona</i> (13)	13	13		7	5
<i>S. Amsterdam</i> (1)	34	35		21	3
<i>S. Blockley</i> (4)	19	19	3	10	7
<i>S. Coeln</i> (3)	38	39	1	12	2
<i>S. Corvallis</i> (4)	49	51		1	5
<i>S. Derby</i> (1)	44	45		16	18
<i>S. Derby</i> (2)	43	44		16	17
<i>S. Hadar</i> (14)	8	8		1	3
<i>S. Havana</i> (1)	53	55		16	12
<i>S. Heidelberg</i> (7)	31	31	1	1	4
<i>S. Indiana</i> (21)	7	7	4	4	5
<i>S. Infantis</i> (13)	5	5	2	1	3
<i>S. Javiana</i> (1)	47	49	7	16	9
<i>S. Kedougou</i> (2)	36	37	3	3	2
<i>S. Kedougou</i> (4)	23	23	3	1	3
<i>S. Kottbus</i> (6)	37	38		20	5
<i>S. Livingstone</i> (1)	41	42		8	4
<i>S. Livingstone</i> (2)	48	50		1	2
<i>S. Manhattan</i> (5)	22	22	3	3	4
<i>S. Mississippi</i> (2)	35	36	5	7	2
<i>S. Montevideo</i> (10)	16	16		8	9
<i>S. Montevideo</i> (9)	17	17		9	10
<i>S. Muenchen</i> (3)	21	21	1	13	7
<i>S. Napoli</i> (1)	32	32		8	13
<i>S. Newport</i> (1)	28	28	1	13	11
<i>S. Newport</i> (2)	18	18	1	2	4
<i>S. Newport</i> (2)	27	27	1	3	11
<i>S. Newport</i> (4)	3	3	1	1	3
<i>S. Newport</i> (4)	4	4	1	3	3
<i>S. Orion</i> (1)	45	46	2	19	14
<i>S. Paratyphi B</i> var. d-tartrate ⁺ (1)	55	57	1	14	5
<i>S. Paratyphi B</i> var. d-tartrate ⁺ (2)	25	25	1	8	4
<i>S. Paratyphi B</i> var. d-tartrate ⁺ (2)	54	56		13	19
<i>S. Poona</i> (1)	51	53	8	16	9
<i>S. Saintpaul</i> (1)	26	26	1	3	2
<i>S. Saintpaul</i> (13)	2	2	1	2	2
<i>S. San Diego</i> (1)	24	24		14	9
<i>S. Senftenberg</i> (10)	12	12		7	6
<i>S. Senftenberg</i> (3)	30	30		3	12
<i>S. Stanley</i> (3)	50	52	1	14	4
<i>S. Tennessee</i> (21)	10	10		6	5
<i>S. Thompson</i> (1)	29	29	3	17	4
<i>S. Thompson</i> (12)	6	6	3	1	4
<i>S. Typhimurium</i> biphasic variant (25)	1	1	1	1	1
<i>S. Typhimurium</i> monophasic variant (21)	11	11		1	1
<i>S. Virchow</i> (3)	20	20	1	11	4
<i>S. Worthington</i> (1)	52	54		13	12
<i>S. IIIb</i> 61:k:1,5,7 (3)	39	40	6	18	15
<i>S. IIIb</i> 59:z10:z53 (1)	40	41		18	16
<i>S. Dublin</i> (6)	15	15		2	8
<i>S. Dublin</i> (7)	14	14		2	7
<i>S. Enteritidis</i> (86)	14	14		2	7
<i>S. Kentucky</i> (3)	42	43		13	2
<i>S. Mbandaka</i> (10)	42	43		13	2
<i>S. Ohio</i> (1)	46	47		15	4
<i>S. Rissen</i> (4)	46	48		15	5
<i>S. Give</i> (3)	33	33	4	16	9
<i>S. Schwarzengrund</i> (3)	33	33	4	16	9
<i>S. Bredeney</i> (5)	33	34	4	8	9

^a Respective reference sequences: *S. Typhimurium* LT2 (AE006468) for HRM-CP 1, *S. Newport* SL254 (CP001113) for HRM-CP 4, *S. Agona* SL483 (CP001138) for HRM-CP 13, *S. Enteritidis* P125109 (AM933172) for HRM-CP 14, and *S. Dublin* CT_02021853 (CP001144) for HRM-CP 15.

TABLE 2 Mutations detected among the 8 different *fljB* STs^a

<i>fljB</i> ST	Nucleotide at position:																															
	566	568	576	577	599	600	603	606	610	611	616	618	621	624	628	630	633	635	636	646	647	651	652	654	655	658	659	666	669	670	673	675
1	A	G	C	A	T	A	G	T	G	A	G	A	T	T	G	G	T	C	G	A	A	T	A	G	G	T	C	C	T	G	G	G
2	C			G					A	C					A						C											
3				G	C	C	A	C				G	C	C				C	T	T						G					A	A
4																							G	A	C	A	G			A		
5				G									C	C			C	T	A		C			C	G		G	A		A	A	
6			T	G			A						G								C		G		C	A	G	C			A	A
7				G	C	C	A	C					C	C		A	C	T	T		C				C	A	G	G	C		A	A
8		A		G	C	C	A	C			A			C				T	A	C	T	C									A	A

^a In the GenBank entry of *S. Typhimurium* LT2 (AE006468), which shows sequence type 1, the amplified fragments correspond to positions 541 to 710 of *fljB*. Numbering starts with the A of the start codon.

into one cluster (Fig. 3). Differences between these MLSTs were only detected in the sequences of *aroC* and *hisD*. ST-11 had the highest sequence similarity to ST-1494 (Table 5).

The five HRM-CPs (HRM-CP 3, HRM-CP 4, HRM-CP 18, HRM-CP 27, and HRM-CP 28) specific for 13 *S. Newport* isolates consisted of seven MLSTs. HRM-CP 3, HRM-CP 18, and HRM-CP 28 could be assigned to ST-31, ST-166, and ST-1497. HRM-CP 4 comprised ST-45 (3 isolates) and ST-1496 (1 isolate), and HRM-CP 27 comprised ST-46 (1 isolate) and ST-223 (1 isolate). Neighbor-joining analysis revealed one cluster for the MLSTs: ST-166, ST-31, ST-45, ST-1496, ST-46, and ST-1497, with the highest sequence homology between ST-46 and ST-1497 (Fig. 3 and Table 5). The 19 *S. Montevideo* isolates investigated belonged to two HRM-CPs and to seven MLSTs. HRM-CP 16 comprised ST-1488 (5 isolates), ST-1489 (2 isolates), ST-1492 (1 isolate), ST-138 (1 isolate), and ST-1493 (1 isolate). HRM-CP 17 comprised ST-1490 (8 isolates) and ST-1491 (1 isolate). All *S. Montevideo* MLSTs could be assigned to a serotype-specific cluster (Fig. 3 and Table 5).

Thirteen *S. Saintpaul* isolates, characterized by HRM-CP 2 and

HRM-CP 26, consisted of three MLSTs. HRM-CP 2 comprised ST-27 (10 isolates) and ST-680 (2 isolates), and HRM-CP 26 was assignable to ST-49. Comparative sequence analysis resulted in a serotype-specific cluster comprising ST-27, ST-680, and ST-49 (Fig. 3 and Table 5).

The three *S. Paratyphi* B var. d-tartrate⁺-specific HRM-CPs were assignable to four MLSTs. HRM-CP 54 and HRM-CP 55 could be assigned to the MLSTs ST-423 and ST-28. HRM-CP 25 was assignable to ST-88 (1 isolate) and ST-1560 (1 isolate). *S. Paratyphi* B var. d-tartrate⁺ ST-423, ST-1560, and ST-28 formed a cluster with *S. Newport* ST-223 (HRM-CP 27) that was adjacent to the *S. Newport* cluster comprising all other *Newport* STs. Finally, the novel alleles (*aroC10*, *aroC411*, *aroC412*, *dnaN387*, and *hisD491*) identified and new sequence types, ST-1487, ST-1488, ST-1489, ST-1490, ST-1491, ST-1492, ST-1493, ST-1494, ST-1496, ST-1497, and ST-1560, were submitted to the MLST database.

DISCUSSION

Conventional *Salmonella* serotyping according to the White-Kauffmann-Le Minor scheme (4, 19) is a time-consuming and

TABLE 3 SNPs detected between the 21 different *gyrB* STs^a

<i>gyrB</i> ST	Nucleotide at position:														
	726	753	756	762	768	780	786	795	807	828	831	837	840	846	
1	T	C	G	A	G	T	C	C	T	C	C	T	T	T	
2	C		C					T		T					
3	C				A								C	G	
4	C							T		T			C	G	
5	C														
6	C	T												G	
7	C	T			A	C							C	G	
8	C		C											G	
9								T					C	G	
10		T	T	T					C	T	T	G			
11	C		T	G									C	G	
12	C							T		T					
13	C					C							C	G	
14	C							T						G	
15	C												C	G	
16	C													G	
17	C				A					A			C	G	
18	C		C	G	A	C	T				T		C	G	
19	C	A	C											G	
20		T											C	G	
21														G	

^a In the GenBank entry of *S. Typhimurium* LT2 (AE006468) which shows sequence type 1, the amplified fragments correspond to positions 698 to 868 of *gyrB*. Numbering starts with the A of the start codon.

TABLE 4 Sequence differences detected among the 19 identified *ycfQ* STs^a

<i>ycfQ</i> ST	Nucleotide at position:																						
	180	186	190	210	243	258	264	279	284	285	288	292	300	315	321	330	333	342	351	369	378	385	386
1	C	C	A	C	A	C	T	A	C	G	G	G	C	C	C	T	A	G	C	C	G	T	C
2			G																				
3		T	G	A	G			G													C		
4			G		G			G													C		
5			G		G			G															
6	T		G		G			G				A								T	C		
7		T	G		G			G													C		
8		T	G		G			G			A										C		
9			G		G			G									G				C		
10			G																		C		
11			G		G			G													T		
12			G		G			G									G						
13		T	G	A	G			G									G				C		
14		T	G	A	G	G		G													C		
15			G		G		C	G	G	C			T	T	T	C			T				
16			G		G		C	G	G	C			T	T	T	C							
17			G		G			G										A			C	T	
18			G																		C	T	
19			G		G																		

^a In the GenBank entry of *S. Typhimurium* LT2 (AE006468), which shows sequence type 1, the amplified fragments correspond to positions 159 to 399 of *ycfQ*. Numbering starts with the A of the start codon.

demanding method and requires vertebrate animals for serum production (5). Therefore, several molecular-based subtyping methods have been developed for *Salmonella* subtyping (5, 7, 14). PFGE and MLST are still expensive and time-consuming methods and are therefore of limited value for routine subtyping (14, 24). Most PCR-based subtyping procedures allow the detection of only a single serotype (17, 20), and even in multiplex-PCR-based approaches, the number of identifiable serotypes is low and requires a multistep protocol (18). Advances in whole-genome sequencing

will further facilitate the identification of suitable markers and therefore improve molecular subtyping procedures (3). Here, we describe an improved protocol for accurate, cost-effective, simple, and fast subtyping of *Salmonella* serotypes in a single closed-tube assay format by parallel high-resolution melting-curve analysis based on serotype-specific SNPs within distinct loci of *fljB*, *gyrB* (26), and *ycfQ* (23). Evaluation of the triplex HRM assay we developed, using an arbitrary collection of 418 *Salmonella* isolates comprising a total of 46 different serotypes, generated 55 distinct

FIG 2 Specific detection of *S. Enteritidis* by amplification of an *edrI* gene fragment (red). No *edrI* fragment was detectable in isolates of all other investigated serotypes (blue).

TABLE 5 Detected MLSTs compared to the observed HRM profiles^a

Serotype (no. of isolates)	Allele							MLST	HRM-ST	HRM profile
	<i>aroC</i>	<i>dnaN</i>	<i>hemD</i>	<i>hisD</i>	<i>purE</i>	<i>sucA</i>	<i>thrA</i>			
<i>S. Enteritidis</i> (21)	5	2	3	7	6	6	11	11	14	14
<i>S. Dublin</i> (7)	5	2	3	491	5	5	10	1494	14	14
<i>S. Dublin</i> (5)	5	2	3	6	5	5	10	10	15	15
<i>S. Dublin</i> (1)	410	2	3	6	5	5	10	1487	15	15
<i>S. Newport</i> (4)	2	2	15	14	15	20	12	31	3	3
<i>S. Newport</i> (3)	10	7	21	14	15	12	12	45	4	4
<i>S. Newport</i> (1)	10	61	18	67	15	12	12	1496	4	4
<i>S. Newport</i> (2)	5	14	6	12	5	14	58	166	18	18
<i>S. Newport</i> (1)	10	7	21	12	15	12	12	46	27	27
<i>S. Newport</i> (1)	16	2	61	43	36	39	42	223	27	27
<i>S. Newport</i> (1)	10	387	21	12	15	12	12	1497	28	28
<i>S. Montevideo</i> (5)	43	31	16	42	35	13	111	1488	16	16
<i>S. Montevideo</i> (2)	43	31	16	13	34	13	4	1489	16	16
<i>S. Montevideo</i> (1)	411	41	18	13	12	13	4	1492	16	16
<i>S. Montevideo</i> (1)	11	41	55	42	34	58	4	138	16	16
<i>S. Montevideo</i> (1)	201	41	16	42	35	13	111	1493	16	16
<i>S. Montevideo</i> (8)	412	204	8	247	216	6	194	1490	17	17
<i>S. Montevideo</i> (1)	201	204	8	247	216	6	194	1491	17	17
<i>S. Saintpaul</i> (10)	5	14	18	9	6	12	17	27	2	2
<i>S. Saintpaul</i> (2)	201	14	18	9	6	12	17	680	2	2
<i>S. Saintpaul</i> (1)	5	14	21	9	6	12	17	49	26	26
<i>S. Paratyphi</i> B var. d-tartrate ⁺ (1)	46	44	46	46	38	18	34	88	25	25
<i>S. Paratyphi</i> B var. d-tartrate ⁺ (1)	2	14	24	14	2	19	140	1560	25	25
<i>S. Paratyphi</i> B var. d-tartrate ⁺ (2)	20	4	23	14	16	19	140	423	56	54
<i>S. Paratyphi</i> B var. d-tartrate ⁺ (1)	15	15	19	17	5	19	18	28	57	55

^a The numbers in boldface represent newly detected alleles or MLSTs.

melting-curve profiles. Subsequent sequencing revealed that these 55 melting curves consisted of 57 different HRM sequence types (sequences of *fljB*, *gyrB*, and *ycfQ* amplicons). All 86 *S. Enteritidis* isolates investigated and 7 out of 13 *S. Dublin* isolates yielded a unique melting-curve profile (HRM-CP 14) due to identical sequences of the three amplification products. Six *S. Dublin* isolates produced HRM profile 15 and could therefore be differentiated from the *S. Enteritidis* isolates. At nucleotide position 288 of *ycfQ*, an SNP (G-to-A transition) specific to these six *S. Dublin* (HRM-CP 15) isolates was detected. This SNP represents the only difference between the six isolates of *S. Dublin* and all tested *S. Enteritidis* isolates that could be detected in the three amplification products mentioned above. The other seven *S. Dublin* isolates investigated lacked this SNP and were therefore indistinguishable from the *S. Enteritidis* isolates. For unambiguous identification of the most frequent serotype, *S. Enteritidis*, a PCR assay specific for *S. Enteritidis* difference region I was developed (2, 23). Evaluation of this *S. Enteritidis*-specific assay with 438 *Salmonella* isolates comprising 48 serotypes revealed 100% specificity and 100% sensitivity.

The triplex HRM assay we developed failed to differentiate between *S. Mbandaka* and *S. Kentucky* (both HRM-CP 42), between *S. Ohio* and *S. Rissen* (both HRM-CP 46), and between *S. Give*, *S. Schwarzengrund*, and *S. Bredeney* (all HRM-CP 33). Sequencing of the three HRM amplification products revealed no sequence variations between the serotypes *S. Mbandaka* and *S. Kentucky* (both HRM-ST 43) or between *S. Schwarzengrund* and *S. Give* (both HRM-ST 33).

However, a single SNP class 3 G-to-C base exchange within the *gyrB* amplicon differentiated *S. Bredeney* (HRM-ST 34) from *S.*

Schwarzengrund and *S. Give* (both HRM-ST 33). Furthermore, a single G-to-C base exchange within the *ycfQ* amplicon differentiated *S. Ohio* (HRM-ST 47) from *S. Rissen* (HRM-ST 48). In both cases, the G-to-C base exchange was not detectable using high-resolution melting analysis. A T_m difference of less than 0.4°C for 412 bp is insufficient for accurate detection of SNP class 3 mutations (13, 21).

All other HRM profiles detected had specific HRM-STs. *S. Newport* had five, *S. Paratyphi* B var. d-tartrate⁺ had three, and *S. Saintpaul*, *S. Thompson*, *S. Senftenberg*, *S. Montevideo*, *S. Kedougou*, *S. Derby*, and *S. Livingstone* each had two different but unique melting-curve profiles. Thus, this triplex assay even allows the subtyping of *S. Dublin*, *S. Newport*, *S. Paratyphi* B var. d-tartrate⁺, *S. Saintpaul*, *S. Montevideo*, *S. Thompson*, *S. Senftenberg*, *S. Kedougou*, *S. Derby*, and *S. Livingstone*.

In-depth molecular analysis of *S. Enteritidis*, *S. Dublin*, *S. Montevideo*, *S. Saintpaul*, *S. Newport*, and *S. Paratyphi* B var. d-tartrate⁺ using multilocus sequence typing showed a good correlation between HRM profiles and MLSTs. However, MLST discriminated better within serotypes than HRM profiling.

In conclusion, this triplex HRM assay, in combination with the amplification of the *S. Enteritidis* difference I region, allows serotype identification of the 15 most frequent human, as well as non-human (including isolates from veterinary, food, and environmental samples), *Salmonella* serotypes found in the years 2008 and 2009 in Austria: *S. Enteritidis*, *S. Typhimurium* biphasic variant, *S. Newport*, *S. Infantis*, *S. Thompson*, *S. Hadar*, *S. Abony*, *S. Tennessee*, *S. Typhimurium* monophasic variant, *S. Senftenberg*, *S. Agona*, *S. Dublin*, *S. Montevideo*, *Salmonella enterica* serotype Virchow, and *S. Paratyphi* B var. d-tartrate⁺ (9, 10). Within a pool

FIG 3 Phylogenetic tree of 84 *Salmonella* isolates based on alignment of the seven MLST genes (*aroC*, *dnaN*, *hemD*, *hisD*, *purE*, *sucA*, and *thrA*) sequenced. Identified MLSTs were compared to the respective HRM-CPs.

of 46 different *Salmonella* serotypes, 39 serotypes could be clearly characterized by this triplex HRM assay (Table 1) when applied in combination with the amplification of a region specific for *S. Enteritidis*. Thus, HRM analysis has the potential to complement classical serotyping of *Salmonella* isolates due to its discriminatory power and simplicity.

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