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Diagnostic Real-Time PCR for Detection of *Salmonella* in Food

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A robust 5′ nuclease (TaqMan) real-time PCR was developed and validated in-house for the specific detection of *Salmonella* in food. The assay used specifically designed primers and a probe target within the *ttrRSBCA* locus, which is located near the *Salmonella* pathogenicity island 2 at centisome 30.5. It is required for tetrathionate respiration in *Salmonella*. The assay correctly identified all 110 *Salmonella* strains and 87 non-*Salmonella* strains tested. An internal amplification control, which is coamplified with the same primers as the *Salmonella* DNA, was also included in the assay. The detection probabilities were 70% when a *Salmonella* cell suspension containing 10^3 CFU/ml was used as a template in the PCR (5 CFU per reaction) and 100% when a suspension of 10^4 CFU/ml was used. A pre-PCR sample preparation protocol including a preenrichment step in buffered peptone water followed by DNA extraction-purification was applied when 110 various food samples (chicken rinses, minced meat, fish, and raw milk) were investigated for *Salmonella*. The diagnostic accuracy was shown to be 100% compared to the traditional culture method. The overall analysis time of the PCR method was approximately 24 h, in contrast to 4 to 5 days of analysis time for the traditional culture method. This methodology can contribute to meeting the increasing demand of quality assurance laboratories for standard diagnostic methods. Studies are planned to assess the interlaboratory performance of this diagnostic PCR method.

The rapid, cost-effective, and automated diagnosis of food-borne pathogens throughout the food chain continues to be a major concern for the industry and public health. Because of these requirements, the PCR became a powerful tool in microbiological diagnostics during the last decade (28). An international expert group of the European Committee for Standardization has been established to describe protocols for the diagnostic detection of food-borne pathogens by PCR (23). A standardized PCR-based method for the detection of food-borne pathogens should optimally fulfill various criteria such as analytical and diagnostic accuracy, high detection probability, high robustness (including an internal amplification control [IAC]), low carryover contamination, and acceptance by easily accessible and user-friendly protocols for its application and interpretation (23). The second generation of PCR methodologies, real-time PCR, has the potential to meet all these criteria by combining amplification and detection in a one-step closed-tube reaction.

Nontyphoidal salmonellosis causes high incidences of infections worldwide due to food poisoning in humans which is associated with contaminated food products of animal origin (30). In many countries, it is the leading cause of food-borne infections and outbreaks (31). Diagnostic real-time PCR for the specific detection of *Salmonella* in foods is increasingly being used as a rapid and reliable tool for the control of contaminated samples along the food production chain. Some real-time PCR-based assays for the detection of *Salmonella* have already been described (9, 13, 15, 17, 18). Most of these assays are not applicable as a diagnostic tool because they lack an IAC. In many instances, the detection is based on nonspecific melting curve analyses, or the selectivity and accuracy were not rigorously tested. The aim of this study is the development and validation of an open-formula, nonpatented, diagnostic real-time PCR-based assay for the detection of *Salmonella* in foods. The selected target region, the *ttrRSBCA* locus, has never been described before for the specific detection of *Salmonella enterica* and *Salmonella bongori*. In combination with two different DNA sample preparation regimes, it is shown that this method is highly accurate for various food matrices. The assay meets the requirements of a diagnostic PCR and, after further interlaboratory validation studies, has the potential to become a standardized method for the rapid detection of *Salmonella* in diagnostic laboratories.

MATERIALS AND METHODS

Bacterial reference strains. A total of 110 *Salmonella* strains were used for inclusivity tests (Table 1). These strains were from a selection recently published for a *Salmonella* validation study representing all known *S. enterica* subspecies *enterica* and *S. bongori* strains (20). *S. enterica* serotype Typhimurium phage type DT104 strain 51K61, isolated in 1996 from pig feces, was used as the reference strain (22). Eighty-seven non-*Salmonella* strains were used for the exclusivity tests (Table 1). Those strains were chosen because of the close relation to *Salmonella* or because they are found in the same environment and grow under the same conditions.

Preparation of DNA samples. For selectivity tests, *Salmonella* or non-*Salmonella* strains were grown aerobically with gentle shaking at 37°C for 18 to 20 h in Luria-Bertani medium (26). A 1-ml aliquot of the enriched culture was prepared as previously described by using a 300-μl aliquot of a 6% (wt/vol) Chelex 100 (catalog no. 142–2832; Bio-Rad, Munich, Germany) suspension (21). A 5-μl aliquot of DNA preparation was diluted 1:10 in TE buffer (10 mM Tris, 0.1 mM

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TABLE 1. *Salmonella* and non-*Salmonella* strains used for selectivity real-time PCR tests and results

Salmonella strains				Non-*Salmonella* strains		
Serotype (subspecies)	Serogroup	No. of strains	Result[b]	Organism	No. of strains	Result[b]
Enteritidis (I)	D_1	21	+	*Campylobacter jejuni*	2	−
Typhimurium[a] (I)	B	20	+	*Campylobacter coli*	2	−
Hadar (I)	C_2-C_3	3	+	*Campylobacter lari*	1	−
Virchow (I)	C_1	3	+	*Citrobacter freundii*	11	−
Infantis (I)	C_1	3	+	*Citrobacter diversis*	2	−
Heidelberg (I)	B	3	+	*Enterococcus faecalis*	3	−
Newport (I)	C_2-C_3	3	+	*Enterobacter agglomerans*	1	−
Brandenburg (I)	B	3	+	*Enterobacter cloacae*	3	−
Saintpaul (I)	B	3	+	*Enterobacter aerogenes*	2	−
Agona (I)	B	3	+	*Escherichia coli*	15	−
Blockley (I)	C_2-C_3	3	+	*Hafnia alvei*	3	−
Bovismorbificans (I)	C_2-C_3	3	+	*Klebsiella pneumoniae*	5	−
Bredeney (I)	B	3	+	*Klebsiella oxytocan*	1	−
Derby (I)	B	3	+	*Listeria monocytogenes*	1	−
Dublin (I)	D_1	3	+	*Listeria innocua*	2	−
Livingstone (I)	C_1	3	+	*Listeria ivanovii*	1	−
Montevideo (I)	C_1	3	+	*Morganella morganii*	1	−
Paratyphi B (I)	B	3	+	*Proteus vulgaris*	3	−
42:r:-[a] (II)	T	2	+	*Proteus mirabilis*	2	−
9,12:z:z39 (II)	D	1	+	*Providencia alcalifaciens*	1	−
48:d:z6 (II)	Y	1	+	*Pseudomonas aeruginosa*	1	−
42:b:e,n,x,z15 (II)	T	1	+	*Serratia marcescens*	1	−
30:1,z28:z6 (II)	N	1	+	*Shigella flexneri* 2a	1	−
21:g,z51:- (IIIa)	L	1	+	*Shigella boydii*	3	−
47:r:- (IIIa)	X	1	+	*Staphylococcus aureus*	4	−
18:z4,z32:-[a] (IIIa)	K	1	+	*Yersinia enterocolitica*	5	−
50:z:z52 (IIIb)	Z	1	+	*Yersinia pseudotuberculosis*	2	−
47:1,v:z[a] (IIIb)	X	1	+	*Yersinia kristensenii*	1	−
18:i,v:z (IIIb)	K	1	+	*Yersinia aldovae*	1	−
16:z4,z32:-[a] (IV)	I	1	+	*Yersinia frederiksenii*	1	−
48:g,z51:- (IV)	Y	1	+	*Yersinia bercovieri*	1	−
11:z4,z23:- (IV)	F	1	+	*Yersinia rohdei*	1	−
44:r:- (V)	V	1	+	*Yersinia mollaretii*	1	−
66:z65:-[a] (V)		1	+	*Yersinia intermedia*	2	−
48:z35:-(V)	Y	1	+			
45:a:e,n,x (VI)	W	1	+			
1,6,14,25:a:e,n,x (VI)	H	1	+			
41:b:1,7 (VI)	S	1	+			
Total no. of strains		110		Total no. of strains	87	

[a] *ttrBCA* regions sequenced. From the serovar Typhimurium strains, the *ttrBCA* region of reference strain 51K61 was sequenced (Genbank accession numbers AY578064 to AY578070).

[b] +, positive test result; −, negative test result.

EDTA [pH 8.0]) and used as a template in the PCR assay (approximately 10^7 CFU per reaction).

For robustness and assay precision tests, *Salmonella* DNA was purified from reference strain 51K61 by using QIAGEN (Hilden, Germany) genomic buffer set in combination with Genomic-tip 100/G columns (QIAGEN) according to the manufacturer's instructions. This method of DNA purification was used to obtain RNA-free pure high-molecular-weight genomic DNA for a precise measurement of the DNA concentration. The concentration of the DNA was determined by measuring the optical density at 260 nm with a GenQuant photometer (Amersham Pharmacia, Uppsala, Sweden). *Salmonella* genomic copy numbers were calculated based on the *S. enterica* serotype Typhimurium genome size (25). Consequently, one *Salmonella* genome weighs about 5.0 fg.

A 1-ml aliquot of a preenriched culture of chicken carcass rinses, minced meat, or raw milk samples was treated with a 6% (wt/vol) Chelex 100 suspension as described above. A 5-μl aliquot of the supernatant containing DNA was directly used as a template in the PCR assay. Preliminary experiments have shown that a Chelex 100 treatment of fish samples was not optimal for efficient removal of PCR inhibitors. Therefore, for the DNA preparation of the fish samples, a QIAmp DNA stool mini kit (QIAGEN) was used according to the manufacturer's instructions, starting with a 1-ml preenrichment aliquot. The purified DNA was eluted in 200 μl of AE buffer. A 5-μl aliquot was used as a template in the PCR assay.

Sequencing. From seven reference strains representing each *S. enterica* subspecies and *S. bongori* (Table 1) the *ttrB* 3′ region, the *ttrC* gene, and the *ttrA* 5′ region were sequenced. For each strain, three specific overlapping fragments were amplified in the DNA sequence. The primers were designed according to the published DNA sequence of the *S. enterica* serotype Typhimurium *ttr* locus (GenBank accession no. AF282268). Primers ttrC-13 (5′-ACT GCC GAT AAA TGC ACG TT-3′ [positions 3018 to 3037]) and ttrB-1 (5′-CTT TTT TCC GCC AGT GAA GA-3′ [positions 3416 to 3435]) gave a 418-bp PCR product, and primers #151 (5′-GTG GGC GGT ACA ATA TTT CTT TT-3′ [positions 3353 to 3375]) and #152 (5′-TCA CGA ATA ATA ATC AGT AGC GC-3′ [positions 4251 to 4263]) gave a 921-bp PCR product from all strains. Primers ttrC-7 (5′-GTT GGC TRA TGC GCT GGA C-3′ [positions 4117 to 4134]) and ttrA-1 (5′-GAC GTC CCG TTT AAC AGG CCA-3′ [positions 4410 to 4430]) gave a 314-bp PCR product from all strains except strain 00-1307 (*Salmonella* 45:a:e,n,x). For this strain, a 363-bp PCR product was generated by using primers ttrC-7 and ttrA-2 (5′-CTC CGG AAT TAA CGC ATT G-3′ [positions 4461 to 4469]). All PCRs were carried out with a GenAmp PCR system 9700 thermocycler (Applied Biosystems, Weiterstadt, Germany). A 50-μl PCR contained 0.4

TABLE 2. Primers, probes, and IAC sequence used for the *Salmonella*-specific assay

Designation	Sequence[c]	Positions[a]	Melting temp (°C)[b]
ttr-6 (forward)	CTCACCAGGAGATTACAACATGG	4287–4309	57
ttr-4 (reverse)	AGCTCAGACCAAAAGTGACCATC	4359–4381	58
Target probe (ttr-5)	FAM-CACCGACGGCGAGACCGACTTT-Dark Quencher	4336–4356	68
IAC probe	Yakima Yellow-CACACGGCGACGCGAACGCTTT-Dark Quencher		68
IAC sequence	GA**CTCACCAGGAGATTACAACATGG**CTCTTGCTGTGCATCATCGCAGAACATC AAAGCGTTCGCGTCGCCGTGTGG**GATGGTCACTTTTGGTCTGAGCT**AC		

[a] Positions correspond to Genbank accession no. AF282268.
[b] Calculated with the Primer Express program (version 1.5) with the default settings.
[c] The sequences marked in boldface type are the primer binding sites, and the underlined sequence is the reverse complement IAC probe binding site.

μM corresponding primers, 200 μM each deoxynucleoside triphosphate (Roche Applied Science, Mannheim, Germany), 1× PCR buffer (20 mM Tris-HCl [pH 8.4], 50 mM KCl), 1.5 mM $MgCl_2$, 1 U of Platinum *Taq* polymerase (Invitrogen, Karlsruhe, Germany), and a 5-μl aliquot of the sample DNA. Reaction conditions for all PCRs were 95°C for 1 min followed by 33 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. A final extension step of 72°C for 4 min was employed. The PCR products were sequenced by automated cycle sequencing with an ABI Prism 310 genetic analyzer (Applied Biosystems) according to the manufacturer's instructions. The sequence primers can be obtained on request. Sequence alignments were performed with the Sequence Navigator software version 1.0 (Applied Biosystems).

Primer and TaqMan probe design. The sequences for the *Salmonella*-specific oligonucleotide primers (ttr-6 and ttr-4) and the *Salmonella* target probe (ttr-5) were designed based on a multiple alignment of the *ttrBCA* sequences (Table 2). Primer Express (version 1.5; PE Biosystems) software was applied with respect to guidelines from PE Biosystems (19) to identify optimal primers and a *Salmonella* target probe within highly conserved DNA regions. The primer ttr-6 is located within the *ttrC* gene, whereas primer ttr-4 and *Salmonella* target probe ttr-5 are located within the *ttrA* gene. The IAC probe sequence was manually designed based on the *Salmonella* target probe sequence by nucleotide exchanges of a 15-mer core sequence. The melting temperatures of both probes were identical due to the same nucleotide composition. The specificity of the sequences were tested by a BLAST search in GenBank, located at the National Center for Biotechnology Information website (BLASTN version 2.2.8) (2). Desalted primers were purchased from Biotez (Berlin, Germany). TaqMan probes were purchased from Eurogentec (Seraing, Belgium). The *Salmonella* target probe was labeled at the 5′ end with the reporter dye 6-carboxyfluorescein (FAM) and at the 3′ end with the Eclipse Dark Quencher. The IAC probe was labeled at the 5′ end with the reporter dye Yakima Yellow and at the 3′ end with the Eclipse Dark Quencher. Yakima Yellow is an alternative to the VIC dye. It possesses an absorbance maximum of 526 nm and an emission maximum of 548 nm.

Internal amplification control. An artificially created DNA fragment was used as an IAC in every reaction mixture. The control DNA (93-bp PCR product) consisted of the IAC probe sequence flanked by the target *Salmonella* primer sequences ttr-6 and ttr-4 (Table 2). The IAC was constructed as previously described (20). The IAC sequence was cloned into the pGEM-T Easy vector (Promega, Mannheim, Germany) and amplified with M13 forward and M13 reverse primers located on the vector resulting in a 303-bp fragment. The PCR product was purified, and the number of copies were calculated and adjusted for use in the assay as described previously (20). The optimal IAC copy number was assessed to be 150 copies.

***Salmonella* 5′ nuclease-based PCR assay.** A typical 50-μl PCR mixture contained 400 nM primers ttr-4 and ttr-6; 250 nM *Salmonella* probe (ttr-5); 250 nM IAC probe; 200 μM each dGTP, dATP, and dCTP; 400 μM dUTP (Roth, Karlsruhe, Germany); 1× PCR buffer (20 mM Tris-HCl [pH 8.4], 50 mM KCl); 4.5 mM $MgCl_2$; 1 U of Platinum *Taq* polymerase (Invitrogen); 150 copies of IAC DNA (purified 303-bp PCR product); and a 5-μl aliquot of the sample DNA. A total of 1 μg of bovine serum albumin fraction V (catalog no. T844.1; Roth)/ml was added when DNA samples prepared from food matrices were analyzed. No template controls that contained 5 μl of TE buffer instead of DNA were included in each run to detect any PCR fragment contamination. PCRs were performed in 8-Strip Low Profile tubes (TLS-0851; MJ Research) and closed with Ultra Clear caps (TCS-0803; MJ Research). The samples were run by using a DNA Engine Opticon 2 system (MJ Research), recorded, and analyzed with the corresponding Monitor software (Version 1.1). Optimization resulted in reaction conditions of 95°C for 1 min (primary denaturation step) followed by 45 cycles of 95°C for 15 s and 65°C for 30 s. For analysis, the baseline subtraction option was always selected. In general, the threshold line for calculating the threshold cycle number (C_T) was set manually to a fluorescence value of 0.06 for reasons of standardization. For selectivity tests, DNA of *Salmonella* strains was cycled in the absence of an IAC. Non-*Salmonella* DNA was cycled in the absence and presence of 150 copies of IAC.

Determination of the detection probability. The probability of detecting *Salmonella* in a suspension of known concentration was assessed as described previously (20). The analysis range was 1 to 10^6 CFU/ml. A 5-μl aliquot of each dilution was added to five separate PCR tubes in the presence of approximately 150 copies of IAC template. The cycle conditions were the same as those described above. The experiment was repeated five times, resulting in 30 PCRs for each cell concentration. Each PCR gave a positive or negative result at the concentration tested. The threshold line was set to a fluorescence value of 0.1. The detection probability was obtained by plotting the relative number of positive PCRs observed against the concentration of the cell suspension. A sigmoidal line fitting was performed with the ORIGIN program (version 4.0; Microcal Software, Northampton, Mass.)

Assay precision and robustness tests. To determine the precision of the assay, three replicates of six 10-fold dilutions of purified *S. enterica* serotype Typhimurium DNA (reference strain 51K61) in TE buffer (10 mM Tris, 1 mM EDTA [pH 8.0]) containing 10^6 to 1 genome equivalent were determined simultaneously in a single run by using the PCR conditions described above. The experiment was repeated three times by the same operator on different days with the same dilutions. The repeatability standard deviation s_r was calculated for each level with outlier tests such as Cochran's test, Grubbs' test, and Mandel's h and k statistics at the 1 and 5% significance levels according to the international standard ISO 5725-2 (3). The repeatability standard deviation is defined as the standard deviation of test results obtained under repeatability conditions.

The robustness of the assay was determined as follows: eight replicates of 100 copies of *Salmonella* DNA from strain 51K61 were made simultaneously in one run at optimized concentrations and suboptimal concentrations of 20% less and 20% more PCR reagent. The IAC template DNA was added to 150 copies ± 20% in the corresponding run. The experiment was performed at annealing-extension temperatures of 62, 65, 67, and 68°C. All other cycle conditions were kept constant as described above.

Investigation of food samples by traditional enrichment. Twenty-two chilled or frozen chickens and 20 various fish fillet samples were purchased in Germany from various local food stores. Minced meat samples were obtained from various locations in France (food stores and producers). The samples were preselected in order to have a maximum amount of naturally positive *Salmonella* samples. Forty-six raw milk samples obtained from one farm in France tested positive for *Salmonella*. The samples were cooled at 4°C for no longer than 24 h before investigation. The traditional enrichment method for the detection of *Salmonella* in artificially and naturally contaminated samples was performed according to international standard ISO 6579:2003, which is the internationally accepted traditional culture method to detect *Salmonella* in foodstuffs (5). As a second selective medium for plating out, brilliant green phenol red lactose saccharose (Merck, Darmstadt, Germany) was used. Whole-chicken carcass rinses were prepared as recommended by international standard ISO 6887-2 (6). For rinsing, 500 ml of buffered peptone water (BPW) was used. A 100-ml aliquot was used as preenrichment culture. A 25-g sample of fish fillet or of minced meat was homogenized in 225 ml of BPW with a stomacher (Seward Medical, London, United Kingdom). A 25-ml sample of raw milk was homogenized in 225 ml of BPW by mixing. All samples were preenriched for 20 h at 37°C without shaking.

Artificial inoculation of whole-chicken carcass rinses and minced meat. A 100-ml aliquot of a whole-chicken carcass rinse was inoculated at four levels (0, 1 to 10, 10 to 100, and 100 to 1,000 CFU per 100 ml of rinse) with *S. enterica* serotype Enteritidis phage type PT4 (BgVV 98–425) and incubated at 37°C for 20 h prior to testing. The inoculate was produced as described previously (21). The experiment was repeated three times on three consecutive days. For the artificial inoculation of minced meat, four different *Salmonella* serotypes (serotype Enteritidis [isolate AFFSA-SE45, France], serotype Typhimurium [isolate AFSSA-STM3, France], serotype Virchow [isolate AFFSA-SV17, France], and serotype Derby [isolate AFFSA-SD1352, France]) were used at five contamination levels (0, 1 to 5, 5 to 10, 10 to 20, and 20 to 100 CFU/25 g). The inoculation procedure was described previously (21).

Detection of *Salmonella* in the presence of background flora. Three meat-associated species, *Pseudomonas aeruginosa* DSM 50071, *Citrobacter freundii* EU-NS26 (isolated in Germany), and *Escherichia coli* ATCC 25922, as well as *S. enterica* serotype Typhimurium reference strain 51K61 were cultured in BPW overnight at 37°C. The number of CFU was determined by 10-fold dilutions in BPW followed by plating onto Luria-Bertani agar plates in triplicate and incubation at 37°C for 24 h. An equal amount of the three non-*Salmonella* bacterial species was mixed in 10 ml of BPW each at low (approximately 10^4 CFU/ml), intermediate (approximately 10^6 CFU/ml), and high (approximately 10^8 CFU/ml) levels. Various concentrations of *S. enterica* serotype Typhimurium strain 51K61 were added (approximately 0, 3, 6, 30, 60, 6×10^2, and 6×10^3 CFU/ml). A DNA extraction of 1 ml of each sample with a 6% Chelex 100 suspension was performed (see above) before and after incubation at 37°C for 20 h, followed by a PCR analysis. The experiment was repeated one time.

Statistical analysis and terms. The following key terms were used and calculated according to the MICROVAL protocol (7). Inclusivity is the ability of the PCR method to detect the target analyte from a wide range of strains. Exclusivity is the lack of interference from a relevant range of nontarget strains of the PCR method. Relative sensitivity, specificity, and diagnostic accuracy describe the ability of the PCR method to detect or not detect the analyte when it is detected or not detected by the reference method. It takes into account the target and nontarget microorganisms in the presence of a biological matrix. The sensitivity, specificity, and accuracy were calculated as described previously (21).

Nucleotide sequence accession numbers. The *ttrBCA* sequences of strains 51K61 (*S. enterica* serovar Typhimurium), 00–1307 (*Salmonella* 45:a:e,n,x), K1354 (*Salmonella* 66:z65:-), 99–1556 (*Salmonella* 18:z4,z32:-), 00–262 (*Salmonella* 16:z4,z32:-), 00–269 (*Salmonella* 47:l,v:z), and 97–565 (*Salmonella* 42:r:-) have been deposited in GenBank under the accession numbers AY578064 to AY578070.

RESULTS

Selectivity. Table 1 shows the results of the inclusivity and exclusivity tests. All 110 *Salmonella* strains tested were identified correctly. When the threshold line was set to a fluorescence value of 0.06 for FAM, the threshold cycle numbers (C_T) of the strains ranged between 14.1 and 17.9, and end-point fluorescence values were between 2.08 and 0.46 (*Salmonella* target probe). All 87 non-*Salmonella* strains tested gave a fluorescence end-point signal below the threshold line. The end-point fluorescence FAM values were between 0.00 and 0.01 in the absence of an IAC. In the presence of 150 copies of an IAC, the FAM values were between 0.01 and 0.05. For the IAC probe, the threshold cycle number of the non-*Salmonella* strains ranged between 30.0 and 32.5, setting the threshold line to a fluorescence signal of 0.03 for Yakima Yellow. The end-point fluorescence Yakima Yellow values of the IAC were between 0.14 and 0.30.

Detection probability. The probability of detecting the reference strain 51K61 (*S. enterica* serotype Typhimurium phage type DT104) was determined at serially 10-fold-diluted cell concentrations in the presence of 150 copies of IAC. Figure 1 shows that the detection probability of a cell suspension at a concentration of 10^3 CFU/ml (5 CFU per reaction) was 70%, and at a concentration of 10^4 CFU/ml (50 CFU per reaction), the detection probability was 100%. By using 10 genome equivalents of purified *Salmonella* DNA (50 fg) as a template per reaction, the detection probability was determined to be 100% (data not shown). This probability corresponds approximately with the probability when cells were used as the template.

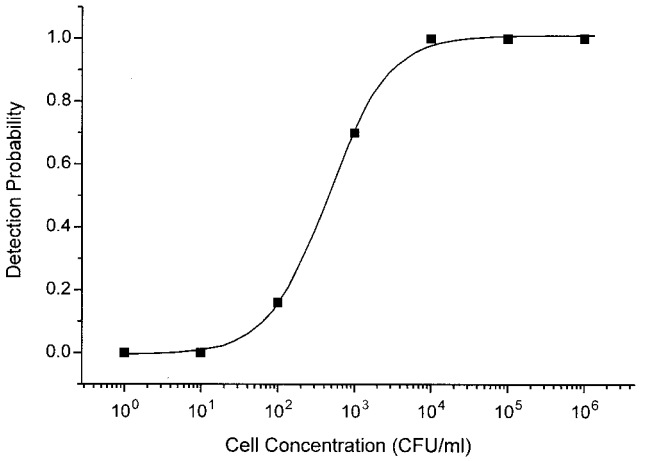

FIG. 1. Detection probability of the *Salmonella* real-time PCR assay at serially 10-fold-diluted cell concentrations of serotype Typhimurium reference strain 51K61. The detection probability was determined in the presence of 150 copies of IAC DNA. Five microliters of each suspension (10^0 to 10^6 CFU/ml) was used as the template in the PCR. The graph shows a sigmoidal fit of data points generated by 30 repetitive PCRs from six independent experiments.

Precision and robustness. The precision of the assay has been determined in four consecutive runs with three replicates within each run by using a *Salmonella* DNA dilution series in the presence of 150 IAC copy numbers. The C_T values for the *Salmonella* target probe (FAM layer) depended on the initial number of *Salmonella* genome equivalents per reaction (Table 3). The s_r has been calculated to be between 0.9 and 3% of the measured mean C_T values, indicating a high precision of the assay (Table 3). However, it was observed that the precision decreases slightly if the *Salmonella* genome equivalents decreases. For example, the relative s_r of the *Salmonella* genome level with 10^6 copies was 0.9% in comparison to 2.1% at the 10-copy *Salmonella* genome level. For the one-copy *Salmonella* genome level, an s_r value was calculated to be 3%, but due to the 45-cycle limit of the assay, the value has no significance. The FAM end-fluorescence values showed a relative repeatability standard deviation of between 12 and 25% of the measured mean fluorescence value, indicating that it is not a precise measure for quantitative analysis of *Salmonella* DNA.

A 20% increase of the concentration of the master mix reagents at various annealing and extension temperatures (62, 65, 67, and 68°C) had no major influence on the FAM C_T value (Table 4). A 20% decrease of the concentrations led to a higher C_T value for approximately three cycles at 67°C compared to annealing temperatures at 65 or 62°C. At 68°C, the signal was below the FAM threshold line of 0.06 for the optimized reaction and the 20% lower concentration reaction.

Influence of background flora on the detection of *Salmonella*. In the presence of the species *P. aeruginosa*, *E. coli*, and *C. freundii* at low (approximately 10^4 CFU/ml), intermediate (ap-

TABLE 3. Mean C_T, end-point fluorescence, and s_r of 10-fold serially diluted *Salmonella* DNA in the presence of 150 IAC copy numbers[b]

No. of *Salmonella* genome copies/PCR	Dye			
	FAM (*Salmonella* probe)		Yakima Yellow (IAC probe)	
	$C_T \pm s_r$	$F_{end} \pm s_r$	$C_T \pm s_r$	$F_{end} \pm s_r$
10^6	19.76 ± 0.183	1.220 ± 0.157	25.64 ± 2.591[a]	0.099 ± 0.0203
10^5	23.58 ± 0.248	0.856 ± 0.114	29.71 ± 1.221[a]	0.089 ± 0.0122
10^4	26.51 ± 0.314	0.844 ± 0.113	30.72 ± 0.880[a]	0.136 ± 0.0229
10^3	29.84 ± 0.411	0.592 ± 0.084	31.60 ± 0.586	0.205 ± 0.0242
10^2	33.69 ± 0.560	0.343 ± 0.055	31.68 ± 0.558	0.229 ± 0.0179
10^1	38.40 ± 0.818	0.150 ± 0.032	31.79 ± 0.520	0.251 ± 0.0154
1	44.42 ± 1.327	0.041 ± 0.020	31.68 ± 0.558	0.263 ± 0.0225
0	45 ± 0	0.023 ± 0.018	31.90 ± 0.483	0.243 ± 0.0312

[a] PCR growing curves did not show exponential increase and only low fluorescence values due to suboptimal amplification.
[b] F_{end}, end-point fluorescence. The s_r is shown for four runs with three replicates each.

proximately 10^6 CFU/ml), or high (approximately 10^8 CFU/ml) levels, *Salmonella* could be detected in a suspension containing 6×10^2 CFU/ml (10 CFU per reaction) as the lowest level (data not shown). After enrichment of the artificial culture mixes for 20 h, the detection limit decreased to less than 3 CFU/ml at the three background flora levels tested (threshold line for FAM, 0.06). The detection limit of the PCR without the background flora mix was 6×10^2 CFU/ml (10 CFU per reaction) in the presence of 150 IAC copy numbers in two independent determinations using Chelex 100-extracted DNA of *S. enterica* serotype Typhimurium cells (strain 51K61). The results show that background microorganisms under the conditions used have no influence on the detection limit of low levels of *Salmonella* even at concentrations of 10^8 CFU of background flora/ml.

Artificial and naturally contaminated samples. The artificial inoculation of 100-ml whole-chicken carcass rinses at four different levels (0, 5, 26, and 474 CFU/100 ml) resulted in 100% agreement of *Salmonella* detection between the traditional and the real-time PCR methods in four independent experiments. Noninoculated carcass rinses were negative, whereas all inoculated samples were positive by both methods. Similarly, the inoculation of 25 g of minced meat at five levels (0, 1 to 5, 5 to 10, 10 to 20, and 20 to 100 CFU/25 g) using four different *Salmonella* serotypes also resulted in 100% agreement between both methods. FAM C_T values (*Salmonella* probe) for positive samples ranged between 17.4 and 31.7, and Yakima Yellow C_T values (IAC probe) were between 29.6 and 34.6 (data not shown).

A total of 110 potentially naturally contaminated samples were analyzed by the traditional culture method according to international standard ISO 6579:2003 and the real-time PCR method. The samples comprised 23 whole-chicken carcass rinses, 20 samples of minced meat from pig and cattle, 20 samples of various fish fillets, and 46 raw milk samples. The results are shown in Table 5. Of these samples, 28 were positive and 82 were negative by both methods. The overall relative diagnostic sensitivity, specificity, and accuracy were 100%. No false-negative or false-positive samples were obtained by PCR.

TABLE 4. Mean C_T values (FAM) and standard deviations of 100 *Salmonella* DNA copy numbers (eight replicates in one run) using optimized or suboptimal (±20%) PCR reagent concentrations

Annealing-extension temp (°C)	Mean C_T value ± SD (FAM)		
	Optimized	20% lower concn	20% greater concn
68	≤45	≤45	34.83 ± 0.923
67	34.35 ± 0.732	38.51 ± 1.647	33.78 ± 0.260
65	34.07 ± 0.532	35.23 ± 0.665	34.41 ± 0.418
62	33.16 ± 0.416	35.31 ± 1.328	33.63 ± 0.495

DISCUSSION

In this study, a 5′ nuclease real-time PCR was developed for the detection of *Salmonella* in food. The method consists of a preenrichment step of the sample in BPW done overnight followed by an extraction-purification step for the bacterial DNA. The DNA is finally analyzed by the real-time PCR assay for the presence of *Salmonella* DNA. The assay has an open formula, and the primers and probe are not patented. The overall analysis time was approximately 24 h, in contrast to 4 to 5 days for the traditional culture method (5). The agreement between both methods was 100%, tested on 110 potentially naturally contaminated samples investigated.

The primers and probe designed for the assay anneal in a highly conserved region of the *Salmonella*-specific *ttr* locus. The forward primer is located at the 3′ end of the *ttrC* gene, whereas the TaqMan probe and the backward primer are located at the 5′ end of the *ttrA* gene. This region has not been reported before for the specific detection of *Salmonella*. The locus consists of five genes organized as an operon. The locus was formerly allocated to *Salmonella* pathogenicity island 2, and it was shown by hybridizations that the operon occurred in all *S. enterica* subspecies and *S. bongori* (12). The genes *ttrA*, *ttrB*, and *ttrC* encode the tetrathionate reductase structural proteins, and the *ttrS* and *ttrR* genes encode the sensor and response regulator components of a two-component regulatory system (11). Tetrathionate respiration is characteristic for certain genera of *Enterobacteriaceae*, including *Salmonella*, *Citrobacter*, and *Proteus* (8), but has not been extensively studied at the molecular level. Phenotypic tetrathionate respiration has been used for the classification of *Enterobacteriaceae* and other gram-negative bacteria (27).

The choice of the *ttr* locus as a target specific for *Salmonella* over other published *Salmonella* targets might have the advantage in the identification of all *Salmonella* strains. The ability to

TABLE 5. Results of the real-time PCR-based method compared to the traditional culture method for detection of *Salmonella* in various food samples

Type of sample	Total no. of samples	Traditional culture method (ISO 6579:2003)		*Salmonella* real-time PCR		Sensitivity (%)	Specificity (%)	Accuracy (%)
		No. of positive samples	No. of negative samples	No. of false-negative samples	No. of false-positive samples			
Fish fillets	20	0	20	0	0		100	100
Carcass rinses, chicken	23	7	16	0	0	100	100	100
Minced meat	21	11	10	0	0	100	100	100
Raw milk	46	10	36	0	0	100	100	100
Total	110	28	82	0	0	100	100	100

respire tetrathionate is likely to be significant within the life cycle of *Salmonella* spp. (11); therefore, the *ttr* genes should be genetically stable in all *Salmonella* strains. Other genetically more unstable targets might occur. It is known, for example, that in single *Salmonella* strains, natural deletions within *Salmonella* pathogenicity island 1 encompassing the *inv*, *spa*, and *hil* loci occur (10). One of these loci is often used as a *Salmonella*-specific target and consequently could lead to false-negative results for such strains.

The selectivity of the primers and probe was tested both by homology searches of a nucleotide database (GenBank, BLASTN version 2.2.6) in combination with sequences obtained from various reference strains and by screening of a number of 87 representative non-*Salmonella* and 110 *Salmonella* strains. No false negatives or false positives were recorded. This result demonstrates the high selectivity of the assay. The combination of the assay with a pre-PCR treatment comprising nonselective preenrichment in BPW followed by either a simple Chelex 100 resin-based DNA extraction or a column-based DNA purification showed that the method is highly accurate compared to the traditional culture method. No false negatives or false positives were observed in the presence of a suitable IAC.

The real-time PCR assay detected a cell suspension of 10^3 CFU/ml (5 CFU per reaction) with a probability of 70% and a cell suspension of 10^4 CFU/ml (50 CFU per reaction) with a probability of 100%. This detection level of *Salmonella* cells was obtained at least through the preenrichment step, even if low levels of *Salmonella* in food were present as determined in artificially contaminated samples of carcass rinses and minced meat. The duration of the preenrichment step was approximately 20 h and should also be sufficient for the recovery and subsequent multiplication of sublethally injured *Salmonella* cells to detectable levels in the real-time PCR.

The use of an IAC in diagnostic PCR is becoming mandatory (14). Such an IAC indicates the presence of DNA polymerase inhibitors (1), errors caused by PCR components, or malfunction of the thermal cycler (29). The IAC used in this assay is a synthetically generated sequence which is recognized by a specific probe labeled with the dye Yakima Yellow. Yakima Yellow can be applied as an alternative to the dye VIC. It possesses an absorbance maximum of 526 nm and an emission maximum of 548 nm, similar to VIC (emission maximum, 552 nm). Therefore, it is not necessary to recalibrate a detection channel already calibrated for VIC. The initial number of IAC copies in a PCR has been optimized to approximately 150. Low numbers of copies reduce the competitive amplification effect between the target and the IAC template. On the other hand, a low number of IAC starting copies causes unstable fluorescence signals leading to inaccurate IAC detection even in the presence of low target copy numbers. A stable signal in *Salmonella*-negative food samples or whether less than 10^4 *Salmonella* target copies are present could be assessed between 30 and 32 cycle numbers with a Yakima Yellow fluorescence end-point signal of 0.1 to 0.3.

It was observed that in the presence of a high excess of *Salmonella* DNA (3 to 4 log), the IAC template DNA had low C_T values (but a low fluorescence signal) compared to the C_T values when little or no *Salmonella* DNA was present (Table 3). High C_T values of the IAC are expected in the presence of a high excess of *Salmonella* DNA due to the competitive PCR. Preliminary results showed that by applying the assay using an ABI Prism 7700 sequence detection system (Applied Biosystems) with ROX as a reference dye in the reaction, the IAC C_T value was later in the presence of a high excess of *Salmonella* DNA than in the presence of low copies of *Salmonella* DNA, as expected. We speculate that due to the competitive PCR, a suboptimal amplification of the IAC template occurred but there are instrument-dependent analysis methods leading to different C_T value analyses. However, it is important that the IAC works reliably at low copy numbers or in the absence of *Salmonella* DNA and for many different types and numbers of food samples.

One major requirement of a diagnostic PCR is the robustness of the method to a range of physical and chemical parameters (23). In addition, it is important to evaluate the limit of the parameters. By using 100 *Salmonella* genome equivalents in the presence of 150 IAC copies for the assay developed here, it was shown that a decrease of the concentrations of the PCR reagents in the master mix has only minor or no influence on the efficiency of the PCR at the optimized temperature of 65°C. However, a 3°C change to higher annealing-extension temperatures inhibited the reaction totally at optimized or 20% lower reagent concentrations in the reactions. In contrast, at a 20% increase of the reagent concentrations, a signal could be still detected at 68°C. This result is probably mainly caused by two parameters, temperature and magnesium ion concentration. Both factors play a major role in stabilizing primer and

probe annealing (24). A malfunction of the thermal cycler of 3°C higher annealing-extension temperatures would therefore lead to ambiguous results under the conditions tested here. If higher initial numbers of *Salmonella* DNA copies were to be used, a positive signal would probably be still detected. The PCR master mix includes dUTP instead of dTTP. This offers the possibility of applying uracil-*N*-glycosylase (UNG) in the reaction to prevent carryover contamination (16) without further optimizing the assay conditions. UNG inactivates all undesired PCR products at an incubation step of 50°C for 2 min prior to the amplification reaction.

Recently, a PCR method for the detection of *Salmonella* was evaluated and validated in international collaborative studies (20, 21). The assay amplifies a 284-bp *invA* fragment followed by agarose gel electrophoresis of the PCR product. The disadvantage of the assay is that the nature of the PCR product is not verified by any appropriate method, e.g., restriction analysis or hybridization, as is recommended by standard protocol (4). The real-time PCR described here uses TaqMan probes for simultaneous detection and PCR product verification by sequence-specific hybridization. The detection probability of a cell suspension with a concentration of 10^3 CFU/ml is increased from 23 to 70%. One *Salmonella* strain serotype, Saintpaul, could be not detected by the *invA* assay. This strain was also positive in the real-time assay.

In conclusion, the real-time method described here demonstrated high selectivity, accuracy, and detection probability. The assay is robust against pipetting and minor temperature variability. Due to the use of an IAC, the assay is suitable for rapid diagnosis of *Salmonella* in food. A study is planned to evaluate the interlaboratory performance in various food matrices at different inoculation levels. Last but not least, the assay will be further developed for quantitation of *Salmonella* DNA.

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