

Multicenter Validation of the Analytical Accuracy of *Salmonella* PCR: towards an International Standard

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As part of a major international project for the validation and standardization of PCR for detection of five major food-borne pathogens, four primer sets specific for *Salmonella* species were evaluated in-house for their analytical accuracy (selectivity and detection limit) in identifying 43 *Salmonella* spp. and 47 non-*Salmonella* strains. The most selective primer set was found to be 139-141 (K. Rahn, S. A. De Grandis, R. C. Clarke, S. A. McEwen, J. E. Galán, C. Ginocchio, R. Curtiss III, and C. L. Gyles, *Mol. Cell. Probes* 6:271-279, 1992), which targets the *invA* gene. An extended determination of selectivity by using 364 strains showed that the inclusivity was 99.6% and the exclusivity was 100% for the *invA* primer set. To indicate possible PCR inhibitors derived from the sample DNA, an internal amplification control (IAC), which was coamplified with the *invA* target gene, was constructed. In the presence of 300 DNA copies of the IAC, the detection probability for primer set 139-141 was found to be 100% when a cell suspension containing 10⁴ CFU/ml was used as the template in the PCR (50 CFU per reaction). The primer set was further validated in an international collaborative study that included 16 participating laboratories. Analysis with 28 coded (“blind”) DNA samples revealed an analytical accuracy of 98%. Thus, a simple PCR assay that is specific for *Salmonella* spp. and amplifies a chromosomal DNA fragment detected by gel electrophoresis was established through extensive validation and is proposed as an international standard. This study addresses the increasing demand of quality assurance laboratories for standard diagnostic methods and presents findings that can facilitate the international comparison and exchange of epidemiological data.

Salmonella species are recognized as major zoonotic pathogens for animals and humans (14). In many countries, *Salmonella* is the leading cause of food-borne outbreaks and infections (23, 24). In order to minimize the risk of infection for consumers, microbiological control of the food chain is being increasingly applied. Thus, the availability of reliable, rapid, and internationally accepted test systems for determination of the presence or absence of food-borne pathogens has become increasingly important for the agricultural and food industry, as well as for legislative regulation of food safety.

In vitro amplification of DNA by the PCR has become a powerful tool in microbiological diagnostics. However, due to the lack of international validation and standard protocols, as well as the variable quality of reagents and equipment, the method produces inconsistent results between expert- and end-user laboratories. For example, many primer sets that have been used for the in vitro amplification of *Salmonella* DNA by PCR differ in their detection limits and accuracy (1, 6, 7, 16). In some cases, the strain collections used for validation do not include all seven known subspecies of *Salmonella enterica* and *Salmonella bongori* and lack epidemiologically important isolates. Furthermore, an internal amplification control (IAC) that is necessary to indicate false-negative results caused by PCR inhibitors is rarely included in the final diagnostic test.

A current European research project (12) is focusing on the

validation and standardization of PCR for the detection of thermophilic *Campylobacter* spp., *Escherichia coli* O157, *Yersinia enterocolitica*, *Listeria monocytogenes*, and *Salmonella* spp. The objective of this project is to facilitate the implementation of diagnostic PCR for both detection and verification of food-borne pathogens by harmonizing, validating, and standardizing PCR methods in Europe (18).

The present study reports a PCR assay for the detection of all *Salmonella enterica* subspecies and *Salmonella bongori* which was developed and validated within the European research project. The results of a collaborative study which included 16 participating laboratories that used reference reagents are presented.

MATERIALS AND METHODS

Terms. In accordance with the MICROVAL protocol (4), several key terms were defined in this study as follows. Selectivity is defined as a measure of the degree of response from target and nontarget microorganisms and comprises inclusivity and exclusivity. Inclusivity is the ability of an alternative method (PCR in this case) to detect the target pathogen from a wide range of strains, and exclusivity is the lack of response from a relevant range of closely related but nontarget strains. Analytical accuracy includes primary validation on purified cell cultures for establishment of selectivity and detection probability (18).

Bacterial reference strains. The *Salmonella* strains used for inclusivity tests are listed in Table 1. A total of 242 strains were selected to represent all known subspecies, with an emphasis on the 10 serotypes most frequently isolated from humans in Europe in 1999 and other epidemiologically important serotypes (Ian Fisher, personal communication). Detailed data on the strains are available on the homepage of the FOOD-PCR project (<http://www.pcr.dk>). Of the 242 strains, 43 were selected for preliminary screening of the primer sets (Table 1). *S. enterica* serotype Typhimurium phage type DT104 strain 51K61, isolated in 1996 from pig feces (17), was used as the reference strain.

The non-*Salmonella* strains used for the exclusivity tests are listed in Table 2. The strains were chosen because they are closely related to *Salmonella* or be-

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TABLE 1. *Salmonella* strains used for inclusivity PCR tests and results of preliminary screening

Serotype (subspecies)	Serogroup	Total no. of strains	No. of strains selected for prescreening	No. of strains testing positive with primer set:			
				P1-P2	S18-S19	ST11-ST15	139-141
Enteritidis (I)	D ₁	60	10	10	9	10	10
Typhimurium (I)	B	60	10	10	10	10	10
Hadar (I)	C ₂ -C ₃	5	1	1	1	1	1
Virchow (I)	C ₁	5	1	1	1	1	1
Infantis (I)	C ₁	5	1	1	1	1	1
Heidelberg (I)	B	5	1	1	1	1	1
Newport (I)	C ₂ -C ₃	5	1	1	1	1	1
Brandenburg (I)	B	5	1	1	1	1	1
Saintpaul (I)	B	20	1	1	1	1	1
Agona (I)	B	5	1	1	1	1	1
Blockley (I)	C ₂ -C ₃	5	1	1	1	1	1
Bovismorbificans (I)	C ₂ -C ₃	5	1	1	1	1	1
Bredeney (I)	B	5	1	1	1	1	1
Derby (I)	B	5	1	1	1	1	1
Dublin (I)	D ₁	5	1	1	1	1	1
Livingstone (I)	C ₁	5	1	1	1	1	1
Montevideo (I)	C ₁	5	1	1	1	1	1
Paratyphi B (I)	B	5	1	1	1	1	1
Senftenberg (I)	E ₄	2	— ^b	—	—	—	—
Litchfield (I)	C ₂ -C ₃	2	—	—	—	—	—
Typhi (I)	D ₁	2	—	—	—	—	—
42:r:- (II)	T	2	1	1	1	1	1
9,12:z:z39 (II)	D	1	1	1	1	1	1
48:d:z6 (II)	Y	1	—	—	—	—	—
42:b:e,n,x,z15 (II)	T	1	—	—	—	—	—
30:1,z28,z6 (II)	N	1	—	—	—	—	—
21:g,z51:- (IIIa)	L	1	1	1	1	0	1
47:r:- (IIIa)	X	1	—	—	—	—	—
18:z4,z32:- (IIIa)	K	1	—	—	—	—	—
50:z:z52 (IIIb)	Z	1	1	1	1	1 ^a	1
47:1,v:z (IIIb)	X	1	—	—	—	—	—
18:i,v:z (IIIb)	K	1	—	—	—	—	—
16:z4,z32:- (IV)	I	1	1	1	1	1	1
48:g,z51:- (IV)	Y	1	—	—	—	—	—
11:z4,z23:- (IV)	F	1	—	—	—	—	—
44:r:- (V)	V	1	1	0	0	1 ^a	1
66:z65:- (V)	—	1	—	—	—	—	—
48:z35:- (V)	Y	1	—	—	—	—	—
45:a:e,n,x (VI)	W	1	1	1	1	1	1
1,6,14,25:a:e,n,x (VI)	H	1	—	—	—	—	—
41:b:1,7 (VI)	S	1	—	—	—	—	—
Total		242	43	42	41	42	43

^a Only faint fragments were obtained but included as positive response.

^b —, not included.

cause they are found in the same environment and grow under the same conditions. A panel of 47 strains was selected for preliminary screening of the primer sets. The remaining 75 strains were selected for further exclusivity tests, as has been recommended previously (13).

Preparation of DNA samples. For selectivity tests, *Salmonella* or non-*Salmonella* strains were grown aerobically without shaking at 37°C for 16 h in Luria-Bertani medium (20). Viable counts were obtained by plating a dilution made in 0.9% (wt/vol) NaCl solution onto plate count agar (Merck, Darmstadt, Germany) in duplicate and incubating the plates for 24 h at 37°C. The concentration was estimated by calculating the average number of CFU. A 1-ml aliquot of the enriched culture was centrifuged at 10,000 × g in a microcentrifuge tube for 5 min at 4°C. The supernatant was carefully discarded, and the cell pellet was suspended in 300 µl of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA [pH 8.0]). The microcentrifuge tube was incubated for 10 min at 100°C in a water bath and immediately chilled on ice. After centrifugation at 14,000 × g at 4°C for 5 min, the supernatant containing DNA was carefully transferred to a new microcentrifuge tube. A 5-µl aliquot was used as the template DNA for the PCR.

Primer sets and PCR. The *Salmonella*-specific primer sets for comparison and the corresponding thermocycler annealing temperatures that were used are presented in Table 3. The primer sets have been published previously and are

commonly used in many studies for detection of *Salmonella*. PCRs were carried out in a GenAmp PCR System 9700 thermocycler (Applied Biosystems, Weiterstadt, Germany). A typical 25-µl PCR mixture contained 0.4 µM concentrations of each primer, 200 µM concentrations of each dNTP (Roche Diagnostics, Mannheim, Germany), 1× PCR buffer (20 mM Tris-HCl [pH 8.4], 50 mM KCl), 1.5 mM MgCl₂, 0.75 U of Platinum *Taq* polymerase (Invitrogen, Karlsruhe, Germany), and 5 µl of sample DNA (approximately 10⁶ CFU per reaction tube). The incubation conditions were 95°C for 1 min, followed by 35 or 38 cycles of 95°C for 30 s, 55 to 64°C (depending on the primer set used [Table 3]) for 30 s, and 72°C for 30 s. A final extension of 72°C for 4 min was employed. For selectivity tests, *Salmonella* DNA was cycled 35 times and non-*Salmonella* DNA was cycled 38 times in order to detect possible nontarget PCR fragments.

A 10-µl aliquot of a PCR product was loaded on a 1.8% agarose gel containing 0.5 µg of ethidium bromide/ml and electrophoresed at 6 V/cm for 90 min. Marker X (Roche Diagnostics) was used in the electrophoresis as the molecular weight standard. The gel was documented with a video camera. A positive response was defined as the presence of a visible band at the expected size, while a negative response was defined as the lack of any band at the expected size.

Construction of the internal controls. The IAC for primer set 139-141 was constructed according to the method described by Ausubel et al. (5), with slight

TABLE 2. *Salmonella*-related strains used for exclusivity PCR tests

Organism	No. of strains	No. of strains selected for preliminary screening
<i>Citrobacter brakii</i>	1	0
<i>Citrobacter diversis</i>	1	1
<i>Citrobacter freundii</i>	19	5
<i>Escherichia coli</i>	16	7
<i>Escherichia fergusonii</i>	1	0
<i>Erwinia herbicola</i>	1	0
<i>Enterobacter aerogenes</i>	4	1
<i>Enterobacter agglomerans</i>	2	1
<i>Enterobacter amnigenus</i>	1	0
<i>Enterobacter asbunae</i>	1	0
<i>Enterobacter asburiae</i>	1	0
<i>Enterobacter cloacae</i>	2	1
<i>Enterobacter gergoviae</i>	2	0
<i>Enterobacter sakazakii</i>	1	0
<i>Enterobacter tarda</i>	1	0
<i>Enterobacter taylorae</i>	2	0
<i>Enterococcus faecalis</i>	3	3
<i>Ewingella americana</i>	1	0
<i>Hafnia alvei</i>	3	1
<i>Klebsiella oxytoca</i>	3	1
<i>Klebsiella pneumoniae</i>	5	4
<i>Kluyvera ascorbata</i>	1	0
<i>Listeria inucua</i>	2	2
<i>Listeria ivanovii</i>	2	1
<i>Listeria monocytogenes</i>	8	3
<i>Micrococcus kristinae</i>	1	0
<i>Morganella morganii</i>	4	1
<i>Obesumbacterium proteus</i>	1	0
<i>Proteus agglomerans</i>	1	0
<i>Proteus mirabilis</i>	4	1
<i>Proteus vulgaris</i>	3	2
<i>Providencia heimbachae</i>	1	0
<i>Providencia stuartii</i>	1	0
<i>Pseudomonas aeruginosa</i>	2	0
<i>Pseudomonas alcaligenes</i>	1	0
<i>Rhanelia aquatilis</i>	1	0
<i>Serratia marcescens</i>	3	1
<i>Serratia odorifera</i>	2	0
<i>Shigella boydii</i>	3	3
<i>Shigella flexneri</i>	1	1
<i>Shigella sonnei</i>	1	0
<i>Staphylococcus aureus</i>	4	4
<i>Yersinia enterocolitica</i>	4	1
Total	122	47

modifications. The method allows the construction of short artificial DNA fragments. Briefly, four, approximately 100-mer oligonucleotides overlapping in 20 bp were hybridized and gaps were filled with Klenow fragment and dNTPs. The product was reamplified using primers malo2-F (GTATTGTTGATTAATG

AGATCCG) and malo2-R (ATATTACGCACGGAAACACG), resulting in a 253-bp fragment which was cloned in pGEM-T Easy vector (Promega, Mannheim, Germany). The cloned internal fragment was amplified as described above at an annealing temperature of 55°C with primers M13 forward and M13 reverse located on the vector, resulting in a 498-bp fragment. The PCR product was purified by using a Centrispin 20 column (Princeton Separations, Adelphia, N.J.), and the concentration was spectrometrically determined at 260 nm. The sequence of the IAC, composed of various *invA* primer sequences between 18 and 26 bp in size and primer sequences (S18-S19) occurring in the *ompC* gene of *Salmonella* spp. (16), was confirmed (Fig. 1). The following equation was used to calculate the copy numbers from a known PCR product concentration: weight of PCR fragment (in grams per microliter)/(660 g per mol × the number of base pairs of the PCR fragment) × (6.023 × 10²³) = the number of genomic copies per microliter.

Determination of the detection probability. The probability of detecting *Salmonella* in a suspension of known concentration in the presence of defined DNA copy numbers of the IAC was determined essentially as described previously (15). Buffered peptone water (Merck) was inoculated with the reference strain 51K61 and incubated for approximately 5 h at 37°C to the exponential phase (approximately 10⁸ CFU/ml). The cell suspension was serially diluted 10-fold in 0.9% (wt/vol) NaCl within the concentration range of 10⁶ to 10⁰ CFU/ml. The dilutions were stored at -20°C until use. The approximate number of CFU per milliliter of cell suspension was determined by plating 100 µl of the dilution containing 10⁴ CFU/ml onto Luria-Bertani agar five times. The average number of CFU from the five plates was used to recalculate the concentration (CFU per milliliter) of the frozen dilutions. Each dilution was thawed and adjusted to approximately 10¹ to 10⁶ CFU/ml. Five microliters of each dilution was added to five separate PCR tubes and was run in the presence of 300 or 30 copies of IAC as 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 detection probability of the PCR assay was obtained by plotting the relative number of positive PCRs observed against the concentration of the cell suspension. A sigmoidal line fitting was performed using the ORIGIN program (version 4.0; Microcal Software, Northampton, Mass.). The determination of the detection probability was repeated by using purified DNA in the concentration range of 10⁵ to 10⁰ genomic DNA copies per PCR. Template DNA for determination of the detection probability was purified from reference strain 51K61 with a Genomic Tip kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. The concentration of the purified DNA was determined by measuring the optical density at 260 nm with a GenQuant photometer (Amersham Pharmacia, Uppsala, Sweden). The number of *Salmonella* genomic copies of purified DNA was calculated as follows: $m = n \times (1.013 \times 10^{-21} \text{ g/bp})$, where m is the mass and n is the number of base pairs. The number of kilobase pairs for one serotype Typhimurium genome was previously determined to be 4,951 (19). Consequently, according to the above equation, one *Salmonella* genome weighs about 5.0 fg. The concentration of DNA (range, 10⁵ to 10⁰ genomic DNA copies/ml) was adjusted in TE buffer.

Interlaboratory study using the primer set 139-141. The collaborative study was designed and analyzed according to the basic guidelines of ISO 5725-1 (2) and ISO 5725-2 (3) and the MICROVAL protocol (4). The formulas used for analysis are shown in Fig. 2 and were calculated as described previously (4, 11). The Kappa index, which indicates the strength of the relationship between the row and column variables of a cross tabulation, was calculated as described previously (22). Kappa values of <0.01 indicate no concordance, those between 0.1 and 0.4 indicate weak concordance, those between 0.41 and 0.60 indicate

TABLE 3. Selected primer sets for selectivity tests

Target	Primer	Sequence	Size of PCR product (bp)	Annealing temp (°C)	Reference
<i>oriC</i>	P1	TTATTAGGATCGCGCCAGGC	163	55	25
	P2	AAAGAATAACCGTTGTTAC			
<i>ompC</i>	S18	ACCGCTAACGCTCGCCTGTAT	159	58	16
	S19	AGAGGTGGACGGGTTGCTGCCGTT			
Random fragment	ST11 ST15	AGCCAACCATTGCTAAATTGGCGCA GGTAGAAATTCCCAGCGGGTACTG	429	60	1
<i>invA</i>	139 141	GTGAAATTATCGCCACGTTTCGGGCAA TCATCGCACCGTCAAAGGAACC	284	64	21

gtattgttga ttaatgagat ccgttaccgc taactcgcoct gtatttgtga
aattatcgcc acgttcgggc aatttgatga attcgtaa gaagttctc
 tatgctcagc ccggttcaat ttacggctata ttttgatttt tgatgcgagt
 ggaaattatt cogatgaagt cgtgtccttt tggttccttt gacggtgcca
tgattaacgg cagcaaccog tocacctctt tegtgtttcc gtcgtaata
 tga

FIG. 1. Cloned artificial 253-bp sequence of the IAC. For the *invA* PCR assay, a 157-bp IAC was amplified by using primer set 139-141. The underlined italic sequence is the binding sequence of primer 139, and the underlined roman sequence is the binding sequence of primer 141.

clear concordance, those between 0.61 and 0.80 indicate strong concordance, and those between 0.81 and 1.00 indicate nearly complete concordance.

A total of 16 laboratories (from Austria, Belgium, the Czech Republic, Denmark, England, Finland, France, Germany, Greece, Slovakia, Spain, Sweden, Italy, and The Netherlands) participated in the interlaboratory study. Each laboratory received 28 coded ("blind") DNA samples, including samples from 12 *Salmonella* and 16 non-*Salmonella* strains (see Table 5), one negative (TE buffer) and one positive control DNA, an IAC template (498-bp PCR product, produced as described above), and reagents for use in PCR, including Platinum *Taq* polymerase (Invitrogen). All DNA samples were prepared according to the thermal cell lysis method as described above. The positive control DNA was purified from reference strain 51K61 with a Genomic Tip kit (Qiagen). Each lab strictly followed a standard operating procedure (available at <http://www.pcr.dk>). The 25- μ l PCR mixture contained 0.4 μ M concentrations of primers 139 and 141, 200 μ M concentrations of each dNTP (Roche Diagnostics), 1 \times PCR buffer, 1.5 mM MgCl₂, 0.75 U of Platinum *Taq* polymerase (Invitrogen), 300 copies of the IAC, and 5 μ l of sample DNA (approximately 10⁶ CFU per reaction tube). The incubation conditions were 95°C for 1 min, followed by 38 cycles of 95°C for 30 s, 64°C for 30 s, and 72°C for 30 s. A final extension of 72°C for 4 min was employed. The choice of the thermal cycler model was unrestricted. Each set of DNA samples was amplified in three independent experiments. Amplicons were detected after electrophoresis by using a 1.8% agarose gel. The choices of the electrophoresis equipment and buffer were unrestricted. The electric field strength was restricted to 5 V/cm. The calculation took into account all single PCRs from each participating laboratory. The criteria for the acceptance of data generated by the individual laboratories were as follows: (i) both the positive control and negative control were detected correctly, (ii) the 157-bp IAC fragment was visible on the agarose gel from PCRs with non-*Salmonella* strains, and (iii) missing data, e.g., where a laboratory omitted one of the samples, were excluded.

RESULTS

Selectivity. Table 1 shows the results of the inclusivity tests of the four primer sets tested with a panel of 43 *Salmonella* strains. All four primer sets amplified a PCR product of the expected size. However, while *invA* primer set 139-141 identified all 43 strains, the other three primer sets failed to identify one or two *Salmonella* strains (Table 1). The exclusivity tests with 47 non-*Salmonella* strains gave unspecific PCR fragments (data not shown). Primer set P1-P2 (*oriC*) (25) produced multiple, non-target-sized fragments of 0.2 to 3 kb in several non-*Salmonella* strains. Primer set ST11-ST15 (random genomic fragment) (1) showed faint fragments of similar size from DNA of *Citrobacter* spp., *Yersinia enterocolitica* (approximately 400 bp), and *Klebsiella oxytoca* (approximately 500 bp). Primer set S18-S19 (*ompC*) (16) produced fragments of similar size from DNA of *Proteus vulgaris* (approximately 120 bp) and *Hafnia alvei* (approximately 140 bp). Other strains produced several non-target-sized fragments ranging in size from 0.3 to 2 kb. Primer set 139-141 (*invA*) (21) produced faint non-target-sized fragments from DNA of *E. coli* (approximately 400 bp)

and *Citrobacter* spp. (approximately 350 bp) strains that could clearly be distinguished from the 284-bp *Salmonella* target fragment.

Based on these data, the *invA* primer set 139-141 was selected for extended inclusivity and exclusivity tests. A total of 199 additional *Salmonella* strains and 75 additional non-*Salmonella* strains were tested (Table 1 and 2). All *Salmonella* strains, except one, were identified correctly. The incorrectly identified *Salmonella* strain belonged to serotype Saintpaul. However, 19 other serotype Saintpaul isolates (collected between 1999 and 2001 from different sources in Germany) were identified correctly. Primer set 139-141 again produced faint non-target-sized fragments from DNA of *E. coli* (approximately 400 bp) and *Citrobacter* spp. (approximately 350 bp) strains. The nonspecific fragments were absent in the presence of an IAC (see below).

IAC. In order to identify false-negative PCR results, an IAC was constructed for primer set 139-141 (Fig. 2). The IAC was coamplified with the target DNA, resulting in a 157-bp amplicon. In the absence of an IAC, the detection limit was determined to be 1 to 10 *Salmonella* genomic DNA copies (38 cycles of amplification). In the presence of an IAC, the detection

	PCR		Σ
	positive	negative	
(culture) positive	a True Positive	b False Negative	a+b
negative	c False Positive	d True Negative	c+d
Σ	a+c	b+d	

$$\text{Inclusivity (\%)} = \frac{a}{(a + b)} \cdot 100$$

$$\text{Exclusivity (\%)} = \frac{d}{(c + d)} \cdot 100$$

$$\text{Positive predictivity (\%)} = \frac{a}{(a + c)} \cdot 100$$

$$\text{Negative predictivity (\%)} = \frac{d}{(b + d)} \cdot 100$$

$$\text{Analytical accuracy (\%)} = \frac{a + d}{(a + b + c + d)} \cdot 100$$

$$\text{Kappa index} = \frac{2(ad - bc)}{(a + c) \cdot (c + d) + (a + b) \cdot (b + d)}$$

FIG. 2. Features of a two-row by two-column contingency table with respect to the reference culture method and the alternative PCR method. The derived formulas used for analysis in the validation study are shown below the table. a, number of samples that are both reference positive and PCR positive (true positive); b, number of samples that are reference negative but PCR positive (false positive); c, number of samples that are reference positive but PCR negative (false negative); d, number of samples that are both reference and PCR negative (true negative).

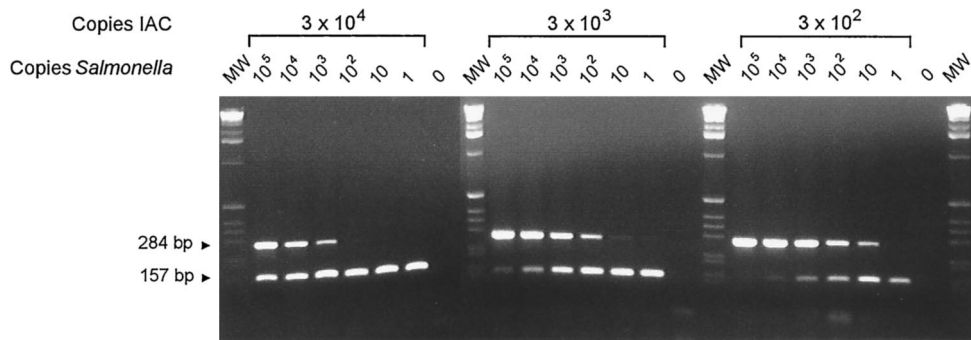


FIG. 3. Coamplification of different copy numbers of the IAC (157-bp fragment) and *Salmonella* genomic DNA (284-bp fragment) by using primer set 139-141. The initial numbers of *Salmonella* DNA and IAC copies per reaction tube are indicated above the gels. The sizes of the PCR products are indicated at left. Marker X (Roche Diagnostics) was used as the molecular weight standard (MW). Ten microliters of the 25- μ l PCR mixture was loaded per well.

limit depended on the initial number of IAC copies (Fig. 3). Since a 10-fold increase in the initial number of IAC copies increased the detection limit of the *Salmonella* target gene by 10-fold as well, the competition between the two templates could be linear, indicating that the IAC is not preferably amplified. No difference in the detection limit was observed when purified plasmid DNA or PCR product was used as the template for the IAC (data not shown).

Detection probability. The probability of detecting serotype Typhimurium phage type DT104 reference strain 51K61 was established at different cell and DNA concentrations by using primer set 139-141 in the presence of 30 or 300 copies of IAC. Figure 4 shows that a 10-fold decrease in the initial number of IAC copies increased the detection probability approximately 10-fold. The detection probability for a cell suspension at a concentration of 10^4 CFU/ml (50 CFU per reaction) was 100% at both 30 and 300 copies of IAC. However, the detection probability of a concentration of 10^3 CFU/ml was approximately 93% for 30 copies of IAC and 23% for 300 copies of IAC. By using 10 copies of purified *Salmonella* DNA (50 fg) per reaction as the template, the detection probability was determined to be 100% in the presence of 300 copies of IAC (data not shown). This is a fivefold increase in the detection probability compared with that when cells were used as the template, assuming that 1 genomic copy per cell occurred during the exponential replication of the cells.

When 30 initial IAC copies were used, the signal intensity of the IAC amplicon varied substantially among identical PCRs, indicating poor reproducibility. Therefore, approximately 300 IAC copies should be used as the template.

Interlaboratory study using primer set 139-141. The objective of this study was to validate the reproducibility of the analytical accuracy of the described *Salmonella* PCR assay in a multicenter investigation. Twelve *Salmonella* and 16 non-*Salmonella* coded (blind) DNA samples were sent refrigerated to 16 international laboratories. The performance of each participating laboratory is shown in Table 4. Four hundred ninety-five of 516 samples expected to be PCR positive for *Salmonella* (inclusivity, 96%) and 681 of 688 samples expected to be PCR negative for *Salmonella* (exclusivity, 99%) were correctly classified by the participating laboratories. The overall analytical accuracy of the study was 98.0%. Laboratory 6 was excluded from the analysis since its Kappa index was significantly lower

than those of other laboratories (Table 4). Furthermore, one negative control DNA sample from laboratory 6 was detected as positive, indicating cross contamination of DNA samples at the receiving laboratory. The results of the classification of the strains are shown in Table 5. It was observed that false positives were generally detected as weak signals, indicating carry-over contamination from positive DNA samples. One DNA sample (*S. enterica* serotype Dublin 98-443) that was expected to be positive was frequently detected as negative (15 of 46 PCRs). This was probably caused by the degradation of the sample during transport or handling during the experiments in some laboratories.

DISCUSSION

The aim of this study was to validate a PCR assay for the detection of *Salmonella* spp. which will contribute to an international standard. It was decided that the *Salmonella*-specific

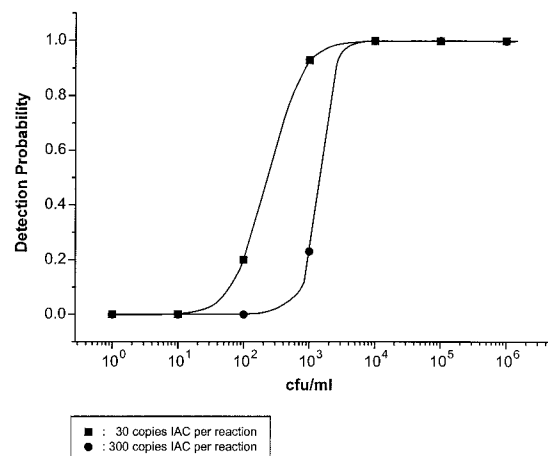


FIG. 4. Detection probability of the *Salmonella* PCR assay using primer set 139-141 at serially 10-fold-diluted cell concentrations of serotype Typhimurium phage type DT104 reference strain 51K61. The detection probability was established in the presence of 30 or 300 copies of IAC DNA. Five microliters of 10-fold-diluted cell suspensions (10^0 to 10^6 CFU/ml) were 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.

TABLE 4. Results of the collaborative study validating the reproducibility of the analytical accuracy using primer set 139-141

Laboratory no.	Inclusivity (%)	Exclusivity (%)	Positive predictivity (%)	Negative predictivity (%)	Analytical accuracy (%)	Kappa index ^a
1	100	100	100	100	100	1.000
2	94	100	100	92	96	0.926
3	96	97	98	94	96	0.927
4	100	100	100	100	100	1.000
4	98	92	94	97	95	0.903
6 ^b	94	67	65	94	77	0.561
7	100	100	100	100	100	1.000
8	100	100	100	100	100	1.000
9	100	100	100	100	100	1.000
10	91	100	100	88	95	0.889
11	100	100	100	100	100	1.000
12	96	100	100	94	98	0.951
13	94	92	94	92	93	0.854
14	100	100	100	100	100	1.000
15	87	100	100	81	92	0.826
16	100	100	100	100	100	1.000
All	97	96	97	96	96	0.926
All but laboratory 6	97	99	99	96	98	0.952

^a Kappa values of <0.01 indicate no concordance, 0.1 to 0.4 indicate weak concordance, 0.41 to 0.60 indicate clear concordance, 0.61 to 0.80 indicate strong concordance, and 0.81 to 1.00 indicate nearly complete concordance.

^b Laboratory 6 was excluded from the final statistical analysis due to a markedly lower Kappa index compared with those of the other laboratories.

amplicon should be detected by gel electrophoresis in order to offer a simple and economical detection method that is available in most laboratories. We compared four *Salmonella*-specific primer sets and, in a collaborative study, validated the most selective and sensitive (in terms of detection limit) PCR system for the detection of all *S. enterica* subspecies and *S. bongori*. The primer set used for specific amplification of a *Salmonella* genomic DNA fragment has previously been published by Rahn et al. (21) and amplifies a 284-bp sequence of the *invA* gene. The *invA* target gene is located on pathogenicity island 1 of *Salmonella* spp., which encodes proteins of a type III secretion system (8). The amplification conditions of the PCR assay described here were optimized and differed considerably from those reported by Rahn et al. (21). For instance, the annealing time was shortened from 2 min to 30 s, the extension time was reduced from 3 min to 30 s, and the annealing temperature was made more stringent by being raised from 53 to 64°C. Furthermore, a hotstart *Taq* polymerase known to reduce amplification of unspecific fragments was used. With these new parameters, the amplification of nonspecific fragments from non-*Salmonella* strains should have been limited without influencing the inclusivity of the PCR. Some unspecific but faint amplicon products were still detected in some *E. coli* and *Citrobacter freundii* isolates when 38 cycles of amplification were performed. However, in the presence of 300 copies of an IAC, which was competitively coamplified with the *invA* target fragment, the unspecific fragments were totally absent. The addition of an IAC in each PCR tube is necessary if the PCR is to be used as a diagnostic tool. If no IAC is used, we recommend that the number of amplification cycles be decreased to a maximum of 33 to 35. Naturally, the initial DNA copy number of the IAC in the PCR should be kept as low as possible but must be detected reproducibly.

We observed that the *invA* PCR assay using primer set 139-141 detected, with a high probability, 5 to 50 *Salmonella* CFU or 10 genomic copies of purified *Salmonella* DNA per

TABLE 5. Results of classification of strains used in the interlaboratory study (laboratory 6 excluded)

Strain and designation	Correct classification of strain (%)
Non-<i>Salmonella</i> strains	
<i>Citrobacter diversus</i> EU-NS25	100.0
<i>Hafnia alvei</i> EU-NS35	93.3
<i>Staphylococcus aureus</i> EU-NS22	100.0
<i>Citrobacter freundii</i> EU-NS24	100.0
<i>Enterobacter cloacae</i> EU-NS32	100.0
<i>Enterococcus faecalis</i> EU-NS3	100.0
<i>Escherichia coli</i> ATCC 25922	97.7
<i>Escherichia coli</i> O128 EU-NS10	100.0
<i>Escherichia coli</i> O157 EU-NS11	100.0
<i>Klebsiella oxytoca</i> EU-NS18	93.3
<i>Klebsiella pneumoniae</i> EU-NS17	100.0
<i>Proteus vulgaris</i> EU-NS28	100.0
<i>Shigella boydii</i> EU-NS38	100.0
<i>Shigella flexneri</i> 2a EU-NS27	100.0
<i>Yersinia enterocolitica</i> EU-NS41	100.0
<i>Serratia marcescens</i> EU-NS36	100.0
<i>Salmonella</i> serotypes^a	
Hadar 99-601	100.0
Enteritidis 00-4	90.9
Bredeney 00-215	100.0
Dublin 98-443	69.7
Blockley 98-1262	100.0
Typhimurium 00-510	100.0
42:b:e,n,x,z15 (II) 98-1308	90.9
18:z4,z32:- (IIIa) 99-1556	97.0
47:1,v,z (IIIb) 00-269	100.0
48:g,z51:- (IV) 98-3722	100.0
66:z65:- (V) K-1354	97.0
1,6,14,25:a:e,n,x (VI) 94-2957	100.0
Controls	
Negative (TE buffer)	100.0
Positive (serotype Typhimurium 51K61)	100.0

^a Numerals in parentheses indicate subspecies.

reaction in the presence of 30 to 300 IAC copies, whereas Rahn et al. previously reported a higher detection limit (300 cells per reaction or 27 pg of extracted chromosomal DNA) (21).

Of the 242 *Salmonella* strains tested, one serotype Saintpaul strain was not detected. We speculate that the *invA* gene, at least, is absent in this strain. Rahn et al. (21) reported that two serotype Litchfield and two serotype Senftenberg strains were not detected when primer set 139-141 was used. However, it has been shown that the *invA* gene is essential for the invasion of epithelial cells by *Salmonella*; consequently, the apparent absence of the *invA* gene suggests that such strains are not invasive or use alternative invasion mechanisms (9, 10). The two serotype Litchfield and two serotype Senftenberg reference strains that we tested were *invA* PCR positive. Absence of the *invA* gene in *Salmonella* seems to be rare.

The collaborative study showed a high selectivity and reproducibility of the PCR assay among the 16 international laboratories when identical batches of reagents were used. The fact that, in this study, inclusivity (96%) was lower than exclusivity (99%) was probably due to the use of partially degraded sample DNA, especially one sample (serotype Dublin 98-443) expected to be positive that was frequently detected as negative (15 of 46 PCRs). DNA used in the study was prepared by the thermal cell lysis method without subsequent purification of the DNA. Potentially active DNase might be responsible for this degradation.

In conclusion, the *invA* PCR assay using the primer set 139-141 that was originally published by Rahn et al. (21) demonstrated inclusivity for a wide range of *Salmonella* serotypes including all subspecies and exclusivity for other species and genera. The assay showed a high (good) detection probability in the presence of 30 or 300 copies of the IAC. The assay was found to be selective and robust by an international collaborative study. The PCR assay will be validated in-house on naturally contaminated samples to investigate the diagnostic accuracy. A second international interlaboratory study is planned to investigate diagnostic accuracy and reproducibility on naturally and artificially contaminated swab samples from pig and poultry rinses.

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