Evaluation of *Salmonella* spp. specific primer-sets for the validation within the Food PCR project

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Summary

A primer-set for the specific detection of *Salmonella* spp. was evaluated targeting the *invA* gene and published by Rahn *et al*. 1992. *invA* is located on the pathogenicity island 1 of *Salmonella* spp. encoding proteins of a type III secretion system (Collazo, C.M and J.E. Galán, 1997). For the indication of possible PCR inhibitors derived from the sample DNA, an internal control was constructed which is co-amplified with the *invA* target gene. 221 *Salmonella* strains and 132 non-*Salmonella* strains were tested for the specificity. The sensitivity was 1.5-15 cfu/ per reaction by the thermal cell digestion and 1-10 copies/ per reaction with purified DNA using Genomic-tip kit (Qiagen).

Introduction

Salmonella spp. cause one of the most important food-borne disease in the world. The identification of *Salmonella* spp. from food by traditional cultural techniques requires 4 to 5 days. The polymerase chain reaction (PCR) offers a simple tool for the rapid detection of *Salmonella* spp.. However, the lack of harmonization and standardization of PCR methologies influence the efficient dissemination from expert research laboratories to end-user laboratories. The European research project ‚Food PCR' (www.pcr.dk) was launched to validate and standardize the use of PCR for detection of food-borne pathogens. Partner 6 (BgVV, Fg 501) is within the project responsible for the validation of a *Salmonella* spp. specific PCR assay.

Material and Methods

PCR reactions were carried out in a GenAmp PCR System 9700 thermocycler. A typical 25 µl PCR reaction contained 0,4 µM of each primer, 200 µM of each dNTP (Roche Diagnostics), PCR reaction buffer (20 mM Tris, 50 mM KCl), 1.5 mM $MgCl_2$ and 0.75 u Platinum *Taq* polymerase (Life Technologies), 5 µL sample DNA (~1 x 10^6 copies per reaction tube). Non-*Salmonella* DNA was cycled 38 times, *Salmonella* DNA was cycled 35 times for specificity tests and 38 times for sensitivity tests. Table 1 shows the primer-sets, thermocycler incubation temperatures and time used for amplification.

Table 1. Selected primer-sets for specificity tests

Primer Set	**Specificity**	**Size (bp)**	**PCR conditions (35 or 40 cycles)**
ST11: AGCCAACCATTGCTAAATTGGCGCA ST15: GGTAGAAATTCCCAGCGGGTACTG (Aabo *et al.* 1993)	Random fragment	429 bp	1 min 95°C 30s 95°C 30s 60°C 30s 72°C
P1: TTA TTA GGA TCG CGC CAG GC P2: AAA GAA TAA CCG TTG TTC AC Widjojoatmodjo *et al.* 1996)	*oriC*	163	1 min 95°C 30s 95°C 30s 55°C 30s 72°C
139: GTG AAA TTA TCG CCA CGT TCG GGC AA 141: TCA TCG CAC CGT CAA AGG AAC C (Rahn *et al.* 1992)	*invA*	284	1 min 95°C 30s 95°C 30s 60 or 64°C 30s 72°C
S18: ACC GCT AAC GCT CGC CTG TAT S19: AGA GGT GGA CGG GTT GCT GCC GTT (Kwang *et al*. 1996)	*ompC*	159	1 min 95°C 30s 95°C 30s 60 or 58°C 30s 72°C
Malo2-F: GTA TTG TTG ATT AAT GAG ATC CG Malo2-Ra: ATA TTA CGC ACG GAA ACA CG TT (Malorny unpublished)	*invA*	373	1 min 95°C 30s 95°C 30s 55 °C 30s 72°C

Results

Pre-screening

For specificity tests five primer-sets were selected (Table 1). Four primer-sets were published previously. A fifth primer-set was designed based on the *invA* gene (Malorny unpublished). The five primer-sets were pre-screened with a panel of 47 non-*Salmonella* strains and 43 *Salmonella* strains including all subspecies. Table 2 shows the specificity of the primer-sets tested. Due to the high template concentration and cycles used for non-*Salmonella* strains unspecific PCR fragments were observed in several strains and primer-sets. However only a few faint unspecific fragments were observed with primer-set malo2-F/malo2-Ra. Based on these data the *invA* primer-sets 139/141 (Fig. 1) and malo2-F and malo2-Ra were selected for further specificity and sensitivity tests.

Table 2. List of *Salmonella* strains used for pre-screening specificity tests

Salmonella Subspecies	No. of strains tested	No. of strains positive				
		oriC (P1/P2)	*ompC* (S18/S19)	ST11/ST15	*InvA* (139/141)	*invA* (malo2-F/malo2-Ra)
I	36	36	**35***	36	36	36
II	2	2	2	2	2	2
IIIa	1	1	1	**0**	1	1
IIIb	1	1	1	1	1	1
IV	1	1	1	1	1	1
V	1	**0**	1	**1***	1	1
VI	1	1	1	1	1	1

*: faint fragments partially

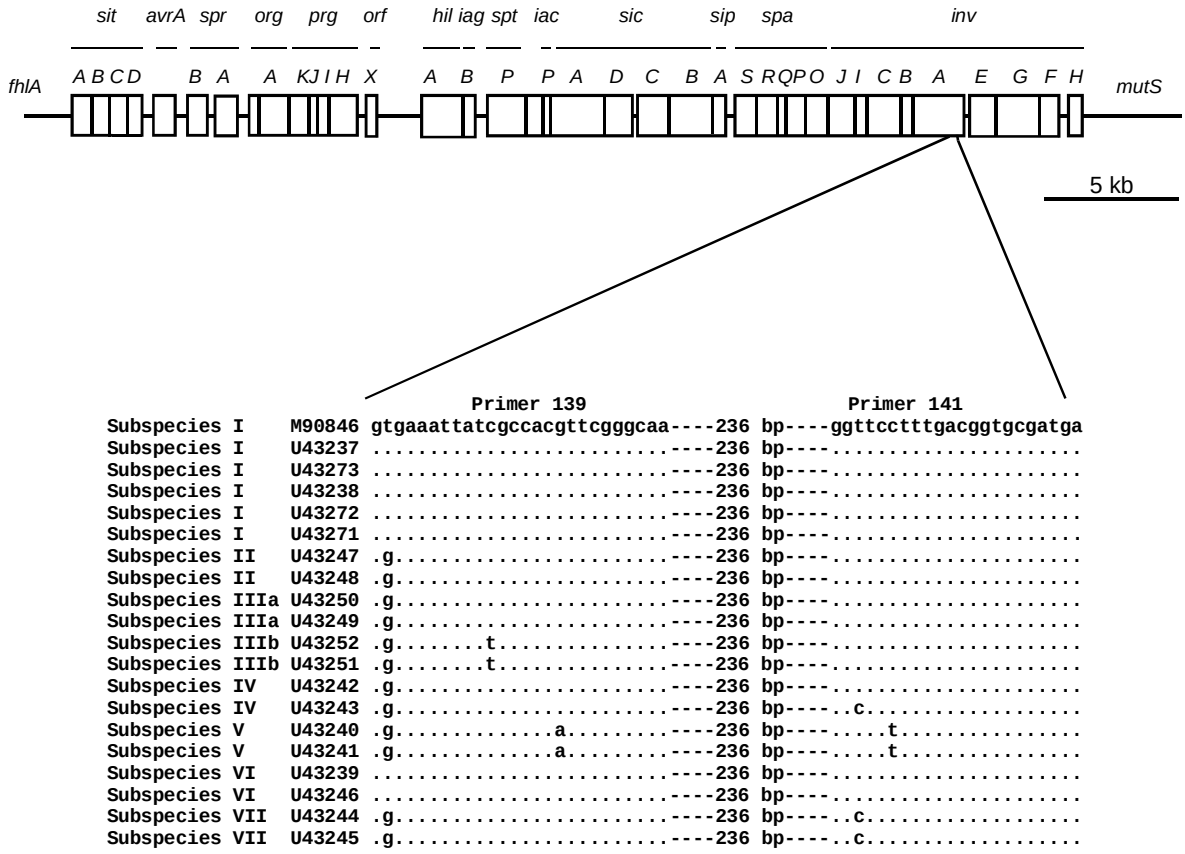

Figure 1. Map of the pathogenicity island 1 (SPI1). A sequence alignment of the primers 139/141 (Rahn *et al*. 1992) in respect to the *Salmonella* subspecies is shown at the bottom. A point indicates an identical nucleotide to the consensus sequence.

Specificity

For further specificity tests 221 *Salmonella* strains (Table 3) and 85 non-*Salmonella* strains (Hoorfar *et al*. 2000) were tested using primer-set 139/141 and malo2-F/malo2-Ra. Primer-set malo2-F/malo2-Ra did not produce any unspecific fragments. Using an annealing tempearature of 60°C the primer-set 139/141 produced from the non-*Salmonella* strains, specially from *E. coli*, *Shigella* and *Citrobacter* spp., unspecific mainly non-targeted size fragments. Using an annealing temperature at 64°C only a few faint non-target size fragments were observed.

220 of 221 *Salmonella* strains yielded an amplificate of the expected size with both primer-sets. The exceptional strain belongs to serotype *S.* Saintpaul. 15 additional tested *S.* Saintpaul strains isolated between 1998 and 2001 were positive

Table 3. List of *Salmonella* reference strains for PCR specificity tests

Serotype	Serogroup	No. of strains	Comment
Enterica Subspecies I			
Enteritidis	D	60	Most important serotype in Europe
Typhimurium	B	60	Most important serotype in Europe
Hadar	C	5	Frequently isolated serotype in Europe
Virchow	C	5	Frequently isolated serotype in Europe
Infantis	C	5	Frequently isolated serotype in Europe
Heidelberg	B	5	Frequently isolated serotype in Europe
Newport	C	5	Frequently isolated serotype in Europe
Brandenburg	B	5	Frequently isolated serotype in Europe
Saintpaul	B	5	Frequently isolated serotype in Europe
Agona	B	5	Frequently isolated serotype in Europe
Blockley	C	5	Other important serotype
Bovismorbificans	C	5	Other important serotype
Bredeny	B	5	Other important serotype
Derby	B	5	Other important serotype
Dublin	D	5	Other important serotype
Livingstone	C	5	Other important serotype
Montevideo	C	5	Other important serotype
Paratyphi B	B	5	Other important serotype
Salamae Subspecies II			
S. 42:r:-		2	
others		4	
Arizonae Subspecies IIIa		3	
Arizonae Subspecies IIIb		3	
Houtanae Subspecies IV		3	
Bongori Subspecies V		3	
Indica Subspecies VI		3	
Total No. of strains		221	

Detection limit

Using chromosomal DNA of reference strain 51K61 extracted by the Genomic-tip kit (Qiagen) the detection limit for primers 139/141 was 1 to 10 copies and for malo2-F/malo2-Ra 10 to100 copies DNA per reaction (38 cycles) (Fig. 2). Using DNA isolated by the thermal cell digested DNA method (www.pcr.dk) the detection limit was 1.5-15 cfu per reaction for primers 139/141.

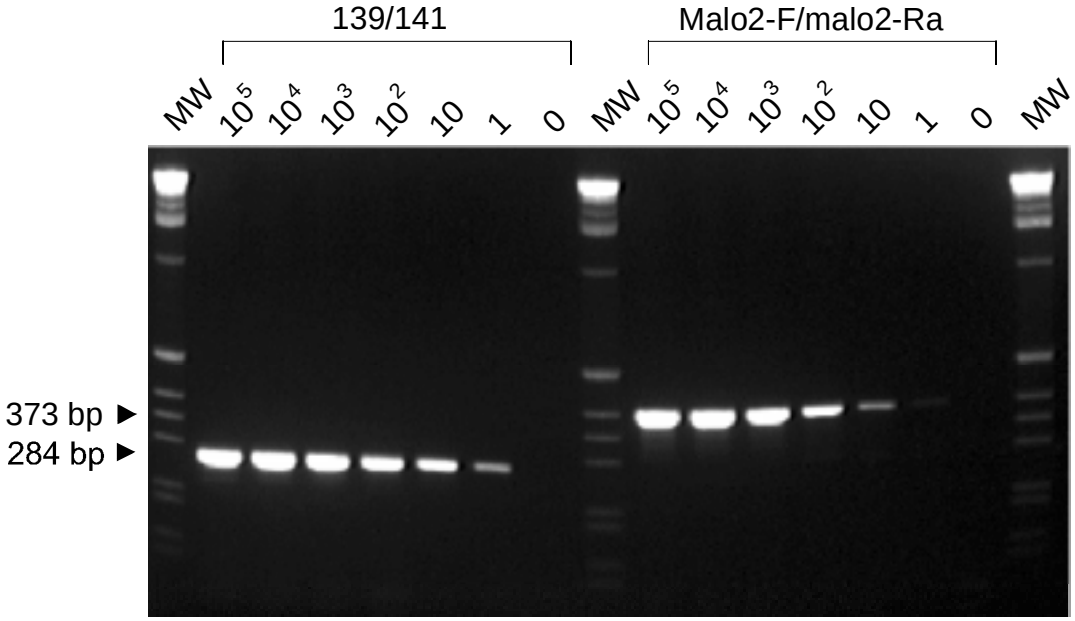

Figure 2. Sensitivity of the PCR detection after 38 cycles amplification using chromosomal *Salmonella* DNA of reference strain *S.* Typhimurium 51K61 extracted by the Qiagen Genomic-tip kit. Above the gels the number of copies are given. Left: primer-set 139/141 (Rahn *et al*. 1992). Right: primer-set malo2-F/malo2-Ra (Malorny unpublished). Left of the gel the size of PCR products is given. As molecular weight standard (MW) marker X (Roche Diagnostics, Germany) was loaded. 10 µl of 25µl PCR product was loaded per well.

Internal control

An internal control was constructed for primer-set 139/141 and cloned in pGEM-T Easy vector (Promega). The target size of the internal control is 157 bp. Experiments using native plasmid DNA or PCR product for the amplification of the internal control and Qiagen purified *Salmonella* DNA as templates were performed. Depending on the amount of template DNA of the internal control added, the assay resulted in different detection limits. In the presence of 1.7×10^5 copies of the internal control PCR product, the detection limit for *Salmonella* DNA was 5×10^3 copies. Decreasing the copy number of the internal control 10 fold (1.7×10^4) increased the detection limit to 5×10^2 copies *Salmonella* DNA. 1.7×10^2 copies internal control resulted in a detection limit of 5 copies *Salmonella* DNA (Fig. 3).

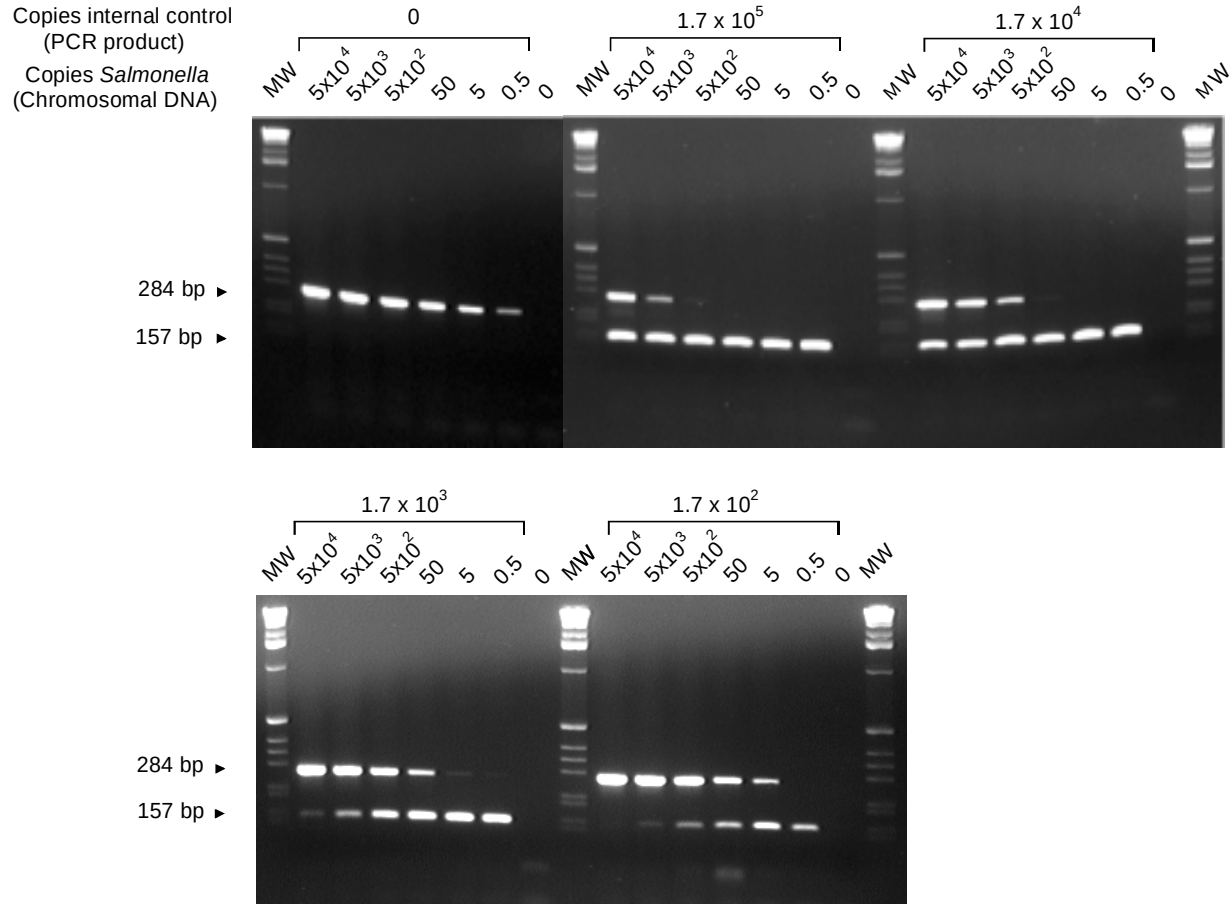

Figure 3. Influence of the internal control using primer-set 139/141 (Rahn *et al.* 1992). Qiagen isolated *Salmonella* DNA and a PCR product as template for the internal control was co-amplified. Above the gels the number of *Salmonella* DNA copies and internal control copies per reaction tube is given. Left of the gels the size of PCR products is given. As molecular weight standard (MW) marker X (Roche Diagnostics, Germany) was loaded. 10 µl of 25µl PCR product was loaded per well.

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