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PCR Assays Based on *invA* Gene Amplification are not Reliable for *Salmonella* Detection

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Abstract

Background: *Salmonella* surveillance relies on *invA* polymerase chain reaction (PCR) assays for the rapid detection of *Salmonella*; however, false-positive results have been reported using this method.
Objectives: To evaluate the performance and specificity of the published and validated PCR protocols targeting *invA* gene for the detection of *Salmonella*.
Methods: The performance and specificity of 11 different PCR primer sets were evaluated using *Salmonella* type strains and *Citrobacter* spp., *Escherichia coli* and *Serratia* spp. isolates recovered during a *Salmonella* surveillance program.
Results: It was revealed that the published PCR protocols using validated primers targeting *invA* and *16S rRNA* genes generated false-positive signals. Importantly, a protocol targeting the *ttrA/C* genes was able to discriminate *Salmonella* and non-*Salmonella* isolates.
Conclusions: Detection of *Salmonella* spp. by means of *invA* PCR amplification is not reliable. In fact, false-positive results are commonly obtained from *Citrobacter*, *E. coli* and *Serratia* isolates. It is recommended to use other loci, such as *ttrA/C* genes, for the accurate and reliable detection of *Salmonella*.

Keywords: *Salmonella*, *invA*, PCR, Detection, *Citrobacter*, *16S rRNA*, *ttrA*, *ttrC*

1. Background

Salmonella is an important pathogen transmitted through food, water or direct contact with animals. Most *Salmonella* surveillance programs rely on polymerase chain reaction (PCR)-based assays for rapid and accurate detection (1, 2). Among these molecular tools, the *invA*-PCR assay has been accepted as the conventional method for *Salmonella* detection (2-4). This PCR protocol amplifies a fragment of the *invA* gene, a *Salmonella*-specific locus (5, 6) proposed as an international standard tool for the accurate detection of this pathogen (7). Nearly, 7,000 scientific reports have used *invA* PCR assays for *Salmonella* detection and ~ 450 of them were published in 2018 (as of December) (8). Nevertheless, some reports have described the occurrence of false-positive results (5, 7, 9, 10).

2. Objectives

The main goal of the present study was to evaluate the performance of previously published *invA* PCR assays using a collection of isolates obtained from a *Salmonella* surveillance program.

3. Methods

3.1. Ethics Statement

School of Chemistry Biosafety Committee revised and approved the experiments under project #FQ-2017-01.

3.2. Bacterial Isolates

Salmonella enterica type strains (ATCC 140028, ATCC 700720, ATCC 23595, ATCC 14028 and ATCC 13076), *Citrobacter* spp., *Escherichia coli*, *Enterobacter* sp., *Serratia* sp., *Hafnia* sp. and *Aeromonas* sp. isolates were retrieved from our frozen-glycerol stock collection. This collection comprises more than 400 bacterial isolates obtained during a *Salmonella* surveillance program for poultry meat. All the selected isolates were grown overnight in Tryptic soy broth at 35°C to reach a concentration of ~ 4×10^9 CFU/mL. After incubation, one milliliter of the culture was used

for genomic DNA extraction (Quick-DNA Miniprep Plus Kit, Irvine, CA).

3.3. PCR Assay Targeting the invA Gene

Genomic DNA from the selected isolates was subjected to *invA* PCR amplification using seven previously published primer sets (5, 11-14) and targeting the *invA* gene (STM2896; Table 1). When the published protocols generated non-specific amplicons, gradient PCR (temperature range: 41 - 64°C) was performed to identify optimum annealing temperatures. Reactions were carried out using maxima hot start Taq DNA polymerase (Thermo Fisher Scientific, Waltham, MA) and 5.0 ng/μL of purified DNA. The optimized PCR protocols consisted of an initial denaturation at 94°C for 3 minutes, 35 cycles of: desaturation at 94°C for 30 secods, annealing at 53 - 69.3°C for 30 seconds (Table 1), extension at 72°C for 30 seconds and a final extension at 72°C for 3 minutes. Specificity of the PCR method was analyzed on 1.5% agarose gel.

3.4. PCR Assay Targeting the 16s rRNA, STM3098, and ttrA/C Genes

To improve the discriminatory power of PCR protocols, alternative *Salmonella*-specific PCR assays were performed. Four additional primer sets (16SF1 + 16SIII, MINf + MINr, STM3098-f2 + STM3098-r2, and ttr-6 + ttr-4) were evaluated using protocols published elsewhere (15-18) (Table 1). Similarly, gradient PCR was carried out to identify optimum annealing temperatures. PCR protocols were carried out as described above with annealing temperatures described in Table 1. Specificity of the PCR methods was analyzed on 1.5% agarose gel.

4. Results and Discussion

4.1. PCR Assay Targeting the invA Gene

During a *Salmonella* surveillance program, it was observed that some bacterial isolates generated false-positive signals using the conventional *invA* PCR assay (5); thus, it was decided to evaluate the performance of other published primers targeting the *invA* gene. Using a collection of *Citrobacter* spp., *E. coli* and *Serratia* sp. recovered from poultry meat, it was revealed that all the selected *invA* gene primers generated non-specific signals (Figure 1); comparable results have been reported in reactions containing genomic DNA from non-*Salmonella* isolates (5, 7, 9, 10). Recent studies have reported a high specificity for *invA* PCR assays (19, 20); however, these analyses were carried out using DNA obtained from type strain collections. The advantage of the present study was that field isolates known to generate conflictive results were used to evaluate the specificity of the assays. Overall, the results of the present study indicate that PCR assays based on *invA* gene amplification are not reliable for *Salmonella* detection.

4.2. Evaluation of Other Salmonella-Specific PCR Assays

Taking advantage of this collection of isolates, the performance of the other four additional primer sets was evaluated. Primers pairs 16SF1 + 16SIII and MINf + MINr targeting the *16S rRNA* gene generated non-specific signals in reactions containing *Citrobacter* spp. and *Serratia* sp. DNA, even after gradient PCR was performed (Figure 1). This lack of specificity has been reported in other studies (15, 21) and could be caused because primer sets 16SF1 + 16SIII and MINf + MINr target the V3 region of the *16S rRNA*, a segment with a high level of homology between members of the *Salmonella*, *Citrobacter* and *Enterobacter* genera (21, 22). Also, the primer set STM3098-f2 + STM3098-r2 targeting locus STM3098, a genomic region coding for a putative transcriptional regulator (17), generated non-specific signals in reactions containing *Citrobacter* spp. and *Serratia* sp. DNA (Figure 1). To the best of our knowledge, only one study has evaluated the specificity of this primer set, showing a high specificity against 37 non-*Salmonella* isolates; however, these isolates belonged to type strain collections (23). These results highlight the importance of using field isolates during PCR protocol validations.

The present study also revealed that the primer set ttr-6 + ttr-4 targeting the *ttrA/C* genes (tetrathionate reductase subunit A/C) was able to discriminate between *S. enterica* and non-*Salmonella* isolates (Figure 1). Comparable results were reported using a set of 110 *S. enterica* strains, representing 38 different serovars and 87 non-*Salmonella* strains (18). Importantly, the primer set ttr-6 + ttr-4 has shown to be an excellent molecular target for quantitative assays (e.g., qPCR) due to its high specificity and amplicon size (~ 90 bp) (24, 25).

5. Conclusions

In sum, the results of the present manuscript indicate that PCR assays based on *invA* gene amplification are not reliable for *Salmonella* detection. False-positive results are commonly obtained from *Citrobacter* spp., *E. coli* and *Serratia* sp. isolates. Other loci, such as *ttrA/C* genes, should be used for the accurate and reliable detection of this pathogen.

Footnotes

Authors' Contribution: Carolina Resendiz-Nava and Yajaira Esquivel-Hernandez performed the experiments;

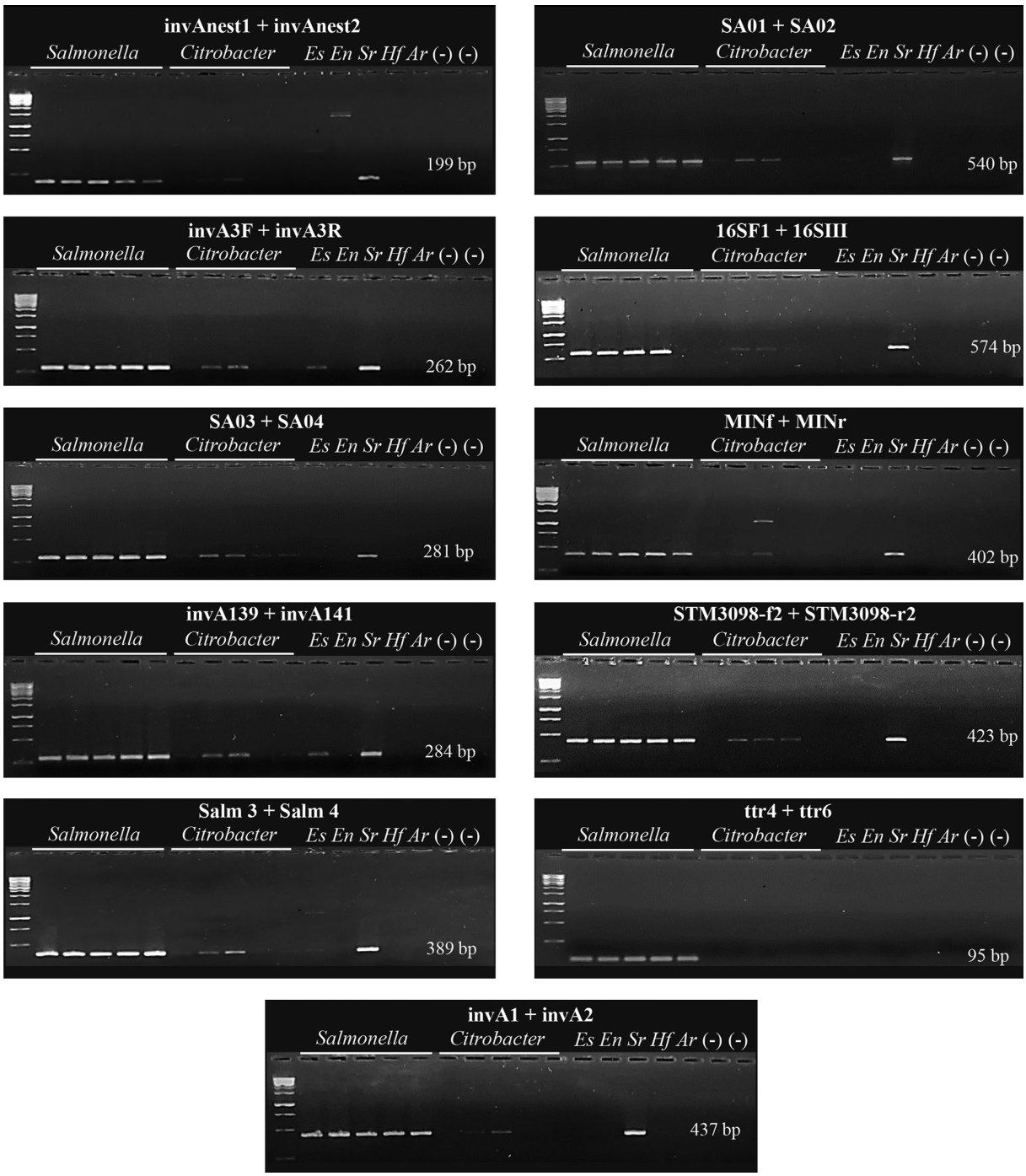

Figure 1. Performance evaluation of PCR assays for *Salmonella* detection. Representative PCR reactions using *Salmonella enterica* type-strains and isolates recovered from poultry meat samples. Eleven primer sets targeting the *invA* (invA1 + invA2, invAnest1 + invAnest2, invA3F + invA3R, SA01 + SA02, SA03 + SA04, invA-139 + invA-141 and Salm 3 + Salm 4), *16S rRNA* (16SF1 + 16SIII and MINf + MINr), STM3098 (STM3098-f2 + STM3098-r2) and *ttrA/C* (ttr-6 + ttr-4) genes were evaluated against *Salmonella enterica* type-strains (placed in this order: ATCC 140028, ATCC 700720, ATCC 23595, ATCC 14028 and ATCC 13076), *Citrobacter* spp., *Escherichia coli* (*Es*), *Enterobacter* sp. (*En*), *Serratia* sp. (*Sr*), *Hafnia* sp. (*Hf*), *Aeromonas* sp. (*Ar*).

Table 1. PCR Primers Pairs Used in the Present Study, Its Amplicon Size, Targets and Annealing Temperature

Primer	Sequence (5' - 3')	Amplicon Size, bp	Locus	Annealing Temperature, °C	References
invA1	CTGTTGAACAACCCATTTGT	437	*invA*	57.4	(11)
invA2	CGGATCTCATTAATCAACAAT				
invAnest1	AACCAGCAAAGGCGAGCAG	199	*invA*	65.0	(11)
invAnest2	GCGCACGCCATAATCAATAAA				
invA3F	AACGTGTTTCCGTGCGTAAT	262	*invA*	65.0	(12)
invA3R	TCCATCAAATTAGCGGAGGC				
SA01	TATCGTACTGGCGATATTGGTGTTTA	540	*invA*	65.0	(13)
SA02	GGACAAATCCATACCATGGCGAGTCA				
SA03	GAAATTATCGCCACGTTCGGG	281	*invA*	65.0	(13)
SA04	TCATCGCACCGTCAAAGGAAC				
invA-139	GTGAAATTATCGCCACGTTCGGGCAA	284	*invA*	64.0	(5)
invA-141	TCATCGCACCGTCAAAGGAACC				
Salm 3	GCTGCGCGCGAACGGCGAAG	389	*invA*	65.0	(14)
Salm 4	TCCCGGCAGAGTTCCCATT				
16SF1	TGTTGTGGTTAATAACCGCA	574	*16s rRNA*	57.4	(15)
16SIII	CACAAATCCATCTCTGGA				
MINf	ACGGTAACAGGAAGMAG	402	*16s rRNA*	53.0	(16)
MINr	TATTAACCACAACACCT				
STM3098-f2	TTTGGCGGCGCAGGCGATTC	423	STM3098	69.3	(17)
STM3098-r2	GCCTCCGCCTCATCAATCCG				
ttr-6	CTCACCAGGAGATTACAACATGG	86	*ttrA/C*	65.0	(18)
ttr-4	AGCTCAGACCAAAAGTGACCATC				

Alejandro Alcaraz-Gonzalez collected the samples and obtained bacterial isolates; Carolina Resendiz-Nava, Pilar Castaneda-Serrano, and Gerardo M Nava designed the experiments and wrote the manuscript. All the authors revised and approved the manuscript.

Conflict of Interests: The authors declare that there is not conflict of interest.

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