An Improved invA-based PCR Method for Rapid and Accurate Detection of *Salmonella* Isolates

Yajaira Esquivel-Hernandez, BS, MS[1]

Carolina Resendiz-Nava, BS[1]

Alejandro Alcaraz Gonzales, BS, MS[1]

Pilar Castañeda-Serrano, DVM, MC, PhD[2]

Gerardo M. Nava, DVM, MC, PhD[1]*

[1]Departmento de Investigación y Posgrado en Alimentos.
Universidad Autónoma de Querétaro, México.

[2]Centro de Enseñanza, Investigación y Extensión en Producción Avícola.
Universidad Nacional Autónoma de México, México.

* Corresponding author address:
Universidad Autónoma de Querétaro.
Cerro de las Campanas S/N, Querétaro. Qro. México. 76010
Phone and fax: (442) 192 13 04. Email: gerardomnava@gmail.com

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ABSTRACT

Salmonella is an important zoonosis and a constant threat to animal and human health worldwide. To prevent spread of infections, veterinary and human health laboratories use molecular assays for the rapid and accurate detection of this pathogen. To this end, numerous laboratories specialized in *Salmonella* surveillance rely on *invA*-based PCR assays for the rapid detection of *Salmonella* spp. in clinical samples. However, it has been extensively documented that current PCR protocols targeting the *invA* gene generate false-positive results. The goal of the present study was to standardize a rapid and feasible PCR protocol for the accurate detection of *Salmonella* isolates. The performance and specificity of five different PCR primers and protocols were evaluated using poultry meat isolates recovered from a *Salmonella* surveillance program. The present study revealed that *Citrobacter* spp. could be an important cause of false-positive results. Furthermore, it is shown that the use of a double PCR approach, using primes targeting the *invA* (*invA*-1 + *invA*-2) and *16S rRNA* (MINf + MINr) genes, is an effective method for accurate and reliable detection of *S. enterica*. Using these assays, *S enterica* isolates produce single, artifact-free, and size-expected amplicons, which are easily distinguishable from non-*Salmonella* isolates. This approach is simple, cost-effective, and easily adaptable to small and medium size laboratories.

INTRODUCTION

Salmonella is an important pathogen transmitted through food, water or direct contact with animals. Recently, it was estimated that this pathogen causes more than 20 million disease episodes and 144,000 worldwide deaths annually.[1] In order to prevent infec-

Table 1. *PCR Primers pairs use in the present study and its gene targets*

Primer	Sequence (5' - 3')	Product size (bp)	Gene	Ref.
invA-139	GTGAAATTATCGCCACGTTCGGGCAA	284	*invA*	11
invA-141	TCATCGCACCGTCAAAGGAACC			
Inva-1	CTGTTGAACAACCCATTTGT	437	*invA*	13
Inva-2	CGGATCTCATTAATCAACAAT			
16SF1	TGTTGTGGTTAATAACCGCA	574	*16s rRNA*	14
16SIII	CACAAATCCATCTCTGGA			
MINf	ACGGTAACAGGAAGMAG	402	*16s rRNA*	16
MINr	TATTAACCACAACACCT			
ttr-6	CTCACCAGGAGATTACAACATGG	86	*ttrRSBCA*	15
ttr-4	AGCTCAGACCAAAAGTGACCATC			

**Ref. = References*

tions, veterinary and human health laboratories have stablished national *Salmonella* surveillance programs oriented to monitor prevalence, distribution and antibiotic resistance profiles of this pathogen.[2,3] The majority of these programs rely on PCR-based assays for the rapid and accurate detection of *Salmonella* spp.[3-5]

Numerous PCR assays have been developed for molecular detection of *Salmonella* spp., many of them revealing different levels of specificity and accuracy.[3,6,7] Among these molecular tools, the *invA*-based PCR assay has been accepted as the conventional method for detection of *Salmonella* spp. in animal and human clinical samples.[3,8-10] This PCR protocol was originally proposed by Rahn et al. in 1992[11] and amplifies a 284-bp DNA fragment of the *invA* gene, a *Salmonella*-specific locus. This PCR assay has been extensively validated for its use as an international standard tool for accurate detection of *Salmonella* spp.[3,8-10] Although the *invA*-based PCR assay is now considered one of the standard methods for detection of *Salmonella* spp., numerous reports have described the occurrence of false-positive results in PCR reactions using DNA obtained from non-*Salmonella* isolates.[3,4,10-12] To this end, our research group has recovered multiple *Citrobacter* spp. isolates from poultry meat samples in which the standard *invA*-based PCR assay[3] generates false-positive results. Thus, the main goal of the present study was to standardize a rapid, feasible, and accurate PCR protocol for the detection of *Salmonella* isolates.

MATERIAL AND METHODS

Bacterial Isolates and Culture Conditions

Salmonella enterica type-strains (Typhimurium ATCC-14028 and Typhi ATCC-6539) and non-type-strains (laboratory collection), as well as *Citrobacter* spp. isolates (laboratory collection) were retrieved from our laboratory frozen-glycerol stock collection. The laboratory collection comprises >200 bacterial isolates obtained during a Poultry Meat *Salmonella* Surveillance Program. Bacteria strains were grown overnight in tryptic soy broth (BD Difco, Mexico) at 35°C. After incubation, one milliliter of the culture was used for genomic DNA extraction using a commercial kit (Quick-DNA Miniprep Plus Kit, Irvine, CA) following manufacturer instructions. DNA quantity and quality were determined using a NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE).

PCR Assay Targeting the *invA* Gene

Genomic DNA extracted from *S. enterica* and *Citrobacter* spp. isolates were sub-

jected to *invA*-PCR amplification using the protocol described for primers *invA*-139 and *invA*-141.[3] Also, an alternative PCR assay with primers *invA*-1 and *invA*-213 was evaluated (Table 1). When published PCR protocols generated non-specific amplicons using DNA from *Citrobacter* spp. as a template, gradient PCR runs (temperature range: 41 to 64 °C) were performed to establish optimum annealing temperature. All PCR reactions were carried out with Maxima Hot Start Taq DNA Polymerase (Thermo Fisher Scientific, Waltham, MA) as described by the manufacturer.

The optimized PCR protocol used with primers *invA*-139 + *invA*-141 consisted of an initial denaturation at 94°C 60 s, 35 cycles of: 94°C 30 s, 64°C 30 s, 72°C 30 s, and a final extension step at 72°C for 4 min. The optimized PCR protocol used with primers *invA*-1 + *invA*-2 consisted of an initial denaturation at 94°C 3 min, 35 cycles of: 94°C 30 s, 57.4°C 30 s, 72°C 30 s, and a final extension step at 72°C for 5 min. Specificity of the PCR method was confirmed by visualizing single bands corresponding to DNA fragments of the expected size via ethidium bromide/agarose gel electrophoresis.

Complementary *Salmonella*-specific PCR Assays

To improve the discriminatory power of the *invA*-based PCR protocol, alternative *Salmonella*-specific PCR assays were evaluated. Three additional primers sets: (16SF1 + 16SIII, MINf + MINr, and ttr-6 + ttr-4) were evaluated using protocols published elsewhere[14-16] (Table 1). Similarly, when published PCR protocols generated non-specific amplicons, gradient PCR runs (temperature range: 41 to 64 °C) were performed to establish optimum annealing temperature. The optimized PCR protocol used with primers MINf + MINr consisted of an initial denaturation at 94°C 3 min, 32 cycles of: 94°C 20 s, 53°C 30 s, 72°C 30 s, and a final extension step at 72°C for 2 min. Specificity of the PCR method was confirmed by visualizing single bands corresponding to DNA fragments of the expected size via ethidium bromide/agarose gel electrophoresis.

16S rRNA Gene Sequencing and Phylogenetic Analysis

Bacterial isolates identified as non-*Salmonella* by the *invA* and *16S rRNA* PCR assays were subject to molecular identification by means of PCR amplification of near full-length *16S rRNA* gene as described elsewhere.[17] PCR products were purified and subjected to Sanger sequencing using an ABI 3730XL capillary sequencer. Inspection, alignment, and trimming of sequences were performed with MEGA6 software.[18] Initial identification was performed using the Ribosomal Database Project (RDP) Classifier and Sequence Match tools.[19] Then, a phylogenetic analysis was performed using *16S rRNA* genes from *Salmonella* and *Citrobacter* species described in the Approved Lists of Bacterial Names published.[20] Phylogenetic inference by means of Maximum Parsimony (MP), Neighbor-Joining (NJ), and Minimum Evolution (ME) tree models was estimated with the MEGA6 software.[18] Statistical significance of branch order was estimated by bootstrap analysis with 1,000 replicates.

RESULTS

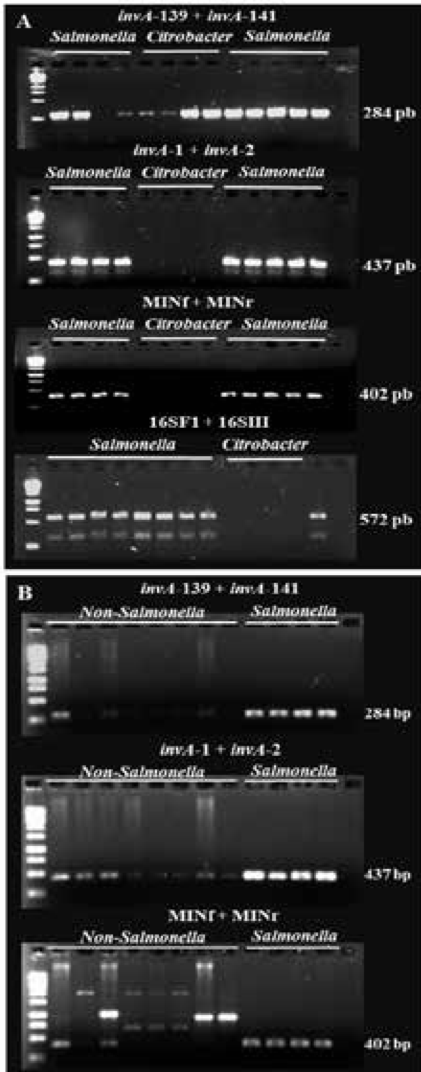
PCR Aassay Targeting the *invA* Gene

A series of PCR runs were carried out to validate the specificity of the *invA*-based PCR assays using protocols published elsewhere.[3,13] Under our laboratory conditions, published protocols using primers *invA*-139 + *invA*-141 and *invA*-1 + *invA*-2 generated non-specific signals in reactions containing *Citrobacter* DNA (data not shown). Therefore, new PCR conditions were established by using temperature gradient assays. After numerous efforts, it was not possible to eliminate non-specific signals with primer pair *invA*-139 and *invA*-141. In contrast, specify of primers *invA*-1 and *invA*-2 was enhanced with an optimized PCR protocol (Figure 1A).

Complementary *Salmonella*-specific PCR Assays

Due to the occurrence of non-specific

Figure 1. *PCR assays for detection of Salmonella spp. Representative PCR reactions using isolates recovered from poultry meat samples. A) Salmonella enterica and Citrobacter spp. isolates were used for PCR primer validation. B) Double PCR approach for detection of Salmonella spp. With this approach, Salmonella isolates produce single, artifact-free, and size-expected amplicons. Citrobacter spp. isolates were subjected to molecular identification (Figure 2).*



amplifications (false-positive results) with the *invA*-based PCR assay, it was decided to establish a double-PCR protocol for accurate detection *S. enterica*. After multiple attempts, primers pairs 16SF1 + 16SIII and ttr-6 + ttr-4 generated non-specific signals in reactions using *Citrobacter* DNA. Importantly, it was possible to eliminate these false-positive reactions with an optimized PCR protocol using primers MINf + MINr (Figure 1A).

To evaluate the effectiveness of this double-PCR protocol for accurate detection *S. enterica*, a collection of >200 poultry meat isolates was subjected to PCR amplification using primers *invA*-139 + *invA*-141, *invA*-1 + *invA*-2, and MINf + MINr. In all PCR assays, non-specific amplifications (weak amplicons and artifacts) were observed in numerous reactions. However, PCR reactions containing DNA extracted from confirmed *S. enterica* produced DNA fragments of the expected size and free of PCR artifacts (Figure 1B). To corroborate this observation, representative isolates generating non-specific amplifications (weak amplicons) and artifacts were characterized by sequencing of the *16S rRNA* gene.

Molecular Identification by Means of *16S rRNA* Gene Phylogenetic Analysis

Initial characterization performed by RDP Classifier and Sequence Match tools[19] identified the selected isolates as members of the genus *Citrobacter* (data not shown). This result was confirmed by a phylogenetic analysis using Maximum Parsimony (MP), Neighbor-Joining (NJ), and Minimum Evolution (ME) tree models. Together, these phylogenetic analyses confirmed that isolates generating non-specific amplifications and artifacts belonged to the genus *Citrobacter* (Figure 2). The *16S rRNA* gene sequences obtained in the present study were deposited at the GenBank under accession numbers: MG597049-MG597056.

DISCUSSION

Worldwide, numerous veterinary and human health laboratories rely on the *invA*-based PCR assay for detection of *Salmonella* spp. in clinical samples.[3,8-10] Despite the fact that this PCR assay is considered one of the standard methods for detection of *Salmonella* spp., numerous reports have documented

Figure 2. *Molecular characterization of isolates recovered from poultry meat samples. Selected isolates generate PCR artifacts using primers invA-139 + invA-141, invA-1 + invA-2 and MINf + MINr. 16s rRNA genes sequences from eight isolates (black circle) were compared against reference Salmonella, Citrobacter, and Escherichia isolates (GenBank accession numbers provided). Similar tree topologies were obtained by Maximum Parsimony, Neighbor-Joining, and Minimum Evolution models. Numbers on branches indicate bootstrap values after 1,000 replicates.*

false-positive results caused by the appearance of non-specific amplifications and artifacts in the PCR reactions.[3,4,10-12]

As reported elsewhere, published PCR protocols with primers *invA*-139 + *invA*-141 and *invA*-1 + *invA*-2 generate non-specific signals in reactions containing DNA from non-*Salmonella* strains.[3,4,10-12] To improve the discriminatory power of *invA*-based PCR protocols, we evaluated the performance of three validated *Salmonella*-specific primers targeting *16S rRNA* and functional genes.[14-16] Using *Citrobacter* isolates selected by their known capability of generating false-positive results, it was revealed that primers MINf + MINr were more reliable for detecting *S. enterica* isolates. This superior specificity was also demonstrated using a set of 78 *S. enterica* strains representing 31 different serovars, and 23 non-*Salmonella* strains.[16] Thus, it is recommended to perform detection of *Salmonella* species by using the combination of PCR protocols targeting the *invA* gene and the selected locus of the *16S rRNA* gene.

The performance of this double PCR approach was evaluated using a large set of bacterial isolates recovered from poultry meat. The analysis revealed that *S enterica* isolates generate single, artifact-free, and size-expected amplicons. In contrast, non-*Salmonella* isolates produce weak amplicons and a series of non-specific PCR products. Interestingly, all selected non-*Salmonella* strains belonged to the genus Citrobacter. We have continued the design and evaluation of alternative PCR protocols for the accurate detection of *S. enterica*. However, so far, none of the evaluated primer sets have been able to eliminate the non-specific amplification generated by this challenging *Citrobacter* isolates.

CONCLUSION

For the rapid, feasible and accurate detection of *Salmonella* isolates, it is recommended to use of a double PCR approach, using primes targeting the *invA* (*invA*-1 + *invA*-2) and *16S rRNA* (MINf + MINr) genes. Under these assays, *S enterica* isolates produce single, artifact-free, and size-expected amplicons, which are easily distinguishable from non-*Salmonella* isolates due to the generation of weak amplicons and PCR artifacts.

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CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this article.

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