Original Article

Distribution of pathogenicity islands among Colombian isolates of *Salmonella*

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Abstract

Introduction: *Salmonella* pathogenicity islands (SPIs) are regions scattered along the bacterial chromosome, with an acknowledged pivotal role during gastrointestinal and systemic infection. The distribution of SPIs has been investigated in reference strains. However, there is a lack of studies on their presence and/or assortment within the genomes of *Salmonella* enterica (*S*. enterica) serovars that circulate in different geographical regions. Therefore, in this study, we aimed to determine the presence of genes of the pathogenicity islands 1 to 5 (SPI-1 to 5), in *Salmonella* clinical isolates from Colombian patients with systemic and enteric outcomes.

Methodology: A total of 125 strains of *S*. enterica belonging to different serovars were isolated from various clinical samples. Strains were identified and screened for the presence of various genes located in pathogenicity islands. The genes tested were selected according to the attributed pathogenic function and detected by PCR for the SPI-1 *hil*A and *inv*A; for SPI-2 *spi*C and *ttr*C; for SPI-3 *mis*L and *mgt*C; for SPI-4 *orf*L and SPI-4R; and for SPI-5 *pip*D and *sop*B.

Results: *Salmonella* pathogenicity islands 1 to 5 were detected in isolates from patients with systemic and gastrointestinal infection. All the systemic isolates possessed all the genes tested; in contrast, 16 isolates from stool samples lacked one or more sequences encoded by the SPI-3 and SPI-4 ($p < 0.000001$).

Conclusions: These results describe the heterogeneous distribution of SPIs-encoded sequences within the genomes of Colombian clinical isolates, and reveal important differences among systemic and stool sample isolates.

Key words: *Salmonella* infections; pathogenicity; genomic islands; PCR; virulence factors, salmonellosis

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Introduction

Salmonellosis is responsible for a large number of infections in both humans and animals [1]. *Salmonella enterica* comprises approximately 2,579 serovars that cause a variety of clinical syndromes [2].

Virulence properties of *S. enterica* are associated with many molecular determinants. Among the other proteins encoded by genes located on *Salmonella*, pathogenicity islands (SPIs) have a very important role during the infection process, since they are either structural components of the secretion system III (SST), or effectors injected through this supramolecular device, and allow bacteria to colonize and invade the host mucosa and to establish the subsequent systemic infection [3].

SPIs are portions of DNA that have been acquired from other microorganisms by horizontal transfer, and are absent in non pathogenic strains [4]. To date, 12 SPIs have been identified in different serovars [5], but only five are well characterized.

The assessment of whether a different assortment of SPI genes occurs in serovars causing systemic versus enteric infection in humans could help to explain the function of this group of genes, and the variability of clinical syndromes produced by *S. enterica* in humans.

The aim of the present work was to determine the distribution of pathogenicity islands SPI-1 to SPI-5 among Colombian isolates of *Salmonella* from patients with systemic and enteric clinical outcomes, inhabitants of an endemic region of salmonellosis. This is the first report on the distribution of SPIs among Colombian *S. enterica* clinical isolates.

Table 1. Sequences of primers and products sizes.

Primer	Sequence	Product size (pb)	References
hilA forward	5'- CGG AAG CTT ATT TGC GCC ATG CTG AGG TAG -3'	854	6
hilA reverse	5'- GCA TGG ATC CCC GCC GGC GAG ATT GTG -3'		
invA forward	5'- TGC CTA CAA GCA TGA AAT GG -3'	450	7
invA reverse	5'- AAA CTG GAC CAC GGT GAC AA -3'		
s*piC* forward	5'- CCT GGA TAA TGA CTA TTG AT -3'	301	
s*piC* reverse	5'- AGT TTA TGG TGA TTG CGT AT -3'		
misL forward	5'- GTC GGC GAA TGC CGC GAA TA -3'	561	
misL reverse	5'- GCG CTG TTA ACG CTA ATA GT -3'		
orfL forward	5'- GGA GTA TCG ATA AAG ATG TT -3'	332	
orfL reverse	5'- GCG CGT AAC GTC AGA ATC AA -3'		
pipD forward	5'- CGG CGA TTC ATG ACT TTG AT -3'	399	
pipD reverse	5'- CGT TAT CAT TCG GAT CGT AA -3'		
ttrC forward	5'- GTG GGC GGT ACA ATA TTT CTT TT -3'	920	8
ttrC reverse	5'- TCA CGA ATA ATA ATC AGT AGC GC -3'		
mgtC forward	5'- TGA CTA TCA ATG CTC CAG TGA AT -3'	655	
mgtC reverse	5'- ATT TAC TGG CCG CTA TGC TGT TG -3'		
SPI4R forward	5'- GAT ATT TAT CAG TCT ATA ACA GC -3'	1.269	
SPI4R reverse	5'- ATT CTC ATC CAG ATT TGA TGT TG -3'		
sopB forward	5'- GAT GTG ATT AAT GAA GAA ATG GC -3'	1.170	
sopB reverse	5'- GCA AAC CAT AAA AAC TAC ACT CA -3'		

Methodology

Bacterial Strains

A total of 125 strains belonging to different serovars of *Salmonella enterica*, were isolated from various clinical samples. Out 125 strains, 91 were isolated from stool samples collected from patients who showed gastrointestinal symptoms, including diarrhoea, vomiting, and fever. Thirty-four strains were isolated from clinical samples of patients having different systemic symptoms such as enteric fever (n = 24), osteomyelitis (n = 3), hepatic abscess (n = 1), meningitis (n = 2), and intestinal perforation (n = 1). Samples included blood, cerebrospinal fluid (CSF) and peritoneal fluid.

Bacterial identification

Salmonella strains were isolated from clinical samples and Xylose Lactose Desoxycholate agar (XLD) (Difco, Becton Dickinson, New South Wales, Australia). All isolates were classified as *Salmonella enterica* by standard biochemical methods using API20E (Biomerieux, Cambridge, MA, USA) strips. Serotyping was performed using mono and polyclonal antibodies (Bio-Rad Hercules, CA, USA) and Pasteur Institute, Paris, France). Bacterial cultures were further grown in Luria Bertani broth (Sigma, St. Louis, MO, USA) for DNA extraction. Genomic DNA was extracted by boiling and used for PCR assays.

Molecular Identification

The isolates were evaluated for the presence of two specific genes on each SPI-1 to SPI-5: *hil*A, *inv*A (SPI-1); *spi*C, *ttr*C (SPI-2); *mgt*C, *mis*L (SPI-3); *spi4*R,*orf*L (SPI-4); *sop*B and *pip*D (SPI-5). The primer sequences and product sizes can be seen in Table 1. The design of primers was made according to *Salmonella* Typhimurium LT2 genome, GenBank (access number NC_ 003197).

PCR protocol

Briefly, 25 µl volume of reaction mixture was made, containing 1.25 µl 10 µM of each primer; 0.5 µl 10mM of deoxynucleotides; 2.5µl buffer 10X; 0.5 µl of Taq polymerase (Invitrogen Carlsbad, CA, USA) 5 U/µl; 1 µl of DNA; and 18 µl of distilled water to complete the reaction volume**.** One positive control (*S*. Typhimurium ATCC 14028) and two negative controls (water and *E. coli* DNA) were included. PCR products were visualized by electrophoresis in 1% agarose gel and stained with Sybr Safe Invitrogen Carlsbad, CA, USA. To guarantee reproducibility of

all experiments, each PCR amplification was repeated three times.

Statistical analysis

Statistical analysis was performed by the calculation of frequencies by percentages. The comparison of variables was done through the proportion comparison using Epi Info 6.0, CDC Atlanta, GA, USA statistical program. The significance of 0.05 was used to establish differences between variables.

Results

Culture Identification

All isolates were confirmed by API as *Salmonella* belonging to 15 different serovars. The most represented serovar was Typhimurium with 53 isolates (42.4%), followed by Enteritidis (n = 27; 21.6%), Typhi (n = 20;16%), Muenchen (n = 5; 4%), Choleraesuis (n = 4; 3.2%), Paratyphi C (n = 3; 2.4%), Dublin (n = 3; 2.4%), Paratyphi B (n = 2; 1.6%), Panama (n = 2; 1.6%), Agona (n = 1; 0.8%), Derby (n = 1; 0.8%), Weltevrenden (n = 1; 0.8%), Javiana (n= 1; 0.8%), Braenderup (n = 1; 0.8%), Virginia (n = 1; 0.8%). The majority of strains were isolated from stool, (72.8%), and from blood (20%). Only nine isolates were obtained from other sources such as bone, cerebral spinal fluid, and peritoneal fluid (7.2%).

All *S. enterica* serovar Typhi strains were isolated from blood and other systemic sources. Table 2 shows serotype distribution according to source of sample

Virulence genes of SPIs

Salmonella serovars isolated from systemic samples were positive to all sequences tested, including *hilA, invA, spiC, ttrC, mgtC, misL, spi4R, orfL, sopB* and *pipD.* In contrast, 16 isolates from stool samples lack one or more probed sequences. Specifically, five isolates, including *S.* Typhimurium (n = 1), *S.* Enteritidis (n = 3), and *S.* Derby (n = 1), lack both *mis*L (SPI-3) and *orfL* (SPI-4). Five Typhimurium isolates and one Enteritidis isolate lack misL (SPI-3). Moreover, three Typhimurium, one Virginia, and one Muenchen isolates did not show *orf*L (SPI-4). Figure 1 and Figure 2 illustrate the amplification products of *invA* and *spi4R* genes.

Statistical analysis

Statistical differences were found comparing the percentages of positive isolates from systemic samples versus positive isolates from stool samples ($p = 0.000001$). Significant differences among presence or absence of the genes tested according to *Salmonella* serovars were not found ($p > 0.05$).

Discussion

During the last decade, knowledge about the biology of *Salmonella enterica* has become more extensive and identification of some structural components such as virulence factors has been made. However, information about the presence of virulence genes in clinical isolates is scarce, especially for those that come from geographical regions where *Salmonella enterica* is endemic, such as Colombia. In this country, the prevalence of disease is not well known; the information that is available is from the National Institute of Health of Colombia, which reports the identification of 3,173 isolates between 1997 and 2008,, mainly *S.* Typhimurium with 1,131 isolates (35.6%) [9].

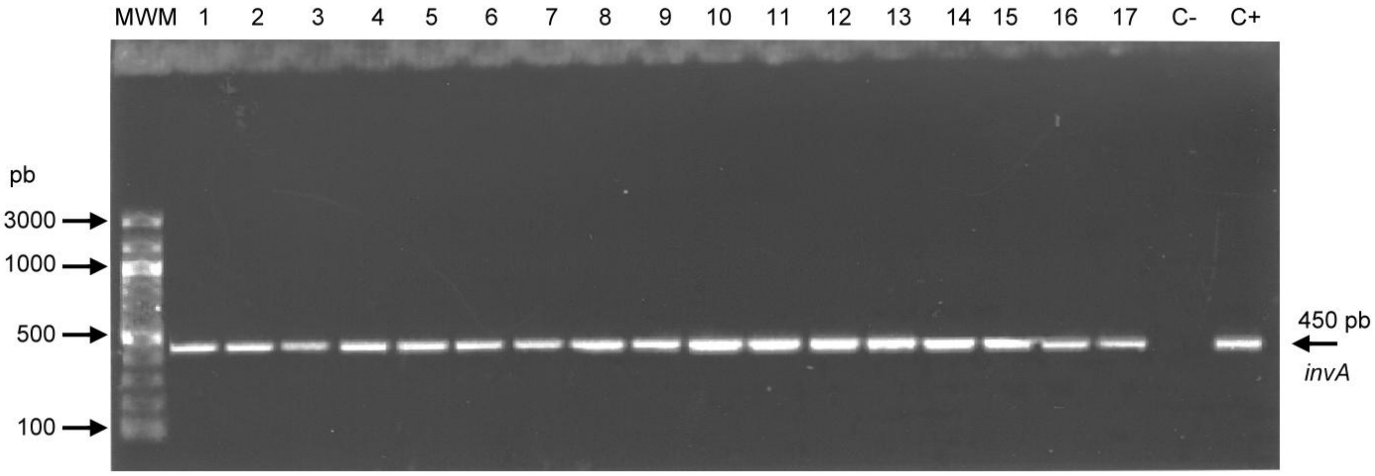
We tested the presence of virulence genes located in SPI-1 to 5 (SPI-1 to 5) in local clinical isolates. A number of studies focusing the same approach have been conducted in other countries including Italy [10], France [11], Brazil [12] and the United States [13]. To our knowledge, however, this is the first study reporting the distribution of SPIs in Colombian isolates.

In this study, all clinical isolates were found positive for SPI-1 genes *hil*A and *inv*A, regardless of serovars and clinical outcome. These results are in agreement with those of Ginocchio *et al*. [14], who reported the occurrence of SPI-1 gene deletions solely in a few environmental isolates of *Salmonella* serovars, which are consistently present in isolates from human source. Ammari *et al*. [15] also reported the occurrence of *inv*A genes in fifteen isolates of *Salmonella* Enteritidis analyzed from Morocco.

Accordingly, we showed that *Salmonella* serovars isolated from systemic infection samples (i.e., blood, bone, cerebral spinal fluid, and peritoneal fluid) were positive to all genes tested. In contrast, the selected panel of SPI-encoded genes was not complete in 16 isolates from stool samples. In particular, SPI-3 and SPI-4 encoded genes were missing (*mis*L and *orf*L respectively). This result could be explained by insertions or deletions within SPI-3 and SPI-4 regions, to be attributed to differences in the evolutionary process of acquisition and loss of corresponding genes.

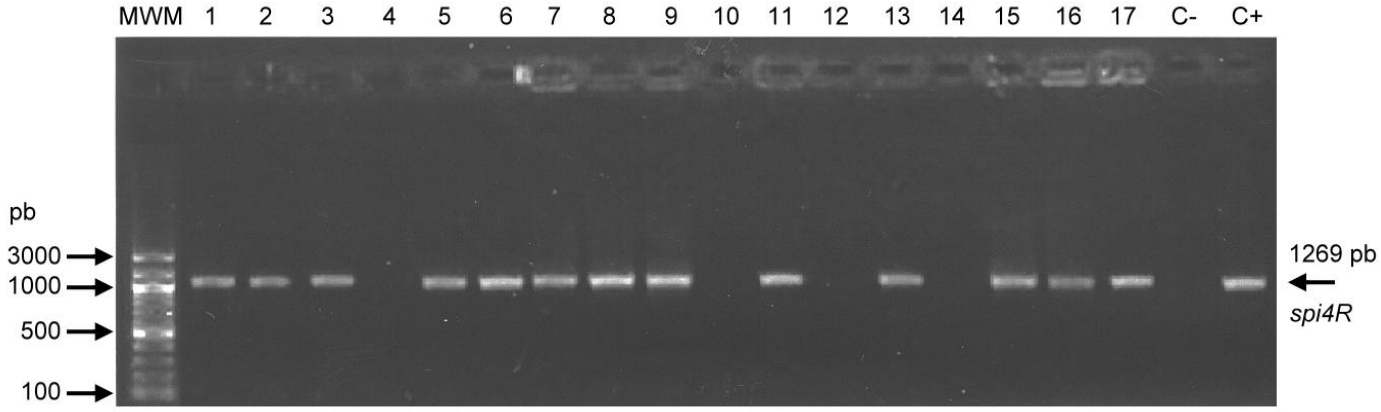
A number of studies reported that *mis*L and *orf*L genes play an important role in Salmonella

Figure 1. Agarose gel for detection of invA gene of SP1 by PCR.



Lanes 1-3, *S.* Typhimurium; lane 4, *S.* Enteritidis; lanes 5-6, *S.* Typhi; lanes 7-8, *S.* Muenchen; lane 9, *S.* Agona; lanes 10-14, *S.* Enteritidis; lanes 15-16 *S.* Choleraesuis; lane 17, *S* Paratyphi C C-, negative control; C+, positive control strain *S.* Typhimurium ATCC 14028. MWM, Molecular weight marker GeneRuler 100 bp DNA Ladder plus (Fermentas).

Figure 2. Agarose gel for detection of *spi4R* gene of SPI4 by PCR.



Lanes 1-2, *Salmonella* Enteritidis; lanes 3-5, *S.* Typhimurium; lanes 6-8, *S.* Typhi; lanes 9-10, *S.* Muenchen; lanes 11-12, *S.* Choleraesuis; lanes 13-14, *S.* Paratyphi C; lanes 15-17, *S.* Agona C-, negative control; C+, positive control strain *Salmonella* Typhimurium ATCC 14028. MWM: Molecular weight marker GeneRuler 100 bp DNA Ladder plus (Fermentas).

pathogenicity. For example, MisL, an auto-transporter protein, is an extracellular matrix adhesin involved in intestinal colonization [16], and *orf*L is required for macrophage survival [17]. The function of both genes is involved directly with the invasion process, which is necessary to produce systemic infection [18]. SPI-3 and SPI-4 encoded genes have been associated with *Salmonella* pathogenicity, due to the impaired virulence of their specific deleted derivatives, as demonstrated with experimental evidence in animal or cellular models of infection [16,18]. In this respect, our data provide epidemiological evidence that misL and orfL, and possibly more virulence SPI-encoded genes, already known to play a "secondary" role during *Salmonella* systemic infection are, indeed, required to establish a systemic infection in humans.

The present study on *Salmonella enterica* isolates from Colombia constitutes the first attempt to learn about the distribution of virulence loci in clinical report demonstrate that molecular epidemiology might effectively contribute to define the clinical isolates in this region. The data collected in this impact of already known SPI-encoded virulence genes, and point to the need for further studies in different epidemiological and geographic contexts.

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