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# Genotyping of *Salmonella enterica* strains from animal and human origin using three molecular techniques

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**Abstract**

This study aims to characterize different *Salmonella enterica* subsp molecularly. *enterica* strains (*n*=49) were isolated from human gastrointestinal cases in the Tolima region and poultry from Santander and Tolima regions using PCR-RFLP, PCR-ribotyping, and PCR-SSCP. The band patterns obtained with each technique were analyzed by building dendrograms based on the Unweighted Pair Group Method with Arithmetic mean (UPGMA) method and using the Dice coefficient. On the other hand, the discriminatory power of each technique was assessed using Simpson's discriminatory index. The genetic profiles of the *gnd* gene obtained with *Aci*I restriction enzyme and the PCR-SSCP carried out with *groEL* gene allowed the inter-and intraserovar differentiation. Finally, the PCR-ribotyping method exhibited the highest discriminatory power (0.8571). In conclusion, we show three PCR-based genotyping methods providing an alternative for identifying similarities and differences within *Salmonella enterica* strains from different geographic and biological regions.

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## Introduction

Salmonellosis is a zoonotic disease that affects different animal species such as cattle, sheep, poultry, and pigs, generating significant economic losses in the animal production industry (1). It is caused by different *Salmonella* serotypes, which are commonly isolated from food products of animal origin (1). More than 2600 *Salmonella* serotypes have been described, 1586 belonging to *Salmonella enterica* subsp. *enterica*, responsible for 99% of the salmonellosis cases in humans and warm-blooded animals (2,3). Annually, 93.8 million non-typhoidal salmonellosis cases and 150,000 deaths are reported worldwide. Likewise, 15.5 million typhoidal salmonellosis cases are reported annually, and 154000 of these cases are fatal (4). The White-Kauffmann-Le Minor scheme is a phenotyping method widely used for *Salmonella* serotyping (5), but it cannot distinguish the possible clonal origin of the isolates (6).

In contrast, PCR-based genotyping methods allows the discrimination of clonal origin, being a useful tool for epidemiological characterization of pathogens isolated from outbreaks at inter-and intraserovar level and determining the relationships within the isolates, all of these good generating reproducibility and discriminatory power (DP) values with a low requirement in time and specialized equipment (6,7). In Colombia, salmonellosis is under permanent surveillance through the programs of control and tracing of foodborne diseases, and despite there being information regarding circulating serotypes, little data regarding relationships of the distinct isolates are available (8). Therefore, this study aimed to molecularly characterize *Salmonella enterica* strains from different origins through three PCR-based genotyping techniques to establish the genetic differences among the isolates and infer the possible phylogenetic relationship of the strains.

## Materials and methods

### Bacterial strains

There are 50 *Salmonella enterica* strains, 49 belonging to 8 different serotypes and one reference *Salmonella enterica* subsp. *enterica* ser. Enteritidis (ATCC 13076®), used as amplification control, were evaluated. These strains were isolated in previous projects of our laboratory, of which ten correspond to isolates from human gastrointestinal cases in the Tolima region (9). Fifteen belong to poultry from the Santander region (10), and 24 are from the Tolima region (11). All strains have been previously characterized via the White-Kauffmann-Le Minor scheme and correspond to *Salmonella enterica* subsp. *enterica* serovar Enteritidis, *Salmonella enterica* subsp. *enterica* serovar Typhimurium, *Salmonella enterica* subsp. *enterica* serovar Braenderup, *Salmonella enterica* subsp. *enterica* serovar Newport, *Salmonella enterica* subsp. *enterica* serovar Grupensis, *Salmonella enterica* subsp. *enterica* serovar Uganda, *Salmonella enterica* subsp. *enterica* serovar Paratyphi B and *Salmonella enterica* subsp. *enterica* serovar Heidelberg.

### Genomic DNA extraction

Genomic DNA was isolated from fresh bacterial colonies using Wizard Genomic DNA Purification Kit (Promega, USA), following the manufacturer's instructions. The isolated DNA was stored at -20°C until its use.

### Molecular confirmation of Salmonella isolates

Molecular confirmation was carried out via PCR by amplifying a fragment of 284 bp of *invA* gene accession number: M90846.1, using specific primers (Table 1). Furthermore, *S.* Enteritidis (ATCC 13076®) was used as a positive control.

Table 1: Primers sequences were used for the three genotyping methods

| Gene or target region | Primer | Sequence (5-3) | Amplicon length (bp) | Reference |
|---|---|---|---|---|
| *fliC* | F | CAAGTCATTAATACAAACAGCC | 1,500 | (12) |
| | R | TTAACGCAGTAAAGAGAGGAC | | |
| *gnd* | F | CTGCGCCTGAATTAAGTTAGCTGG | 1,266 | (13) |
| | R | GAAAGCCGTGGTTATACCGTCTCC | | |
| Ribosomal operon | F | GAGCAAACAGGATTAGATACCC | Variable | This study |
| | R | TCGTGCAGGTCGGAACTTAC | | |
| *groEL* | F | CGCTCGTGTGAAAATGCTGC | 1,598 | This study |
| | R | TACCACCCATACCACCCAT | | |
| *invA* | F | GTGAAATTATCGCCACGTTCGGGCAA | 284 | (14) |
| | R | TCATCGCACCGTCAAAGGAACC | | |

### Genotyping

A total of 5 molecular markers were used: *invA*, *fliC*, *gnd*, *groEL* genes, and the 16S-23S rRNA Intergenic Spacer Region (ISR). In order to design specific primers and select the most suitable endonucleases, *in silico* analyses were performed using the GenBank database (Table 1). PCR experiments carried out for PCR-RFLP, PCR-ribotyping, and PCR-SSCP techniques were performed using 25 µl of total reaction volume, composed by 1 µl of template DNA, 5 µl of Flexi Buffer 5x colorless GoTaq® (Promega, USA), 1 µl of dNTPs (Invitrogen, USA), 5 µl of each primer (Table 1) at 10 pmol/mL (Macrogen, Korea), 1 µl of $MgCl_2$ (25 mM) (Promega, USA), 0,125 µl of GoTaq Flexi DNA Polymerase (Promega, USA) and 14,875 µl of nuclease-free water. An initial denaturation at 95°C for 3 minutes, followed by 35 cycles comprising 30 seconds of denaturation at 95°C, 30 seconds of annealing step at 55°C for *fliC*, *groEL*, *invA*, and 16S-23S ISR rRNA genes and 60°C for *gnd* gene, an extension step at 72°C for 90 seconds for *fliC* and *gnd* genes, 120 seconds for *groEL* gene, 210 seconds for 16S-23S ISR rRNA and 30 seconds for *invA* gene, and a final extension at 72°C and 7 minutes were used. Electrophoresis was carried out in a vertical and continuous system in 10% non-denaturing polyacrylamide gels using 0,5X TBE as running buffer, the Mini PROTEAN Tetra Cell device (Bio-Rad, USA), and Diamond™ Nucleic Acid Dye (Promega, USA) as an intercalating agent. Conditions were 120V, 60 minutes for PCR-RFLP, and 70 minutes for PCR-ribotyping and PCR-SSCP.

### PCR-RFLP

*fliC* and *gnd* genes were digested with *Hha*I and *Aci*I, and *the groEL* gene was cleaved using *Hha*I and *Pst*I. Restriction reactions followed the manufacturer's instructions (NEB, USA).

### PCR-ribotyping

16S-23S rRNA ISR was amplified for each strain. Then, double enzymatic digestion was carried out with *Hae*III and *Sph*I, following the manufacturer's instructions (NEB, USA).

### PCR-SSCP

A 284 bp fragment of *the invA* gene and the *groEL-Pst*I restriction fragments were subjected to heat denaturation at 95°C for 15 minutes and then stored at -20°C until electrophoresis.

**Sanger sequencing**

The *gnd* gene of two *S*. Enteritidis isolates was sequenced by the Sanger method. The sequences were deposited in GenBank with accession numbers MZ028205 and MZ028206. Bioinformatic analyses were performed with Geneious Prime Software version 2021.1.

**Fingerprinting**

The genetic profiles generated by each technique were analyzed with BioNumerics software version 8.0 (Applied Maths NV, Belgium), calculating the genetic distances with Dice coefficient (15). In addition, to generate dendrograms, the UPGMA method was used. Furthermore, combined analysis using the genetic profiles obtained with the three techniques were also performed. Finally, the DP of each technique was measured through Simpson's discriminatory index (16).

**Results**

**PCR amplification of molecular markers**

PCR amplified all molecular markers from all *Salmonella enterica* isolates (*n*=49).

**PCR-RFLP**

The RFLP analysis of *the fliC* gene restricted with *Hha*I showed three commons and five specific patterns (SP) (Table 2). *fliC* cleaved with *Aci*I endonuclease exhibited five common and 3 SP (Table 2). The DP of *fliC* patterns generated with *Hha*I and *Aci*I was 0.6471 and 0.6718, respectively. Furthermore, *gnd* digested with *Hha*I showed four standard and 2 SP (Table 2), while the restriction of this gene with *Aci*I enzyme generated four standard (RP-C1 to RP-C4) and 5 SP (RP-U1 to RP-U5) (Figure 1). For *gnd-Hha*I, the calculated DP was 0.6088 and 0.6726 for *gnd-Aci*I. Finally, *the groEL* gene cleaved with *Pst*I generated one profile for all strains and a DP of 0 (Table 2). On the other hand, *groEL* restriction with *Hae*III produced four common and 1 SP, with a DP of 0.6446 (Table 2).

**PCR-ribotyping**

Twelve genetic profiles were obtained, with seven common (RT-C1 to RT-C7) and five specific ribotypes (RT-U1 to RT-U5) (Figure 2.); the DP was 0.8571, yielding the highest value out of the three methods.

**PCR-SSCP**

The *invA* PCR-SSCP fingerprints showed five common (SS-C1 to SS-C5) and one specific profile (SS-U1) (Figure 3), yielding a DP of 0.6675. Furthermore, *groEL-Pst*I SSCP fingerprints generated four common and 5 SP, describing a DP of 0.6726 (Table 2).

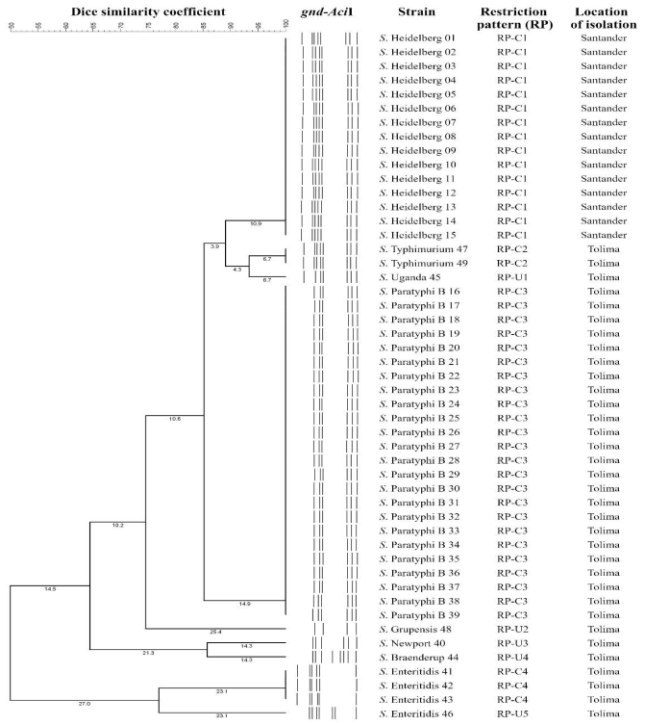


Figure 1: Dendrogram from restriction patterns of *gnd* gene cleaved with *Aci*I endonuclease. The tree was generated through UPGMA and Dice coefficient.

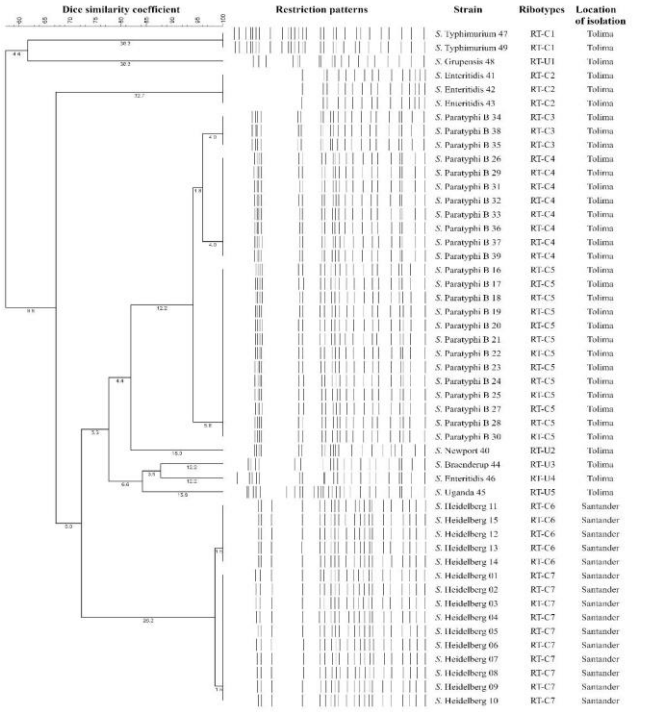


Figure 2: Dendrogram from restriction patterns of 16S-23S rRNA ISR cleaved with *Hae*III and *Sph*I endonucleases. The tree was generated through UPGMA and Dice coefficient.

Table 2: The discriminatory power of individual and combined genotyping methods

| Genotyping method | Molecular marker | Restriction enzyme | Common profiles | Specific profiles | Number of strains per profile | DP |
|---|---|---|---|---|---|---|
| RFLP | *fliC* | *Hha*I | 3 | 5 | 17,1,3,1,1,1,1,24 | 0.6471 |
| | | *Aci*I | 5 | 3 | 3,1,1,2,1,24,2,15 | 0.6718 |
| | *gnd* | *Hha*I | 4 | 2 | 15,27,1,2,1,3 | 0.6088 |
| | | *Aci*I | 4 | 5 | 15,2,1,24, 1,1,1,3,1 | 0.6726 |
| | *groEL* | *Hha*I | 4 | 1 | 3,25,5,1,15 | 0.6446 |
| | | *Pst*I | 1 | 0 | 49 | 0 |
| PCR-ribotyping | 16S-23S rRNA ISR | *Hae*III-*Sph*I | 7 | 5 | 2,1,3,3,8,13, 1,1,1,1,5,10 | 0.8571 |
| SSCP | *groEL* | *Pst*I | 4 | 5 | 24,1,2,1,1,3,1,1,15 | 0.6726 |
| | *invA* | - | 5 | 1 | 4,3,2,1,24,15 | 0.6675 |
| RFLP+RFLP | *fliC-Hha*I+*fliC-Aci*I | | 4 | 5 | 15,2,1,1,1,1,3,1,24 | 0.6726 |
| | *fliC-Hha*I+*gnd-Hha*I | | 4 | 5 | 15,2,1,1,1,1,24,3,1 | 0.6726 |
| | *fliC-HhaI*+*groEL-HhaI* | | 4 | 5 | 3,1,24,1,1,1,1,2,15 | 0.6726 |
| | *fliC-Aci*I+*groEL-Hha*I | | 4 | 5 | 24,1,3,1,1,1,1,2,15 | 0.6726 |
| SSCP+RFLP | SSCP *invA*+*fliC-Hha*I | | 4 | 5 | 15,2,1,3,1,1,1,1,24 | 0.6726 |
| All genotyping methods combined | | | 7 | 5 | 3,1,1,1,1,3,8, 13,1,2,5,10 | 0.8571 |

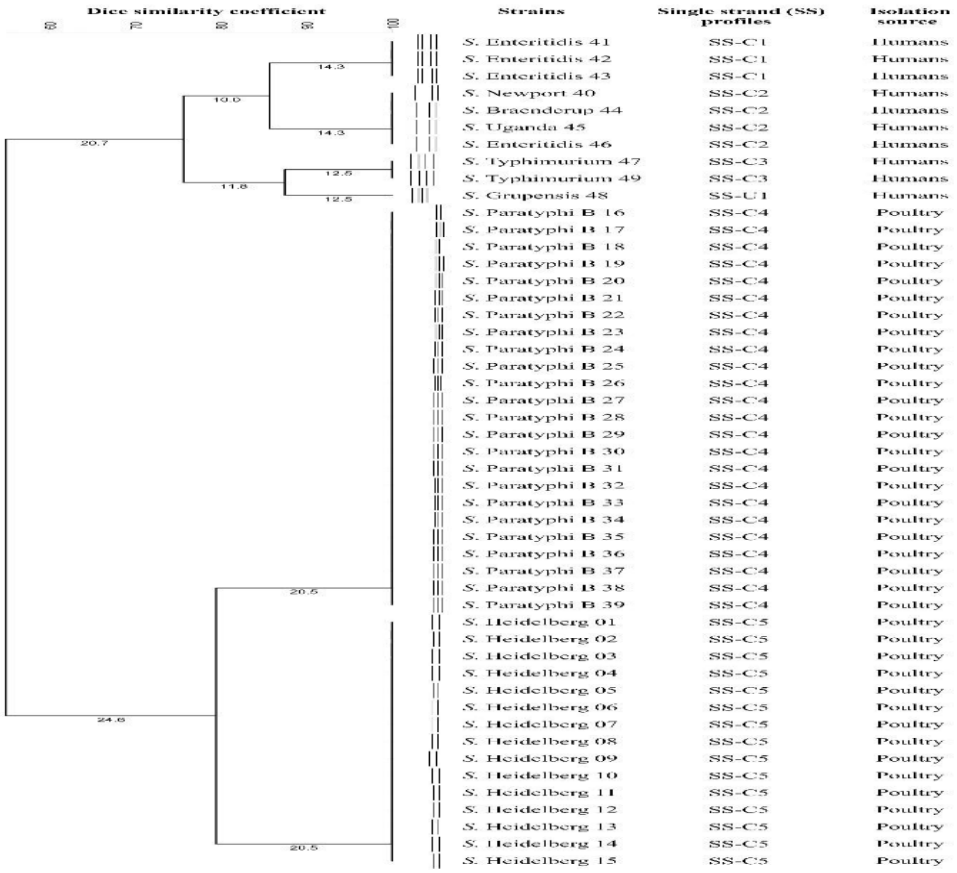


Figure 3: Dendrogram from denatured fragments of *invA* gene. The tree was generated through UPGMA and Dice coefficient.

**Sanger sequencing**

Obtained sequences were different in 62 nucleotides, which represents an identity of 89.99%. Furthermore, for *Aci*I, 5 and 13 restriction sites were found in *S*. Enteritidis isolates 43 and 46, respectively. For *Hha*I, 7 and 12 restriction sites were found for isolates 43 and 46, respectively. On the other hand, 2 *Aci*I and 7 *Hha*I restriction sites are conserved in both strains (Figure 4).

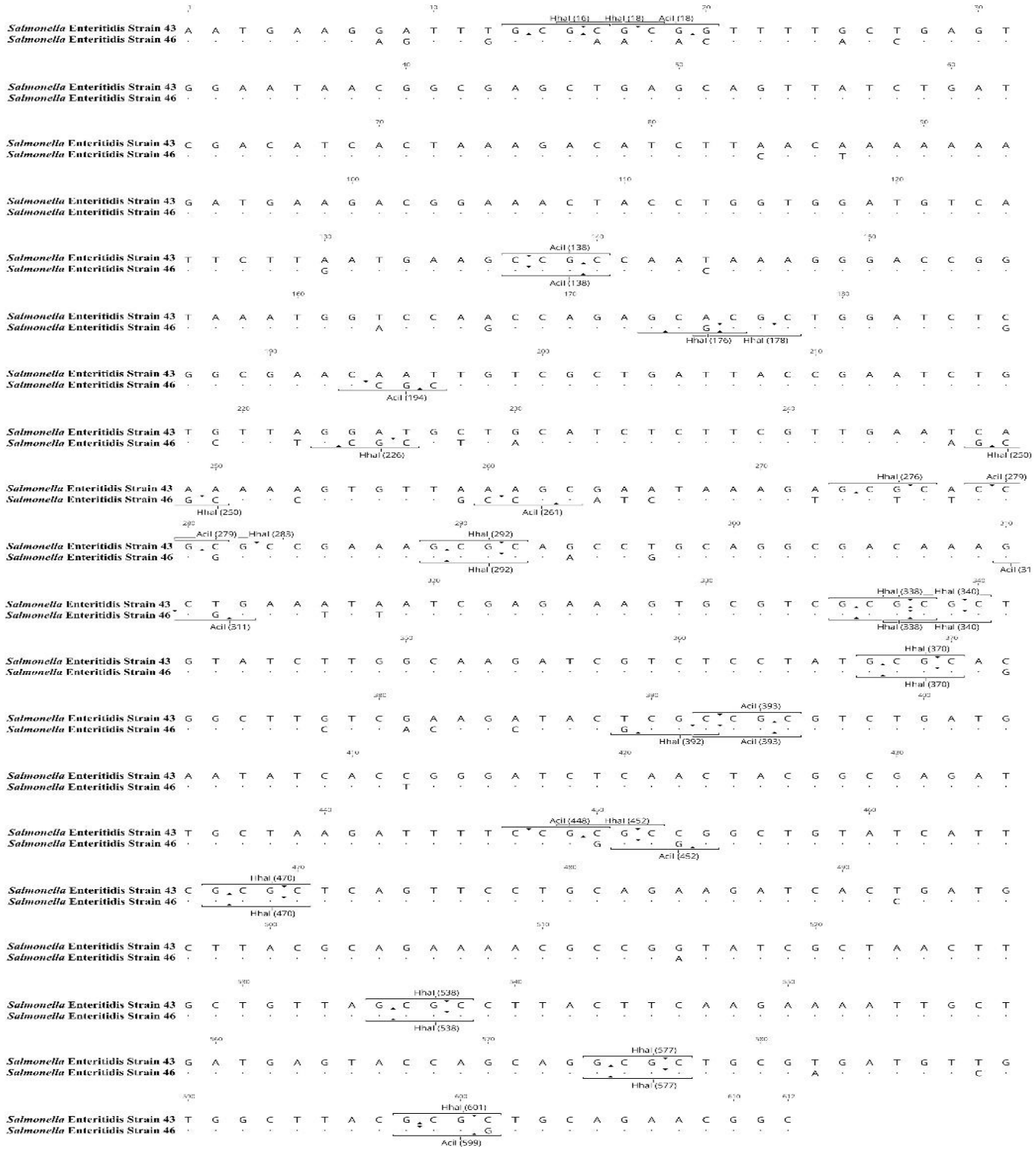


Figure 4: Pairwise alignment and restriction map of the *gnd* gene from 2 *S*. Enteritidis strains.

**Combined analysis**

*fliC-Hha*I+*fliC-Aci*I; *fliC-Hha*I+*gnd-Hha*I; *fliC-Hha*I+*groEL-Hha*I and *fliC-Aci*I+*groEL-Hha*I combined analyses yielded a value of 0.6726 and generated dendrograms with 9 clusters and one branch for each serotype, excluding *S*. Enteritidis, which were grouped in 2 branches. SSCP *invA*+*fliC-Hha*I yielded a 0.6726 DP value and a dendrogram with 9 clusters; finally, by combining the three techniques as follows, *fliC-Hha*I+*fliC-Aci*I+*gnd-Hha*I+*gnd-Aci*I+*groEL-Hha*I+SSCP *invA*+SSCP *groEL-Pst*I+ribotyping, 12 genetic profiles were generated, and a DP of 0.8571 was described (Table 2).

**Discussion**

*fliC* gene encodes for phase 1 flagellin, and it has been previously reported as present throughout the whole *Salmonella* genus (12). Furthermore, this gene has a hypervariable central region flanked by two conserved regions at 5 and 3 ends, representing suitable regions for primers annealing (7). In addition, the PCR-RFLP technique using this gene and *Hha*I enzyme allowed the differentiation of 6 out of 8 serotypes due to its lack of discrimination between *S*. Heidelberg and *S*. Typhimurium. Nonetheless, *S*. Braenderup and *S*. Newport isolates were differentiated as opposed to the reported by (7).

On the other hand, *the gnd* gene encodes the 6-phosphogluconate dehydrogenase enzyme, which belongs to the pentose phosphate pathway (15). RFLP fingerprints generated with *gnd* gene and *Hha*I were capable of distinguishing between *S*. Heidelberg, *S*. Typhimurium, *S*. Grupensis, and *S*. Enteritidis, which agrees with the *in silico* analyses, while *S*. Paratyphi B, *S*. Newport, *S*. Braenderup, and *S*. Uganda were not experimentally discriminated, which is opposite to bioinformatic analyses. This methodology yielded a DP of 0.6088. Moreover, the cleavage of the *gnd* gene with the *Aci*I enzyme yielded a DP of 0.6726, representing a low value as stated by (16). However, it was the only RFLP methodology to assign one specific pattern to each serotype, the highest type ability of all RFLP methods.

Additionally, *S*. Grupensis, *S*. Typhimurium, and *S*. Heidelberg's distinctive genetic profiles obtained through *groEL-Hha*I and the intraserovar differentiation within *S. enteritidis* strains could be explained variable regions distributed intermittently between the conserved zones in *the groEL* gene (17). The patterns generated by cleaving the *groEL* gene with *Hha*I described a DP of 0.6446. Finally, all of the PCR-RFLP methods were able to describe an intraserovar differentiation within *S*. Enteritidis isolates, and their DP was less than 0.9 (16), represents low values, cannot be described as suitable genotyping methods.

Interserovar differences for the eight serotypes analyzed and intraserovar differentiation observed only with this method for *S*. Heidelberg and *S*. Paratyphi B could be due to point mutations and insertion/deletions (indels) of more significant segments (18) in the multiple copies of ISR. In the same way, previous studies have reported that ISR is polymorphic among some *Salmonella enterica* serotypes (19). Under our conditions, a DP of 0.8571 for PCR-ribotyping was calculated. It can be considered close to optimal for genotyping techniques (16,19), who reported a DP of 0.167. It is essential to highlight that (19) only amplified 16S-23S ISR and did not perform a restriction digest step. Additionally, we obtained intraserovar differentiation for the 4 *S*. Enteritidis strains (18), who described a shared pattern for 41 *S*. Enteritidis strains. However, the methodology used by these authors does not include an enzymatic digestion step, suggesting that enzymatic restriction with two enzymes can increase the DP of this method.

*Salmonella enterica invA* gene encodes for the invasion protein A, which has a vital role in the bacterial binding to the intestinal epithelium during infection. Therefore, this gene is highly conserved, is also described as specific for *the Salmonella* genus, and is used to identify several serotypes (14). In this study, a 284 bp fragment of *the invA* gene was amplified in all strains before denaturation. Using this method, we identified a DP of 0.6675, which differs from the DP of 0.799 (19). Furthermore, our DP results could be considered lower than the fair values for genotyping methods (16). Additionally, (14) described a correlation between the different PCR-SSCP profiles and the variations found by sequencing the *invA* gene from several *Salmonella* isolates. However, the PCR-SSCP patterns obtained with this gene could not identify a specific profile for each serotype, although the strains were grouped according to the isolation source.

On the other hand, being the PCR-SSCP sensitivity of up to 89% in amplicons shorter than 450 bp (20), the *groEL* gene PCR products were subjected to enzymatic restriction with *Pst*I endonuclease in order to obtain suitable length fragments before the denaturation step. The denatured fragments from *S*. Typhimurium isolates showed the same pattern, while two band patterns were generated for the 4 *S*. Enteritidis isolates, describing intraserovar differentiation for this serotype (21), who performed enzymatic digestion of a 1.6 kb fragment of *groEL* gene with *Hae*III endonuclease, describing three profiles among 11 *S*. Enteritidis strains one shared profile for 5 *S*. Typhimurium isolates. The DP value of our methodology was 0.6726, with nine patterns for the eight serotypes.

On the other hand, the PCR-RFLP results indicated no different *Pst*I restriction sites on the *groEl* gene of the studied strains. In contrast, PCR-SSCP showed that the nucleotide composition of this gene is heterogeneous within the strains, which generated different single-stranded patterns and inter-and intraserovar differentiation. Moreover, this method generated nine profiles for the eight serotypes and inter-and intraserovar differentiation, equal to the PCR-RFLP results from the *gnd* gene cleaved with *Aci*I, both methods yielding the same typeability percentage and

DP. Finally, the PCR-SSCP of *the invA* gene required less time to be carried out than the other techniques.

In general, combined analysis with PCR-RFLP increased the DP compared to the individual methods, which agrees with (22), who suggested that more than two genes and restriction enzymes could increase the heterogeneity of genetic profiles, thereby improving the DP. In the same way, the combination of several genotyping methods may increase the discrimination of *Salmonella enterica* serotypes (23), which agrees with our results of SSCP *invA+fliC-Hha*I, but it is opposite to the composite analysis with the three methods. Additionally, none of the combinations yielded a DP or genetic profile number higher than PCR-ribotyping, PCR-RFLP of *gnd* gene with *Aci*I enzyme, or *groEL-Pst*I PCR-SSCP as individual methods. *Salmonella* Enteritidis differentiation with all methods. The *Salmonella* Typhimurium homogeneity found in this study contrasts with the serotypes data in Colombia since *S*. Enteritidis has been reported as a clonal group, while *S*. Typhimurium is a highly genetically diverse serotype (24). Noteworthily, the profiles assigned by the three genotyping methods and their combinations were able to describe a specific fingerprint for each serotype, agreeing with the previous serotyping of the isolates, and allowed us to describe intraserovar differentiations that are not perceivable with serotyping. Similarly, PCR-based genotyping methods have been previously used to differentiate *Salmonella enterica* strains at and below serotype level (7,25). However, our purpose is not to replace but to complement the traditional typing methods for *Salmonella enterica* to provide valuable data regarding the relationships within isolates, information that can be used in epidemiological surveillance.

## Conclusion

Our study showed three PCR-based genotyping methods as tools for *Salmonella enterica* inter-and intraserovar discrimination, generating clusters according to the different geographical origins and isolation sources.

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## Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

## Author's contributions

Cruz-Méndez JS and Ortiz-Muñoz JD have equal authors contribution.

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# التوصيف الجزيئي لأنواع السالمونيلا المعوية في الحيوانات والانسان باستخدام ثلاثة تقنيات جزيئية

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**الخلاصة**

تهدف هذه الدراسة إلى توصيف جزيئي لمختلف أنواع السالمونيلا المعوية باستخدام مؤشر تباين أطوال قطع التقييد ومؤشر التنميط الرايبي ومؤشر تباين أطوال الشريط المفرد للدنا لتقنية تفاعل البلمرة المتسلسل . عزلت السلالات المعوية (عددها = ٤٩ ) من حالات التهاب الجهاز الهضمي البشري في منطقة توليما وكذلك من الحالات المرضية للدواجن في منطقة سانتاندير وتوليما في كولومبيا . تم تحليل أنماط الحزم التي تم الحصول عليها من كل مؤشرعن طريق بناء مخططات شجرية على طريقة مجموعة الأزواج غير الموزونة بالمتوسط الحسابي وباستخدام معامل النرد للحصول على ارقام عشوائية . من ناحية أخرى ، تم تقييم القوة التمييزية لكل مؤشرباستخدام معامل سيمبسون التمييزي . اظهرت الصورة الجينية لجين gnd التي تم الحصول عليها باستخدام إنزيم التقييد AciI و مؤشرتباين أطوال الشريط المفرد للدنا لتقنية تفاعل البلمرة المتسلسل باستخدام جين groEL والتي سمحت بالتمايز بين وداخل الانماط المصلية . أخيرًا ، أظهرمؤشر التنميط الرايبي لتقنية تفاعل البلمرة المتسلسل أعلى قوة تمييزية (٠,٨٥٧١). نستنتج من هذه الدراسة بان هناك ثلاث مؤشرات للتنميط الجيني المعتمدة على تقنية تفاعل البلمرة المتسلسل والتي توفر بديلاً لتحديد أوجه التشابه والاختلاف ضمن سلالات السالمونيلا المعوية المأخوذة من مناطق جغرافية وبيولوجية مختلفة.

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