Molecular identification of fluoroquinolone resistance in Salmonella spp. isolated from broiler farms and human samples obtained from two regions in Colombia

Maria Paula Herrera-Sánchez1, Rafael Enrique Castro-Vargas1,2, Luz Clemencia Fandiño-de-Rubio1,2, Roy Rodríguez-Hernández1,2 and Iang Schroniltgen Rondón-Barragán1,2

1. Research Group in Immunobiology and Pathogenesis, Laboratory of Immunology and Molecular Biology, Faculty of Veterinary Medicine and Zootechnics, University of Tolima, Santa Helena Highs, Ibagué 730006299, Tolima, Colombia; 2. Poultry Research Group, Laboratory of Immunology and Molecular Biology, Faculty of Veterinary Medicine and Zootechnics, University of Tolima, Santa Helena Highs, Ibagué 730006299, Tolima, Colombia.

Corresponding author: Iang Schroniltgen Rondón-Barragán, e-mail: isrondon@ut.edu.co
Co-authors: MPH: mpherreras@ut.edu.co, REC: recastrov@ut.edu.co, LCF: lfandino@ut.edu.co, RR: rayrodriguezh@ut.edu.co

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Abstract

Background and Aim: Salmonella is one of the most common foodborne pathogens, the emergence of antibiotic-resistant strains of which is increasing. The aim of this study was to phenotypically and genotypically characterize the fluoroquinolone resistance of Salmonella isolates from broiler and humans in two regions of Colombia.

Materials and Methods: Salmonella strains (n=49) were evaluated. The phenotype of antibiotic resistance was assessed by an automated method and agar diffusion method, as well as the presence of the quinolone resistance genes qnrA, qnrB, qnrC, qnrD, qnrS, and aac(6’)-Ib as determined by polymerase chain reaction.

Results: Strains were resistant to ciprofloxacin (75%), levofloxacin (57.1%), and enrofloxacin (38.8%). Molecular identification showed that 24 out of 49 strains possessed the qnrB gene (48.9%), while only one isolate from the Santander region possessed the aac(6’)-Ib gene. Regarding Class 1 integron, it was present in 11 out of the 49 strains (22.44%).

Conclusion: This study reports the presence of the gene qnrB as well the presence of Class 1 integrons in broiler Salmonella isolates, which may contribute to the resistance to fluoroquinolones.

Keywords: antibiotic resistance, broiler, resistance genes, Salmonella.

Introduction

Salmonella spp. is one of the most common foodborne pathogens globally and has a major impact on public health [1,2]. Salmonella enterica comprises a diverse group classified according to their antigens; some serotypes can cause major systemic infections [1]. In the case of humans, salmonellosis can induce different clinical conditions varying widely in their severity, such as typhoid fever, paratyphoid fever, septicemia, and gastroenteritis, especially in immunocompromised individuals such as children and the elderly [3]. In terms of global numbers, Salmonella spp. is responsible for 93.8 million human infections and 155,000 deaths annually [4]. In Colombia between 1997 and 2017, the National Institute of Health reported Salmonella spp. strains isolated from 12,055 human samples [5]. Salmonella is frequently transmitted to humans by the consumption of food of animal origin, especially avian products such as eggs and chicken meat [2,6]. As an aggravating factor, most clinical isolates have been reported to be multi-drug-resistant, associated with the inappropriate use of antibiotics such as enrofloxacin as growth promoters in animal production [7,8].

At present, quinolones and fluoroquinolones are prescribed as the first therapeutic option for patients with acute bacterial diarrhea, but Salmonella has been increasingly developing resistance to this family of antibiotics [2,3]. The genetic basis of resistance to fluoroquinolones and quinolones in Salmonella spp. is mediated mainly by mutations in DNA gyrase and topoisomerase IV, which are the target structures of these antibiotics. In addition, modifications in the permeability of the external membrane, frequently transmitted by plasmids (plasmid-mediated quinolone resistance [PMQR]), and efflux bombs can confer resistance; this indicates that several biochemical mechanisms are involved in the resistance to quinolones and fluoroquinolones [9].

Our research group has reported the antibiotic resistance patterns of Salmonella spp. in poultry...
production as well as in other sectors in Colombia, as characterized by different genotyping methods [10-15]. However, these studies did not address the molecular mechanisms associated with resistance to quinolones and fluoroquinolones. The present study aims to characterize the phenotype behind resistance and the resistance genes qnrA, qnrB, qnrC, qnrD, qnrS, and aac(6')-Ib, which mediate antimicrobial resistance to fluoroquinolones, in Salmonella isolates from poultry and humans from the Colombian regions of Tolima and Santander.

Materials and Methods

Ethical approval

No ethical approval was required for this study because Salmonella spp. strains were from the Bacterial Strain Collection of the Laboratory of Immunology and Molecular Biology of the Universidad del Tolima, which were obtained from previous research projects made by the Poultry Research Group of the Universidad del Tolima and they were approved by Bioethics Committee of the Central Office of Research from Universidad del Tolima and complied with the guidelines for animal care and use in research and teaching [11,16].

Study period and location

This study was carried out from August 2018 to May 2019. Microbiological culture and molecular experiments were done at Laboratory of Immunology and Molecular Biology of the University of Tolima. In addition, Antibiotic resistance assay was done at Tolima Clinic.

Salmonella spp. strains

A total of 49 strains of Salmonella spp. isolated from broiler farms and humans were included in this study. Overall, 15 strains were serotyped as Salmonella Heidelberg isolated from broiler farms in Santander; 24 strains were serotyped as Salmonella Paratyphi B isolated from broiler farms in Tolima; while 10 were serotyped as Newport (n=1), Enteritidis (n=4), Braenderup (n=1), Uganda (n=1), Typhimurium (n=2), and Grupensis (n=1) isolated from humans with gastroenteritis in Ibagué-Tolima.

Phenotypic resistance

The phenotypic resistance to ciprofloxacin and levofloxacin was determined using an automated MicroScan system (Beckman Coulter, Porterville, CA, USA) and BD Phoenix NMIC/ID-94 (Becton Dickinson, Franklin Lakes, NJ, USA) through the minimum inhibitory concentration (MIC) method following the recommendations of the CLSI [17]. The resistance to enrofloxacin (5 μg) was determined by the Kirby–Bauer disk diffusion susceptibility test. A bacterial suspension was spread in Mueller-Hinton agar (Oxoid, Wesel, Germany) according to the McFarland turbidity scale of 0.5; then, bacterial growth inhibition was evaluated at 37°C for 18 h according to the CLSI guidelines [17].

Genomic DNA (gDNA) extraction

gDNA was extracted from fresh colonies using the Invisorb® Spin Universal Kit (Stratec, Berlin, Germany); then, the samples were dissolved in 50 μL of TE buffer and maintained at −20°C until further use. In addition, all isolates were confirmed by polymerase chain reaction (PCR) through amplification of the invA gene using the following primers, forward 5′-TGAAATATTACGCCACGTTCGGCGAAC-3′ and reverse 5′-TCATCGACCGTCGCAAAGGACG-3′, with an amplicon size of 284 bp [10]. S. enterica ATCC® 13076 strain (ATCC, Manassas, VA, USA) was used as a positive control. The reaction was carried out in a total volume of 25 μL, composed of 14.87 μL of distilled-deionized water, 5 μL of 5× colorless GoTaq® Flexi Buffer (Promega, Madison, WI, USA), 1 μL of dNTPs (1.5 mM) (Invitrogen, Waltham, MA, USA), 1 μL of each primer (forward and reverse) (10 pmol/μL), 1 μL of MgCl2 (25 mM), 0.125 μL of GoTaq® Flexi DNA polymerase (Promega), and 1 μL of gDNA as a template. The amplification was carried out in a T100 thermocycler (Bio-Rad, Hercules, CA, USA) with an initial denaturation step at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 30 s, and a final extension step at 72°C for 7 min. Amplicons were revealed on 2% agarose gel by electrophoresis (PowerPac™ HC, Bio-Rad, Hercules) using the 100 bp DNA ladder Load Ready™ (Amplysus, Cambridge, MA, USA). The gel was stained with HydraGreen™ (ACTGene, Piscataway, NJ, USA) and visualized under UV light using the ENDURO™ GDS gel documentation system (Labnet International, Inc., Edison, NJ, USA).

PMQR and Class 1 integron detection

For PMQR and Class 1 integron detection, gDNA from isolates was used as a template for the reaction, using gene-specific primer sets (Table-1) [18-22]. PCR conditions were as described above and the annealing temperature was adjusted depending on the melting temperature of each primer set.

Results

Phenotypic resistance to antibiotics

Enrofloxacin resistance was present in 19/49 isolates (38.8%), while 75% of the isolates showed resistance to ciprofloxacin (36/49). In the case of levofloxacin, among 28 isolates tested for this antibiotic, 57.1% (16/28) were resistant. None of the strains isolated from humans showed resistance to these three antibiotics (Table-2).

PMQR and Class 1 integron detection

The qnrA, qnrC, qnrD, and qnrS genes were not detected in any of Salmonella spp. isolates. In contrast, 49% of the samples were positive for the qnrB gene (24/49), which were distributed in two serotypes belonging to S. Heidelberg from broiler farms in Santander (n=3) and S. Paratyphi B from broiler
farms in Tolima (n=21) (Table-2; Figure-1). In addition, one S. Heidelberg was positive for the aac(6′)-Ib gene (Figure-2), and the Class 1 integron was present in 11 strains isolated from Santander broiler farms (Table-2 and Figure-3).

**Discussion**

Quinolones and fluoroquinolones have great importance due to their use in both human medicine and veterinary medicine all over the world. These groups of antibiotics are classified as being of the “highest priority” according to Critically Important Antimicrobials for Human Medicine published by the World Health Organization [23]. In terms of the criteria for settling on this classification, it is mentioned that quinolone and fluoroquinolone can be the sole or one of few available therapies to treat serious bacterial infections in people infected from non-human sources.

Regarding phenotypic resistance to members of the fluoroquinolone family, the rate of levofloxacin resistance was 57.1% (n=16/28), which is higher than that reported by Donado et al. [24] in poultry farms from Colombia, where the antibiotic resistance levels showed values of 2.3% and 0% in the regions of Cundinamarca and Santander, respectively. The antibiotic resistance found in this study was similar to that in reports on poultry meat marketed in six Colombian cities, where the rate of resistance was 44.2% [25]. Regarding ciprofloxacin, 75% of the isolates were resistant (n=36/49), which was higher than reported in poultry farms from Brazil [26]. The ciprofloxacin resistance identified in this study agrees with the results in Colombian poultry farms from the regions of Cundinamarca (56.8%) and Santander (40.9%) [24] and in chicken carcasses marketed in Ibagüé (Colombia), where the resistance was more than 42% (n=20/47) [10].

In this study, 38.8% (n=19/49) of the isolates showed resistance to enrofloxacin, which is a higher rate than reported in poultry farms in Canada (0%) [27] and Brazil, where the resistance rates ranged from 4.9% [28] to 18.2% [29]. In addition, the rate of resistance to enrofloxacin was lower than reported in two poultry-producing regions in Colombia by Donado et al. [24], where the resistance rates were 84.1% and 66.7% in the regions of Cundinamarca and Santander, respectively.

### Table-1: Primers used to evaluate the presence of PMQR genes in *Salmonella* spp. strains.

| Target gene     | Primer sequence          | Annealing temperature (°C) | Amplicon size (bp) | Reference |
|-----------------|--------------------------|----------------------------|--------------------|-----------|
| qnrA            | F- CCGCTTTTATCAGTGTGACT  | 55                         | 188                | This study |
|                 | R- ACTCTATGGCAAAAGCGTGTG |                            |                    |           |
| qnrB            | F- GATGATGGAAGGCAAGG     | 54                         | 469                | [18]      |
|                 | R- ACGATGACCCTTGTGTTGCC  |                            |                    |           |
| qnrC            | F- GGGTTTATGCTATTGATGC   | 54                         | 308                | [19]      |
|                 | R- CACCTACTCCTTTTATTT    |                            |                    |           |
| qnrD            | F- CGAGATCATTACAGGGGAA   | 57                         | 582                | [20]      |
|                 | R- AAACAGCTGAAGGCCTCG    |                            |                    |           |
| qnrS            | F- ACGACTCTGTCAAACTGCAA  | 55                         | 417                | [18]      |
|                 | R- TAAATTGACCCCTGCTAGGCC |                            |                    |           |
| aac(6′)-Ib      | F- TTGGGATGTCATGATAGGTCTA | 57                       | 482                | [21]      |
|                 | R- CTGGATGTCCTGCTGTTT    |                            |                    |           |
| Class 1 Integron (Integrase) | F- TCCAGCGATCGTCAAGC | 55                             | 280               | [22]      |
|                 | R- CCTCAGCGACGAGTAC      |                            |                    |           |

**Figure-1:** Polymerase chain reaction amplification of *qnrB* gene of seven *Salmonella* isolates. Lane C+: positive control; lane 1: *Salmonella* Heidelberg; lanes 2-7: *Salmonella* Paratyphi B; MW: 100 bp DNA Ladder (Corpogen, Colombia).

**Figure-2:** Polymerase chain reaction amplification of aac(6′)-Ib gene from *Salmonella* Heidelberg isolate. Lane C+: positive control; lane 1: *Salmonella* Heidelberg; MW: 100 bp DNA ladder (Solis BioDyne, Estonia).
In the case of genotypic resistance, the qnr genes encode proteins of the pentapeptide repeat family, which have the ability to bind to DNA gyrase and topoisomerase IV and protect them against fluoroquinolones when the antibiotic concentration is low (0.75 μM) and Qnr protein is high (2.01 μM) [30], due to the protection is inversely proportional [31,32]. Regarding these genes, qnrA, qnrB, qnrS, qnrC, and qnrD have been described [9], but in our study, only the qnrB gene was detected in 24 out of 49 strains (49%), corresponding to S. Heidelberg and S. Paratyphi B isolated from Santander and Tolima broiler farms (Table-2). Our results showed a high rate of this gene in comparison to the results obtained in isolates from chicken products and human patients in 13 European countries, where 11.35% (n=138/1215) of isolates were positive for qnrB variants [32]. This rate is also high compared with that in strains isolated from food, animals, and humans, where the gene was present in 4.7% (n=6/129) of isolates [33]. In the case of Colombia, our results are higher than reported by Karczmarczyk et al. [34] in Salmonella isolated from commercial food, where 30% of the strains (n=4/13) belonging to Salmonella Infantis and Salmonella Uganda showed the presence of the qnrB gene. The high frequency of the qnrB gene in this study agrees with the previous studies in which this gene was the most commonly found in the qnr family of Enterobacteriaceae [35].

In addition, 14 strains corresponding to S. Heidelberg and S. Paratyphi B showed phenotypic resistance to fluoroquinolone antibiotics without the presence of the PMQR gene, which may indicate that the resistance could be mediated by other genes different from those assessed in this study, but resistance to this antibiotic family can also be conferred through mutations in DNA gyrase, topoisomerase IV, or efflux pumps [31]. In contrast, one of the strains of S. Paratyphi B was not resistant to the fluoroquinolone antibiotics evaluated in this study, but was found to possess a qnrB gene. Correia et al. [36] mentioned that this could be because the PMQR genes confer low-level quinolone resistance that alone do not allow being an effective resistance mechanism.

Aminoglycoside 6’-N-acetyltransferase (AAC(6’)-Ib) belongs to subfamily C of N-acetyltransferase (AAC), whose members are all monomeric enzymes [37]. AAC is known to catalyze the addition of an acetyl group from AcCoA to the 6’-N of aminoglycosides, disrupting the crucial electrostatic and
hydrogen bonding interactions [38,39]. In our study, the $aac(6')$-$Ib$ gene was found only in one isolate ($n=1/49$) of S. Heidelberg from Santander broiler farms (Table-2). This prevalence is low with that compared in a report from Brazil [40], where 23 out of 129 strains showed the presence of the gene. The isolate that carried the $aac(6')$-$Ib$ gene was phenotypically resistant to levofloxacin and ciprofloxacin. Vetting et al. [41] showed that the $aac(6')$-$Ib-cr$ variant of this gene can confer resistance to fluoroquinolones, such as ciprofloxacin and levofloxacin, and this resistance can be related to stimulation of chromosomal mutations of the fluoroquinolones’ targets, topoisomerase IV, and DNA gyrase [42].

Class 1 integron is an important mobile element with a role in antibiotic resistance of bacteria [43]. It has been reported in Salmonella spp. isolates derived from poultry and other sources [44-46]. In this study, Class 1 integron was found in 11 out of the 49 strains (22.44%), which is a higher rate than reported in poultry farms in Uganda, where six out of 54 isolates showed the presence of the gene (11%) [44]; in chicken farms in Egypt, where 4.4% of S. Typhimurium strains were positive for this gene ($n=3/67$) [45]; and in Morocco, where one out of 26 isolates carried the integron (3.84%), corresponding to S. Infantis isolated from turkey meat [46]. These findings raise concerns about Class 1 integrons contributing to the acquisition and propagation of resistance genes in different serotypes, such as S. Typhimurium, S. Enteritidis, and S. Heidelberg [47].

Conclusion

This study reports the presence of the $qnrB$ gene in S. Heidelberg and S. Paratyphi B strains isolated from broiler farms in Santander and Tolima, Colombia, which may contribute to resistance to fluoroquinolones. In addition, the presence of mobile elements such as Class 1 integrons may contribute to the dissemination of resistance genes between strains.

Authors’ Contributions

ISR and MPH: Study design. MPH: Performed the experiments, the laboratory analyses. LCF: Responsible for the phenotypic resistance experiments. ISR: Administered the project. REC, LCF, and RR: Collected the strains. ISR and MPH: Wrote the manuscript. ISR, MPH, REC, and RR: Reviewed and edited the paper. ISR: Revised the manuscript critically. All authors read and approved the final manuscript.

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Competing Interests

The authors declare that they have no competing interests.

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