Brazilian Journal of Poultry Science
Revista Brasileira de Ciência Avícola
ISSN 1516-635X Apr - Jun 2014 / v.16 / n.2 / 93-96
http://dx.doi.org/10.1590/1516-635x160293-96

Phenotypic and Genotypic Resistance Profile of Salmonella Typhimurium to Antimicrobials Commonly Used in Poultry

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■Keywords

Antimicrobial, gene, resistance, *Salmonella*.

Submitted: May/2013
Approved: February/2014

ABSTRACT

Isolates of *Salmonella* sp (104) from poultry samples were isolated and serotyped where eleven were identified as *Salmonella* Typhimurium (ST). ST isolates were phenotypically tested by disk diffusion and minimum inhibitory concentration (MIC). Four genes related to enrofloxacin (GyrA, GyrB, ParC and ParE), two to gentamicin (AadA and AadB) and two to ceftiofur (BlaCMY-2 and AmpC) resistance were searched by PCR. Our results showed ST resistance to all three antibiotics tested (18.1% for ceftiofur, 45.4% for gentamicin, and 18.1% for enrofloxacin) according to the diffusion test. In the MIC test, the ST isolates showed higher levels of resistance (27.2% for ceftiofur, 54.5% for gentamicin, and 18.2% for enrofloxacin). Three resistance genes out of four searched genes for enrofloxacin were found in the ST isolates. Regarding gentamicin and ceftiofur, resistance genes were found mainly in samples with resistant phenotypic profile. Interestingly, some phenotypically-resistant strains did not present the resistance gene, which suggests an alternative route of resistance. Also, sensitive strains had the presence of the gene. It is possible to conclude that the ST isolates evaluated in this study have a multidrug resistance profile to the antibiotics routinely used in poultry production, and potential of greater levels of resistance in the near future.

INTRODUCTION

Salmonellosis is considered a common cause of foodborne illnesses in humans, representing a significant public health problem in many countries. Studies show that poultry products have been recognized as a major transmitter of these bacteria, taking an important role in disease control (Carrasco *et al*., 2012).

Due to the increasing incidence of infections by *Salmonella* sp and frequent reports of multiresistant strains, it is necessary to investigate the mechanisms of resistance used by this microorganism. According to Guerra *et al.* (2000), the spread of genes that confer resistance to microorganisms is due to the indiscriminate use of antibiotics in veterinary medicine. Those authors found that 31% of the isolates tested were resistant to all antimicrobials tested, and the species with the highest resistance was *S*. Typhimurium.

The main objective of this study was to investigate the genotypic and phenotypic antimicrobial resistance profile to gentamicin, ceftiofur and enrofloxacin of *S.* Typhimurium of poultry origin.

MATERIALS AND METHODS

Isolation

Salmonella sp isolates (104 samples) of poultry origin from Brazil were obtained from a private and accredited laboratory. These isolates

had various sources, such as cloacal swabs, drag swabs, poultry houses, shipping boxes, chicks, fertile eggs, meconium, feces, organs, feed ingredients, and poultry feed. These isolates were serotyped at Osvaldo Cruz Foundation (FIOCRUZ)) and an aliquot of these samples was frozen in brain heart infusion (BHI) and glycerol for further use.

Disk diffusion test

The antimicrobials used to verify *S*. Typhimurium susceptibility are some of the most commonly used in the poultry industry: enrofloxacin (5 mg), gentamicin (10 mg) and ceftiofur (30 µg).

Disk diffusion test was performed according to the methodology approved by the NCCLS (National Committee for Clinical Laboratory Standards, USA) and ANVISA (Brazilian National Health Surveillance Agency) (Brasil, 2003).

Minimum Inhibitory Concentration (MIC)

MIC tests were carried out according to the Normative M-2 A-8 (Brasil, 2003). Dosages were those recommended by the manufacturers of the antimicrobial drugs: 10 mg/kg for enrofloxacin, and 5 mg/kg for ceftiofur and gentamicin.

Polymerase Chain Reaction (PCR)

DNA extraction was performed by the boiling-centrifugation technique, as described by Borsoi *et al*. (2009). DNA samples were then stored at -20 °C.

Four genes related to enrofloxacin (GyrA, GyrB, ParC and ParE), two to gentamicin (AadA and AadB) and two to ceftiofur (BlaCMY-2 and AmpC) resistance were searched by PCR on all eleven *S*. Typhimurium samples using primers and previously established protocols (Table 1).

STATISTICAL ANALYSIS

All results were statistically tested by Fisher exact test at 5% significance level.

RESULTS

Disk diffusion test and Minimum Inhibitory Concentration (MIC)

There was resistance to all three antibiotics tested (18.1% ceftiofur, 45.4% gentamicin, and 18.1% enrofloxacin) according to the disk diffusion test. However, the isolates of *S*. Typhimurium showed higher levels of resistance: ceftiofur (27.2%), gentamicin (54.5%), and enrofloxacin (18.2%) in the MIC test. All susceptibility results for both methodologies tested are described on Table 2.

Table 2 – *S.* Typhimurium phenotypic profiles determined by disk diffusion tests and MIC to antimicrobials commonly used in poultry.

	Disk diffusion test		MIC	
	S (%)	R (%)	S (%)	R (%)
Ceftiofur	9 (81,8)	2 (18,1)	8 (72,7)	3 (27,2)
Gentamicin	6 (54,5)	5 (45,4)	5 (45,4)	6 (54,5)
Enrofloxacin	9 (81,8)	2 (18,1)	9 (81,8)	2 (18,2)

S - Sensitive, R - Resistant

Statistical analyses using Fisher exact test for enrofloxacin and ceftiofur showed that the presence or absence of a resistance gene does not interfere with the phenotypic response, meaning that phenotype and genotype are independent variables. On the other hand, gentamicin results analyzed also by Fischer exact test demonstrated dependence between phenotype and genotype.

Table 1 - Resistance genes to each antibiotic tested, specific primer sequence, gene size and references used.

Resistance genes	Primers	Size (pb)	Reference
GyrA	5'-CGTTGGTGACGTAATCGG-3'(F) 5'-CCGTACCGTCATAGTTAT-3'(R)	251	Randall *et a*l. (2005)
GyrB	5'-GCGCTGTCCGAACTGTACCT-3'(F) 5'-CGGTGATCAGCGTCGCCACTTCC-3' (R)	181	Eaves *et al*. (2004)
ParC	5'-CTATGCGATGTCAGAGCTGG-3'(F) 5'-TAACAGCAGCTCGGCGTATT-3'(R)	260	Randall *et al*. (2005)
ParE	5'-TCTCTTCCGATGAAGTGCTG-3'(F) 5'-ATACGGTATAGCGGCGGTAG-3'(R)	237	Randall *et al*. (2005)
AadA	5'- GTGGATGGCGGCCTGAAGCC-3'(F) 5'- ATTGCCCAGTCGGCAGCG-3'(R)	526	Ribeiro *et al.* (2011)
AadB	5'- TCCAGAACCTTGACCGAAC-3'(F) 5'- GCAAGACCTCAACCTTTTCC-3'(R)	700	Ribeiro *et al*. (2011)
BlaCMY-2	5'-TGGCC GAACTGACAGGCAAA-3'(F) 5'-TTTCTCCTGAACGTGGCTGGC-3'(R)	354	Alcaine *et al*. (2005)
AmpC	5'-AACACACTGATTGCGTCTGAC-3'(F) 5'-CTGGGCCTCATCGTCAGTTA-3'(R)	1226	Alcaine *et al*. (2005); Pérez-Pérez & Hanson (2002)

PCR

All enrofloxaxin-sensitive ST strains showed at least three out of the four search genes. We were unable to find AadA and AadB gentamicin genes in two resistant ST strains. On the other hand, as expected, all gentamicin-sensitive strains lacked both genes. Resistance genes for ceftiofur (AmpC and BlaCMY) were found in samples presenting both resistant and sensitive phenotypic profile. All results obtained from the genotypic isolates of *S*. Typhimurium are shown in Table 3.

DISCUSSION

The emergence of antimicrobial resistance in zoonotic bacteria has important implications for public health. Data from several researchers suggest that the indiscriminate use of antimicrobials can lead to resistance of several bacteria that, which can reach consumers through products of animal origin (Ribeiro *et al*., 2011).

A third-generation cephalosporin is used to treat animals infected with *Salmonella* sp as well as humans, especially children (Frye & Cray, 2007). These authors reported a growing global concern due to the emergence of multidrug-resistant strains, and isolates of *S*. Typhimurium accounted for 23.5% of the observed resistance to ceftiofur with values very close to those found in this study.

The genetic element responsible for most of the resistance to ceftiofur in *Salmonella* sp isolated from animals in the USA seems to be related to the BlaCMY gene (Frye & Cray, 2007). This was also reported by Alcaine *et al*. (2005), who observed that nineteen resistant Salmonella isolates carried the ceftiofur gene BlaCMY. Studies conducted by Frye & Cray (2007) reported that 17% of resistant strains did not have the BlaCMY gene or some other β-lactamase resistant genes detected by PCR, raising a concern that other mechanisms are associated to ceftiofur resistance. Our results, however, showed that all ceftiofur resistant strains carried at least one of the resistant genes.

Studies by Peirano *et al*. (2006) in Brazil showed that the number of *Salmonella* sp isolates resistant to ceftiofur was 16.3%, out of which only 13.6% had the BlaCMY gene, indicating that some other genes that may also be responsible for resistance.

ST samples with a sensitive phenotype and the presence of a resistance gene may be explained by the study of Alberts (2004), who suggested the possibility that the gene may not be expressed at the time of the analysis.

Our results for gentamicin resistance in disk diffusion test (45.4%) and in MIC (54.5%) differ from those found by Medeiros (2006), who worked mainly with animal and food samples (12.4%). It was not possible to detect a resistance gene in one ST sample with resistant phenotype for gentamicin. Ribeiro *et al*. (2011) and Peirano *et al*. (2006) also reported that it was not always possible to correlate phenotype and genotype. The absence of the gene in isolates showing phenotypic resistance suggested that there are other mechanisms related to resistance, warranting further research.

ST samples showed 18% resistance to enrofloxacin, confirming multidrug resistance to all three antibiotics

Table 3 – Presence of antimicrobial resistance genes to enrofloxacin, ceftiofur, and gentamicin in *S*. Typhimurium isolates.

Source/State	ENROFLOXACIN						GENTAMICIN				CEFTIOFUR			
	MIC	ATB	Gene ParC	Gene ParE	Gene GyrA	Gene GyrB	MIC	ATB	Gene AadA	Gene AadB	MIC	ATB	Gene AmpC	Gene BlaCMY
FB/SC	S	S	P	P	P	P	S	S	N	N	S	S	P	N
SA/PR	S	S	P	P	P	P	R	R	N	P	R	R	P	N
SA/PR	S	S	P	P	P	P	R	R	N	N	S	S	N	N
SA/PR	S	S	P	N	P	P	R	R	P	N	S	S	N	N
SA/PR	S	S	P	P	P	P	R	R	P	N	S	S	N	P
SA/PR	S	S	P	P	P	P	S	S	N	N	S	S	N	P
SA/PR	S	S	N	P	P	P	S	S	N	N	S	S	P	N
SA/PR	S	S	P	P	P	P	R	R	P	N	R	R	P	N
SC/PR	R	R	P	P	P	P	S	S	N	N	R	S	P	P
SA/SC	S	S	P	P	N	P	R	S	N	N	S	S	P	N
SA/PR	R	R	P	P	P	P	S	S	N	N	S	S	N	N

FB - feed for breeders, SA - drag-swab, SC - cloacal swab; SC - Santa Catarina, PR - Paraná; S - Sensitive R - Resistant; P - gene present; N - No gene

tested in most of the samples. Interestingly, all samples presented resistance genes to enrofloxacin, independently of their phenotype, showing their genetic potential to become resistant in the future.

Borsoi *et al*. (2005) and Ribeiro *et al*. (2011) found high resistance values for enrofloxacin (69.2% and 15%, respectively) in *Salmonella* sp samples. It appears that mutations in the GyrA gene may be responsible for the resistance to fluoroquinolones. In contrast, San Martin *et al*. (2005), evaluating 39 samples of *Salmonella* sp, found no resistance to enrofloxacin. According to these authors, phenotypic resistance to this antibiotic only occurs when there are double-single point mutations in the GyrA gene, which may explain why the isolates evaluated in the present study presented the GyrA gene, but did not express it phenotypically.

CONCLUSIONS

Our study showed that tested ST isolates were resistant to three antibiotics evaluated; however, the most significant resistance was observed relative to ceftiofur and gentamicin. The use of molecular tests is important because it shows the future antimicrobial resistance profile. In this study, we observed that most isolates presented the genes of resistance, although these were not being expressed yet, demonstrating the future potential for these strains to become resistant to the evaluated antimicrobial agents.

ACKNOWLEDGEMENTS

We would like to thank FIOCRUZ (Osvaldo Cruz Foundation), CEDIMA (Animal Microbiological Diagnostic Center - CAV/UDESC), Mercolab Laboratories, and Laboratory for Hemoparasites and Vectors (CAV/UDESC).

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