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***Salmonella* Heidelberg resistant to ceftiofur and disinfectants routinely used in poultry**

***Salmonella* Heidelberg resistente ao ceftiofur e desinfetantes rotineiramente utilizados na avicultura**

Lenita Moura Stefani^{1*}; Gabriella Bassi das Neves²; Maiara Cristiane Brisola³; Regiane Boaretto Crecencio³; Eduarda Caroline Pick³; Denise Nunes Araujo³

Abstract

Bacteria of the genus *Salmonella* may infect humans and domestic animals, causing a serious public health problem worldwide. Nowadays, *Salmonella enterica* serovar Heidelberg (SH) is among the top three serovars isolated from people with salmonellosis and it is present in the poultry production chain. Moreover, it seems to be more invasive than other serotypes that cause enteritis. The overall status of the antimicrobial resistance of Brazilian strains of SH is still unknown. The bacterium may use similar mechanisms of resistance to antibiotics, as well as disinfectants such as the efflux system and enzymatic degradation of chemical compounds. Thus, the objective of this study was to identify the Minimal Inhibitory Concentration (MIC) for ceftiofur of SH isolated from different materials of poultry origin, as well as to verify the relation between antibiotic resistance and disinfectant resistance. In addition, the screening efflux system was performed, using ethidium bromide to determine the presence of this mechanism of resistance. MIC results indicated high levels of SH resistance to ceftiofur, indicating the need for alternative drugs to treat salmonellosis. The concentration of ceftiofur needed to eliminate SH resistant isolates were 32 times higher than the therapeutic dose. Regarding disinfectants, most of the disinfectants tested were efficient to eliminate SH, however one isolate was resistant to glutaraldehyde-quaternary ammonia. All isolates were negatives in the screening efflux system, which suggest a different mechanism of resistance. It is possible to conclude that SH shows a real threat to poultry production, and caution should be taken when choosing the right antibiotic and disinfectant against this serovar.

Key words: Ceftiofur. Disinfectant. Public health. Resistance. *S. Heidelberg*.

Resumo

Bactérias do gênero *Salmonella* podem infectar o homem e animais domésticos, causando sérios problemas de saúde pública no mundo todo. Hoje, a *Salmonella enterica* serovar Heidelberg (SH) está entre os três sorovares mais comumente isolados de pessoas com salmonelose, e presente na cadeia produtiva avícola. Além disso, SH parece ser mais invasiva que outros sorovares que causam enterite. O perfil geral de resistência aos antimicrobianos de cepas brasileiras de SH ainda é desconhecido. Esta bactéria parece usar mecanismos de resistência aos antibióticos similares aos usados para desinfetantes, tais como a bomba de efluxo e a degradação enzimática de compostos químicos. Por isso, o objetivo

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deste trabalho foi de identificar a Concentração Mínima Inibitória (CMI) para o ceftiofur de isolados de SH oriundas de produtos avícolas diversos, bem como de verificar a relação entre resistência a antibióticos e desinfetantes. Além disso, o sistema de bomba de efluxo foi realizado usando o brometo de etídio para determinar se esse era o mecanismo de resistência utilizado. Os resultados do MIC indicaram altos níveis de resistência para ceftiofur, demonstrando a necessidade de formas alternativas para o tratamento da salmonelose. Desinfetantes rotineiramente utilizados pela indústria avícola foram eficientes no combate da SH, porém um isolado mostrou-se resistente ao desinfetante glutaraldeído associado à amônia quaternária. Todos os isolados foram negativos para o sistema de bomba de efluxo, sugerindo que há outro mecanismo de resistência envolvido. É possível concluir que os isolados brasileiros de SH representam uma ameaça para a avicultura, e cautela deveria ser tomada na hora da escolha do antibiótico e do desinfetante a ser utilizado contra este sorovar.

Palavras-chave: Ceftiofur. Desinfetante. Resistência. Saúde pública. S. Heidelberg.

Introduction

Poultry production currently stands out in the Brazilian agribusiness by generating millions of jobs in the country and raising billions of dollars from the exportation. There is an increasing consumption of poultry meat worldwide, which requires continue supply of poultry products of good quality, where Brazil is the largest chicken meat exporter, and this production is concentrated mainly in three states in the South: Paraná, Santa Catarina, and Rio Grande do Sul. As for the world production of poultry meat, the largest producer is the USA, followed by Brazil (ABPA, 2015).

Salmonella has worldwide distribution being found on a variety of environments, but mainly those of animal production. The ingestion of contaminated food is the main form of human contamination, especially by undercooked or raw eggs. Therefore, *Salmonella* constitutes a serious public health problem in many countries (BARROW et al., 2010; QUINN et al., 2005). The gastrointestinal illness caused by this bacteria is called salmonellosis (BERCHIERI AND OLIVEIRA, 2006; TERZOLO, 2010).

S. Heidelberg (SH) is among the top three serovars isolated from people infected with salmonellosis in Canada. Moreover, it seems to be more invasive than other serotypes that cause enteritis. In Canada, the Canadian Integrated Program for Antimicrobial Resistance Surveillance reported a strong correlation ($r = 0.9$) between SH isolates resistant to ceftiofur from chicken scraps and the incidence of SH

infections resistant to ceftiofur in humans (DUTIL, 2010). For the treatment of Salmonellosis in farm animals, ceftiofur, a third-generation cephalosporin, is currently the only approved drug (WEBSTER, 2005). In addition to antibiotic treatment, it is also important to use cleaning and disinfecting measures in order to destroy pathogenic microorganisms, including *Salmonella*. According to Grezzi (2007), quaternary ammonium compounds, glutaraldehyde, and hypochlorite are the main available and active chemical compounds used in poultry.

The abuse and indiscriminate use of antibiotics and even residual concentrations of disinfectants used routinely may lead to the survival of resistant microorganisms (DUTIL, 2010). Some mechanisms, such as the efflux system and enzymatic degradation of the chemical compound are responsible for the resistance to antibiotics and disinfectants, increasing the possibility of cross-resistance (RUSSEL, 2012). There are only a few reports available on the resistance profile specifically for SH regarding ceftiofur (NEVES et al., 2016), and none about disinfectants routinely used in the poultry industry for Brazilian strains of SH. Thus, the objective of this study was to identify the Minimal Inhibitory Concentration (MIC) for ceftiofur of SH isolated from poultry in the state of Paraná, as well as to verify the relation between resistance to ceftiofur to three of the most common disinfectants used in poultry. In addition, the screening efflux system was performed, using ethidium bromide to detect if this mechanism of resistance was involved.

Materials and Methods

Samples

Salmonella enterica serovar Heidelberg (n=17) of poultry origin were kindly provided by Mercolab Laboratories after serotyping using specific antibodies against O and H antigens according to Kauffmann-White method (TRABULSI; ALTERTHUM, 2008). They were isolated from Paraná State during 2012 to 2014. Sample source can be seen on Table 2.

Minimum inhibitory concentration (MIC)

The determination of the Minimal Inhibitory Concentration (MIC) was performed following the recommendations of CLSI (2008). According to the legislation, the isolates were classified as resistant to ceftiofur when the critical point was $\geq 8 \mu\text{g/mL}$ and sensitive for concentrations \leq than $2 \mu\text{g/mL}$. MIC was performed using a commercial product (EXCENEL®) containing 50 mg of ceftiofur per mL of the product. Ten dilutions ranging from 0.064 mg/mL to 0.000125 mg/mL were used.

Testing disinfectants

The same 17 strains of SH underwent the dilution method into tubes to determine SH resistance to disinfectants. *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (ATCC 25922) were used as controls. Data regarding the disinfectants are described in Table 1. The methodology used to analyze the effectiveness of disinfectants was adapted from the Ordinance number 101 of 11 August 1993 of MAPA using bacterial total count plate (BRASIL, 1993).

The culture medium used for the cultivation of microorganisms was brilliant green agar for SH, nutrient agar for *S. aureus*, and eosin methylene blue (EMB) for *E. coli*. Gram staining was performed to confirm the presence of *S. aureus* (ISENBERG, 1998). To obtain the suspension of microorganisms, the colonies were subjected to serial dilutions of 10^1 to 10^7 , using peptonated water, obtaining a final count of 10^{10} CFU/mL for each microorganism. The disinfectants (A, B and C) were diluted according to the manufacturer's instructions using sterile distilled water as described in Table 1. The samples were tested in the presence and absence of organic matter (milk) diluted (1:5000) in sterile distilled water.

Table 1. Characteristics of the disinfectants.

Product	Active compounds	Manufacturing date	Expiration date	Dilution
Disinfectant A	benzalkonium chloride 100% (QAC and polyethylene glycol ether	Apr/14	Apr/17	1:3000
Disinfectant B	benzalkonium chloride (QAC) and gluteraldehyde 100%	Jun/14	Jun/16	1:1500
Disinfectant C	sodium hypochlorite 2%	Jun/14	Dec/14	1:100

For the assay, 9 mL of each sample of the disinfectant was placed into a tube with 0.1 mL of the SH suspension. After contact between the inoculum and the disinfectant, the mixture was incubated for 10 minutes at room temperature. The

same procedure was repeated for the controls and in the presence of organic matter (1 mL) (BRASIL, 1993). Each dilution was plated on PCA (plate count agar) and incubated at 37°C for 24 hours to verify bacterial counts.

Screening procedure of the efflux system

Bacterium might be resistant using a pump to exclude chemicals from its inside by a system called efflux system. To evaluate whether the resistance to quaternary ammonium compounds was mediated by the efflux system, the technique described by Sundheim et al. (1992) was used. Briefly, 0.1 mL of Brain Heart Infusion broth (BHI) containing SH isolates resistant to ceftiofur as verified by the MIC test, were individually seeded on Mueller-Hinton agar containing ethidium bromide (0.5mg/mL) with the aid of a Drigalsky handle. The plates were incubated for 24 h at 37 °C. After incubation, plates were examined under UV light. Bacterial cells that had accumulated the ethidium bromide would emit a red fluorescence and were considered sensitive to QAC by the absence of efflux pump system.

Results and Discussion

MIC results for the 17 isolates of SH to ceftiofur showed result up to 64 µg/mL, where only two samples were sensitive to the antibiotic. The respective values of MIC for each SH isolate are shown in Table 2. Ceftiofur is a third generation cephalosporin of great importance in veterinary medicine, being the only approved cephalosporin for livestock, commonly used to treatment salmonella infections in broilers (WEBSTER, 2005). As can be noted by MIC results, high levels of resistance were identified, indicating that ceftiofur is inefficient on killing this infectious agent, suggesting the need of an alternative drug to treat the infection. To treat salmonellosis in poultry, the concentration of ceftiofur needed is 2 mg/kg. Our results show that the concentration needed to eliminate SH isolates was 32 fold higher than the therapeutic dose. Frye and Cray (2007) evaluated the susceptibility of isolates of *Salmonella* spp. from production animals for a period of five years and found that the resistance to ceftiofur increased from 4% in 1999 to 18% in 2003. In this same study, MIC values ranged from 1 to 16 µg/mL, differently from our findings. Zhao

(2008), also analyzed SH isolates from chicken meat scraps, and found lower levels of resistance (9%) to ceftiofur. In addition, Medeiros et al. (2011) found higher levels of ceftiofur resistance in isolates of SH from carcasses and chicken meat, to similar levels as observed in this study. The increased resistance to broad spectrum, third generation cephalosporins in isolates of *Salmonella* spp is of significant interest to public health, since ceftriaxone is an important drug used to treat children with severe salmonellosis. Considering that ceftiofur resistant microorganisms may cross-resistant to ceftriaxone, the use of this antimicrobial agent in food animals is under increased scrutiny for being a potential agent responsible for the emergence and spread of resistance to ceftriaxone in *Salmonella* spp. and other enteric pathogens in humans (FOLEY; LYNNE, 2007; DUTIL, 2010).

Of the 17 isolates of SH that underwent dilution test with disinfectants, 16 were sensitives to all three disinfectants at the recommended dilution, regardless of the presence of organic matter (milk). However, a single isolate of *S. Heidelberg* (ID 81) inoculated in the absence of organic matter grew even in the presence of the disinfectant B (benzalkonium chloride (QAC) and glutaraldehyde 100%). The quaternary ammonium compounds (QACs) are synthetic cationic detergents that have antimicrobial activity. This sanitizer is soluble in water, with low toxicity and efficient against Gram-positive bacteria and thermophilic microorganisms, but poorly effective against Gram-negative bacteria, coliforms, and spores (WALIA et al., 2017). Disinfectant A (quaternary ammonium) used in this study, has polyethylene glycol ether in its composition. According to Kich et al. (2004), this compound is characterized by the surfactant action, able to remove biofilms. It was concluded that the 17 isolates of SH were killed *in vitro* in the presence of this disinfectant, both in the presence and absence of organic matter (milk). Glutaraldehyde and quaternary ammonium (disinfectant B) have a synergistic effect, associated with a higher

success in the control of bacteria, exerting lower selection pressure in microbial populations (FIGUEIROA et al., 2017). Both compounds have been used routinely by the Brazilian poultry industry. Ammonia-based products, and quaternary aldehydes are very effective in eliminating *E. coli* in contaminated environments (GILBERT; MCBAIN, 2003). However, in the present work disinfectant B was not fully efficient to eliminate SH, since one isolate (ID 81) was resistant. According to Gilbert

and Mcbain (2003), many Gram negative pathogens might be resistant to disinfectants, since they have a relatively impermeable outer membrane of the cell wall (SUNDHEIM, 1992), which could justify our findings. We observed that the disinfectant C (2% sodium hypochlorite) was effective in killing SH isolates either in the presence or absence of organic matter (milk). Overall chlorinated compounds are widely used due to its low cost (BUNCIC; SOFOS, 2012).

Table 2. Identification, source and Minimum Inhibition Concentration (MIC) results using ceftiofur against Brazilian strains of *Salmonella* Heidelberg.

ID strain	Source	MIC (µg/mL)
52	Meat product	32
53	Meat product	64
54	Meat product	>64
55	Culture	2
56	Culture	64
57	Culture	64
62	Meat product	64
69	Meat product	64
70	Meat product	64
77	Drag swab	32
79	Meat product	64
80	Drag swab	64
81	Drag swab	32
82	Drag swab	64
91	Dead birds	32
103	Drag swab	>64
104	Drag swab	<2

Several authors have reported that bacterial resistance to disinfectants might be related to the presence of the efflux system to pump out toxic substances from inside the bacterial cells (PAIXÃO et al., 2009). Our findings support this hypothesis, since the strains were sensitive to disinfectants and efflux mechanism was not observed in any of the isolates. The same was observed by Bjorland et al. (2005) while analyzing isolates of *Staphylococcus*

spp. from milk of cattle and goats that were resistant to quaternary ammonium compounds. Therefore, we conclude that a different mechanism might be related to this type of resistance, and further tests should be performed such as sequencing to verify gene mutations in genes associated with the mechanism of action of the compound, and the presence of genetic mobile elements through horizontal gene transfer by conjugation assays.

Humans once infected by a bacterium resistant to antibiotics that are commonly used to treat infections or resistant to disinfectants routinely used in animal production systems or hospitals, may have impaired treatment, higher costs and often need hospitalizations for longer periods (SHIRAKI et al., 2004; SAN MARTIN et al., 2005; JOHNSON et al., 2007; RIBEIRO et al., 2011). Our results indicate a problem of resistance to antimicrobials and disinfectants with severe consequences to public health, since most of the isolates were from meat products, showing the possibility of transmission of these bacteria to humans by the consumption of contaminated poultry-derived products.

Conclusions

Only very high doses of ceftiofur would be effective to treat salmonellosis caused by SH, which would be too costly. Since ceftriaxone is also a third generation cephalosporin used to treat children with this disease, resistance to ceftiofur found in this study raises a major public health concern. Other studies have reported that SH is more invasive and persistent in the environment compared to other serotypes, and our study shows that SH can also be resistant to well-known disinfectants used in the poultry industry. More studies should be conducted to investigate the mechanisms involved on SH resistance, besides the pump efflux system.

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