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Antimicrobial and disinfectant resistance of *Salmonella* Heidelberg from Brazilian flocks did not increase for ten years (2006-2016)[1]

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ABSTRACT.- Bassani J., Paravisi M., Wilsmann D.E., Borges K.A., Furian T.Q., Salle C.T.P., Moraes H.L.S. & Nascimento V.P. 2021. **Antimicrobial and disinfectant resistance of *Salmonella* Heidelberg from Brazilian flocks did not increase for ten years (2006-2016)**. *Pesquisa Veterinária Brasileira 41:e06818, 2021*. Centro de Diagnóstico e Pesquisa em Patologia Aviária, Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves 9090, Porto Alegre, RS 91540-000, Brazil. E-mail: wilsmanndaia@gmail.com

Salmonella is a major cause of foodborne illness worldwide, and poultry and its derived products are the most common food products associated with salmonellosis outbreaks. Some countries, including Brazil, have experienced an increased prevalence of *Salmonella* Heidelberg among their poultry flocks. Some isolates have also presented high resistance to antimicrobial agents and persist in the poultry farm environment. This study aimed to compare the susceptibility of *S.* Heidelberg strains isolated in 2006 with those isolated in 2016 against disinfectants and antimicrobial agents. The results showed that all the strains were highly susceptible to sodium hypochlorite, regardless of the conditions and year of isolation. Resistance to benzalkonium chloride varied according to the conditions applied, but not to the year of isolation. Increased antimicrobial resistance from 2006-2016 was observed only for tetracycline. The results suggest that the antimicrobial and disinfectant resistance of *S.* Heidelberg did not increase for ten years (2006-2016). However, further analysis should include a larger number of S. Heidelberg isolates from poultry origin and additional antimicrobial agents for more precise conclusions about the increasing in antimicrobial resistance in the last years.

INDEX TERMS: Antimicrobial, disinfectant resistance, *Salmonella* Heidelberg, poultry flocks, Brazil.

RESUMO.- [**A resistência a desinfetantes e a antimicrobianos não aumentou em um período de 10 anos (2006 a 2016) em *Salmonella* Heidelberg isoladas de granjas avícolas brasileiras.**] *Salmonella* é uma das principais causas das doenças transmitidas por alimento em todo o mundo, e a carne de frango e produtos derivados são os principais alimentos associados com surtos de salmonelose em humanos. Alguns países, incluindo o Brasil, têm observado um aumento da ocorrência de *Salmonella* Heidelberg nas suas granjas avícolas. Além disto, alguns isolados têm apresentado alta resistência aos antimicrobianos e têm persistido no ambiente de produção avícola. Neste contexto, o objetivo deste estudo foi comparar a susceptibilidade de cepas de *S.* Heidelberg isoladas em 2006 com aquelas isoladas em 2016 contra desinfetantes e agentes antimicrobianos. Os resultados demonstraram que as cepas foram altamente resistentes a hipoclorito de sódio, independentemente das condições e do ano de isolamento. A resistência ao cloreto de benzalcônio variou de acordo com as condições testadas, mas não com o ano de isolamento. Um aumento da resistência aos antimicrobianos de 2006 a 2016 foi observado apenas para tetraciclina. Os resultados sugerem que a resistência aos desinfetantes e aos antimicrobianos não aumentou em um período de dez anos (2006-2016). Entretanto, novas análises devem incluir um número maior de cepas de *S.* Heidelberg isoladas de fontes avícolas e outros agentes antimicrobianos para uma conclusão mais precisa sobre o aumento da resistência antimicrobiana nos últimos anos.

TERMOS DE INDEXAÇÃO: *Salmonella* Heidelberg, avicultura, desinfetante, antimicrobiano, resistência, Brasil.

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INTRODUCTION

Salmonellosis is the second main cause of bacterial enteritis in the European Union (EFSA 2019), and the first in the United States (CDC 2020a). *Salmonella* is a major cause of foodborne illness worldwide, affecting nearly one in ten persons, resulting in approximately 550 million infected people per year (WHO 2020). The US Centers for Disease Control and Prevention (CDC) reports that poultry and its derived products are the most common food products associated with salmonellosis outbreaks and are responsible for more than 47% of all infections (CDC 2020b).

Data from the Brazilian Ministry of Health show that *Salmonella* is among the most common pathogens isolated from foodborne diseases in Brazil (Brasil 2019b). *S.* Heidelberg is considered one of the most commonly found serotypes in poultry farming worldwide, and previous studies have shown that strains isolated in recent years are highly resistant to antimicrobials (CDC 2016, EFSA 2019, Neves et al. 2020). In recent years, there has been an increase in the prevalence of *Salmonella* Heidelberg, especially in the United States and Brazil (CDC 2016, Brasil 2019a).

Bacterial resistance to antimicrobials and disinfectants has increased considerably over the years, mainly because of their inadequate use in human and veterinary medicine (FAO 2016). Recent studies have demonstrated increasing resistance of *Salmonella* strains isolated from humans and animals to the most commonly used antibiotics (Borges et al. 2019, EFSA 2020, Elhariri et al. 2020, Souza et al. 2020). Despite the development of several alternative products for pathogen control in the food processing chain, the best viable protection against foodborne pathogens are disinfectants and biosecurity (Bragg et al. 2018, Mc Carlie et al. 2020). Thus, it is of concern that disinfectant resistance could rapidly increase (Mc Carlie et al. 2020). However, unlike antimicrobials, the possible resistance to disinfectants traditionally used in the poultry industry has not been elucidated.

In recent years, the specific resurgence of the serotype *S.* Heidelberg, followed by its resistance to control programs, has worsened worldwide, including in Brazil (Colla et al. 2012, Gieraltowski et al. 2016, Stefani et al. 2018, Etter et al. 2019, Voss-Rech et al. 2019). In view of the epidemiological situation in Brazil, the present study aimed to compare the susceptibility of *S.* Heidelberg strains isolated in 2006 with those isolated in 2016 from poultry sources against two disinfectants and six antimicrobials.

MATERIALS AND METHODS

Salmonella Heidelberg isolates

A total of 40 *S.* Heidelberg strains were selected for this study. The strains were isolated in two periods: 2006 (n=20) and 2016 (n=20) from poultry sources (drag swabs, cloacal swab, and chicken carcasses) in Southern Brazil (Table 1). The strains were previously isolated, biochemically characterized, and serotyped. All strains were stored frozen at -80°C in brain heart infusion broth (BHI) (Merck, Darmstadt, Germany) supplemented with 15% glycerin (Synth, Diadema, Brazil). The bacteria were retrieved from frozen culture stocks and cultured overnight at 37°C in xylose lysine deoxycholate (XLD) agar (Merck, Darmstadt, Germany) and incubated at 37°C for 24h. One colony morphologically characteristic of *Salmonella* spp. was selected and inoculated in BHI and incubated again at 37°C for 24h.

Disinfectant test

Inoculum preparation. To prepare the inoculum, McFarland standard no. 0.5 (Probac do Brasil, São Paulo, Brazil) was used as a reference to adjust the turbidity of the bacterial suspension in 0.1% buffered peptone water (BPW) (Oxoid, Basingstoke, England) to a concentration of 10^8CFU/mL, corresponding to an optical density interval of 0.08-0.1 in a spectrophotometer (SP 22; Biospectro, Curitiba, Brazil) at a wavelength of 625nm (CLSI 2013a). The bacterial suspension was diluted in 0.1% BPW to a concentration of 10^6CFU/mL.

Concentrations, time of exposure, temperature conditions, and neutralizer solution. Two commercially available disinfectants were evaluated: sodium hypochlorite (MediQuímica, Juiz de Fora, Brazil) and chloride benzalkonium (Exodus Científica, Sumaré, Brazil). Sodium hypochlorite was evaluated at 0.5% and 1.0%, and benzalkonium chloride at 100 and 200ppm, following the manufacturer's recommendations for use in poultry industries. Dilutions were performed in sterile distilled water. The products were evaluated at two temperatures: 25°C, to simulate a cleaning scenario, and 12°C, the maximum temperature allowed in cutting rooms, according to Brazilian legislation (Brasil 1998). A solution of 1% bovine fetal serum (Gibco, Thermo Fisher Scientific, Waltham, USA) was used to simulate the presence of organic matter. Two exposure times (5 and 15 min) were tested to mimic the cleaning and disinfection processes in the poultry industry. A neutralizer solution was used to inactivate the antimicrobial effect of the disinfectant. The neutralizer was composed of polysorbate Tween 80 (Neon, São Paulo, Brazil), 2g of soy lecithin (Stem, Porto Alegre, Brazil), and 2g of sodium thiosulfate (Dynamic, Diadema, Brazil).

Evaluation test. The evaluation test was performed according to Brazilian legislation guidelines (Brasil 1993) for suspension test in planktonic cells. Briefly, 0.1mL of the inoculum was inoculated in 9.9mL of each disinfectant containing 1% bovine fetal serum. After the exposure time, 10µL of the suspension was inoculated in BHI with a neutralizer solution. The materials were incubated at 37°C for 96h. Tubes presenting turbidity, surface film formation, or background precipitate were considered positive (non-susceptible strains). The tube was considered negative (susceptible strains) when no growth was observed after 96h of incubation. Bacterial viability was confirmed by reseeding an aliquot in the XLD. Plates were incubated at 37°C for 24h.

Minimum inhibitory concentration (MIC)

Inoculum preparation. The bacteria were cultured overnight at 37°C in trypticase soy agar (TSA) (Oxoid, Basingstoke, England). To prepare the inoculum, McFarland standard no. 0.5 (Probac do Brasil, São Paulo, Brazil) was used as a reference to adjust the turbidity of the bacterial suspension in 0.85% saline solution (NaCl; Synth, Diadema, Brazil) to a concentration of 10^8CFU/mL, corresponding to an optical density interval of 0.08-0.1 in a spectrophotometer (SP 22; Biospectro, Curitiba, Brazil) at a wavelength of 625nm (CLSI 2013a). The bacterial suspension was diluted in cation-adjusted Muller-Hinton broth (CAMBH) (Honeywell Fluka - Fisher Scientific, Loughborough, UK) to reach a concentration of 10^7CFU/mL.

Antimicrobials and MIC determination. As described by the Clinical and Laboratory Standards Institute (CLSI 2013b), a broth microdilution test was performed to determine the MIC for antimicrobial agents (Sigma-Aldrich, St. Louis, US): gentamicin (0.25-128µg/mL), chloramphenicol (2-128µg/mL), nalidixic acid (1-128µg/mL), ciprofloxacin (0.008-16µg/mL), enrofloxacin (0.008-16µg/mL), and tetracycline (0.5-64µg/mL). The strains were classified as susceptible

or non-susceptible (including intermediate strains), according to the breakpoints described in the CLSI standards (CLSI 2013b, 2020). The interpretation of MIC values for enrofloxacin was performed according to previously available data (Hao et al. 2013). The strains were also classified as wild type (WT) or non-wild type (nWT) based on their epidemiological MIC cutoff (ECOFFs), which were determined according to the EUCAST guidelines available at the time of data analysis (EUCAST 2020). An *Escherichia coli* reference strain (ATCC 325922) was selected to ensure the validity of the tests. The strains that were resistant to three or more classes of antimicrobials were classified as multidrug-resistant (MDR) strains (Schwarz et al. 2010). The multiple antibiotic resistance (MAR) index was determined as previously described (Krumperman 1983). Both MDR and MAR were defined according to CLSI breakpoints.

Statistical analysis

The data were subjected to descriptive statistical analysis using PASW Statistics software (IBM, Hong Kong). Fisher's exact test was used to compare the periods (2006 and 2016), temperatures (12°C and 25°C), concentrations (100 and 200ppm), and time of exposure (5 and 15min) in the disinfectant test. A 5% level of significance was applied for all tests. The kappa index (Landis & Koch 1977)

Table 1. *Salmonella* Heidelberg strains: identification, source of isolation, and antimicrobial resistance results (phenotypic resistance profiles, multiple antibiotic resistance, and multidrug resistance)

Identification	Year of isolation	Source of isolation	Phenotypic antimicrobial resistance profile		Multiple antibiotic resistance (MAR)[a]	Multidrug resistance[a]
			CLSI breakpoints	EUCAST breakpoints		
1	2006	Cloacal swab	-	-	0	No
2	2006	Cloacal swab	NAL	NAL, CIP	0.2	No
3	2006	Drag swab	-	-	0.0	No
4	2006	Carcass	NAL	NAL, CIP	0.2	No
5	2006	Carcass	-	-	0.0	No
6	2006	Drag swab	NAL	NAL	0.2	No
7	2006	Drag swab	GEN, TET	GEN, TET	0.3	No
8	2006	Cloacal swab	-	GEN	0.0	No
9	2006	Carcass	-	-	0.0	No
10	2006	Carcass	NAL	NAL, CIP	0.2	No
11	2006	Cloacal swab	-	-	0.0	No
12	2006	Carcass	-	-	0.0	No
13	2006	Cloacal swab	NAL	NAL, CIP	0.2	No
14	2006	Carcass	-	-	0.0	No
15	2006	Carcass	NAL	NAL, CIP	0.2	No
16	2006	Carcass	NAL	NAL, CIP	0.2	No
17	2006	Carcass	NAL	NAL, CIP	0.2	No
18	2006	Carcass	ENR, NAL, CIP	ENR, NAL, CIP	0.5	No
19	2006	Cloacal swab	NAL	NAL, CIP	0.2	No
20	2006	Carcass	-	-	0.0	No
21	2016	Drag swab	NAL, TET	NAL, CIP, TET	0.3	No
22	2016	Drag swab	NAL, TET	NAL, CIP, TET	0.3	No
23	2016	Drag swab	GEN, NAL, TET	GEN, NAL, CIP, TET	0.5	Yes
24	2016	Drag swab	-	-	0.0	No
25	2016	Drag swab	GEN, NAL, TET	GEN, NAL, CIP, TET	0.5	Yes
26	2016	Drag swab	GEN, ENR, NAL, TET	GEN, NAL, CIP, TET	0.7	Yes
27	2016	Drag swab	NAL, TET	NAL, CIP, TET	0.3	No
28	2016	Drag swab	ENR, NAL, TET	NAL, CIP, TET	0.5	No
29	2016	Drag swab	GEN, NAL, TET	GEN, NAL, CIP, TET	0.5	Yes
30	2016	Drag swab	-	-	0.0	No
31	2016	Drag swab	NAL, TET	NAL, CIP, TET	0.3	No
32	2016	Drag swab	-	-	0.0	No
33	2016	Drag swab	NAL, TET	NAL, CIP, TET	0.3	No
34	2016	Drag swab	NAL, TET	NAL, CIP, TET	0.3	No
35	2016	Drag swab	GEN, ENR, NAL, TET	GEN, NAL, CIP, TET	0.7	Yes
36	2016	Drag swab	GEN, ENR, NAL, TET	GEN, NAL, CIP, TET	0.7	Yes
37	2016	Drag swab	NAL, TET	NAL, CIP, TET	0.3	No
38	2016	Drag swab	-	-	0.0	No
39	2016	Drag swab	-	-	0.0	No
40	2016	Drag swab	NAL, TET	NAL, CIP, TET	0.3	No

CIP = Ciprofloxacin, GEN = gentamicin, ENR = enrofloxacin, NAL = nalidix acid, TET = tetracycline; [a] According to CLSI breakpoints.

was used to evaluate the concordance between the classifications based on the CLSI breakpoints and ECOFF values.

RESULTS

Disinfectant tests

The individual results of the susceptibility tests of the disinfectants are described as supplementary material. The strains were highly susceptible to sodium hypochlorite. Among the 20 strains of *Salmonella* Heidelberg isolated in 2006, only two (10%) were non-susceptible to sodium hypochlorite 0.5%, both at 25°C and after 5 min of contact. All the strains isolated in 2016 were susceptible, regardless of the temperature, concentration of disinfectant, and contact time. In relation to benzalkonium chloride susceptibility, different frequencies of non-susceptible strains were observed for both groups (Table 2). The comparison of *S.* Heidelberg susceptibility to benzalkonium chloride between strains isolated in 2006 and those isolated in 2016 showed a significant increase in the number of non-susceptible strains only at 200ppm, after a contact time of 5min at 25°C. There were no differences (p>0.05) between the frequencies of the non-susceptible strains isolated in different years when other conditions of temperature, exposure time, and disinfectant concentration were considered (Table 2). Comparisons among the tested conditions for benzalkonium chloride revealed no differences (p>0.05) in the total number of non-susceptible strains between temperatures. The increase in the exposure time led to a significant (p<0.05) decrease in the number of non-susceptible strains isolated in 2016, regardless of the temperature and the concentration of the disinfectant. For strains isolated in 2006, a significant difference (p<0.05) was observed only at 25°C, with a concentration of 100ppm.

Minimum inhibitory concentration

The phenotypic antimicrobial resistance profiles of each strain are described in Table 1. The MIC results are described in Figure 1 and 2. According to the CLSI breakpoints, 45% (9/20) of the strains isolated in 2006 and 20% (4/20) of those from 2016 were susceptible to all the antimicrobials. When ECOFF values were considered, 40% (8/20) of the strains isolated in 2006 and 25% (5/20) of those isolated in 2016 were classified as wild type to all the antimicrobials. There was no significant (p>0.05) difference in overall resistance between the two periods of isolation, regardless of the breakpoint evaluated. Considering all the strains of *S.* Heidelberg, regardless of the year of isolation, chloramphenicol, nalidix acid, and tetracycline presented similar results between the CLSI and EUCAST breakpoints (Fig.1 and 2). Ciprofloxacin, gentamicin, and enrofloxacin presented significant (p<0.05) differences in antimicrobial susceptibility according to the breakpoint evaluated. Resistance to ciprofloxacin and gentamicin was higher according to the EUCAST parameters compared to the CLSI breakpoints. Resistance to enrofloxacin was lower according to the EUCAST breakpoints. To measure the agreement between the EUCAST and the CLSI breakpoints, *a kappa* test was applied when the results were different. It showed almost perfect agreement for gentamicin (κ = 0.846), substantial agreement for enrofloxacin (κ = 0.780), and fair agreement (κ = 0.246) for ciprofloxacin (Landis & Koch 1977). The statistical analyses results showed a significant (p<0.05) increase in resistance to tetracycline in strains isolated in 2016 compared to those isolated in 2006, regardless of the breakpoint evaluated. However, there was no significant difference (p>0.05) in resistance to chloramphenicol, ciprofloxacin, enrofloxacin, gentamicin, and nalidixic acid. For both breakpoints, a higher resistance to nalidix acid was observed among strains isolated in 2006. Among strains from 2016, the highest resistance rates were observed for tetracycline and nalidixic acid. Multidrug-resistant strains were identified only among strains from 2016 and represented 20% (4/20) of the total. The individual maximum and minimum multiple-antibiotic resistance (MAR) indices for the strains isolated in 2006 were 0.3 and 0.1, respectively, with an average index of 0.1. Among those isolated in 2016, the maximum and minimum MAR indices were 0.7 and 0.3, respectively, with an average index of 0.3.

DISCUSSION

Brazil is a leading supplier of poultry meat in the world, and the states of Rio Grande do Sul, Santa Catarina, and Paraná in the southern region are responsible for more than 64% of the total poultry slaughter (ABPA 2020). The spread of diseases, especially foodborne diseases, such as salmonellosis, represents a major economic and public health problem for these states. In the last few years, studies have reported the presence of persistent environmental *Salmonella* Heidelberg in Brazilian broiler farms, especially in the southern region (Duarte 2018, Voss-Rech et al. 2019). In addition, increased antibiotic resistance of *S.* Heidelberg has been reported in this region (Neves et al. 2020).

Previous studies have shown that the *S.* Heidelberg strains isolated in 2016 and 2017 were capable of producing biofilms at 25°C (Lucca et al. 2020). However, it is important to note that strains isolated from 1996-2006 also have the ability to produce biofilms at similar temperatures (28°C) (Borges et al. 2018). Thus, it is probable that the surface adherence capability is not enough to explain and justify the increased

Table 2. Non-susceptible *Salmonella* Heidelberg strains (2006 and 2016) to benzalkonium chloride

Time of contact	Relative frequencies (%) of non-susceptible strains (n/N)							
	12°C				25°C			
	2006		2016		2006		2016	
	100ppm	200ppm	100ppm	200ppm	100ppm	200ppm	100ppm	200ppm
5 minutes	70% (14/20)[a]	25% (5/20)[a]	85% (17/20)[a]	35% (7/20)[a]	70% (14/20)[a]	20% (4/20)[a]	75% (15/20)[a]	55% (11/20)[b]
15 minutes	40% (8/20)[a]	5% (1/20)[a]	35% (7/20)[a]	5% (1/20)[a]	30% (6/20)[a]	10% (2/20)[a]	25% (5/20)[a]	5% (1/20)[a]

[a,b] Different letters on the same line indicate that there is statistical difference (p<0.05) between relative frequencies of non-susceptible strains in 2006 and 2016, considering the same disinfectant concentration, temperature and contact time.

isolation and difficulty of the Brazilian poultry chain in removing this serotype from flocks in recent years.

In this context, we evaluated and compared the disinfectant and antibiotic resistance between strains isolated in two periods of time (2006 and 2016). The disinfectant test was performed to evaluate the susceptibility of the tested isolates in the planktonic phase. Sodium hypochlorite and benzalkonium chloride concentrations and contact times were tested as recommended by the manufacturer. In addition, we evaluated a lower concentration, simulating under dosage situations. The antibiotics were selected based on their importance for human and veterinary use.

Sodium hypochlorite is widely used in the food industry as a disinfectant, despite the increasing availability of other products. The main advantages are the low cost, broad antimicrobial spectrum, rapid bactericidal action, and low toxicity to humans and animals (Fukuzaki 2006). The antimicrobial effectiveness of sodium hypochlorite is based on its high pH (hydroxyl ion action), which interferes with the cytoplasmic membrane integrity with an irreversible enzymatic inhibition, biosynthetic alterations in cellular metabolism, and phospholipid degradation observed in lipidic peroxidation (Estrela et al. 2002). In the current study, the strains seemed to be highly susceptible to sodium hypochlorite, regardless of the year of isolation and the conditions employed. It is important to highlight that sodium hypochlorite efficacy is highly dependent on organic load (Köhler et al. 2018), which was simulated by 1% bovine fetal serum. Although specific strains and species differences have been observed (Köhler et al. 2018), the higher susceptibility of the strains to this disinfectant has already been described by other researchers for several pathogens, including against multidrug-resistant bacteria (Köhler et al. 2018, Borges et al. 2020).

Benzalkonium chloride is a broad spectrum quaternary ammonium antibacterial agent with widespread applications. Several mechanisms of action have been described, mostly related to the cell membrane, such as changes in the overall membrane composition and downregulation of porins (Pereira & Tagkopoulos 2019). In the current study, a large number of the strains were not susceptible to benzalkonium chloride for both years of isolation. However, a significant increase in the resistance of the strains isolated in 2016 was observed only when the strains were exposed to 200ppm for 5 min at 25°C. Low susceptibility to benzalkonium chloride has been previously described for some *Salmonella* serotypes (Long et al. 2016). Its effectiveness is reduced against Gram-negative bacteria,

Antimicrobial agent[a]	Minimum inhibitory concentration (MIC) – (n)[b]															Total n (%)[c]	
	≤0.007	0.016	0.031	0.062	0.125	0.25	0.5	1	2	4	8	16	32	64	≥128	nWT	NS
CHL									20	0	0	0	0	0	0	0	0
CIP	0	10	0	1	0	8	0	1	0	0	0	0				9 (45)	1 (5)
ENR	1	0	9	1	0	3	5	0	0	1	0	0				1 (5)	1 (5)
GEN						1	0	13	4	0	0	1	0	1	0	2 (10)	1 (5)
NAL								0	4	6	0	0	0	0	10	10 (50)	10 (50)
TET							0	16	3	0	0	0	0	1		1 (5)	1 (5)

Fig.1. Minimum inhibitory concentration (MIC) results for the strains isolated in 2006: non-susceptible strains (CLSI breakpoints) and non-wildtype strains (ECOFF values). [a] Chloramphenicol (CHL), ciprofloxacin (CIP), enrofloxacin (ENR), gentamycin (GEN), nalidixic acid (NAL), tetracycline (TET). [b] MIC breakpoints, according to CLSI guidelines, also include "intermediate" strains, which are considered non-susceptible. [c] Non-wild type (nWT), according to ECOFF values (EUCAST breakpoints). Non-susceptible (NS), according to CLSI breakpoints. Continuous lines indicate CLSI breakpoints. Dotted lines indicate ECOFF values (EUCAST breakpoints). Double lines indicate that CLSI breakpoints and ECOFF values are the same. Shaded areas indicate the tested concentrations.

Antimicrobial agent[a]	Minimum inhibitory concentration (MIC) – (n)[b]															Total n (%)[c]	
	≤0.007	0.016	0.031	0.062	0.125	0.25	0.5	1	2	4	8	16	32	64	≥128	nWT	NS
CHL									4	14	2	0	0	0	0	0	0
CIP	0	5	0	0	0	11	4	0	0	0	0	0				15 (75)	0
ENR	0	1	3	1	0	4	7	4	0	0	0	0				0	4 (20)
GEN						0	0	11	3	0	0	0	1	3	2	6 (30)	6 (30)
NAL								0	4	1	0	1	1	0	13	15 (75)	15 (75)
TET							0	5	0	0	0	0	0	15		15 (75)	15 (75)

Fig.2. Minimum inhibitory concentration (MIC) results for the strains isolated in 2016: non-susceptible strains (CLSI breakpoints) and non-wildtype strains (ECOFF values). [a] Chloramphenicol (CHL), ciprofloxacin (CIP), enrofloxacin (ENR), gentamycin (GEN), nalidixic acid (NAL), tetracycline (TET). [b] MIC breakpoints, according to CLSI guidelines, also include "intermediate" strains, which are considered non-susceptible. [c] Non-wild type (nWT), according to ECOFF values (EUCAST breakpoints). Non-susceptible (NS) according to CLSI breakpoints. Continuous lines indicate CLSI breakpoints. Dotted lines indicate ECOFF values (EUCAST breakpoints). Double lines indicate that CLSI breakpoints and ECOFF values are the same. Shaded areas indicate the tested concentrations.

such as *Salmonella* spp., owing to the outer membrane, once the lipopolysaccharide layer act as a barrier against harmful external conditions (Bragg et al. 2014). The efflux pump system may be another possible mechanism of resistance, and at least nine genes have been described for the *Salmonella* genus. Among them, three (*acrAB*, *acrEF*, and *mdsABC*) are known to expel benzalkonium chloride from the cell (Møretrø et al. 2012). Most studies did not find significant differences in resistance based on the temperature of incubation in *in vitro* tests with *Salmonella* serotypes (Kich et al. 2004, Stringfellow et al. 2009, Jaenisch et al. 2010). However, Camilotti et al. (2015) found that benzalkonium chloride activity was reduced at 8°C in relation to 20°C when tested against *S.* Hadar. In addition, the mechanisms of resistance to benzalkonium chloride may also be related to the conditions of use, especially according to the amount of organic matter, not only by the resistance profile of *Salmonella* strains (Kich et al. 2004).

Brazilian reports have shown decreased susceptibility to chlorhexidine, but higher susceptibility to sodium hypochlorite and benzalkonium chloride among *S.* Heidelberg strains (Colla et al. 2012, Stefani et al. 2018). Studies evaluating the susceptibility of *S.* Heidelberg serotype to disinfectants are still uncommon. Our findings demonstrate that the resistance to disinfectants did not increase over time among the analyzed strains, although this result has been observed previously. Riazi & Matthews (2011) evaluated the susceptibility of foodborne pathogens to common disinfectants after repeated exposure, and they observed that the bacterial pathogens tested remain susceptible under the conditions evaluated.

Antimicrobial resistance represents a public health risk owing to the decrease in available treatment options for patients. In addition, it increases the costs of healthcare for these patients. Thus, this is a serious threat that requires immediate action worldwide (WHO 2020). It has already been proven that the increase in antimicrobial resistance is closely related to the use of these substances, which leads to selection pressure and consequently to the emergence of non-susceptible strains (Balsalobre et al. 2014, WHO 2020).

To determine whether resistance increased over time and may have contributed to the persistence of *S.* Heidelberg in the flock, the current study evaluated the antimicrobial resistance of both groups to commonly used antimicrobials. Our results indicate that despite the overall increase in resistance from 2006-2016, the differences were not significant for both of the breakpoints evaluated (CLSI and EUCAST). As the misuse of antimicrobials in humans and animals over time may accelerate the process of resistance, the results were unexpected. However, these findings are not unique. A recent study analyzing the antimicrobial resistance of *Salmonella* strains isolated over 20 years reported increased resistance rates to third-generation cephalosporins, but not to quinolones and sulfonamides (Lo et al. 2020). The detection of MDR strains among the *S.* Heidelberg strains isolated in 2016 was an indicator of an increase in antimicrobial resistance over time. However, this situation is probably due to a higher number of resistant strains to only one antimicrobial, tetracycline, in 2016.

The MAR index can be applied to differentiate low (MAR < 0.2) and high-risk (MAR > 0.2) regions where antimicrobials are overused (Proroga et al. 2016). Strains isolated in 2016 presented an average index of 0.3, which could indicate high antibiotic usage and high selective pressure. However, the widespread use of antibiotics in developing countries, including Brazil, probably reduces the practical significance of this finding (Davis & Brown 2016).

The MIC results are based on pre-established breakpoints, including those from EUCAST and CLSI agencies. The variations in the breakpoints can result in significant changes in the final MIC, which will affect clinical decisions and official data reports (Kassim et al. 2016). A previous study compared the MIC results for *Campylobacter jejuni* strains according to the EUCAST and CLSI values and showed an agreement between these guidelines, which indicates that data based on both parameters could be compared (Paravisi et al. 2020). A similar analysis was performed in the current study, and the results showed good agreement for gentamicin and enrofloxacin, indicating that the results from both breakpoints were similar. Tetracycline, nalidix acid, and chloramphenicol presented equal results. However, a fair agreement was obtained for ciprofloxacin, which implies a separate analysis and discussion of this antimicrobial. The *kappa* analysis for ciprofloxacin resulted in a fair agreement, which means that it was significantly (p<0.05) variable according to the breakpoint evaluated. When the CLSI breakpoint was applied, only one strain, isolated in 2006, was resistant. In contrast, analysis with the EUCAST breakpoint resulted in 45% (9/20) and 75% (15/20) of non-susceptible strains in 2006 and 2016, respectively. The majority of previous studies evaluating the resistance of *S.* Heidelberg for ciprofloxacin used the CLSI breakpoint, which is a possible reason for the lower resistance rate found elsewhere for this substance (Elhariri et al. 2020, Souza et al. 2020). Our results suggest that the guidelines for the breakpoints should be chosen carefully as well as the comparison of results with previously published studies.

All the strains were susceptible to chloramphenicol, which is probably related to its banishment in production animals since 2003 in Brazil (Brasil 2003). The resistance to gentamicin in the present study varied from 5% (2006) to 30% (2016). Although not significant, this high resistance rate is not commonly observed in *Salmonella* strains, and especially among *S.* Heidelberg (Pandini et al. 2014, Mendonça 2016, Saifuddin et al. 2016, Neves et al. 2020), probably because the use of gentamicin in poultry production is restricted (Giacomelli et al. 2014).

Tetracycline was the only antibiotic that showed a significant increase over the period from 2006-2016, and 75% of the strains isolated in 2016 were non-susceptible. In contrast, only one strain (5%) was non-susceptible among those isolated in 2006. The resistance of *Salmonella* to tetracycline is variable in the literature (Mion et al. 2016, El-Tayeb et al. 2017, Nair et al. 2018, Borges et al. 2019). A recent study found high non-susceptibility rates among *S.* Heidelberg isolated from Brazilian flocks (Neves et al. 2020). The resistance of *Salmonella* to tetracycline may be related to the widespread of *tet* resistance genes among *Salmonella* serotypes (Khoshbakht et al. 2018). Tetracycline was commonly used as a feed additive for prophylactic purposes in broiler rations in Brazil, and its use was restricted for therapeutic purposes in animals since 2009 (Brasil 2009). Even if its use has been considerably reduced, it contributed to the maintenance of the circulation of resistance genes. In addition, opportunistic pathogens and commensal bacteria can serve as reservoirs for these genes, through mobile genetic elements, and may transfer them to

Salmonella strains and other pathogens. Thus, its increased resistance may also be a result of the presence of plasmids or transposons that carry antimicrobial resistance genes for other substances, which continue to suffer selective pressure (Frye & Jackson 2013).

Resistance to nalidixic acid (quinolone) and enrofloxacin (fluoroquinolone) did not vary according to the year of isolation or the breakpoint evaluated. However, resistance to nalidix acid was higher than that of enrofloxacin. High resistance rates to nalidix acid among *S.* Heidelberg isolated from Brazilian poultry flocks have been previously described (Giuriatti et al. 2017, Neves et al. 2020, Souza et al. 2020), and it is probably related to its wide use in poultry therapy for many years (Neves 2014). In contrast to what happens with first-generation quinolones, the resistance levels to fluoroquinolones, such as enrofloxacin and ciprofloxacin, are usually lower among *Salmonella* serotypes (Dallal et al. 2010, Panzenhagen et al. 2016, Proroga et al. 2016, Danish 2018).

Resistance to disinfectants is probably because of the excessive use of these substances, imposing selective pressure and, consequently, vertical and horizontal gene transfer from resistant bacteria. However, the mechanisms of transfer of disinfectant resistance genes remain unclear, and only a few have been described (Mc Carlie et al. 2020). Cross-resistance between antimicrobial substances is becoming an increasing concern because of the possible positive correlation between resistance to disinfectants and resistance to antibiotics (Cadena et al. 2019, Mc Carlie et al. 2020). The major concern is based on the hypothesis that disinfectant exposure can promote antibiotic resistance, thus generating MDR bacteria (Mc Carlie et al. 2020). Jin et al. (2020) demonstrated that the process of chlorination promoted the horizontal transfer of plasmids and the exchange of antimicrobial resistance genes, highlighting its potential risk to public health. According to Joynson et al. (2002), increased resistance to benzalkonium chloride does not confer cross-resistance to antibiotics, but increased MIC to some antibiotics resulted in a slightly increased MIC of this disinfectant. However, this cross-resistance is not a consensus among researchers, and some studies have shown that bacteria remain susceptible to disinfectant products when they are correctly used. According to Maertens et al. (2019), disinfectants are generally used at concentrations above the MIC of wild-type isolates. In contrast, antibiotics are commonly used in concentrations closer to their MIC.

In the current study, there was no relationship between antimicrobial and disinfectant resistance. The reasons for the emergence of *S.* Heidelberg in broiler farms in southern of Brazil are still not clear. Complementary studies, including the whole-genome sequencing analysis and the pulsed-field gel electrophoresis patterns determination could elucidate the predominance of this serotype (Voss-Rech et al. 2019).

Unfortunately, the Brazilian government does not have an integrated program for monitoring antimicrobial and disinfectant resistance in human and animal foodborne pathogens, making the adoption of new measures to control and restrict the use of antimicrobials difficult (Borges et al. 2019). The benefits of monitoring antimicrobial resistance in foodborne pathogens are well-known. However, monitoring disinfectant resistance is also important to define sanitation programs, which include appropriate compounds, specific contact times, and concentrations of these products.

CONCLUSIONS

Salmonella Heidelberg strains were highly susceptible to sodium hypochlorite, regardless of the conditions applied and the year of isolation.

Increased resistance to benzalkonium chloride from 2006 to 2016 was observed only under specific conditions (200ppm for 5min at 25°C), and probably it is not related to the continuous use of this substance.

Similar results were obtained for antimicrobial resistance since increased resistance from 2006-2016 was observed only for tetracycline.

Further analysis should include a larger number of *S.* Heidelberg isolates from poultry origin and additional antimicrobial agents for more precise conclusions about the increasing in the antimicrobial resistance in the last years.

Conflict of interest statement.- The authors have no competing interests.

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