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Antimicrobial susceptibility of *Salmonella* spp and *Staphylococcus aureus* isolated from beef sold in Campo Grande, Mato Grosso do Sul, Brazil

Suscetibilidade antimicrobiana de Salmonella spp e Staphylococcus aureus isolados de carnes bovinas comercializadas em Campo Grande, Mato Grosso do Sul, Brasil

Daniele Bier[1*], Carina Elisei de Oliveira[1], Eduarda de Cássia Lima Brugeff[1], Michele Silva Areco[1], Isabella Nunes de Araújo Ramos[1], Agatha Alecxandra Pinesso Brunetta[1], Dhanielly Pereira Andrade[1]

[1]Universidade Católica Dom Bosco (UCDB), Campo Grande, Mato Grosso do Sul, Brasil
[*]Correspondente: danielebier@ucdb.br

Abstract
Hygiene failures in meat can be identified based on the evaluation of pathogenic microorganisms, which compromise the microbiological quality of food and can transmit food-borne diseases. The aim of the present study was to evaluate the hygienic quality of beef sold at supermarkets, butcher shops and public markets in the city of Campo Grande, state of Mato Grosso do Sul, Brazil, through the phenotypic and genotypic characterization of *Salmonella* spp. and Shiga toxin-producing *Escherichia coli* (STEC) as well as the investigation and quantification of *Staphylococcus aureus*. Seventy-one samples of beef from 17 commercial establishments were evaluated. Isolates were tested for antimicrobial susceptibility using the disk diffusion method recommended by the Clinical & Laboratory Standards Institute. *Salmonella* was found in 7.04% of the samples and 70.0% of the isolates were sensitive to the antimicrobials tested. A total of 25.35% of the samples were positive for *Staphylococcus aureus*, with counts ranging from 1.0 x 10^2 to 4.3 x 10^4 CFU/g; these isolates exhibited resistance to penicillin (87.5%), tetracycline (18.75%) and chloramphenicol (6.25%). None of the samples was positive for STEC. The detection of these pathogens in food poses a danger to public health, mainly due to the presence of antimicrobial-resistant isolates. These findings underscore the need for good hygiene and manufacturing practices at retail establishments.
Keywords: antibiotic; retail trade; food pathogens; resistance.

Resumo
As falhas na qualidade higiênico-sanitária da carne podem ser identificadas a partir da avaliação de microrganismos patogênicos que comprometem a qualidade microbiológica do alimento e podem veicular doenças de origem alimentar. O presente estudo objetivou avaliar a qualidade higiênica-sanitária de carnes bovinas comercializadas em supermercados, açougues e mercados públicos da cidade de Campo Grande (Mato Grosso do Sul, Brasil) por meio da pesquisa e caracterização fenotípica e genotípica de *Salmonella* spp. e *Escherichia coli* produtora de toxina *Shiga* (STEC) e pesquisa e contagem de *Staphylococcus aureus*. Foram avaliadas 71 amostras de carne bovina de 17 estabelecimentos comerciais que foram submetidas a pesquisa de detecção de *Salmonella* spp., *Escherichia coli* produtora de toxina *Shiga* (STEC) e pesquisa e contagem de *Staphylococcus aureus*. Os isolados obtidos foram submetidos ao perfil de sensibilidade aos antimicrobianos pelo teste de difusão em disco, de acordo com o *Clinical & Laboratory Standards Institute* (*CLSI*). Constatou-se a presença de *Salmonella* em 7,04% das amostras avaliadas, sendo que 70,0% dos isolados foram sensíveis aos antimicrobianos testados. Em relação ao *Staphylococcus aureus*, 25,35% das amostras foram positivas com contagens variando entre 1,0 x 10^2 a 4,3 x 10^4 UFC/g, sendo que os isolados apresentaram resistência para penicilina (62,5%), tetraciclina (18,75%) e cloranfenicol (6,25%). Nenhuma amostra apresentou-se positiva para STEC. A detecção desses patógenos em alimentos representa um perigo a saúde pública, principalmente, devido a presença de isolados resistentes a antimicrobianos. Além disso, ressalta-se a necessidade do emprego das boas práticas de higiene e fabricação nos estabelecimentos varejistas.
Palavras-chave: antibiótico; comércio varejista; patógenos alimentares; resistência.

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Introduction

In Brazil, the sale of raw beef must meet microbiological requirements determined by the National Health Surveillance Agency (ANVISA)[1] as well as the good practices stipulated and monitored by this agency.[2] Cattle are symptomatic carriers of enteric pathogens, such as *Salmonella* spp. and Shiga toxin-producing *Escherichia coli* (STEC) O157:H7 and have bacteria in the microbiota on the hide, such as *Staphylococcus aureus*, that can be transferred to the carcass during the slaughtering process and contaminate the meat.[3] Beef has intrinsic factors that contribute to bacterial multiplication and can be a vehicle for pathogens to humans,[4] increasing the risk of food-borne diseases.

Raw beef is reported to be one of the main vehicles for the transmission of food-borne diseases.[5] However, the contribution of meat to such diseases varies from country to country and is dependent on three main factors: the transmitted pathogenic agent, the consumption *per capita* of beef products and meat cooking and consumption habits in the country.[6] Epidemiological data reveal that meat was one of the most incriminated in food-borne illness outbreaks in Brazil, according for 5.3% of cases.[7] Some characteristics of retail establishments are considered improper practices that contribute significantly to bacterial development in meat, such as a lack of training of product handlers in good practices, a lack of hygiene in the work area, the use of poorly cleaned utensils and equipment, inadequate temperatures and cross-contamination.[8-4]

The aim of the present study was to evaluate the hygienic quality of beef sold at supermarkets, butcher shops and public markets in the city of Campo Grande (state of Mato Grosso do Sul, Brazil) through the phenotypic and genotypic characterization of *Salmonella* spp. and Shiga toxin-producing *Escherichia coli* (STEC) as well as the investigation and quantification of *Staphylococcus aureus*.

Material and methods

Sample collection

During the period from August 2016 to August 2018, 71 beef samples were collected from different supermarkets, butcher shops and public markets chosen randomly in the city of Campo Grande. The samples were acquired in an approximate quantity of 300 grams each. All products were weighed and wrapped by employees of the establishment using their standard materials in the traditional form of sale to reproduce what normally occurs in the merchant/consumer relationship. Samples were obtained from the butcher section of the stores or directly from refrigerated shelves near the butcher section. All samples were transported in coolers containing recyclable ice.

Preparation of initial sample

A total of 225 mL of 1% buffered peptone water (BPW) were added to 25 ± 0.2 g of each meat sample homogenized for approximately 60 seconds in a "stomacher" and incubated at 37 ± 1º C for 18 ± 2 h. This was considered the initial sample (dilution: 10^{-1}) for all techniques described below.

Detection of Salmonella spp.

For the detection of *Salmonella* spp. in the meat samples, the method described in the International Organization for Standardization (ISO 6579:2002)[9] was used with modifications. After enrichment (with BPW), selection (with Muller-Kauffmann tetrathionate broth with novobiocin and Rappaport-Vassiliadis soya broth) and differentiation (with xylose-lysine-deoxycholate agar and *Salmonella-Shigella* agar), colonies suspected of being *Salmonella* spp. isolated in nutrient agar were submitted to complementary biochemical tests (indole, urea, motility, lysine decarboxylation, H_2S production, methyl red, Voges-Proskauer, carbohydrate fermentation, citrate and β-galactosidase).

PCR for confirmation of Salmonella spp.

After incubation at 37 °C for 18-24 horas, strains of *Salmonella* spp. were sown in TSA medium. A portion of this culture was transferred to microtubes containing 100 µL of sterilized ultrapure water (Milli-Q, Millipore) and centrifuged at 14000 x g for 3 seconds. The extraction of bacterial DNA was performed using the DNeasy Blood & Tissue Kit (QIAGEN, Valencia, CA, USA) following the manufacturer's instructions. The isolated strains of *Salmonella* spp. were investigated for the presence of the virulence gene *invA*, based on Skyberg et al.[10] The amplified products were applied to 1.5% agarose gel in TBE 0.5 X followed by electrophoresis for approximately 40 minutes at 70 V in a horizontal cube containing TBE 0.5 X. The gel was stained with SYBR Gold (Invitrogen, USA) and the image was recorded using a photodocumentation system.

Detection of Shiga toxin-producing Escherichia coli

The method described in the *Compendium of Methods for the Microbiological Examination of Foods* (2001)[11] was used for the detection of STEC in the beef samples.

PCR for investigation of toxin-producing genes

Strains confirmed as *E. coli* were submitted to

PCR for the investigation of the *stx1* and *stx2* genes following the method described by Paton & Paton.[12] The isolated collected from the beef samples and control strains were sown in BHI broth and incubated at 35 °C for 18-24 h. Aliquots of 1 mL of broth were submitted to centrifugation (14,000 x g) for two minutes. Bacterial DNA extraction was performed using the DNeasy Blood & Tissue Kit (QIAGEN, Valencia, CA, USA) following the manufacturer's instructions. For multiplex PCR, a solution was prepared containing 2.5 µL of *Taq* buffer 10x, 0.75 µL of $MgCl_2$ (50 mM), 1.0 µL of dNTP (5 mM), 0.15 µL de *Taq* DNA polymerase and 3 µL of DNA (approximately 20 ng), with the addition of four pairs of primers (IDT, Integrated DNA Technologies, USA) at concentrations of 10 pmoles and free DNase and RNase ultrapure water (Invitrogen, USA) for a final reaction volume of 25 µL. The amplified products were applied to 1.0% agarose gel in TBE 1 X followed by electrophoresis for approximately 60 minutes at 100 V in a horizontal cube containing TBE 1 X. The gel was stained with SYBR Gold (Invitrogen, USA) and the image was recorded using a photodocumentation system.

Staphylococcus aureus count

The detection and quantification of *Staphylococcus aureus* were performed using the method described in Normative Instruction n° 62, from August 2003 – Official Analytical Methods for Microbiological Analyses for the Control of Products of an Animal Origin and Water.[13]

Antimicrobial susceptibility

The antimicrobial susceptibility of the strains of *Salmonella* spp. and *Staphylococcus aureus* was determined according to the Clinical and Laboratory Standards Institute,[14] employing the following antimicrobials: cefepime (30 µg), ciprofloxacin (5 µg), chloramphenicol (30 µg), gentamicin (10 µg) and tetracycline (30 µg). Ampicillin (10 µg) and penicillin (10 µg) were also used for *Salmonella* spp. and *Staphylococcus aureus*, respectively. The results of the susceptibility profile were interpreted according to the manufacturer's instructions. Descriptive statistical analysis was performed of sensitivity and resistance to the antimicrobials with the calculation of absolute and relative frequencies. Multiple antibiotic resistance (MAR) was determined following the method described by Krumperman.[15]

Control strains

Strains from the Health Surveillance Reference Microorganism Collection (CRMVS, FIOCRUZ-INCQS, Rio de Janeiro, RJ, Brazil) were used as negative and positive controls for all techniques: *Escherichia coli* INCQS 00033 (ATCC 25922), *Escherichia coli* INCQS 00171 (CDC EDL-933), *Salmonella enterica* subsp. *enterica* serovar Enteritidis INCQS 00258 (ATCC 13076) and *Salmonella enterica* subsp. *enterica* serovar Typhimurium INCQS 00150 (ATCC 14028). The *Staphylococcus aureus* ATCC 25923 strain was also used.

Results and discussion

Among the 71 samples of beef acquired from 17 different establishments, five (7.04%) were positive for *Salmonella* spp. These five samples were from two supermarkets (A and I) (Table 1). Several studies have reported the occurrence of this pathogen in raw meat sold at retail establishments, with rates ranging from 7.10% to 86.67%.[17,18,19,20,21] This bacterium in meat products poses a risk to consumer health[16] and may demonstrate improper conditions in the obtainment, processing, handling and/or sale of the raw material.[22] Hussain et al.[23] reported the absence of this microorganism in beef samples from supermarkets with the use of good hygiene practices.

Brazilian legislation determines the absence of *Salmonella* spp. in 25 grams of raw beef analyzed,[1] indicating that the positive meat samples found in the present study were not safe for consumption. The presence of this pathogen in food can cause gastroenteritis, fever and stomach cramps and can lead to more severe cases, especially in children, older people and immunosuppressed individuals.[24]

None of the samples tested in this study was positive for Shiga toxin-producing *E. coli* (STEC) (Table 1). Only one biochemically positive *E. coli* isolate was found, which was tested using PCR for the *Stx*1 and *Stx*2 genes associated with virulence, but did not present specific fragments for *Stx* genes. Most of the cases and outbreaks caused by STEC have been attributed to the consumption of beef and pork.[25,26] The two *Shiga* enterotoxins (*stx*1 and *stx*2) produced by this bacterial line are responsible for clinical manifestations in patients, such as bloody diarrhea and hemolytic uremic syndrome.[27,30] The absence or low prevalence of STEC in beef has also been reported in previous studies.[24,28,29,30] One reason for the absence of these strains in meat may be the capacity of this bacterium to enter a viable but non-culturable (VBNC) state, which hinders its detection using conventional methods.[31] Meyer-Broseta et al.[32] argue that the prevalence of cattle contaminated by strains of STEC is likely underestimated due to inefficient sampling procedures.

Table 1. Results of microbiological analysis of raw beef obtained from commercial establishments in Campo Grande, state of Mato Grosso do Sul, Brazil.

Sample	Cut	Establishment	*Salmonella spp. in 25 g*	*S. aureus (CFUs/g)*	*Escherichia coli (STEC)*
1	Striploin	Market A	Presence	---	Absence
2	Knuckle	Market A	Presence	---	Absence
3	Brisket point	Market A	Absence	---	Absence
4	Rump steak	Market A	Absence	---	Absence
5	Shank	Market A	Absence	---	Absence
6	Chuck	Market B	Absence	14.8×10^3	Absence
7	Topside	Market B	Absence	11×10^3	Absence
8	Shoulder clod	Market B	Absence	---	Absence
9	Brisket point	Market B	Absence	---	Absence
10	Brisket point	Market B	Absence	---	Absence
11	Outside flat	Market C	Absence	---	Absence
12	Striploin	Market C	Absence	---	Absence
13	Topside	Market C	Absence	---	Absence
14	Topside	Market C	Absence	---	Absence
15	Sirloin cap	Hypermarket D	Absence	---	Absence
16	Topside	Hypermarket D	Absence	---	Absence
17	Striploin	Hypermarket D	Absence	---	Absence
18	Trimmings	Market E	Absence	---	Absence
19	Shank	Market E	Absence	---	Absence
20	Outside flat	Hypermarket F	Absence	---	Absence
21	Topside	Hypermarket F	Absence	---	Absence
22	Rump steak	Hypermarket F	Absence	33×10^3	Absence
23	Striploin	Hypermarket F	Absence	---	Absence
24	Knuckle	Hypermarket F	Absence	---	Absence
25	Outside flat	Market G	Absence	2.0×10^4	Absence
26	Brisket point	Market G	Absence	---	Absence
27	Topside	Market G	Absence	---	Absence
28	Shank	Market G	Absence	30×10^3	Absence
29	Rump steak	Market G	Absence	---	Absence
30	Topside	Hypermarket H	Absence	---	Absence
31	Striploin	Hypermarket H	Absence	---	Absence
32	Rump steak	Hypermarket H	Absence	---	Absence
33	Outside flat	Hypermarket H	Absence	---	Absence
34	Knuckle	Hypermarket H	Absence	---	Absence
35	Topside	Market I	Presence	---	Absence
36	Outside flat	Market I	Presence	---	Absence
37	Knuckle	Market I	Absence	---	Absence
38	Shank	Market I	Presence	---	Absence
39	Rump steak	Market I	Absence	---	Absence
40	Brisket point	Supermarket J	Absence	---	Absence
41	Striploin	Supermarket J	Absence	---	Absence
42	Rump steak	Supermarket J	Absence	---	Absence
43	Knuckle	Supermarket J	Absence	---	Absence
44	Knuckle	Butcher shop K	Absence	---	Absence
45	Rump steak	Butcher shop K	Absence	---	Absence
46	Brisket point	Butcher shop K	Absence	---	Absence
47	Striploin	Butcher shop K	Absence	---	Absence
48	Knuckle	Supermarket L	Absence	18×10^4	Absence
49	Rump steak	Supermarket L	Absence	---	Absence
50	Brisket point	Supermarket L	Absence	---	Absence
51	Striploin	Supermarket L	Absence	---	Absence
52	Knuckle	Market M	Absence	---	Absence
53	Rump steak	Market M	Absence	---	Absence
54	Brisket point	Market M	Absence	---	Absence
55	Striploin	Market M	Absence	---	Absence
56	Knuckle	Hypermarket N	Absence	---	Absence
57	Rump steak	Hypermarket N	Absence	9.2×10^3	Absence
58	Brisket point	Hypermarket N	Absence	---	Absence
59	Striploin	Hypermarket N	Absence	---	Absence
60	Brisket point	Market O	Absence	---	Absence
61	Striploin	Market O	Absence	43×10^3	Absence
62	Rump steak	Market O	Absence	50×10^3	Absence
63	Knuckle	Market O	Absence	21×10^3	Absence
64	Brisket point	Butcher shop P	Absence	17×10^3	Absence
65	Striploin	Butcher shop P	Absence	21×10^3	Absence
66	Rump steak	Butcher shop P	Absence	43×10^3	Absence
67	Eye round	Butcher shop P	Absence	10×10^3	Absence
68	Brisket point	Butcher shop Q	Absence	19×10^3	Absence
69	Topside	Butcher shop Q	Absence	21×10^3	Absence
70	Rump steak	Butcher shop Q	Absence	91×10^3	Absence
71	Knuckle	Butcher shop Q	Absence	47×10^3	Absence
Tolerance			Absence	-	Absence

Regarding *Staphylococcus aureus*, 25.35% (18/71) of the samples were positive, with counts ranging from 1.0 x 10^2 to 4.3 x 10^4 colony-forming units (CFUs)/g (Table 1). Likewise, Naas et al.[33] identified the presence of this bacterium in 35.5% of raw beef samples found at retail establishments. Baghbaderani et al.[34] related the high incidence of *S. aureus* in beef to poor hygiene practices at retail establishments and the excessive, inadequate handling of these products. Although Brazilian legislation does not determine limits for the presence of this pathogen in raw beef,[1] the investigation of *S. aureus* in food products can serve as an indicator of the hygiene and processing practices of commercial establishments.[35] *S. aureus* counts up to 10^3 CFUs/g may indicate inappropriate hygiene and/or ineffective processing, whereas counts between 10^3 and 10^4 CFUs/g suggest a public health risk and counts of 10^5 CFUs/g are considered critical, indicating an epidemiological risk, as the production of enterotoxins by the bacterium can occur at this quantity.[36]

The analysis of the susceptibility of *Staphylococcus aureus* isolates to antimicrobials (Figure 1) revealed 62.5% resistance to penicillin (10 µg). However, 100% sensitivity to cefepime (30 µg), ciprofloxacin (5 µg) and gentamicin (10 µg) was found. Among all isolates, the multiple antibiotic resistance (MAR) index ranged from 0.16 to 1.6. Other studies also report the resistance of *S. aureus* isolates to β-lactam antibiotics, including penicillin[33,36] as well as sensitivity to aminoglycosides, quinolones and tetracyclines.[34, 37] Most isolates (87.5%) were sensitive to chloramphenicol (30 µg), which is in agreement with results described in the study conducted by Irkin et al.,[37] in which all *S. aureus* isolates obtained from meat products were sensitive to this antibiotic.

The sensitivity profile to antimicrobials enables the screening of the propagation of multi-resistant strains.[38,39] The occurrence of multi-resistance strains of *Salmonella* spp. in meat at retail establishments has been reported in some studies.[17,19] The high percentage of sensitivity found among the isolates in the present study explains the absence of these strains in the samples analyzed. Likewise, Ekli et al.[21] reported the inhibition of isolates of *Salmonella* spp. in beef when testing gentamicin and ciprofloxacin. Bergamo et al.[19] and Thung et al.[17] also reported susceptibility to these antimicrobials. This pathogen has lower resistance to fluroquinolone and aminoglycoside antibiotics compared to other enterobacteria.[40]

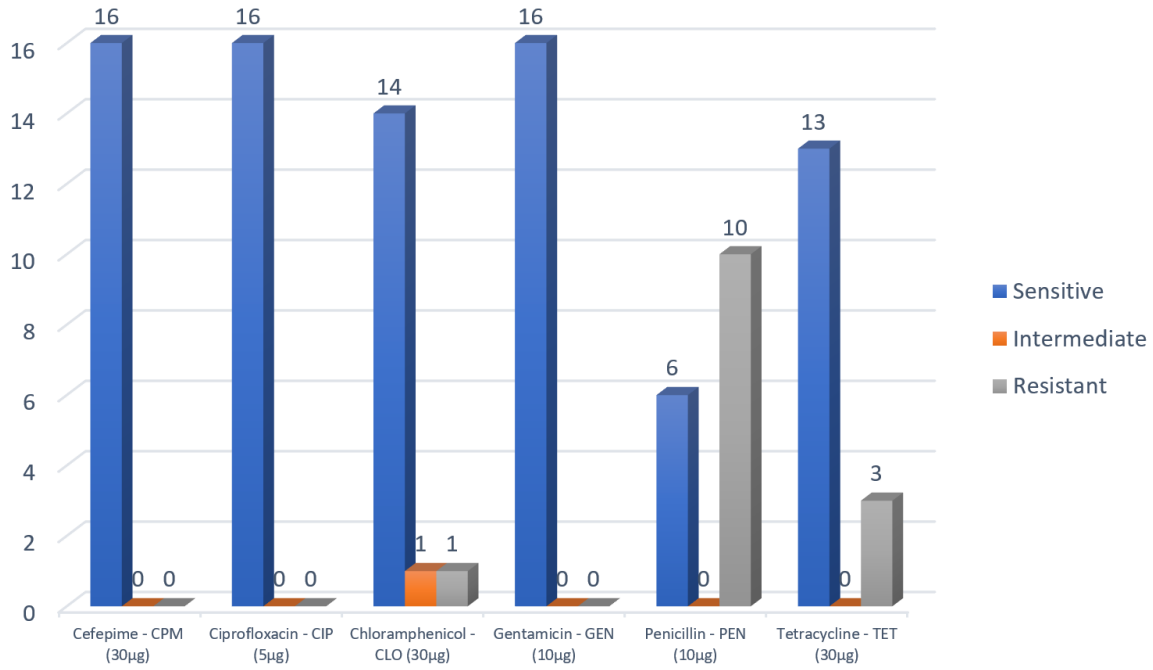

Figure 1. Number of *Staphylococcus aureus* isolates from raw beef sold in city of Campo Grande (Mato Grosso do Sul, Brazil) with sensitivity, intermediate resistance and resistance to antimicrobials tested.

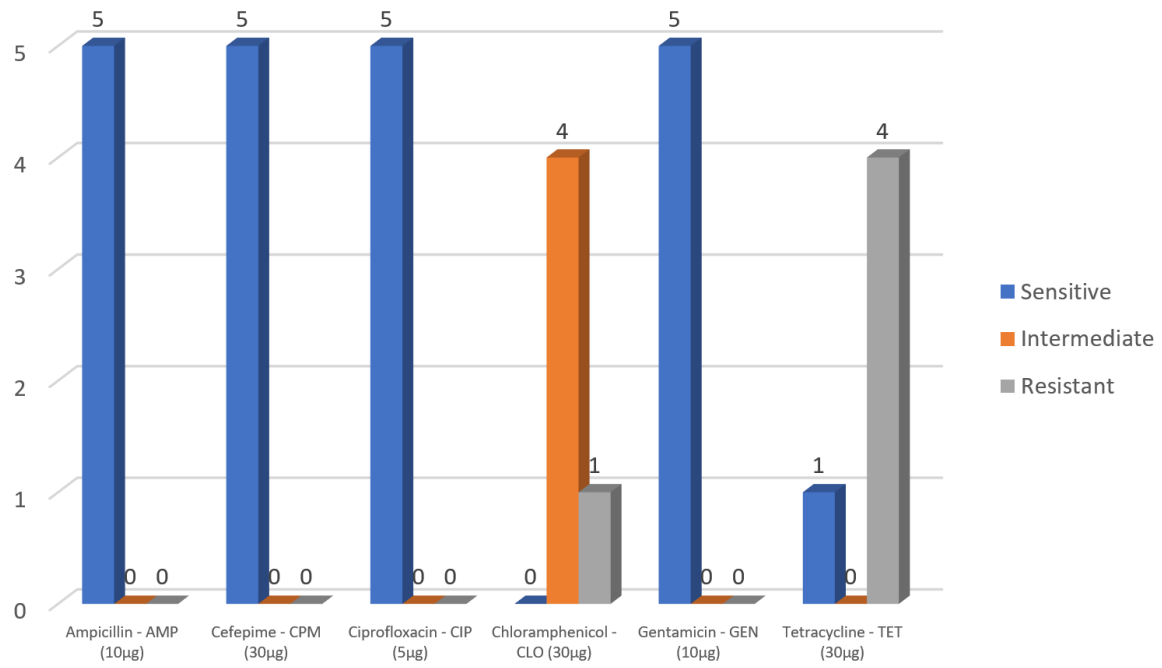

Figure 2. Number of *Salmonella* spp. isolates from raw beef sold in city of Campo Grande (Mato Grosso do Sul, Brazil) with sensitivity, intermediate resistance and resistance to antimicrobials tested.

Conclusion

The presence of *Salmonella* spp. and *Staphylococcus aureus* in beef sold at supermarkets poses a direct risk for consumers and may indicate the improper handling of this food. In ten of the 17 establishments sampled, at least one sample of meat was positive for one of the two pathogens studied, underscoring the need for the adoption of more rigorous hygiene practices to reduce the occurrence of contamination of the final product. The only strain of *E. coli* isolated did not have the *stx*1 or *stx*2 genes associated with the virulence of this bacterium. The isolates presented variability in terms of sensitivity to antimicrobials, exhibiting high sensitivity to the majority of antibiotics tested. However, the resistance to some antibiotics underscores the risk of these contaminated foods for consumers.

Declaration of conflicts of interest

The authors declare no conflicts of interest.

Author contributions

Conceptualization: D. Bier. *Project administration*: D. Bier. *Supervision*: D. Bier. *Writing (review and editing)*: D. Bier. *Writing (original draft)*: D. Bier and E.C.L. Brugeff. *Resources*: C.E. Oliveira. *Investigation*: C.E. Oliveira, E.C.L. Brugeff, M.S. Areco, I.N.A. Ramos, A.A.P. Brunetta and D.P. Andrade.

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