Salmonella Schwarzengrund, Akufo, and O:16 isolated from vacuum-packaged beef produced in the state of Mato Grosso, Brazil

Bárbara Muller1, Adelino Cunha-Neto1, Vinicius Silva Castro2, Ricardo Cesar Tavares Carvalho1,3, Larrayane Albuês Carvalho Teixeira1, Dália dos Prazeres Rodrigues4, Eduardo Eustáquio de Souza Figueiredo1,5

1 Postgraduate program in Nutrition, Food and Metabolism, Faculty of Nutrition, Federal University of Mato Grosso, Cuiabá, Mato Grosso, Brazil
2 Postgraduate program in Food Science, Chemistry Institute, Federal University of Rio de Janeiro, Rio de Janeiro, Rio de Janeiro, Brazil
3 Postgraduate Program in Animal Bioscience, Faculty of Veterinary Medicine, Cuiabá University, Cuiabá, Mato Grosso, Brazil
4 National Reference Laboratory for Diagnosis of Enteric Bacteria, Oswaldo Cruz Fundation, Rio de Janeiro, Rio de Janeiro, Brazil
5 Postgraduate program of Animal Science, Faculty of Agronomy and Zootechnics, Federal University of Mato Grosso, Cuiabá, Mato Grosso, Brazil

Abstract
Introduction: Salmonella spp. is a pathogen associated with foodborne infections, mainly in foods of animal origin. In this context, the present study investigated the occurrence of Salmonella serotypes, genotypes and the antimicrobial resistance profiles of strains in fresh beef produced in Mato Grosso, Brazil.

Methodology: A total of 107 samples from 13 different slaughterhouses in the Mato Grosso were analyzed. Suggestive Salmonella spp. colonies detected during the biochemical screening were submitted to DNA extraction, and hila gene amplification was used for the PCR reaction. Antimicrobial resistance analyses were performed using 17 antimicrobial agents from eight different classes by the disk diffusion method. Strains exhibiting multiple drug resistances were submitted to PCR genotyping based on repetitive sequences (rep-PCR), using a commercial semiautomatic DiversiLab® system.

Results: A total of 5.6% (6/107) of the samples tested positive by the conventional method and were confirmed by PCR, namely two S. Akuafo, two non-typable Salmonella enterica strains, one Salmonella O:16 serovar, and one S. Schwarzengrund. The antimicrobial resistance profiles indicated resistance to gentamicin (30%), tetracycline, nitrofurantoin, and trimethoprim + sulfamethoxazole (16%). Genotyping indicated a 70% difference between S. Schwarzengrund and the non-typable Salmonella strains. No genetic similarities were observed between the six Salmonella isolates based on rep-PCR, including two S. Akuafo.

Conclusions: The results obtained herein corroborate that Salmonella serovar Schwarzengrund is commonly isolated in animal products in the state of Mato Grosso, Brazil, also highlighting the presence of two unusual Salmonella serovars in beef (Akufo and O:16).

Key words: Food production; microbial contamination; foodborne illness; beef contamination; beef exports.


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Introduction
Salmonella are a public health concern in both high- and low-income countries [1]. Typhoid fever is mainly endemic in low to middle income countries (LMICs), although non-typhoidal Salmonella, including invasive serovars that cause systemic diseases, are becoming a public health concern worldwide and comprise the third leading cause of death from foodborne illnesses [1,2]. This bacterium is commonly associated with meat consumption, such as beef, pork and poultry, and derived products [2]. Food chain contamination by Salmonella in Brazil has been noted, and S. Schwarzengrund has been described for both chicken and pork [3], as well as for cheese [4]. The importance of beef to the spread of Salmonella spp. in different Brazilian regions, including the south [5,6], northeast [7] and southeast [8] is clear, although more data are required.

Moreover, the presence of multidrug-resistant (MDR) Salmonella strains has been reported in animal production [9]. Antimicrobial resistance in bacteria can lead to human infections that often require prolonged
and more expensive treatments and lead to higher disability and death rates compared to sensitive strains [10]. In this regard, a comprehensive study on Salmonella spp. in chicken slaughterhouses conducted by our group in the state of Mato Grosso indicated the occurrence of multidrug-resistant strains [11] and a review of available genome sequences demonstrates the paucity of current data [12]. Therefore, assessments in this regard are indispensable to the elaboration of effective disease control plans, and the accurate identification of Salmonella spp. in animals and food products is the key to understanding the epidemiological dynamics of salmonellosis [13,14].

The state of Mato Grosso is located in the central-west region of Brazil and is currently the largest beef producer in the country [15]. Ninety percent of the cattle herds reared in the region are destined to beef production for both the internal and external markets [16]. This state was responsible for 15.4% (1.1 million tons) of all Brazilian exports, generating 4.3 billion dollars [15]. In this context, the present study aimed to investigate the occurrence of Salmonella spp. in fresh vacuum-packaged beef, through serotyping and genetic profile determinations and the identification of the presence of antimicrobial-resistant strains.

Methodology

Sample collection

A total of 107 fresh beef samples were assessed, represented by different meat cuts, with an average weight of 1.5 kg. All fresh beef samples were analyzed chilled and vacuum-packaged. The samples were collected randomly from 13 different slaughterhouses, which undergo either federal or state inspection services, located in 12 different cities in Mato Grosso (Figure 1). The selected beef cuts were: paleta (supraspinatus), acém (trapezius thoracis), picanha (biceps femoris), alcatra (medium gluteos), filet mignon (psosas major), lagarto (semitendinosus) and lombo (longissimus dorsi). The samples were received at the Food Molecular Microbiology Laboratory, at the Federal University of Mato Grosso (LABMMA/UFMT), chilled between 1 °C and 8 °C, from April to October 2016.

Salmonella spp. detection

The samples were submitted for Salmonella detection based on an adapted ISO-6579:2002 protocol [17]. Briefly, ten grams of each sample were pre-enriched in a suspension containing 90 mL of Buffered Peptone Water broth (Oxoid®, Basingstoke, United Kingdom), and incubated at 41.5 °C for 24 hours. Subsequently, 0.1 mL were added to 10 mL of Rappaport-Vassiliadis broth (Oxoid®, Basingstoke, United Kingdom) and 1 mL of the same culture in 9 mL of Muller Kauffmann Tetrathionate broth (Himedia®, Mumbai, India), incubated at 42 °C and 37 °C, respectively, for 24 hours. After incubation, one microliter of each presumptive Salmonella growth medium was inoculated onto Xylose Lysine Deoxycholate agar (Himedia®, Mumbai, India) and Brilliant Green Agar (Himedia®, Mumbai, India) plates and incubated at 37 °C by 24 hours. Typical colonies (red colony with a black center and red translucent halo) were picked and cultivated again on Nutrient agar incubated at 37 °C for 24 hours. Subsequently, suspected colonies were subjected to biochemical tests on Triple Sugar Iron Agar (TSI), Lysine Decarboxylase Agar (LIA) and Urea Agar. Strains with typical reactions were considered suggestive for Salmonella spp.

DNA extraction and quantification

Suggestive Salmonella spp. colonies detected in the biochemical screening were inoculated in 10 mL of Brain Heart Infusion Broth - BHI (Himedia®, Mumbai, India), and incubated at 35 °C for 24 hours. Subsequently, a 1.5 mL aliquot was centrifuged at 14,000 × g for 5 minutes. The bacterial pellet was then resuspended in 500 µL of ultrapure water, heated to 95 °C for 15 minutes and quickly cooled to 0 °C ± 1 °C for 20 minutes. Subsequently, an aliquot was centrifuged at 14,000 × g for 5 minutes, and 100 µL of the supernatant were collected and stored at -20 °C. DNA was quantified by the fluorimetry method using the QUBIT 2.0 system (Invitrogen®, St. Louis, USA), according to the manufacturer’s recommendations. DNA
quantification is recommended to avoid possible false negative results associated to a low amount of target DNA in the PCR solution.

**PCR assays**

The *hiA* gene (*Salmonella* spp. specific) was used for the PCR reaction [18,19]. The reaction was carried out in a final volume of 25 μL, containing 1x of buffer solution, 3.5 mM MgCl2 (Fermentas®, Walthan, USA), 0.2 mM dNTPs (Fermentas®, Walthan, USA), 1.0 mM of each primer (Invitrogen®, St. Louis, USA), 1U of Taq DNA Polymerase Platinum (Invitrogen®, St. Louis, USA) and approximately 40ng/μL of bacterial DNA. The following conditions were applied: initial denaturation at 94° C for 5 minutes, followed by 35 cycles at 94 °C for 1 minute, 58 °C for 1 minute and 72 °C for 1 minute, and a final step at 72 °C for 10 minutes. The reactions were performed employing a Veriti® thermocycler (Applied Biosystems®, Massschusetts, USA), 0.2 mM dNTPs (Fermentas®, Walthan, USA), 1.0 mM of each primer (Invitrogen®, St. Louis, USA), 1U of Taq DNA Polymerase Platinum (Invitrogen®, St. Louis, USA) and approximately 40ng/μL of bacterial DNA. The following conditions were applied: initial denaturation at 94° C for 5 minutes, followed by 35 cycles at 94 °C for 1 minute, 58 °C for 1 minute and 72 °C for 1 minute, and a final step at 72 °C for 10 minutes. The reactions were performed employing a Veriti® thermocycler (Applied Biosystems®, Massschusetts, USA), and results were observed by the electrophoresis technique using a 10 μL aliquot of the PCR product on 1.5% agarose gels with TBE buffer (45 mM Tris pH 8.3, 45 mM borate and 2 mM EDTA). A standard 100 bp ladder or gel ruler and 3 μL of GelRed (Biotium®, Fremont, USA) were used.

**Salmonella serotyping**

All the isolated strains were sent to the National Reference Laboratory on the Diagnosis of Enteric Bacteria at the Oswaldo Fiocruz Institute for serotyping. Somatic and flagellar antigens were assessed using mono and polyvalent antisera with and without flagellar-phase induction [20].

**Antimicrobial Susceptibility Tests**

The antimicrobial resistance analyses were performed using 17 antimicrobial agents from eight different classes by the disk diffusion method, as described by the Clinical and Laboratory Standards Institute [21]. Briefly, the isolates were spiked in 5 mL of BHI broth and incubated at 37 °C for 2 hours. Subsequently, the solutions were inoculated on Mueller Hinton 2 agar with a sterile swab. The disks containing the antibiotics (Cefar Diagnóstica®, São Paulo, Brazil) were distributed on Petri dishes (Table 1) and incubated at 37 °C for 18 hours. Subsequently, the inhibition zones were measured, and the strains were classified as either resistant, intermediate resistance or sensitive, according to the CLSI guide [22]. Strains resistant to three different classes of antimicrobials were classified as multi-resistant strains (MDR).

**MDR genotyping strains**

Strains exhibiting multiple drug resistances were submitted to PCR genotyping based on repetitive sequences (rep-PCR), using a commercial semiautomatic system DiversiLab® (bioMérieux, Craponne, France). The rep-PCR reaction was applied according to the manufacturer’s instructions using the Diversilab® *Salmonella* kit. The rep-PCR products were separated and detected on a microfluidic chip using a Bioanalyzer 2100 (Agilent, Santa Clara, USA). All analyzed samples were compared by a Pearson’s correlation analysis using the DiversiLab® software (version vr3.3.40). The distance from the matrices and the unweighted pair group were used to create a

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Code</th>
<th>Class</th>
<th>Disk Content (µg)</th>
<th>Zone diameter breakpoints (mm) for each serotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>AMP</td>
<td>Penicillin</td>
<td>10</td>
<td>S:  ≥ 17, I: 14 – 16, R:  ≤ 13</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>ATM</td>
<td>Monobactam</td>
<td>30</td>
<td>S:  ≥ 21, I: 18 – 20, R:  ≤ 17</td>
</tr>
<tr>
<td>Cefepime</td>
<td>CPM</td>
<td>Cephem</td>
<td>30</td>
<td>S:  ≥ 25, I: 19 – 24, R:  ≤ 18</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>FOX</td>
<td>Cephem</td>
<td>30</td>
<td>S:  ≥ 18, I: 15 – 17, R:  ≤ 14</td>
</tr>
<tr>
<td>Ceftiofur</td>
<td>TIO</td>
<td>Cephem</td>
<td>30</td>
<td>S:  ≥ 21, I: 18 – 20, R:  ≤ 17</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>CL</td>
<td>Phenicol</td>
<td>30</td>
<td>S:  ≥ 18, I: 13 – 17, R:  ≤ 12</td>
</tr>
<tr>
<td>Florfenicol</td>
<td>FEN</td>
<td>Phenicol</td>
<td>30</td>
<td>S:  ≥ 19, I: 15 – 18, R:  ≤ 14</td>
</tr>
<tr>
<td>Imipenem</td>
<td>IPM</td>
<td>Carabapenems</td>
<td>10</td>
<td>S:  ≥ 23, I: 20 – 22, R:  ≤ 19</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>GEN</td>
<td>Aminoglycoside</td>
<td>10</td>
<td>S:  ≥ 15, I: 13 – 14, R:  ≤ 12</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>TET</td>
<td>Tetracycline</td>
<td>300</td>
<td>S:  ≥ 15, I: 12 – 14, R:  ≤ 11</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>NAL</td>
<td>Quinolone</td>
<td>30</td>
<td>S:  ≥ 19, I: 15 – 18, R:  ≤ 14</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>CIP</td>
<td>Fluoroquionline</td>
<td>5</td>
<td>S:  ≥ 31, I: 21 – 30, R:  ≤ 20</td>
</tr>
<tr>
<td>Enrofloxacin</td>
<td>ENR</td>
<td>Fluoroquionline</td>
<td>5</td>
<td>S:  ≥ 21, I: 17 – 20, R:  ≤ 16</td>
</tr>
<tr>
<td>Sulfamethoxazole/Trimethoprim</td>
<td>SXT</td>
<td>Folate Pathway Inhibitor</td>
<td>25</td>
<td>S:  ≥ 16, I: 11 – 15, R:  ≤ 10</td>
</tr>
<tr>
<td>Sulfonamides</td>
<td>SSS</td>
<td>Folate Pathway Inhibitor</td>
<td>300</td>
<td>S:  ≥ 19, I: 15 – 18, R:  ≤ 14</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>TMP</td>
<td>Folate Pathway Inhibitor</td>
<td>5</td>
<td>S:  ≥ 16, I: 11 – 15, R:  ≤ 10</td>
</tr>
<tr>
<td>Nitrofurantoin</td>
<td>NIT</td>
<td>Nitrofuran</td>
<td>300</td>
<td>S:  ≥ 17, I: 15 – 16, R:  ≤ 14</td>
</tr>
</tbody>
</table>

S: susceptible; I: Intermediate; R: Resistant; Adapted from CLSI [22].
Results

Of the 107 evaluated samples, 5.6% (6/107) tested positive. The serotype results comprise two non-typable Salmonella enterica subspecies strains, two S. Akufo, one Salmonella serovar (O:16), and one S. Schwarzengrund. The antimicrobial susceptibility profiles of the six isolated strains indicate that all displayed multi-drug resistance (MDR). Strains exhibited 100% resistance (0% susceptibility) or intermediary resistance to ceftiofur, nalidixic acid, ciprofloxacin, and chloramphenicol (Figure 2). Concerning antibiotic resistance, strains were sensitive to imipenem (100%), tetracycline (83.3%), cefepime (83.3%), and gentamicin (66.6%) (Figure 2).

A method based on repetitive sequences (rep-PCR) was applied to compare genetic similarities between the detected strains using the commercial semiautomatic system DiversiLab® (BioMérieux). The results indicate a difference of over 70% between S. Schwarzengrund (S01) and Non-Typable (NT) Salmonella (S02) and Salmonella (S04). In addition, differences are phenotypically highlighted by S. Schwarzengrund’s sensitivity to ampicillin (AMP), cefoxitin (FOX), aztreonam (ATM), florfenicol (FFN) and resistance to (TET) tetracycline (Figure 2), which differs from the aforementioned Salmonella NT strains. A 92% genotypic non-clonal similarity was observed between S. Akufo and Salmonella (O:16), respectively. It is important to note that all antibiotic results were different between the S05 and S06 strains. Further whole genome analyses will aid in discriminating phylogenetic relationship among strains.

Discussion

The absence of Salmonella spp. is a criterion established in different standard methods and country requisites for beef importation. The absence criterion is noted in the Brazilian legislation and in importing countries, such as those belonging to the European Union [23,24]. To meet this criterion, slaughterhouses apply Good Manufacturing Practices (GMP) tools and the Hazard Analysis and Critical Control Points (HACCP) plan. These control tools aim to monitor and prevent bacterial cross-contamination of beef during slaughter, processing, and commercialization. These programs are applied continuously by companies to achieve safe products, avoiding embargoes and improving the competitiveness of Brazilian beef [25].

Previous studies carried out in slaughterhouses, and beef butcher shops in Cuiabá and Barra do Garças, Mato Grosso have indicated the occurrence of Salmonella spp. (8.3% and 17%, respectively) in samples prior to processing [26,27]. The present study in the state of Mato Grosso demonstrated the presence of viable Salmonella in the final product packaged under a vacuum atmosphere. Survival of Salmonella in vacuum-packed products has been previously reported in the literature. For example, Djordjević et al. [28] estimated that the use of both vacuum and modified packaging was not sufficient to obtain a Salmonella-free product. These results reinforce the need to improve quality control tools to detect potential quality control program operation deficits, as vacuum packaging is the main form of beef product export and represents an actual risk to consumers.

To describe the prevalence of Salmonella serovars, our group has previously carried out different studies in the state of Mato Grosso in animal (fish, beef and chicken slaughterhouses) and milk derivative (cheeses) products [4,11,25,29]. It is interesting to note that the Schwarzengrund serovar was present in all analysed matrices, including those assessed in the present study. Therefore, this serovar seems to be commonly found in the state of Mato Grosso. Moreover, the presence of Schwarzengrund contamination in final products indicates an imminent risk of foodborne illness, as infections by this serovar have been previously reported in beef [30]. Another serovar isolated in the present study, S. Akufo, has been described in environmental samples from Portugal [31], and in farmed fish from Mato Grosso, Brazil [32]. However, there is no reference to this serovar as a animal origin food...
contaminant or associated to human infections. Another unusual contamination is noted herein concerning the Salmonella enterica subsp. enterica (O:16) serovar.

Serovars that cause enteric fever were not isolated in the present study. However, it is estimated that non-typhoid Salmonella is responsible for 155,000 deaths among 94 million annual infection cases worldwide [33]. The global expansion of genes that regulate the multidrug resistance (MDR) to antibiotics, mainly ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline is correlated to this increase, through horizontal transmission between Salmonella serovars, resulting in MDR strains [33]. A common characteristic for all the Salmonella spp. strains detected herein was resistance towards three or more antimicrobial classes (Figure 3), with all strains classified as multidrug-resistant (MDR). The hypothesis for this is that strains presenting a wild profile or containing several resistance genes are able to better overcome certain hurdles present both intrinsically and extrinsically in the final meat product, such as cooling temperatures and an anaerobic environment, favoring the survival of resistant strains compared to other sensitive strains [34].

This resistance may be associated with the use of antibiotics in veterinary medicine for therapeutic or preventive purposes, or their application as growth promoters in animal feed, leading to the emergence of resistant Salmonella strains [35]. Hong et al. [36] observed that Salmonella strains isolated from cattle from Minnesota, in the USA, displayed resistance to Sulfadimethoxine (79 - 87%), Ampicillin, Cefotiofur, Florfenicol and Oxytetracycline (30 - 70%), and Neomycin, Sulfamethoxazole/trimethoprim (15%). In the present study, resistance was lower compared to the results reported by Hong et al. [36], except for neomycin and sulfadimethoxine (not evaluated). Other Salmonella strains isolated from food produced in Mato Grosso, such as beef and chicken, also displayed resistance to nitrofurantoin, sulfonamides-generic, trimethoprim, trimethoprim/sulfamethoxazole, tetracycline, gentamicin, and chloramphenicol [11,25].

Moreover, the rep-PCR employed for the detection of Salmonella strains in the present study found no evidence of clonal expansion for a single strain (Figure 3), although the sample size is very small. However, it is important to note that, in contrast to the results reported by Cunha-Neto et al. [11], in which genetic homology was related to the horizontal transmission of resistance genes, the genetic similarities observed herein were not related to phenotypic characteristics. This suggests that the Salmonella present in the beef production environment may exhibit a more heterogeneous resistance profile than strains present in chicken meat production, although larger studies, including genome sequencing, are required.

**Conclusions**

The results reported herein indicate the presence of Salmonella strains in 5.6% of final packaged products. Although this rate is lower compared to other studies carried in the state of Mato Grosso, all detected strains were classified as multidrug-resistant (displaying resistance to 3 or more antibiotic classes). In addition, this study highlights the presence of two rare Salmonella strains in beef (Akuafo and O:16) and reinforces that Salmonella serovar Schwarzengrund is commonly isolated in animal products in Brazil. Moreover, a genetic heterogeneity between the detected isolates was also observed, demonstrating the heterogenic presence of Salmonella strains in beef production. Finally, novel studies investigating the presence of Salmonella in beef and vegetal foods in the state of Mato Grosso are encouraged, to fully characterize the spread of this food pathogen, one of the leading public health problems worldwide.

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**Corresponding author**
Professor Eduardo Eustáquio de Souza Figueiredo, PhD.
Fernando Correa da Costa. Avenue, Boa Esperança, Federal University of Mato Grosso – Campus Cuiabá, Mato Grosso, 78060-900, Brazil.
Phone: +55-65-3615-8589
Fax: +55-65-3615-8852
Email: figueiredoeduardo@ufmt.br

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