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Research Paper

Prevalence and Antimicrobial Resistance of *Salmonella* spp. Isolated From Chilled Chicken Meat Commercialized at Retail in Federal District, Brazil

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ABSTRACT

Salmonella represents one of the most common foodborne pathogens, frequently associated with the contamination of poultry products, constituting a prominent worldwide public health concern. This study determined the prevalence and antimicrobial resistance of *Salmonella* spp. in chilled chicken meat (115 samples) commercialized at retail in the Federal District, Brazil. Microbiological tests were performed to screen for *Salmonella* spp. in the chicken meat samples, and the isolated strains were confirmed by the *invA* gene presence (PCR technique). The strains were evaluated for antimicrobial susceptibility by the disk diffusion technique (Kirby-Bauer method) and tested for the presence of the *sul2*, *blaCTX,* and *tetB* antimicrobial resistance genes. The *Salmonella* spp. prevalence in chilled chicken meat sold at retail in the Federal District, Brazil, was 46.1% (53 of 115 chicken meat samples analyzed had *invA* gene-positive strains). Seventy-eight strains of *Salmonella* spp. isolated from the 53 contaminated samples showed higher resistance to amoxicillin/clavulanic acid (83.3%), followed by sulfonamide (64.1%) and tetracycline (46.2%); 53.8% of the isolates were multidrug-resistant (MDR). The *sul2* gene that confers resistance to sulfonamide was found in 53 strains (68.0%), the *blaCTX* gene that confers resistance to beta-lactams was identified in 39 strains (50.0%), and the *tetB* gene that confers resistance to tetracycline was identified in 29 strains (37.2%). The high percentage of *Salmonella* contamination in chicken meat can pose a risk to consumers' health due to the possibility of causing salmonellosis. In addition, many isolates were MDR and carried antimicrobial resistance genes. Public agencies can use these results to develop effective public health policies and strategies to ensure the safety of these food products.

Brazil is the world's largest exporter and second-largest chicken meat producer, exporting its products to more than 150 countries (US Department of Agriculture, 2021; Brazilian Association of Animal Protein, 2021). Nontyphoidal salmonellosis cases are often related to consuming contaminated food of animal origin, mainly poultry products, such as eggs and raw chicken meat (Perin et al., 2020; Borges et al., 2019; Antunes et al., 2016; Castro-Vargas et al., 2020). Nonetheless, *Salmonella* spp. remain the leading pathogens responsible for foodborne diseases worldwide (Brasil Ministry of Health, 2019; Centers for Disease Control and Prevention, 2020; European Food Safety Authority/European Centre for Disease Prevention and Control, 2021).

Poultry populations are frequently colonized with *Salmonella* without noticeable symptoms (subclinical infections or healthy carriers) by horizontal and vertical transmission at the production level (Antunes et al., 2016; Choi et al., 2014). *Salmonella* transmission in the poultry production chain may occur directly or indirectly from animal feed, at the farm, within the slaughterhouse or packing plant, and in the manufacturing, processing, and retailing of chicken meat. So, healthy poultry carrying *Salmonella* can act as a reservoir and contaminate chicken meat and eggs, allowing the bacteria to be easily transmitted to the final consumers (Antunes et al., 2016; Choi et al., 2014; Borges et al., 2019).

Another critical concern is the presence of antimicrobial-resistant *Salmonella* strains in chicken meat. Recent studies have demonstrated increasing resistance of *Salmonella* strains isolated from humans and animals to the most used antimicrobials (Perin et al., 2020; Borges et al., 2019; Yamatogi et al., 2016; Lee et al., 2016; Vilela et al., 2019; Cunha-Neto et al., 2018). *Salmonella* has also been identified as an important resistance-coding genes carrier often involved in infec-

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tion outbreaks with multidrug-resistant (MDR) bacteria (Castro-Vargas et al., 2020; Yamatogi et al., 2016; Cunha-Neto et al., 2018).

Considering that most Brazilian studies assess the *Salmonella* prevalence in chicken meat from chicken slaughterhouses (Borges et al., 2019; Cunha-Neto et al., 2018; Mattiello et al., 2015; Baptista et al., 2018), the present study evaluated the prevalence of *Salmonella* spp. isolated from retail chicken meat in the Federal District, Brazil. In addition, the antimicrobial resistance and the presence of resistance genes *blaCTX*, *sul2,* and *tetB* were investigated in *Salmonella* spp. strains isolated from chicken meat.

Material and Methods

Sample collection

Between February 2019 and March 2021, 115 samples of chilled chicken cuts (thigh, drumstick, breast, wing, and wing drumstick) representing the main Brazilian slaughterhouse brands were collected in 83 different supermarkets in the Federal District, Brazil. These samples were exposed to refrigerated counters in the establishments and packed in polystyrene trays within the expiration date. All samples were transported refrigerated (5-8°C) to the laboratory within a maximum period of 1 h, and then, the microbiological analyzes were initiated.

Salmonella isolation and biochemical characterization

The *Salmonella* isolation from chicken meat followed the methodology described in the Technical Guide for Laboratory Detection of *Salmonella* spp. (Brasil Ministry of Health, 2011). In triplicate, 25 g of each sample was inoculated in 225 mL of buffered peptone water (BPW) (Kasvi), homogenized for 5 min, and incubated at 37°C for 24 h. Then, 1.0 mL each of the preenrichment BPW aliquots was transferred into 10 mL of tetrathionate broth (Himedia) and selenite cystine broth (Acumedia,), respectively, and incubated at 37°C for 24 h. A loopful (10 μL) of enriched broth was streaked onto the xylose lysine deoxycholate (XLD) agar (Himedia) and *Salmonella Shigella* (SS) agar (Himedia) and incubated at 37°C for 24 h. Presumptive *Salmonella* colonies in XLD and SS agars were confirmed biochemically using triple sugar iron (TSI) agar (Himedia) and lysine iron (LIA) agar (Himedia) slants. These slants were incubated at 37°C for 24 h. The presumptive *Salmonella* isolates, which tested positive on TSI and LIA biochemical testing, were confirmed by amplifying a targeted *Salmonella*-specific invasive (*invA*) gene by polymerase chain reaction (PCR) (Table 1).

Confirmation of Salmonella and detection of antimicrobial genes using PCR

The presumptive *Salmonella* isolates were confirmed by identifying the *invA* gene. The confirmed *Salmonella* strains (n = 78) were then screened for antimicrobial resistance genes: *blaCTX* for beta-lactams, *tetB* for tetracycline, and *sul2* for sulfonamide. Bacterial DNA was extracted with the PureLink® Genomic DNA kit (Thermo Fisher Scientific), starting from strains cultivated in Mueller-Hinton broth (Kasvi) at 37°C for 18 h. The extracted DNA quality was evaluated by electrophoresis in a 2% agarose gel, and the DNA concentration was quantified using NanoDrop 2000 (Thermo Fisher Scientific).

Gene fragments amplification was performed using the Life Express Thermal Cycler (model TC-98/G/H(b), BIOER). Amplified PCR products' electrophoresis was carried out in 2% (w/v) agarose gel (Invitrogen Life Technologies), stained with ethidium bromide. The DNA fragments separated on the gel were visualized under ultraviolet (UV) lighting with a 100 bp marker (Ludwig Biotecnologia Ltda). The primers' design and the PCR's thermocycling conditions are presented in Table 1.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed according to the Brazilian Committee on Antimicrobial Susceptibility Testing Guidelines, following the standard Kirby–Bauer disk diffusion method (Brazilian Committee on Antimicrobial Susceptibility Testing, 2019). The bacterial inoculum was prepared using a direct suspension from microbial growth in Mueller-Hinton broth with turbidity equivalent to 0.5 McFarland standard (1.0×10^8 UFC/mL), adjusted between the optical density of 0.08 and 0.10 on a spectrophotometer (625 nm). The following antimicrobial agents obtained from Newprov (Brazil) were tested: amoxicillin/clavulanic acid (AMC, 20/10 μg), ceftazidime (CAZ, 30 μg), cefotaxime (CTX, 30 μg), gentamycin (GEN, 10 μg), chloramphenicol (CLO, 30 μg), tetracycline (TET, 30 μg), imipenem (IMP, 10 μg), sulphonamide (SUL, 300 μg), and ciprofloxacin (CIP, 5 μg). The isolates were classified as susceptible (S), intermediate (I), or resistant (R), according to the CLSI guidelines (CLSI, 2020). *Salmonella* isolate resistant to three or more antimicrobials were defined as multidrug-resistant (MDR) isolates.

Statistical analysis

The chi-square test determined if there was a difference between the occurrence of *Salmonella* spp. in different types of chicken cuts. Values of $p < 0.05$ were considered significant. The analysis was performed using the IBM® SPSS Statistics version 28.0 program.

Results and Discussion

Salmonella spp. prevalence in chilled chicken meat sold at retail in the Federal District, Brazil, was 46.1% (53 of 115 chicken meat samples analyzed had *invA* gene-positive strains). Table 2 shows *Salmonella* spp. prevalence in different types of chicken cuts, and the *Salmonella* occurrence did not differ between the different chicken cuts ($p > 0.05$). Perin et al. (2020) reported similar results in frozen chicken meat samples collected in retail stores in Paraná state, Brazil,

Table 1
Primers' sequence and the polymerase chain reaction (PCR) thermocycling conditions for the *invA* virulence gene and the *blaCTX*, *tetB*, and *sul2* resistance genes

Target gene	Primer sequence (5'→ 3')	Product size (bp)	PCR conditions	Reference
invA	CATTGGTGATGGTCTTGTCG CTCGCCTTTGCTGGTTTTAG	298	denaturation for 2 min at 95°C, followed by 35 cycles for 1 min at 95°C, annealing for 1 min at 60°C, with final extension for 1 min at 72°C	Cruz et al. (2019)
blaCTX	CGATGTGCAGTACCAGTAA AGTGACCAGAATCAGCGG	585	denaturation for 5 min at 94°C, followed by 30 cycles for 30 s at 94°C, annealing for 30 s at 55°C, 50 s at 72°C with final extension for 7 min at 72°C	Li et al. (2013)
tetB	TTGGTTAGGGGCAAGTTTTG GTAATGGGCCAATAACACCG	659	denaturation for 5 min at 94°C, followed by 34 cycles for 25 s at 94°C, annealing for 30 s at 55°C, 50 s at 72°C with final extension for 7 min at 72°C	*(21)*
sul2	GCGCTCAAGGCAGATGGCATT GCGTTTGATACCGGCACCCGT	285	denaturation for 10 min at 95°C, followed by 35 cycles for 45 s at 94°C, annealing for 50 s at 55°C, 50 s at 72°C with final extension for 10 min at 72°C	*(22)*

Table 2
Overall prevalence of *Salmonella* spp. in chilled chicken meat and different types of chicken cuts

Types of chicken cuts	Number of samples (%)	Number of samples with *Salmonella* spp. (%)	p value
Thigh	22 (19.1)	13 (59.1)	
Wing drumstick	35 (30.4)	17 (48.6)	
Drumstick	27 (23.5)	8 (29.6)	0.33
Breast	23 (20.0)	11 (47.8)	
Wing	8 (7.00)	4 (50.0)	
Total	115 (100.0)	53 (46.1)	

where they found 30.0% of *Salmonella* spp. contamination with also no statistical difference between the analyzed cuts. Frozen chicken cuts, in general, have a lower *Salmonella* occurrence than chilled chicken cuts; however, Borges et al. (2019) detected *Salmonella* in frozen chicken carcasses even after long periods of storage.

The Brazilian government has officially reported the prevalence of *Salmonella* spp. in whole chicken carcasses after being slaughtered in the slaughterhouses and before being transformed into cuts or transported and distributed for sale in supermarkets: 17.97% in 2017, 12.61% in 2018, and 15.08% in 2019 (Ministry of Agriculture, 2021). Baptista et al. (2018) found 26.7% of chicken carcasses contaminated by *Salmonella* spp. in slaughterhouses in Rio de Janeiro state, Brazil. Overall, chicken carcasses at slaughterhouses are less contaminated by *Salmonella* than chicken cuts at retail establishments, as they are handled less, which reduces cross-contamination (Sodagari et al., 2015). According to Sodagari et al. (2015), the variations observed between the studies that reported the *Salmonella* prevalence might also be due to the differences in isolation methods applied to detect *Salmonella*. Golden and Mishra (2020) also reported an increased *Salmonella* spp. prevalence in the United States from 14.3% in broiler chicken samples to 19.0–23.0% in retail chicken samples.

According to Golden and Mishra (2020), the analyses of retail chicken meat samples are crucial because they are the sample types available to consumers for direct use. Although raw chicken is cooked before consumption, *Salmonella* spp. control in chicken meat is vital to avoid cross-contamination of surfaces and other foods in the kitchen and potential risks of salmonellosis from undercooking the meat.

The virulence gene *invA* was used to confirm *Salmonella* spp. in our study. Other studies also used the *invA* gene as a molecular marker to confirm *Salmonella* spp. isolated from chicken meat samples (Perin et al., 2020; Borges et al., 2019; Zishiri et al., 2016; Zhu et al., 2017). The *invA* gene encodes a protein in the inner membrane of bacteria, which is necessary to invade the host's epithelial cells. This gene is essential for virulence in *Salmonella*, so the *invA* gene is a specific method for detecting *Salmonella* at the genus level in a variety of samples (Ammar et al., 2016; Amini et al., 2010).

Table 3 shows the antimicrobial susceptibility profile of 78 *Salmonella* spp. strains isolated from chicken meat samples. The highest resistance rates observed were for amoxicillin with clavulanic acid (83.3%), sulfonamide (64.1%), tetracycline (46.2%), and ciprofloxacin had 65.4% of intermediate and resistant results.

S: sensitive; I: intermediate; R: resistant; n (%) = number and percentage in relation to the total of 78 strains; AMC: amoxicillin-clavulanic acid; CAZ: ceftazidime; CTX: cefotaxime; IPM: imipenem; SUL: sulfamethoxazole; GEN: gentamycin; TET: tetracycline; CLO: chloramphenicol; CIP: ciprofloxacin. *Interpretation of zones of inhibition according to the CLSI guidelines (CLSI, 2020)

Perin et al. (2020) reported similar results from *Salmonella* strains isolated from frozen chicken meat samples collected in retail stores in Paraná state, Brazil, which presented high resistance to tetracycline (94.0%), amoxicillin with clavulanic acid (84.0%), and ciprofloxacin (76.0%). A meta-analysis conducted in Brazil compared the antimicrobial resistance of poultry-origin nontyphoidal *Salmonella* isolated from 1995 to 2014. The highest antimicrobial resistance levels were verified for sulfonamides (44.3%), nalidixic acid (42.5%), and tetracycline (35.5%) (Voss-Rech et al., 2017).

In addition to the high resistance to amoxicillin-clavulanic acid (83.3%) presented by the *Salmonella* strains, resistant and intermediate values were also observed for cefotaxime (41.1%), imipenem (32.1%), and ceftazidime (23.1%). In *Enterobacteriaceae*, the resistance to beta-lactamases antimicrobials is generally attributed to the production of beta-lactamases that cleaves the beta-lactam ring to inactivate the drug as ESBL (extended-spectrum beta-lactamase) (Chon et al., 2015). The resistance to beta-lactam antimicrobials, such as cephalosporins, the drugs of choice to treat clinical salmonellosis, limit the available therapeutic options (Chon et al., 2015; Crump et al., 2015).

Sulfonamides were, starting in 1948, the first drugs used in therapeutic doses in veterinary medicine and, for many years, as prophylaxis for avian diseases. Tetracyclines and sulfonamides were used as additives in animal feed in Brazil until 1998, when their use became restricted to therapeutic purposes. However, these drugs still exert selection pressure on microorganisms (Voss-Rech et al., 2017; Lees et al., 2021).

The increased resistance to quinolones has been found in human-origin nontyphoidal *Salmonella* in the United States since 1996 (Voss-Rech et al., 2017; Centers of Disease Control and Prevention, 2019). *Salmonella* isolates from poultry displayed increased resistance to nalidixic acid, which in *Enterobacteriaceae* generally correlates to reduced susceptibility to ciprofloxacin. Ciprofloxacin is a recognized first-line drug for treating severe human salmonellosis cases, and treatments with fluoroquinolones have failed in patients infected with *Salmonella* spp. resistant to nalidixic acid. The reduced susceptibility of nontyphoidal *Salmonella* to quinolones seems attributed to the widespread use of these antimicrobials in both human and veterinary medicine (Voss-Rech et al., 2017; Centers of Disease Control and Prevention, 2019; Lai et al., 2014).

Only 1.3% (1/78) of the isolates were sensitive to all tested antimicrobials. The remaining isolates were resistant to one or more classes of antimicrobials. The present study observed that 53.8% (42/78) of *Salmonella* isolates were multidrug-resistant (MDR) (Table 4). Other studies have reported high rates of MDR *Salmonella* isolated from

Table 3
Antimicrobial susceptibility profile of 78 *Salmonella* spp. strains isolated from chicken meat samples

Antimicrobials	R n (%)	I n (%)	S n (%)	R *	I *	S *
AMC	65 (83.3)	3 (3.8)	10 (12.8)	< 13	14-17	> 18
SUL	50 (64.1)	1 (1.3)	27 (34.6)	< 12	13-16	> 17
TET	36 (46.2)	6 (7.7)	36 (46.2)	< 11	12-14	> 15
CTX	23 (29.5)	9 (11.5)	46 (59.0)	< 22	23-25	> 26
CAZ	17 (21.8)	1 (1.3)	60 (76.9)	< 17	18-20	> 21
CIP	13 (16.7)	38 (48.7)	27 (34.6)	< 20	21-30	> 31
GEN	11 (14.1)	11 (14.1)	56 (71.8)	< 12	13-14	> 15
IPM	8 (10.3)	17 (21.8)	53 (67.9)	< 19	20-22	> 23
CLO	4 (5.1)	11 (14.1)	63 (80.8)	< 12	13-17	> 18

Table 4
Number of antimicrobial classes to which *Salmonella* spp. strains showed resistance

Number of antimicrobial classes with resistance	*Salmonella* isolates (n)	*Salmonella* isolates (%)
0	1	1.3
1	9	11.5
2	26	33.3
3	21	26.9
4	13	16.7
5	8	10.3
MDR (sum of 3, 4 and 5)	42	53.8

MDR = multidrug-resistant; strains resistant to 3, 4, and 5 antimicrobials classes were defined as MDR; % = percentage in relation to the total of 78 strains

chicken meat, with values of 85.7% (Brazil) (Perin et al., 2020); 84.8% (Japan) (Moe et al., 2017); 62.2% (Iran) (Sodagari et al., 2015), 60.8% (China) (Zhu et al., 2017); and 52.2% (Myanmar) (Furukawa et al., 2017).

In recent years, multidrug-resistant (MDR) phenotypes have been increasingly described among *Salmonella* species in animal products worldwide. Outbreaks of human salmonellosis have been associated with antimicrobial-resistant *Salmonella* isolates. The emergence of MDR *Salmonella* is a worldwide concern, representing increased disease severity leading to higher hospitalization rates and possibly death (Antunes et al., 2016; Castro-Vargas et al., 2020; Cunha-Neto et al., 2018).

The *blaCTX*, *sul2*, and *tetB* resistance genes were searched in the 78 *Salmonella* strains isolated from chicken meat samples (Table 5). In total, the *sul2* gene was identified in 53 strains (68.0%), the *blaCTX* gene in 39 strains (50.0%), and the *tetB* gene in 29 strains (37.2%).

Most of the phenotypically sulfonamide-resistant *Salmonella* spp. strains (64.1%) in this study carried the *sul2* resistance gene (44.9%). Four *sul* genes (*sul1*, *sul2*, *sul3,* and *sul4*) encode insensitive dihydropteroate synthase to escape the sulfonamide activity; however, dissemination of *sul1* and *sul2* genes among *Salmonella* spp. is reported more often than the *sul3* and *sul4* genes. *Sul* genes are transferred between bacteria via integrons, transposons, or plasmids (Maka et al., 2015; Xu et al., 2019). Deng et al. (2018) reported that *sul1* and *sul2* gene prevalence was equal in trimethoprim-resistant *Salmonella* strains isolated from chicken meat in China (20.4%, $n = 20$). Maka et al. (2015) studied the *sul* genes dissemination among sulfonamide-resistant *Salmonella* spp. isolated from food in Poland and found that 44.0% of isolates carried the *sul1* gene, 46.4% were *sul2* positive, while the *sul3* gene was undetected.

The resistance gene *blaCTX* was present in 43.6% of the *Salmonella* isolates phenotypically resistant to amoxicillin-clavulanic acid. Extended-spectrum beta-lactamase (ESBL) with the ability to hydrolyze penicillins and most cephalosporins are synthesized by an increasing number of *Enterobacteriaceae*, and *blaCTX* is one of the most prevalent ESBL genes worldwide (Chon et al., 2015). The emergence of *Salmonella* ESBL has been attributed to the acquisition of genes that encode beta-lactamase enzymes, commonly located in mobile genetic elements, such as plasmids, transposons, and integrons. Consequently, resistance can spread horizontally between the isolates (Crump et al., 2015; Bythwood et al., 2019). Lee et al. (2016) reported that the *Salmonella* strains isolated from duck carcasses in South Korea phenotypically resistant to both cefotaxime and ceftazidime harbored the *blaCTX* gene, while genes encoding TEM and SHV enzymes were not detected. Djeffal et al. (2017) reported ESBL presence in avian- and human-origin *Salmonella* in northeastern Algeria (12 avian and six human strains), and the *blaCTX* gene as the most prevalent (66.7% of the strains), 27.8% contained both *blaTEM* and *blaCTX* genes and 5.5% contained the *blaTEM* gene.

Table 5
Percentage of resistance genes in the 78 *Salmonella* spp. strains isolated from chicken meat samples

Resistance genes	Antimicrobial resistance profile			Total n (%)
	R n (%)	S n (%)	I n (%)	
blaCTX-M	34 (43.6)	2 (2.6)	3 (3.8)	39 (50.0)
sul2	35 (44.9)	18 (23.1)	0	53 (68.0)
tetB	14 (17.9)	13 (16.7)	2 (2.6)	29 (37.2)

R: resistant, S: sensitive, I: intermediate; n (%) = number and percentage in relation to the total of 78 strains

Among this study's 36 phenotypically tetracycline-resistant strains (46.2%), 14 strains (38.9%) carried the *tetB* resistance gene. The tetracycline resistance frequently found in *Salmonella* spp. is mainly due to the tet genes' presence in these bacteria (Maka & Popowska, 2016; McMillan et al., 2019). The most frequent types of *tet* genes belong to classes A, B, C, D, and G (Xu et al., 2020), and the *tetB* gene is specific for Gram-negative bacteria (Roberts & Schwarz, 2016). The *tet* genes are responsible for encoding membrane-associated tetracycline efflux pumps and are usually associated with plasmids, transposons, or both and are often conjugative (Maka & Popowska, 2016; Roberts & Schwarz, 2016). Zhu et al. (2017) evaluated the presence of tetracycline resistance genes in *Salmonella* strains isolated from broiler chickens along the slaughtering process in China and found the *tetB* gene in 50% of the total 98 strains phenotypically resistant to tetracycline.

Resistance genes were also detected in phenotypically susceptible and intermediate *Salmonella* strains. Our study found 18 strains (23.1%) sensitive to sulfonamides with the *sul2* resistance gene, 15 strains (19.3%) sensitive and intermediate to tetracycline with the *tetB* gene, and five strains (6.4%) sensitive and intermediate to β-lactams with the *blaCTX* gene. Deekshit et al. (2012) also reported silent genes in *Salmonella* strains (40% of chloramphenicol-susceptible strains carried the *catA1* gene). Silent genes are DNA sequences usually unexpressed or expressed at an insufficient level but still can spread through horizontal gene transfer (Stasiak et al., 2021). According to Adesiji et al. (2014), some antimicrobial resistance genes are silent in bacteria *in vitro* and activate *in vivo*, especially under the selective pressure of antibiotic use. Silent genes have also been proven to become active after being transferred to a new host (Stasiak et al., 2021).

Due to the limited number of resistance genes (*sul2*, *blaCTX*, and *tetB*) tested in this study, *Salmonella* strains phenotypically resistant to sulfonamides, β-lactams, or tetracycline may be carrying other genes that encode resistance to these antimicrobials. This study's findings indicate that retail chilled chicken meat may act as a reservoir of *Salmonella*-harboring antimicrobial resistance genes. Therefore, the need for the rational use of antimicrobial agents in poultry production systems is evident to reduce the proliferation of multidrug resistance among species.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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