


Molecular Detection Of Multidrug-Resistant *Salmonella* Isolated From Livestock Production Systems In South Africa

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Background: Antibiotic-resistant bacterial pathogens associated with livestock remain a major concern worldwide as they get transmitted from animals to humans and cause foodborne and zoonotic diseases.

Methods: Antimicrobial resistance in livestock-associated *Salmonella* spp in South Africa was investigated using molecular DNA methods. Three hundred and sixty-one environmental faecal samples were randomly collected from avian (chicken and ducks), cows, pigs, goats, and sheep. *Salmonella* spp. were isolated on selective media and were confirmed using the polymerase chain reaction. Antimicrobial susceptibility testing against ampicillin, chloramphenicol, ciprofloxacin, ceftriaxone, azithromycin, tetracycline, amoxicillin-clavulanate and trimethoprim-sulfamethoxazole was determined using the Kirby–Bauer disk diffusion method. Isolates were screened for the presence of *bla*_{TEM-1}, *bla*_{CMY-2}, *tetA*, *tetC*, *sul2* and *dfpA7* resistance genes by PCR.

Results: Most of the isolates were resistant to ampicillin (64%), tetracycline (63%), amoxicillin-clavulanate (49%), trimethoprim-sulfamethoxazole (38%), and ceftriaxone (20%). Eight percent of the tested isolates were ciprofloxacin-resistant *Salmonella* spp. Multidrug resistance was observed with the mean multiple antibiotic resistance (MAR) index of 0.31. The study demonstrated that 43% of the isolates were multiple drug resistant. The prevalence rates of resistance genes were 44% for *bla*_{TEM-1}, 35% for *bla*_{CMY-2}, 21% for *sul2*, 18% for *tetC*, 14% for *dfpA7* and 8% for *tetA*.

Conclusion: Resistance to ceftriaxone, detection of *bla*_{CMY-2} gene and the high level of intermediate susceptibility (33%) against ciprofloxacin suggested that livestock carry problematic *Salmonella* spp. This study used the global one-health initiative to report the potential public health risks of livestock-associated pathogens and highlights the importance of monitoring the trends of antimicrobial resistance for sustainability of antibiotics.

Keywords: *Salmonella*, food-borne pathogens, zoonotic, animals, antimicrobial, resistance, *bla*_{TEM-1}, *bla*_{CMY-2}, bacteria, ciprofloxacin

Introduction

The emergence of zoonotic bacterial infections and the ever-increasing occurrence of antimicrobial-resistant bacteria is a global concern.¹ The adverse effects caused by antimicrobial-resistant bacteria in both humans and domestic animals draw significant attention to these pathogens and pose a need for them to be monitored to ensure human biosecurity.^{2–4} Antimicrobial-resistant bacteria decrease the efficiency of available antibiotics, resulting in infection treatment failures which sometimes lead to high morbidity and mortality.⁵ *Salmonella* is a zoonotic bacterial

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pathogen that is responsible for gastroenteritis, focal infection, enteric fever (typhoid) and bacteremia in humans.⁶⁻⁸ Enteric fever is caused by *Salmonella typhi* and its subtypes, which infect only humans while the other diseases are caused by the non-typhoidal *Salmonella*.⁷ Gastroenteritis (or food poisoning) is the most common disease caused by non-typhoidal *Salmonella*, which is normally treated by fluid supplementation.⁹ Antimicrobial agents are recommended only in severe cases in children under the age of 5, elderly people (over 65 years) and immunocompromised individuals.⁵ Antibiotics are categorized according to their mode of action in bacteria. Depending on the type of the antimicrobial agent, the activities of the antibiotics result in bacterial cell wall synthesis disruption, inhibition of nucleic acids synthesis, inhibition of protein synthesis or folic acid pathway inhibition.^{10,11} The use of antibiotics for treatment of salmonellosis is challenged by the continuous emergence of antimicrobial-resistant strains.¹²

The antibiotic resistance challenge dates back to the 1960s when antimicrobial-resistant *Salmonella typhimurium* was reported.¹³ Ampicillin (beta-lactam), sulfamethoxazole (sulfonamide), furazolidone (nitrofurans) and tetracyclines were reported to have decreased efficiency against *S. typhimurium* treatment in calves and humans.¹⁴ Resistance to antibiotics such as gentamicin, kanamycin, streptomycin, chloramphenicol and trimethoprim was detected in the subsequent years.¹⁵⁻¹⁷ In the late 1990s, resistance to nalidixic acid (fluoroquinolone) and ceftriaxone (third-generation cephalosporin) was reported following an outbreak in cattle and humans in the USA.^{14,18} Antibiotic-resistant *Salmonella* spp. were reported to newer drugs such as azithromycin and ciprofloxacin.^{19,20} In addition, fluoroquinolone-resistant *Salmonella* spp. are listed among the high priority list of the World Health Organization (WHO).^{21,22} The major concern is that once a drug is discovered it takes a very short period of time for the bacteria to develop resistance to it as a result of quick response to environmental stimuli, short generation time by bacteria and horizontal sharing of resistance genes between bacterial species.^{23,24} On the other hand, it takes a many years for a new antibiotic to be developed, and approved for clinical use.²⁵⁻²⁸ Antibiotic resistance is mainly attributed to the improper and injudicious use of antibiotics as growth promoters in animal feeds as well as during treatment and for prophylactic purposes.^{29,30}

Administration of small doses of antibiotics to healthy animals causes the microbiota in the guts of animals to be familiar with the drugs and gives them a chance to develop

resistance towards the given substances.^{19,30,31} The bacteria then share the antibiotic resistance determinants via mobile genetic elements (MGEs) such as plasmids, transposons, integrons and phages.^{32,33} This renders the healthy animals as carriers of antibiotic-resistant bacteria.³⁴ Since antibiotic-resistant bacteria are found in the gut, faecal contamination is the main route of transmission of these pathogens to humans. The environments in which the animals are reared and direct contact with animals are possible sources of transmission of resistant zoonotic pathogens to humans.^{35,36}

The mechanisms encoded by antimicrobial resistance determinants include antimicrobial modification and inactivation, alteration of the antimicrobial target site, efflux pumps and membrane impermeability.^{37,38} These protect the bacteria from being attacked by antibiotics.³⁷ Bacteria which are resistant to the beta-lactam class of antibiotics produce beta-lactamase enzyme which destroys the beta-lactam ring thus deactivating the antibiotic.^{10,23} Macrolide repressor protein produced by *Salmonella* spp. inactivates azithromycin and erythromycin.³⁹ Resistance to quinolones is achieved by mutations in DNA gyrase coding genes (*gyrA* and *gyrB*) thus modifying the target site of the antibiotic.⁴⁰ Efflux pumps transport substances out of the cell, including detergents, dyes and antibiotics.⁴¹ *Salmonella* spp. uses the well-studied AcrAB-TolC efflux pump to extrude antibiotics such as tetracycline, chloramphenicol and quinolones.^{42,43} Bacteria resist antibiotic entry into the cell by reducing or modifying porin channels in the outer membrane which are used by antibiotic molecules to enter the bacterial cell to reach their targets.^{44,45} Genes which encode for antibiotic resistance are either located on the chromosome or plasmids within a bacterial cell and are mobilized by transposons and integrons during conjugation or phages through transduction.^{33,46}

Antibiotics used for the treatment of salmonellosis include ampicillin, tetracycline, amoxicillin-clavulanate, chloramphenicol, trimethoprim-sulfamethoxazole, ciprofloxacin, ceftriaxone and azithromycin.^{47,48} *Salmonella* spp. utilize several resistance mechanisms to escape the antimicrobial activities. The alarming rates of antimicrobial resistance led to the investigation of the genetic determinants in bacteria. Several studies focused on the detection of antimicrobial resistance genes which are the drivers of antimicrobial resistance.^{5,37,40}

The use of antibiotics for growth promotion in livestock decreases their efficiency and therefore the effectiveness of the antibiotics differs between countries depending on the available antibiotic regulatory practices.^{30,49} It was

reported that a decrease in resistance to antibiotics such as tylosin, which were banned for use as growth promoters in European countries while resistance rates are still rising in countries where this antibiotic is used as a growth promoter.⁵⁰ Screening bacterial pathogens for the presence of antibiotic resistance genes and detection by molecular methods enable researchers to determine whether a drug will be effective in a certain area. This aids in appropriate prescription of the correct treatment to patients with a particular bacterial infection, rather than prescription of an antibiotic which may result in the development of antimicrobial resistance.⁴ Against this background, the current study aimed to determine the antimicrobial susceptibility profiles and to detect resistance determinants of *Salmonella* spp. isolated from livestock production systems in South Africa.

Materials And Methods

Ethical Approval

The study was approved by the Animal Research Ethics Committee of the University of Kwa-Zulu Natal (Reference numbers AREC/051/017M, AREC 071/017 and AREC 014/018). The field sampling protocols, samples collected from animals, and the research were conducted in full compliance with Section 20 of the Animal Diseases Act of 1984 (Act No 35 of 1984) and were approved by the South African Department of Agriculture, Forestry and Fisheries DAFF (Section 20 approval reference number 12/11/1/5 granted to Prof ME El Zowalaty).

Samples And Pre-Enrichment

A total of three hundred and sixty-one (361) faecal and environmental samples were collected from different animal hosts including cattle, sheep, goats, pigs, ducks and chickens which were housed in small-scale commercial farms in Flagstaff (O.R Tambo, Eastern Cape), Verulam (eThekweni, KwaZulu-Natal) and South Coast (Amandawe and Mtwalume, UGU, KwaZulu-Natal) as depicted in Figure 1. All samples as shown in Table 1 were randomly collected between May and August 2018. In the farms, livestock are bought from large-scale farms and sold to the local communities. Oral and rectal swab samples were collected from animals using sterile collection swabs and transferred into 10 mL of 0.1% buffered sterilized peptone water (Merck, South Africa). Fresh environmental faecal samples emanating from purportedly healthy livestock, as well as samples from their environments including soil,



Figure 1 Map of South Africa showing the geographic locations of the farms where the samples used in this study were collected.

feed and water, were also randomly collected using sterile collection swabs. All swab samples were transferred into 10 mL of 0.1% buffered sterilized peptone water (Merck, South Africa). Water samples were collected from the containers inside the livestock housing. Samples were transported on ice to the discipline of genetics laboratories at University of KwaZulu-Natal Westville campus where enrichment of the samples was done by incubating the swabs in 10 mL of 0.1% buffered peptone water at 37°C overnight.

Selective Enrichment

From each of the enriched samples, 0.1 mL was aseptically transferred into 10 mL of Rappaport Vassiliadis (RV) broth (Sigma-Aldrich, India) and incubated for 24 hrs at 42°C. RV is a selective medium that is enriched with malachite green which inhibits the growth of microorganisms other than *Salmonella*. A previously identified and confirmed *Salmonella enterica* was used as a positive control.⁵¹ Bacterial isolation was performed on Xylose-Lysine-Deoxycholate (XLD) agar (Sigma-Aldrich, Switzerland) by aseptically streaking a loopful of the culture from RV broth onto the plates. *S. enterica* is differentiated from *Escherichia coli* and *Shigella* spp. by producing red colonies with black centers on XLD agar. After 24 hrs of incubation at 35°C, the plates were observed for the growth of the expected colonies. Representative single colonies of the correct morphology were randomly picked from each plate and transferred into tubes containing 10 mL of tryptose soy broth (Merck, South Africa) and incubated at 37°C for 18–24 hrs. A

Table 1 Number And Type Of Samples Collected From Farms In Flagstaff, Verulam And South Coast In South Africa

Animal Host	Flagstaff					Verulam					South Coast					Total
	Oral	Fecal	Feed	Soil	Water	Oral	Fecal	Feed	Soil	Water	Oral	Fecal	Feed	Soil	Water	
Chicken	0	0	0	0	0	0	24	0	0	0	40	40	0	5	5	114
Ducks	0	10	0	0	0	0	0	0	0	0	0	0	0	0	0	10
Cow	0	5	0	5	0	0	20	0	0	0	0	10	0	5	5	50
Goats	10	9	0	6	6	0	10	0	0	0	17	16	0	0	5	79
Sheep	12	10	0	6	0	0	10	0	0	0	4	8	0	0	0	50
Pigs	17	17	9	9	6	0	0	0	0	0	0	0	0	0	0	58
Total	39	51	9	26	12	0	64	0	0	0	61	74	0	10	15	361

2 mL of the culture was used for DNA extraction. Equal amounts of 0.5 mL each of 60% glycerol and *Salmonella* pure culture were mixed in 1.5 mL cryotubes and stored at -80°C for future use.

DNA Extraction

Total genomic DNA was extracted from *Salmonella* cultures using the conventional boiling method. One milliliter of the cultured sample was transferred into 1.5 mL Eppendorf tube and centrifuged at $14,000 \times g$ for 5 mins. The supernatant was discarded and another 1 mL of culture was added to the pellet and centrifuged again to get a larger pellet. Six hundred μL of sterile distilled water was added to the pellet and centrifuged for 5 mins at $14,000 \times g$. The supernatant was discarded, 200 μL of sterile distilled water was added again and incubated in a heating block at 100°C (Labnet, USA). The sample was boiled for 10 mins with immediate cooling on ice for 5 mins. After cooling, the sample was centrifuged at $14,000 \times g$ for 5 mins; the resulting supernatant was transferred into a fresh Eppendorf tube and stored at -20°C for further use.

Molecular Confirmation Of *Salmonella* spp. Using PCR

Salmonella spp. were confirmed by amplifying the *invA* gene, which is genus-specific gene and the *iroB* gene for identification of *S. enterica* using specific primers as previously reported^{52,53} as shown in Table 2. The PCR reaction volume

was 25 μL which consisted of 12.5 μL Dream Taq master mix (Thermo-Fischer Scientific, South Africa), 0.5 μL each of forward and reverse primers, 6.5 μL sterile distilled water and 5 μL template DNA. The PCR reaction conditions consisted of 34 amplification cycles for *invA* gene using the following conditions: denaturation for 30 s at 95°C , annealing for 30 s at 58°C , extension for 1 min at 72°C and final extension for 5 mins at 72°C . The same amplification parameters were used for the *iroB* gene using a different annealing temperature of 58°C . The PCR amplicons were stored at -20°C till future use.

Antimicrobial Susceptibility Testing

Salmonella spp. isolates were cultured into 10 mL of tryptone soy broth at 37°C for 18 hrs. Isolates were tested for their antimicrobial susceptibility profiles according to the CLSI guidelines.⁵⁴ Antibiotic disks containing ampicillin (10 μg), trimethoprim and sulfamethoxazole (25 μg), amoxicillin-clavulanate (20 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), tetracycline (30 μg), ceftriaxone (30 μg) and azithromycin (15 μg) were placed on the inoculated Mueller-Hinton agar plates. *E. coli* ATCC 25922 reference strain was used as a positive control as recommended in the CLSI guidelines. The plates were incubated at 37°C for 16 hrs to examine susceptibility of the isolates to the antibiotics. The zone of inhibition (in millimeters) were determined by measuring the diameter of the area of no bacterial growth around the disk. The results were

Table 2 Forward And Reverse Primer Sequences Of *invA* And *iroB* Genes

Gene	Primer Sequence (5'-3')	Amplicon Size (bp)	Annealing Temperature ($^{\circ}\text{C}$)	Reference
<i>invA</i>	F: TCATCGCACCGTCAAAGGAACC R: GTGAAATTATCGCCACGTTCTGGGCAA	284	61	52
<i>iroB</i>	F: TGC GTATTCTGTTTGTCTGGTCC R: TACGTTCCCACCATTTCTCC	606	55	53

interpreted as susceptible, intermediate or resistant in compliance with CLSI recommendations.

PCR Amplification Of Antibiotic Resistance Genes

Screening of antimicrobial resistance genes in *Salmonella* isolates was performed by PCR methods using previously reported primers as shown in Table 3. The reaction tube contained 25 μ L which was made up of 12.5 μ L Dream Taq master mix (Thermo-Fischer Scientific, South Africa), 0.5 μ L each of forward and reverse primers, 6.5 μ L sterile distilled water and 5 μ L template DNA. The negative control had sterile distilled water instead of DNA. *E. coli* ATCC 25922 reference strain was used as a positive control for some of the investigated resistance genes and previously extracted and known DNA from a pure *S. enterica* culture was used as positive control. PCR was conducted in a thermocycler (BioRad, Singapore) using the following conditions consisting of an initial denaturation for 3 mins at 95°C, followed by 30 amplification cycles of denaturation for 30 s at 95°C, annealing for 30 s at a specific temperature for each set of primers, extension for 1 min at 72°C and final extension for 8 mins at 72°C.

Statistical Analysis

Multiple antibiotic resistance (MAR) indices were determined to assess the risk level of the isolates using a previously reported method.⁶⁰ The number of antibiotics that each isolate was resistant to was divided by the total number of antibiotics tested to determine the MAR index.

Indices greater than 0.2 were regarded as high risk, meaning that the isolates were multidrug resistant and representing an origin of the isolates from a source of high use of antibiotics as was reported previously.⁶⁰ Furthermore, inferential statistics (Fisher's exact and correlation tests) using IBM SPSS version 25 and Microsoft excel 2010 were used to analyze the results. The Fischer's exact test was implemented to investigate whether there was a relationship between antimicrobial resistance genes with animal host and farm location (area where samples were collected). The magnitude and direction of the correlation between antimicrobial resistance genes were evaluated using Pearson's correlation test. Furthermore, the effect of farm location as a predictor of the presence of the genes in the present study was investigated by binary logistic regression. South coast was used as a reference category for comparing Flagstaff and Verulam. The statistical models evaluated the null hypothesis that there was no association between location, animal host and the presence of the antimicrobial resistance genes. The critical value of 0.05 was used to decide whether to reject or not reject the null hypothesis.

Results

Out of 361 samples, 195 (54%) showed positive growth on RV medium and XLD agar, this was observed by cloudiness of the RV broth after 24 hrs growth on XLD agar plates. The positive control showed red colonies with black centers, as expected for *S. enterica* on XLD agar. Out of 195 presumptive *Salmonella* spp., 106 were confirmed to be positive by the *invA* gene amplification (284

Table 3 Primers And Annealing Temperatures For Antimicrobial Resistance Genes Amplification

Gene	Primer Sequence	Annealing Temperature (°C)	Reference
<i>bla_{CMY-2}</i>	F: 5'-ATAACCACCCAGTCACGC-3' R: 5'-CAGTAGCGAGACTGCGCA-3'	50	55
<i>bla_{TEM-1}</i>	F: 5'-TTGGGTGCACGAGTGGGT-3' R: 5'-TAATTGTTGCCGGAAGC-3'	56	56
<i>tetA</i>	F: 5'-GCTACATCCTGCTTGCCCTTC-3' R: 5'-CATAGATCGCCGTGAAGAGG-3'	52	57
<i>tetC</i>	F: 5'-CTTGAGAGCCTTCAACCCAG-3' R: 5'-ATGGTCGTCATCTACCTGCC-3'	42	57
<i>sul2</i>	F: 5'-CGG CAT CGT CAA CAT AACCC-3' R: 5'-GTG TGC GGA TGA AGT CAG-3'	60	58
<i>dfxA7</i>	F: 5'-AAATGGCGTAATCGGTAATG-3' R: 5'-GTGAACAGTAGACAAATGAAT-3'	52	59

bp). *S. enterica* is responsible for infections in humans, with serovars such as Enteritidis and Typhimurium being the most reported.⁶ In this study, 30.2% of the *Salmonella* isolates were confirmed to be *S. enterica* by *iroB* gene amplification (606 bp). The 106 *Salmonella* isolates that were confirmed by *invA* gene amplification were further analyzed for antimicrobial susceptibility profiling and detection of resistance genes.

The results from Kirby–Bauer disk diffusion method demonstrated that the *Salmonella* isolates in this study were resistant to all the tested antibiotics (Figure 2). The prevalence of resistance was high to ampicillin (64%) followed by tetracycline (63%), amoxicillin-clavulanate (49%), trimethoprim-sulfamethoxazole (38%) and ceftriaxone (20%). Resistances to ciprofloxacin, chloramphenicol and azithromycin were low with proportions of 8%, 8% and 5%, respectively. It was found that 33% of the isolates were intermediate-resistant to ciprofloxacin. As illustrated in Figure 2, resistance in *Salmonella* spp. isolated was high in AMP, TE, AMC, SXT and CRO. The mean of MAR index was 0.31 (range 0–0.875) with 43% (n=46 out of 106) of the isolates displaying resistance to three or more antibiotics and were considered multidrug resistant. It was found that one isolate was resistant to seven antibiotics, while five, seven, 15 and 18 isolates were resistant to six, five, four, and three antibiotics out of the eight tested drugs. The multidrug resistance patterns are depicted in Table 4.

All tested isolates collected from cows, pigs, sheep, and goat, and chickens possessed at least five of the six screened antimicrobial resistance genes using PCR except for isolates collected from ducks which had only four genes. Most of the AMR genes were found in *Salmonella* isolated from chicken

Table 4 Multidrug Resistance Patterns Of *Salmonella* Isolates Resistant To Two Or More Antibiotics

Number Of Isolates	Multidrug Resistance Patterns
4	SXT, TE
5	AMP, TE
19	AMP, AMC
1	Cip, TE
1	AMP, CRO
1	TE, AMC
1	SXT, AMC
2	TE, CRO
6	AMP, SXT, TE
1	SXT, TE, CRO
5	AMP, TE, AMC
1	AMP, CRO, AMC
2	AMP, TE, CRO
1	C, SXT, TE
1	Cip, TE, AMC
1	SXT, TE, AMP
9	AMP, SXT, TE, AMC
2	AMP, SXT, CRO, AMC
1	Cip, AMP, TE, AMC
1	Cip, SXT, TE, AZM
1	Cip, AMP, AMC, AZM
1	Cip, AMP, SXT, TE
1	AMP, SXT, TE, AMC, AZM
1	C, AMP, SXT, TE, AMC
4	AMP, SXT, TE, CRO, AMC
1	Cip, AMP, SXT, TE, CRO
1	Cip, C, AMP, SXT, TE, CRO
4	C, AMP, SXT, TE, CRO, AMC
1	C, AMP, SXT, TE, CRO, AMC, AZM

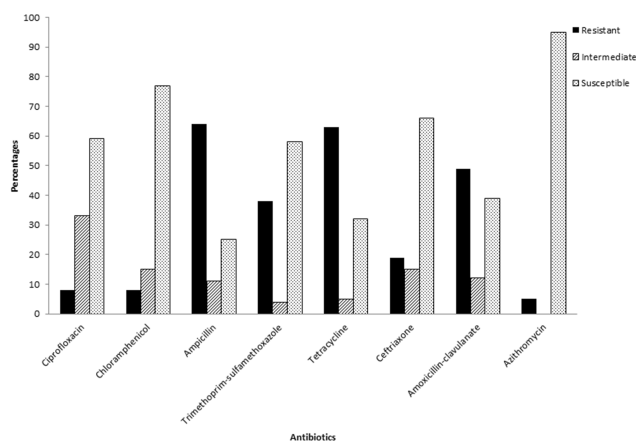


Figure 2 The number of samples that were susceptible, intermediate or resistant to the tested antibiotics.

as shown in Figure 3. The prevalence of the genes was as follows: *bla*_{TEM-1} (44%), *bla*_{CMY-2} (35%), *sul2* (21%), *tetC* (18%), *dfrA7* (14%) and *tetA* (8%).

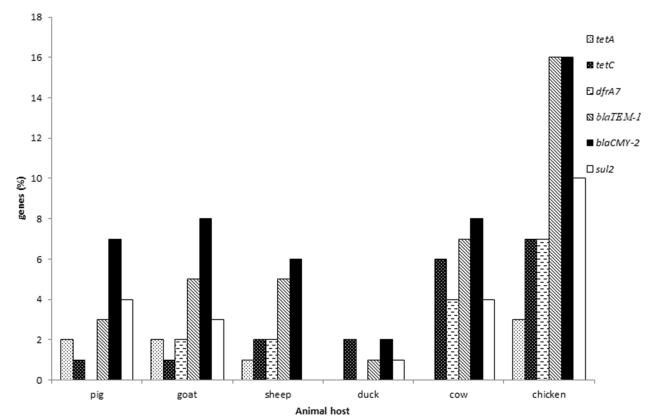


Figure 3 Distribution of antimicrobial resistance genes in *Salmonella* spp. isolated from livestock.

There was no statistically significant ($p > 0.05$) association between sample material and the animal host in the presence of antimicrobial resistance genes. However, farm location was significantly ($p < 0.05$) associated with the presence of *tetC*, *dfrA7* and *bla_{TEM-1}* as shown in Table 5.

A significant ($p < 0.05$) positive correlation was observed between *tetC* and *bla_{CMY-2}*, moreover, *bla_{CMY-2}* was positively correlated to *bla_{TEM-1}* and *sul2*. The *sul2* gene was also positively correlated to *dfrA7* and *bla_{TEM-1}* as the p-values were less than the critical values of 0.05 and 0.01 (Table 6).

The effect of farm location as a predictor for the presence of antimicrobial resistance genes was further assessed by binary logistic regression. Although the majority of the results were not significant ($p > 0.05$) it was observed that the presence of genes in the South coast was more related to Flagstaff than Verulam with regard to the odds ratios (Table 7). Higher odds ratios were observed when comparing South coast to Flagstaff than when comparing South coast to Verulam. The effect of farm location as a predictor of the presence of *dfrA7* was significant ($p < 0.05$) when Verulam was compared to South coast and the effect on the presence of *bla_{TEM-1}* was significant ($p < 0.05$) when South Coast was compared to Verulam as shown in Table 7. The presence of

tetC was significant ($p < 0.05$) when all the three farm locations were compared.

Discussion

The findings of the current study indicate the presence of multidrug-resistant *Salmonella* spp. isolated from livestock in South Africa. High rates of resistance were observed against ampicillin, tetracycline, amoxicillin-clavulanate, trimethoprim-sulfamethoxazole and ceftriaxone with frequencies of 64%, 63%, 49%, 38% and 20%, respectively. There was also decreased activity against ciprofloxacin, chloramphenicol and azithromycin as some of the isolates were also resistant to these antibiotics (Figure 2). These high resistance rates detected in livestock-associated *Salmonella* spp. in South Africa may be explained that antibiotics use as growth-promoting agents is legally allowed in the country.⁶¹ In addition, imprudent use of antibiotics in livestock farms was recently reported in South Africa.⁶²

The findings in the current study were in line with other studies which reported high resistance rates towards ampicillin, tetracycline, trimethoprim-sulfamethoxazole.^{63–65} Higher resistance rates against azithromycin (16.9%) and ceftriaxone (24.07%) were previously reported⁶⁶ as compared to the current study. A decrease in the efficiency of chloramphenicol and ciprofloxacin was previously reported with intermediate susceptibilities of 67% and 33%, respectively.⁶⁷ Tetracycline, ampicillin and amoxicillin are commonly used for the treatment of infections in cattle, pigs, poultry, sheep and goats.⁶⁸ During treatment, antibiotics are administered to all livestock herd on the farm thus triggering antimicrobial resistance and affects the intestinal microflora of healthy animals. Other than disease treatment and prevention, antibiotics such as tetracyclines, sulfonamides, macrolides, fluoroquinolones and beta-lactams are mainly used as growth promoters in farms.^{30,50,69} This can be an explanation for the high rates of resistance towards these antibiotics. Even though the resistance frequencies were low for ciprofloxacin,

Table 5 Fisher's Exact Test Results Showing Whether There Is A Significant Association (p-Values) Between The Antimicrobial Resistance Genes And Location, Animal Host As Well As Sample Material

Genes	Farm Location	Animal Host	Sample Material
<i>tetA</i>	0.807	0.872	0.474
<i>tetC</i>	0.000*	0.091	0.052
<i>dfrA7</i>	0.000*	0.208	0.095
<i>bla_{CMY-2}</i>	0.723	0.860	0.745
<i>bla_{TEM-1}</i>	0.008*	0.312	0.279
<i>sul2</i>	0.358	0.259	0.818

Note: An asterisk denotes a significant association as the $p < 0.05$.

Table 6 The Relationship Between The Detected Antimicrobial Resistance Genes As Statistically Assessed By Pearson's Correlation

Genes	Statistical Tests	<i>tetA</i>	<i>tetC</i>	<i>dfrA7</i>	<i>bla_{CMY-2}</i>	<i>bla_{TEM-1}</i>	<i>sul2</i>
<i>tetA</i>	Pearson's correlation Sig. (2-tailed)	1	-0.134 0.172	-0.014 0.890	0.033 0.740	0.165 0.090	-0.058 0.554
<i>tetC</i>	Pearson's correlation Sig. (2-tailed)	-0.134 0.172	1	0.093 0.345	0.227* 0.020	0.174 0.075	0.125 0.203
<i>dfrA7</i>	Pearson's correlation Sig. (2-tailed)	-0.014 0.890	0.093 0.345	1	0.182 0.061	0.043 0.659	0.193* 0.048
<i>bla_{CMY-2}</i>	Pearson's correlation Sig. (2-tailed)	0.033 0.740	0.227* 0.020	0.182 0.061	1	0.303** 0.002	0.339** 0.000
<i>bla_{TEM-1}</i>	Pearson's correlation Sig. (2-tailed)	0.165 0.090	0.174 0.075	0.043 0.659	0.303** 0.002	1	0.260** 0.007
<i>sul2</i>	Pearson's correlation Sig. (2-tailed)	-0.058 0.554	0.125 0.203	0.193* 0.048	0.339** 0.000	0.260** 0.007	1

Notes: *Correlation significant at level 0.05 (2-tailed), **Correlation is significant at the level 0.01 (2-tailed).

Table 7 The Effect Of Farm Location As A Predictor Of The Presence Of The Investigated Antimicrobial Resistance Genes

Genes	Location	
	Flagstaff	Verulam
<i>tetA</i>	Sig: 0.894 OR: 0.886 95% CI: (0.150, 5.235)	Sig: 0.616 OR: 1.682 95% CI: (0.221, 12.802)
<i>tetC</i>	Sig: 0.998 OR: 0.000 95% CI: (0.000, -)	Sig: 0.998 OR: 0.000 95% CI: (0.000, -)
<i>dfrA7</i>	Sig: 0.998 OR: 1.469 95% CI: (0.000, -)	Sig: 0.036* OR: 0.182 95% CI: (0.037, 0.895)
<i>bla_{CMY-2}</i>	Sig: 0.617 OR: 1.294 95% CI: (0.472, 3.550)	Sig: 0.824 OR: 0.891 95% CI: (0.321, 2.468)
<i>bla_{TEM-1}</i>	Sig: 0.004* OR: 5.170 95% CI: (1.702, 15.707)	Sig: 0.311 OR: 1.699 95% CI: (0.609, 4.738)
<i>sul2</i>	Sig: 0.264 OR: 2.056 95% CI: (0.58, 7.276)	Sig: 0.955 OR: 0.967 95% CI: (0.300, 3.116)

Note: An asterisk denotes a significant association as the $p < 0.05$.

Abbreviations: OR, odds ratio; 95% CI, 95% confidence interval.

chloramphenicol and azithromycin, the high level of intermediate susceptibility towards ciprofloxacin, is concerning as it indicates the possibility of resistance towards this antibiotic in the future. The multidrug resistance phenotype displayed by 43% of the isolates reveals that humans in proximity to these farms are at risk of serious zoonotic infections with difficult-to-treat *Salmonella* spp. The occurrence of antimicrobial-resistant *Salmonella* spp. in this study indicates that food animals and their environments are the source of antimicrobial resistance.

Antibiotic resistance is mediated by genetic elements which code for different mechanisms which are used by the bacteria to escape the effects of antibiotics.³⁷ Beta-lactam resistance genes (*bla_{TEM-1}*, *bla_{CMY-2}*), sulfonamide resistance gene (*sul2*), tetracycline resistance genes (*tetA* and *tetC*) and trimethoprim resistance determinant (*dfrA7*) were observed in this study. The *bla_{TEM-1}* and *bla_{CMY-2}* genes code for enzymes which degrade beta-lactam antibiotics by hydrolyzing the beta-lactam ring.^{10,70} The *sul2* and *dfrA7* genes are involved in synthesizing the insensitive forms of dihydropteroate and dihydrofolate reductase to escape the activity of trimethoprim-sulfamethoxazole, respectively.⁷¹ *TetC* and *tetA* are

responsible for transporting tetracycline out of the cell.⁷² The antimicrobial resistance genes were distributed in all livestock animals; however, higher prevalence was observed in chickens (Figure 3). Chickens are reared in large numbers in closed environments thus creating favourable conditions for pathogens and disease dissemination leading to frequent treatment. Poultry production systems such as battery cages, litter systems and automated feeders also promote antimicrobial resistance dissemination in industrialized farms.⁷³ Similar studies which screened antimicrobial resistance genes from food animals reported lower rates of *bla_{TEM-1}* of 19.3%⁶³ and 24.73%⁷⁴ as compared to the current study. Very low prevalence of *tetA* gene was previously reported.^{51,67} The prevalence of *bla_{CMY-2}* in the current study was similar to a previous study which reported a rate of 38.88%.⁷⁵ The high prevalence of beta-lactam degrading genetic determinants in this study is of concern because extended beta-lactams such as ceftriaxone are the drug of choice for salmonellosis treatment in children and pregnant women.⁴²

The prevalence of *tetC*, *dfrA7* and *bla_{TEM-1}* genes was significantly ($p < 0.05$) associated with farm location (Table 5). These genes were highly prevalent in Verulam, which is more urbanized as compared to South coast and Flagstaff (Table 7). A possible explanation is that livestock in urban areas are mostly fed on preserved foods with traces of chemicals and antibiotics while in rural areas livestock are normally fed on natural grass and grains. As a result of high demand for meat, over-population and limited space, food animals in urban areas are commonly fed antibiotics for fast growth.^{29,76} The prevalence of *tetC*, *dfrA7* and *bla_{TEM-1}* suggested that tetracycline, trimethoprim and ampicillin are frequently used on the farms in this study, particularly Verulam.

There was a significant ($p < 0.05$) weak positive correlation (ranging from 0.34 to 0.19) between *tetC*, *bla_{CMY-2}*, *bla_{TEM-1}*, *sul2* and *dfrA7* (Table 6). The roles of these genes and their locations in *Salmonella* could explain their correlation. The *sul2* and *dfrA7* genes are cassettes found in class 1 integrons which are associated with antimicrobial resistance and are responsible for biosynthetic processes in *Salmonella*.⁵⁹ The *tetC* gene and *bla_{CMY-2}* are related by their involvement in cell wall synthesis and membrane transport activity.^{10,43} The *bla_{TEM-1}* and *bla_{CMY-2}* genes have the same activity of degrading beta-lactams and extended beta-lactams which could explain their association.¹⁰ Furthermore, *bla_{TEM-1}* and *bla_{CMY-2}* are located on transferable plasmids.⁷⁰ The association between *sul2* and beta-

lactamase-producing genes (*bla_{TEM-1}* and *bla_{CMY-2}*) can be explained by their location in *Salmonella*.³⁷

The associations between resistance genes in the current study are in accordance with the previously reported results where it was found that class I integrons are related to transposons and insertion sequence (IS) elements which carry antimicrobial resistance genes.⁷⁷ Resistance genes are usually carried by integrons as gene cassettes.³³ The *dfrA7* gene is one of the classic genes carried by the class I integron.⁷⁸ The *sul2* gene is also found in the class I integron which further explains the relationship between these genes. Beta-lactamase genes (*bla_{TEM-1}* and *bla_{CMY-2}*) are located in the IS elements which are linked to the class I integrons⁷⁷ which explained the correlation between these genes in the current study.

High levels of antimicrobial resistance phenotypes were observed compared to the resistance genetic determinants. Highlighted in this study is the risk that humans are exposed to as a result of livestock-associated antibiotic-resistant *Salmonella*. A high level of intermediate susceptibility towards ciprofloxacin provokes questions about the efficiency of this antibiotic in future. In addition, in the current study the detection of fluoroquinolone-resistant *Salmonella* spp. which is listed in the WHO high priority list²² is very concerning. The presence of *bla_{CMY-2}* gene which confers *Salmonella* resistance to cephalosporins (ceftriaxone) is also a matter of concern as this antibiotic is one of the lately introduced antibiotics for medicinal use. The beta-lactam class of antibiotics is very important for treatment of salmonellosis in children, who are at risk of being infected. Another matter to point out is that these antibiotics are not only used to treat salmonellosis, but also infections by other bacterial pathogens.

The lack of human samples and the limited number of screened samples are among the limitations in the present study, which can be used to draw conclusions on the spread of *Salmonella* from livestock to humans. Typing the *Salmonella* isolates to the serovar level in order to determine the dominant *Salmonella* serovars in farms and human samples in South Africa require further investigation. Whole-genome sequence of selected samples will provide in-depth genomic characterization of resistome and virulome of the isolates. Frequent monitoring and enhanced surveillance programs will serve as an early warning system of antibiotic-resistant *Salmonella*, helping us find any potential disease much more quickly, control antibiotic resistance at the farm level, and minimize the public health burden.

Conclusions

The results of this study indicate the importance of monitoring the effects of antimicrobial use in livestock to minimize the public health risks. The findings of this study highlights the importance of monitoring the trend of antimicrobial resistance in South Africa and will contribute to the global One Health initiative to contain antimicrobial resistance.

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Disclosure

The authors report no conflicts of interest in this work.

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