Running title: **C**haracterization of *S. aureus* from chickens

Molecular and phenotypic characterization of *Staphylococcus aureus* strains isolated from carcass swabs and carcass drips of chickens slaughtered in the informal market in Gauteng Province, South Africa

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Abstract

The study was conducted to characterize *S. aureus* strains from swabs and drips of dressed chicken carcasses sold at outlets in six townships in the informal market in Gauteng province, South Africa, using molecular and phenotypic methods. Seven genes (6 toxins and 1 antimicrobial resistance) comprising of staphylococcal enterotoxin A (SEA), B (SEB), C (SEC), D (SED), exfoliative toxin A (ETA), toxic shock syndrome toxin (TSST) and MecA

encoding methicillin resistance were assayed using polymerase chain reaction (PCR). The resistance of the *S. aureus* strains to 18 antimicrobial agents was determined using the disc diffusion method. The frequency of detection of the six toxin genes was *sea* (52.2%), followed by *seb* (10.9%), s*ec* (6.5%), *sed* (2.2%), *eta* (93.5%) and *tst* (19.6%). The *mecA* gene was detected in 4.3% of the isolates. The predominant profiles of toxin genes detected were *sea-eta* (37.0%). All 63 isolates of *S. aureus* were resistant to one or more antimicrobial agents. The frequency of resistance was high to spectinomycin (98.4%), nalidixic acid (85.7%), and penicillin (84.1%), but low to gentamycin (1.6%) and cefotaxime (1.6%). The high frequency of toxin genes and antimicrobial resistance gene observed in *S. aureus* isolates from chicken could pose a challenge to food safety and may have therapeutic and zoonotic implications.

Keywords: Toxigenicity, Antimicrobial resistance, *S. aureus*, Chicken, South Africa

1. INTRODUCTION

In South Africa, the broiler industry was the largest individual agricultural sector contributor in 2016, with a contribution of 16% to the gross domestic product (GDP). Formal broiler production is controlled by a small number of large commercial producers providing 80% of the total broiler production while the informal sector makes up the remaining 20% (https://www.daff.gov.za).

Reports have suggested that chicken from retail outlets can be carriers of bacterial pathogens such as, *Salmonella* spp., verocytotoxigenic *Escherichia coli* (VTEC) and *Staphylococcus aureus* among others; and may be incriminated in human infections, diseases and epidemics (Ahlstrom et al., 2017; Umeda et al. 2017; Yamasaki et al., 2017). Processed raw chicken sold at outlets on 'road-sides', at 'wet markets', at 'pluck shops' and by cottage poultry processors in several countries have been reported to have a high prevalence of

pathogens. This has been attributed to poor sanitary practices, including poor water quality at these outlets (Nhung et al., 2017; Oguttu, McCrindle, Makita, & Grace, 2014).

In South Africa, published reports on staphylococcal contamination of chicken and chicken products are rare. Most studies have focussed on milk contamination with *S. aureus,* since *S. aureus* is known to cause mastitis in cattle and is a huge challenge to the dairy industry (Mkize, Zishiri, & Mukaratirwa, 2017). Likewise, comprehensive information on the volume and patterns of antibiotic use in food animals is lacking. This may be attributed to the fact that antibiotics intended for use by non-veterinarians (mainly farmers), are considered as livestock cure-all remedies and are available over the counter as per Act 36 of 1947 of Fertilizers, Farm Feeds, Agricultural Remedies and Stock Remedies. No record on the use these antimicrobial agents is kept. However, Eagar, Swan, & van Vuuren (2012) have reported that data collected between 2002-2004, showed the most widely used antibiotics by weight are those utilized as growth promoters, for disease treatment and prevention in poultry and pigs. Macrolides, pleuromutilins, tetracyclines, sulphonamides, penicillins were found to be among the most commonly used antimicrobial classes in food animals in South Africa (Henton, Eagar, Swan, & van Vuuren (2011). Resistance of *S. aureus* and other bacteria to antimicrobial agents have been reported in the livestock industry and has been attributed to the indiscriminate (abuse or over-use) of these agents as feed additives to promote growth, and in prophylaxis and therapy (Abreu et al., 2019; Ferri et al., 2017; Manishimwe, Nishimwe, & Ojok, 2017).

The potential zoonotic significance is that strains of resistant *S. aureus* in chicken could be transmitted to human handlers at processing facilities (Abdalrahman, Stanley, Wells, & Fakhr, 2015; Igbinosa, Beshiru, Akporehe, Oviasogie, & Igbinosa, 2016) or in kitchens, thereby posing potential therapeutic problems (Wendlandt, Schwarz, & Silley, 2013). Genetic characterisation targeting toxin using PCR (Li et al., 2018) and resistance genes (Sallam, Abd-Elghany, Elhadidy, & Tamura, 2015) provide information on the potential of S. *aureus* strains

to produce toxins and exhibit resistance to antimicrobial agents with therapeutic implications (Li et al., 2018; Sallam, Abd-Elghany, Elhadidy, & Tamura, 2015).

In Gauteng province, South Africa, the informal chicken market, with numerous outlets throughout the province, serves as a popular, convenient and cost-effective source of protein for the population, particularly in the townships. It has been reported that the microbial quality of chicken from these outlets is poor, primarily due to insanitary practices, and may pose a health risk to consumers, if improperly cooked (Mosupye, & von Holy, 2000). Oguttu, McCrindle, Makita, & Grace (2014) reported that chicken from the informal market in the province was contaminated with *S. aureus* but the strains were not characterized. Adigun (2017) conducted a study at informal market outlets in seven areas in Gauteng province to determine the microbial quality (*S. aureus*, non-staphylococcal *S. aureus*, coliforms and total aerobic plate count) of carcass swabs, carcass drips and rinse water as a measure of the level of sanitation. The study detected high prevalence and counts of these indicator microorganisms. Although Mkize, Zishiri, & Mukaratirwa (2017) conducted a genetic characterization of antimicrobial resistance and virulence genes in *S. aureus* isolates from commercial broiler chickens in the formal outlets in the Durban metropolis area of South Africa, there is a dearth of information on the characteristics of *S. aureus* strains recovered from chickens processed and retailed in the informal chicken market in the province.

The current study was therefore conducted to characterize the *S. aureus* strains isolated from the informal chicken market outlets in Gauteng province using a molecular method (PCR) to detect toxin and resistance genes and a conventional phenotypic method (disc diffusion) to determine their resistance to antimicrobial agents. The study also related the frequency of detection of toxin and resistance genes, and resistance to antimicrobial agents, to the types of samples (carcass swabs and carcass drips) and their sources (outlets across five townships).

2 MATERIALS AND METHODS

2.1 Sample collection and isolation of *S. aureus* strains used in the study

Overall, 63 *S. aureus* isolates were characterized in the current study. These isolates originated from 151 samples each of carcass swabs and carcass drips collected from 47 informal chicken outlets across six townships in Gauteng province, South Africa, namely: Atteridgeville/Phomolong, Garanguwa, Tembisa/Modisa, Alexandra, Germiston and Soweto (Adigun, 2017). The isolates were phenotypically confirmed to be *S. aureus* using standard methods (Fijałkowski, Peitler, & Karakulska, 2016). Briefly, swabs of chicken carcasses and carcass drips were inoculated on Baird-Parker agar (BPA) plates and streaked for isolation followed by aerobic incubation at 37°C for 48 h. Greyish-black or black colonies were tentatively classified as staphylococci. Suspect staphylococcal colonies on BPA plates were initially subjected to the following identification tests: Gram-stain, catalase, oxidase, indole and coagulase tests (Abdalrahman & Fakhr, 2015). A StaphTex latex agglutination test kit was used to detect coagulase production (Thermo Fisher Scientific, Australia). Fermentation of mannitol and maltose was determined using the tube test as earlier described (Abdalrahman & Fakhr, 2015). DNAse production was detected by inoculating DNAse agar (Oxoid, Basingstoke, UK) plates with pure cultures of the suspect staphylococci which were then incubated at 37°C for 24 h after which agar was flooded with 1N Hydrochloric acid and left for a few minutes. Susceptibility of the staphylococcal isolates to Polymycin B (300 Units) and Novobiocin (5 µg) (Oxoid, Basingstoke, United Kingdom) was also determined on Mueller-Hinton agar using standard methods (Buzón-Durán, Capita, & Alonso-Calleja, 2018). All isolates that were Gram-positive cocci, catalase-positive, indole-negative, oxidase-negative, fermenters of maltose and mannitol, coagulase-positive, Polymycin B-resistant, novobiocin-sensitive and DNAse-positive were phenotypically identified as *S. aureus*. These *S. aureus* isolates were also confirmed using PCR amplification of *femA* gene (Asadollahi et al., 2014).

Cultures were maintained in either blood tryptose agar plates or in 50% brain heart infusion broth/50% glycerol broth at -20^{o}C for subsequent assays.

Overall, a total of 63 isolates were used for the phenotypic determination of the antibiograms, but only 46 isolates for molecular studies. This was because 17 isolates of *S. aureus* were accidentally lost during the months of refrigerated storage between conducting the phenotypic and molecular studies.

2.2 PCR for toxin and resistance genes

2.2.1 DNA extraction

DNA from individual colonies was extracted using the heating method as earlier described (Reischl et al., 2000; van Tongeren et al., 2002). Briefly, colony suspensions were prepared by mixing a loopful of bacterial culture with 200 µl of PCR grade water. The suspensions were then heated at 95°C in a heating block for 25 min. The culture lysates were stored at -20°C until further analysis or were immediately used as DNA template in subsequent PCR protocols.

2.2.2 *In vitro a*mplification of toxin and resistance genes

Multiplex polymerase chain reaction (PCR) assays were performed in two sets, SET A to amplify *sea*, *seb, sec, sed* and *femA* gene fragments and SET B for amplification of *femA, mecA, eta,* and *tst-1*. Primer sequences and expected amplicon sizes were as described by Asadollahi et al. (2014) and are presented in Table 1. The PCR master mix for each set was made to a total volume of 45 µl and contained 25 µl of Red mix (Lasec, South Africa), 0.4 uM of each primer and PCR grade water to make the final volume. Five microlitre of DNA was added. The PCR reactions for SET A multiplex were preceded by denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 5 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min. The cycles were followed by the final elongation step at 72 °C for 7 min. The

PCR conditions for SET B differed from those of SET A only by the annealing temperature, which was 58 °C. The PCR products were separated by gel electrophoresis on 2% agarose gel.

TABLE 1 Oligonucleotide sequences and expected amplicon sizes for *S. aureus* toxin gene PCR

Gene	Primer sequence	PCR product size (bp)[†]
sea	5' ggt tat caa tgt gcg ggt gg 3' 5' cgg cac ttt ttt ctc ttc gg 3'	102
seb	5' gta tgg tgg tgt taa ctg agc 3' 5' cca aat agt gac gag tta gg 3'	164
sec	5' aga tga agt agt tga tgt gta tgg 3' 5' cac act ttt aga atc caa ccg 3'	451
sed	5' cca ata ata gga gaa aat aaa ag 3' 5' att ggt att ttt ttt cgt tc 3'	278
femA[‡]	5' aaa aaa gca cat aac aag cg 3' 5' ctg gtg aag ttg taa tct gg 3'	132
mecA	5' act gct atc cac cct caa ac 3' 5' ctg gtg aag ttg taa tct gg 3'	163
eta	5' gca ggt gtt gat tta gca tt 3' 5' aga tgt ccc tat ttt tgc tg 3'	93
tst	5' acc cct gtt ccc tta tca tc 3' 5' ttt tca gta ttt gta acg cc 3'	326

[†] Mehrotra et al. (2000)
[‡] *femA* gene, although not a toxin gene, was used as an internal control for the identification of *S. aureus*.

2.3 Antimicrobial profiling

A total of 63 isolates of *S. aureus* were available to determine their antimicrobial profiles. Antimicrobial susceptibility or resistance to spectinomycin (SPEC, 100 µg), nalidixic acid (NA, 30 µg), penicillin (P, 10 µg), amoxicillin (AMOX, 10 µg), oxytetracycline (OXY, 30 µg), sulphamethoxazole-trimethoprim (SXT, 30 µg), ampicillin (AMP, 10 µg), enrofloxacin (ENRO, 5 µg), erythromycin (ERY, 15 µg), amoxicillin-clavulanic acid (AMC, 30 µg), doxycycline (DOX, 30 µg), ceftazidime (CEFT, 30 µg), ciprofloxacin (CIP, 5 µg), kanamycin (K, 30 µg), streptomycin (STR, 10 µg), gentamycin (CN, 10 µg), cefotaxime (CEF, 30 µg) and

chloramphenicol (C, 30 µg) (Oxoid Ltd., UK) was determined using Kirby Bauer disk diffusion method as described by the National Committee for Clinical Laboratory Standards, NCCLS (2013). Briefly, cultures were inoculated on Mueller - Hinton agar plates and incubated for 24 h at 37°C, after which the zones of inhibition were measured.

2.4 Statistical analyses

The frequency of detection of toxin and resistance genes and the occurrence of resistance (single and multi-drug) to individual or classes of antimicrobial agents were related to the source (area) and type (carcass swab and carcass drip) of sample, as well the frequency of possession of toxin genes and the occurrence of resistance to antimicrobial agents. The differences were subjected to statistical analyses. The p-values were calculated, and the results obtained from R software. The null hypothesis used was that all proportions are equal for the Goodness of fit chi-square. The Fisher's exact value was used where the program suggested that the use of the calculated Chi-square might be incorrect, especially in small data size. For the study, the level of significance was set at alpha level of 0.05.

3 RESULTS

3.1 Frequency of detection of toxin and resistance genes in *S. aureus* strains

The detection of toxin genes in 46 isolates of *S. aureus* recovered from carcass swabs and carcass drips based on the location of the informal chicken outlet is shown in Table 2. Amongst the isolates of *S. aureus* positive for staphylococcal enterotoxin genes (*sea, seb, sec,* and *sed*), the frequency of detection, regardless of type of sample, ranged from 42.9% in Atteridgeville/Phomolong to 60.0% in Soweto for *sea*; 0.0% in Garankuwa and Germiston to 42.9% in Atteridgeville/Phomolong for *seb*; 0.0% in Garankuwa, Germiston and Soweto to 22.2% in Alexandra for *sec* while *sed* was detected in Alexandra, 11.1%. The antimicrobial

TABLE 2 Frequency of detection of toxin and resistance genes in *S. aureus* strains from carcass swabs and drips

Township	Type of samples	No. of samples tested	No. (%) of samples positive for toxin and resistance genes[†]							
			sea[‡]	*seb*	*Sec*	*sed*	*mecA*	*eta*	*tst*	p-value
Atteridgeville/ Phomolong	Carcass swab and drip	7	3 (42.9)	3 (42.9)	1 (14.3)	0 (0.0)	0 (0.0)	6 (85.7)	3 (42.9)	1
Garankuwa	Drip[§]	8	4 (50.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	8 (100.0)	1 (12.5)	NA[¶]
Alexandra	Carcass swab and drip	9	4 (44.4)	1 (11.1)	2 (22.2)	1 (11.1)	0 (0.0)	9 (100.0)	1 (11.1)	0.5623
Germiston	Carcass swab and drip	2	1 (50.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (100.0)	1 (50.0)	1
Soweto	Carcass swab and drip	20	12 (60.0)	1 (5.0)	0 (0.0)	0 (0.0)	2 (10.0)	18 (90.0)	3 (15.0)	0.9813
Total	Carcass swab	12	5 (41.7)	2 (16.7)	1 (8.3)	0 (0.0)	0 (0.0)	11 (91.7)	4 (33.3)	0.8311
	Drip	34	19 (55.9)	3 (8.8)	2 (5.9)	1 (2.9)	2 (5.9)	32 (94.1)	5 (14.7)	
	Carcass swab and drip	46	24 (52.2)	5 (10.9)	3 (6.5)	1 (2.2)	2 (4.3)	43 (93.5)	9 (19.6)	
p-value			0.2345	0.2345	1	1	1	0.4673	1	

[†]*sea*: Staphylococcal enterotoxin A (SEA) gene, *seb*: Staphylococcal enterotoxin B gene, *sec*: Staphylococcal enterotoxin C gene, *sed:* Staphylococcal enterotoxin D gene, *mecA* : gene encoding for penicillin binding protein 2A , eta: Exfoliative toxin A gene and *tst:* Toxic Shock Syndrome gene.

[‡]Of a total of 46 isolates of *S. aureus* strains tested, 29 (63.0%) possessed enterotoxin genes producing the following patterns: *sea*, 22 (47.8%), *seb*, 4 (8.7%), *sec*, 1 (2.2%), *sea-sec*, 1 (2.2%) and *sea-seb-sec-sed*, 1 (2.2%)

[§]No *S. aureus* strain was isolated from carcass swabs

[¶]Not applicable

gene, *mecA,* was detected in samples (10%) from Soweto only. The frequency of detection of *eta* was comparatively high, ranging from 85.7% in Atteridgeville/Phomolong to 100.0% in the following three townships of Garankuwa, Alexandra and Germiston. The *tst* gene was detected at a frequency that ranged from 11.1% in Alexandra to 50.0% in Germiston. Overall, the frequency of detection of *sea*, *seb*, *sec*, *sed*, *mecA*, *eta* and *tst* was 52.2%, 10.9%, 6.5%, 2.2%, 4.3%, 93.5% and 19.6% respectively. The frequencies of detection of staphylococcal toxins in carcass swabs and carcass drips across the five townships are shown in Appendix 1. For the 46 isolates of *S. aureus*, the patterns of staphylococcal enterotoxin (SE) genes were *sea*, 22 (48.7%), *seb*, 4 (8.7%), *sec*, 1 (2.2%), *sea-sec*, 1(2.2%) and *sea-seb-sec-sed*, 1 (2.2%). Therefore, a total of 29 (63.0%) *S. aureus* isolates were positive, either singly or in combination, for staphylococcal enterotoxin (A, B, C, and D) genes, and *sea* was the predominant enterotoxin gene detected, with 24 (52.2%) isolates being positive.

For all genes (toxin and antimicrobial resistance) detected, a total of 13 patterns were detected with the predominant ones being *sea-eta*, 17 (37.0%), *eta*, 15 (32.6%), *sea-eta-tst*, 3 (6.5%) and *sea*, 2 (4.3%). The following patterns were exhibited by one isolate each i.e. 1 (2.2%): *eta-tst, sec-tst, sea-seb-sec-sed, seb-tst, sea-sec-eta-tst, seb-eta*, *mecA-tst, seb-eta-tst* and *sea-mecA-eta*. All the 46 isolates of *S. aureus* were positive for one or more of the eight genes assayed.

Overall, the differences in the frequency of detection of toxin and resistance genes *sea, seb, sec, sed, eta, tst* and *mecA*) among the five townships were not statistically significantly ($P>0.05$).

3.2 Frequency of resistance to antimicrobial agents amongst *S. aureus* strains.

Table 3 shows the frequency of resistance of *S. aureus* isolates to 18 antimicrobial agents. All 63 isolates of *S. aureus* from carcass swabs and carcass drips were resistant to one or more

TABLE 3 Frequency of resistance to antimicrobial agents by *S. aureus* strains from carcass swabs and drips

				No. (%) of isolates resistant to:											
Township	**Type of sample**	**Resistant**[†]	**SPEC**[‡]	**NA**	**P**	**AMOX**	**OXY**	**SXT**	**AMP**	**ENRO**	**ERY**	**ACA**	**DOX**	**CEFT**	**p-value**
Atteridgeville/ Phomolong	Carcass swab and drip	5 (100.0)	5 (100.0)	2 (40.0)	2 (40.0)	1 (20.0)	3 (60.0)	2 (40.0)	0 (0.0)	1 (20.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (20.0)	0.673
Garankuwa	Carcass swab[§]	9 (100.0)	9 (100.0)	9 (100.0)	7 (77.8)	7 (77.8)	8 (88.9)	6 (66.7)	5 (55.6)	1 (11.1)	0 (0.0)	2 (22.2)	0 (0.0)	0 (0.0)	NA[¶]
Alexandra	Carcass swab and drip	14 (100.0)	14 (100.0)	12 (85.7)	14 (100.0)	10 (71.4)	13 (92.9)	11 (78.6)	6 (42.9)	10 (71.4)	1 (7.1)	0 (0.0)	1 (7.1)	0 (0.0)	0.91
Germiston	Carcass swab and drip	8 (100.0)	7 (87.5)	7 (87.5)	6 (75.0)	1 (12.5)	8 (100.0)	4 (50.0)	4 (50.0)	6 (75.0)	6 (75.0)	1 (12.5)	1 (12.5)	0 (0.0)	1.00
Soweto	Carcass swab and drip	27 (100.0)	27 (100.0)	24 (88.9)	24 (88.9)	23 (85.2)	9 (33.3)	16 (59.3)	16 (59.3)	2 (7.4)	0 (0.0)	2 (7.4)	1 (3.7)	1 (3.7)	1.00
Total	Carcass swab	16 (100.0)	16 (100.0)	14 (87.5)	14 (87.5)	9 (56.3)	9 (56.3)	7 (43.8)	8 (50.0)	5 (31.3)	3 (18.8)	0 (0.0)	1 (6.3)	0 (0.0)	1.00
	Drip	47 (100.0)	46 (97.9)	40 (85.1)	39 (83.0)	33 (70.2)	32 (68.0)	32 (68.0)	23 (48.9)	15 (31.9)	4 (8.5)	5 (10.6)	2 (4.3)	2 (4.3)	
	Total	63 (100.0)	62 (98.4)	54 (85.7)	53 (84.1)	42 (66.7)	41 (65.1)	39 (61.9)	31 (49.2)	20 (31.7)	7 (11.1)	5 (7.9)	3 (4.8)	2 (3.2)	
p-value		NA	0.31	0.67	0.22	1.00	0.60	0.01	0.71	1.00	1.00	1.00	1.00	1.000	

[†]Resistant to one or more antimicrobial agents

[‡]Spectinomycin (100 µg), NA: Nalidixic acid (30 µg), Penicillin (10 µg), Amoxicillin (10 µg), OXY: Oxytetracycline (30 µg), SXT: sulphamethoxazole-Trimethoprim (30 µg), AMP: Ampicillin (10 µg), ENRO: Enroflaxicin (5 µg), ERY: Erythromycin (15 µg); ACA: Amoxicillin-Clavulanic acid (30 µg), DOX: Doxycycline (30 µg), CEFT: Ceftazidime (30 µg), CIP: Ciprofloxacin (5 µg), K: Kanamycin (30 µg), STR: Streptomycin (10 µg), CN: Gentamycin (10 µg), CEF: Cefotaxime (30 µg) and C: Chloramphenicol (30 µg);

[§]No *S. aureus* strain was recovered from carcass swabs;

[¶]Not applicable

Note that not all the results of the antimicrobial resistance testing are presented in this table: The following antimicrobials Ciprofloxacin (CIP), Kanamycin (K),

Streptomycin (STR), Gentamycin (CN), Cefotaxime (CEF) and Chloramphenicol (C) have location-based resistance of < 10% and have been collapsed in the current table.

The full details can be viewed in the online supplementary

antimicrobial agents (Supplementary material: Appendix 1). For the isolates of *S. aureus* recovered from carcass swabs and carcass drips from each of the five townships, the frequency of resistance to each of the 18 antimicrobial agents was not statistically significantly different (P>0.05).

Regarding the frequency of resistance by *S. aureus* strains from both carcass swabs and carcass drips to specific antimicrobial agents, it was found to be high to spectinomycin and ranged from 87.5% in Gemiston to 100.0% in Atteridgeville/Phomolong, for nalidixic acid, 40.0% in Atteridgeville/Phomolong to 100.0% in Garankuwa; for penicillin, 40.0% in Atteridgeville/Phomolong to 100.0% in Alexandra, for amoxicillin, 12.5% in Gemiston to 85.2% in Soweto, for oxytetracycline, 33.3% in Soweto to 100.0% in Gemiston and for sulphamethoxazole-trimethoprim, 40.0% in Atteridgeville/Phomolong to 78.6% in (Alexandra). Across the five areas, resistance was generally low (<10.0%) to each of the following seven antimicrobial agents namely, ceftazidime, ciprofloxacin, kanamycin, streptomycin, gentamycin, cefotaxime and chloramphenicol. Overall, among the five townships and the isolates of *S. aureus* recovered from carcass swabs and carcass drips, the differences in the frequency of resistance to antimicrobial agents were not statistically significant (P>0.05).

The frequency of resistance of all the *S. aureus* isolates to the classes of antimicrobial agents tested, as shown in Table 4, was high to antimicrobial agents in the Aminoglycoside class (gentamycin, spectinomycin, streptomycin and kanamycin): 98.4%, to the β-lactams (amoxicillin-clavulanic acid, amoxicillin, penicillin and ampicillin): 88.9%, to the Fluoroquinolones (ciprofloxacin, nalidixic acid and enrofloxacin): 88.9%, to the Tetracyclines (doxycycline and oxytetracycline): 65.1% and to the Sulphonamide (sulphamethoxazole-trimethoprim): 61.9%. For four of the antimicrobial classes (aminoglycoside, β-lactams, fluoroquinolones and tetracyclines) the differences in the frequency of resistance exhibited to

TABLE 4 Prevalence of resistance to classes of antimicrobial agents

Antimicrobial class ( )[‡]	Type of antimicrobial agents used	No. (%)[†] of isolates resistant	*P-value*
Aminoglycoside (n=4)	Spectinomycin,	62 (98.4)	<0.00001
	Streptomycin	2 (3.2)	
	Kanamycin	2 (3.2)	
	Gentamycin	1 (1.6)	
	Subtotal	*62 (98.4)*	
Beta lactams (n=4)	Penicillin	53 (84.1)	<0.00001
	Amoxicillin	42 (66.7)	
	Ampicillin	31 (49.2)	
	Amoxicillin-clavulanic acid	5 (7.9)	
	Subtotal	*56 (88.9)*	
Fluoroquinolones (n=3)	Nalidixic acid,	54 (85.7)	<0.00001
	Enrofloxacin	20 (31.7)	
	Ciprofloxacin	2 (3.2)	
	Subtotal	*56 (88.9)*	
Tetracycline (n=2)	Oxytetracycline	41 (65.1)	<0.00001
	Doxycycline	3 (4.8)	
	Subtotal	41 (65.1)	
Cephalosporin (n=2)	Ceftazidime	2 (3.2)	0.599
	Cefotaxime	1 (1.6)	
	Subtotal	*3 (4.8)*	
Phenicols (n=1)	Chloramphenicol	1 (1.6)	**NA**
Macrolides (n=1)	Erythromycin	7 (11.1)	NA
Sulponamide (n=1)	Sulphamethoxazole-trimethorim	39 (61.9)	NA

[†]Of a total of 46 isolates of *S. aureus* and resistant to any of the antimicrobial agents

[‡]( ): Number of antimicrobial agents tested in each class

individual antimicrobial agents within each class were statistically significant ($p<0.0001$). The frequency of resistance was however very low to Macrolides (erythromycin): 11.1%, Cephalosporins (cefotaxime and ceftazidime): 4.8% and Phenicols (chloramphenicol): 1.6%.

Overall, a total of 47 resistance patterns were exhibited by the *S. aureus* strains tested. Of these patterns, 45 were multi-drug resistant (3 or more antimicrobial agents). The extent of multidrug resistance, as reflected by the number of antimicrobial agents in the patterns and the number of isolates involved, are as follows: 9 antimicrobial agents (3 isolates), 8 (5 isolates), 7 (11 isolates), 6 (12 isolates), 5 (6 isolates), 4 (6 isolates) and 3 (2 isolates). The predominant antimicrobial multi-drug resistance patterns exhibited were SPEC-NA-P-AMOX-SXT: 5 (7.9%), SPEC-NA-P-AMOX-SXT-AMP: 4 (6.3%), SPEC-NA-P-AMOX-OXY-SXT-ENRO: 3 (4.8%), SPEC-NA-P-AMOX-OXY-SXT-AMP: 3 (4.8%), SPEC-P-AMOX-OXT-AMP-ENRO: 2 (3.2%), SPEC-NA-P-AMOX-AMP: 2 (3.2%), SPEC-NA-P-OXY: 2 (3.2%), SPEC-NA-P-AMP: 2 (3.2%) and SPEC-NA-AMOX-OXY: 2 (3.2%). The remaining 38 patterns were exhibited by one isolate each.

4 DISCUSSION

In this study, we characterised *S aureus* isolates from chickens originating from informal market outlets in Gauteng Province, South Africa, using toxin gene identification and antimicrobial resistance (AMR) profiles. There are more than 40 staphylococcal toxin genes encoded in the core genome as well mobile genetic elements, including genomic islands, bacteriophages, plasmids and pathogenicity islands (Grumann, Nübel & Bröker, 2014). Six (*sea*, *seb*, *sec*, *sed*, *eta* and *tst*) of these genes known to be encoded in mobile genetic elements were assayed in this study. The 46 isolates tested were positive for at least, one of the six toxin genes.

Up to 63.0% of the isolates tested possessed enterotoxin genes encoding SEA, SEB, SEC and SED. The predominance of *sea* (52.2%) was an indication of potential food safety consequences because enterotoxin-expressing *S. aureus* strains produce these particular enterotoxins (Moghassem et al., 2015). In addition, outbreaks of foodborne intoxication has been associated with staphylococcal enterotoxins, particularly SEA (Denayer, Delbrassinne, Nia, & Botteldoorn 2017; Umeda et al., 2017). In this study, the detection of predominantly *sea* gene, agrees with previous reports (El Bayomi et al., 2016; Li et al., 2018; Madahi, Rostami, Rahimi, & Dehkordi, 2014). The frequency of detection of enterotoxin genes in *S. aureus* strains in the current study was higher than those reported in other studies in the USA (0.0%; Abdalrahman & Fakhr, 2015), Egypt (10.4%; El Bayomi et al., 2016) and Nigeria (18.0%; Ayeni et al., 2018). Hence, the findings in the current study have potential public health significance for consumers of improperly cooked chickens from studied outlets.

In a 2017 study, of the 104 *S. aureus* isolates from commercial broiler chicken slaughterhouses and retail outlets in Durban, South Africa none was positive for *sea* and *see* genes (Mkize, Zishiri, & Mukaratirwa, 2017). In the current study, 52.2% of the chickens sampled from the informal market outlets tested positive for *sea*. We suggested that the sample sources may be responsible for this difference in the two findings. While Mkize, Zishiri, & Mukaratirwa (2017) sampled the formal market and included only the broilers, we collected samples from the informal markets including broilers, culled breeders and spent hens. Akindolire, Babalola, & Ateba (2015) have earlier found staphylococcal enterotoxin genes (*sec* gene but not *sea, seb, sed* or *see* genes) in 32.7% of the *S. aureus* strains isolated from milk obtained from retail outlets in the North-West, South Africa. The present study may be considered the first documented evidence of enterotoxin genes found in isolates of *S. aureus* from informal market-sourced chicken in South Africa.

The finding in our study agrees with the report of Sallam, Abd-Elghany, Elhadidy, & Tamura (2015) who found that isolates of *S. aureus* (n = 76) from raw chicken samples in Egypt were positive for staphylococcal enterotoxin genes. Considerably lower frequency of detection of enterotoxin genes in *S. aureus* strains from chicken have been reported in China, 46% (Li et al., 2018).

In our study, a total of 13 toxin profiles were detected with *sea-eta* (37.0%) and *eta* (32.6%) being the predominant ones compared with the findings of Li et al. (2018) who detected 29 toxin gene profiles with Panton-Valentine leukocidin (PVL), *sea* and *seb* being 10.7%, 5.9% and 4.8% respectively. It should be noted that we assayed for a limited number of toxin profiles in this investigation.

The overall frequency of *eta* gene was very high (93.3%) for the isolates tested ranging from 75.0% to 100.0% across the outlets and townships. Though, limited number of samples were assayed, the frequency was considerably higher than the 0.0% and 1.2% reported elsewhere (Abdalrahman, & Fakhr, 2015; Li et al., 2018; Marek et al., 2018). In a previous assessment in the North-West Province, South Africa, Akindolire, Babalola, & Ateba (2015) assayed 156 *S. aureus* isolates from milk (raw, bulk and pasteurised) from retail outlets but failed to detect *eta* and *etb* genes. The detection of *eta* gene in high frequency in this study implies that if expressed, the toxins may pose a zoonotic risk to chicken handlers and consumers during processing and retailing of chickens, as such exfoliative toxin-producing *S. aureus* may cause clinical diseases in humans (Hennigan, Kourtney, & Cheryl-Riley, 2016; Saida et al., 2015).

A total of 19.6% of all *S. aureus* isolates in our study were positive for *tst* gene, a much higher frequency than those reported by other researchers (0.0% to 3.5%) (Abdalrahman, & Fakhr, 2015; Li et al., 2018; Marek, Pyzik, Stępień-Pyśniak, Urban-Chmiel, & Nowaczek,

2018; Sallam, Abd-Elghany, Elhadidy, & Tamura, 2015). The presence of *tst* gene in high frequency of *S. aureus* poses a zoonotic risk to chicken handlers as previous works have confirmed that TSST-producing *S. aureus* had caused human diseases (Klüter et al., 2015; Sharma et al., 2018). This is the first documentation of *S. aureus* strains with *tst* positive genes from animal, food or human sources in South Africa.

In this study , the *mecA* gene was detected in only 2 (4.3%) of the 46 isolates, with both isolates having originated from carcass drip samples from one township. In South Africa, the *mecA* gene was recently detected in *S. aureus* strains circulating in poultry and from farm workers at an intensive poultry production system (Amoako et al., 2019) but there was a failure to detect the gene in *S. aureus* isolated from clinical cases in humans (Antiabong, Kock, Bellea, & Ehlers, 2017). The *mecA* gene encodes for methicillin resistance and is indicative of potential occurrence of MRSA strains in our findings (Osman et al., 2016; Sallam, Abd-Elghany, Elhadidy, & Tamura, 2015). Although we did not test was done to specifically for methicillin resistance, the two *mecA* gene-positive *S. aureus* isolates were resistant to other β-lactam antibiotics (penicillin, ampicillin, amoxicillin and amoxicillin-clavulanic acid) used in the study. MRSA and some strains of methicillin sensitive *S. aureus* (MSSA) strains may or may not carry the *mecA* genes (Aqib et al., 2018; Elhassan, Ozbak, Hemeg, Elmekki & Ahmed 2015; Osman et al., 2015). Other studies have documented various frequencies for *mecA* gene: 0.0% in Oklahoma, USA (Abdalrahman & Fakhr, 2015), 10.0% in England (Dhup, Kearns, Pichon, & Foster, 2015), 30.8% in Poland (Marek, Pyzik, Stępień-Pyśniak, Urban-Chmiel, & Nowaczek, 2018*)* and 38.0% in Egypt (Sallam, Abd-Elghany, Elhadidy, & Tamura, 2015).

The detection of toxin and resistance genes in this study should be taken with caution as the presence of such genes does not always correspond to toxin gene expression. Furthermore, our sample size was limited to generalise for the country. A spatially distributed, nationwide detailed study, with investigation into gene expression for this category of food in South Africa

is warranted. We tested a panel of 18 antimicrobial agents against 63 isolates of *S. aureus* and all the isolates exhibited resistance to one or more antimicrobials. These results were of therapeutic, zoonotic and public health significance. Therapeutic failures against staphylococcal infections in live chickens and the potential for the transmission of antimicrobial resistant strains from poultry to human handlers may occur in view of the above. In other studies in South Africa and elsewhere, 99.7% to 100.0% of the *Staphylococcus aureus* isolates exhibited resistance to antimicrobial agents (Akindolire, Babalola, & Ateba, 2015; Buzón-Durán, Capita, & Alonso-Calleja, 2017; Li et al., 2018). These findings are likely to be a consequence of the easy access to antimicrobial agents in South Africa, including the commonly used ones in animals such as macrolides, pleuromutilins, tetracyclines, sulphonamides, and penicillins (Henton, Eagar, Swan, & van Vuuren, 2011).

Chickens evaluated in this study originated from poultry farms in Gauteng province and elsewhere, and the implications of such high frequency of resistance to antimicrobial agents on therapeutic intervention and residues that can be passed to human cannot be ignored. Similar study by Mkize, Zishiri, & Mukaratirwa (2017) had reported that between 66.7% - 100.0% (for retail outlets) and 46% - 79.4% (from chicken abattoirs) of all isolates of *S. aureus* were resistant to 10 antimicrobial agents, with the highest specific resistance against tetracyclines (Table 3).

The remaining eight antimicrobial agents (doxycycline, ceftazidime, ciprofloxacin, kanamycin, streptomycin, gentamycin, cefotaxime and chloramphenicol) have comparatively low resistance levels (1.6% to 4.8%), an indication that these antimicrobials might be effective in the treatment of *S. aureus* infections in chicken in South Africa.

Amongst the β-lactams, the overall resistance exhibited by *S. aureus* strains was high (88.9%). This class of antibiotics is commonly used for the treatment of *S. aureus* infections in

the poultry industry in Gauteng province and throughout South Africa, hence the result is unsurprising. Equally, high frequencies of resistance were detected to ampicillin (49.2%), amoxicillin (66.7%) and to penicillin (84.1%). It has been established that *S. aureus* strains developed resistance readily to penicillin, due to its overuse and the mechanism used to develop resistance has been documented (Yılmaz & Aslantaş, 2017). High frequency of resistance to penicillin have been documented against *S. aureus* from chickens in other countries (Buzón-Durán, Capita, & Alonso-Calleja, 2017; Osman, Amer, Badr, & Saad, 2015; Sallam, Abd-Elghany, Elhadidy & Tamura, 2015). Mkize and colleague (2017) have earlier detected a higher frequency (65.1%) of resistance to ampicillin has been reported for *S. aureus* strains from chicken from commercial broiler abattoirs in South Africa. The informal market outlets mostly obtain their chicken from small poultry farms, which may use reduced antimicrobials due to less intensification compared to the large poultry farms.

Similarly, our frequency of detection of resistance to oxytetracycline was somewhat similar to what other study had documented in South African commercial broiler abattoirs (65.1% versus 79.4%) (Mkize, Zishiri & Mukaratirwa, 2017). The commercial abattoirs essentially slaughter all broilers originating from the large broiler farms where intensive use of antimicrobials like oxytetracycline and sulphonamides, is higher than for the backyard and small-scale farm-sourced chickens (culled breeders, spent hens and broilers) which are mostly designated for informal market outlets. Similar observations are true for the sulphonamide in our study.

The frequency of resistance to the macrolide (erythromycin) was low (11.1%) compared to those reported for *S. aureus* isolates recovered from commercial broiler abattoirs (57.1%) and from chicken retail outlets (83.3%) in South Africa (Mkize, Zishiri & Mukaratirwa,

2017). Perhaps, differences in the sources and types of chicken from where *S. aureus* strains were isolated influenced this observation.

Generally, the resistance patterns exhibited by *S. aureus* strains tested in this study against 18 antimicrobial agents varied slightly from the findings in the limited published reports from South Africa and considerably from those findings from other countries. Caution should be observed here in comparing the variously sourced data on antimicrobial resistance of *S. aureus* because the test methods and their sensitivities, seasonal prevalence, management systems of sources of chickens, location of samples and national policy on AMR may influence point prevalence and cannot be over-emphasized.

A total of 47 resistance patterns were detected and 45 (95.7%) of these patterns were multi-drug resistant in nature, with resistance to at least three classes of antimicrobial agents, and 71.4% (45/63) of all the multi-drug resistant strains of *S. aureus* exhibited resistance to 3 - 9 antimicrobial agents. Similar profiles have been confirmed for chicken-originated *S. aureus* strains from commercial broiler chicken in Durban, South Africa (100%; Mkize, Zishiri, & Mukaratirwa, 2017), in China (87.2%; Li et al., 2018), and in Korea (≤ 100%; Kim, Seo, Jeon, Lim, & Lee, 2018). Inappropriate use of antimicrobial agents on poultry farms will continue to fuel increasing resistance of *S. aureus* and other pathogens to antimicrobial agents leading to multi-drug resistance (Kim, Seo, Jeon, Lim & Lee, 2018; Manishimwe, Nishimwe, & Ojok, 2017; Sirdar, Picard, Bisschop, Jambalang, & Gummow, 2012).

5 CONCLUSIONS

The detection of six toxin genes (*sea, seb, sec, sed, eta* and *tst*) encoding for staphylococcal enterotoxins, exfoliative toxin A and toxic shock syndrome toxin-1, and one gene (*mecA*) that encodes for methicillin resistance, in S. *aureus* strains in the current study has ramifications for

food safety due to potential staphylococcal intoxications in humans. The findings also have zoonotic and antimicrobial resistance significance since strains of *S. aureus* which are carriers of *eta*, *tst* and *mecA* genes can be transmitted to poultry handlers in Gauteng province. Finally, the study demonstrated that *S. aureus* strains from raw chicken obtained from the informal market in Gauteng province exhibited a high frequency of multi-drug resistance to antimicrobial agents which poses both zoonotic and therapeutic hazards to humans exposed to the chickens. The need for good hygienic practices during the processing of chicken at these outlets and the prudent use of antimicrobial agents in the poultry industry in Gauteng province, South Africa would be invaluable to food safety and public health.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding publication of this paper.

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