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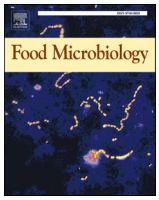

Antimicrobial resistance profiles of *Pseudomonas aeruginosa*, *Escherichia coli* and *Klebsiella pneumoniae* strains isolated from broiler chickens

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ABSTRACT

Globally, the spread of multidrug-resistant *Pseudomonas aeruginosa, Escherichia coli*, and *Klebsiella pneumoniae* from food to humans poses a severe threat to public health. The aim of this study was to assess the co-occurrence of colistin and *β*-lactamase resistance genes in *E. coli*, *K. pneumoniae*, and *P. aeruginosa* strains isolated from faeces of abattoir broiler chickens. The *E. coli*, *P. aeruginosa* and *K. pneumoniae* isolates were successfully detected from faecal samples by polymerase chain reaction (PCR) at infection rates of 60.7%, 22.5% and 16.7% respectively. The isolates displayed the highest levels of antibiotic resistance (AR) against ampicillin (82.3%) and amoxicillin-clavulanic acid (74.2%) for *E. coli*, followed by cefoxitin (70.6%) for *K. pneumoniae*, whilst *P. aeruginosa* displayed 26.1% antibiotic resistance (AR) against both ampicillin and colistin sulphate. The colistin *mcr-1* gene was harboured by 46.8%, 47.1% and 21.7%, *E. coli*, *K. pneumonia* and *P. aeruginosa* isolates respectively. Ten out of 62 (16.1%), 6/17 (35.3%), 4/23 (17.4%) isolates were phenotypically classified as ESBL *E. coli*, *K. pneumoniae*, and *P. aeruginosa* respectively. The ESBL-*E. coli* isolates respectively possessed $bla_{CTX\text{-}M}$ (60%), bla_{TEM} (20%) and $bla_{CTX\text{-}M\text{-}9}$ (10%) genes. The ESBL-*K. pneumoniae* harboured, $bla_{CTX\text{-}M}$ (50%), bla_{OXA} (33%), bla_{CARB} (17%), and $bla_{CTX\text{-}M\text{-}9}$ (17%) genes respectively, whilst, *P. aeruginosa* isolates respectively carried bla_{TEM} (75%), $bla_{CTX\text{-}M}$ (50%), bla_{OXA} (25%) and bla_{CARB} (25%) genes. Molecular analysis identified the $bla_{CTX\text{-}M}$ *β*-lactamase-encoding genes collectively from *E. coli*, *P. aeruginosa*, *K. pneumoniae* isolates. Colistin and *β*-lactamase genes were present in only 16.7%, 6.9%, and 2.9% of *E. coli*, *K. pneumoniae*, and *P. aeruginosa* isolates, respectively. A total of 17, 7 and 3 isolates for *E. coli*, *K. pneumoniae* and *P. aeruginosa* respectively carried both colistin and *β*-lactamase antibiotics resistant genes. This is a public health threat that points to a challenge in the treatment of infections caused by these zoonotic bacteria. Data generated from this study will contribute to formulation of new strategies for combating spread of *E. coli*, *K. pneumoniae*, and *P. aeruginosa* isolates as well as prevention of their AR development.

1. Introduction

Antibiotics are secondary metabolites produced by microbes, chemically synthesized equivalents, and semi-synthesized chemical analogues that inhibit both growth and survival in other microbes (Kumar et al., 2019; Karungamye et al., 2023). The development of resistant bacteria occurs naturally over time (Sabri et al., 2020). It is common to use antibiotics in livestock production as therapeutics, prophylactics at sub-therapeutic doses to prevent infection by bacterial pathogens in animals under high stress, or sub-therapeutically to increase growth (Chee-Sanford et al., 2009). It is not possible for these compounds to be metabolized fully when they are used for any given purpose. Studies have shown that only a small portion of these compounds are fully metabolized both in humans and animals, and that 20–90% of them are excreted as part of urine and faeces as the parent compound or metabolite (Boxall et al., 2004; Sabri et al., 2020; Karungamye et al., 2023).

Developing countries face the greatest challenges due to the emergence and spread of infectious diseases caused by multidrug-resistant (MDR) bacteria such as *E. coli*, *P. aeruginosa* and *K. pneumoniae* (Dadgostar et al., 2019; Ejikeugwu et al., 2021). The proportion of Gram-negative bacteria, including *E. coli*, *P. aeruginosa*, and *K. pneumoniae*, carrying extended-spectrum beta-lactamases (ESBLs), continues to increase globally (Yezli et al., 2015; Alatoom et al., 2017; Ramatla et al., 2023a). Production of various *β*-lactamases (enzymes

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that hydrolyze β-lactams) has been shown to be the primary cause of β-lactamase resistance, particularly in Gram-negative bacilli, where it has been shown to be an increasing concern (Khalifa et al., 2021; Ramatla et al., 2022; Sethuvel et al., 2023).

One of the first antibiotics with appreciable efficacy for treating infections caused by Gram-negative bacteria was the polymyxin-class antibiotic colistin, initially used in clinical settings in the 1950s (Poirel et al., 2017; Doremus et al., 2022). Colistin, a last-resort antibiotic used to treat hard-to-treat bacteria like *Acinetobacter baumanni*, *K. pneumoniae,* and *P. aeruginosa*, is experiencing increased development of bacterial resistance (Maamar et al., 2018; Ramatla et al., 2022).

There are few studies on the genetic epidemiology of antibiotic-resistant *E. coli* from farm to fork in intensive poultry production by McIver et al. (2020), however there is a significant lack of information regarding zoonotic bacteria from broiler chickens in South Africa. There is also a limited information available about colistin and β-lactamase resistance in *K. pneumoniae*, *E. coli*, and *P. aeruginosa*, particularly in broiler chickens (Balkhair et al., 2023). In addition, details on how common colistin resistance in β-lactamase Gram-negative bacteria are extremely scarce in South Africa. Colistin and β-lactamase resistance genes in Gram-negative bacteria continue to be a significant global food and public health burden, particularly in chicken production. Therefore, this study was conducted to examine the prevalence of colistin and β-lactamases resistance in *K. pneumoniae*, *E. coli* and *P. aeruginosa* isolated from broiler chicken faecal samples.

2. Materials and methods

2.1. Sample collection

Chicken faeces were collected randomly from broiler chickens (n = 480) after the removal of gastrointestinal tract at four abattoirs (n = 120 samples per abattoir) around Mahikeng City in the North West Province of South Africa. Faeces were collected from the caeca/rectum and placed into sterile faecal containers for further processing in the laboratory. An overall total of 96 pooled faecal samples were used in this study.

2.2. Isolation of Pseudomonas aeruginosa

A total of 5 g of each faecal sample was mixed with 45 mL of buffered peptone water (BPW) and incubated for 24 h with agitation at 37 °C. A 100 μL of the BPW was spread on a Sorbitol McConkey agar (SMA). The spread plate method was carried out in duplicate to ensure that the results were harmonized. All the inoculated plates were incubated at 37 °C for 24 h for culture isolation. All presumptive *P. aeruginosa* colonies were identified and sub-cultured on SMA. The *P. aeruginosa* colonies with a white appearance (lactose-negative) were considered as presumptive isolates.

2.3. Isolation of Escherichia. Coli

Using sterile forceps, 5 g of each faecal sample was mixed with 45 mL of peptone enrichment broth for Enterobacteriaceae and incubated at 37 °C for 24 h. The enriched broth was inoculated onto SMA selective plates using the spread plate method. After 24 h of incubation at 37 °C, the isolates were confirmed as presumptive pathogenic *E. coli* by phenotypic identification. *Escherichia coli* appears yellowish on MacConkey agar because it does not ferment sorbitol or ferments at very slow rates.

2.4. Isolation and identification of Klebsiella pneumoniae

The bacterial mass of 5g of each faecal sample collected from broiler chickens was transferred to a sterile test tube containing 10 mL of enrichment broth. The enrichment broth was then homogenised by vortexing for about 5 min, followed by incubation at 37 °C for 24 h. About 100 mL of the broth was spread plated on Eosin methylene blue [EMB] (Hardy Diagnostics agar, USA). The EMB plates were incubated overnight at 37 °C. The isolates were sub-cultured on EMB agar plates and incubated at 37 °C for 24 h for isolation and characterization. Mucoid pink to purple colonies were characterized as presumptive identification of *K. pneumoniae*.

2.5. Bacterial genomic DNA extraction

Bacterial cells of *P. aeruginosa*, *E. coli* and *K. pneumoniae* isolates were grown overnight, thereafter gDNA was extracted using Quick-DNA Microbe Mini-prep DNA kit following the manufacturer's protocol (ZYMO Research, USA). A NanoDrop Lite 1000 spectrophotometer (ThermoFisher Scientific, USA) was used to determine the concentration and purity of DNA, which was ultimately stored at −20 °C until further analysis.

2.6. Genetic identification of P. aeruginosa, E. coli and K. pneumoniae isolates

The species-specific PCR assays were conducted to confirm identity of the presumptive *P. aeruginosa*, *E. coli* and *K. pneumoniae* isolates targeting housekeeping genes *gyrB*, *uidA* and *rpoB* gene, respectively. Table 1 shows the primers for bacterial species used in this study. Each PCR reaction consisted of a total volume of 25 μL consisting of 12.5 μL of a 2X DreamTaq Green PCR Master Mix (4 mM $MgCl_2$, and loading buffer and 0.4 mM each of dATP, dCTP, mM dGTP, mMdTTP) (ThermoFisher Scientific, USA), 8.5 μL of nuclease-free water, 2 μL of the template DNA, and 1 μL of each oligonucleotide primer (10 μM each). The following conditions were used: 94 °C for 5 min, 94 °C for 1 min, 55–66 °C for 1 min (Table 1), 72 °C for 1 min, 72 °C for 7 min with an infinite hold at 4 °C using the ProFlex PCR System (Applied Biosystems, USA). The DNA free template (nuclease-free water) was included as a negative control while *E. coli* (ATCC:259622™), *P. aeruginosa* (ATCC 27853™) and *K. pneumoniae* (ATCC 1705 ™) were used as positive controls. PCR products were electrophoresed on 2% (w/v) agarose gel for 60 min at 80 V using 1x TAE buffer (40 mM Tris, 1 mM EDTA, and 20 mM glacial acetic acid, pH 8.0). The 1 kb and 100 bp ladders were used (PROMEGA, Madison, WI, USA) to determine the size of all PCR amplicons. The gel was stained in ethidium bromide (0.1 μg/mL) for 15 min and amplicons were visualized under UV light.

Furthermore, 16S rRNA PCR assay and sequencing were used to confirm the identity of the *P. aeruginosa*, *E. coli* and *K. pneumoniae* isolates with the following bacterial universal primers: 27F- AGA GTT TGA TCM TGG CTC AG and 1492R- GGT TAC CTT GTT ACG ACT T (Mariam, 2021). Each PCR reaction consisted of a total volume of 25 μL consisting of 12.5 μL of a 2X DreamTaq Green PCR Master Mix (4 mM $MgCl_2$, and loading buffer and 0.4 mM each of dATP, dCTP, mM dGTP, mMdTTP) (ThermoFisher Scientific, USA), 8.5 μL of nuclease-free water, 2 μL of the template DNA, and 1 μL of each oligonucleotide primer (10 μM each). The PCR conditions were 96 °C initial denaturation for 4 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s and extension at 72 °C for 1 min, and final extension step of 10 min at 72 °C. The amplified 16S rRNA gene fragments were sequenced with the BigDye Terminator (ThermoFisher Scientific) cycle sequencing kit (v 3.1) on the SeqStudio genetic analyzer at UESM sequencing facility at North-West University. The representative sequences were aligned on nucleotide Basic Local Alignment Search Tool (BLASTn) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) in order to confirm the isolates' identity.

2.7. PCR amplification of O-serogroups for E. coli

For the detection of O-serogroups, Table 1 shows a list of primers as well as the conditions for each reaction as described by previous studies (Adefisoye and Okoh, 2016; Ranjbar et al., 2017; Montso et al., 2019) for the detection of *E. coli* O-serogroups. Each PCR reaction consisted of a

Table 1
Oligonucleotide primers used in this study.

Genes	Primer name	Primer sequence (5′–3′)	Length (bp)	Annealing temp (°C)	References
E. coli					
UidA	UidA-F	AAAACGGCAAGAAAAAGC	147	60 °C	Adefisoye and Okoh, 2016
	UidA-R	ACGCGTGGTTACAGTCTTGCG			
0177	wzy –F	GGTCAGGAGCATGGAGCATT	457	55 °C	Montso et al., 2019
	wzy –R	AATCCATCCGGTGTATCGGC			
O157	O157–F	CGGACATCCATGTGATATGG	259	58 °C	Ranjbar et al., 2017
	O157-R	TTGCCTATGTACAGCTAATCC			
O145	O145–F	CCATCAACAGATTTAGGAGTG	609	58 °C	Ranjbar et al., 2017
	O145-R	TTTCTACCGCGAATCTATC			
O103	O103–F	TTGGAGCGTTAACTGGACCT	321	58 °C	Ranjbar et al., 2017
	O103-R	GCTCCCGAGCACGTATAAG			
stx2	stx2-F	ATCAGTCGTCACTCACTGGT	110	55 °C	Hinenoya et al., 2014
	stx2-R	CTGCTGTCACAGTGACAAA			
stx1	Stx1-F stx1-R	CAACACTGGATGATCTCAG	349	55 °C	Hinenoya et al., 2014
		CCCCCTCAACTGCTAATA			
P. aeruginosa					
gyrB	gyrB –F	CCTGACCATCCGTCGCCACAAC	222	66 °C	Mulamattathil et al., 2014
	gyrB –R	CGCAGCAGGATGCCGACGCC			
algD	algD –F	CGTCTGCCGCGAGATCGGCT		60 °C	Faraji et al., 2016
	algD –R	GACCTCGACGGTCTTGCGGA			
lasB	lasB –F	GGAATGAACGAAGCGTTCTCCGAC		60 °C	Faraji et al., 2016
	lasB –R	TTGGCGTCGACGAACACCTCG			
toxA	toxA –F	CTGCGCGGGTCTATGTGCC	270	63 °C	Faraji et al., 2016
	toxA –R	GATGCTGGACGGGTCGAG			
K. pneumoniae					
rpoB	rpoB –F	CAACGGTGTGGTTACTGACG	108	55 °C	Chander et al., 2011
	rpoB –R	TCTACGAAGTGGCCGTTTTC			

total volume of 25 μL consisting of 12.5 μL of a 2X DreamTaq Green PCR Master Mix (4 mM $MgCl_2$, and loading buffer and 0.4 mM each of dATP, dCTP, mM dGTP, mMdTTP) (ThermoFisher Scientific, USA), 8.5 μL of nuclease-free water, 2 μL of the template DNA, and 1 μL of each oligonucleotide primer (10 μM each). The following conditions were used: 94 °C for 5 min, 94 °C for 1 min, 55–58 °C for 1 min (Table 1), 72 °C for 1 min, 72 °C for 7 min with an infinite hold at 4 °C using the ProFlex PCR System (Applied Biosystems, USA). All runs included a negative DNA control consisting of PCR grade water.

2.8. Antimicrobial susceptibility testing

The antimicrobial susceptibility of all *P. aeruginosa*, *E. coli* and *K. pneumoniae* isolates was evaluated using the Kirby-Bauer disk diffusion method on Cation-Adjusted Mueller Hinton agar (Merck, Germany), as recommended by the Clinical and Laboratory Standards Institute (CLSI, 2018). Antibiotic discs used were as follows: β-lactamases; ampicillin (AMP; 10 μg), and amoxicillin-Clavulanic acid (AMC; 20/10 μg), fluoroquinolones; nalidixic acid (NA; 30 μg), cephalosporin; cefoxitin (CTX; 30 μg), cefotaxime (CT; 30 μg), aminoglycoside; streptomycin (S; 10 μg), and colistin sulphate (CS; 300 μg) polymyxins, the antibiotics disks were purchased from ThermoFischer Scientific™ (ThermoFisher, South Africa). *Escherichia coli* ATCC 25922 was used as a reference for quality control in antimicrobial susceptibility tests. The organism was classified as multidrug-resistant (MDR) if it was resistant to at least three antibiotic classes (Ramatla et al., 2019).

2.9. Detection of ESBL producing bacterial isolates

The ESBL producing *E. coli*, *K. pneumoniae* and *P. aeruginosa* isolates were confirmed phenotypically on CHROMagar™ ESBL (CHROMagar, France). The agar differentiates between ESBL *E. coli* (red colony presentation), ESBL *P. aeruginosa* (pigmentation cream to green) and ESBL *Klebsiella* species (metallic blue). The selection of these three bacteria was based on the ability to produce the extended-spectrum-β-lactamases enzyme. The reference strains that were used in this study were *E. coli* ATCC 10536, pathogenic *E. coli* and ESBL producing *E. coli* ATCC 35218 (Microbiologics, USA).

2.10. Molecular screening of β-lactamase-encoding genes

The *P. aeruginosa*, *E. coli* and *K. pneumoniae* isolates were screened for the β-lactams: bla_{SHV}, bla_{OXA}, *blaCARB*, bla_{TEM}, $bla_{CTX\text{-}M}$, $bla_{CTX\text{-}M\text{-}1}$ group, $bla_{CTX\text{-}M\text{-}2}$ group, $bla_{CTX\text{-}M\text{-}8}$ group, $bla_{CTX\text{-}M\text{-}9}$ group, $bla_{CTX\text{-}M\text{-}15}$ group, and $bla_{CTX\text{-}M\text{-}25}$ group β-lactamase resistance encoding genes using primers described previously (Table 2). To set up PCR assays for virulent genes, a total of 25 μL reaction mixture consisted of 12.5 μL of the 2X DreamTaq Green PCR Master Mix (New England Biolabs, USA), 10 μM of each primer, 2 μL of template DNA, and 8.5 μL nuclease-free water.

2.11. Molecular detection of colistin resistance genes

Five colistin resistance genes (*mcr*-1, *mcr*-2, *mcr*-3, *mcr*-4 and *mcr*-5) were screened in *P. aeruginosa*, *E. coli* and *K. pneumoniae* isolates using PCR assays. PCR screening for *mcr-1* to 5 colistin-encoding genes was performed as described previously by Jousset et al. (2019). A total volume of 25 μl PCR reaction was prepared consisting of 12.5 μl 2X DreamTaq Green PCR Master Mix (New England Biolabs, USA), 2.5 mM of each primer, 2 μl of template DNA and double distilled water was added to make the final volume. The PCR conditions were set as follows: initial denaturation at 95 °C for 10 min, followed 35 cycles of 95 °C for 30 s, annealing at 56 °C (Table 2), elongation at 72 °C for 30 s and final elongation at 72 °C for 1 min. PCR products were electrophorized on a 1% agarose gel along with a 100bp DNA ladder, followed by visualization under UV light.

2.12. Data analysis

A descriptive statistical analysis was conducted using percentages and ratio methods. Bar graphs were developed in Microsoft Excel to display antimicrobial susceptibility data. The 16S rRNA sequences of the 26 isolates were aligned with nucleotide sequences available in the National Centre for Biotechnology Information database (NCBI) GenBank using BLASTn (http://www.ncbi.nlm.nih.gov/BLAST/).

Table 2
List of antibiotic resistance genes primers used in this study.

Target gene	Primer	Primer sequence (5′ → 3′)	Amplicon size (bp)	Annealing temp (°C)	References
SHV	SHV-F	CACTCAAGGATGTATTGT G	885	55	Ramatla et al., 2022
	SHV-R	TTAGCGTTGCCAGTGCTCG			
OXA	OXA-F	ACACAATACATATCAACTTCGC	813	55	Ramatla et al., 2022
	OXA –R	AGTGTGTTTAGAATGGTGATC			
CARB	CARB-F	CAAGTACTTTYAAAACAATAGC	534	46	Jiang et al., 2013
	CARB-R	GCTGTAATACTCCKAGCAC			
TEM	TEM-F	TTC TTG AAG ACG AAA GGG C	1150	55	Ramatla et al., 2022
	TEM-R	ACGCTCAGTGGAACGAAAAC			
CTX-M	CTX-*M*-F	GTTACAATGTGTGAGAAGCAG	550	55	Liu et al., 2018
	CTX-*M*-R	CCGTTTCCGCTATTACAAAC			
CTX-M-1 group	CTX-*M*-1-F	GTT ACA ATG TGT GAG AAG CAG	1041	55	Liu et al., 2018
	CTX-*M*-1-R	CCGTTTCCGCTATTACAAAC			
CTX-M-2 group	CTX-*M*-2-F	ATGATGACTCAGAGCATTCGCCGC	876	56	Gundran et al., 2019
	CTX-*M*-2-R	TCAGAAACCGTGGGTTACGATTTT			
CTX-M-8 group	CTX-*M*-8-F	TGATGAGACATCGCGTTAAG	666	52	Gundran et al., 2019
	CTX-*M*-8-R	TAACCGTCGGTGACGATTTT			
CTX-M-9 group	CTX-*M*-9-F	GTGACAAAGAGAGTGCAACGG	856	55	Gundran et al., 2019
	CTX-*M*-9-R	ATGATTCTCGCCGCTGAAGCC			
CTX-M-15 group	CTX-*M*-15	CACACGTGGAATTTAGGGACT	995	50	Gundran et al., 2019
	CTX-*M*-15-R	GCCGTCTAAGGCGATAAACA			
CTX-M-25 group	CTX-*M*-25-F	GCACGATGACATTCGGG	327	52	Gundran et al., 2019
	CTX-*M*-25-R	AACCCACGATGTGGGTAGC			
mcr-1	mcr-1-F	TATCGCTATGTGCTAAAGCCTG	1139	56	Jousset et al., 2019
	mcr-1-R	CGTCTGCAGCCACTGGG			
mcr-2	mcr-2-F	TATCGCTATGTGCTAAAGCCTG	816	56	Jousset et al., 2019
	mcr-2-R	AAAATACTGCGTGGCAGGTAGC			
mcr-3	mcr-3-F	CAATCGTTAGTTACACAATGATGAAG	676	56	Jousset et al., 2019
	mcr-3-R	AACACATCTAGCAGGCCCTC			
mcr-4	mcr-4-F	ATCCTGCTGAAGCATTGATG	405	56	Jousset et al., 2019
	mcr-4-R	GCGCGCAGTTTCACC			
mcr-5	mcr-5-F	GGTTGAGCGGCTATGAAC	207	56	Jousset et al., 2019
	mcr-5-R	GAATGTTGACGTCACTACGG			

3. Results

3.1. Occurrence of P. aeruginosa, E. coli and K. pneumoniae isolates

Ninety-one presumptive *E. coli*, 23 *K. pneumoniae*, and 26 *P. aeruginosa* isolates were found after screening 90 pooled faecal samples. The *E. coli*, *P. aeruginosa* and *K. pneumoniae* isolates were successfully detected from faecal samples by species-specific PCR at infection rates of 60.7%, 22.5% and 16.7% respectively. The 16S rRNA gene sequences from the *P. aeruginosa*, *E. coli*, and *K. pneumoniae* isolates had a high nucleotide similarity ranging from 98.7 to 99.9% with the reference GenBank sequences. The representative isolates were deposited in GenBank with the following accession numbers: P. aeruginosa (OR462073, OR462074, OR462075 and OR462076), E. coli (OR462157, OR462158, OR462159 and OR462160) and K. pneumoniae (OR123585, OR123586 and OR123587).

3.2. Detection E. coli O-serogroups and their virulence genes

A total of 62 *E. coli* isolates were amplified by the *UidA* gene PCR assay. The strains tested negative for the *E. coli* O145 serogroup. About 37.1% of the isolates were classified as *E. coli* O177, while 19.4% as *E. coli* O157, 17.7% as *E. coli* O103, and 25.8% were unclassified. Table 3 shows the distribution of O-serogroups in *E. coli* isolates obtained from faecal samples of broiler chickens. Sixty-two *E. coli* isolates were examined for the presence of the virulence genes for the Shiga toxins (*stx1* and *stx2*). Only 46.8% of the 62 *E. coli* isolates had the *stx1* gene, which was present in 37.1% of *E. coli* 0177, 6.5% of *E. coli* 0157, 8.1% of *E. coli* 0145, and 16.2% of unclassified *E. coli* serogroups. In contrast, 61.3% of the isolates had the *stx2* gene, which was present in 24.2% of *E. coli* 0177, 4.8% of *E. coli* 0157, 9.7% of *E. coli* 0145 and 14.5% of unclassified *E. coli* serogroups.

Table 3
The distribution of O-serogroups and virulence genes in *E. coli* and *P. aeruginosa* strains isolated from faecal samples of broiler chickens.

	No. Isolates	Genes	Positive (%)
E. coli	62		
		0177	23 (37.1%)
		O157	12 (19.3%)
		O145	–
		O103	11(17.7%)
		stx1	29 (46.8%)
		stx2	38 (61.3%)
P. aeruginosa	23		
		algD	8 (34.7%)
		lasB	6 (26.1%)
		toxA	9 (39.1%)

3.3. Detection of virulence genes in P. aeruginosa

The *toxA*, *algD* and *lasB* virulence genes were examined among *P. aeruginosa* isolates by PCR assays. Out of 23 *P. aeruginosa* isolates, 9 (39.1%) harboured *toxA* genes, 8 (34.7%) possessed *algD* gene while 6 (26.1%) carried *lasB* gene (Table 3).

3.4. Antimicrobial resistance profiles of the E. coli, K. pneumoniae and P. aeruginosa isolates

Table 4 shows the distribution of antibiotic resistance profile for *E. coli, K. pneumoniae* and *P. aeruginosa*. The isolates displayed the highest levels of resistance against ampicillin (n = 51; 82.3%) and amoxicillin-clavulanic acid (n = 46; 74.2%) for *E. coli*, cefoxitin (n = 12; 70.6%) for *K. pneumoniae* and ampicillin (n = 6; 26.1%) and colistin sulphate for *P. aeruginosa*. On the contrary, all *E. coli* isolates were susceptible to streptomycin. Furthermore, all *K. pneumoniae* isolates were susceptible to nalidixic acid while *P. aeruginosa* isolates were susceptible

Table 4
Antibiotic resistance patterns of bacterial isolates in faecal samples from broiler chickens based on CLSI.

Bacterial species	No. Isolates	AMP	AML	CS	NA	CTX	CT	S
E. coli	62	51 (82%)	46 (74%)	17 (27%)	11 (18%)	9 (15%)	5 (8%)	0
K. pneumoniae	17	5 (29%)	4 (24%)	6 (35%)	0	6 (35%)	12 (71%)	7 (41%)
P. aeruginosa	23	6 (26%)	1 (4%)	6 (26%)	0	4 (17%)	5 (22%)	0

ampicillin = AMP, amoxicillin-Clavulanic acid = AML, colistin sulphate = CS, Nalidixic acid = NA, cefoxitin = CTX, cefotaxime = CT, streptomycin = S.

to nalidixic acid and streptomycin. Respectively, (n = 7; 6.9%), (n = 6; 5.9%) and (n = 2; 2.0%) of *K. pneumoniae*, *E. coli* and *P. aeruginosa* isolates from faecal samples of broiler chickens showed MDR phenotypes to at least three classes of antimicrobials.

3.5. Prevalence of ESBL producing bacteria

Out of 62 *E. coli* isolates, 16.1%were phenotypically classified as ESBL producing, of which 4.8% were classified as *E. coli* O177, 4.8% as *E. coli* 0103, 3.2% as *E. coli* O157 and only 3.2% as unclassified *E. coli* serogroup. Six out of seventeen (35.3%) were confirmed to be ESBL *K. pneumoniae*, whilst only 17.4% (4/23) classified as ESBL producing *P. aeruginosa*.

3.6. Identification of colistin-resistant genes

All *E. coli, K. pneumoniae* and *P. aeruginosa* isolates were screened for the presence of *mcr-1* and *mcr-2* genes encoding for colistin antibiotic. The *E. coli, K. pneumoniae* and *P. aeruginosa* isolates possessed *mcr-1*, with 46.8%, 47.1% and 21.7%, respectively (Fig. 1). About 62.3% of *E. coli* isolates harboured the *mcr-2* gene. Of which 37.1% were *E. coli* O177 serogroup, 9.7% were *E. coli* O157 serogroup, and 4.8% were *E. coli* O103 serogroup. None of the isolates of *K. pneumoniae* and *P. aeruginosa* carried the *mcr-2* gene.

3.7. Identification of β-lactamase -resistant genes

Table 5 shows the total distribution of ESBL genes amongst ESBL producing and non-producing *P. aeruginosa*, *E. coli* and *K. pneumoniae* isolates. The ESBL-*E. coli* possessed 60%, 20%, and 10% of $bla_{CTX\text{-}M}$, bla_{TEM} and $bla_{CTX\text{-}M\text{-}9}$ gene groups respectively. The ESBL- *K. pneumoniae* harboured 50%, 33%, 17%, and 17%, of $bla_{CTX\text{-}M}$, bla_{OXA}, bla_{CARB}, and $bla_{CTX\text{-}M\text{-}9}$ gene groups, respectively. While 75%, 50%, 25% and 25% of *P. aeruginosa* carried bla_{TEM}, $bla_{CTX\text{-}M}$, bla_{OXA} and bla_{CARB} gene groups, respectively. The β-lactamase-encoding $bla_{CTX\text{-}M}$ gene was identified in *E. coli*, *P. aeruginosa* and *K. pneumoniae* isolates. The bla_{TEM} gene was only carried by *E. coli* and *P. aeruginosa* isolates, while bla_{OXA} and bla_{CARB} were harboured by *P. aeruginosa*, and *K. pneumoniae* isolates. *E. coli*, and *K. pneumoniae* isolates also possessed the narrow-spectrum β-lactamase-encoding gene $bla_{CTX\text{-}M\text{-}9}$.

3.8. The co-occurrence of colistin and β-lactamase

A total of 17 *E. coli* isolates carried both colistin and β-lactamase genes from which 3 isolates carried *mcr-1, mcr-2* and β-lactamase genes. Six isolates were considered as ESBL producing *E. coli*. The results further show that 7 *K. pneumoniae* isolates harboured colistin and β-lactamase genes, with only 3 isolates considered as ESBL producing *K. pneumoniae*. After screening colistin and β-lactamase genes, 3 *P. aeruginosa* isolates possessed both genes, one ESBL producing *P. aeruginosa* isolate harboured *mecr-1,* bla_{OXA}, and bla_{TEM} genes.

4. Discussion

In this study, *E. coli* was the most common isolate detected from chicken faecal samples. The high prevalence of *E. coli* was similarly observed in a study conducted in Ecuador from humans and backyard animals (Bastidas-Caldes et al., 2023). In contrast, a study conducted by Karungamye et al. (2023) from Tanzania in hospital wastewater, the *K. pneumoniae* was the most common isolate detected.

In this study, we report the first case of co-existing colistin and β-lactamase resistant *E. coli*, *K. pneumoniae*, and *P. aeruginosa* from broiler chickens in South Africa. Colistin and β-lactamase resistant *E. coli* isolates were highly resistant to ampicillin followed by colistin sulphate, cefoxitin, cefotaxime and amoxicillin-clavulanic acid. Resistance to nalidixic acid was only observed in *E. coli*, while streptomycin resistance was also observed in *K. pneumoniae*. A recent study in Malaysia found that colistin-resistant *E. coli* isolates were in addition extremely resistant to tetracycline, streptomycin, nalidixic acid, ciprofloxacin, gentamicin, tobramycin, chloramphenicol, and cefotaxime (Karim et al., 2023). Interestingly, a study conducted in Egypt reported that *K. pneumoniae* isolates were resistant to ampicillin, followed by amoxicillin-clavulanic acid, nalidixic acid and trimethoprim-sulfamethoxazole (Elmonir et al., 2021). Different drug legislation in the various countries may possibly account for the observed antibiotic resistance disparities.

The presence of ESBL was high in *E. coli* isolates, followed by

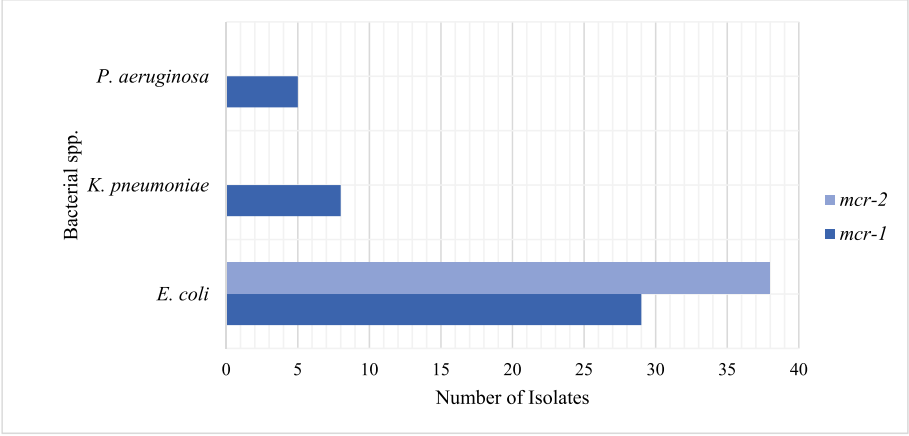

Fig. 1. Colistin-resistant genes detected on bacterial isolates isolated from faecal of broiler chickens.

Table 5
Distribution of ESBL genes amongst ESBL producing and non-producing *P. aeruginosa*, *E. coli* and *K. pneumoniae* isolates.

Bacterial species	ESBL[a]	No	SHV	OXA	CARB	TEM	CTX-M	CTX-*M*-1 group	CTX-*M*-2 group	CTX-*M*-8 group	CTX-*M*-9 group	CTX-*M*-15 group	CTX-*M*-25 group
E. coli	+	10	0	0	0	6	6	0	0	0	1	0	0
	–	52	3	0	0	1	16	0	0	1	2	0	1
K. pneumoniae	+	6	0	1	1	0	3	0	1	0	1	0	0
	–	11	0	2	3	0	6	0	0	0	2	0	0
P. aeruginosa	+	4	0	1	1	3	2	0	0	0	0	0	0
	–	20	0	0	0	2	5	0	1	0	0	1	0

[a] = (+) ESBL-producing and (−) non-ESBL-producing *E. coli* and *K. pneumoniae* isolates.

P. aeruginosa, and *K. pneumoniae* in the current study. It is well known that one of the major mechanisms through which Gram-negative bacteria are able to overcome penicillin-derived antibiotics as a result of their resistance is the production of *β*-lactamases (Gawish et al., 2021). In our study, the narrow-spectrum *β*-lactamase-encoding gene $bla_{CTX\text{-}M}$ was identified in *E. coli*, *P. aeruginosa* and *K. pneumoniae* isolates. In South Africa, only one study screened for *β*-lactamase-encoding genes ($bla_{CTX\text{-}M}$, $bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}9}$, and bla_{TEM}) in Gram-negative bacteria isolated from chickens (Ramatla et al., 2022; Ramatla et al., 2023b). This indicates that there is a lack of studies on antibiotic resistance in chickens. However, different studies have been conducted globally on *β*-lactamase-encoding genes in chicken. In Lebanon, bla_{TEM}, bla_{SHV}, and $bla_{CTX\text{-}M}$ were identified in *E. coli*, *K. pneumoniae*, *Proteus mirabilis* and *Enterobacter cloacae* (Dandachi et al., 2018). The $bla_{CTX\text{-}M}$ gene is one of the most widespread ESBLs found in both humans and poultry (Gundran et al., 2019; Ayinla and Mateus, 2023).

In our study, only 62.3% of *E. coli* isolates harboured the *mcr-2* gene, whilst, all *K. pneumoniae and P. aeruginosa* isolates did not possess the *mcr-2* gene inconsistent with prior investigations whereby *E. coli* and *K. pneumoniae* isolates were negative for presence of *mcr-1* and *mcr-2* genes in Portugal and German and (Kieffer et al., 2017; Roschanski et al., 2017; Clemente et al., 2019). It is unknown why in this investigation *mcr-2* gene was only discovered in *E. coli* and not in *P. aeruginosa* and *K. pneumoniae* isolates. Colistin has recently been re-introduced as a last-line antibiotic to treat severe infections, notably those brought on by carbapenems manufacturers, as a result of the advent of MDR bacteria such as ESBL-producing Enterobacteriaceae. (Le et al., 2021). There are still many Gram-negative bacteria for which colistin is the last line of defence (Sharahi et al., 2021). Colistin resistance encoded by plasmid-mediated *mcr* genes has recently been posing as a threat to global health (Le et al., 2021), In spite of this, Gram-negative bacilli that are colistin-resistant and even multidrug-resistant have already been detected and reported (Paterson et al., 2016; Sharahi et al., 2021).

It is thought that some antibiotic resistance genes in bacteria present in poultry and poultry products pose a serious threat to human health (Karim et al., 2023). An earlier Canadian study revealed the possible danger of eating poultry leading to the spread of drug-resistant bacteria to human (Manges et al., 2007). In the current study, we report the first case of co-existing colistin and *β*-lactamase resistance genes from *E. coli*, *K. pneumoniae*, and *P. aeruginosa* of broiler chickens, in South Africa. Noteworthy, colistin and *β*-lactamase genes co-existed in 17 *E. coli* isolates, of which 6 were ESBL-producing *E. coli*, seven were *K. pneumoniae*, and 3 of the isolates were ESBL-producing *K. pneumoniae*. Three *P. aeruginosa* isolates possessed both genes and one isolate was ESBL-producing *P. aeruginosa*. Among them, 9 isolates co-harbored *mcr-1* and *mcr-2* which were identified as *E. coli* (Ayinla and Mateus, 2023), *K. pneumoniae* (Alatoom et al., 2017) and *P. aeruginosa* (Abunna et al., 2023) respectively. Different *mcr* and *β*-lactamase genes co-existing in a single bacterial isolate have only been discovered up to this point (Feng et al., 2023). Similar to this study, a report from Switzerland demonstrated the presence of the *mcr-1* gene and ESBLs in water-derived Enterobacteriaceae (Zurfuh et al., 2016) in lower Himalayan region of India (Singh et al., 2021). A hospitalized patient in France was infected by *K. pneumoniae* with both extended-spectrum *β*-lactamase−producing *K. pneumoniae* strain and *mcr-1* colistin resistance genes (Caspar et al., 2017).

In Malaysia, *E. coli* isolates harbouring the *mcr-1* gene were co-harbouring multiple antibiotic resistance genes including *β*-lactamase genes (Karim et al., 2023). A Portuguese study also found that 45.7% of the *mcr-1*-positive *E. coli* isolated from meat and animals used for food production were ESBL producers (Clemente et al., 2019). It has been suggested that the presence of the *mcr-1* gene is connected to ESBLs occurrence (Dalmolin et al., 2018). The co-existence of the ESBL and *mcr-1* genes on plasmids may contribute to the spread of colistin resistance (Dandachi et al., 2018). Due to increasing findings regarding the co-existence of *mcr* and ESBL genes in Enterobacteriaceae, surveillance of such combinations is crucial in both clinical and non-healthcare contexts (Le et al., 2021). The MDR *K. pneumoniae* isolate infections are typically treated with carbapenems and polymyxin E (colistin), especially when the isolate is generating extended-spectrum -lactamase (ESBL) enzymes (Alizadeh et al., 2018; Petrosillo et al., 2019; Elmonir et al., 2021).

The MDR is one of the worldwide public health concerns (Karim et al., 2023). Remarkably, we observed that *K. pneumoniae* (6.9%), *E. coli* (5.9%) and *P. aeruginosa* (2.0%) isolates were MDR. This could be due to intensive poultry farming where antimicrobials are utilized more frequently. In places where infectious disease mortalities are common, the presence of colistin-resistant MDR *K. pneumoniae*, *E. coli*, and *P. aeruginosa* pose as a health problem hazard (Karim et al., 2023). In South Africa and other regions of the world, various MDR strains of *K. pneumoniae*, *E. coli*, and *P. aeruginosa* have been reported (Horcajada et al., 2019; Kotsoana et al., 2019; Iqbal et al., 2021; Ramatla et al., 2022; Abunna et al., 2023). *Escherichia coli* is a prominent source of the resistance genes associated with treatment failure in both humans and animals (Karim et al., 2023).

5. Conclusion

To the best of our knowledge, this is the first report showing the presence of co-existing colistin and *β*-lactamase resistance genes from *E. coli, K. pneumoniae*, and *P. aeruginosa* isolated from broiler chickens in South Africa. This study emphasises the significance of broiler chickens as a reservoir of colistin and *β*-lactamase resistant *E. coli*, *K. pneumoniae*, and *P. aeruginosa* which may potentially spread to humans through the food chain. This is a public health threat that highlights the challenge of treating infections caused by these zoonotic bacteria. Considering "One Health" perspective, it is crucial to pay more attention to ongoing surveillance of the presence of these zoonotic bacteria and *β*-lactamase resistance. To address such novel concerns, it is vital to keep the public informed and to provide training on responsible antibiotic use in the agricultural sector.

Availability of data and materials

The data and materials of the study will be available from the corresponding author on reasonable request.

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Ethics approval and consent to participate

The ethical approval was received from the Ethical Committee of the Animal Health Research Division at the North-West University, South Africa (NWU-00511-18-A5).

CRediT authorship contribution statement

Tsepo Ramatla: Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft. **Prudent Mokgokong:** Formal analysis, Writing – review & editing. **Kgaugelo Lekota:** Conceptualization, Supervision, Validation, Writing – review & editing. **Oriel Thekisoe:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

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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