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Detection of Extended-Spectrum Beta-Lactamase (ESBL) Producing *Enterobacteriaceae* from Diseased Broiler Chickens in Lusaka District, Zambia

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Abstract: Poultry products in Zambia form an integral part of the human diet in many households, as they are cheap and easy to produce. The burden of poultry diseases has, however, remained a major challenge. Growing consumer demand for poultry products in Zambia has resulted in non-prudent antimicrobial use on farms, intending to prevent and treat poultry diseases for growth optimization and maximising profits. This cross-sectional study aimed to identify the different types of bacteria causing diseases in chickens in Lusaka and to detect the extended-spectrum-lactamase (ESBL)-encoding genes. We collected 215 samples from 91 diseased chickens at three post-mortem facilities and screened them for gram-negative bacteria. Of these samples, 103 tested positive for various clinically relevant *Enterobacteriaceae*, including *Enterobacter* (43/103, 41.7%), *Escherichia coli* (20/103, 19.4%), *Salmonella* (10/103, 9.7%), and *Shigella* (8/103, 7.8%). Other isolated bacteria included *Yersinia, Morganella, Proteus,* and *Klebsiella,* which accounted for 21.4%. *E. coli, Enterobacter, Salmonella,* and *Shigella* were subjected to antimicrobial susceptibility testing. The results revealed that *E. coli, Enterobacter,* and *shigella* were highly resistant to tetracycline, ampicillin, amoxicillin, and trimethoprim-sulfamethoxazole, while *Salmonella* showed complete susceptibility to all antibiotics. The observed resistance patterns correlated with antimicrobial usage estimated from sales data from a large-scale wholesale and retail company. Six (6/14, 42.9%) *E. coli* isolates tested positive for *bla*$_{\text{CTX-M}}$, whilst eight (8/14, 57.1%) *Enterobacter* samples tested positive for *bla*$_{\text{TEM}}$. Interestingly, four (4/6, 66.7%) of the *E. coli* isolates carrying *bla*$_{\text{CTX-M}}$-positive strains were also positive for *bla*$_{\text{TEM}}$. Sanger sequencing of the PCR products revealed that five (5/6,83.3%) of the abovementioned isolates possessed the *bla*$_{\text{CTX-M-15}}$ allele. The results suggest the presence of potentially pathogenic ESBL-producing *Enterobacteriaceae* in poultry, threatening public health.

Keywords: Enterobacteriaceae; bla$_{\text{CTX-M}}$; bla$_{\text{TEM}}$

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1. Introduction

Poultry production in Zambia is one of the most important activities in the livestock sector. The chicken population is estimated at 94 million broilers,15 million village chickens, and 5.8 million layers [1]. Moreover, poultry products in Zambia, like in other developing African countries, form an integral part of the human diet in many households, as it is a cheaper source of animal protein and is easier to produce compared to other foods of animal origin [2,3].

As the Zambian population progressively expands, the demand for meat and other foods increases, leading to food security problems [1]. As a result, the government relies on agricultural industries to heighten animal production and address food insecurity. These industries usually raise large numbers of animals by boosting production through extensive farming methods that involve Antimicrobial Growth Promoters [4]. Although such livestock intensification approaches are essential for alleviating food shortages, they are also associated with the emergence and spread of antimicrobial resistance (AMR) [5]. This is further exacerbated by poorly monitored animal husbandry practices that result in frequent infections requiring antimicrobial use, leading to AMR. Besides, farmers sometimes deliberately underdose their livestock because of the high cost associated with antibiotics, worsening the problem.

AMR can be defined as the inability of bacteria parasites and viruses to respond to medicines making infection treatment difficult [6]. AMR may be encoded chromosomally, but the production of plasmid-mediated extended-spectrum lactamases (ESBLs) is more common. There are nine ESBL classes, but common ones include variants of the CTX-M-type and derivatives of SHV-1, TEM-1 and TEM-2 [7]. ESBL-producing *Enterobacteriaceae*, resistant to third generation cephalosporins like cefotaxime (CTX), are dreaded profoundly because of their extensive geographic distribution and adverse health impacts. While ESBLs are more prevalent among hospital isolates, poultry has emerged as an important reservoir for possible zoonotic transmission [7]. This reservoir includes ESBL-encoding genes harboured by commensal and pathogenic strains and may disseminate to humans via two main mechanisms. Firstly, ESBL genes may be transmitted by horizontal gene transfer, and the treatment implications depend on the pathogenicity of the recipient bacterial strain [8]. Of greater concern is the direct transmission of disease-causing pathogens by clonal expansion, potentially leading to clinical disease and treatment failure. Therefore, understanding the zoonotic transmission of poultry associated ESBLs requires a multipronged approach that considers both healthy and diseased chickens. In Zambia, most studies have focused on non-pathogenic bacteria isolated from asymptomatic chickens, leaving a gap in the ESBL status among sick chickens. Thus, this study was undertaken to estimate antimicrobial usage, isolate bacterial pathogens and confirm the presence of ESBL-encoding genes by PCR and sequencing in bacteria isolated from poultry.

2. Results

2.1. Antibiotics sales

Antibiotic importation data from 2015 to 2022 showed that tetracyclines were the most imported antibiotics, followed by sulphonamides and then penicillins (Figure 2). The group of tetracyclines imported included doxycycline, oxytetracycline, and chlortetracycline. The high influx of tetracyclines was consistent with the inexpensive nature, broad-spectrum activity, and minimal side effects of this antibiotic class. The sulphonamides included sulfamethoxazole, trimethoprim, and the sulfadiazine/trimethoprim combination. The high influx of sulphonamides could be attributed to their use in the treatment of coccidiosis and colibacillosis, which are among the most common poultry infections. Finally, the penicillin group included amoxicillin and ampicillin. The high importation levels of tetracyclines, sulphonamides, and penicillins coincide with sales data (Figure 4) which equally show high sales volumes of the named antimicrobials. Seventy percent (70%) of these antimicrobials are sold to Lusaka and Copperbelt provinces whilst the remaining 20% is shared amongst the other provinces. The high antibiotic consumption in Lusaka and the Copperbelt is consistent with the observation that that these two provinces have the highest number of poultry farms in the country coupled with high population densities [1].

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All poultry antimicrobials sold were for oral administration following presentation of a valid licence (for companies) or prescription (for farmers). Throughout the year, the trend in the sales had minimal fluctuation, with tetracyclines being the most sold antibiotics, followed by sulphonamides, penicillins, and aminoglycosides (Figure 3).

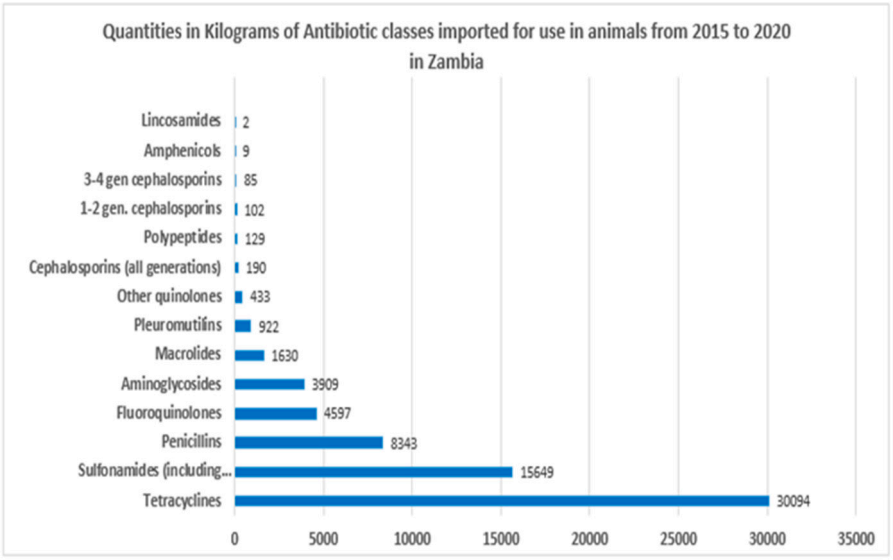

Figure 1. Importation of antibiotics from 2015 to 2020.

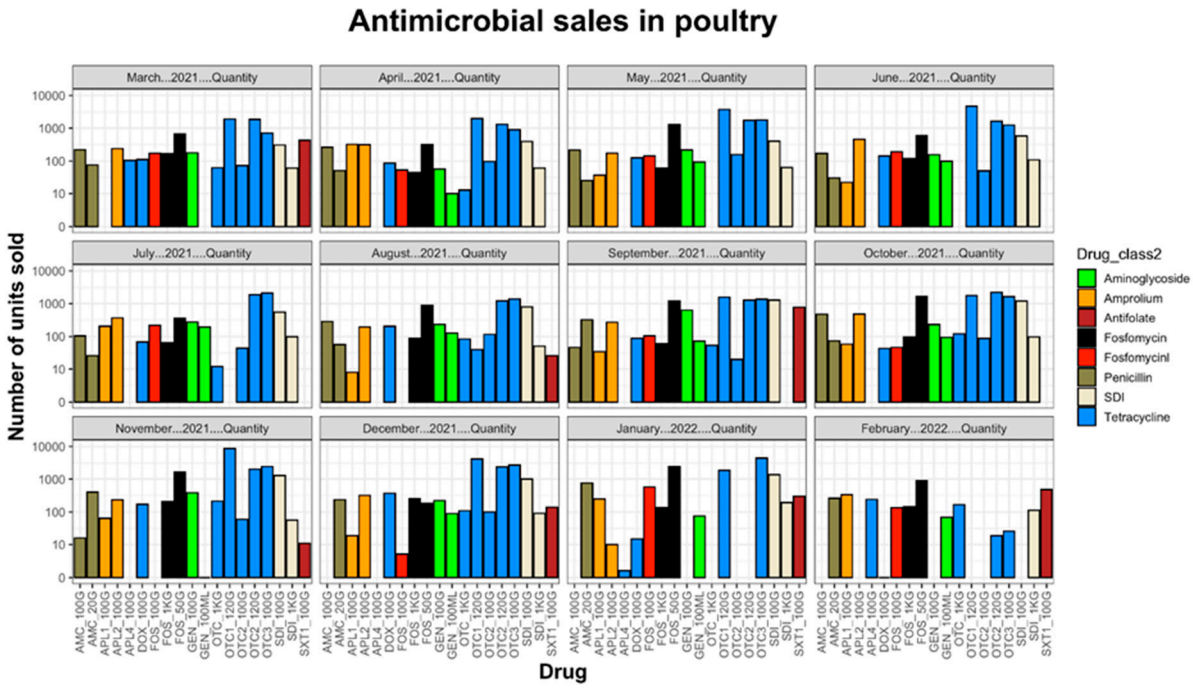

Figure 2. Sales data from March 2021 to February 2022.

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2.2. Prevalence of Enterobacteriaceae

A total of 215 samples were screened from 91 diseased chickens aged three to five weeks across Lusaka (Figure 5), of which 103 (103/215, 43%) tested positive for potentially pathogenic gram-negative bacteria, mostly belonging to the *Enterobacteriaceae* family. The major pathogenic species of clinical relevance identified were *Enterobacter* (43/103, 41.7%), *E. coli* (20/103, 19.4%), *Salmonella* (10/103, 9.7%), *Proteus* (10/103,9.7%) and *Shigella* (8/103, 7.8%) (Table 2). Other isolated bacteria included *Yersinia, Morganella,* and *Klebsiella,* which accounted for 19.5% (Table 2).

Table 2. Species isolated at the three facilities.

Species	**Facility**			**Total**
	A	B	C	
Enterobacter	2	26	15	43
E. coli	5	11	4	20
Salmonella	2	8	0	10
Klebsiella	0	1	0	1
Shigella	0	5	3	8
Yersinia	0	2	0	2
Citrobacter	0	4	0	4
Vibrio	1	0	0	1
Proteus	4	5	1	10
Morganella	0	4	0	0
Total	**14**	**66**	**23**	**103**

2.3. Antimicrobial sensitivity showed multidrug resistance (MDR) among Enterobacteriaceae

From the identified *Enterobacteriaceae* strains (n=103), 16 were randomly picked, including *Enterobacter* (n = 5), *E. coli* (n = 5), *Salmonella* (n = 4), and *Shigella* (n = 2). These strains were subjected to antibiotic sensitivity testing (AST) against five antibiotics, and the results showed the highest resistance to tetracycline (11/16, 68.8%), followed by amoxicillin (10/16, 62.5%), ampicillin (9/16, 56.2%), cotrimoxazole (7/16,43.8 %) and gentamicin (1/16, 6.2%) (Figure 3). In addition, MDR was observed in *E. coli* (3/5, 60%), *Enterobacter* (2/5, 40%), and *Shigella* (1/2, 50%), while *Salmonella* showed complete susceptibility to all antibiotics (Figure 4). By area sampling, most of the samples showing resistance were from lab A (Table 3), which is centrally located compared to the other sites.

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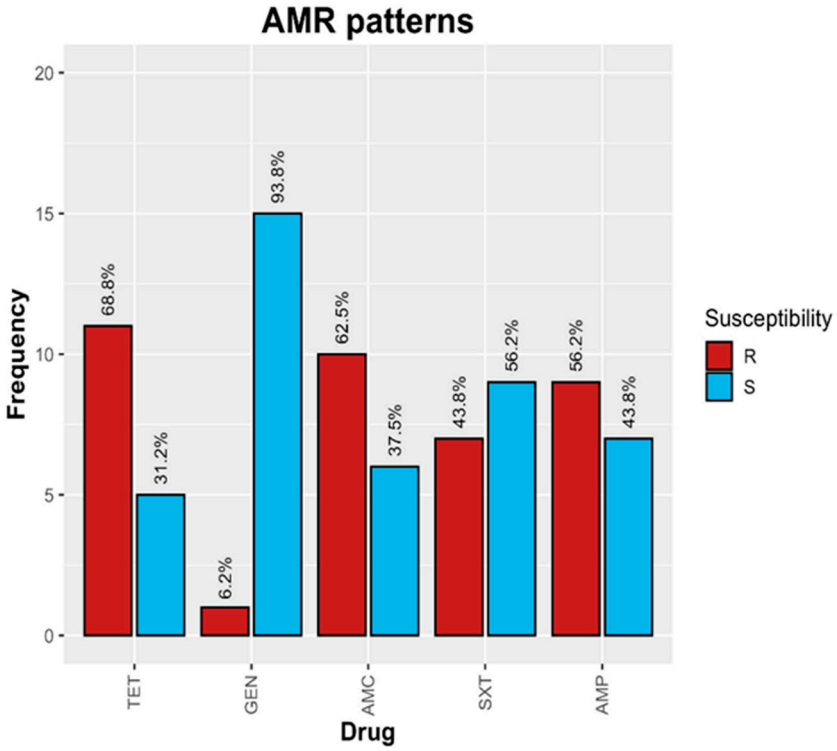

Figure 3. Overall AST for 16 *Enterobacteriaceae* strains.

Table 3. Sampling points for isolates for AST.

FACILITY	PATHOGENS			
	Enterobacter	*E. coli*	*Salmonella*	*Shigella*
A	3	3	3	1
B	1	1		1
C	1	1	1	

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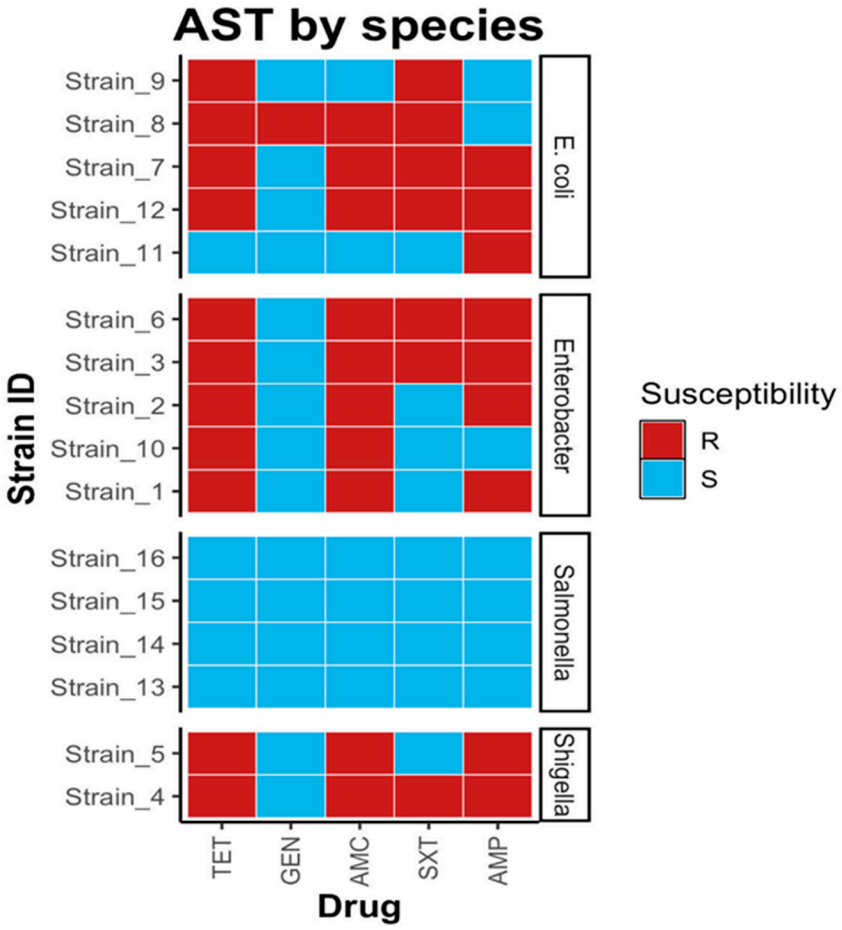

Figure 4. AMR patterns for the various bacteria.

2.4. Association between cefotaxime (CTX) resistance and ESBL genes

To quantify CTX resistance, the isolates were subjected to broth microdilution. The results indicated that 14 out of 103 (13.6%) isolates were CTX-resistant, of which 57.1% (8/14) were *Enterobacter*, and 42.9% (6/14) were *E. coli*. Notably, one *Enterobacter* and three *E. coli* strains exhibited high-level of resistance with CTX MICs of at least 512 µg/ml (Table 4). In addition, all the *E. coli* isolates (n = 6) were *bla*$_{CTX-M}$ gene positive on PCR and 66.7% (4/6) carried the *bla*$_{TEM}$ gene. Furthermore, all the eight Enterobacter isolates possessed only *bla*$_{TEM}$. However, none of the 14 isolates harboured the genes *bla*$_{OXA}$ and *bla*$_{SHV}$. Sequence analysis of the six *E. coli bla*$_{CTX-M}$ positive isolates showed the presence of the *bla*$_{CTX-M-15}$ allele in all the isolates with accession number (MN096663.1).

Table 4. CTX MICs for 14 *Enterobacteriaceae* strains.

SAMPLE ID	ORGANISM	CTX MIC	*bla*$_{TEM}$	*bla*$_{CTX-M}$
LS 1	*Enterobacter*	**2**	**+**	**-**
LS 2	*E. coli*	**4**	**-**	**+**
LS 3	*Enterobacter*	**2**	**+**	**-**
UZ 1	*E. coli*	**≥ 512**	**+**	**+**
LS 4	*Enterobacter*	**2**	**+**	**-**
AV 1	*E. coli*	**2**	**+**	**+**
LS 5	*E. coli*	**≥ 512**	**+**	**+**
LS 6	*Enterobacter*	**128**	**+**	**-**
AV 2	*Enterobacter*	**≥ 512**	**+**	**-**

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AV 3	*Enterobacter*	**2**	**+**	**-**
UZ 2	*Enterobacter*	**2**	**+**	**-**
LS 7	*E. coli*	**≥ 512**	**-**	**+**
LS 8	*E. coli*	**16**	**+**	**+**
LS 9	*Enterobacter*	**128**	**+**	**-**

3. Discussion

The documentation of antimicrobial importation and sales data is vital as it serves as a basis for intervention programs and policy decisions. In this study, antibiotic disk selection was based on sales data from March 2021 to February 2022 from one of the country's largest wholesale and retail outlets of animal pharmaceuticals. The highest group of antibiotics sold belonged to the tetracyclines, followed by the penicillins, which corresponds to the AST results that showed that 68.8% of the isolates were resistant to tetracyclines, 62.5% to amoxicillin, and 56.2% to ampicillin. The high levels of tetracycline and penicillin resistance observed in this study are similar to what has been reported previously [9]. This could be attributed to huge quantities of tetracyclines and penicillins being imported and sold in the country, as reflected in the sales data (Figure 2). These results also concur with a study done in Tanzania and Cameroon where tetracyclines, penicillins and sulfonamides were the most used antimicrobials in poultry production [10,11].

The cumulative rise in AMR could firstly be attributed to the use of antibiotics for infection control rather than treatment of disease by poultry farmers. This is usually done by introducing antibiotics in the first week of the chick's life to counter infections that may rise due to breaches in biosecurity [12].In Zambia, farmers avoid economic losses from infections by medicating healthy chickens upto to the sixth week despite being aware of the withdrawal periods [13]. Secondly, despite large agro shops dispensing antimicrobials by prescription, farmers still have access to antimicrobials in smaller outlets in the central business district [14]. Furthermore, the lack of knowledge by farmers is a major attribute contributing to the development of MDR as some farmers are of the belief that the use of different antibiotics lowers the chances of AMR development [15].

Over the past few years, AMR among the *Enterobacteriaceae* family has skyrocketed in worldwide [16]. Studies pertaining to AMR have frequently been reported in *E. coli* and *Salmonella* from healthy chickens [2,17].In this study, the most predominant species isolated was *Enterobacter* (43/103,47%) which showed multidrug resistance to tetracyclines, sulphonamides, ampicillin and amoxicillin. Sulfonamide and penicillin resistance are commonly reported among *Enterobacteriaceae* in other studies [18], probably due to the overuse of these two drug classes in poultry. For instance, previous studies have revealed a corelation between tetracycline concentration and resistance in the environment [19], as well as a relationship between tetracycline usage and resistance in poultry [20].

In our study, the prevalence of *E. coli* was 19.4% (20/103), which was lower than what has been reported elsewhere. For instance, Ibrahim et al (2019) and Ameen-Ur-Rashid et al (2016) found about 34% and 35% *E. coli* isolates in diseased chickens in Jordan and Pakistan, respectively [21,22]. Also, a higher percentage of *E. coli* (75.5%) isolates was observed in a study by Engy Ahmed Hamed et al [23]. Despite the lower *E. coli* prevalence, 60% (3/5) of the tested *E. coli* isolates exhibited MDR, involving tetracyclines, penicillins, and suphomonamides. Since this observation could suggest the clonal expansion of one strain or the horizontal transfer of an MDR mobile element (e.g., a plasmid), detailed characterization by whole-genome sequencing will be required to confirm the hypothesis.

Ten out of the 103 (9.7%) were *Salmonella*. A recent study on the Copperbelt Province in Zambia reported a higher *Salmonella* prevalence of 17.7% in commercial poultry farms [24]. Interestingly, the four randomly analyzed *Salmonella* strains in our study showed susceptibility to all tested antibiotics. While this finding could suggest a lower AMR burden in *Salmonella* from diseased chickens, the sample size was too low to allow valid prevalence estimation. Therefore, future studies must target larger numbers to account for rare phenotypes and genotypes.

Over the recent years, *Enterobacter* has emerged as the third *Enterobacteriaceae* showing resistance to third generation cephalosporins (3GCs) after *E. coli* and *Klebsiella* [18]. This study revealed the presence of CTX resistance not only in *E. coli* but *Enterobacter* as well. One out of eight (12.5%)

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Enterobacter and 3/6 (50%) *E. coli* showed significantly high CTX MICs of at least 512 µg/ml. Globally, 3GCs are administered to the parent flock or day-old chicks, which contributes to increased levels of CTX resistance [25]. Additionally, CTX resistance could result from the selection pressure created by other antibiotics if ESBL genes coexist with other AMR genes on the same mobile genetic element [8]. Also, the administration of antibiotics through feed and water has contributed to increased levels of resistance as this allows the uptake of antimicrobials by both infected and healthy chickens [26]. Such oral treatment regimens in chickens are prone to contamination with antimicrobials either by application or exposure to excreted faeces from treated chickens.

This is the first study to describe the presence of ESBL-encoding genes in diseased chickens in Zambia. Studies in healthy chickens in Zambia have reported high prevalence of the *bla*$_{\text{CTX-M}}$ gene among CTX-resistant *E. coli*. Accordingly, our study also revealed the presence of *bla*$_{\text{CTX-M}}$ in all CTX-resistant *E. coli*, although none of the *Enterobacter* strains harboured this gene. Conversely, all *Enterobacter* isolates exhibited the *bla*$_{\text{TEM}}$ gene. Interestingly, the *bla*$_{\text{CTX-M}}$ and *bla*$_{\text{TEM}}$ genes coexisted in two-thirds of the CTX-resistant *E. coli* isolates. This could be attributed to the presence of the genes on the same plasmid, as reported previously in Lusaka, Zambia [27] and in rural Nepal were *E.coli* co-harboured the genes *bla*$_{\text{CTX-M}}$ and *bla*$_{\text{TEM}}$ [28]. Studies done in Zambia reported 13% prevalence of *bla*$_{\text{CTX-M}}$ in *E. coli* isolated from market ready chickens [2]. Another study done on commercial poultry farms in Zambia's Copperbelt Province revealed 12.8% occurrence of *bla*$_{\text{CTX-M}}$ in *Salmonella* [24].

The detection *bla*$_{\text{CTX-M}}$ genes in diseased chickens has public health implications as transmission of drug-resistant pathogenic strains to humans may cause hard-to-treat severe infection. It is assumed that not only is poultry a zoonotic risk to humans, but poultry also acts as a reservoir for ESBL-producing bacteria. Several studies suggest the transmission of *bla*$_{\text{CTX-M}}$-producing *Enterobacteriaceae* between humans and animals through horizontal gene transfer and clonal expansion [29,30]. For instance, the *bla*$_{\text{CTX-M}}$ gene has been detected on plasmids shared by human and poultry *E. coli* strains [31]. Also, similar *bla*$_{\text{CTX-M}}$-positive *E. coli* strains have been found in humans and poultry, suggesting clonal dissemination. This transmission could be attributed to the poor handling of poultry in abattoirs or the increase in backyard poultry barns.

The increase in the presence of *bla*$_{\text{CTX-M}}$ could be attributed to the general increase in antimicrobial usage over the recent years. In our study, amplicon sequencing of *bla*$_{\text{CTX-M}}$ revealed the predominance of *bla*$_{\text{CTX-M-15}}$ (5/6, 83%) among the CTX-resistant *E. coli* isolates. These findings are in line with the fact that *bla*$_{\text{CTX-M-15}}$ is the most widely spread ESBL genotype globally [32]. The emergence of *bla*$_{\text{CTX-M-15}}$ has been attributed to the clonal spread of the *E. coli* O25b:H4-ST131 pandemic clone. However, we did not perform multilocus sequence typing on our strains.

MDR bacteria pose significant danger to the public as common infections which were once easily treatable become fatal owing to the expensive nature of stronger antimicrobials. The *bla*$_{\text{CTX-M-15}}$-positive *E. coli* isolates analysed in this study also portrayed a MDR phenotype which included tetracyclines and sulfonamides. The observed MDR could be attributed to the fact that in Zambia, farmers are still using antibiotics to optimise their production. This is supported by antibiotic sales data that shows that large amounts of antibiotics belonging to various classes are sold indiscriminately (Figure 3). Moreover, MDR can also be selected by only one antibiotic since AMR genes usually reside together on mobile genetic elements, allowing for simultaneous selection by a single drug [33].

Unlike *E. coli*, the clinical relevance of *Enterobacter* in poultry has not been well documented in Zambia, which can be attributed to the fact that *Enterobacter* rarely causes disease in immunocompetent chickens [18]. Data from this study has shown that *Enterobacter* does indeed carry the gene *bla*$_{\text{TEM}}$, which may in turn be transmitted to humans. The MDR phenotype profile of the *bla*$_{\text{TEM}}$-positive *Enterobacter* is alarming and calls for the urgent need for diagnostics before dispensing antimicrobials. This will allow for the more prudent use of antimicrobials and in turn limit the spread of AMR. Since *Enterobacter* rarely causes disease in immunocompetent chickens, its presence probably goes unnoticed. Therefore, appropriate biosecurity measures in poultry houses would play a vital role in preventing its spread.

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Study limitations

The study lacked MLST and whole-genome-based comparison analysis to assess the possibility of transmission between humans and poultry. In addition, this study only assessed a limited number of AMR genes. WGS would identify other AMR genes, mutations, serogroups, phylogroups, virulence genes, plasmids and mobile genetic elements. The identification of various virulence factors would allow the confirmation of the pathogenicity of the strains.

4. Materials and Methods

4.1. Study area and sampling.

The study was conducted in Lusaka district (Lusaka Central, Kanyama, Munali, Matero, Chawama, and Mandeveu areas) (Figure 5) the capital city of Zambia, with a total population of 3,042,000 [1]. Between April 2021 to December 2021, a total of 215 samples from 91 diseased chickens were aseptically collected from three different post-mortem facilities in Lusaka.

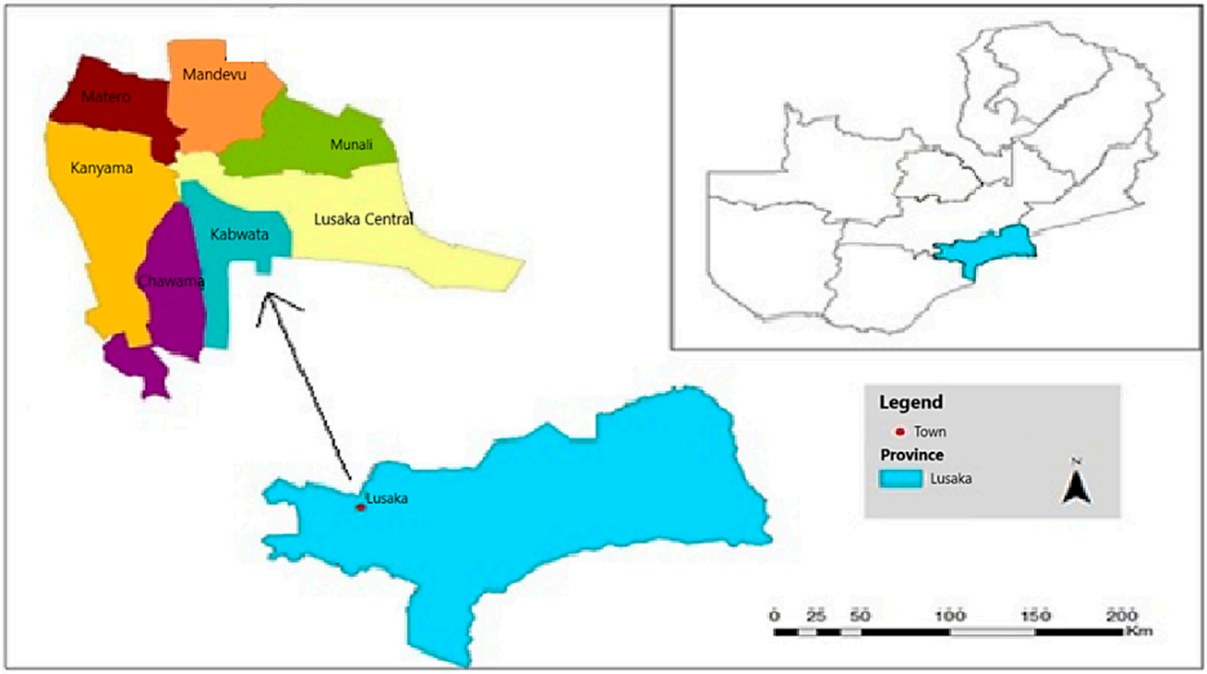

Figure 5. Map of Zambia showing the study areas.

4.2. Antimicrobial usage data.

Antimicrobial usage (AMU) was estimated indirectly from antibiotic imports and sales recorded by one of Lusaka's major suppliers of livestock antibiotics. The company sells antibiotics to retail agrovet shops as well as directly to farmers on a prescription basis. The importation and sales data was used to provide information on AMU and guide the classes of antibiotics for use in susceptibility tests.

4.3. Bacterial isolation.

The samples were inoculated on MacConkey agar (Oxoid LTD, Hamphshire UK), blood agar, and xylose lysine deoxycholate plates (Oxoid LTD, Hamphshire UK). The plates were incubated at 37°C for 24 hours followed by phenotypic identification using biochemical tests.

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4.4. Antibiotic sensitivity testing.

Antimicrobial sensitivity was done using the Kirby-Bauer disk diffusion method on Mueller Hinton Agar (Becton, Dickinson and Company). The antibiotics disks (Becton, Dickinson and Company) used included tetracycline (30µg), ampicillin (10µg), erythromycin (10µg), cotrimoxazole (10µg), amoxicillin (10µg), gentamicin (10µg), and penicillin (30µg). All results were interpreted according to the Clinical and Laboratory Standards Institute (CSLI) guidelines.

4.5. Cefotaxime (CTX) minimum inhibitory concentration (MIC).

Briefly, CTX resistance was confirmed by inoculating bacteria on Luria Bertani (LB) (Oxoid LTD, Hamphshire UK) agar plates supplemented with 1 µg/ml of CTX at 37°C for 18hrs. Next, a single colony was picked from each plate and transferred to LB broth supplemented with 1 µg/ml CTX at 37°C for 18hrs, at 175 rpm. Finally, the cultures were diluted 10,000-fold and added to two-fold serial dilutions of CTX in a 96-well plate in triplicate. MIC was defined as the lowest CTX concentration that inhibited visible bacterial growth. *E. coli* strain MG1655 was used for quality control.

4.6. Detection of ESBL genes by PCR

To screen for various ESBL genes (*bla*$_{CTX-M}$, *bla*$_{TEM}$, *bla*$_{SHV}$, and *bla*$_{OXA}$), *Enterobacteriaceae* strains were subjected to PCR using primers in Table 1. gDNA was extracted using the ZymoBIOMICS® DNA Miniprep Kit following the manufacturer's instructions. PCR was performed using ExTaq HS (TaKaRa, Japan) with a total reaction volume of 50µl consisting of 5µl 10x ExTaq buffer, 4µl of dNTP mixture, 2µl of DNA template, 5µl of both forward and reverse primers (10µM each)(Table 2), 28.75µl of nuclease free water, and 0.25µl of Takara Ex Taq HS. PCR conditions for *bla*$_{CTX-M}$, *bla*$_{SHV}$, and *bla*$_{OXA}$ were: denaturation at 98°C for 2 minutes, followed by 25 cycles of template denaturation at 98°C for 10 seconds, annealing at 60.5°C for 5 seconds and extension at 72°C for 1 minute with a final extension at 72°c for 8 minutes. The PCR conditions for *bla*$_{TEM}$ were 94°C for 7 minutes, followed by 30 cycles of template denaturation at 94°C for 30 seconds, primer annealing at 57°C for 30 seconds and 72°C for 5 minutes, whilst the final extension of 72°C was for 5 minutes. PCR products were visualised under UV light after electrophoresis using 1.5% agarose gel.

Table 1. Primers used in this study.

PRIMERS	TARGET GENE	SEQUENCE 5'-3'	EXPECTED AMPLICON SIZE
TEM1F **TEM1R**	*bla*$_{TEM}$	ATGAGTATTCAACATTTCCG CTGACAGTTACCAATGCTTA	864
SHVF **SHVR**	*bla*$_{SHV}$	GGTTATGCGTTATATTCGCC TTAGCGTTGCCAGTGCTC	865
CTX-MA1 **CTX-MA2**	*bla*$_{CTX-M}$	*SCSATGTGCAG≠YACCAGTAA CCGC¥RATATGRTTGGTGGTG	544

Note: *S = G or C, ≠Y = C or T, ¥R = A or T.3.6 Sequencing of PCR Products [34].

4.6. PCR product purification and Cycle sequencing

PCR products were purified using MinElute PCR purification kit (QIAGEN) according to the manufacturer's instructions. BigDye Terminator v3.1 (Applied Biosystems, USA) was then used for sequencing PCR followed by purification of excess buffers and unincorporated dNTPs using the ethanol precipitation method. Capillary electrophoresis was then performed using the ABI 3500 Genetic Analyzer (Applied Biosystems, USA).

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4.7. Data analysis

4.7.1. AMR data analysis

Sales data was collected from the largest wholesale and retail supplier, inputted into an Excel sheet (Microsoft Excel, 2010), and cleaned. For antimicrobial susceptibility data, the inhibition zones were interpreted according to CSLI guidelines and the data was manipulated using dplyr v1.0.7 [35] and reshape2 v1.4.4 [35] in Rstudio (Version 3). Finally, the data was displayed as tables and also visualised in ggplot2 v3.3.5 [36].

4.7.2. ESBL gene sequence analysis

The sequences were edited and assembled using the ATGC plug-in in Genetyx ver. 12 (GENETYX corporation, Tokyo, Japan). Sequences obtained in this study were then subjected to the BLAST analysis on the National Centre for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/BLAST) for identity confirmation. The nucleotide sequences were then aligned with other nucleotide sequences obtained from GenBank using Clustal X2 [37]. All the sequences obtained in this study have been submitted to the National centre for bitochenology institute.

5. Conclusions

This is the first study in Zambia focusing on AMR patterns among *Enterobacteriaceae* strains isolated from diseased chickens. The study revealed a relationship between the estimated AMU and AMR, particularly with reference to tetracyclines and penicillins, highlighting the need to restrict unjustified access to antibiotics. Furthermore, MDR was identified among the isolates, and genotypic characterization identified *bla*$_{\text{CTX-M}}$ and *bla*$_{\text{TEM}}$ genes.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org.

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