



Research article

Detection of CTX-M-type extended-spectrum beta-lactamase-producing *Salmonella* Typhimurium in commercial poultry farms in Copperbelt Province, Zambia

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Abstract

In Zambia, poultry is a rapidly increasing sector, contributing 4.8% of the Agricultural Gross Domestic Product (GDP), thus providing a significant income-generating activity. Worldwide, poultry is a major reservoir of *Salmonella* with an increasing incidence of extended-spectrum beta-lactamase (ESBL) producing strains. ESBLs are enzymes produced by bacteria and are capable of inactivating a wide range of beta-lactam antibiotics. *Salmonella enterica* serovars Enteritidis and Typhimurium are the most common food-borne serotypes in many countries, infecting both humans and animals and are transmitted to humans through the food supply chain. CTX-M ESBLs have been described in *Salmonella* Typhimurium isolates with resistant genes located on transferable plasmids. This study aimed to detect *S. Typhimurium*, its antimicrobial resistance, and CTX-M-type ESBL-producing strains in commercial poultry farms in Copperbelt province, Zambia. Five districts were considered for this study, where one poultry farm per district was randomly selected for sampling. An overall number of 384 fecal samples were analyzed using microbiological and molecular methods. *S. Typhimurium* was detected at 17.7% (CI: 14.2%-21.8%) in commercial poultry farms in Copperbelt province, of which 12.8% (CI: 9.8%-16.5%) were found harboring the CTX-M-type ESBL genes. *S. Typhimurium* isolates showed 88.2% resistance to at least one antimicrobial compound. All the isolates showed 100% resistance to tetracycline, followed by ampicillin and amoxicillin at 91.2%. These isolates also showed 58.8% resistance to cefotaxime and 54.4% to ceftazidime. Detection of CTX-M ESBL-producing *S. Typhimurium* suggests the contamination of the chicken food chain at the farm level and calls for public health protection measures.

Keywords: *Salmonella* Typhimurium, CTX-M Extended-Spectrum Beta-Lactamase, Copperbelt, Poultry

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Introduction

In Zambia, poultry is a rapidly increasing sector, contributing 4.8% of the Agricultural Gross Domestic Product (GDP), thus providing significant income-generating activities (Bronkhorst and Chongo, 2015). Despite this rapid increase, the poultry industry still faces challenges associated with the emergence of pathogenic bacterial strains. Moreover, the emergence of antimicrobial-resistant bacterial strains throughout the production process is threatening the growth of the industry. Previous studies have shown that poultry is a major reservoir of *Salmonella* worldwide, associated with increased incidences of enterobacterial strains producing extended-spectrum β -lactamases (ESBLs) (Gelinski et al., 2014; Ziech et al., 2016). *Salmonellae* are facultative anaerobic intracellular pathogens of medical importance. They are the causative agents of

numerous diseases, such as typhoid fever, bacteremia, enteric fever, salmonellosis, and enterocolitis in a broad range of organisms (Wilson et al., 2000).

Some serovars like *S. Gallinarum* and *S. Pullorum* have a host range restricted to avian and cause severe fowl typhoid and pullorum disease, respectively. While *S. Typhi*, *S. Paratyphi* A, and C cause typhoid fever exclusively in humans and closely related primates (Kisiela et al., 2012). *Salmonella enterica* serovars Enteritidis and Typhimurium are the most important food-borne serotypes in many countries, infecting humans and animals and transmitting to humans through the food supply chain (Zhang et al., 2019). CTX-M (Cefotaximase-Munich) ESBLs have been described in *S. Typhimurium* isolates with resistant genes located on transferable plasmids (Tzouveleki et al., 2000).

The cefotaximases can be transmitted by horizontal gene transfer mechanisms that include conjugation, transformation, and transduction (Vaidya, 2011). The emergence and spread of antibiotic resistance among *Salmonella* serovars originating from food-producing animals have become a serious challenge in human and veterinary medicine globally and pose a serious community threat (Silva et al., 2013). Antibiotic resistance has been associated with antibiotic usage during the animal production process. Easy access to antibiotics by Zambian farmers contributes to the abuse of these drugs in animal production and leads to the emergence of resistant pathogens (MNAP-AR, 2017). In Zambia, studies have been conducted to assess the magnitude of bacteria associated with poultry farming and backyard rearing chicken. These studies have mainly been carried out in Lusaka province, which is the capital city of Zambia. However, the detection of CTX-M type ESBL-producing *Salmonella* Typhimurium and the rates of antimicrobial resistance in commercial poultry farms in the Copperbelt province has not been established.

Therefore, this study has gone further into the animal production processes in poultry farms to rule out issues of contaminating factors from sources other than birds. This study aimed to detect *S. Typhimurium*, its antimicrobial resistance, and CTX-M-type ESBL-producing strains in commercial poultry farms in Copperbelt province, Zambia.

Materials and methods

Study Area and study design

The study was carried out in the Copperbelt province, the second-largest province in Zambia. The total population size was 2,480,657, covering an estimated area size of 31,328 Km² in ten districts (CSO, 2018) (Figure 1). The province is the mining hub of Zambia, with copper being the most predominant mineral, hence the name Copperbelt. Of the ten districts in the province, Ndola, Kitwe, Chingola, Mufulira, and Luanshya were considered for this study, and only commercial poultry farms were sampled. These are urban areas and the most populated, where poultry farming is widely practiced at small-scale farming and backyard chicken rearing, with bird population sizes ranging from 50-1000. Therefore, this study only focused on commercial poultry farms in the Copperbelt province. These farms are few (8 commercial poultry farms) in the province but commercially supply their products to different parts of the country. The poultry sector in Zambia is governed by the Poultry Association of Zambia, which groups small-scale and large-scale farms together. The chick producers also subscribe to the Poultry Association of Zambia. Both small and large poultry farms are dependent on the Government's Veterinary services, even though large farms are now hiring their veterinarians. The market-ready poultry products are usually directly sold to consumers.

Sample collection

A cross-sectional study design was conducted from March 2020 through May 2020, where one commercial poultry farm was selected for sampling from each of the five districts in the Copperbelt Province. From these districts, 78 cloacal swabs were collected from Ndola, 76 from Kitwe, 77 from Chingola, 76 from Luanshya, and 77 from Mufulira. Cloacal swab samples were carefully collected to avoid contamination from the outside of the cloaca and were placed in Amies with charcoal transport medium (Zimbro and Power, 2009). The samples were transported on ice packs to the Microbiology laboratory at Tropical Diseases Research Centre. For data collection, face to face questionnaire interview was used. Chicken population size per poultry farm, husbandry practices, antimicrobial usage, and administration therapy, bio-security and hygiene practices, manure handling, and feeding patterns were considered.

Culture, isolation, and identification of *Salmonella* Typhimurium

Isolation of *S. Typhimurium* was done using bacteriological methods as previously described (Zimbro and Power, 2009; Merck, 2010). Cloacal swabs were first inoculated in Selenite-F broth (HiMedia Laboratories Pvt. Ltd. India) to enrich *Salmonella* species and incubated for 15 hrs at 37°C. The cultures were inoculated and streaked on *Salmonella-Shigella* Agar (SSA) plates (HiMedia) (Merck, 2010), which is selective and differential media that differentiates between colonies of *Salmonella* from some *Shigella* species and incubated at 37°C for 24 hrs. Suspected *Salmonella* isolates were then inoculated on Brilliant Green Agar Base Modified (BGABM) plates (HiMedia) (Zimbro and Power, 2009) and incubated at 37°C for 24 hrs. For quality control purposes, *S. Typhimurium* ATCC14028 was used.

Characterization of *Salmonella* isolates

Suspected *Salmonella* isolates were characterized through biochemical tests, which included Triple Sugar Iron (TSI) (HiMedia) and Urease (HiMedia). Isolates were inoculated in TSI and Urease slant tubes aseptically using a heat-flamed wire loop and incubated for 24 hrs at 37°C. The isolates were examined for gas production, hydrogen sulfide, and color change in TSI, while in urease, isolates were examined for color change.

Antimicrobial susceptibility testing (AST) of *S. Typhimurium*

The AST was carried out using the Kirby-Bauer disc diffusion method according to the CLSI guidelines (CLSI, 2018). The antibiotic discs (HiMedia) included Cefotaxime 30 µg, Ceftazidime 30 µg, Ampicillin 10 µg, Tetracycline 30 µg, Gentamicin 10 µg, Chloramphenicol 30 µg, Norfloxacin 10 µg and Amoxicillin 25 µg and Nalidixic acid 30 µg.

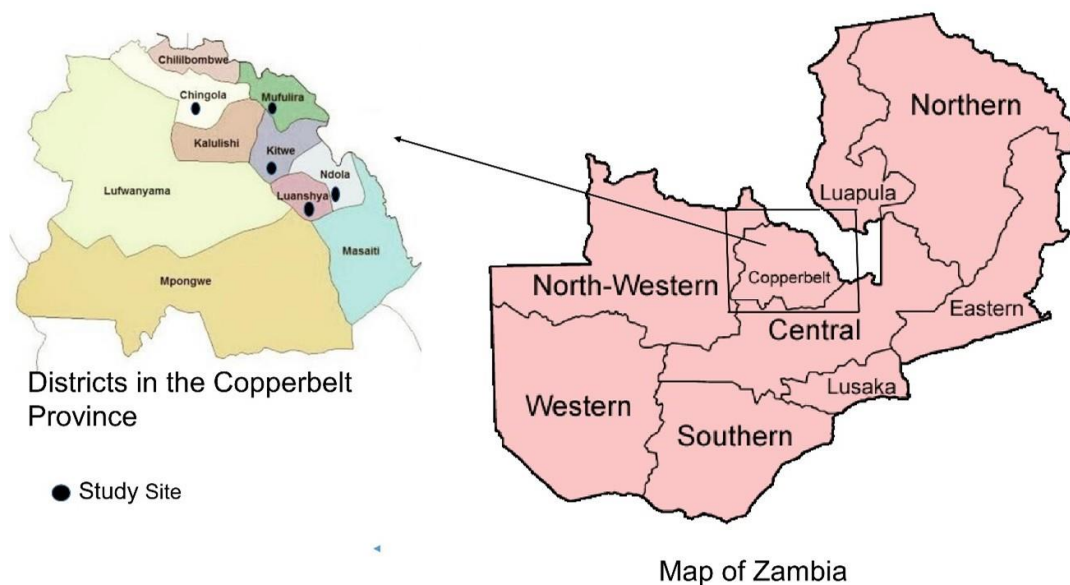


Figure 1: A map of Copperbelt province showing the study area (CSO, 2018).

Phenotypic detection of CTX-M-type ESBL-producing *S. Typhimurium*

The CTX-M-type ESBL-producing *S. Typhimurium* isolates were identified using the phenotypic disc combination method based on CLSI directions (CLSI, 2018). Combination discs of ceftazidime-clavulanic acid (CAZ30-CA10) were used with single discs of cefotaxime (30 µg) and ceftazidime (30 µg). Direct colony suspension was employed by suspending *Salmonella* colonies in 2 mL 0.85% (w/v) normal saline and adjusting the inoculum to a turbidity equivalent to a 0.5 McFarland Standard (1.5×10^8 CFU/ml). These colonies were then evenly streaked on MHA plates, and discs were placed 2.5 cm from each other and incubated for 24 hrs at 37°C. A difference in the zones of inhibition of 5 mm of either cefotaxime or ceftazidime discs and their clavulanic acid discs indicated the production of ESBLs. Confirmation of CTX-M-ESBLs was done using PCR.

DNA extraction

DNA was extracted using the boiling method described by Reischl et al. (2000), where a single pure bacterial colony was suspended in a lysis buffer containing a detergent (0.1% Tween 20) of 300 µL and a buffer solution (10 mM Tris-HCl pH 8) of 300 µL in an Eppendorf tube (Reischl et al., 2000). These cell suspensions were boiled at 100°C in a boiling water bath for 10 min. Afterward, the Eppendorf tubes were then removed from the water bath and centrifuged for 5 min to separate the debris from the supernatant. At this point, the samples were ready to be used for PCR. The DNA concentration of samples was measured using the BioDrop (BioDrop Ltd, UK) and ranged from 140 to 190 µg/mL.

Detection of *S. Typhimurium* and CTX-M-type genes by Polymerase Chain Reaction (PCR)

The detection of *S. Typhimurium* and CTX-M-type genes was achieved by serovar-specific *Typhimurium*

specific primers as described in Table 1. The amplification was carried out in a final volume of 25 µL with the following optimized PCR contents: 12.5 µL of one- Taq Master Mix (BioLabs®Inc, England), 1.5 µL of each primer, 5 µL of template DNA, and 4.5 µL of Nuclease free water. The PCR protocol was conducted under the following steps: an initial denaturation step for 4 min at 94°C, 40 cycles of 30 seconds at 94°C, 30 seconds at 58°C, and 1 min at 72°C and the final extension step for 4 min at 72°C. The positive control *S. Typhimurium* ATCC 14028 was used following the cycling protocol of Anbazhagan et al. (2019). Molecular confirmation of CTX-M genes was done using two sets of primers (Table 1). Both multiplex and conventional PCR protocols were used. The *bla*CTX-M with 590 bp could not be amplified in the multiplex PCR due to differences in annealing temperatures.

Amplification products were detected in 1.5% agarose gel electrophoresis performed at a voltage of 100 V and a current of 400 A for 60 min, and visualized under UV trans-illuminator (UVP, Upland, USA).

Statistical data analysis

Data were entered and analyzed using the statistical package EPI INFO version 7.2.3.1. Frequencies and proportions in terms of percentages were computed for categorical outcomes. Fisher's exact test was performed to test the association between the occurrence of *Salmonella Typhimurium* and other variables such as antibiotic usage, the purpose of antibiotic use, withdrawal period, antibiotic administration and veterinarian consultation, manure handling, hygiene, and biosecurity practices at the p-value of <0.05 at 95% confidence level.

Ethical clearance

Ethical approval to conduct this study was obtained from the Research Ethics and Science Converge Committee (ERES) Institutional Review Board with reference number 2019-Dec-012.

Table 1: Primer sequences and sizes for Typhi and *bla*CTX-M genes.

| Primer | Sequence (5'-3') | Size bp | Reference |
|------------------|---------------------------|---------|--------------------------------|
| Typh | F: TTGTTCACTTTTTACCCCTGAA | 401 | Anbazhagan et al. (2019) |
| | R: CCCTGACAGCCGTTAGATATT | | |
| <i>bla</i> CTX-M | F: ACGCTGTTGTTAGGAAGTG | 759 | Mansouri and Ramazanzad (2009) |
| | R: TTGAGGCTGGGTGAAGT | | |

In addition, permission to visit farms was obtained from the Ministry of Livestock and Fisheries at Provincial (with reference number PFLC/CBP/101/15/1) and district levels before data collection.

Results

Culture, isolation, and characterization of *Salmonella* Typhimurium

The preliminary identification of *S. Typhimurium* gave an overall total of 130 suspected isolates from all the farms. The identification was based on overnight cultures on differential and selective media and biochemical tests. About 146 tested positive for TSI, and 130 tested negative for the Urease test.

Detection of *Salmonella* Typhimurium by PCR

Results of analysis of the 130 suspected *S. Typhimurium* isolates by PCR revealed that 68 of the isolates were *S. Typhimurium* (Figure 2). Amongst the districts, Chingola reported a prevalence of 7.3% *S. Typhimurium* followed by Ndola 5.2%, Luanshya 2.9%, Kitwe 1.6% and Mufulira 0.8% (Table 2). The total prevalence of *S. Typhimurium* in commercial poultry farms in the Copperbelt province was 17.7% (CI: 14.2%-21.8%).

Association between occurrence of *S. Typhimurium* and different variables

The overall occurrence of *S. Typhimurium* isolated from commercial farms of the Copperbelt province was tested for associated with eight risk factors that included antibiotic usage, the purpose of use, veterinarian consultation, antibiotic administration, withdrawal period, biosecurity practice, hygiene, and manure handling (Table 3). The association between the occurrence and purpose of antibiotic usage, withdrawal period, hygiene, and biosecurity practices was significant (p-value= 0.00578499, CI: 0.0194-0.7197) with Fisher's exact test-value of 7.6164 (2, N=384). There was also an association between antibiotic usage and manure handling with the overall occurrence (p-value= 0.00000025, CI: 0.0000-0.1497 with Fisher's exact test of 26.592).

Antimicrobial resistance patterns of *S. Typhimurium* isolated from commercial poultry farms in the Copperbelt province

A total of the 68 *S. Typhimurium* isolates tested for antimicrobial susceptibility, 88.2% of the isolates showed resistance to one or more antimicrobial compounds. Interestingly, all 68 *S. Typhimurium* isolates showed 100% resistance to tetracycline, followed by ampicillin and amoxicillin at 91.2%.

The diversity of the antimicrobial resistance and susceptibility, as well as multidrug resistance of the isolates, are presented in Table 4 and Table 5.

Phenotypic and molecular detection of CTX-M-type ESBL producing *S. Typhimurium*

Phenotypic ESBL detections are represented in (Table 6). The molecular detection of CTX-M-type ESBL-producing *S. Typhimurium* revealed that of the 68 *S. Typhimurium* confirmed isolates, 49 were ESBL producers carrying -lactamase genes *bla*CTX-M (Figure 3). Therefore, the presence of CTX-M-type ESBL-producing *S. Typhimurium* in commercial poultry farms in the Copperbelt province was detected at 12.8% (CI: 9.8%-16.5%).

Discussion

Findings from this cross-sectional study show that *S. Typhimurium* in commercial poultry farms of the Copperbelt province was detected at the rate of 17.7%. These findings were slightly higher than findings in a study conducted in Lusaka, Zambia, which reported a prevalence of 3.74% and 4.7% *S. Enteritidis* in egg yolk and chicken carcasses, respectively (Hang'ombe et al., 1999). In their study, Hang'ombe et al. (1999) only used biochemical tests to identify *Salmonella*, while our study used both biochemical and molecular tools, therefore improving the validity of the findings.

The occurrence of *S. Typhimurium* in a poultry farm in Nigeria was reported as 16.0% (Ahmed et al., 2019), similar to our findings but contrary to the findings in Egypt, where *S. Typhimurium* was detected at higher rates of 44%, 40% and 48% in chicken meat, liver, and heart, respectively (El- Aziz, 2013). From these studies, the occurrence of *Salmonella* in poultry, chicken carcasses, and eggshells ranges from 3% to 48%, and findings from the present study are within this range.

In this study, detection of CTX-M-type ESBL-producing *S. Typhimurium* isolates was at 12.8% in commercial poultry farms in the Copperbelt province. The prevalence was associated with the administration of antibiotics to flocks. These findings were similar to a study conducted in Zambia, which reported a prevalence of 13% CTX-M-type ESBL-producing *E. coli* in market-ready chickens (Chishimba et al., 2016). Another study conducted in China on foodborne animals reported a prevalence of 17.7% CTX-M-type producing *Salmonella* (Zhang et al., 2019), which is slightly higher than the prevalence reported in this study.

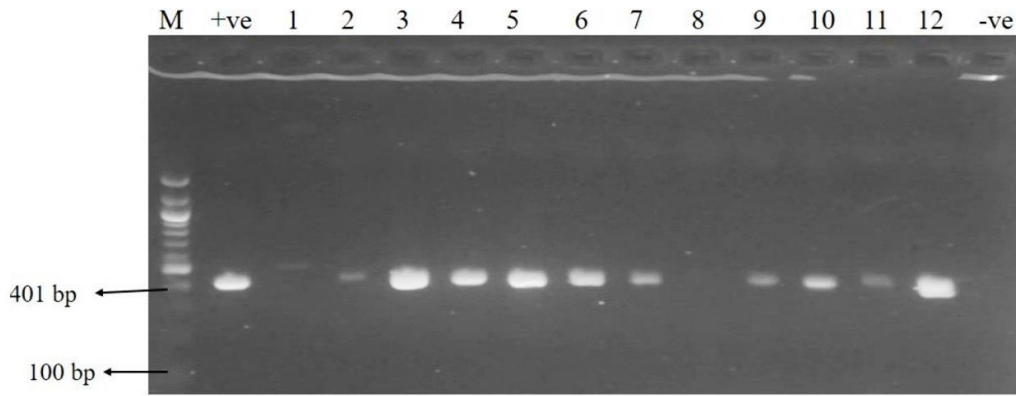


Figure 2: Detection of *S. Typhimurium* by conventional PCR at 401bp expected band size. Key: M = 100bp DNA ladder, +ve = positive control (*S. Typhimurium* ATCC 14028), and -ve = negative control, 1-12 are isolates loaded for amplification.

Table 2: Distribution of *Salmonella* Typhimurium isolated from commercial poultry farms of the Copperbelt province per district (n=384)

| District | Total samples collected | Number of positive isolates | Prevalence | Confidence interval (95%) | |
|----------|-------------------------|-----------------------------|------------|---------------------------|-------------|
| | | | | Low limit | Upper limit |
| Chingola | 77 | 28 | (7.3%) | 5.09% | 10.34% |
| Kitwe | 76 | 6 | (1.6%) | 0.72% | 3.37% |
| Mufulira | 77 | 3 | (0.8%) | 0.27% | 2.27% |
| Luanshya | 76 | 11 | (2.9%) | 1.61% | 5.06% |
| Ndola | 78 | 20 | (5.2%) | 3.40% | 7.91% |

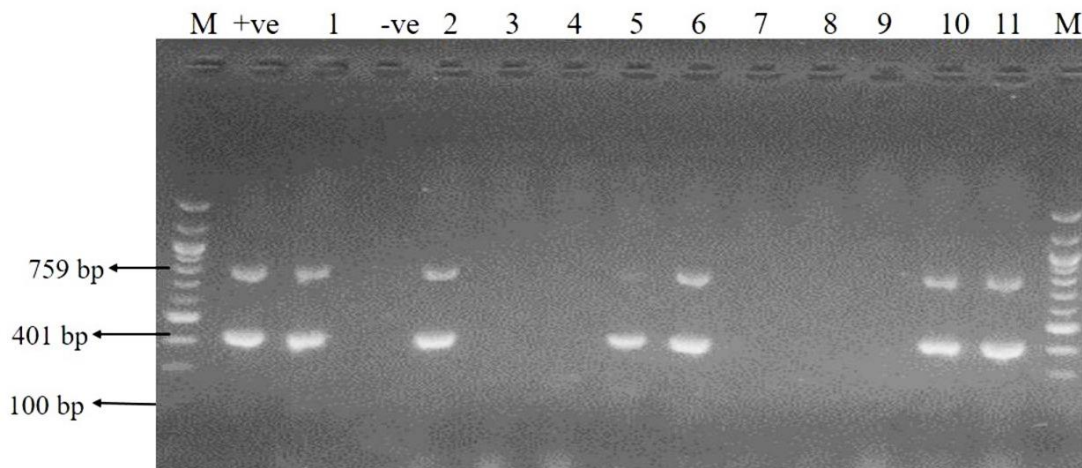


Figure 3: Detection of CTX-M-type ESBL-producing *S. Typhimurium* by Multiplex PCR at 759bp and 401bp expected band sizes. Key: M = 100bp DNA ladder, +ve = positive control (*S. Typhimurium* ATCC 14028), 1-11 are isolates loaded for amplification. Double bands on one lane indicate the presence of CTX-M-type ESBLs. Lanes 3, 4, and 8 show no amplification.

The presence of CTX-M-type ESBLs is often associated with co-resistance to other family phenotypes of antibiotic compounds, in particular to fluoroquinolones trimethoprim-sulfamethoxazole and aminoglycosides (Zeynudin et al., 2018). Therefore, in the present study, the isolates showed antimicrobial resistance to other classes of antibiotics (chloramphenicol (75.0%),

gentamicin (20.6%), and nalidixic acid (17.6%)) other than -lactams. A previous study in Zambia reported resistance of *Salmonella* isolates to nalidixic acid at 35.9% and chloramphenicol at 15.4% (Phiri et al., 2020).

Table 3: Association between occurrence of *S. Typhimurium* and different variables

| Risk factor | Category | Frequency | Fisher's exact test value | p-value | CI (95%) | |
|---------------------------|------------------|-----------|---------------------------|------------|-------------|-------------|
| | | | | | Lower limit | Upper limit |
| Antibiotic usage | Yes | 5 | 26.592 | 0.00000025 | 0.0000 | 0.1497 |
| | No | 0 | | | | |
| Purpose of antibiotic use | Prophylaxis | 2 | 7.6164 | 0.00578499 | 0.0194 | 0.7197 |
| | Growth promoter | 3 | | | | |
| Veterinarian consultation | Yes | 1 | 0.0945 | 0.75822758 | 0.0780 | 6.4342 |
| | No | 4 | | | | |
| Antibiotic administration | Veterinarian | 1 | 0.0945 | 0.75822758 | 0.0780 | 6.4342 |
| | Self | 4 | | | | |
| Withdrawal period | Yes | 2 | 7.6164 | 0.00578499 | 0.0194 | 0.7197 |
| | No | 3 | | | | |
| Biosecurity practice | No | 3 | 7.6164 | 0.00578499 | 0.0194 | 0.7197 |
| | Yes | 2 | | | | |
| Hygiene | Disinfectant use | 2 | 7.6164 | 0.00578499 | 0.0194 | 0.7197 |
| | Water | 2 | | | | |
| Manure handling | Farming purpose | 5 | 26.592 | 0.00000025 | 0.0000 | 0.1497 |
| | Disposal | 0 | | | | |

Table 4: Antimicrobial resistance patterns of *Salmonella Typhimurium* isolated from commercial poultry farms in the Copperbelt province by the zone of inhibition of the isolates (N= 68 Isolates)

| Antimicrobial agent % | (n/N) | | |
|-----------------------|---------------|---------------|----------------|
| | Susceptible | Intermediate | Resistant |
| Ampicillin | 2.9% (2/68) | 5.9% (4/68) | 91.2% (62/68) |
| Amoxicillin | 0.0% (0/68) | 8.8% (6/68) | 91.2% (62/68) |
| Chloramphenicol | 7.4% (5/68) | 17.6% (12/68) | 75.0% (51/68) |
| Gentamicin | 44.1% (30/68) | 35.3% (24/68) | 20.6% (14/68) |
| Nalidixic Acid | 27.9% (19/68) | 54.4% (37/68) | 17.6% (12/68) |
| Norfloxacin | 97.1% (66/68) | 2.9% (2/68) | 0.0% (0/68) |
| Tetracycline | 0.0% (0/68) | 0.0% (0/68) | 100.0% (68/68) |

In another study, the detection of ESBL-producing *E. coli* in chickens in Zambia showed resistance to gentamicin at 37.7%, chloramphenicol at 57.1%, and norfloxacin at 54.5% (Chishimba et al., 2016). The present study only focused on one class of ESBL, while Chishimba et al. (2016) included several ESBL classes, therefore, the differences in percentages. Antibiotic resistance profiles of the current study have shown similarities with results reported by (Ak-iba et al., 2008; Ahmed et al., 2019).

This study also reported 58.8% resistance of *S. Typhimurium* isolates to cefotaxime and 54.4% resistance to ceftazidime, similar to the findings of Burke et al. (2014), who reported the prevalence of 58% resistance of *Salmonella enterica* to cefotaxime but contrary to the findings in Nigeria where 100% resistance of *S. Typhimurium* to cefotaxime and ceftazidime was reported in poultry farms (Ahmed et al., 2019). Resistance to the third-generation cephalosporins was due to the production of CTX-M-type ESBLs.

Therefore, the dissemination of ESBL genes of *Salmonella* isolated from commercial poultry farms in Copperbelt province, Zambia, is of great concern. The data suggest that *S. Typhimurium* may transmit antimicrobial resistance from chicken to humans, the environment, or the food supply chain. Manure handling, hygiene, and biosecurity practices could be other sources of ESBL contaminating factors in these poultry farms.

Conclusions

To our knowledge, the current study is the first to be conducted in the Copperbelt province in Zambia. The study reports the presence of CTX-M-type ESBL-producing *S. Typhimurium* in commercial poultry farms, which are also resistant to numerous antimicrobial agents. The contamination of chickens at the primary production level poses a public health risk and calls for appropriate measures to reduce the usage of antimicrobial agents. Biosecurity measures should strictly be followed to minimize contamination levels.

Table 5: Multi-drug resistance patterns of *S. Typhimurium* isolates per district

| District | Number of isolates | Multi-Drug Resistance |
|----------|--------------------|---|
| Chingola | 28 | Ampicillin, tetracycline, and amoxicillin. |
| Kitwe | 3 | Tetracycline and amoxicillin. |
| Mufulira | 1 | Tetracycline and ampicillin. |
| Luanshya | 9 | Tetracycline, ampicillin, chloramphenicol, and amoxicillin. |
| Ndola | 19 | Tetracycline, ampicillin, and amoxicillin. |

Table 6: Cephalosporin resistance patterns of *S. Typhimurium* isolated from the commercial poultry farms of Copperbelt province by the zone of inhibition of the isolates (N = 68 Isolates)

| Antimicrobial agent % | (n/N) | | |
|-----------------------------|----------------|---------------|---------------|
| | Susceptible | Intermediate | Resistant |
| Ceftazidime-clavulanic acid | 100.0% (68/68) | 0.0% (0/68) | 0.0% (0/68) |
| Cefotaxime | 26.5% (18/68) | 14.7% (10/68) | 58.8% (40/68) |
| Ceftazidime | 29.4% (20/68) | 14.7% (10/68) | 54.4% (37/68) |

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Conflict of Interest. The authors declare no conflict of interest.

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