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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, their 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 chicken food chain at 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 (Tzouvelekis 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, 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 had gone further to 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, their 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 10 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 size 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 Veterinary services, even though large farms are now hiring their own veterinarians. The market-ready poultry products are usually directly sold to the 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.

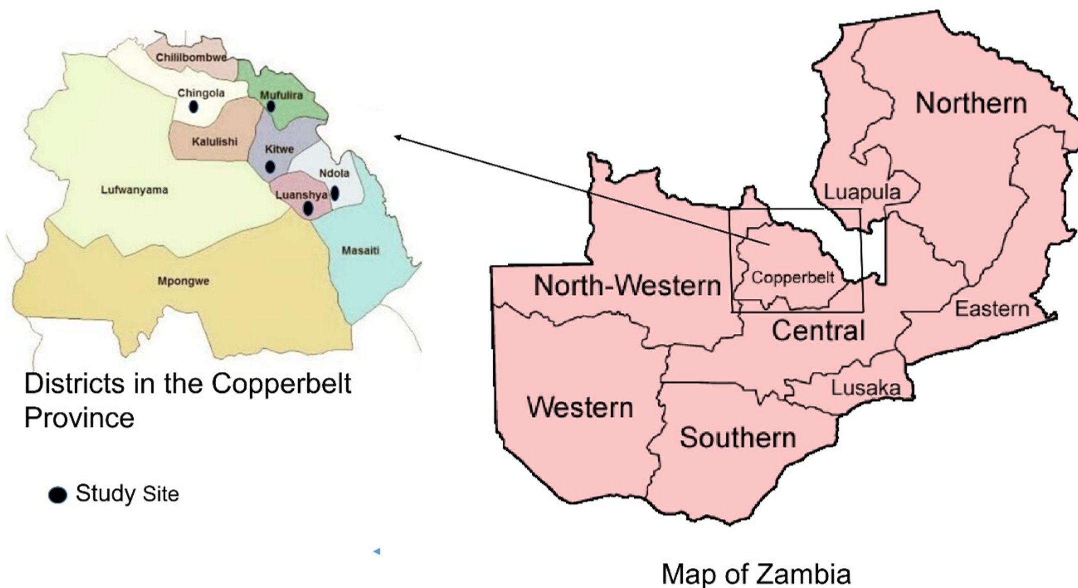

Figure 1: A map of Copperbelt province showing the study area (CSO, 2018).

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 adjust 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 in 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. Afterwards, 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 were 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 minutes at 94°C, 40 cycles of 30 seconds at 94°C, 30 seconds at 58°C, and 1 minute at 72°C and the final extension step for 4 minutes 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 *blaC*TX-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, 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 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. In addition, permission

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

Primer	Sequence (5'-3')	Size bp	Reference
Typh	F: TTGTTCACTTTTTACCCCTGAA R: CCCTGACAGCCGTTAGATATT	401	Anbazhagan et al. (2019)
blaCTX-M	F: ACGCTGTTGTTAGGAAGTG R: TTGAGGCTGGGTGAAGT	759	Mansouri and Ramazanzad (2009)

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 to TSI, and 130 tested negative to 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 the 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 8 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 the 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 reported 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 eggshell 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.

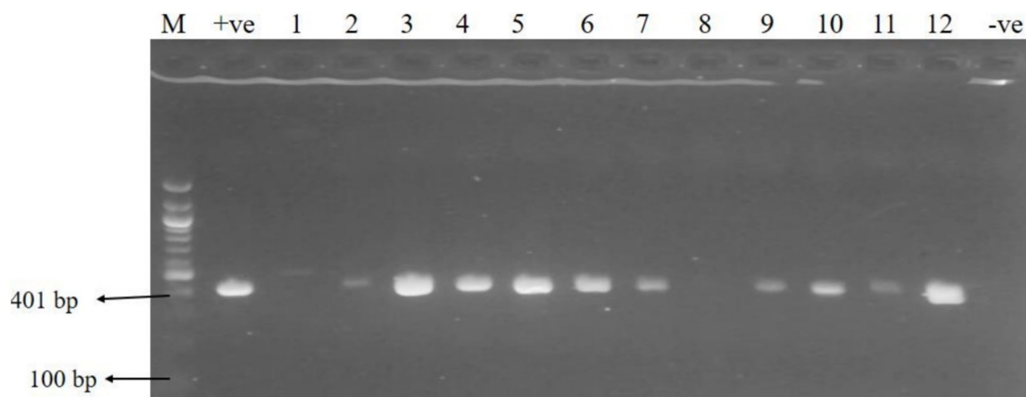

Figure 2: Detection of *S.* Typhimurium by conventional PCR at 401bp expected band size. Key: M = 100bp DNA ladder, +ve = positive control (*S.* Typhimurium ATCC 14028) and -ve = negative control, 1-12 are isolates loaded for amplification.

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%

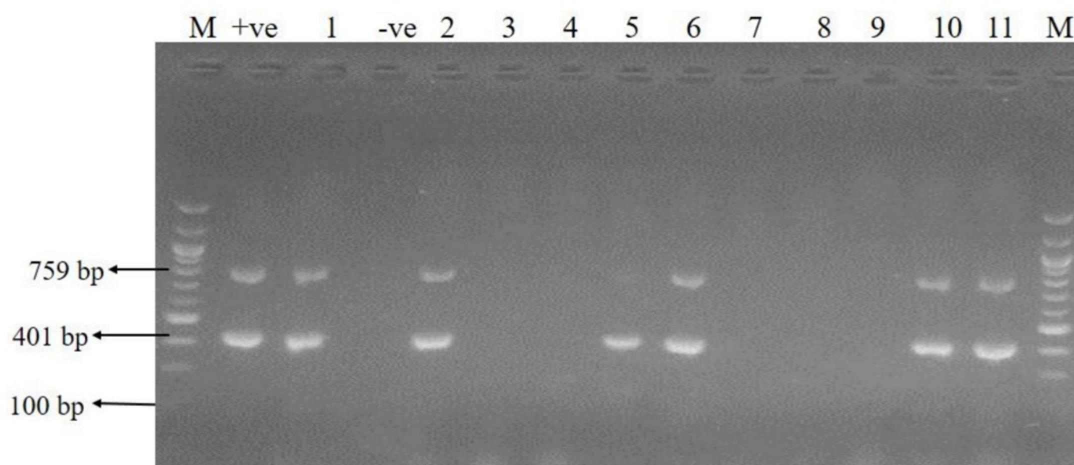

Figure 3: Detection of CTX-M-type-ESBL producing *S.* Typhimurium by Multiplex PCR at 759bp and 401bp expected band sizes. Key: M = 100bp DNA ladder, +ve = positive control (*S.* Typhimurium ATCC 14028), 1- 11 are isolates loaded for amplification. Double bands on one lane indicates the presence of CTX-M- 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

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 Salmonell*a Typhimur*ium 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)

nalidixic acid at 35.9% and chloramphenicol 15.4% (Phiri et al., 2020). 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 (Akiba 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)

Article Information

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

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