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Extended-spectrum beta-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae* in local and imported poultry meat in Ghana

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ABSTRACT

Antibiotic use in animal husbandry has raised concerns on the spread of resistant bacteria. Currently animal products are traded globally with unprecedented ease, which has been challenging the control of antimicrobial resistance. This study aims to detect and characterize extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* and *Klebsiella pneumoniae* from imported and locally produced poultry products sold in Ghana.

Local and imported chicken meat was collected from 94 stores and markets throughout Kumasi (Ghana) and cultured on selective ESBL screening agar. Phenotypic ESBL-producing *E. coli* and *K. pneumoniae* isolates were confirmed by combined disc test and further characterized by antibiotic susceptibility testing, amplification of the bla_{CTX-M}, bla_{TEM} and bla_{SHV} genes as well as multilocus sequence typing (MLST) and linked to the country of origin.

Out of 200 meat samples, 71 (36%) samples revealed 81 ESBL-producing isolates (46 *E. coli* and 35 *K. pneumoniae*), with 44% (30/68) of local poultry and 31% (41/132) of imported products being contaminated. Most ESBL-producing isolates harboured the $bla_{CTX-M-15}$ gene (61/81, 75%) and the dominant Sequence Types (ST) were ST2570 (7/35, 20%) among *K. pneumoniae* and ST10 (5/46, 11%) among *E. coli*.

High numbers of ESBL-producing bacteria, particularly on local but also imported poultry meat, represent a potential source for human colonization and infection as well as spread within the community. Surveillance along the poultry production-food-consumer chain would be a valuable tool to identify sources of emerging multidrug resistant pathogens in Ghana.

1. Background

The use of antimicrobial drugs in animal husbandry for the treatment and prevention of infectious diseases or as growth promoters have contributed to the spread of multidrug resistant bacteria (Chantziaras et al., 2014). As common causes of nosocomial and community infections among humans, multidrug resistant *Escherichia coli* and *Klebsiella pneumoniae* are of particular interest (Mathers et al., 2015). Worryingly, prevalence rates for extended-spectrum beta-lactamase (ESBL)/AmpC-producing *E. coli* in poultry meat have exceeded 90% in industrialized countries such as France, the Netherlands and Spain (Leverstein-van Hall et al., 2011; Egea et al., 2012; Casella et al., 2017).

There is increasing evidence for the transmission of ESBL genes, plasmids and *E. coli* isolates from poultry to humans, most likely through the food chain (Leverstein-van Hall et al., 2011). Other studies have shown that ESBL-producing *E. coli* causing extraintestinal infections in humans originated from meat products, particularly poultry (Lazarus et al., 2014). Consequently, the European Food Safety Authority (EFSA) declared the presence of ESBL-producing *E. coli* in poultry meat a significant hazard to public health (EFSA, 2012) and jointly, the EFSA and the European Medicines Agency (EMA) recently advocated measures to reduce antimicrobial agents in animal husbandry in the European Union (Murphy et al., 2017).

In contrast to industrialized countries, the extent of poultry meat contamination with ESBL-producing *E. coli* in Sub-Saharan Africa is not sufficiently monitored and the use of antimicrobials remains largely unregulated (Maron et al., 2013). Apart from inappropriate drug applications without prescriptions, antimicrobial use for growth

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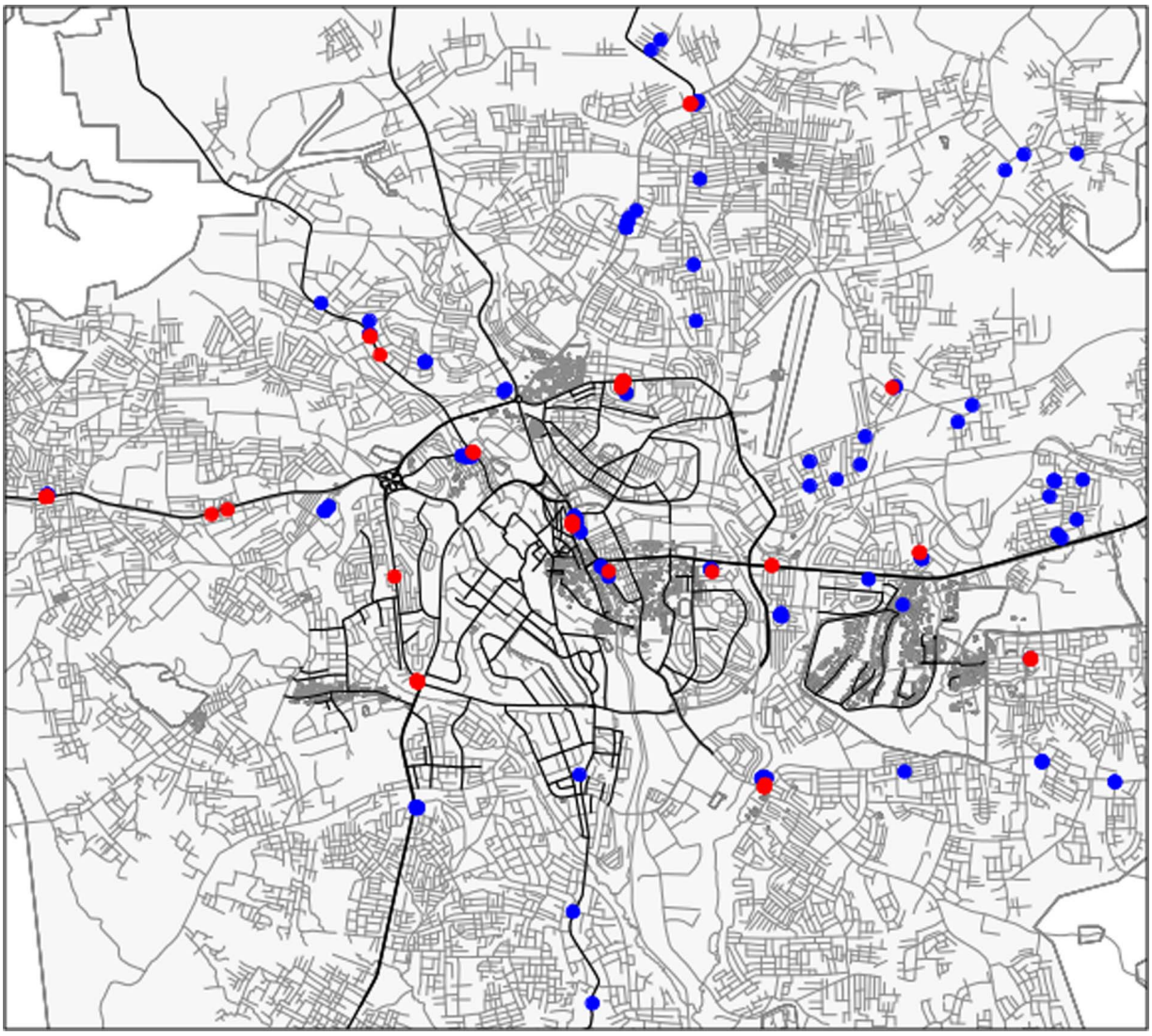

Fig. 1. Sampling locations in the city of Kumasi, Ghana. Blue dots represent supermarkets (n = 75), where imported meat was collected. Red dots represent the open markets (n = 19), on which local meat was purchased. Map data was retrieved from https://www.openstreetmap.org. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

promotion has been regarded as a major problem (Okeke et al., 1999; Adzitey, 2013; Van Boeckel et al., 2015). Despite this, recent studies from Sub-Saharan Africa found only 11% of Ghanaian and no Nigerian poultry samples to be contaminated with ESBL-producing *E. coli* (Rasmussen et al., 2015; Ojo et al., 2016). Imported poultry meat from industrialized countries may therefore rather be contributing to the spread of ESBL-producing bacteria within Sub-Saharan Africa, as proposed by studies from Gabon and Ghana (Schaumburg et al., 2014; Rasmussen et al., 2015). The significance of the poultry trade for the international dissemination of antimicrobial resistances has been illustrated not only for the transmission of ESBL-producing bacteria, but also just recently for *mcr-1*-mediated colistin resistance (Dhanji et al., 2010; Grami et al., 2016). With poultry products being traded globally with unprecedented ease, the control of antimicrobial resistance is challenging, in particular in Sub-Saharan Africa, where surveillance systems do not exist.

This study aims to characterize and compare ESBL-producing *E. coli* and *K. pneumoniae* from imported and locally produced poultry products sold in Ghana in order to identify potential sources for the emergence of antimicrobial resistant bacteria.

2. Methods

2.1. Study site and sampling procedure

The study was conducted from May to December 2015 in Kumasi, with roughly 2.5 million inhabitants the second largest city in Ghana and the capital of the Ashanti region. Throughout the city 75 supermarkets, selling imported frozen poultry meat, were identified (Fig. 1, blue location marks). Each supermarket was visited once during the study period. One piece of frozen poultry meat was purchased in each store. In case stores sold meat imported from different countries, one piece from each country was acquired. The origin of the imported meat was retrieved from the packaging. Local Ghanaian poultry meat was purchased from 19 different open markets, where each trader was visited only once (Fig.1, red location marks). Approximately 15 g of each meat sample were immediately put into sterile homogenizer bags and kept refrigerated during transport to the laboratory.

2.2. ESBL detection and antibiotic susceptibility testing

On arrival in the laboratory, the sample was homogenized with a pestle and incubated overnight in 100 mL of Brain Heart Infusion (BHI)

broth. 100 µL were then plated onto two selective MacConkey agar plates containing 1 mg/L ceftazidime and 1 mg/L cefotaxime. All morphologically different colonies (never exceeding four colonies) were identified biochemically using the analytical profile index test (API 20E, bioMérieux, Marcy L'Etoile, France) and confirmed by MALDI-TOF mass spectrometry (Bruker, Billerica, USA). For all *E. coli* and *K. pneumoniae* isolates ESBL production was confirmed by the combined disk test using cefotaxime and ceftazidime alone and in combination with clavulanic acid (Becton, Dickinson and Company, Sparks, MD, USA) as described before by the European Committee on Antimicrobial Susceptibility Testing (EUCAST, the EUCAST guideline on detection of resistance mechanisms v 1.0 (2013-12-11)).

Antimicrobial susceptibility testing was performed with the VITEK 2 system using AST-N111 cards (bioMérieux, Marcy L'Etoile, France) for cefotaxime, ceftazidime, imipenem, meropenem, gentamicin, ciprofloxacin and trimethoprim/sulfamethoxazole (cotrimoxazole). Breakpoints for *Enterobacteriaceae* were applied according to the 2016 EUCAST guidelines (http://www.eucast.org).

2.3. ESBL genotyping and multilocus sequence typing (MLST)

All isolates with ESBL phenotypes were screened for the presence of the bla_{CTX-M}, bla_{TEM} and bla_{SHV} genes by Polymerase Chain Reaction (PCR) and subsequent sequencing, as described before (Belmar Campos et al., 2014). Group specific primers were used for amplification and sequencing to further distinguish bla_{CTX-M} positive isolates (Belmar Campos et al., 2014). The resulting sequences were identified by comparison with known sequences using the NCBI BLAST (http://blast.ncbi.nlm.nih.gov) and the Lahey Clinic Database (http://www.lahey.org/studies/). MLST was conducted for all ESBL-producing *E. coli* and *K. pneumoniae* isolates according to previously published 7-loci protocols (Diancourt et al., 2005; Wirth et al., 2006).

2.4. Epidemiological analysis

Categorical variables were described as frequencies and percentages. The association of ESBL positivity with meat import was calculated using the prevalence ratio (PR) along with the 95%-confidence interval (CI). All data analyses were performed with Stata 14 (StataCorp LP, College Station, USA). The map was created with R statistical software (https://www.R-project.org/) using the osmar package (Eugster and Schlesinger et al., 2012). Data for the Kumasi map (Fig. 1) was retrieved from https://www.openstreetmap.org.

3. Results

A total of 200 meat samples, consisting of 68 samples from local Ghanaian poultry and 132 samples from imported poultry meat, were collected from open markets and supermarkets across Kumasi (Fig. 1). The majority of imported meat samples originated from the Netherlands (n = 38; 29%), Brazil (n = 31; 23%) and the United States (n = 31; 23%) (Fig. 2). Imported poultry meat from Belgium (n = 8), Germany (n = 3) Poland (n = 3), Ireland (n = 2) and Turkey (n = 1) was less commonly purchased. For 15 imported products the country of origin could not be identified.

Among the 200 meat samples, 71 (36%) were screened positive for ESBL-producing bacteria, with 46 *E. coli* and 35 *K. pneumoniae* isolates being detected. Nine meat samples were positive with both species, and one sample revealed two different *K. pneumoniae* isolates. With 44% (n = 30) of Ghanaian meat samples and 31% (n = 41) of imported meat samples being contaminated with ESBL-producing bacteria, no clear difference between those two sampling groups was detected (PR 0.81, 95% CI 0.64–1.03), although country-specific prevalences varied slightly. Looking at meat-exporting countries from which more than five samples have been collected, the highest ESBL rates were detected in Belgian (n = 7/8; 88%), Brazilian (n = 10/31; 32%) and Dutch (n = 10/38; 26%) meat products (Fig. 2).

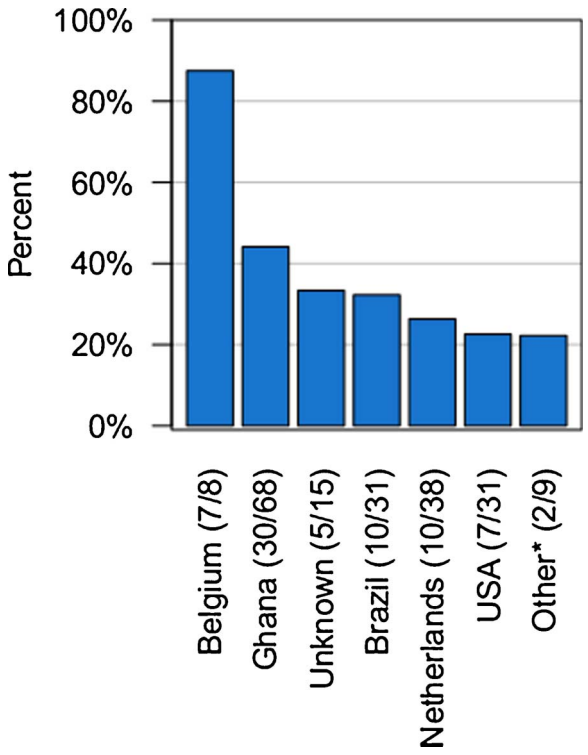

Fig. 2. ESBL prevalence by country. Proportions of ESBL-producing *Escherichia coli* or *Klebsiella pneumoniae* detected in meat samples are seperated by country of origin.
*Countries from which less than five meat samples were collected are summarized under "Other": One ESBL-producing isolate each was found in meat samples from Germany (n = 3) and Poland (n = 3). No ESBL-producing isolate was found among meat from Ireland (n = 2) and Turkey (n = 1).

The most frequently identified ESBL genotype was CTX-M-15, with 67% of *E. coli* (31/46) and 86% of *K. pneumoniae* (30/35) isolates harbouring $bla_{CTX-M-15}$ (Table 1). CTX-M-15 was followed in frequency by CTX-M-1 (11%; 5/46) and CTX-M-2 (9%; 4/46) genotypes among *E. coli* and CTX-M-14 (6%; 2/35) and SHV-12 (6%; 2/35) genotypes among *K. pneumoniae* (Table 1).

Concomitant resistance to ciprofloxacin was detected in 56 of the 81 ESBL-producing isolates (69%) distributed to 59% of *E. coli* and 83% of *K. pneumoniae* isolates. In comparison to *E. coli*, *K. pneumoniae* isolates also revealed higher resistance rates for the antibiotics gentamicin (13% vs. 49%) and trimethoprim/sulfamethoxazole (72% vs. 91%). All tested isolates were susceptible to carbapenems (Table 1).

MLST analysis revealed high strain diversity with 22 and 32 different sequence types (ST) detected in *K. pneumoniae* and *E. coli* isolates, respectively. The most frequent STs in *K. pneumoniae* were ST2570 (n = 7; 20%), ST147 (n = 3; 9%) and ST15 (n = 3; 9%), which comprise 65% of all Ghanaian *Klebsiella* isolates. One *Klebsiella* isolate presented with a new allele (pgi allele 182) and two other isolates revealed a new allele profile, which could not be allocated to any known ST. These strains were assigned the new ST numbers ST2734, ST2741 and ST2956 in the *K. pneumoniae* MLST database (http://bigsdb.pasteur.fr/klebsiella/klebsiella.html). Among *E. coli* ST10 (n = 5; 11%), ST38 (n = 3; 7%) and ST155 (n = 3; 7%) predominated. Based on the MLST analysis no ST clusters could be attributed to any specific importing country (Table 2).

4. Discussion

The study results demonstrate high contamination rates with ESBL-producing *E. coli* and *K. pneumoniae* for local Ghanaian (44%) and imported poultry meat (31%). A previous study from Ghana found

Table 1
ESBL genotypes and antibiotic susceptibility of ESBL-producing *Escherichia coli* and *Klebsiella pneumoniae* by country of orgin.

Country of origin	ESBL genotypes (n)	Resistance types[§]						
		only ESBL n(%)	Cip n(%)	Gen n(%)	Sxt n(%)	CipGen n(%)	CipGenSxt n(%)	CipSxt n(%)	GenSxt n(%)
ESBL *E. coli*									
Belgium (n = 6)	CTX-M-1 (1), CTX-M-15 (1), CTX-M-32 (1), SHV-2a (2), TEM-52c (1)	1 (17)	0 (0)	0 (0)	1 (17)	0 (0)	1 (17)	3 (50)	0 (0)
Brazil (n = 8)	CTX-M-1 (1), CTX-M-2 (2), CTX-M-15 (5)	0 (0)	0 (0)	2 (25)	2 (25)	0 (0)	1 (13)	3 (38)	0 (0)
Germany (n = 1)	CTX-M-1 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)
Ghana (n = 19)	CTX-M-1 (1), CTX-M-15 (17), CTX-M-27 (1)	1 (5)	4 (21)	0 (0)	6 (32)	0 (0)	1 (5)	7 (37)	0 (0)
Netherlands (n = 3)	CTX-M-15 (3)	1 (33)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2 (67)	0 (0)
Poland (n = 1)	CTX-M-15 (1)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)
USA (n = 4)	CTX-M-2 (2), CTX-M-15 (1), CTX-M-27 (1)	0 (0)	2 (50)	0 (0)	0 (0)	0 (0)	1 (25)	1 (25)	0 (0)
Unknown (n = 4)	CTX-M-1 (1), CTX-M-15 (3)	1 (25)	0 (0)	0 (0)	3 (75)	0 (0)	0 (0)	0 (0)	0 (0)
ESBL *K. pneumoniae*									
Belgium (n = 1)	CTX-M-15 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)
Brazil (n = 5)	CTX-M-15 (5)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	4 (80)	0 (0)	1 (20)
Ghana (n = 17)	CTX-M-14 (2), CTX-M-15 (13), SHV-12 (2)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	4 (24)	13 (76)	0 (0)
Netherlands (n = 7)	CTX-M-15 (7)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	4 (57)	1 (14)	2 (29)
USA (n = 4)	CTX-M-15 (3), SHV-2a (1)	1 (25)	1 (25)	0 (0)	0 (0)	0 (0)	2 (50)	0 (0)	0 (0)
Unknown (n = 1)	CTX-M-3 (1)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

§ Cip: ciprofloxacin; Gen: gentamicin; Sxt: trimethoprim/sulfamethoxazole; all isolates were tested susceptible to imipenem and meropenem.

significantly less ESBL bacteria among local poultry (11%) compared to imports (21%) from Brazil and the United States, suggesting the international food trade as an important source for the spread of multidrug resistant bacteria (Rasmussen et al., 2015). With an even higher ESBL rate among international poultry products, the present study apparently reinforces this hypothesis. However, in contrast to other studies, the results presented here show that ESBL contamination of local meat exceeds that of most importing countries. Considering the variable ESBL prevalences from each country, it might be misleading to hold the international poultry trade in general responsible for introducing ESBL-carrying bacteria into Ghana. Instead rather country-specific data should be monitored. According to United Nations data (https://comtrade.un.org) the USA, Belgium, Brazil and the Netherlands are the main poultry meat importers, having contributed to 66% of poultry imports to Ghana in 2016 and comprising 82% of imported specimens in this sample collection. The high ESBL rate from Belgium meat samples is particularly alarming. Previously, not only on-farm antimicrobial therapy, such as off-label ceftiofur use until 2010 (Dierikx et al., 2013), but also management factors, such as hygienic conditions and feeding procedures, have been considered responsible for the emergence of multidrug resistant bacteria on Belgian farms (Persoons et al., 2011). A study from France suggested that even five years after a drastic reduction of ceftiofur use, high ESBL prevalences were still detectable in farm animals (Casella et al., 2017).

Table 2
Multilocus sequence types for ESBL-producing *Escherichia coli* and *Klebsiella pneumoniae* isolates by country of origin.

Country of origin	Sequence Type (ST)
ESBL *E. coli*	
Belgium (n = 6)	ST10 (1), ST46 (1), ST101 (1), ST162 (1), ST665 (1), ST2197 (1)
Brazil (n = 8)	ST10 (1), ST38 (1), ST48 (1), ST131 (1), ST617 (1), ST648 (1), ST1706 (1), ST3268 (1)
Germany (n = 1)	ST174 (1)
Ghana (n = 19)	ST10 (2), ST38 (1), ST69 (2), ST155 (2), ST193 (2), ST215 (1), ST218 (1), ST540 (1), ST617 (1), ST656 (1), ST710 (1), ST1421 (1), ST1722 (1), ST1973 (1), ST2325 (1)
Netherlands (n = 3)	ST38 (1), ST196 (1), ST1487 (1)
Poland (n = 1)	ST540 (1)
USA (n = 4)	ST156 (1), ST354 (1), ST2309 (2)
Unknown (n = 4)	ST10 (1), ST57 (1), ST155 (1), ST196 (1)
ESBL *K. pneumoniae*	
Belgium (n = 1)	ST1418 (1)
Brazil (n = 5)	ST39 (1), ST753 (1), ST1526 (1) ST2734 (1), ST2956 (1)
Ghana (n = 17)	ST15 (3), ST39 (1), ST147 (2), ST261 (1), ST307 (1), ST336 (1), ST1798 (1), ST2390 (1), ST2570 (6)
Netherlands (n = 7)	ST16 (1), ST29 (1), ST307 (1), ST429 (1), ST1436 (1), ST2171 (1), ST2741 (1)
USA (n = 4)	ST147 (1), ST394 (1), ST1035 (1), ST2570 (1)
Unknown (n = 1)	ST1035 (1)

Equally worrying are the high ESBL rates among local poultry. So far two studies from West Africa have investigated the contamination of poultry meat with ESBL *E. coli*, with 0% (sample size: n = 116) detected in Nigeria between 2008 and 2014 and 11% (sample size: n = 36) in Ghana in 2015 (Rasmussen et al., 2015; Ojo et al., 2016). Hence, the 41% of ESBL-producing *E. coli* found in the present study may indicate an increasing trend, however proportions are still lower than reported from European countries, such as Spain (93%) or the Netherlands (80%) (Overdevest et al., 2011; Egea et al., 2012). The easy, unregulated access and inappropriate use of antibiotics has recently been suggested to contribute to increasing antimicrobial resistance in Ghana (Yevutsey et al., 2017). This alarming development should encourage Ghanaian policy makers and public health authorities to enforce regulations on food hygiene and use of antibiotics, as proposed before (Annan-Prah et al., 2012).

As one of the globally predominant STs in the poultry population, ST10 is the most frequently detected ST among *E. coli* isolates (Huijbers et al., 2014; Rasmussen et al., 2015; Ojo et al., 2016). Only one *E. coli* isolate from Brazil belonged to ST131, the ST responsible for an estimated 40–80% of extra-intestinal infections with ESBL-producing *E. coli* worldwide (Nicolas-Chanoine et al., 2014). Among *K. pneumoniae,* ST2570 is most prevalent with six isolates from Ghanaian and one isolate from imported meat (United States). To the authors' knowledge ST2570 has never been isolated in poultry or patients from Sub-Saharan Africa before, but due to insufficient data from this region its relevance is difficult to determine. Due to the high diversity of STs and the relatively low case number for each country, the phylogenetic analysis is not able to identify any country-specific ST clusters.

CTX-M-15 is the most prevalent ESBL genotype among *E. coli* and *K. pneumoniae* in this study, similar to what has been previously reported for poultry meat and faecal samples from West Africa (Rasmussen et al., 2015; Ojo et al., 2016). Interestingly, all the Dutch isolates harboured the $bla_{\text{CTX-M-15}}$ gene, although studies from the Netherlands show a predominance of $bla_{\text{CTX-M-1}}$ among retail poultry meat (Leverstein-van Hall et al., 2011; Overdevest et al., 2011). This finding could be explained by changing epidemiological patterns, but also by the

complexity in tracing back an isolate to a definite animal, environmental or human source. Local as well as imported poultry could have been contaminated at all stages of the food processing chain including processing, packing and distribution, a factor requiring consideration when attributing a geographical source to isolates. Nevertheless, regardless of a contaminating source, ESBL-producing bacteria on poultry meat may represent a risk for the end consumer, eventually leading to the colonization of the intestinal tract or severe infections (Lazarus et al., 2014).

Highly relevant for clinical practice is the substantial number of isolates with coexisting ESBL production and resistance to trimethoprim/sulfamethoxazole (80%) or ciprofloxacin (69%) in this study. A recent meta-analysis from Tanzania revealed very similar co-resistance numbers in animal samples and several studies have demonstrated the coexistence of plasmid-mediated quinolone resistance (PMQR) and ESBL genes on the same plasmid (Strahilevitz et al., 2009; Fortini et al., 2011; Seni et al., 2017). This study however, does not allow any conclusions on a potential linkage of different plasmid-mediated resistance genes. Nevertheless, this issue needs to be addressed in future studies, as those multidrug resistant strains may spread rapidly under antibiotic-specific selective pressure and would drastically reduce the already limited spectrum of available antibiotics for severe infections in the Sub-Saharan region (Eibach et al., 2016). If transmission of antibiotic resistance between poultry and human reservoirs is driven mainly by the spread of plasmids, as suggested previously, the importance of the bacteria's genetic background, including STs, may be negligible (de Been et al., 2014).

5. Conclusion

High numbers of ESBL-producing bacteria, particularly on local but also imported poultry meat, represent a potential source for human exposure. Spread within the community might lead to severe infections with multidrug resistant bacteria for which no antibiotic treatment is available in Ghana. In order to monitor the sources of emerging multidrug resistant pathogens, surveillance along the poultry production-food-consumer chain, integrating animal and human health, should be implemented. Surveillance activities should be combined with a more stringently regulated use of antibiotics on local poultry farms and increased awareness of food hygiene amongst the population. Internationally, advocacy for a reduction of antimicrobial drug use in animal husbandry must be reinforced.

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Conflict of interest

The authors declare no conflict of interest

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