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Prevalence of Type II Topoisomerase Mutations Among Quinolone Resistant *Escherichia coli* from Broiler Chickens in Dar es Salaam, Tanzania

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Abstract

Background: Quinolone-resistant *Escherichia coli* may easily be transmitted from poultry to humans and animals. Quinolone misuse in veterinary medicine, therefore, poses a significant public health risk. This study aimed to assess the prevalence of type II topoisomerase mutations commonly associated with resistance to quinolones in *E. coli*; in Dar es Salaam, Tanzania.

Methods: One hundred sixty E. coli samples isolated from broiler cloacal swabs in Dar es Salaam between March and August 2022 were evaluated and analyzed for quinolone resistance by disc diffusion and genetic methods. The study used two Polymerase Chain Reaction (PCR) techniques, one a Mismatch Amplification Mutation Assay (MAMA-PCR) and the other employing enzyme digestion of amplified products (PCR-RFLP). Both approaches targeted regions in type II topoisomerases (*gyr*A and *par*C) to determine quinolone resistance (QRDR).

Results: Results showed considerable levels of quinolone resistance among *E. coli* isolated from broiler chickens, where 42.5% of the strains demonstrated non-susceptibility to both ciprofloxacin and norfloxacin. Moreover, a significant minority of isolates (5%) were non-susceptible only to ciprofloxacin. All isolates non-susceptible to both drugs harboured a substitution mutation (S83L) at the *gyr*A Ser-83 target site (a resistance marker) as revealed by PCR-RFLP. The genotypic-to-phenotypic agreement ratio for norfloxacin was 100%, in contrast to that of ciprofloxacin, which was 89.47%. However, as revealed by MAMA-PCR results, none of the isolates under study harboured parC80 (S80I) substitution mutation (a resistance marker) on the topoisomerase IV gene.

Conclusion: Type II topoisomerase mutations other than the ones assayed in this study (*gyr*A Ser-83 or *par*C80) or other mechanisms of resistance might be contributing to the resistance against quinolones in *E. coli* strains circulating in broiler chickens in Dar es Salaam. The level of quinolone resistance revealed by this study calls for immediate intervention to mitigate its spread.

Keywords: Quinolone resistance; *Escherichia coli*; MAMA-PCR; Dar es Salaam; Tanzania

Introduction

Escherichia coli is a bacterial species widespread among humans and animals. Like other enterobacteriaceae, it is part of the intestinal microbiota, commonly released in the environment, contaminating food and water (Malabadi *et al.,* 2024). Because of its location in the body and its high capacity for acquiring antimicrobial resistance, indiscriminate use of antimicrobials both in human and animal husbandry increases the risk of spread of anti-microbial resistant *E. coli* strains but also transmission of the resistance to other bacteria through horizontal gene transfer (Lawal *et al.,* 2024). In developing countries, antibiotics are often empirically prescribed for *E. coli* diseases which include gastrointestinal and extra-intestinal infections (Diarra *et al.,* 2024).

Since the introduction of fluoroquinolones such as norfloxacin and ciprofloxacin in the mid 1980's, they have been widely used both in human and animals (Tang & Zhao, 2023). This has sparked the emergency of bacterial resistance against them by several mechanisms. Such mechanisms include chromosomal mutations in type II topoisomerase enzymes, the DNA gyrase and DNA topoisomerase IV (Bush *et al.*, 2020). Other chromosomal mutations have been reported in genes that regulate the

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expression of efflux pumps localized in bacterial membranes or porin genes. Among them are mutations in the Mar regulon which influence the expression of both the AcrAB-TolC pump complex in *E. coli* and the OmpC and ompF porin diffusion channels affecting quinolone permeation into the cytoplasm (Millanao *et al*., 2021). In addition, plasmid mediated quinolone resistance mechanisms (PMQR) are also very common. Even though these tend to impart low-level resistance, they are known for selecting for higher-level mutation-based resistance and significantly contribute to treatment failure (Aworh *et al*., 2023). These PMQR mechanisms include those coded for by *qnr* (*qnr*A, *qnr*B, *qnr*C, *qnr*D, *qnr*S) genes, the aminoglycoside acetyltransferase (*aac(6')-Ib-cr*), and the quinolone efflux pumps (QepA and OqxAB) (Bush *et al*., 2020).

DNA gyrase and DNA topoisomerase IV are both heterotetramers, each made up of two domains of GyrA and B or ParC and ParE respectively. They both catalyze double strand breaks and resealing in DNA to allow DNA replication, transcription and recombination. The DNA passing domains for these two enzymes are the GyrA and ParC domains. Quinolones block the resealing activity (Spencer & Panda, 2023). However, point mutations in either enzyme can lead to resistance against quinolones. These point mutations tend to occur in quinolone resistance determining regions (QRDR) which span amino acid residue 67 to 106 in *gyr*A and residue 63 to 102 in *par*C gene, based on the *E. coli* genomic sequence numbering. The DNA gyrase of *E. coli* is more readily inhibited by fluoroquinolones than the topoisomerase IV (Neyestani *et al*., 2023). In addition, the mutations that change the 83 serine to leucine (S83L) and the 87 aspartate to asparagine (D87N) are among the most commonly observed in quinolone resistant gram negative bacteria. In quinolone resistant *E. coli*, the mutation at 83 serine is the most common (Das *et al*., 2023).

In a previous study conducted in Dar es Salaam, Tanzania, where the *qnr*A gene was used as a marker for quinolone resistance, only 1.61% of quinolone-resistant isolates from broiler chickens harboured the gene (Kiula and Makene, 2023). The *qnr*A gene is a plasmid mediated form of resistance. This study embarked on analyzing isolates from broiler chicken for chromosomal mediated resistance mechanisms.

Materials and Methods

Study Area

This study was done in Dar es Salaam, a Tanzanian city with a population of 5.3 million people (The Population and Housing Census, 2022). The city has the highest poultry meat and egg consumption in the country.

Study design

This cross-sectional study was conducted from March to August 2022. Broiler chickens were selected for the study because they are reared mostly in densely populated settings. In addition, there is a significant misuse of antimicrobials for prophylaxis, growth enhancement, and infection management in poultry.

Sample size calculation

The minimum sample size (n) of 156 broiler chickens was obtained after assuming a prevalence (p) of 88.5% and a Z-score (Z) of 1.96 for a 95% confidence level within 5% margin of error (d). The following formula "$(n) = Z^2pq/d^2$" was applied, where $q = (1 - p)$.

Sample collection

A total of 160 cloacal swab samples were collected from major six poultry slabs in Dar es Salaam, namely Kisutu and Buguruni in Ilala district, Mwenge and Magomeni in Kinondoni district, Stereo in Temeke district, and Shekilango in Ubungo district. All cloacal swab samples were collected aseptically and kept in separate sterile tubes containing 5 ml of prepared tryptic soy broth (Oxoid, Basingstoke, UK). Immediately the samples were transported to the University of Dar es Salaam, Department of Molecular Biology and Biotechnology laboratory and incubated for 24 hours at 37°C.

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Isolation and Identification of Enterobacteria

Following overnight culture, one loop full of each culture was streaked on MacConkey agar (Oxoid, Basingstoke, UK) and incubated overnight at 37°C. Subsequently, pink or red colonies were assumed to be lactose-fermenting *E. coli* colonies. Three colonies were picked for inoculation on eosin methylene blue (EMB) agar, followed by overnight incubation at 37°C. *E. coli* colonies that peaked for further characterisation displayed a metallic sheen appearance. Characterization was done by catalase, indole, methyl red, oxidase, and the Voges-Proskauer tests. Isolates tentatively identified as *E. coli* were then inoculated into 4 ml of nutrient broth for overnight enrichment. Two aliquots were made, one for immediate antimicrobial susceptibility analysis and another stored in nutrient broth containing 15% glycerol at -20°C for later DNA extraction and molecular characterization. The *E. coli* ATCC 25922 obtained from the department culture collection was used as a positive control.

Antimicrobial susceptibility testing

Following overnight enrichment, 150µl of 0.5 McFarland standards matching *E. coli* suspension was inoculated onto Mueller-Hinton agar by even distribution using sterile swabs. The standard Kirby-Bauer disk diffusion method was used to screen for susceptibility to ciprofloxacin and norfloxacin, representing quinolone antibiotics, following the Clinical and Laboratory Standards Institute (CLSI) guidelines 2021. A disc dispenser was used to place ciprofloxacin (5µg) and norfloxacin (10µg) antibiotic discs on the agar plates. Following overnight incubation at 37°C, the zones of growth inhibition were scored and interpreted as per CLSI guidelines.

Molecular characterization of E. coli isolates

E. coli culture aliquots stored in glycerol, representing all isolates screened for drug susceptibility, were characterized further by PCR using primers described in Table 1.

DNA extraction

E. coli culture aliquots from storage were recovered by resuspension in 4 ml of nutrient broth and incubated at 37°C for 24 h. From each nutrient broth culture, 1.5ml was centrifuged at 13,000xg for 10 minutes to pellet the cells. The pellets were then washed with a sterile 0.9% sodium chloride solution and ready for DNA extraction using the boiling method (Ahmed and Dablool, 2017). Briefly, the pellets were reconstituted in 300µl of sterile distilled water. Subsequently, the suspensions were heated for 10 minutes at 100 °C. A final centrifugation at 13,000xg for 10 minutes was done to obtain supernatant and discard debris. The concentration and purity of DNA in the supernatant were determined using the NanoDrop One (Thermo Fisher Scientific, USA). The samples with a DNA concentration ≥100ng/µl were stored at -20°C for further analysis by PCR.

Species confirmation by PCR

E. coli species confirmation was done by PCR targeting the 16S rRNA gene on all DNA samples using species-specific primers as described by Yousef et al. (2023). Amplification reactions were done in a total volume of 25µl each. The reaction mixture contained 3µl of DNA template, 400µM each primer, 12.5µl of Taq 2X Master Mix (New England Biolabs), and 7.5µl of nuclease-free water (BioConcept). The thermal cycling (Applied Biosystems, USA) conditions included a denaturation step of 10 minutes at 95°C, followed by a 30s annealing time at a temperature specific for each primer pair, and an extension temperature at 72°C for 1 minute. The reaction was finally extended for 10 minutes at 72°C. Visualization and documentation of the PCR products were done under the Gel. LUMINAX Gel documentation system (BioZen Labs/Zenith).

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Table 1: Primers for Molecular Characterization of *E. coli* isolates

Primer	Gene	Primer sequence 5'-3'	Size (bp)	Ta (°C)
Species specific PCR	*16S rRNA*[a]	F- GACCTCGGTTTAGTTCACAGA	585	56
		R- CACACGCTGACGCTGACCA		
PCR -RFLP	*gyrA*[b]	F- GACCTTGCGAGAGAAATTACAC	540	55
		R- GATGTTGGTTGCCATACCTACG		
MAMA-PCR	*par*C80[b]	F- CGGAAAACGCCTACTTAAACTA	466	55
		R- GTGCCGTTAAGCAAAATGT		
		R- ATCGCTTCATAACAGGCTCT	217	

[a]Yousef *et al*., 2023; [b]Jazeela *et al*., 2019

MAMA-PCR of the topoisomerase IV gene

Mismatch Amplification Mutation Assay Polymerase Chain Reaction (MAMA-PCR) assay was done in a total volume of 25µl to target the *par*C80 mutation site using primers described in Table 1 according to Jazeela *et al*., (2019). Briefly, the reaction mixture contained 3µl of DNA template, 400µM each primer, 12.5µl of Taq 2X Master Mix (New England Biolabs), and 7.5µl of nuclease-free water (BioConcept). The thermal cycling conditions included a denaturation step of 5 minutes at 95°C, followed by 35 cycles of denaturation at 95°C, annealing at 55°C and extension at 72°C, each for 40s. The reaction was finally extended for 10 minutes at 72°C.

PCR-RFLP of DNA gyrA gene of E. coli

PCR amplification was done in a total volume of 25µl using primers described in Table 1, according to Jazeela et al. (2019). Briefly, the reaction mixture contained 3µl of DNA template, 400µM each primer, 12.5µl of Taq 2X Master Mix (New England Biolabs), and 7.5µl of nuclease-free water (BioConcept). The thermal cycling conditions included a denaturation step of 5 minutes at 95°C, followed by a 40s denaturation step at 95°C, a 30s annealing time at 55°C, and an extension of 40s at 72°C for a total of 35 cycles. There was a final extension step of 10min at 72°C.

The obtained *gyr*A PCR products were digested with *Hinf*I (Thermo Fisher Scientific) to screen for point mutations at position Ser-83. Enzyme digestion was performed in a 20µl mixture containing 16µl (0.1–0.5µg) of the PCR product, 0.5µl (2IU) of enzyme, 2µl of 10x buffer, and 1.5l µl of sterile ultrapure water at 370C for 2h, followed by termination of enzyme activity at 650C for 10 min on a thermocycler (Applied Biosystems, USA).

Visualization and documentation of the PCR and restriction products

Visualization and documentation of the PCR products were done under the Gel. LUMINAX Gel documentation system (BioZen Labs/Zenith). Agarose gel electrophoresis was run on 1.5% agarose gels at 100V for 45min and was stained with Safe view™ and classic dye. GeneRuler 1kb Plus DNA Ladder (Thermo Scientific™) was used for sizing PCR and digestion products.

Data analysis

The data were entered into Microsoft Excel; proportions of isolates showing phenotypic resistance and carriage of the respective genetic marker were calculated. Descriptive statistical analysis and charts were constructed.

Results

Prevalence of antibiotic resistant E. coli isolated from broiler chickens

All 160 samples grew colonies that were presumptively identified as *E. coli* by culture and biochemical methods. Following antimicrobial susceptibility testing, the observed rates of phenotypic resistance against ciprofloxacin and norfloxacin were 47.5 and 42.5% respectively. Of the resistant isolates, 5% were resistant only for ciprofloxacin.

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Species confirmation by PCR

DNA from all 160 samples produced an expected PCR product of 585bp for *E. coli* following amplification by *E. coli* specific PCR primers targeting the 16S rRNA region (Fig. 1).

MAMA-PCR of the topoisomerase IV gene

Following MAMA-PCR amplification of all 120 PCR confirmed *E. coli* isolates targeting the topoisomerase IV gene mutation site (*par*C80), two PCR products were observed for each one of them irrespective of their susceptibility profiles. The two products, 466bp and 217bp are only expected for isolates with no mutation at this site. Isolates with mutation at this site only show one product of 466bp (Fig. 2).

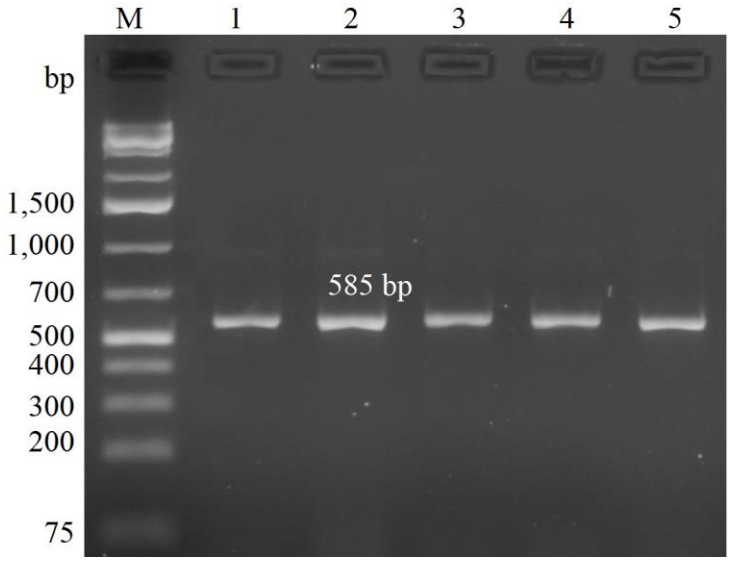

Figure 1: Representative agarose gel electrophoresis image of *E. coli* specific 16S rRNA PCR products. Lane 1-4: All isolates show the expected 585bp product. Lane 5: The *E. coli* ATCC 25922 positive control. Lane M is GeneRuler 1kb Plus DNA Ladder.

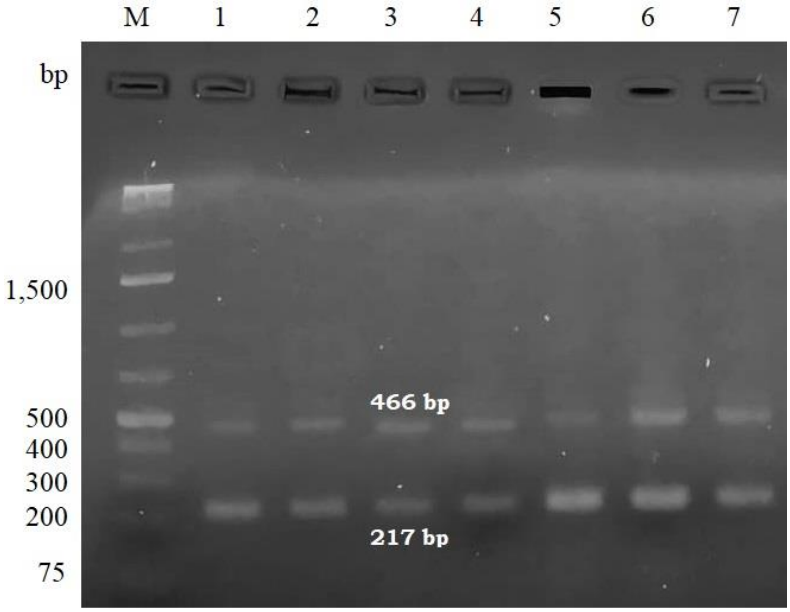

Figure 2: Representative agarose gel electrophoresis image of MAMA-PCR products generated for *par*C80. Lane 1- 7: All isolates showing 466 and 217bp products expected for *E. coli* strains wild-type for this mutation. Lane M is GeneRuler 1kb Plus DNA Ladder.

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PCR-RFLP of DNA gyrA gene of E. Coli

Following PCR amplification of all 120 PCR confirmed *E. coli* isolates targeting the *gyr*A gene, an expected PCR product of 540bp was observed (Fig. 3-A). After *Hinf*I digestion of the PCR products, three digestion products of 239, 202, and 99bp were observed for 57.5% of isolates (Fig. 3-B). This was the expected result for isolates wildtype at the target mutation site (*gyr*A Ser-83). However, 42.5% of isolates showed two digestion products of 337 and 203bp as expected for isolates with a mutation at the target site (Fig. 3-C).

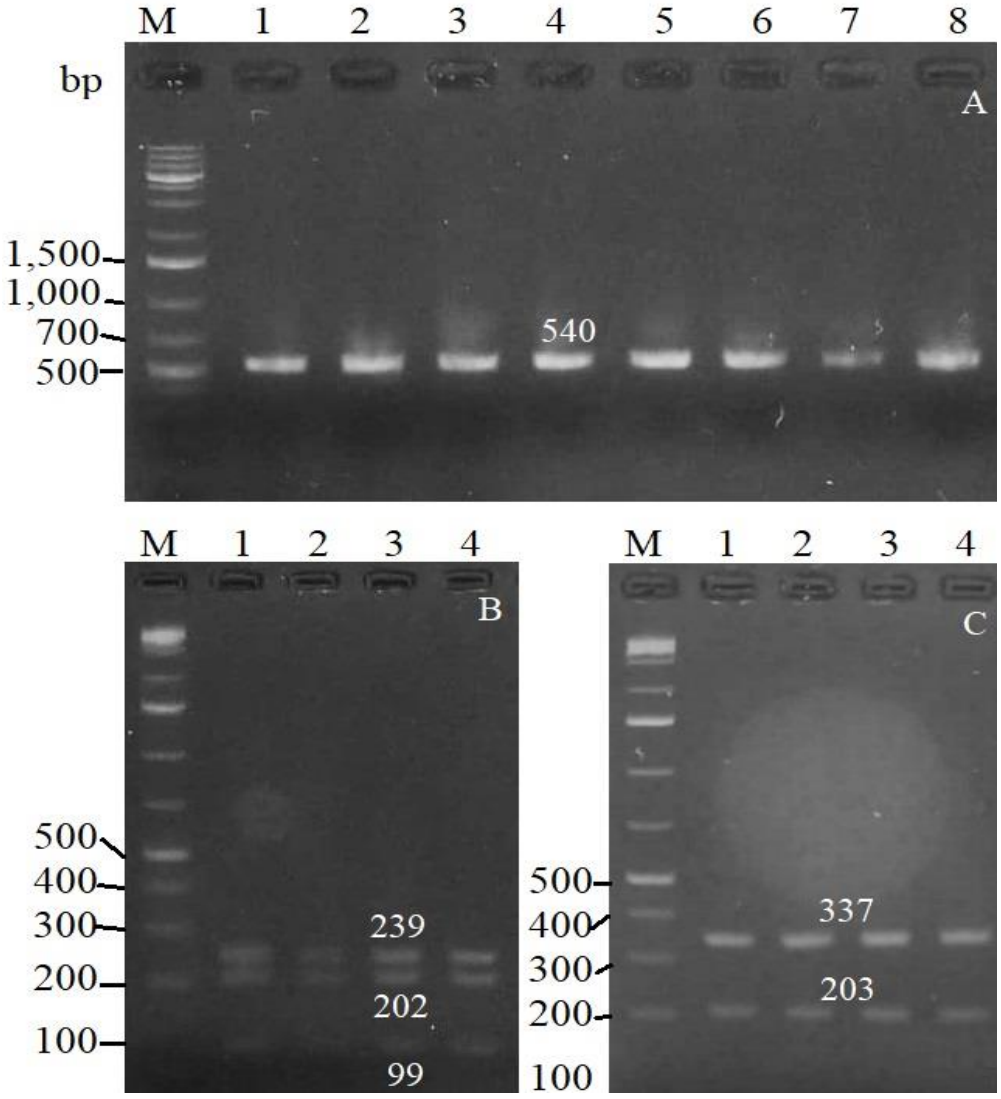

Figure 3: Representative agarose gel electrophoresis images showing results of PCR-RFLP targeting the *gyr*A 83 with mutation. (A) Lane 1-8: isolates showing an expected 540bp PCR product for the targeted region. (B) PCR-RFLP for *gyr*A. Lane 1-4: showing expected *Hinf*I digestion products of *gyr*A (540bp) for isolates wildtype for the targeted *gyr*A 83 mutations. (C) PCR-RFLP for *gyr*A. Lane 1-4: showing expected *Hinf*I digestion products of *gyr*A (540bp) for isolates mutant for the targeted *gyr*A 83 mutations. For A-C: Lane M is the GeneRuler 1kb Plus DNA Ladder.

Genotypic to phenotypic agreement

Comparisons between results from phenotypic susceptibility assays and genetic analysis showed very high concordance for the *gyr*A target mutation. All isolates observed to be phenotypically resistant for both ciprofloxacin and norfloxacin (42.5%) had a mutation at the assayed target site (*gyr*A Ser-83) giving a genotypic to phenotypic ratio of 100% for norfloxacin (Table 2). However, no such mutation was demonstrated for the 5% of isolates that showed phenotypic resistance only against ciprofloxacin, which led to a genotypic to phenotypic ratio for ciprofloxacin resistance to be 89.47%

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(Table 2). In contrast, no isolate, irrespective of the phenotypic resistance profile, was observed to have mutation at the analysed mutation site (*par*C80) on the topoisomerase IV gene (Table 2).

Table 2. Prevalence of genetic resistance markers among isolates under study

Antibiotic	Phenotypic resistance	Target Gene Mutation		Genotypic/Phenotypic agreement
Norfloxacin	68 (42.5%)	*gyr*A *(Ser-83)*	68 (42.5%)	100.00%
		*par*C80	0	0
Ciprofloxacin	76 (47.5%)	*gyr*A *(Ser-83)*	68 (42.5%)	89.47%
		*par*C80	0	0

Discussion

The emergence and spread of antimicrobial resistance pose significant challenges to public health worldwide (Asghar *et al*., 2024; Sartorius *et al*., 2024). In this study, the prevalence and mechanisms of chromosomal-mediated quinolone resistance among *Escherichia coli* isolates obtained from broiler chickens in Dar es Salaam, Tanzania were investigated. The findings from this study were comparable to two recent studies in Dar es Salaam (Kimera *et al*., 2021; Kibwana *et al*., 2023). The Kibwana *et al*., (2023) study associated the observed resistance to chromosomal mutations in *gyr*A, *par*C, and *par*E, whereas the Kimera *et al*., (2021) associated the resistance with plasmid mediated markers at 15% for *aac(6)-lb-cr*, 10% for *qnr*B, and 5% for *qep*A. In addition, studies in Arusha and Mwanza (Tanzania) have also reported ciprofloxacin resistance among *E. coli* isolates from chickens and humans at the rates of 40% and 68.6% (Kiiti *et al*., 2021; Sonola *et al*., 2022). Extending our comparison to East Africa, similar resistance patterns were observed. For instance, a sample-level prevalence for quinolone resistance of 48.3% among food animals was documented in Rwanda (Manishimwe *et al*., 2021). Furthermore in Kenya, rates of resistance against ciprofloxacin as high as 68% were recently reported, and these were mostly associated with PMQR genes at 60% for *aac(6')lb-cr*, 24% for *qnr*B, 22% for *oqx*AB, 16% for *qnr*S and 6% for *qep*A (Kariuki *et al*., 2023).

Expanding the comparison beyond East Africa, similar trends were observed across the continent. In Ghana, 50% and 51.1% resistance rates against ciprofloxacin and 38.8% against norfloxacin among *E. coli* isolates were reported (Deku *et al*., 2022; Sah & Fegio, 2022), while in Nigeria, an even higher rate of resistance (68.2%) was recently reported (Aworh *et al*., 2023). This later study observed PMQR genes in 63.6% of isolates and among isolates with mutations in the quinolone-resistance determining regions (QRDRs), 46.5% had the S83L and D87N substitutions in *gyr*A and S80I substitution in *par*C as the most common mutations. Earlier studies in Nigeria had reported high rates of quinolone resistance and associated the rates with PMQR genes (Ayandiran *et al*., 2018; Eghieye *et al*., 2020; Nsofor *et al*., 2021). Similar findings have also been reported from other countries in Africa, including Cameroon (Mbamyah *et al*., 2020), Egypt (Khalil *et al*., 2017; Kotb *et al*., 2019; Esmaeel *et al*., 2020; Masoud *et al*., 2021), Malawi (Choonara *et al*., 2022), Mozambique (Faife *et al*., 2020), South Africa (McIver *et al*., 2020; Abdalla *et al*., 2021) and Togo (Salah *et al*., 2019).

Numerous studies outside Africa have also extensively explored fluoroquinolone resistance in *Escherichia coli* isolates, uncovering a multifaceted landscape of genetic mutations linked to resistance. Notably, investigations in Australia (Cheng *et al*., 2012), China (Qiu *et al*., 2018), Germany (Juraschek *et al*., 2021), India (Jazeela *et al*., 2019; Dasgupta *et al*., 2018; Varughese *et al*., 2018), Iran (Rezazadeh *et al*., 2016; Lorestani *et al*., 2018; Mirzaii *et al*., 2018; Shenagari *et al*., 2018; Yousefi *et al*., 2018; Hajihasani *et al*., 2022), Japan (Uchida *et al*., 2010), Philippines (Belotindos *et al*., 2021), South Korea (Na *et al*., 2019), Thailand (Onseedaeng & Ratthawongjirakul, 2016) and in the USA (Fuzi & Sokurenko, 2023) have consistently revealed QRDR mutations within *gyr*A and *par*C genes, such as Ser83Leu and Asp87Asn in *gyr*A, as contributing significantly to heightened resistance levels. In addition to elucidating genetic markers of resistance, some studies highlight the effectiveness of policy interventions in curbing resistance rates. For instance, Cheng *et al*., (2012) illustrate how stringent regulations on quinolone use in Australia have led to reduced resistance rates. However, despite these efforts, challenges persist, as

evidenced by findings from Thailand (Boueroy *et al*., 2023) and North Macedonia (Kerluku *et al*., 2023), where high resistance rates are still being reported alongside multiple resistance determinants.

The extensive body of research presented in this discussion underscores the global challenge posed by fluoroquinolone resistance in *Escherichia coli* populations. The prevalence of quinolone-resistant strains, as demonstrated across diverse geographic regions, emphasizes the urgent need for multifaceted strategies to combat antibiotic resistance effectively. Particularly in developing countries with limited healthcare resources, where quinolone-resistant strains often exceed 50%, the challenges for treatment and control are formidable. This study's findings, alongside those from numerous investigations worldwide, provide critical insights into the genetic basis of resistance and transmission pathways, informing strategies for antimicrobial stewardship, infection control with minimal use of antibiotics, and targeted interventions. Urgent action at local, national, and global levels is imperative to address antimicrobial resistance comprehensively, with continued surveillance, research, and evidence-based interventions essential for preserving antimicrobial efficacy and safeguarding public health.

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Conflict of Interest

No conflict of interest

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