Occurrence of Mutidrug Resistant *Escherichia Coli* in Raw Meat and Cloaca Swabs in Poultry Processed in Slaughter Slabs in Dar Es Salaam, Tanzania

Fauster X Mgaya (fauster1973@yahoo.co.in)
Muhimbili University College of Health Sciences: Muhimbili University of Health and Allied Sciences
https://orcid.org/0000-0002-9166-0041
Mecky I Matee
Muhimbili University of Health and Allied Sciences
Amandus P Muhairwa
Sokoine University of Agriculture
Abubakar S Hoza
Sokoine University of Agriculture

Research

Keywords: MDR E. coli, chicken meat, cloaca, Dar es Salaam, Tanzania

Posted Date: November 20th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-111022/v1

Abstract

Background Increased prevalence of multiple drug resistant *Escherichia coli* in chicken meat indicates excessive use of antimicrobials in poultry production systems. We determined the occurrence of multiple drug resistant *E. coli* in raw meat and cloaca swabs in poultry processed in slaughter slabs in Dar es Salaam, Tanzania.

Methods This cross-sectional study was conducted between January and June 2020, in five large slaughter poultry slabs in Dar Es Salaam, Tanzania. Purposive sampling was used to select broilers and spent layers, from which meat and cloaca swabs were collected. MacConkey agar was used to isolate *E. coli* and identification was done using API 20E. Kirby Bauer disc diffusion technique was used in antimicrobial susceptibility testing and results were interpreted as per CLSI (2018) guidelines. EBSL (*CTX- M)* and plasmid mediated quinolone genes *(qnr A, qnr B, qnr S and aac(6')-Ib-cr)* were detected using PCR.

Results Out of the 384 samples, 212 (55.2%) were positive for *E. coli*, of which 147(69.3%) were resistant to multiple drugs (MDR). Isolates were very highly resistant to tetracycline (91.9%), followed by sulfamethoxazole-trimethoprim (80.5%), ampicillin (70.9%), and moderately resistant to ciprofloxacin (40.2%) and 25% cefotaxime (CTX), but had low resistance to 10.8% gentamycin (CN) and 8.6% imipenem (IMP) (95% CI, *P< 0.01*). Ten (10/212) (4.7%) isolates were confirmed as extended spectrum beta lactamase (ESBL) producing *E. coli*, and were all MDR. The MDR *E coli* strains were highly resistant to cefotaxime (100%), ampicillin (90%) and moderately resistant to tetracycline (40%) and sulfamethoxazole-trimethoprim (40%) and ciprofloxacin, lowly resistant to imipenem (10%) but fully susceptible to gentamycin. Quinolones resistant genes were seen in 8 out of the 10 ESBL producers.

Conclusion The high levels of resistance to the commonly used antibiotics in poultry production means these agents are ineffective, add unnecessary cost to farmers and facilitate emergence and spread of resistance. The uncontrolled disposal of waste, seen in all the poultry slabs, has the potential to contaminate the environment with MDR bacteria, with eventual spread to humans and animals and demands intensified surveillance. Advocacy for prudent use of antimicrobials cannot be underscored in poultry production systems, in Tanzania.

Introduction

In Tanzania, the demand of chicken meat was projected to increase from 130,000 tons in 2017 to 465,600 tons in 2020 [1], largely due to increase in urbanization and trade liberation of live animals and products [2]. Dar es Salaam, which is the commercial city of the country, with an estimated population of 4, 364, 541 people, is by far the largest consumer of poultry meat in Tanzania [3].

Poultry farming in Dar es Salaam is done both in urban and peri-urban areas, often in overcrowding and unhygienic conditions [4]. Such conditions are frequently associated with occurrence of diseases and use of excessive antimicrobials. Several studies conducted in Tanzania have shown both over use of antibiotics and high level of resistant organisms in poultry production systems [2, 5, 7]. Antibiotics are commonly used for disease prevention and therapeutic purposes in poultry production systems are obtained over the counter and are administered without advice of veterinary officers [6]. The knowledge of most poultry keepers on prudent use of antibiotics and their effect is low, antimicrobial prescribers and unregistered veterinary drug dealers also have little prescription knowledge, which all together create an environment for emergence and spread of antimicrobial resistance [5]. Metaphylaxis is also very common among poultry keepers [8], exposing even health chicken to unnecessary antimicrobials.

We conducted this study in Dar es Salaam, where the demand for poultry meat and products is highest in the country and the use of antimicrobials among poultry keepers is very high [5, 15]. We aimed at determining the occurrence of multidrug resistant *E. coli* in raw chicken meat and in cloaca, including the occurrence of extended spectrum beta lactamase specifically *CTX-M* and plasmid mediated quinolone resistant genes (*qnrA, qnrB, qnrS* and *aac (6')-Ib-cr*).

Materials And Methods

Study Area

The study was conducted in Dar es Salaam, the commercial city of Tanzania, which has a population of 4 364 541 people [3], with highest production and consumption of chicken meat and eggs in Tanzania. The study involved five large poultry slabs in four Districts (Ilala, Ubungo, Temeke and Kinondoni). Approximately 20,000 chicken are slaughtered daily in these five poultry slaughter slabs, which provides about 80% of chicken consumed in Dar es Salaam.

Study Design

This was a cross sectional study conducted between January to June 2020 in four districts, which have largest poultry slabs in Dar es Salaam. The slabs were; Manzese, and Shekilango in Ubungo district, Kisutu in Ilala district, Mtambani in Kinondoni district and Stereo in Temeke district. In this study we targeted broilers and spent layers because they are raised intensively in overcrowded environment, and use of antimicrobials for prophylaxis, growth promotion and in management of infections is very high. Other types of poultry such as indigenous chicken was excluded from the study.

Sampling technique

Using purposive sampling technique, we selected 96 broilers and 96 spent layers, making total of 192 chicken in all the five poultry slabs. Two samples (i.e. cloaca and meat swab) were collected from each chicken, making a total of 384 samples. Cloaca swabs were collected before chicken were slaughtered (at the entry point), while chicken meat swabs were collected after slaughtered (at the poultry slabs).

Specimen collection

Chicken meat and cloaca swabs were collected aseptically using sterile cotton swabs and placed into a sterile tube containing 5 ml of Cary Blair transport medium (Oxoid, Basingstoke, UK). The collected samples were transported in a cool box at 2 to 8 ^{o}C containing a thermometer and processed within 2 hours of collection in the Microbiology Teaching Laboratory of the Muhimbili University and Allied Sciences (MUHAS).

Isolation and identification of enterobacteria

In the laboratory, swabs were inoculated onto the MacConkey agar (Oxoid Basingstoke, UK) without antibiotics and incubated aerobically at 37ºc for 24 hours. Identification of *E. coli* was using colonial morphology, lactose fermentation and Gram stain. Lactose fermenters were subjected to conventional phenotypical identification using a set of biochemical tests including Triple Sugar Iron Agar (TSI), Sulphur indole Motility (SIM) agar and citrate utilization test. Confirmation was done using API 20E identification system for Enterobacteriaceae according to the instructions of the manufacturer (BioMérieux, Marcy-Etoile, France).

Screening and confirmation of ESBL production

Confirmed *E. coli* were inoculated onto MacConkey agar containing 2 mg/l cefotaxime for preliminary screening of ESBL production. ESBL producers were confirmed using a combination disk method of cefotaxime 30µg alone; combination with clavulanic acid (10 µg) and ceftazidime 30µg alone and combination with clavulanic acid 10 µg. The difference of inhibition zone of more than or equal to 5mm were confirmed as ESBL positive [9]. *Klebsiella pneumoniae* ATCC 700603 was used as a positive control and *E. coli* ATCC 25922 as a negative strain.

Antimicrobial susceptibility testing

The antimicrobial susceptibility testing was done using the Kirby-Bauer disc diffusion method on Mueller Hinton Agar (Oxoid, Basingstoke, UK) based on CLSI 2019 guidelines [9]. Seven antibiotics from different classes were used, which included, ampicillin (10µg), tetracycline (30µg), gentamycin (10µg), ciprofloxacin (5µg), imipenem (10µg), sulfamethoxazole-trimethoprim (1.25/23.5µg), and cefotaxime 30 µg [9].

Colonies of lactose fermenters identified as *E. coli* were emulsified into sterile saline to achieve turbidity equivalent to 0.5 McFarland standard, which is equivalent to 10^8 cfu/ml [10]. Suspensions were spread onto Muller Hinton Agar (MHA) using sterile cotton swabs and incubated aerobically at 37 °C for 16 to 18 hours. The inhibition zone of each antimicrobial agent was measured after 16 to 18-hours of incubation and results were interpreted according to the 2018 CLSI guidelines [9]. *E. coli* strain ATCC 29522 was used as a control strain. A strain was referred to be multidrug resistance (MDR) if it exhibited resistance to at least three different antimicrobial classes [10].

DNA Extraction and Polymerase Chain Reaction (PCR)

ESBL producing *E. coli* isolates were inoculated on nutrient agar and incubated aerobically at 37 ^{o}C for 24 hours. DNA was extracted by boiling in a water bath at 100^{o}C for 10 minutes, followed by centrifugation at 1500 rpm for 3 minutes. The supernatant containing DNA was transferred into sterile Eppendorf PCR tube (Eppendorf AG, Hamburg, Germany) and centrifugation and separation of supernatant were repeated three times. The concentration of DNA was determined by Nano drop spectrophotometer (Biochrom LTD, Cambridge, England) at 260/280 and 260/230 wavelength. DNA were stored at -20^{o}C, before being used for detection of *CTX- M* and PMQR genes *(qnr A, qnr B, qnr S and aac(6')-Ib-cr).*

The Dream Tag DNA polymerase kit (Thermo Fisher scientific, US) was used in detection of resistance genes. Total PCR reaction volumes were 25µL, consisting of 10X dream Tag Buffer 5 µL, dNTP 2mM 5 µL, forward and reverse primers were 1 µL each, DNA extract were 2 µL, Dream Tag DNA Polymerase (1.25 U) 1 µL and nuclease free water 10 µL. The primers used in amplification of respective *E. coli* resistance genes are listed in Table 6.

Molecular detection of *CTX -M* genes

All ESBL producing *E. coli* isolates were screened for the *CTX-M* gene using Uniplex PCR based technique [11]. The process involved initial denaturation at 98 ^{o}C for 3 minutes, followed by 35 cycles of denaturation at 98 ^{o}C for 15 seconds, annealing at 60 ^{o}C for 5 seconds, extension at 72 ^{o}C for 45 seconds and final extension at 72 ^{o}C for 5 minutes (11).

Detection of PMQR genes (*qnr A, qnr B* and *qnr S*)

PMQR resistant genes (*qnr A, qnr B* and *qnr S*) were amplified and detected using multiplex PCR assay [12]. The process involved initial denaturation at 94 ^{o}C for 5 minutes, followed by 32 cycles of denaturation at 94 ^{o}C for 45 seconds, annealing at 53 ^{o}C for 1 minute, extension at 72 ^{o}C for 1 minute and final extension at 72 ^{o}C for 10 minutes (12).

Detection of *aac (6')-Ib-cr gene.*

Aac (6')–Ib-cr gene were screened by uniplex PCR based assay [11], using the following the amplification conditions: initial denaturation at 94 ^{o}C for 5 minutes, followed by 34 cycles of denaturation at 94 ^{o}C for 45 seconds, annealing at 55 ^{o}C for 45 seconds, extension at 72 ^{o}C for 45 seconds and final extension at 72 ^{o}C for 10 minutes [11]. The amplified PCR products were viewed with gel red stain during electrophoresis through 1.5% agarose gel at 100 voltages for 35 minutes.

Data Analysis

The data were entered into Microsoft Excel; proportions were analyzed by *Chi-square test*. A paired *t-test* assuming unequal variance was used for comparing overall prevalence and comparing resistance rate among tested antibiotics in SPSS version16 software. A P- value (< 0.05) was considered to be statistically significant.

Results

Prevalence of *E. coli* in raw chicken meat and cloaca in broiler and spent layers

A total of 384 chicken meat and cloaca swabs samples were collected in the five selected poultry slabs in Dar es Salaam. Out of which, 212 (55.2%) were positive for *E. coli.* Of the isolated strains, 147 (69.3%) were resistant to more than three tested antibiotics of different classes. The slab with the highest proportion of MDR isolates was at Stereo in Temeke District18/19 (94.7%), followed by Shekilango in Ubungo district 37/43 (86%), Manzese in Ubungo district 28/40 (70%), Mtambani in Kinondoni district 14/20(70%) and Kisutu in Ilala District 50/90 (55.6%) (Table 1).

Table 1: Frequency of MDR and none MDR *Escherichia coli* isolated from the selected Poultry slabs in Dar es Salaam (n= 384)

Poultry Slabs	MDR		Not MDR	
	N	**%**	**N**	**%**
Stereo	18	94.7	1	5.3
Manzese	28	70.0	12	30.0
Mtambani	14	70.0	6	30.0
Shekilango	37	86.0	6	14.0
Kisutu	50	55.6	40	44.4
Total	**147**	**69.3**	**65**	**30.7**

MDR- multidrug resistant

Antibiotic resistance rates in *E. coli* isolates

Overall, the highest resistance was detected in Tetracycline (91.9%), followed by Trimethoprim-Sulfamethoxazole (80.5%), Ampicillin (70.9%), Ciprofloxacin (40.2%), Cefotaxime (22.5%), 10.8% Gentamycin (10.8%) and Imipenem (3.3%) (Table 2).

Table 2: Antibiotic resistance pattern of *E. coli* isolates in five poultry slabs (n = 212)

Poultry slabs	Antibiotics tested													
	TE (n)	**%**	**CN (n)**	**%**	**CIP (n)**	**%**	**IMP (n)**	**%**	**SXT (n)**	**%**	**CTX (n)**	**%**	**AMP (n)**	**%**
Stereo	18	95.0	3	16.7	9	46.7	3	16.7	17	89.4	19	100.0	22	100.0
Manzese	38	95.7	3	7.3	25	63.7	0	0.0	34	84.7	0	0.0	24	62.9
Mtambani	18	88.1	0	0.0	7	25.0	0	0.0	15	82.1	0	0.0	14	73.8
Shekilango	42	97.5	5	22.4	16	36.1	0	0.0	37	86.3	5	12.5	30	70.1
Kisutu	75	83.1	7	7.4	29	29.7	0	0.0	56	59.9	0	0.0	45	47.9
Total	**191**	**91.9**	**18**	**10.8**	**86**	**40.2**	**3**	**3.34**	**159**	**80.5**	**24**	**22.5**	**135**	**70.9**

TE=Tetracycline, CN=Gentamycin, CIP=Ciprofloxacin. IMP=Imipenem, SXT=Trimethoprim-sulfamethoxazole, CTX=Cefotaxime, AMP=Ampicillin

Of the 147 MDR *E. coli* isolates isolated in this study, 49% showed resistance to at least three classes of antibiotics, 33.3% to at least four classes, 14.3% resistant to five classes, 2.7% resistant to six classes and one isolate (0.7%) was resistant to all seven tested antibiotics (Table 3, refer table 6 at the end).

Table 3: Classes of antimicrobial patterns resisted n (%)

MDR *E. coli* isolates	Classes of antibiotics				
	3	4	5	6	7
147	72 (49)	49 (33.3)	21(14.3)	4(2.7)	1(0.7)

As shown in Table 4, the isolation of MDR E coli was higher in cloaca than chicken in both types of chicken.

Table 4: MDR *E. coli* by location of poultry slabs and chicken category

Poultry slabs	Meat Br		Cloaca Br		Meat SL		Cloaca SL		Total
	N	%	N	%	N	%	n	%	n
Stereo	2	11.1	7	38.9	4	22.2	5	27.8	**18**
Manzese	6	21.4	7	25.0	6	21.4	9	32.1	**28**
Mtambani	3	21.4	1	7.1	2	14.3	8	57.1	**14**
Shekilango	6	16.2	10	27.0	10	27.0	11	29.7	**37**
Kisutu	16	32.0	22	44.0	8	16.0	4	8.0	**50**
Total	**33**	**22.4**	**47**	**32.0**	**30**	**20.4**	**37**	**25.2**	**147**

Meat Br =broiler meat, Cloaca Br=broiler cloaca, Meat SL=spent layers' meat, Cloaca SL=Spent layers' cloaca

Extended Spectrum Beta Lactamase (ESBL) producing *E. coli*

Out of 212 identified *E. coli*, ten (4.7%) isolates were screened and confirmed to be ESBL producing *E. coli.* All 10 isolates were found to be MDR. All (100%) the ESBL producers were resistant to Cefotaxime and Ampicillin, 90% were resistant to Tetracycline and Trimethoprim-Sulfamethoxazole, 40% were resistant to Ciprofloxacin and 10% were resistant to Imipenem. However, all ten (100%) *E. coli* isolates were susceptible to Gentamycin. All confirmed ESBL producing *E. coli* were isolated from one Poultry slab at Stereo in Temeke district, and were mostly from spent layers.

Detection of *CTX- M* and PMQR genes (*qnrA, qnrB, qnrS and aac (6')-Ib-cr)*

Plasmid mediated quinolones resistant genes were detected in 8/10 ESBL producing *E. coli* either as single genes or in combination with *CTX-M*. The *qnr S* were present in eight (80%) of the isolates isolated from four spent layers' meat, two spent layers' cloaca, one broiler meat and one cloaca of broilers. PMQR determinants *qnrA*, *qnrB* and *aac (6')-Ib-cr* were not be detected in any of the *E. coli* isolates tested (Table 5, Fig.1 and Fig.2).

Table 5: Presence of *CTX- M* and PMQR encoding genes by PCR (n = 10)

Detected genes	*E. coli* No (%)	Spent layers meat	Spent layers cloaca	Broiler meat	Broiler cloaca
CTX- M	2/10 (20)	1	1	0	0
Qnr A	0/10 (0.0)	0	0	0	0
Qnr B	0/10 (0.0)	0	0	0	0
Qnr S	8/10 (80)	4	2	1	1
Aac (6')-Ib-cr	0 (0.0)	0	0	0	0
Total	**10/10(100)**	**5**	**3**	**1**	**1**

Table 6
Antimicrobial resistance pattern of *E. coli*

Antibiotic Combination	Number of Isolates	%	Number of antibiotics
TE, CN, CIP	1	0.7	3
TE, CN, SXT	1	0.7	3
TE, CIP, SXT	17	11.6	3
TE, CN, AMP	1	0.7	3
TE, IMP, SXT	3	2.0	3
TE, CIP, AMP	8	5.4	3
TE, CN, CIP, SXT	3	2.0	4
TE, IMP, AMP	1	0.7	3
TE, SXT, AMP	39	26.5	3
TE, CIP, IMP, SXT	1	0.7	4
CIP, SXT, AMP	1	0.7	3
TE, CN, SXT, AMP	4	2.7	4
TE, CIP, IMP, AMP	1	0.7	4
TE, CIP, SXT, AMP	29	19.7	4
TE, IMP, SXT, AMP	1	0.7	4
TE, CN, CIP, IMP, AMP	1	0.7	5
TE, IMP, CTX, AMP	1	0.7	4
TE, CN, CIP, SXT, AMP	4	2.7	5
TE, SXT, CTX, AMP	9	6.1	4
TE, CIP, IMP, SXT, AMP	3	2.0	5
TE, CN, SXT, CTX, AMP	3	2.0	5
TE, CIP, SXT, CTX, AMP	9	6.1	5
TE, CN, CIP, IMP, SXT, AMP	2	1.4	6
TE, IMP, SXT, CTX, AMP	1	0.7	5
TE, CN, CIP, SXT, CTX, AMP	2	1.4	6
TE, CN, CIP, IMP, SXT, CTX, AMP	1	0.7	7

TE = tetracycline, AMP = ampicillin, SXT = sulfamethoxazole-trimethoprim, CIP = ciprofloxacin, CTX = cefotaxime, CN = gentamycin, IMP = iminepem.

Discussion

The findings of this study indicate that the overall frequency of *E. coli* isolated from both meat and cloaca swabs of broilers and spent layers was 55.2% (212/384), and more than two-third of the isolates (69.3%) were multidrug resistance strains.

We found no significant differences in MDR *E. coli* between broilers and spent layers, even though broilers are raised in a relatively short period (four to six weeks) compared with spent layers (up to two years). This could be explained by the fact that antibiotics are used more intensely for growth promotion and prophylaxis in raising broilers than in spent layers [13]. For both types of chicken, cloaca had higher isolation frequency of MDR *E. coli* (25.2–32%) than in meat samples (20.4–22.4%), a trend that has also been observed in ducks' fecal samples [14]. which indicates the epidemiological significant of chicken droppings in contaminating the environment, and acting as a potential driver of AMR spread [15]. We found significant difference in antimicrobial resistance rates between poultry slabs, indicating possible contribution of the slaughtering environment in contaminating poultry meat with MDR bacteria, which has been cited to be a factor [16–18]. %), in fact, all ESBL producing *E. coli* were isolated from one Poultry slab. However, in this study we did not investigate the sources of contamination.

Isolates were highly resistant to Tetracycline (91.9%), Sulfamethoxazole-Trimethoprim (80.5%), Ampicillin (70.9%), which are the commonly used antibiotics compared to ciprofloxacin, cefotaxime, gentamycin and imipenem, which are less used due to cost [19]. Previous studies conducted in Tanzania have also shown high resistance rates to ampicillin, tetracycline (75.8%) and sulfamethoxazole-trimethoprim (62.3%) [7, 20–21], even in *Campylobacter* species isolated from poultry and other animal products [22–23]. Most of the resisted antibiotics are relatively and easily accessible over the counters and administered without proper diagnosis or dosage [24–27] and often farmers not following withdraw period [4, 5].

In this study, ESBL producing *E. coli* were detected in 10/212 (4.7%) and quinolone resistance genes in 80% of them, supporting observation of several studies that have found a strong association between qnr-positive and ESBL-positive isolates [28–31]. However, unlike a study done in Niger that showed several qnr genes (qnrA, qnrB and qnrS) [32], we only found qnrS. The finding of quinolone resistance, which can rapidly spread along the food chain and in other ecosystems through plasmids [33–35] is significant and need to be highly strictly and if possible, banned from use in animal food production. Tanzania has a number of acts and policies that are intended to control the quality of livestock production. Unfortunately, The Meat Industry Act 2006 that gives a legal backing to support meat inspection to ensure quality does not explicitly address issues of drug residues in meat and meat products. Equally, the National Livestock Policy of 2006 and the National Agriculture Policy of 2013 do not address issues of antimicrobial use (AMU) and antimicrobial resistance (AMR) in livestock and agriculture sectors, respectively.

Of environmental concern, we found improper handling of chicken carcasses and unregulated waste disposal from slaughter poultry slabs including blood, faeces and wastewater disposed into Municipal drains without either monitoring or treatment. Unfortunately, the National Environmental Policy 1997 that is supposed to ensure food security through the promotion of production systems that are environmentally sound does not address the issues of environmental contamination with antimicrobials. Likewise, The Animal Diseases Act, 2003 that make provisions for monitoring of production of animal products, for disposal of animal carcasses is silent on issues related to antimicrobials.

We strongly suggest the existing Acts and Policies, some of which are more than ten years, be critically reviewed by stakeholders from human health, veterinary and environment sectors in order to curb AMU and AMR in livestock production and for protection of humans and the environment. The revised Acts and policies should be reinforced legislation. We also advocating for judicious use of antimicrobials in poultry, through improved hygiene, vaccinations and provision of extensive farmers education.

Conclusion

The high levels of antimicrobial resistance revealed by this study pose a major public health threat to both human and animals as well as the danger of contamination of the environment. We recommend improved animal biosecurity and vaccinations in poultry production at the farm level to reduce the incidence of infections and the need for antimicrobial agents and improved hygiene practices at poultry slaughter. The present acts and policies governing animal food production should be revised to provide legislation for judicious use of antimicrobials.

Abbreviations

MDR multiple drug resistance, ESBL extended spectrum beta lactamase, PMQR plasmid mediated quinolones resistance, AMU antimicrobial use, AMR antimicrobial resistance, PCR polymerase chain reaction

Declarations

Ethics approval and consent to participate

The permit to conduct this study was approved by Sokoine University of Agriculture Ethical Review Committee with reference number: SUA/DPRTC/R/186 VOL.III, dated 2/12/2019.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request

Competing interests

The authors declare no conflicts of interest.

Funding

The study was supported by the grants from the Government of Tanzania in collaboration with the World Bank to the SACIDS foundation for One Health, Sokoine University of Agriculture, Tanzania.

Authors' contributions

FXM: Designed the study, data collection, data analysis, interpretation of data, generated the first draft and wrote the Manuscript.

APM: Reviewed the first draft and final draft of manuscript.

MIM: Designed the study, outlines of manuscript, reviewed the first and final draft of manuscript.

ASH: Designed outline for data analysis, reviewed the first draft to final work and provided the relevant editing.

All Authors read and approved the manuscript.

Acknowledgements

I would like to thank you the Dar es Salaam regional and district authorities including the Regional Administrative Secretary, District Executive Directors, District Veterinary Officers for their permission to conduct the study. I extend thanks to Microbiology and Immunology Department at Muhimbili University of Health and Allied Sciences and all staff members in the Department for their supports, we also thank you Eleonora Haule and Miriam Makange for their technical support. The study was financially supported by Government of Tanzania in collaboration with the World Bank to the SACIDS foundation for One Health.

Authors' information

FXM: MSc Student in one Health molecular Biology

MIM: MD, PhD, and Professor of Clinical Microbiology

APM: BVM, PhD and Professor of veterinary Medicine

ASH: BVM, PhD, Senior Lecturer in Medical Microbiology and President of Academic Staff Association.

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Figures

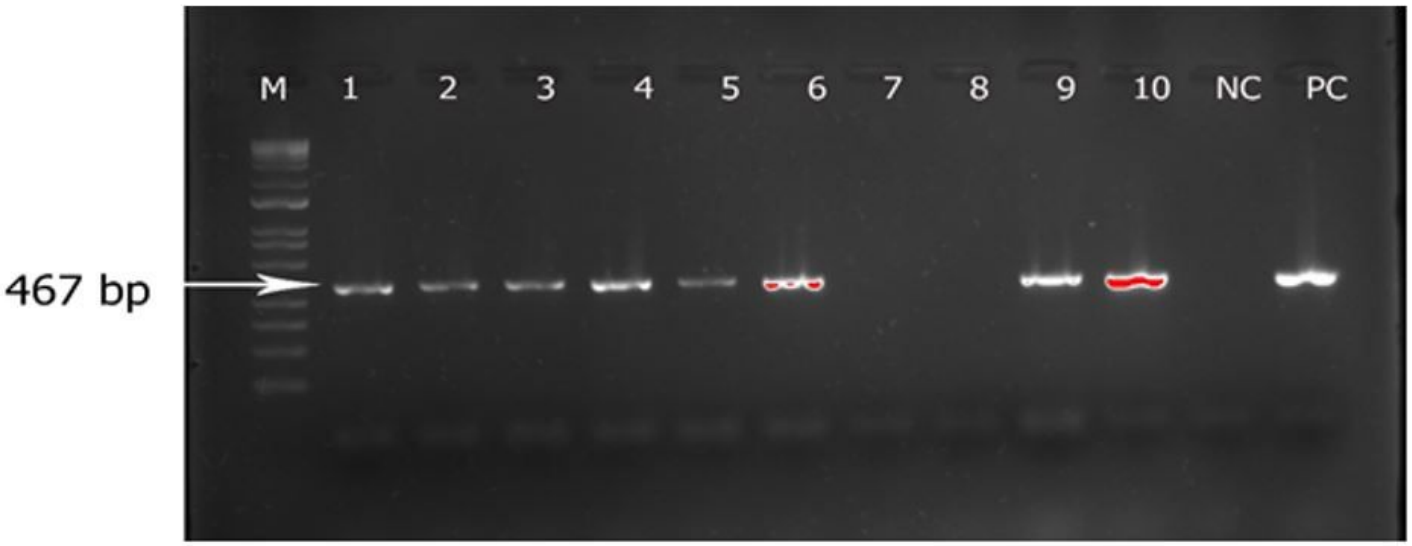
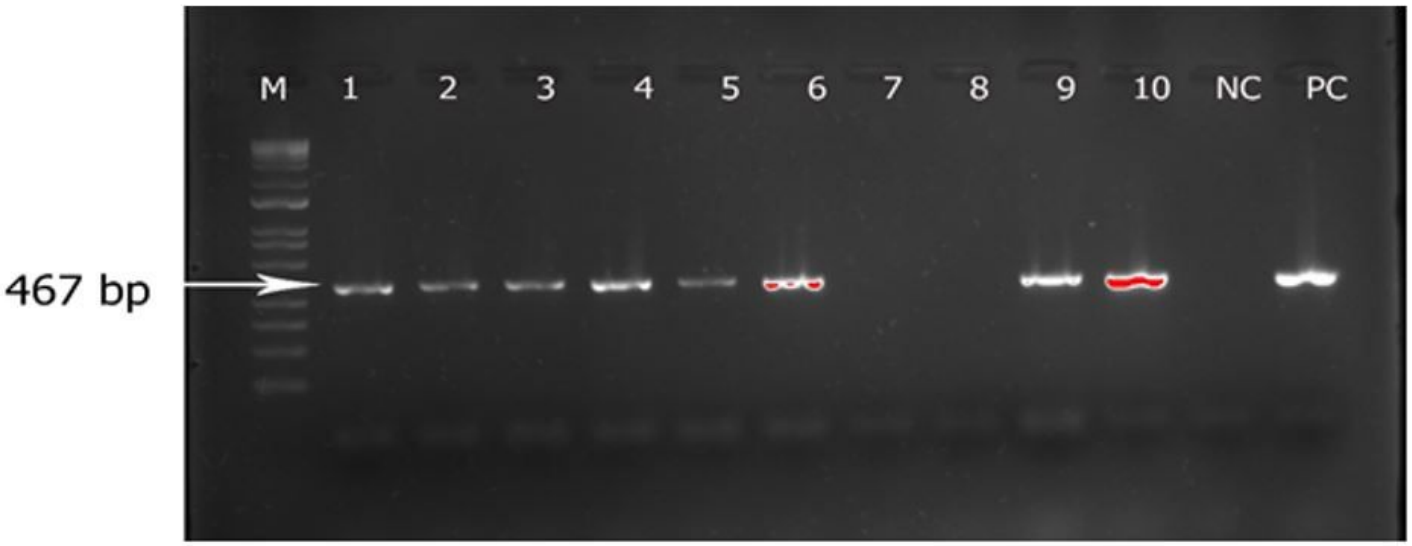


Figure 1

Shows amplified qnrS gene in sample 1- 6, 9 and 10, M- 1 kb Ladder, NC- Negative control, PC- Positive control



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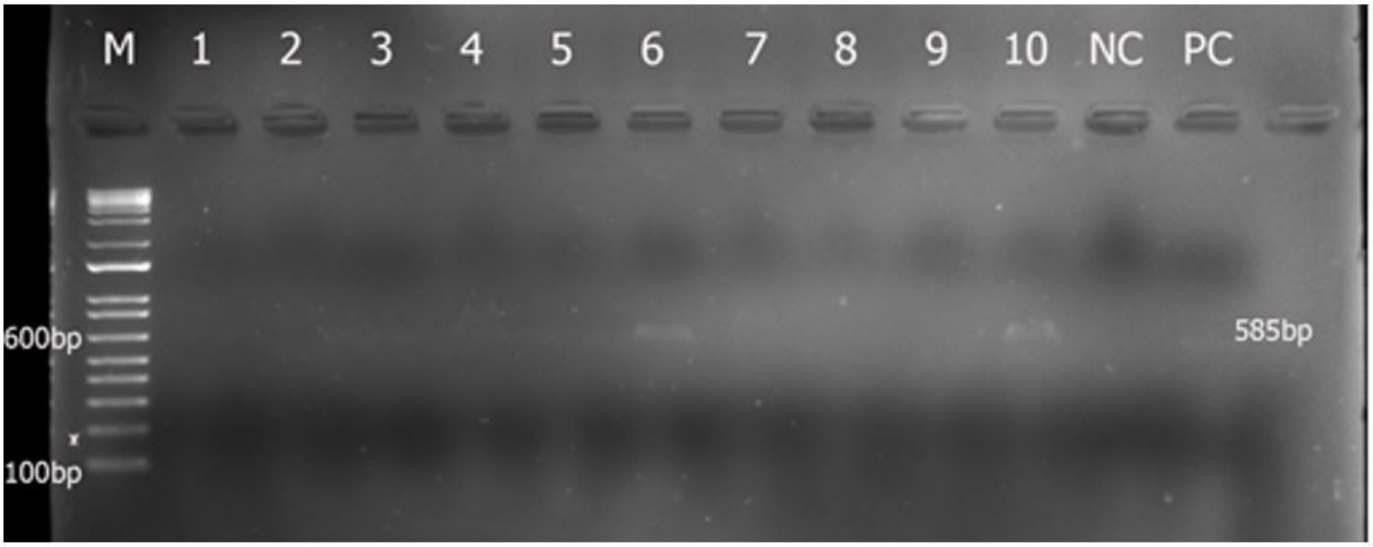

Figure 2

Shows amplified CTX- M in sample 6 and 10, M- 1kb Ladder, NC- Negative control and PC- Positive control

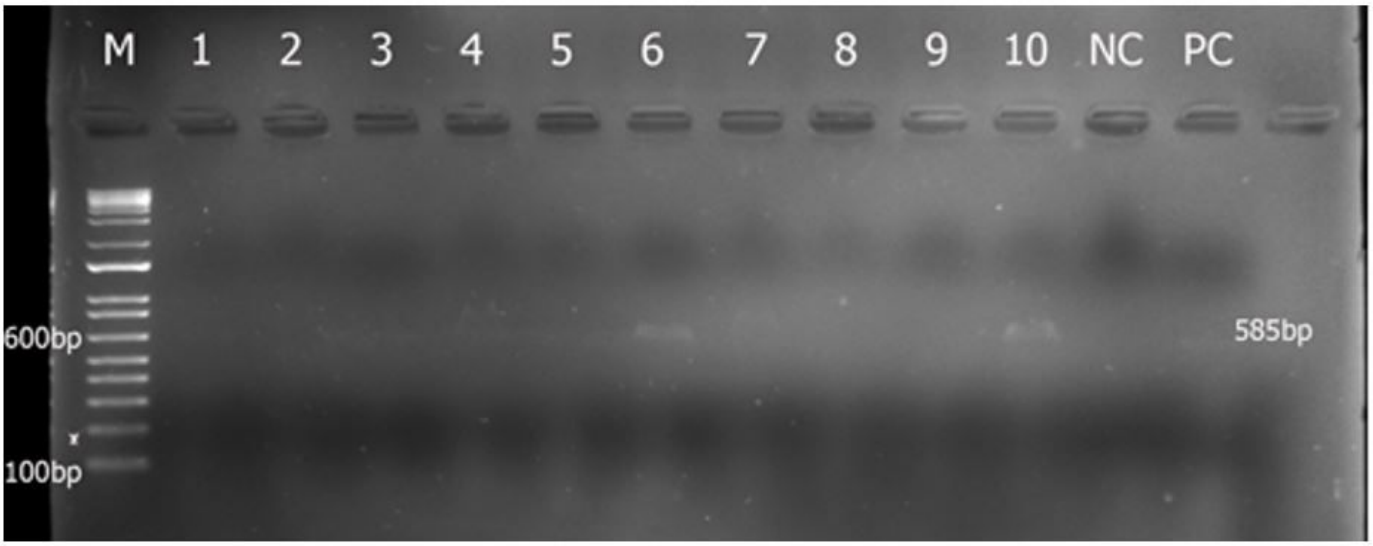

Figure 2

Shows amplified CTX- M in sample 6 and 10, M- 1kb Ladder, NC- Negative control and PC- Positive control