Multiple resistance in clinical isolates of *Escherichia cColi* and plasmid-mediated Quinolone resistancegenes

Abstract

Quinolone antibiotics have been ~~the most~~ commonly used to treat cases of multiple antibiotic resistance. Unfortunately, quinolone antibiotics have so much been resisted by infectious bacterial agents. This study aimeds to evaluate the susceptibility of some clinical isolates of *E. coli* to some commonly used quinolone antibiotics andthe determination of the plasmid-encoded quinolone resistancegenes.

Our results showed the plasmid quinolone-resistancet genes in the following prevalence: Qnr genes: Qnr S (71.4 %); Qnr B (15.4 %); Qnr S and B (12.1 %); aac (6) lb-cr (4 %); Efflux genes: OqxA (7.7 %); OqxB (25.3 %); QepA (12.1 %); OqxA and OqxB (5.5 %). We conclude thatthereis a high frequency of Plasmid-mediated quinolone resistancegenes in *Escherichia coli* isolates from clinical samples in South-Eastern Nigeria. These could be responsible for thea high incidence of quinolone resistance reportedin Enugu. There is a need for whole-genome sequencing to map out all resistance genes.

Keywords: *Escherichia coli;* Quinolone antibiotics; plasmid; antibiotics; Quinolone genes

1. Introduction

Resistance of infectious microorganisms have been a great concern to all globally.[1] Antibiotic resistance occurs when any drug meant to hinder bacterial growth or kill the bacteria fail to exhibit any lethal action on the microorganism or hinder its growth, such that the bacteria grow and multiply in the presence of that drug.[2]The first serious consequence of antibiotic resistance in medical therapy was the gross dissemination of strains of *Staphylococcus aureus*, which were resistant to penicillin, described as penicillin-resistant *Staphylococcus aureus*.[3] Currently, Multi-Drug Resistance(MDR) has become major public health concern all over the world.[1, 4]Among the present-day superbugs are the multidrug-resistant *Escherichia coli (E. coli).*

Quinolones are a family of synthetic broad-spectrum antibiotics, which are drugs of choice commonly used to treat bacterial infections worldwide.[5, 6]Resistance to the first-generation

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quinolones led to the manufacture and introduction of stronger members of the quinolones, otherwise known as the fluoroquinolones.[7, 8]

Many studies have attributed the frequency of quinolone resistance tocarriageof p~~P~~lasmid, which has been found to contain genes for quinolone resistance.[9, 10] The Plasmid-mediated quinolone resistance includes the Qnr genes, majorly *qnrA*,*qnr* B and *qnr* S; the aminoglycoside acetyltransferase gene (aac-6'-Ib-cr) and specific efflux pumps' genes, QepA, OqxA and OqxB.[11, 12]. Owing to the high resistance of *E. coli* to the quinolones, there is an urgent need to find out how *Escherichia coli* is resisting these drugs. This will enable further research for modifications on the drugs.

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In this work, clinical isolates of *E. coli* were screened for quinolone resistance, after which the plasmid-mediated quinolone resistance was investigated. The resistance genes mediated by p~~P~~lasmids may be of epidemiologic importance in the rapid~~fast~~ spread of antibiotic resistance even to bacteria of other species and consequently, elevate antibiotic resistance.

2. Materials and methods

2.1 Study Population and Sampling method

A total of 274 isolates of presumptively identified *E. coli*, were collected from 13 Medical Laboratories/Diagnostic Centers in Enugu State; and from 10 (%) different clinical samples of patients. Notclear?The clinical sample sources of the isolates include aspiratesource?, catheter tip, ear swab, Endocervical swab (ECS), High Vaginal Swab (HVS), semen, stool from infants, throat Swab, urine and wound swab. A convenience method of non-probability sampling was employed.

2.2 Identification and Characterization of the *E. coli* Isolates

For quality control, a competent *E. coli*, American Type Culture Collection (ATCC) 25922, imported and stored at -80 ^{o}C was included in all the test treatments. *E. coli* Chromagar medium (Bio Connections, UK), was prepared and used according to the manufacturer's instructions. A small amount(what is asmall amount?)of the *E. coli* isolate from Brain Heart Infusion agar storage or directly from MacConkey or Blood agar medium was aseptically picked and streaked on Chromagar to enable the growth of pure colonies. This was incubated at 37 ^{o}C for 24 h. The colonies of *E. coli* would appear dark-blue while the non-*E. coli* (other coliforms) would appear colorless/ white. A pure colony identifiable as *E. coli* was streaked on Mueller- Hinton agar MHA (Oxoid) plate for luxuriant colonies. Further identification?

2.3 Antimicrobial Sensitivity Tests

Confirmed *E. coli* isolates(not stated howthey were confirmed) were tested for quinolone resistance using the disk diffusion method for carrying out the cultivation and sensitivity test of the *Enterobacteriaceae* as shown in Fig. 1. The standard guidelines employed were those of the Clinical and Laboratory Standards Institute (2019), and that of the British Society for Antimicrobial Chemotherapy (BSAC), as highlighted by Howe and Andrews (2012).[13] The bacterial colony was inoculated in 5ml sterile normal saline and adjusted to a 0.5 McFarland Standard. A sterile cotton wool swab was dipped in the prepared bacterial suspension~~olution~~ and used to streak the surface of Mueller Hinton Agar plates, ensuring the entire smooth uniform coverage of the plates. Filter paper disks impregnated with known concentrations of the antibiotics: Ciprofloxacin (10 ug), Ofloxacin (30 ug), Pefloxacin (10 ug), Sparfloxacin (10 ug), Moxifloxacin (5 ug) and Levofloxacin (20ug) obtained commercially (Maxi-care) were used and deposited on the agar plate surface. The plates were incubated at 37 ^{o}C for 24 h. The result was interpreted using BSAC standard for Quinolone antibiotic breakpoint for Enterobacteriaceae using zone of inhibition.[13]

Fig. 1: Sensitivity pattern of the *E. coli* isolates(quality of plates are poor)

2.4 Extraction of Plasmid DNA

Extraction of Plasmid DNA from the isolates was done using Zymo Research Plasmid Miniprep *Classic* kit (ZR, USA). The plasmid wash buffer was constituted by adding ethanol (100%) to the supplied buffer. 0.5 ml of bacterial culture (bacterial colony emulsified in Tris EDTA buffer pH 8.0) stored in a clear 1.5 ml tube was centrifuged at full speed for 20 seconds in a micro-centrifuge. 200 µl of P1Buffer was added to the tube and the pellet was re-suspended~~resuspended~~ completely (by vortexing). Then 200 µl of P2 Buffer was added and the

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contents mixed by inverting the tube 4 times, without vortexing, and then incubated for 2 minutes to ensure complete lysis of the cells. 400 µl of P3 Buffer was added and contents mixed gently but thoroughly, without vortexing. The sample turned yellow, indicating complete neutralization. The lysate was then allowed to incubate at room temperature for 2 mins and then centrifuged for 2 mins at 15,000 rpm.

3. Results

3.1 Escherichia coli Isolates

Table 1 shows the sample types and the corresponding numbers of confirmed *E. coli* isolates. A total of 274 suspected *E. coli* isolates were collected from various medical laboratories. The characterization(how?) of these isolates indicated that 138 (50.4 %) were actually *E. coli* isolates, while 136 (49.6 %) belonged to other Enterobacteriaceae groups. Urine specimens gave the highest number of *E. coli* 102 (37.2 %).

Table 1: Clinical Sources of Samples for Isolation of *Escherichia coli*

Sample type	Presumed *E. coli*	Confirmed *E. coli*	Percentage
Aspiratesource?	1	1	0.4
Catheter tip	2	1	0.4
Ear swab	1	0	0
ECS	4	0	0
HVS	11	5	1.8
Semen	5	1	0.4
Stool	24	15	5.5
Throat Swab	1	0	
Urine	176	102	37.2
Wound Swab	49	13	4.7
TOTAL	**274**	**138**	50.4

ECS is an Endocervical swab, HVS is a high vaginal swab. About 50.4 % of pre-diagnosed E. coli from participating laboratories were confirmed E. coli using the E. coli Chromagar.

3.2 Susceptibility Test Result of the *E. coli* Isolates to Quinolone Antibiotics

Great majority of the ~~study~~*E. coli* isolates showed resistance to ciprofloxacin 88 (96.7 %); Ofloxacin 91 (100 %); Pefloxacin 84 (92.3 %); spafloxacin 85 (93.4 %) and moxifloxacin 90 (98.9 %) (Table 2).

Table 2: Susceptibility of *E. coli i*solates to Quinolone Antibiotics

Antibiotic	Sensitive	Intermediate	Resistant	Percentage of the Resistant Isolates
Ciprofloxacin	2 (≥20mm)	1(17-19mm)	88 (≤ 16mm)	96.7
Ofloxacin	0 (≥29mm)	0(26-28mm)	91 (≤ 25mm)	100.0
Pefloxacin	4 (≥20mm)	3(17-19mm)	84 (≤16 mm)	92.3
Pefloxacin	6 (≥20mm)	0 (17-19mm)	85 (≤16 mm)	93.4
Moxifloxacin	0 (≥20mm)	1 (17-19mm)	90 (≤ 16mm)	98.9
Levofloxacin	33 (≥17mm)	16(14-16mm)	41 (≤ 13mm)	45.1

3.3 Plasmid Mediated Resistance Gene

3.3.1 Qnr Genes

Qnr S genes were found the most prevalent quinolone (*Qnr*) gene in the clinically isolated *E. coli*, with a prevalence rate of 71.4 %. Genes arenormally written usinga three letteredlower case)Qnr B was present in fifteen of the isolates at the rate of 15.4 %. *Qnr* A was not detected in any of the isolates. Eleven (12.1 %) of the isolates showed both qnrS and qnr B.Table 3 shows the frequency of plasmid-mediated quinolone (*qnrA*, *qnrB*, *qnrS*) genes and Fig. 2a shows the *qnr* genes on agar gel. *OqxB* of the Efflux pump gene recorded the highest prevalence (25.3 %), followed by QepA (12.1 %), and then, *OqxA* (7.7 %) (Table 3).

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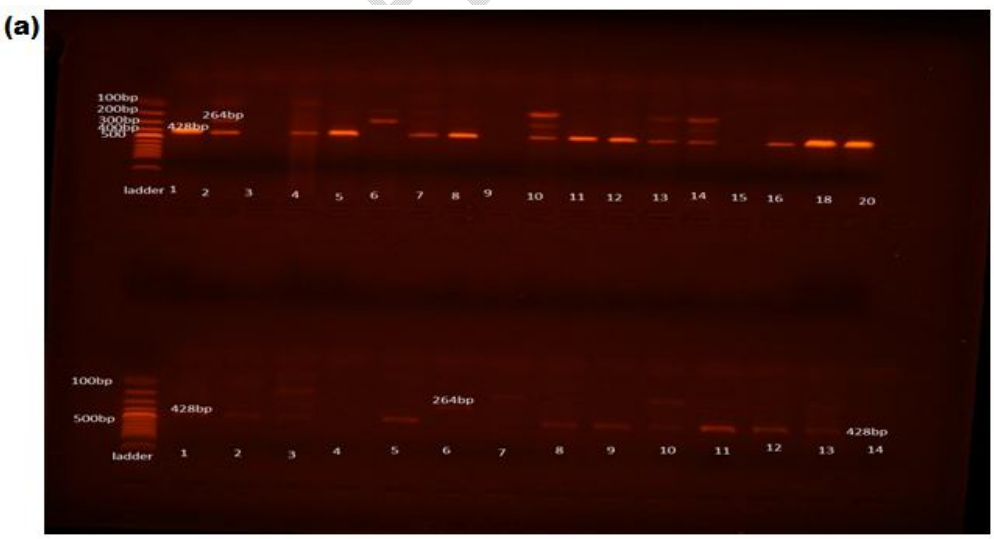

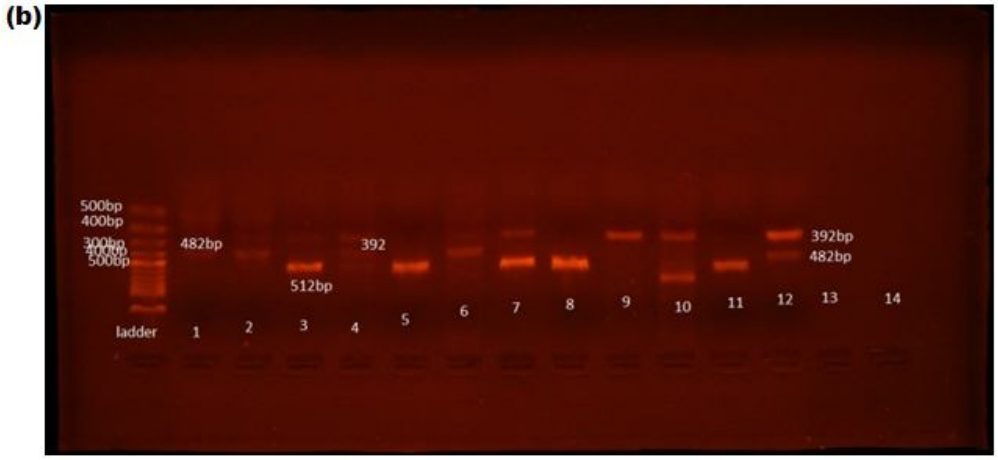

Fig. 2. (a) QNR genes (b) Aminoglycosyl acetyltransferase and Efflux genes

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Table 3: Frequency of Plasmid-Mediated Quinolone Resistance Genes in *E. coli*

Type of gene	Specific gene	Positive Cases	Percentage
Qnr	qnrS	65	71.4
	qnrB	14	15.4
	qnrA	0	
	qnrS & B	11	12.1
AMG	aac (6) lb-cr	4	4.4
Efflux	OqxA	7	7.7
	OqxB	23	25.3
	QeqA	11	12.1
	OqxA & OqxB	5	5.5
No Gene		18	

3.3.2 The Aminoglycoside Acetyltransferase Gene

The Aminoglycoside acetyltransferase gene was seen only in four isolates (4%) (Table 3). Fig. 2b shows the aac (6) lb-cr, and the three efflux genes, *OqxA, OqxB and qepA*. The aac-(6) *lb-cr* is 482 bp and lies close to the 500 bp of the ladder; *OqxA* is 392 bp and is located close to the fourth bar (400 bp) of the ladder; *OqxB* is 512 bp and is found above the 500 bp of the ladder, while *qepA* is 199 bp and lies close to 200 bp of the ladder.

3.3.3 Clinical *E. coli* Isolates and PMQR Gene Carriage

Given the total of 91 *E coli* isolates subjected to Plasmid quinolone-resistant genes, 68 (74.7 %) were positive for *qnr* genes while 23 (25.3 %) were negative for qnr genes; 36(39.6 %) were positive for the *Efflux* pump genes, while 54 (59.3 %) were negative; only 4 (4.4 %) were positive for the *aac (6') lb-cr* while the great majority 87 (95.6 %) were negative as presented in Table 4.

Table 4*:* No of the *E coli* isolates Positive and Negative for the PMQRGenes ***(N=91)***

PMQR Gene	No positive	% age	No Negative	% age
Qrn	68	74.4	23	25.3
Efflux	36	39.6	54	59.3
Aac(6') ib-cr	4	4.4	87	95.6
Total Positive for PMQR Gene	73	80.2		93.4
Total negative for PMQR Gene			18	19.8

3.3.4 Plasmid Gene and the Antibiotic Profiles of the *E. coli* Isolates

Tables 5 and 6 showed the plasmid gene profile of the *E. coli* Isolates and their frequencies. The profile with the highest frequency is *Qnr* alone 36 (39.6 %), followed by *QNR /Efflux*, 28 (30.8 %), and then efflux alone 5(5.5 %). A total of 18(19.8 %) of the population were found to possess none of the genes. Going down the analysis into the *PMQR* gene profile in Table 6, Qnr S occurring alone has the highest-profile(30.8 %), followed by *QnrS/OqxB (QNR /Efflux*),12.1 % and then followed by *Qnr S/ QepA* (also *QNR /Efflux*), 8.8 %. How were these genes detected?

Table 5: Frequency Table of the Plasmid Gene Profile of the *E. coli* Isolates

Gene profile	Frequency	Percentage frequency
QNR/ EFFLUX/ AMG	1	1.1
QNR / EFFLUX	28	30.8
QNR/ AMG/	1	1.1
EFFLUX/ AMG	1	1.1

EFFLUX/	5	5.5
QNR /	36	39.6
AMG/	1	1.1
None Present	18	19.8

Table 6: Quinolone Resistance~~t~~ Gene Profile of the *E. coli* Isolates

Gene profile	Frequency	Percentage frequency
QNR S/QNRB/OqXB	2	**2.2**
QNR S/QNRB/OqXA	0	**0.0**
QNR S/QNRB/qepA	2	**2.2**
QNR S/QNRB/AA	1	**1.1**
QNR B/OqxB	1	**1.1**
QNR B/OqxA	0	**0.0**
QNR B /qEpA	0	**0.0**
QNR S/OqxB	11	**12.1**
QNR S/ qEpA	8	**8.8**
QNR S/ qEpA/AA	1	**1.1**
QNR S/OqxA	1	**1.1**
QNR S/OqxA/AA	1	**1.1**
QNRS/OqxA/OqxB	3	**3.3**
QNR B/OqxA/OqxB	0	**0.0**
QNRS/OqxA/OqxB/AA	1	**1.1**
QNR S/QNRB	6	**6.6**
OqxA/OqxB	1	**1.1**
QNR S/	28	**30.8**
QNR B/	2	**2.2**
OqxA/	0	**0.0**
OqxB/	4	**4.4**
qEpA/	0	**0.0**
AA/	0	**0.0**
None /	18	**19.8**
Total	**91**	**100**

3.3.5 Quinolone Drugs and *E. coli* Resistance: Plasmid Gene Predominance and Frequency

Qnr S is shown to be the leading gene in all the Resisted quinolones. There is also a low frequency of Qnr S coexisting with Qnr B in all the drugs. The efflux pump gene, OqxB was the most frequent of the efflux pump genes and was found to occur alone or in combination with the other efflux or Qnr genes. In almost all the drugs (except in Levofloxacin that was least resisted), all the PMQR genes, Qnr, aac (6') lb-cr, and efflux pumps were all fully represented, alone or in combination with others as shown in Table 7.

Table 7: Frequency of the Plasmid Quinolone Genes in the Quinolone Drugs

Quinolone	**Aac (6') lb-cr + Efflux Genes**											
	Qnr S	**Qnr B**	**QNR B+ S**	**AA**	**Efflux Gene Absent**	**OqxA**	**OqxB**	**QepA**	**AA +OqxA**	**OqxA + OqxB**	**AA+ OqxA +OqxB**	**AA+ QepA**
cip (n=88)	64	14	11	4	52	6	22	11	1	3	1	1
oflo (91)	65	14	11	4	51	7	23	11	1	4	1	1
Pef (n=84)	61	12	10	4	47	7	23	11	1	4	1	1
spa(n=85)	61	12	10	4	49	6	22	11	1	3	1	1
Moxi(n=90)	53	13	10	4	52	6	22	11	1	4	1	1
levo(n=41)	30	6	5	1	22	3	14	4	0	1	1	0

Cip = ciprofloxacin; Oflo= Ofloxacin; pef= pefloxacin; Spa= Spafloxacin; Moxi = moxifloxacin; Levo= levofloxacin; AA=aac (6') lb-cr (aminoglycosyl acetyltransferase gene)

Table 8(a) and (b) show the antibiotic profile of the clinical *E. coli* isolates and the summary, respectively. The six and the five-antibiotic resistance profiles had the highest frequency of 27 (29.7 %) and 42 (46.2 %), respectively, with all the different genes elaborated.

Table 8(a): Antibiotic Profile of *E. coli* Isolates and their Plasmid Genes.

Antibiotic Profile	Total No of Isolate	Qnr gene(s) Present	Efflux gene(s) Present	AMG gene Present
Cip/Oflo /pef / Spa Moxi/ Levo	27	19	8	1
Cip/ Oflo/ pef /Spa/ Moxi	41	31	22	2
S/N	1	1	0	0
Cip/ pef /Spa/ Moxi	1	1	1	0
Cip/ Oflo/ Spa/ Moxi	2	2	0	1
Cip/ Oflo/ pef// Spa	8	7	3	0
Cip Oflo/ pef	1	1	0	0
Cip/ pef/ Moxi	1	0	0	0
Cip/ Spa/ Moxi	1	0	0	0
Oflo/ Moxi/ levo	1	1	1	0
Pef/ Spa/ moxi	1	1	1	0
Spa/ Moxi	1	0	0	0
Cip/ moxi	1	1	0	0
Cip	4	4	0	0

Cip = ciprofloxacin; Oflo= Ofloxacin; pef= pefloxacin; Spa= Spafloxacin; Moxi = moxifloxacin; Levo= levofloxacin; ATCC= American Typed; EC=*E. coli*

Table 8(b): Antibiotic Profile of the Resistant *E. coli* Isolates and their Plasmid Genes

Number of Antibiotics Resistant To	Number of Strains Showing Pattern	Qnr Gene Present	Efflux Gene Present	AMG Gene Present
One Antibiotic (Cip Only)	4	4	0	0
Two Antibiotics	3	1	0	0
Three Antibiotics	5	3	2	0
Four Antibiotics	11	10	4	1
Five Antibiotics	42	32	22	2
Six Antibiotics	27	19	8	1

4. Discussion

The results of the susceptibility testinggivesaportraysthepicture of quinolone resistance in the study area.~~our society.~~ Five of the 6 quinolone drugs were resisted by the greater majority (>90 %) of the *E. coli* isolates.

The frequency of the PMQR genes 73(80.2 %) obtained in this study is much higher than what was found among *E. coli.* in Lome, Togo, the frequency of 67.03 % was reported by Salah *et al.* (2019).[14]. Much lower frequencies were obtained in many other places; in Mexico, it was reported in 32.1 % ;[15] in the United Kingdom (UK) in 35.2 %.[16] In China in 43.6 %,[17] while in Niger, it was reported in 44.4 %.[18]In the Republic of Korea, the frequency was even lower, 5 %.[19] Similarly, the frequency of the *qnr* genes obtained here varied from what was reported in the other countries. In Mexico, *qnr* A was obtained in clinical isolates of *E coli* in 22.7 %., qnr B in 20.9 % and qnr S in 6.4 %. In Iran, Firoozeh *et al.*, (2014) obtained 22.2 % of qnr A and 14.3 % of qnr B from 63 ciprofloxacin-resistant isolates. While similar results of low qnr A and B were said to have been obtained in Jamaica, UK and Spain; in Japan, a lower frequency of 6.5 % was obtained of qnr A, while qnr S and B were not found.[20] This is a clear contradiction of the current study where qnr S and qnr B were prevalent and qnr A not detected. In Korea, Pallecchi *et al.* (2009)[21] found qnr B dominant while qnr A was not found. Qnr S and qnr B as found out in this study, often occurred together in the same isolates with a prevalence of 12 %. The prevalence of aac (6) lb-cr (4 %) found in our study is quite low compared to the prevalence of 34.5 % found in the UK by Ciesielczuk *et al.* (2013) and 18 % detected in Southern Nigeria among species of Gram-negative bacteria. However, their presence indicates their availability in our society and their importance in reducing the activity of Ciprofloxacinby N-acetylation at the amino nitrogen on its piperazine substituent. [22]

The frequency of the efflux pump genes obtained in this study, 41(18.55 %), is very similar to the percentage frequency of 18.7 % obtained by Ogbolu *et al.*, (2016)[23] in Southern Nigeria. However, the frequency of *OqxB* and *QepA* is by far higher than what Ciesielczuk *et al* (2013)[16] obtained in the UK. By implication, these genes contribute to the flushing out of the quinolone antibiotics from the cell membrane of the organism, thereby contributing to the high resistance of the *fluoroquinolones.*

5. Conclusion

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We report a very high frequency of the Plasmid-mediated quinolone resistance genes in multi-resistant clinical isolates of Escherichia coli. Qnr S is the leading gene, while Qnr A was not detected. The plasmid genes, therefore, contribute to the high quinolone resistance being recorded in our society since more of the genes were detected in those isolates that resisted five and six of the quinolone drugs. The fact that these genes are carried on the Plasmid may facilitate their easy spread to other bacterial species. Whole-genome sequencing will be required to expose the molecular mechanisms of quinolone antibiotic resistance, which will enable better interventions in antimicrobial resistance.

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Fig. 1: Sensitivity pattern of the *E. coli* isolates

Fig. 2. (a) QNR genes (b) Aminoglycosyl acetyltransferase and Efflux genes