Research Article

Prevalence of multidrug resistance genes in Escherichia coli isolates from patients attending four hospitals in Minna, Nigeria

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Sri Lankan Journal of Infectious Diseases 2020 Vol.10 (1):53-64

DOI: http://dx.doi.org/10.4038/sljid.v10i1.8269

Abstract

Objectives: This study determined the prevalence, antibiotic susceptibility and resistance genes among multidrug resistant *Escherichia coli* isolates from diarrheic patients in four hospitals within Minna, Nigeria.

Methods: Stool samples from one hundred and seven (107) diarrheic patients were collected and analysed to check for *E. coli* using spread plate techniques. The resistance pattern of the *E. coli* isolates to ten (10) antibiotics using disc diffusion techniques was determined. Five isolates with multidrug resistant index ≥ 0.5 were screened for antibacterial resistant genes (Tn3bla, GyrA, ParC, aadA2 and Sul1) using polymerase chain reaction.

Results: Seventy (70) samples representing 65.4% were *E. coli* positive while fifty-five (55) were resistant to at least one antibiotic. The highest level of resistance was against ampicillin (38.57%) while the least was against cefalexin (4.29%). Multidrug resistant isolates were 58.7% (41/70), while 28.6% (20/70) were resistant to three or more antibiotics. The result of the molecular characterization identified the five multidrug resistant isolates to be *E. coli* strain RAD34, *E. coli* strain CUSMBN2, *E. coli* strain CAU3471, and *E. coli* strain BYPFP. *Tn3bla/GyrA/ParC* resistant genes were detected in all the five isolates. *E. coli* isolate RAD34, *E. coli* strain CUSMBN2, *E. coli* strain CAU3471 and *E. coli* strain BYPFP possessed *Sul1* gene. However, *E. coli* strain BYPFP, *E. coli* strain CAU3471 and *E. coli* strain CUSMBN2 had *aadA2* gene.

Conclusion: The result of this study established the active circulation of *Tn3bla*, *GyrA*, *ParC*, *aadA2* and *Sul1* resistant genes among *E. coli* in the study area.

Keywords: Diarrhoea, Specific primers, Multidrug resistance genes

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Received 16 October 2019 and revised version accepted 18 February 2020

Introduction

Antibiotics continue to play a crucial role in the treatment of bacterial diseases since they were first discovered in 1929.[1] However, the emergence of resistance, which was first reported in 1940,[1] and the continuing evolution of resistance even to newer drugs, remains a great challenge for control of both bacterial diseases as well as many other diseases that affect mankind.

Drug resistance among bacteria that cause either community acquired infections or hospital acquired infections has increased tremendously in the last few decades and is currently generating serious concern within the global health community.[2] *E. coli* have been identified as one of the most important multidrug resistant bacteria by the World Health Organization's (WHO) report of priority pathogens, because of their distinct ability to resist antibiotics as well as transfer the mechanism to other bacteria.[3] They are the most common cause of urinary tract infections (UTIs) in humans[4], as well as a leading cause of food-borne infections primarily due to the Shiga-toxin producing strains.[5-7]

The problem of antibiotic resistance among bacteria globally and particularly in Nigeria calls for urgent attention.[2,8] It was reported by Larsson et al.[8] that bacterial infections alone accounted for as much as 45% of death witnessed at the turn of the century. A number of mechanisms have been identified as being involved in bacterial resistance to certain antibiotics and this knowledge is already being translated into positive action to improve our antibiotic arsenal against resistance. One such example is the conjugation of clavulanic acid with β-lactams to overcome the hydrolytic activity of β-lactamase enzymes utilized by bacteria against β-lactam drugs.[9]

To effectively prevent the spread of antibiotic resistance and/or treat persistent infections, the mechanism of resistance as well as other molecular epidemiologic information of the causative agent(s) are required, hence the need for this study.

Methods

Study area

Minna is the capital of Niger State, Nigeria. It is situated on Latitude 9.61 N and Longitude 6.56 E at an elevation of 299 m above sea level. It is bordered to the North by Sokoto State, West by Kebbi State, and South by Kogi and South-West by Kwara State. Niger State has a common boundary with the Republic of Benin along New Bussa, Agwara and Wushishi Local Government Area. Samples were collected from the microbiology laboratories of selected hospitals in Minna, which included the Nigeria General hospital (GH), Ibrahim Babangida Specialist hospital (IBBSH), Standard Hospital (SH) and Top Medical Hospital (TMH) as shown in Figure 1.

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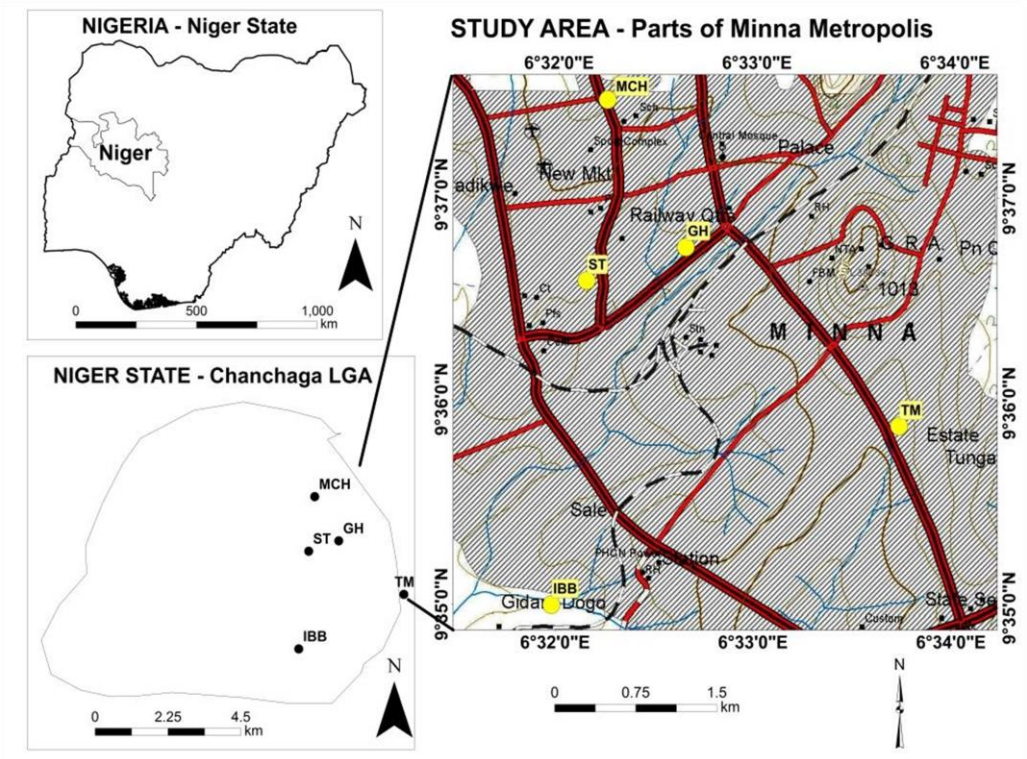

Figure 1: Study area and distribution of hospitals included in the study

GH= General Hospital, IBBSH= IBB Specialist Hospital, SH=Standard Hospital, TMH= Top Medical Hospital.

Sample collection

Stool samples from one hundred and seven (107) diarrheic patients were collected from patients attending four hospitals in Minna, Nigeria between February and June 2017. Ethical clearance was obtained from the research and ethics committee of the Niger State Hospitals Management Board for the study. Sixty (60) stool samples from GH, twenty-five (25) from IBBSH, fifteen (15) from SH and seven (7) from TMH were included in the study. Patients within the age range of 10-65 years who were attendees of the selected hospitals and consented to take part were included.

Isolation and Identification of *Escherichia coli*

An aliquot of stool sample was inoculated into 10 mL sterile peptone water and incubated for 8 hours at 37 ˚C. A loopful from the above was streaked onto Eosin Methylene Blue (EMB) agar plates and incubated at 37 ˚C for 18 hours. Colonies (one per plate) that showed characteristic green metallic sheen on EMB agar were further sub-cultured onto nutrient agar slants for further analysis. The pure isolates obtained were identified and characterized based on their cultural (colony) characteristics, Gram stain and biochemical properties.[10]

Antimicrobial susceptibility test

Kirby Bauer's disc diffusion technique was used for antimicrobial susceptibility testing.[10] The results were interpreted using Clinical and Laboratory Standards Institute (CLSI) guidelines (2015).[11] Briefly, individual colonies were suspended in normal saline to 0.5 McFarland standard. Using a sterile swab, the suspensions was inoculated on Muller Hinton agar and incubated at

37 ˚C for 18 hours. Commercially available antibiotic (streptomycin (10 µg), ampicillin (10 µg), co-trimoxazole (23.75 µg), erythromycin (15 µg), ofloxacin (5 µg), cefalexin (30 µg), ciprofloxacin (5 µg), amoxicillin/clavulanic acid (10 µg), gentamicin (15 µg)) discs of the drugs commonly used for treatment of Gram-negative bacterial infections were tested. The zone of inhibition was measured and used to classify the organisms as susceptible, intermediate or resistant to a specific antibiotic.[11]

Multidrug-resistant (MDR) *E. coli* isolates were defined by resistance to ≥1 agent in ≥3 antimicrobial classes. Five MDR *E. coli* isolates resistant to ≥3 antimicrobials, with at least one antibiotic per antimicrobial group were selected for molecular identification and antibiotic resistance gene detection using PCR with primers specific for *E. coli* and antibiotic resistance genes (*Tn3bla, GyrA, aadA2* and *Sul1*).

Multiple Antibiotic Resistance Index (MARI) in respect to a single isolate defined as a/b, where "a" represents the number of antibiotics to which the isolate was resistant and "b" represents the number of antibiotics to which the isolate was exposed, was also calculated.[12]

Molecular characterization of multidrug resistant *E. coli* isolates

The molecular characterization of five *E. coli* isolates with multidrug resistant index ≥ 0.5 were confirmed using PCR with primers specific for *E. coli*. DNA was extracted by the protocol described by Zhang et al.[3] The integrity of the extracted DNA was checked on a 1% agarose gel to confirm extraction.[3] The ribosomal RNA primer (16SF: GTGCCAGCAGCCGCGCTAA and 16SR: AGACCCGGGAACGTATTCAC) used for the molecular characterisation of the *E. coli* isolates was obtained from the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria. PCR was carried out on a GeneAmp 9700 PCR System Thermalcycler (Applied Biosystem Inc., USA) following the protocol described by Luo et al.[13]

The PCR product was loaded on 1.5% agarose gel. The ladder used was Invitrogen™ 1 Kb Plus DNA Ladder. The expected base pair of the amplicon was 1500bp. The amplified fragments were sequenced using a Genetic Analyzer 3130xl sequencer (Applied Biosystems). The sequencing kit used was that of BigDye terminator v3.1 cycle sequencing kit. Bio- Edit software and molecular evolutionary genetics analysis (MEGA 6) were used for all genetic analysis. Identities and accession numbers of the *E. coli* isolates were determined by Basic Local Alignment Search Tool (BLAST) from the GENE BANK at www.ncbi.nlm.nih.gov.[14]

Detection of antibiotic resistance genes

PCR technique using antibiotic resistance genes specific primers was used to screen five (5) isolates with multidrug resistance index ≥ 0.5 for the presence of five resistance genes, namely *th3bla, gyrac, aadA2* and *sul1* in their template DNA.[6] The primers and their sequences are shown in Table 1.

Table 1: Primers used for identifying the multidrug resistance genes of *E. coli* isolates

Group of antibiotics	Resistance genes	Product size	Primer sequence
Penicillin	*Tn3bla*	800bp	Forward 5'-*CGCGGAACCCCTATTTGTTT*-3'
			Backward 5'-*GGTCTGACAGTTACCAATG*-3'
Quinolone	*gyrA*	200bp	Forward 5'-*ACGTACTAGGCAATGACTGG*-3'
			Backward 5'-*AGAAGTCGCCGTCGATAGAAC*-3'
Aminoglycoside	*aadA2*	533pb	Forward 5'-*ATTTGCTGGTTACGGTGACC*-3'
			Backward 5'-*CTTCAAGTATGACGGGCTGA*-3'
Sulfonamide	*Sul1*	400bp	Forward 5'-*TGAGATCAGACGTATTGCGC*-3'
			Backward 5'-*TTGAAGGTTCGACAGCACGT*-3'
Quinolone	*parC*	287bp	Forward 5'-*GCCTTGCGCTACATGAATTT*-3'
			Backward 5'-*ACCATCAACCAGCGGATAAC*-3'

Reaction cocktail used for all PCR per primer set included (reagent volume µl) - 10X PCR SYBR green buffer (2.5), 25mM $MgCl_2$ (1.0), 5pMol forward primer (1.0), 5pMol reverse primer (1.0), DMSO (1.0), 2.5Mm DNTPs (2.0), Taq 5u/uL (0.1), made up to 22 with sterile distilled water to which 3 µl template was added. The amplicon from the PCR was loaded on 1.5% agarose gel. The bands that appeared were compared with that of the ladder and the observation were recorded.

Results

Distribution of *E. coli* isolated from the four hospitals

The number of positive samples for *E. coli* is presented in Table 2. The highest number of *E.coli* isolates (n=45; 64.3%) were from GH, while only 3 (4.3%) were from TMH.

Table 2: Distribution of *E. coli* isolated from the 4 hospitals

Hospital	Samples collected	No	*E. coli* isolates. % +ve for each hospital	% of total no of *E. coli*
General	60	45	75.0	64.3
Ibrahim Babangida	25	15	60.0	21.4
Standard	15	7	46.7	10.0
Top Medical Hospitals	7	3	42.9	4.3
Total	**107**	**70**		**100**

Antibiotics susceptibility screening

The isolates showed a varied pattern of susceptibility and resistance as shown in Table 3. The highest resistance (38.6%) was against ampicillin while the least (4.3%) was recorded against cefalexin.

Table 3: Antibiotics Susceptibility Screening

Antibiotics	µg	No. resistance	%	No. intermediate	%	No. Susceptible	%
S	10	12	17.1	17	24.3	41	58.6
PN	10	27	38.6	23	32.9	20	28.6
SXT	23.75	19	27.2	35	50.0	16	22.9
E	15	6	8.6	22	31.4	42	60.0
OFX	5	22	31.4	22	31.4	26	37.1
PEF	5	9	12.9	8	11.4	53	75.7
CPX	5	6	8.6	4	5.7	60	85.7
AU	10	22	31.4	28	40.0	20	28.6
CEP	30	3	4.3	23	32.9	44	62.9
CN	15	8	11.4	10	27.1	43	61.4

S (streptomycin), PN (ampicillin), SXT (cotrimoxazole), E (erythromycin), OFX (ofloxacin), PEF (perfloxacin), CPX (ciprofloxacin), AU (Amoxycillin/clavulanic acid), CEP (cefalexin), CN (gentamicin)

Antibiotic susceptibility profile of the isolated *E. coli*

Of the seventy isolated *E. coli* strains, 78.6% (55/70) were resistant to at least one antibiotic (Table 4). MDR *E. coli* isolates accounted for 58.7% (41/70) of the isolated *strains* while 28.6% (20/70) were resistant to three or more antibiotics. The five isolates (H, B, E 3 and A) with multidrug resistant index ≥ 0.5 were selected for further molecular analysis.

Table 4: Single and multidrug resistance pattern in *E. coli* isolates

ID	RP	MDRI	ID	RP	MDRI	ID	RP	MDRI	ID	RP	MDRI
Q	OFX	0.1	I	S/PN	0.2	2YS	PN/AU	0.2	M	PN/SXT/CEP	0.3
Z	PN	0.1	BI	OFX/AU	0.2	U30	S/PN	0.2	S12	S/PN/PEF	0.3
18	CN	0.1	80	PN/E	0.2	S37	S/OFX	0.2	17C	PN/SXT/AU	0.3
44	E	0.1	S40	E/OFX	0.2	S	PN/PEF	0.2	Ve	S/PN/SXT	0.3
32	PN	0.1	38	S/E	0.2	78	PN/SXT/AU	0.3	8C	PN/SXT/CPX/AU	0.4
69	S	0.1	C13	PN/AU	0.2	70	PEF/AU/CN	0.3	A	PN/SXT/OFX/AU/CN	0.5
Li	OFX	0.1	53A	SXT/OFX	0.2	52	SXT/PEF/AU	0.3	H	PN/SXT/OFX/AU/CEP	0.5
S11	OFX	0.1	E1	S/PN	0.2	34	OFX/CPX/AU	0.3	3	SXT/PEF/CPX/AU/CN	0.5
N	OFX	0.1	L	S/OFX	0.2	S14	OFX/PEF/CPX	0.3	B	PN/SXT/OFX/PEF/AU/CEP	0.6
L2	OFX	0.1	9B3	CPX/CEP	0.2	31	PN/SXT/E	0.3	E	PN/SXT/PEF/CPX/AU/CN	0.6
9	PN	0.1	E8	R/AU	0.2	TS	SXT/OFX/AU	0.3			
G	PN	0.1	4T	S/AU	0.2	P	S/PN/OFX	0.3			
S10	CN	0.1	28	PN/AU	0.2	F	PN/SXT/AU	0.3			
32	OFX	0.1	E2	PN/E	0.2	F8	PN/SXT/AU	0.3			

RP – Resistance Profile
MDRI – Multidrug resistance index

S (streptomycin), PN (ampicillin), SXT (cotrimoxazole), E (erythromycin), OFX (ofloxacin), PEF (perfloxacin), CPX (ciprofloxacin), AU (amoxycillin/clavulanic acid), CEP (cefalexin), CN (gentamicin),

Molecular characterization of five (5) *E. coli* isolates

The result of the 16S rRNA gene sequencing of the five (5) isolates with multidrug resistance index ≥ 0.5 isolates revealed them to be E. *coli* strain RAD34 (n=2), *E. coli* strain CUSMBN2, *E. coli* strain CAU3471, and *E. coli* strain BYPFP.

Antibiotic resistance gene presents in the *E. coli* isolates

PCR result using extracted DNA from isolate A indicated the presence of *Tn3bla, GyrA* and *ParC* gene whereas the presence of *aadA2* and *Sul1* were absent. Similarly, isolates B, H and E were positive for the genes*Tn3bla*, *GyrA*, *ParC*, *aadA2* and *Sul1* with the presence of ~ 800 bp, 200 bp, 287 bp, 433 pb and 400 bp bands respectively as shown in Figures 2, 3 and 4 respectively. Only *aadA2* was absent in isolate 3. This was indicated by the presence of multiple bands approximately 800 bp, 200 bp and 287 bp in length corresponding to *Tn3bla* , *GyrA, and ParC* genes.

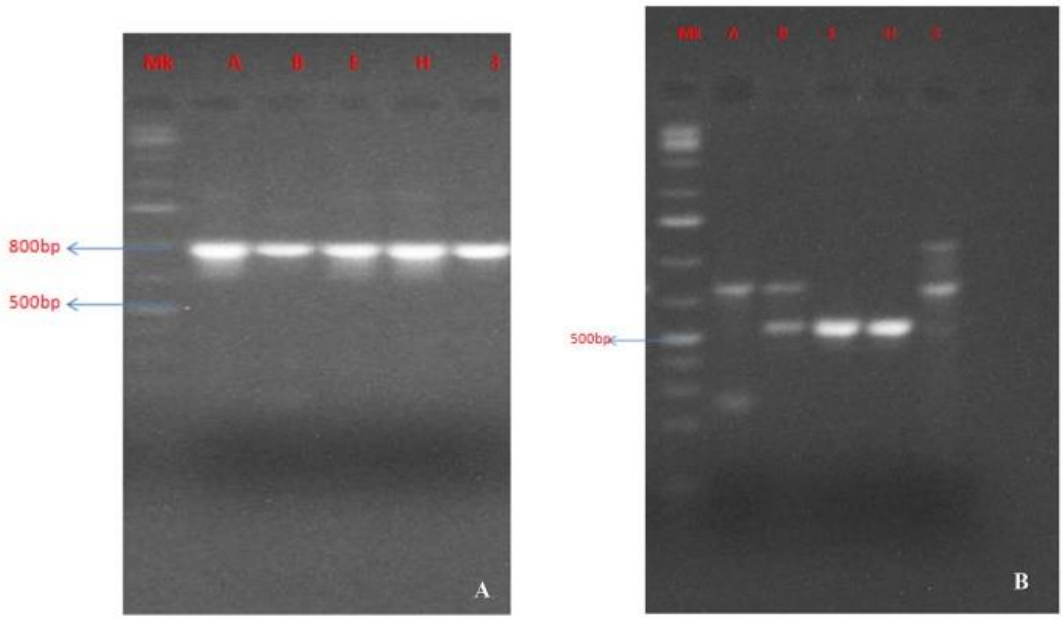

Figure 2: Agarose gel electrophoresis of the amplified antibiotic resistance genes
A = *Tn3bla*; B= *aadA2*

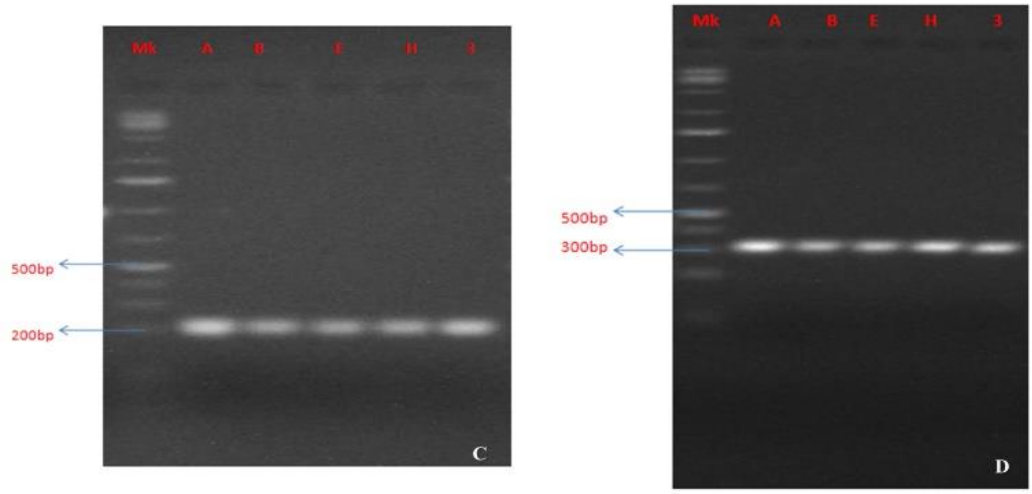

Figure 3: Agarose gel electrophoresis of the amplified antibiotic resistance genes
C = *GyrA* D = *ParC*

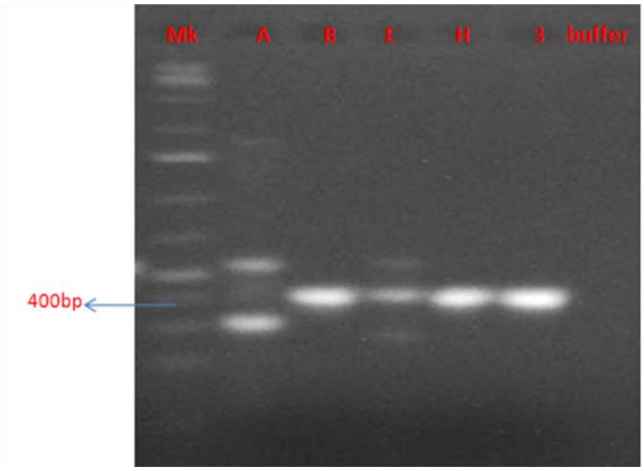

Figure 4: Agarose Gel Electrophoresis of the Amplified *Sul1* Antibiotic Resistance Genes

Distribution of antibiotic resistance genes in the selected *E. coli* isolates

The detection of the antibiotic resistance genes in the five (5) selected *E. coli* isolates revealed that 100% of the isolates had the *Tn3bla*, *GyrA* and *ParC* gene. Similarly, 80% of the isolates possessed the *Sul1* gene as only 4 isolates were amplified with *Sul1* specific primers. The *aadA2* gene had the least occurring because only 60% of the isolates namely B, E and H possessed the gene (Table 5).

Table 5: Antibiotic resistance genes in multidrug resistant *E. coli* isolates

Isolate code	Tn3bla	GyrA	aadA2	Sul1	ParC
A	+	+	-	-	+
B	+	+	+	+	+
E	+	+	+	+	+
H	+	+	+	+	+
3	+	+	-	+	+

+ indicates presence - indicated absence

B= *E. coli* strain BYPFP; A = *E. coli* isolate RAD34**;** H = *E. coli* strain CAU3471

E = *E. coli* strain CUSMBN2; 3 = *E. coli* isolate RAD34

Discussion

E. coli is the main cause of opportunistic or community acquired infections in humans.[15] The emergence and increasing rate of MDR *E. coli* is challenging our ability to treat community acquired infections. This is because *E. coli* can colonize multiple sites in human and animal' bodies, thereby horizontally transmitting or acquiring resistant antimicrobial genes.[16] The highest number of samples (45/60) positive for *E. coli* observed from GH could be due to the fact that the

highest number of samples used for this study was obtained from it as well as the high patronage of this hospital by patients compared to the other study hospitals (Table 2).

The highest phenotypic resistance observed against ampicillin (38.6%), ofloxacin (31.4%) and amoxicillin/clavulanic acid (31.4%) suggests the possible abuse and widespread resistance to these agents in the study area. The presence of *Tn3bla* and *gyrA* in the phenotypically resistant *E. coli* isolates (Table 5) means the resistance observed can become widespread by any of the available mechanisms. Therefore, the use of these antimicrobials as first line antibiotics in the treatment of infections needs to be discouraged since the prevalence of resistance exceeds 10%.[17] The ability of pathogenic isolates of *E. coli* to develop resistance, especially to first line broad spectrum antibiotics amongst the enteric pathogens has been recorded from surveillance studies in Nigeria.[18-21] The higher resistance to ampicillin corroborates previous studies by Rodriguez et al.[18-19], where 40.8% and 74.2% of their *E. coli* isolates exhibited resistance to ampicillin. The common use of these broad-spectrum antibiotics for the treatment of infections by clinicians could be the possible explanation for these observations. The sale of these drugs by non-professionals in Nigerian communities encourages self-medication, possibly resulting to inappropriate dosage, which no doubt, could be responsible for the pattern of resistance observed in this study.

Of the fifty-five (55) resistant *E. coli* isolates, 58.7% were resistant to at least 2 or more classes of antibiotics (Table 3). This may be a result of the presence of multiple resistance genes in *E. coli* isolates as observed in this study. Previous reports in Nigeria have highlighted high multidrug resistance in *E. coli*. Percentages of multidrug resistant *E. coli* from diarrheal stools in previous reports[2,22] were 56% and 69.6% respectively. Hence, the findings of this study are in line with the previous reported studies in Nigeria.

Analysis of results showed the presence of ≥ 4 resistance genes in the five *E. coli* isolates with ≥ 0.5 MDRI (Table 4). This confirms the phenotypic resistance patterns observed for these isolates. The five (5) *E. coli* isolates possess *Tn3bla, gyra* and *pac* genes. Isolates B, E, 3 and H had *sull* gene while only B, E and H possessed *aadA2* gene (Table 5). These genes are known to code for sulfonamide, quinolone, aminoglycoside and penicillin resistance. The detection of these genes in *E. coli* isolates in this study suggests that resistance to these antimicrobials is genetically mediated possibly as a result of long-term use or abuse of these antimicrobials.[2] The presence of *Tn3bla, GyrA* and *Pac* in all the studied *E. coli* isolates agrees with the reports of Momtaz et al[6] who reported that the possession of these resistant genes confer resistance to the penicillin and quinolones groups of antibiotics.

Reports of antibiotic resistant genes found in *E. coli* isolates might serve as a pointer to the possible presence of other drug resistant genes conferring resistance to other classes of antibiotics that were not targeted in this study. The use of antibiotics in poultry farming, poverty and illiteracy are factors that encourage indiscriminate use of antibiotics and the increasing trend of using leftover drugs[23] could be the possible explanation for the emergence of multidrug resistance genes.

Conclusion

Based on the findings of this study, ciprofloxacin and perfloxacin are the most effective against *Escherichia coli* isolated from Minna, Nigeria. Resistant genes, *Tn3bla, GyrA, ParC, aadA2* and

Sul1 coding for penicillin, quinolone, aminoglycoside and sulfonamide resistance are present in *E. coli* isolates from Minna, Nigeria and are responsible for the multidrug resistance observed in isolates.

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