Molecular Detection of Plasmid-mediated Quinolone Resistance Genes (qnrA and *aac*(*6′*)-*Ib-cr)* in Drug Resistant *Escherichia coli,* Sudan

Abstract

Background: Quinolones are group of widely used molecules in the treatment of an array of infectious diseases. Mechanisms of quinolone resistance are mutations in chromosomal gene-containing DNA gyrase, topoisomerase IV, overexpression of the AcrAB efflux system and the acquisition of plasmid resistance genes**. Objective:** The present study reports on the isolation of *Escherichia coli* from patients attending some Hospitals in Wad Medani city, identification of drug resistance patterns and detection of the frequency of quinolones resistance genes; qnrA and *aac*(*6′*)-*Ib-cr* among isolated strains. **Methods:** A cross-sectional descriptive, hospital-based study included 119 *Escherichia coli* strains was conducted. A structured questionnaire was given to study subjects for demographic information and the attitude toward antibiotics administration. Samples were collected and processed according to the standard procedure and isolated bacteria were tested for their antimicrobial susceptibilities by disc diffusion technique according to the CLSI guidelines. Presence of qnrA and *aac*(*6′*)-*Ib-cr* genes was assessed by multiplex PCR. **Results:** Most strains of *Escherichia coli* originated from urine 53.8% (64/119) and wounds 42.9% (51/119) specimens. Meropeneme had the best effect against tested strains with susceptibility of 85% (101/119). Multiplex PCR assay, using specific primers, demonstrated that 41.2% (49/119) and 37.8% (45/119) of isolated *Escherichia coli* possessed qnrA and *aac*(*6′*)-*Ib-cr* genes respectively**. Conclusion:** The high rate of qnrA and aac (6)-Ib-cr genes among *Escherichia coli* necessitate the usage of molecular tools in detecting the genetic determinants of drug resistance microorganisms in countries such as Sudan.

Key Words: *Escherichia coli*, Quinolone resistance**,** qnrA, *aac*(*6′*)-*Ib-cr*, Multiplex-PCR, Sudan

UNDER PEER REVIEW

Introduction:

Pathogenic microbes that are becoming resistant to antimicrobials treatment is a growing public health problem. The situation in Sudan is of particular concern because self-medication and the use of antibiotics without medical guidance is so prevalent due to insufficient regulation of the distribution and sale of prescription drugs [1]. *Escherichia coli* can cause clinical infections including pneumonia, respiratory tract infection, urinary tract infection, wound infection, and bacteremia [2]. Resistance to quinolones in clinical isolates from the family of enterobacteriaceae was first studied in *K.pneumoniae* strains [3]. Resistance has been observed to essentially all of the antimicrobial agents currently approved to be used in human and veterinary clinical medicine [4]. This, in addition to the variety of antimicrobial agents currently available, makes selecting the appropriate antibiotic a more difficult process [5].

Quinolones are a class of molecules that are widely used around the world to treat a range of infectious diseases, they are synthetic antibiotics used for infections involving Gram-negative bacteria such as Enterobacteriaceae [6]. Mechanisms of quinolone resistance are mutations in chromosomal gene-containing DNA gyrase, DNA topoisomerase IV, overexpression of the AcrAB efflux system and the acquisition of plasmid resistance genes [7, 8].

Recently, other plasmid-mediated quinolone resistance (PMQR) mechanism has been described; *aac*(*6'*)-*Ib-cr*, a variant aminoglycoside acetyltransferase capable of modifying ciprofloxacin and reducing its activity, is now recognized to be widely prevalent and circulated together with qnr genes [9, 10]. The aims of this study was to isolate and identify *Escherichia coli,* determine the drug resistance patterns and asses the frequency of quinolones resistance genes (qnrA and *aac*(*6'*)-*Ib-cr*) in different hospitals in Wad Medani city.

UNDER PEER REVIEW

Methods

Study setting and population

This was cross-sectional descriptive, hospital-based study ran between August 2019 and March 2020 from patients attended the major teaching hospitals in Wad-Madani city (Wad Medani Emergency Hospital, Wad Medani Teaching Hospital, Gezira Hospital for Renal Diseases and Surgery, National Cancer Institute, Wad Medani Pediatric Hospital, Wad Medani Teaching Hospital for Obstetrics And Gynecology). This study included patients presented to hospital with clinical findings suggestive of bacterial infection. The selection of participants depends on their voluntary consent. All guardians have been selected to participate after agreement of their parents.

Inclusion and exclusion criteria

All isolates from different types of samples (urine, wound swabs, throat swabs, ear swabs and sputum) that identified as *Escherichia coli* by 16 RNA gene and conventional biochemical methods, at the period of this study were included.

Isolation, identification and susceptibility testing

One hundred and nineteen strains of *Escherichia coli* were isolated. A structured questionnaire was given to study subjects for demographic information (age, gender, marital status) and the attitude toward antibiotics administration. The samples included urine, wound swabs, throat swabs, ear swabs and sputum. The collected the samples were transported to the Microbiology Laboratory within one hour of collection to prevent drying [11]. Sample were immediately inoculated on Chocolate agar, blood agar MacConkey agar and CLED media. Incubation was done aerobically at 37 for 24 hours. Bacterial isolates were identified based on colonial characteristics, Gram staining and biochemical tests [12]. All isolated bacteria were tested for their antimicrobial susceptibilities by disc diffusion technique according to the CLSI guidelines

UNDER PEER REVIEW

[13]. The following antimicrobial discs were used: (Meropeneme, Cefixime, Amoxicillin/Clavulanic acid, Ceftriaxone, Ciprofloxacin, Gentamicin) manufactured by (Bioanalysis Co. Italy).

DNA extraction

DNA was isolated from bacterial colonies using the boiling lysis method. 400 µL of bacterial suspension was boiled at 100 °C for 30 min. The suspension centrifuged at 14000 rpm for 10 min. The supernatant containing the DNA was transferred to a new tube and precipitated in 800µL of absolute cold ethanol (incubated at -4 °C for 20 min. Then centrifugation done at 14000 rpm for 15 min. The pellet washed in 1000µL of ethanol 70%, dried and re-suspended in 100µL of sterile water.

Multiplex PCR

M-PCR is the simultaneous amplification of more than one target sequence in a single reaction tube using more than one primer pair. This co-amplification of two or more targets in a single reaction is dependent on the compatibility of the PCR primers used in the reaction. PCR amplification was performed using published primer pairs which are as shown in (Table 1).

Table 1. Primer sets for amplification of quinolones resistance determine genes and molecular confirmation of *Escherichia coli* isolates

Gene	Sequence	Product Size
qnrA	F ATTTCTCACGCCAGGATTTG R GATCGGCAAAGGTTAGGTCA	516
aac(6')-Ib-cr	F TTG CGA TGC TCT ATG AGT GGC TA R CTC GAA TGC CTG GCG TGT TT	482
***Escherichia coli* 16 rRNA**	F CCA ATC AGT TAA CAC CAG GT R GAT TGC ATC GAC CAG AAC CT	600

UNDER PEER REVIEW

For primers preparation 100 pmol/ml from each primer we dissolved them in sterile DW as instructed by manufacture, then for 10 pmol/ml we dissolved 10 µl of each primer in 90 µl sterile DW [13].

The following were used to obtain final reaction mixture of 20 µl; 2.2 µl deionized sterile water, 3 µl Master mix (Solis Biodyne, Korea), 0.2 µl QnrA forward primer (Macrogen Company, Seoul, Korea), 0.2 µl QnrA reverse primer (Macrogen Company, Seoul, Korea), 0.2 µl AcrA forward primer (Macrogen Company, Seoul, Korea), 0.2 µl AcrA reverse primer (Macrogen Company, Seoul, Korea), 0.3 µl KPC forward primer (Macrogen Company, Seoul, Korea), 0.3 µl KPC reverse primer (Macrogen Company, Seoul, Korea), 0.2 µl K16RNA forward primer (Macrogen Company, Seoul, Korea), 0.2 µl K16RNA reverse primer (Macrogen Company, Seoul, Korea) and 3 µl DNA (template DNA).

Amplification was achieved using the following thermal cycling conditions: five minutes at 94°C for the initial denaturation and for 38 cycles of amplification consisting of 45 seconds at 94°C, 45 seconds at 58°C, and 45 seconds at 72°C with 3 minutes at 72°C for the final extension.

Statistical analysis

Statistical data analysis was performed for descriptive statistics including frequencies, cross tabulation of microbiological and clinical features, and demographic characteristics using the computer software program SPSS version 16 (SPSS Inc., Chicago, IL, USA).

Ethical approval

The study was approved by the Faculty of Medical Laboratory Sciences, University of Gezira and Ministry of Health, Gezira State.

UNDER PEER REVIEW

Results:

Baseline data:

The 119 strains of *Escherichia coli* were recovered from a total of 819 clinical specimens in different hospitals in Wad Medani city, the overall bacterial growth revealed 57.8% (473/819) while *Escherichia coli* constituted 25.2% (119/473). Table 1 represented number of *Escherichia coli* isolates according to sample types, most strains were from Wad Medani Teaching Hospital for Obstetrics and Gynecology and Gezira Hospital for Renal Diseases and Surgery, and urine was the most frequent sample. Number of strains isolated from male were patients were 33% (40/119) and female patients were 77% (79/119). The study subjects distributed to three age groups; children (1 – 15 years), adults (16-55 years) and geriatric (above 56 years) represented 20% (24/119), 38% (45/119) and 42% (50/119) respectively.

Table 2. Distribution of *Escherichia coli* isolate in subject hospitals, and sample types. No 119:

Hospital	Sample type					Total
	wound Swab	Urine	Throat Swab	Ear Swab	Sputum	
Wad Medani Emergency Hospital	0	8	0	0	2	10
Wad Medani Teaching Hospital (Surgery department)	15	0	0	0	0	15
Wad Medani Teaching Hospital for Obstetrics and Gynecology.	4	26	0	0	0	30
Gezira Hospital For Renal Diseases and Surgery	4	20	0	0	0	24
National Cancer Institute	08	06	0	0	0	14
Wad Medani Pediatric Hospital	4	04	0	03	0	8
National Center for Pediatric Surgery	10	0	0	0	0	10

UNDER PEER REVIEW

Wad Medani Teaching Hospital (ENT department)	04	0	2	0	0	4
Total	51	64	2	3	2	119

Antimicrobial susceptibility profile:

It was noted that 41.3% (338/819) of study participants reported that they had received antimicrobials without medical prescription at least one time. From collected data, the most frequently used antimicrobial for self-medication was azithromycin, followed by ceftriaxone and amoxicillin. Also, 55.9% (458/819) of the participants reported having a drug in the home. Meropeneme against *Escherichia coli* had the best effect in antimicrobial susceptibility tests. The ciprofloxacin resistance rate among the isolates was 55%. Antibiotic susceptibility testing results for clinical isolates of *Escherichia coli* is shown in (Table 3).

(Table 3). Antimicrobial susceptibility testing of isolated *Escherichia coli* against commonly used drugs

Antibiotic	Resistance %	Intermediate %	Sensitive %
Ciprofloxacin	55	05	40
Gentamicin	30	03	67
Cefixime	61	12	27
Ceftriaxone	68	20	12
Meropeneme	13	01	85
Amoxicillin/Clavulanic Acid	85	03	12

Multiplex PCR assay

The distribution of the antibiotic resistance genes in the *Escherichia coli* isolates is shown in (Table 4). The multiplex PCR assay, using specific primers, demonstrated that among the 119 isolates, 49 (41.2%) and 45 (37.8%) isolates were positive for the qnrA and *aac (6)-Ib-cr* gene, respectively, showing that qnrA was circulating with a high frequency.

UNDER PEER REVIEW

Table 4. Frequency and distributed of genes out of isolated *Escherichia coli*

No of Genes detected	Type of Genes	No of isolated organisms	Percentage %
2 Genes	qnrA + aac (6)-Ib-cr	21	17.6 %
1 Gene	qnrA	28	23.5 %
1 Gene	aac (6)-Ib-cr	24	20.1 %
Zero gene	No genes	43	36.1 %

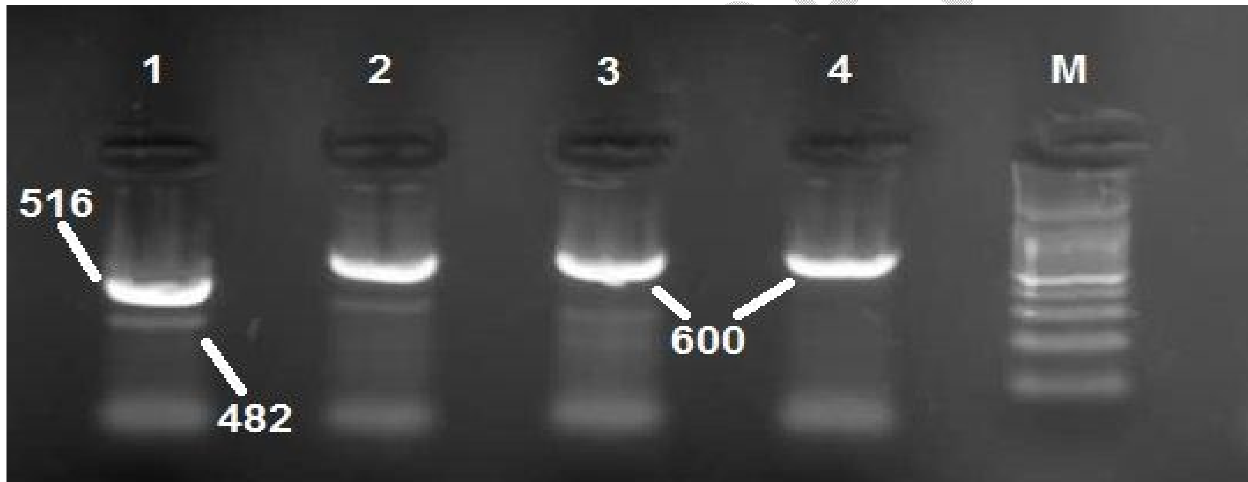

Figure 1. Genotyping of resistant genes of *Escherichia coli* using multiplex PCR. Lane 1: The 482 bp band for *aac (6)-Ib-cr* and 516 bp for qnrA genes. Lanes 2, 3 and 4: 600 bp band for 16 RNA gene. Lane M: DNA ladder (100-1000 bp).

Discussion

Molecular detection of antibiotics resistance determinants is of a great value in the efforts to combat the multi drugs resistant microorganisms. Resistance to quinolone may occur as a consequence of overexpression of the AcrAB efflux system and the acquisition of plasmid resistance genes (qnr) and aac (6′)-Ib-cr [14]. The current study included 112 isolates of drugs

UNDER PEER REVIEW

resistance *Escherichia coli.* In addition to the phenotypic identification, the isolates were confirmed genetically by using *Escherichia coli* 16 rRNA gene. The study aimed to evaluate the frequency of qnrA and acrA among isolated strains.

The results showed that females had a higher incidence for *Escherichia coli* (77%) as compared to males (33%) considering gender classification. This finding agreed with the study carried out in hospitals of Anyigba, Nigeria by Mofolorunsho *et al.,* in 2021 [15], but disagreed with the study conducted by Deshmukh et al. in 2014 whom found that males had a higher prevalence rate [16]. Risk factors such sexual activities and personal hygiene have been attributed to the high infection rates among female patients [17, 18]. The anatomical structure of females genital tract (shorter urethras and closeness to anus), gives the *Escherichia coli* the ability to infect the bladder in ascending manner, and might explain the intersex variation.

The perception of community towards antibiotic usage was fully observed and documented as high percentage of study participants practiced self-medication with antibiotics, obtaining the antibiotics over the counter in community pharmacies. The antimicrobials most frequently used for self-medication included broad spectrum agent such as ceftriaxone which may have many side effects. Females were 1.6 times more likely to use self-medication than males. This finding partially agree with a local study in Sudan conducted by Elmahi et al., 2018 (19). The study discussed the reasons for using over-the-counter treatment and stated that the main reason is the long distance to health services [19]. The size of self-medication problem with antimicrobials, as previously mentioned, is much higher in developing countries as compared to developed ones [20]. Furthermore the high percentage of self-medication reported in this study, may be explained by patients' inability to afford consultation fees and the poor governmental control on drugs trading.

UNDER PEER REVIEW

Meropeneme against *Escherichia coli* as demented in this study had the best effect, and is higher than a recent report from Khartoum by Elbadawi et al. 2017 whom found a resistance rate of 9 % [21]. The quinolone (ciprofloxacin) resistance rate among the isolates was 55%, similar results were obtained in studies conducted in Sudan [22, 23]. All results in Sudan were in agreement with studies conducted in Thailand where high resistance to ciprofloxacin, ceftazidime and cefotaxime were reported [24. 25]. It can be said that, resistance to ciprofloxacin varies geographically and is an emerging problem in both developed and developing countries [26].

In the current study, 65% of participants whom used ciprofloxacin reported that they didn't completed the recommended dose due to the side effects of the drug which may explain the increasing rate of resistance. According to multi resistance (MDR) classification established by European Centre for Disease Prevention and Control (ECDC) and the Centers for Disease Control and Prevention (CDC), 94.8% of Escherichia coli isolated in this study were considered MDR. Similar results obtained from recent study conducted by Azab et al 2021 in three countries; Sudan Egypt and KSA, The ratios of the multidrug-resistant strains for Egypt, Saudi Arabia, and Sudan were 74.4%, 90.1%, and 97.5%, respectively [27]. The behavior of Sudanese and Egyptian citizens toward antibiotic usage is almost similar in addition to the geographical location. This may explain the similar result obtained from Sudan and Egypt.

Plasmid-mediated genes, such as qnr and aac (6) may facilitate spread and increase the prevalence of quinolone-resistant strains [28]. The Multiplex PCR assay, demonstrated that among the 119 isolates, 49 (41.2%) isolates were positive for the qnrA gene. This frequency found in this study is higher than those reported in Eygept, where qnrA genes were found at 26.6 % in Klebsiella pneumoniae isolates [299]. But was in accordance with reports from Togo and Niger where Qnr gene prevalence was 47% and 44.4% respectively [30, 31].

UNDER PEER REVIEW

The aac (6′)-Ib-cr (cr for ciprofloxacin resistance) is a variant of aac (6')-Ib with two amino acid substitutions compared to the wild-type allowing it to acetylate and subsequently reduce the activity of norfloxacin and ciprofloxacin [32. 33], in the current study the frequency of aac (6′)-Ib-cr was found to be 37.8%, this finding is higher than that reported in Iran where Zahra et.al estimated the prevalence of the gene by 25% [34]. The source of the qnr genes might associated with the selective pressure caused by the quinolones used in medical setting, or horizontal transmission. And these genes are usually transported by the plasmid and can easily spread among the members of enterobacteriaceae.

On the other hand, a significant portion of isolates not carrying those qnrA and aac (6′)-Ib-cr genes also showed phenotypic resistance to the ciprofloxacin. The inconsistency of the genotype-phenotype association could be explained by other resistance genes or factors that have not been addressed in this study.

Conclusion

This is the first report of qnrA and aac(6′)-Ib-cr gene among *Escherichia coli* using multiplex PCR in Sudan depending upon similar results in many countries supporting the wide occrance of qnrA and aac(6′)-Ib-cr genes. The study suggests that the multiplex PCR method can be highly sensitive and specific in detecting genetic determinants of multidrug-resistant microorganisms.

UNDER PEER REVIEW

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UNDER PEER REVIEW

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UNDER PEER REVIEW

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UNDER PEER REVIEW

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UNDER PEER REVIEW