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Multiplex PCR Detection of Quinolones Resistance Genes (QnrA and AcrA) among Multi Drugs Resistance Klebsiella pneumoniae

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Abstract: Background*: Quinolones are group of massively used molecules worldwide 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.* ***Aim:*** *The current study reports on Isolate and identifies the K. pneumoniae in patients attending wad Medani teaching hospitals, Determine the drug resistance patterns and asses the Prevalence of quinolones resistance genes (qnrA and acrA) among Klebsiella pneumonea clinical isolates.* ***Methodology:*** *This was cross-sectional descriptive, hospital-based study included 102 Klebsiella pneumonea clinical isolates. A structured questionnaire was given to study subjects for demographic information (age, gender, marital status) 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. The presence of qnrA and acrA genes was assessed by multiplex PCR.* ***Results:*** *Meropeneme against K. pneumoniae had the best effect in antimicrobial susceptibility tests with resistance rate of 15%. The ciprofloxacin resistance rate among the isolates was 56.9%. The multi-drug resistance (MDR) isolates represent 92.9%. The Multiplex PCR assay, using specific primers, demonstrated that among the 102 isolates, 19 (18.6%) and 84 (82.3%) isolates were positive for the qnrA and acrA gene, respectively.* ***Conclusion:*** *This is the first report of qnrA and acrA gene among K. pneumoniae using multiplex PCR technique in Sudan. There is a high prevalence of acrA genes among K. pneumoniae isolates which necessitate the use of molecular tools in detecting the genetic determinants of multidrug resistance microorganisms.*

Keywords: Multi-drug resistance, Klebsiella pneumoniae, Ciprofloxacin**,** Sudan.

Introduction:

Pathogenic microbes that have become resistant to antibiotic agents therapy are an increasing Public health problem. The situation in Sudan is of special concern because self-medication and the use of antibiotics without medical guidance are largely propagated by inadequate regulation of the distribution and sale of prescription drugs [(1)]. K. pneumoniae can cause clinical infections including pneumonia, respiratory tract infection, urinary tract infection, wound infection, and bacteremia [(2)]. Resistance to quinolones in clinical isolates of Enterobacteriaceae family was first studied in K. pneumoniae strain [(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 the selection of an appropriate antibiotic an increasingly more difficult process [(5)].

Quinolones are class of massively used molecules worldwide for the treatment of an array of infectious diseases. Quinolones 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, topoisomerase IV, overexpression of the AcrAB efflux system and the acquisition of plasmid resistance genes [(7, 8)]. Since the appearance of plasmid quinolone resistance genes, an outsized number of qnr alleles are found on plasmids or bacterial chromosome. About 100qnr genes variant have been described mainly from Enterobacteriaceae, and grouped into 5 distinct families: qnrA, qnrB, qnrC, qnrD and qnrS [(9)]. Efflux pumps are transport proteins involved within the transport of toxic substrates from intracellular into the extracellular environment. AcrAB and QepA efflux pumps are reported in clinical isolates of E. coli and K. pneumoniae. These efflux proteins can change the permeability of the

bacterial membrane by drug extrusion to outside, therefore the antibiotic resistance occurs. AcrAB and QepA multidrug-resistant efflux proteins belong to the major facilitator superfamily group and associate with decreased susceptibility to quinolone [(10)]. The aims of this study is to Isolate and identify the K. pneumoniae, Determine the drug resistance patterns of the isolated bacteria and asses the Prevalence of quinolones resistance genes (qnrA and acrA) among Klebsiella pneumonea clinical isolates from Wad Medani Teaching Hospitals, Wad Medani, Sudan.

Materials and 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 Madani emergency hospital, Wad Madani Teaching Hospital, Gazira hospital for renal diseases and surgery, National cancer institute, Wad Medani pediatric Hospital, Wad Medani Teaching Hospital for obs And Gyn). 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 K. pneumoniae by conventional biochemical methods, at the period of this study were included in this study. While Isolates of other klebsiella species were excluded from this study.

Data collection and Processing

One hundred and four strains of K. pneumoniae 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. 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 [(13)]. The following antibiotic discs were used: (Meropeneme, Cefixime, Amoxicillin/Clavulanic Acid, Ceftriaxone, Ciprofloxacin, Gentamicin, Azithromycin) 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 centrifuged 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 K. pneumoniae isolates**

Gene	Sequence	Product Size
AcrA	F TCTGATCGACGGTGACATCC R TCGAGCAATGATTTCCTGCG	157
QnrA	F ATTTCTCACGCCAGGATTTG R GATCGGCAAAGGTTAGGTCA	516
K. pneumoniae 16 rRNA	F TGTTGCTGAAGGAGTTGGGC R ACGACGGCATAGTCATTTGC	340

A. Preparation of primers

For 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)].

B. Preparation of reaction mixture

The following reagents were used the following volumes (total reaction volume was 20 µl) in 0.2 ml PCR tube;

- 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).
- 3 µl DNA (template DNA).

C. Thermo-cycler conditions:

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).

Results:

Demographic Characteristics:

The 102 strains of Klebsiella pneumoniae were recovered from different hospitals in wad-Medani city with different types of samples **(Table 2)**. A total of 43 strains were isolated from male patients (42%) and 59 from female patients (57%). The study subjects distributed to 3 age groups: pediatric (1 – 15 years), adults (16-55 years) and geriatric (above 56 years) representing 20%, 38% and 42% respectively.

(Table 2) Distribution of K. pneumoniae isolate among different hospitals and samples types:

Hospital	Sample type					Total
	Wound Swab	Urine	Throat Swab	Ear Swab	Sputum	
Wad Medani Emergency Hospital	0	20	0	0	4	24
Wad Medani Teaching Hospital (Surgery department)	14	0	0	0	0	14
Wad Medani teaching hospital for Obs. and Gyn.	0	4	0	0	0	4
Gazira Hospital For Renal Diseases and surgery	4	16	0	0	0	20
National Cancer Institute	10	4	0	0	0	14
Pediatric Hospital	4	4	0	0	0	8
National Center for Pediatric Surgery	14	0	0	0	0	14
ENT Hospital	0	0	2	2	0	4
Total	46	48	2	2	4	102

41.2% of study participants reported that they had received antibiotics without medical prescription at least one time. The antibiotics most frequently used for self-medication were azithromycin, Ceftriaxone and Amoxicillin representing 21.5%, 19.3% and 18% respectively. Also **55.9 %** of participants reported that they have home drug store.

Antimicrobial susceptibility profile:

Meropeneme against K. pneumoniae had the best effect in antimicrobial susceptibility tests. The ciprofloxacin resistance rate among the isolates was 56.9% Antibiotic susceptibility testing results for clinical isolates of Klebsiella pneumoniae is shown in (table 3).

According to multi drug resistance (MDR) classification established by European Centre for Disease Prevention and Control (ECDC) and the Centers for Disease Control and Prevention (CDC), 92.9% of K. pneumoniae isolated in this study were MDR.

(Table 3) Antibiotic susceptibility testing results:

Antibiotic	Resistance %	Intermediate %	Sensitive %
Ciprofloxacin	57	06	37
Gentamicin	27	05	68
Cefixime	62	25	13
Ceftriaxone	78	10	12
Meropeneme	15	02	83
Amoxicillin/Clavulanic Acid	73	17	10
Azithromycin	63	25	12
Vancomycin	94	01	05

Multiplex PCR assay:

The distribution of the antibiotic resistance genes in the K. pneumoniae isolates is shown in (Table 4). The Multiplex PCR assay, using specific primers, demonstrated that among the 102 isolates, 19 (18.6%) and 84 (82.3%) isolates were positive for the qnrA and acrA gene, respectively, showing that t acrA was circulating with a high prevalence. Also the PCR assay included a confirmatory gene (K.P16 rRNA) to exclude the other species in the genus klebsiella. The accuracy of the conventional biochemical tests used in this study found to be 89.4%.

(Table 4) Frequency and distributed of Genes out of Isolated K. Pneumoniae:

No of Genes detected	Type of Genes	No of isolated organisms	Percentage %
2 Genes	qnrA + acrA	16	15.6 %
1 Genes	qnrA	19	18.6 %
	acrA	84	82.3
Zero gene	No genes	14	13.7

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) [(14)]. The current study included 102 isolates of multi drugs resistance Klebsiella pneumoniae. In addition to the phenotypic identification, the isolates were confirmed genetically by using Klebsiella pneumoniae 16 rRNA gene. The study aimed to evaluate the frequency of qnrA and acrA among Klebsiella pneumoniae isolates.

The results showed that females had a higher incidence of K. pneumoniae (57%) as compared to males (42%) considering gender classification. This finding agreed with the study carried out in Kenya by Nibogora et al., in 2016 [(15)] but disagreed with the study conducted by Deshmukh et al. in 2014 whom found that males had a higher prevalence of K. pneumoniae as compared to females [(16)]. The anatomical structure of Females genital tract (shorter urethras and closeness to anus), gives the K. pneumonia the ability to infect the bladder in ascending manner, and might explain the intersex variation in K. pneumoniae incidence. High frequency in K. pneumoniae was observed in geriatrics (age groups above 56 years) in the current study which was in accordance with others studies (Jesmin et al., 2014, Lina et al., 2007; Meatherall et al., 2009) [(17,18,19)]. These observations may indicate that age can be considered as risk factor for K. pneumoniae infection. The perception of community towards antibiotic usage was fully observed and

documented as high percentage of study participants (41.2%) practiced self-medication with antibiotics, obtaining the antibiotics over the counter in community pharmacies. The antibiotics most frequently used for self-medication were azithromycin, Ceftriaxone and Amoxicillin representing 21.5%, 19.3% and 18% respectively. The commonest symptom which associated with self-medication was pneumoniae (31%). 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 reported that the prevalence of self-medication was 60% and concluded the major cause behind taking antibiotics without prescription was the long distances to healthcare facilities [(20)]. The prevalence of self-medication with antibiotics, as previously mentioned, is much higher in developing countries as compared to developed ones [(21)]. 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, considering that 55.9 % of participants reported that they have home drug store. Meropeneme against K. pneumoniae had the best effect on antimicrobial susceptibility tests with resistance rate of 15%. The present rate of Meropeneme resistance is lower than a recent report from Khartoum by Elbadawi et al. 2017 whom found a resistance rate of 23.2% [(22)]. The quinolone (ciprofloxacin) resistance rate among the isolates was 56.9%. Similar results were obtained by Shatalov 2015 in Equatorial Guinea who reported (53.7%) resistance rate [(23)]. Another study in Sudan 2020 reported a 77% resistance rate for Ciprofloxacin [(24)]. This was alarming result demonstrate an increasing in K. pneumoniae ability to overwhelm the defense lines of healthcare providers. Resistance to ciprofloxacin varies geographically and is an emerging problem in both developed and developing countries [(25)]. In the current study, 65% of participants whom used Ciprofloxacin reported that they don't complete the recommended dose due to the side effects of the drug which may explain the increasing rate of resistance. According to multi drug resistance (MDR) classification established by European Centre for Disease Prevention and Control (ECDC) and the Centers for Disease Control and Prevention (CDC), 92.9% of K. pneumoniae 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 [(26)]. 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. The Multiplex PCR assay, using specific primers, demonstrated that among the 102 isolates, 19 (18.6%) isolates were positive for the qnrA gene. This result was in partial agreement with a study from Khorramabad (Iran), reported that (12.1%) isolates of Klebsiella were positive for qnrA gene [(27)]. These frequencies found in this study are higher than those reported in Côte d'Ivoire where qnrA genes were found at 9.9 % in Klebsiella pneumoniae isolates [(28)]. In Morocco, qnrA was found at 10% Klebsiella spp. [(29)]. Quinolones are the most common antimicrobial used in urinary tract infection; hence 47% of K. pneumoniae in the present study was isolated from urine samples, the source of the qnr gene might associated with the selective pressure caused by the quinolones used in medical setting , or horizontal transmission.

AcrAB efflux pump is one of the main chromosomal mechanisms of resistance to quinolones in Enterobacteriaceae family [(30, 31, 32)]. This efflux protein is one of the important mechanisms in multidrug-resistant E. coli and K. pneumoniae isolates. This study showed high prevalence rate of acrA (84.3%). These results was in accordance with a study conducted in Iran 2017 demonstrated that prevalence rate of acrA was (94%) [(33)]. Another study conducted by Hasdemir et al. in 2004 demonstrated that the AcrAB efflux pump system participated in resistance to fluoroquinolones in multidrug resistant K. pneumoniae strains isolated from Turkey [(34)]. On the other hand, a significant portion of isolates not carrying those qnrA and acrA 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 acrA gene among K. pneumoniae using multiplex PCR technique from Sudan depending upon similar results in many countries supporting the wide distribution of qnrA and acrA genes. (18.6%) and 84 (82.3%) of K. pneumoniae isolates were positive for the qnrA and acrA gene respectively. The study suggests that multiplex PCR method can be highly sensitive and specific in detecting the genetic determinants of multidrug resistance microorganisms.

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