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ORIGINAL ARTICLE

Detection of *TEM, AmpC, SHV, CTX-M,* and *MCR-1* Genes in Gram Negative Isolates of Urinary Tract Infections

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SUMMARY

Background: **Urinary tract infection (UTI) is an infection caused by the presence and growth of microorganisms anywhere in the urinary tract. It is usually due to bacteria from the digestive tract which climb the opening of the urethra and begin to multiply to cause infection. However, UTI is more frequent in female than male, because of the short urethra, absence of prostatic secretion, and pregnancy.**
Methods: **This study was aimed to detect extended spectrum beta lactamase and *MCR-1*genes from Gram negative bacterial clinical isolates from urinary tract infections. Ninety-one urine samples were collected in this study, then cultured on CLED agar and identified by conventional biochemical methods. Modified Kirby-Bauer method was used for sensitivity testing. Genomic DNA extracted by boiling method, and multiplex PCR was conducted to amplify *TEM, AmpC, MCR-1, SHV,* and *CTX-M* genes from all Gram-negative isolates.**
Results: **The result of the susceptibility test revealed that the highest resistant rates were 73% for Ceftazidime, followed by 63%, 56%, and 55% for Ciprofloxacin, Gentamicin, and Co-trimoxazole, respectively, and 21% were resistant to Imipenem. Moreover, for the presence of resistance genes, multiplex PCR results displayed that the *TEM* gene was present in 34% of bacteria, *AmpC* gene was found in 49.4% of isolates. Also, 38.5% and 6.6% were positive for *MCR-1* gene and *SHV* gene, respectively. All *Proteus* species were negative to *MCR-1* and *TEM* genes. Fifty *E. coli,* 7 *Klebsiella pneumonia,* two *Pseudomonas aeruginosa,* and five *Proteus* species were positive for *CTX-M* gene and all *Citrobacter* spp. were negative for *CTX-M* gene. Eighty-nine isolates were positive for one or more ESBL genes, while two isolates were negative to all genes.**
***CTX-M* gene is predominant among uropathogenic bacteria and imipenem is the best effective antibiotic.**
Conclusions: **This recent study proved that the result of the susceptibility test revealed that the highest resistant rate were 73% for Ceftazidime, followed by 63%, 56%, and 55% for Ciprofloxacin, Gentamicin, and Co-trimoxazole, respectively, and 21% were resistant to imipenem.**
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INTRODUCTION

Urinary tract infection (UTI) is an infection caused by the presence and growth of microorganisms anywhere in the urinary tract. It is usually due to bacteria from the

digestive tract which climb the opening of the urethra and begin to multiply to cause infection [1,2]. However, UTI is more frequent in female than male, because of the short urethra, absence of prostatic secretion, and pregnancy [3].

The "mobilized colistin resistance" (*mcr-1*) gene confers plasmid-mediated resistance to colistin, a polymyxin, and one of a number of last-resort antibiotics for treating infections [1,2]. The gene is found in no less than ten species of the Enterobacteriaceae: *Escherichia coli*, *Salmonella*, *Klebsiella pneumoniae*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Cronobacter sakazakii*, *Shigella sonnei*, *Kluyvera* species, *Citrobacter* species, and *Raoultella ornithinolytica*.

The mechanism of resistance of the MCR-1 gene is a phosphatidylethanolamine transferase. The enzyme transfers a phosphoethanolamine residue to the lipid A present in the cell membrane of Gram-negative bacteria. The altered lipid A has much lower affinity for colistin and related polymyxins resulting in reduced activity of the antimicrobial. This type of resistance is known as target modification. Although the same mechanism has been observed before with enzymes like *eptA*, *mcr-1* is the first polymyxin resistance gene known to be capable of horizontal transfer between different strains of a bacterial species [1].

The gene was first discovered in *E. coli* (strain SHP45) from a pig in China April 2011 and published in November 2015 [5,6]. It was identified by independent researchers in human samples from Malaysia, China [1], England [7,8], Scotland [9], and the United States [10]. Moreover, antibiotic-resistant gram-negative bacteria are a challenge for clinicians due to their limited therapeutic options. Specifically, *Enterobacteriaceae* are common causes of both hospital-acquired and community-acquired UTIs. Antibiotics resistance genes include extended-spectrum-lactamases (ESBLs), ampC-β-lactamase, and carbapenemases [7,8]. Since 1998 most of the commonly derived ESBLs genes are from TEM and SHV, recently CTX-M types are more often reported worldwide [20].

Also, increasing antibiotic resistance complicates its treatment by increasing patient morbidity, costs of reassessment and retreatment, rates of hospitalization, and use of broader spectrum antibiotics [7]. Unfortunately, despite the widespread availability of antibiotics, UTI remain the most common bacterial infection among the human population. It is assumed that although being considered benign diseases, in near future UTI will probably once become deadly disease and will be hard to treat. Culture and antimicrobial drug susceptibility testing are needed for surveillance purposes to guide the clinicians on the proper management and prevent empirical treatment to minimize further burden on antibiotic resistance [8,9].

Besides, extended spectrum beta lactamase genes (ESBL) producers have a wide clinical significance and high impact in healthcare systems especially in developing countries, where ESBL dissemination higher [10]. It is to be noted that ESBL producers have been reported to be associated with urinary tract infections among other kinds of infections such as pneumonia, septicemia, intra-abdominal infections, and meningitis [11,12]. In Khartoum state, the prevalence status of ESBLs and MCR-1 genes in UTI are not well documented, consequently, this study aimed to detect extended spectrum beta lactamase and MCR-1genes from Gram-negative bacterial clinical isolates from urinary tract infections (UTI) in Khartoum State and to point out the sensitivity profile for isolated bacteria.

MATERIALS AND METHODS

Samples

Urine samples were collected from males and females. All samples were collected randomly from Khartoum city, Sudan. Only midstream urine was collected in sterile containers, then immediately transferred to laboratory in ice-packs.

Bacterial isolation

The specimens were immediately inoculated on CLED agar (Himedia laboratories Ltd, Mumbai, India). Specimens were incubated aerobically for 24 hours at 37°C. Pure colonies were sub cultured on nutrient agar medium (Himedia laboratories Ltd, Mumbai, India) aerobically for 24 hours at 37°C. All isolates were subjected to standard confirmatory tests (kliger iron agar, tryptophan peptone water, semisolid media, Simmon's citrate agar, Christensen's urea agar) (Himedia laboratories Ltd, Mumbai, India) [13]. *E. coli* ATCC 25922 was used for quality control.

Antimicrobial susceptibility testing

The standard disc diffusion method was used (Kirby-Bauer) for sensitivity tests. The following antibiotics discs were used: Gentamicin (10 µg), Ciprofloxacin (5 µg), Ceftazidime (30 µg), Imipenem (10 µg), and Co-trimoxazole (30 µg) (Himedia Laboratories Pvt. Ltd, Mumbai, India), according to CLSI guidelines [1].

DNA extraction and detection of resistant genes by multiplex PCR

Genomic DNA template was extracted by boiling method [14]. Briefly, from overnight growth of bacterial isolates, several colonies were suspended in 500/µL of sterile deionized water and boiled for 15 minutes. After centrifugation of the boiled samples at 14,000 g for 10 minutes, supernatant was stored at -20°C as a template DNA stock. The purity of the extracted DNA was determined by running the DNA sample on 1.5% gel agarose stained with ethidium bromide (20 mg/dL) and then visualized under UV light [15].

Multiplex PCR was carried out using TECHNE® Ltd, peltier thermal cycler (Germany), DNA amplification was done using 5 µl Maxime PCR PreMix kit (iNtRON, Korea), 1 µl DNA template and 0.5 µl (10 pmol) of each

Table 1. Amplicon sizes and primers used in this study.

Primer	Sequence (5 - 3)	Amplicon sizes bp
TEM-F	**ATGAGTATTCAACATTTCCGTG**	**861**
TEM-R	**TTACCAATGCTTAATCAGTGAG**	**861**
SHV-F	**ATTTGTCGCTTCTTTACTCGC**	**1,050**
SHV-R	**TTTATGGCGTTACCTTTGACC**	**1,050**
AmpC-F	**ATCAAAACTGGCAGCCG**	**550**
AmpC-R	**GAGCCCGTTTTATGGACCCA**	**550**
CTX-M-F	**SCSATGTGCAGYACCAGTAA**	**500**
CTX-M-R	**CCGCRATATGRTTGGTGGTG**	**500**
MCR-1-F	**CGGTCAGTCCGTTTGTTC**	**309**
MCR-1-R	**CTTGGTCGGTCTGTAGGG**	**309**

Table 2. Results of the association between the presence of ESBLs genes and age group.

Gene	Age group (year)				p-value
	1 to 20	21 to 40	41 to 60	61 to 80	
AmpC	**5**	**18**	**12**	**10**	**0.12**
CTX-M	**6**	**24**	**11**	**23**	**0.03**
TEM	**2**	**15**	**6**	**8**	**0.12**
SHV	**1**	**2**	**1**	**2**	**0.21**
SHV	**4**	**14**	**8**	**9**	**0.11**

gene specific primer (5 µl) and 14 µl distilled water up to a final volume of 25 µl. The following primers were used: bla$_{SHV}$, *bla*$_{TEM}$, *bla*$_{CTX-M}$, bla$_{MCR-1}$, bla$_{AmpC}$ (Macrogen; Korea) (Table 1) [16]. PCR amplification conditions were as follows: initial denaturation step at 95°C for 5 minutes; 30 cycles of denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute, extension at 72°C for 1 minute, followed by a final extension step at 72°C for 10 minutes as designed in the current study. The PCR product was visualized in 1.5% gel agarose stained with ethidium bromide (20 mg/dL) [15]. DNA ladder (100 bp DNA ladder, iNtRON, Korea) used as marker. It was then photographed by U.V. transilluminator (Uvitec - UK).

Ethical approval

All patients (or co-patients) have been asked to sign consent prior to specimen collection. Verbal consent and approval of the study was given by the laboratory administration.

Statistical Analysis

Data were introduced to Statistical Package for Social Sciences 16.0 software (SPSS Inc., Chicago, IL) for analysis. p-value ≤ 0.05 was considered significant using chi squared test. In addition, means, frequencies and averages were also calculated.

RESULTS

Demographic data of samples

A total of 91 urine samples were collected from 42 males (46.3%) and 49 females (53.7%) and cultured during the study. The mean of the age distribution of the study population was 45 ± 24 years old.

The association between the presence of ESBLs genes and age group

The majority of participants (46; 50.54%) of UTI patients infected with Gram-negative bacteria were in the 21 - 40 age group. Age group 1 - 20 contains only 8 participants (8.7%), 20 participants (21.97%) were

Table 3. The association between the presence of ESBL genes and gender.

Gene	Positive n (%)		Negative n (%)		p-value
	Male	Female	Male	Female	
AmpC	19	26 (53.06%)	23	23	1.00
CTX-M	28	36 (73.46%)	14	13	0.04
TEM	20	11 (22.44%)	22	38	0.02
SHV	3	3 (6.12%)	39	46	0.61
MRC-1	17	18 (36.73%)	25	31	0.10

Table 4. Detection of ESBLs among study isolates.

Isolate		AmpC	CTX-M	TEM	SHV	MCR-1
Citrobacter species	positive	0	0	1	0	1
	negative	1	1	1	1	0
	p-value	1.3	1.3	0.87	1.3	0.08
E. coli	positive	32	50	24	5	31
	negative	40	22	48	67	41
	p-value	0.07	0.03	0.08	0.12	0.09
K. pneumoniae	positive	6	7	2	0	1
	negative	3	2	7	9	8
	p-value	0.04	0.03	1.20	1.31	1.25
P. auroginosa	positive	2	2	4	0	2
	negative	2	2	0	4	2
	p-value	1.2	1.2	0.00	1.24	1.2
P. mirabilis	positive	2	2	0	0	0
	negative	0	0	2	2	2
	p-value	0.05	0.05	1.3	1.3	1.3
P. vulgaris	positive	3	3	0	1	0
	negative	0	0	3	2	3
	p-value	0.05	0.05	1.3	1.2	1.3
Total	positive	45	64	31	6	35
	negative	46	27	60	85	56

found belonging to the age group 41 - 60 while the age group 61 - 80 has 16 participants (17.58%). The mean of the age distribution of the study population was 45 years old. The presence of bla_{CTXM} gene has been found to be significantly associated with age (p-value = 0.03). All results were summarized in Table 2.

The association between the presence of ESBLs genes and gender

There was significant association between the presence of TEM and CTX-M genes and gender (p-value = 0.02 and 0.04, respectively) (Table 3).

Identification of isolates

Ninety-one samples revealed positive culture results under aerobic conditions. All positive culture specimens showed single microbial growth. The most common organism isolated was *E. coli* as 72 isolates (79.12%), *Citrobacter* one isolate, *K. pneumoniae* 9 isolates, *P. auroginosa* 4 isolates, *P. mirabilis* 2 isolates, *P. vulgaris* 3 isolates (Table 4).

Among 72 *E. coli* isolated; 50 isolates showed a positive result for the acquisition of $bla_{CTX\text{-}M}$ gene (p-value = 0.03). Regarding *K. pnumoniae*, a statistically significant relationship was found between the organism and

Table 5. Association between the presence of resistance genes and susceptibility to Antibiotics.

Antibiotic	Gene	Sensitive number		Resistant number		Intermediate number		p-value
		+ve	-ve	+ve	-ve	+ve	-ve	
Imipenem	*AmpC*	37	42	9	2	0	1	0.06
	CTX-M	55	25	8	3	1	0	0.11
	TEM	29	50	1	10	1	0	0.09
	SHV	6	73	0	11	0	1	0.13
	MCR-1	31	48	4	7	0	1	0.16
Gentamicin	*AmpC*	21	23	15	28	2	2	0.08
	CTX-M	27	33	10	18	4	0	0.06
	TEM	11	19	25	32	1	3	0.08
	SHV	0	6	25	46	0	4	0.11
	MCR-1	11	22	29	25	2	2	0.03
Ciprofloxacin	*AmpC*	18	25	19	26	2	1	0.09
	CTX-M	20	42	17	9	2	1	0.04
	TEM	8	23	29	28	0	3	0.09
	SHV	0	6	37	45	0	3	0.14
	MCR-1	8	26	29	25	1	2	0.08
Cotrimoxazole	*AmpC*	15	30	19	37	0	0	0.11
	CTX-M	17	47	17	20	0	0	0.05
	TEM	9	22	15	45	0	0	0.08
	SHV	1	5	23	62	0	0	0.18
	MCR-1	11	24	13	43	0	0	0.19
Ceftazidime	*AmpC*	11	31	16	29	3	1	0.09
	CTX-M	20	41	7	19	3	1	0.03
	TEM	6	24	36	21	1	3	0.07
	SHV	0	6	27	54	0	4	0.18
	MCR-1	8	27	19	33	0	4	0.16

the presence of both $bla_{CTX\text{-}M}$ and bla_{AmpC} (p-value = 0.03, 0.04, respectively). All results are summarized in Table 4.

Antimicrobial susceptibility testing

Five antibiotic discs were used to perform antimicrobial susceptibility testing: Imipinem, Gentamicin, Ciprofloxacin, Co-trimoxazole, and Ceftazidime.

A statistically significant relationship was observed between the presence of MCR-1 gene and the resistance to Gentamicin (p-value = 0.03) and between the presence of CTX-M gene and the resistance to Ceftazidime, Ciprofloxacin, and Co-trimoxazole (p-value = 0.03, 0.04, and 0.05, respectively) as indicated in Table 5.

DISCUSSION

For the frequency of ESBL genes, it is found that bla_{CTXM} is the most frequent among study isolates, as 64 isolates (70%) possess the gene. Forty-five isolates (50%) possess the bla_{AmpC} gene, 31samples (34%) possess the bla_{TEM} gene, and 6 isolates (6.5%) possess the bla_{SHV} gene. Our findings, in contrast to a recently published study in India, concluded different frequencies of the tested ESBL genes as bla_{TEM} predominated as 48.7%, followed by bla_{CTXM} (7.6%) and bla_{SHV} (5.1%). This difference may be due to their small sample size (38 samples) [16]. In Sudan Ahmed et al. [17] demonstrated different frequencies as only 22% were found positive for bla_{CTXM}, 17% for bla_{TEM} and almost similar results for bla_{SHV} as 6.8% of isolates were found positive. These differences might be due to the differences of the study time and population sample size. Another

study for detection of ESBL and AmpC by Yousif [18] stated that 78 (52%) strains produced ESBLs, whereas 4 (2.7%) organisms produced AmpC β-lactamase and 6 (4%) coproduced ESBLs and AmpC β-lactamases. This difference in AmpC gene prevalence might be due to the methodological difference adopted in the current study compared to their study. They tested the isolates for AmpC beta-lactamases using the D68C *AmpC* and ESBL detection set as well as testing the inducible *AmpC* beta-lactamases using the disc antagonistic test. No study to our knowledge has been found in literature describing the prevalence of *MCR-1* genes in Sudan. In the present investigation, the most prevalent isolated bacterial uropathogen was *E. coli*, with an isolation rate of 30%, which is similar with previous studies [19,20]. The major contributing factor for isolating at a higher rate of *E. coli* is due to a number of virulence factors specific for colonization and invasion of the urinary epithelium, such as the P-fimbria and S-fimbria adhesions [21].

Gender appeared to be a risk factor for the emergence of ESBL-producing bacteria, and the proportion of ESBLs in the current study was about 9% higher in females than in males, although some studies have shown no statistical difference in gender or a slightly higher incidence of ESBL-producing bacterial infection in men [22-25]. For instance, Shah et al. [26] studied the relationship of ESBL-producing *Enterobacteriaceae* with respect to gender and reported more ESBL-positive isolates in males (65.33%) than females (34.67%). Das and Borthakur also found a slight male preponderance for ESBL production in their study [27]. Our findings disagreed with those studies as we observed female preponderance in the present study. After applying the *Z*-test for proportions with 95% CI, this difference was found to be statistically significant ($p = 0.03$). However, the low sample size in the current study should not be underestimated.

The current situation of resistance to antibiotics has reached a serious point in urinary tract infection and presently, multidrug-resistant bacteria including ESBL-producing bacteria can be readily encountered in clinics. Antibiotics that can be used for the treatment of multidrug-resistant bacteria including ESBL-producing bacteria in urinary tract infection are limited [28]. In our study, excluding imipenem, antibiotics had a sensitivity higher than 60% to ESBL-producing gram negative isolates. Only 21 isolates (58%) from ESBL-producing organisms were sensitive to Gentamicin, 49.6% to Ciprofloxacin, and 80% to Imipinem. Ceftazidime was effective only against 26.19% of ESBL-producing organisms in the study. Thus, low sensitivity of ESBL producing *Enterobacteriaceae* was observed for Gentamicin, Ciprofloxacin, and Ceftazidime. Rudresh and Nagarathnamma [29] reported similar susceptibility patterns for ESBL isolates with 46.9% isolates sensitive to gentamicin followed by Ciprofloxacin (29.5%) and Ceftazidime (23.4%), while Das and his team in their study had showed 30.96% sensitivity toward Ceftazidime [27]. In Sudan, Ibrahim et al. [30] concluded that 81% of ESBL producing isolates were resistant to Ciprofloxacin compared to 49.06% observed in the current study. Nevertheless, as Ibrahim and colleagues adopted the double-disk diffusion method to confirm ESBL production using antimicrobial disks of Ceftazidime and Cefotaxime, there might be a source of false positive results.

CONCLUSION

The results of the susceptibility test showed that Ceftazidime had the highest resistance rate of 73%, followed by Ciprofloxacin, Gentamicin, and Co-trimoxazole at 63%, 56%, and 55%, respectively. Imipenem resistance was found in 21% of the patients.

Availability of Data:
The datasets generated during the current study are available from the corresponding author on reasonable request.

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Declaration of Interest:
The authors declare no conflicts of interest in this work.

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