Annals of Biology **39** (2) : 236-243, 2023

Impact Score : 0.32 (Scopus)

Prevalence of *pks*-positive *Escherichia coli* in Iraqi Patients with Urinary Tract Infections or Bladder Cancer

SARAH ALI NEHMAA*, AALAA A. CHMAGH AND KHAIRALLAH A. S. MOHAMMED

Medical Laboratory Techniques, College of Health and Medical Technology, Southern Technical University, Basra, Iraq

**(e-mail: barakatya94@gmail.com; Mobile: 964 0 77233 54137)*

(Received: January 4, 2023; Accepted: February 11, 2023)

ABSTRACT

Escherichia coli has been considered as one of the most common microorganisms associated with UTIs. Colibactin is a bacterial toxin encoded by *pks* pathogenicity island which is composed of *clbA*-Sgenes. The aim of this study was to investigate the prevalence of *pks*+*E. coli* among patients with urinary tract infections and bladder cancer. *E. coli* isolates were identified by routine microbiological methods. PCR with specific primers were used to confirm the *E. coli* identification and for phylogenetic grouping and *pks* genes. *clbB* gene was used as a main marker in addition to *clbQ* and *clbA* genes, which are closely located near to the 5′ and 3′ ends of the *pks* island. Antibiotics susceptibility tests were performed by using disc diffusion and VITEC methods. One hundred & thirty six *E. coli* isolates were identified containing 50, 25.5, 23.5 and 1% belonging to B2, A, B1 and D phylo-groups, respectively. Out of 94 *E. coli* strains showed 100% resistance to ceftizoxime, cefsulodin, cefuroxime and norfloxan, followed by piperacillin (94.73%), ticarcillin (94.63%), trimethoprim (91.13%) and tetracycline (90%) and high susceptibility (> 85%) to Ceftazidime/Avibactam, Amikacin, Imipenem, Meropenem and gentamycin. Out of 84 strains, 12 *E. coli* strains (8.82%) showed positive results for all tested genes (*ClbA*, *ClbB*, *ClbQ*), of which 9 (75%) strains were isolated from UTIs and 3 (25%) strains from patients with bladder cancer. Most of the *pks*+ strains (75%) belonged to phylogenic B2 group. These findings provided essential baseline data, which would contribute to understanding facts of the epidemiology of possibly genotoxic phenotypes of *E. coli* and their clinical consequence.

Key words: *E. coli*, *clbB, clbA, clbQ,* B2 phylogroup

INTRODUCTION

The most common bacterial infections that yearly affect 150 million individuals worldwide are the infections of urinary tract (McLellan and Hunstad, 2016). Although both men and females are infected by urinary tract infections; more frequently are in females, among whom more than 60% are affected during their lifetime (Klein and Hultgren, 2020). The severity of UTIs ranges from asymptomatic bacteriuria to urosepsis and 30% of the infected women suffer from recurrent infections (Klein and Hultgren, 2020). In addition to morbidity, mortality, the continuous use of antibiotics in UTIs treatment strictly contributes to the universal issue of antibiotic resistance. Uropathogenic *E. coli* (UPEC) causes about 80% of urinary tract infections. These organisms mostly belong to B2 phylogenic group (Belas *et al.*, 2022). Several virulence factors such as cytolethal distending toxins (CDT) and cytotoxic necrotizing factors (CNF) are involved in the pathogenicity of UPEC (Firoozeh *et al.*, 2022). Recent studies demonstrated that large numbers of UPEC strains harbored a specific genomic island called polyketidesynthetase (*pks*) island. This genomic island comprises cluster of genes (*clbA-S* genes) that is responsible for encoding peptide-polyketide hybrid genotoxic-cyclomodulin which is termed as colibactin (*clb*) (McErlean *et al.*, 2019). This toxin (collabtin) initiates DNA interstrand cross links causing DNA damage which can result in gene mutations (Prasad, 2023). Recently, many studies demonstrated the association *E. coli* producing colibactin with bladder cancer and colorectal cancer (Dejea *et al.*, 2018; Pleguezuelos-Manzano *et al.*, 2020). Hence, *pks* island can play a role as carcinogenic risk factor and could be used as a foretell biomarker for cancer development. Until now, there is no existing information about the prevalence of these *pks*+*E. coli* amongst Iraqi patients. In this study, we investigated the prevalence and

the phenotypic characterization of *pks*+UPEC among patients suffering from urinary tract infections and bladder cancer.

MATERIALS AND METHODS

Total of three hundred and seventy midstream urine samples were collected from patients with UTI (250) and bladder cancer (50) who attended different hospitals in Basra province, whereas 70 samples were collected from healthy people.

The collected samples were inoculated on MacConkey agar and eosin methyl blue agar at 37°C for 24 h incubation. A single pure isolated colony was transferred to Brain-Heart infusion agar medium to carry out other biochemical tests and for the preservation and identification of isolates. All the isolates were examined for their shape, size, colour and Gram stain reaction. The *Escherichia coli* isolates were identified according to their morphological features on culture medium (MacConkey agar and eosin methyle blue agar), biochemical tests (IMVC tests) and Gran stain. Polymerase chain reaction (PCR) was used to confirm the identification of isolated *E. coli* (Neamah *et al.*, 2022). PCR technique was also used to classify *E. coli* according to their phylogenetic groups (Kirtikliene *et al.*, 2022).

Susceptibility tests were determined for *E. coli* isolates to 28 different antibiotics by disc diffusion method as recommended by Clinical and Laboratory Standard Institute (CLSI 2019) and by VITEK 2 compact system (bioMérieux, Inc., Durham, NC, software ver-sion 8.01 and AST-GP580) VITEK.

PCR was used to detect selected *pks* genes (*clbA*, *clbB*, *clbQ;* Shimpoh *et al.*, 2017). The primers used in this study are shown in Table 1.

In order to extract the genomic DNA, the bacterial isolates were cultured overnight in 10 ml of broth at 37°C. The extraction was carried out by using a commercial kit (Promega kit), according to manufacturer's instructions. The polymerase chain reaction mix used a final volume (25 µl) containing 2 µl of DNA, 1 µl of each primer (12.5 µl) master mix and (8.5 µl) nuclease free water. The (PCR) amplification was done under the following situation conditions: initial denaturation at 94°C for 4 min (1 cycle), then 30 cycles were performed: denaturation 94°C for 30 sec, annealing temperature 56°C for 30 sec (annealing temperature changed depending on primer as shown in Table 1), followed by extension of 72°C for 1 min. The final extension was at 72°C for 4 min. PCR products were subjected to electrophoresis on 2% agarose gels and visualized using a UV light trans-illuminator.

For DNA sequencing, 20 µl of PCR products of selected *E. coli* isolates were sent to Macrogen Company (Seoul, South Korea). According to Table 1, forward primer was used for DNA sequencing (Alfinete *et al.,* 2022; Wong *et al.,* 2022). The sequences obtained were analyzed and aligned to reference gene recorded in the National Center for Biotechnology using the Bio Edit program (Octaviana *et al.*, 2023).

The sequences of the *clbA*, *clbB*, *clbQ* genes from representative iso-lates were recorded in

Table 1. Primers used in this study

Name of primers	Sequence of primers	Product size pb	Annealing temp.	Reference
	Primers for *E. coli* identification			
F3	F-GCCATCTCCTGATGACGC	204	56	12
B3	R-ATTTACCGCAGCCAGACG			
	Primers for phylogenetic groups			
Chua	F-GACGAACCAACGGTCAGGAT R- TGCCGCCAGTACCAAAGAC	279	56	13
Yaja	F- TGAAGTGTCAGGAGACGCT G RATGGAGAATGCGTTCCTCAAC	211	56	13
TCPESC2.1	F-GAGTAATGTCGGGGCATTCA R-CGCGCCAACAAAGTATTACG	152	56	13
	Primers for *pks* genes			
clbA	F-AAGCCGTATCCTGCTCAAAA R-GCTTCTTTGAGCGTCCACAT	342	55	14
clbB	F-GCGCATCCTCAAGAGTAAATA R-GCGCTCTATGCTCATCAACC	283	57	14
clbQ	F-GCAC GATCGGACAGGTTAAT R-TAGTCTCGGAGGGATCATGG	308	57	14

the GenBank data base under accession numbers OP341741, OP341742, OQ116747, OQ116748, OQ116749 and OQ116750.

RESULTS AND DISCUSSION

Out of 370 urine samples, only 250 samples were grown; 136 (38.7%) *E. coli* isolates were identified of which 98/250 (39.2%) were found in patients infected with urinary tract infection, 18/50 (36%) were found in bladder cancer and 20/70 (28.6%) were isolated from healthy people. The age average that showed the higher isolation rate was from group 31-40 years among people suspected with urinary tract infections and from group 61-70 years among cancer bladder.

A simple rapid phylogenetic grouping technique based on conventional PCR was used. The method, employed specific primers for chua, yaja and TCPEs genes. The phylogenetic grouping of the tested strains (136) was based on the presence or absence of these gene (Table 2). Phylogenetic analysis showed that *E. coli* was composed of four main phylogenetic groups (A, B1, B2 and D) and that virulent extra-intestinal *E. coli* strains mainly belonged to group B2 (58.8%; Table 3).

Table 2. Phylogenetic grouping based on genetic markers

Phylogenetic group	*ChuA*	*YjaA*	*TSPES*
B2	+	+	+/-
D	+	-	+/-
B1	-	-	+
A	-	+/-	+

Susceptibility tests were determined for *E. coli* isolates to 28 different antibiotics by disc diffusion and VITEK methods. The 94 studied *E. coli* strains showed 100% resistance to ceftizoxime, cefsulodin, cefuroxime, and norfloxan, followed by piperacillin, (94.73%), ticarcillin (94.63%), trimethoprim (91.13%) and tetracycline (90%) and moderate resistance (< 70%) to aztroznam, tetracycline, doxycycline, rimethoprim/sulfamethoxazole, ceftazidime, piperacillin/tazobactam, FEP, cefepime, moxifloxacin, minocycline, ticarcillin/clavulanic acid, levofloxacin, ticarcillin clvulanicacid (100+10mg), and ciprofloxacin. The resistance to tobramycin, amikacin, gentamycin and imipenem was 22.97, 17.56, 14.86 and 10.81%, respectively. While high susceptibility (> 85%) to ceftazidime/avibactam, amikacin, imipenem, meropenem and gentamycin was 100, 95.94, 89.18, 86.48 and 85.13%, respectively (Fig. 1). All of the 13 tested *E. coli* isolated from bladder cancer patients were extensively drug resistant. They were resistant to ticarcillin, piperacillin, cefepime, aztreonam, ceftazidime, norfloxacin, cefsulodin, tetracycline, trimethoprim, cefuroxime and ceftizoxime, but none of them was resistant to amikacin.

clbB gene was used as a main marker in addition to *clbQ* and *clbA* genes in order to confirm the presence of a complete *pks* island, which are closely located near to the 5′ and 3′ ends of the *pks* island. So, presence of *clbB* jointly with *clbQ* and *clbA* genes implied the presence of whole *pks* island and was used in the following statistical analysis. Other isolates which showed positive results for one or two genes were considered as potential *pks* positive strains and will be investigated in further study. Twelve UPEC strains (8.82%) showed confirmed positive results for all tested genes (*ClbA*, *ClbB*, *ClbQ*), of which 9 (75%) strains were isolated from UTIs, and 3 (25%)

Table 3. Distribution of phylogenic group of *E. coli* isolates among sample sources

		Type of sample			Total	*P*-value
		UTI	CA	Control		
Phylogenetic groups	A	25	2	1	28	0.013
		25.5%	11.1%	5.0%	20.6%	
	B1	23	2	1	26	
		23.5%	11.1%	5.0%	19.1%	
	B2	49	13	18	80	
		50.0%	72.2%	90.0%	58.8%	
	D	1	1	0	2	
		1.0%	5.6%	0.0%	1.5%	
Total		98	18	20	136	
		100.0%	100.0%	100.0%	100.0%	

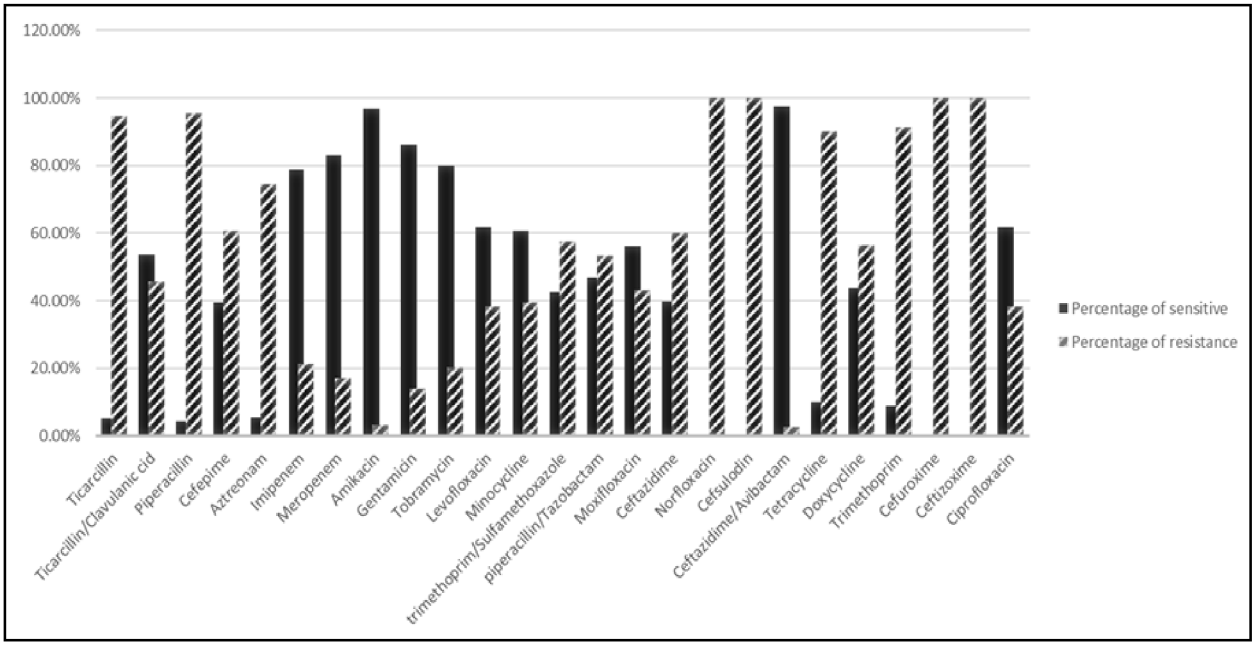

Fig. 1. Antibiotic susceptibility profile of *Escherichia coli* strains isolated from different samples (n=94).

strains were isolated from patients with bladder cancer. Most of the *pks*+ strains (75%) belonged to phylogenic B2 group, followed by B1 group (16.66%) and A group (8.33%; Table 4).

Out of 136 tested isolates, 72 (52.29%) strains had positive results for one or two of tested PKS genes, of which 43 (59.72%), 13 (18.05%) and 16 (22.22%) were from UTI, bladder cancer and healthy people, respectively (Table 4). Out of the 72 potential pks+ *E. coli,* 46 strains (63.88%) belonged to phylogenetic B2. Also, within the phylogenic groups, phylogenetic B2 group showed the highest potential pks+strains (68.75%). The DNA sequences of *clbA, clbB,* and *clbQ* genes showed 99% similarity with reference genes in National Center for Biotechnology Information and deposited in the GenBank database under accession numbers OP341741, OP341742,

Table 4. Distribution of *pks* among phylogenic groups and sample types

Phylogenic groups n (%)	Distribution of phylogenic groups among sample types n (%)		Distribution of potential *pks*+among phelygenic groups n (%)	Distribution of potential *pks*+among sample types n (%)		*pks* genes n (%)			Distribution of confirmed *pks*+among sample types n (%)	Distribution of confirmed *pks*+among phylogenic groups n (%)
						Clba	*CLbB*	*ClbQ*		
A	Healthy	1 (3.57)	12 (46.42)	Healthy	1 (100)	0 (0)	1 (100)	1 (100)	0	1 (3.57)
20 (20.58)	cancer	2 (7.14)		cancer	2 (100)	1 (50)	0 (0)	1 (50)	0	
	uti	25 (89.28)		uti	10 (40)	1 (4)	3 (12)	9 (36)	1 (4)	
B2	Healthy	18 (22.5)	46 (67.72)	Healthy	15 (83.33)	0 (0)	4 (22.22)	16 (88.88)	0	9 (11.25)
80 (58.82)	cancer	13 (16.25)		cancer	11 (84)	3 (23.07)	5 (38.46)	11 (84.61)	3 (23.07)	
	uti	49 (61.25)		uti	29 (59)	13 (26.53)	6 (12.5)	18 (36)	6 (12.24)	
B1	Healthy	1 (3.8)	13 (57.69)	Healthy	0 (0)	0 (0)	0 (0)	0 (0)	0	2 (7.69)
26 (19.11)	cancer	2 (7.69)		cancer	2 (100)	2 (100)	0 (0)	1 (50)	0	
	uti	23 (11.53)		uti	13 (56.52)	2 (8.69)	6 (26.08)	13 (56.52)	2 (8.69)	
D	Healthy	0	1 (50)	Healthy	0 (0)	0 (0)	0 (0)	0 (0)	0	0 (0)
2 (1.47)	cancer	1 (50)		cancer	0 (0)	0 (0)	0 (0)	0 (0)	0	
	uti	1 (50)		uti	1 (100)	1 (100)	0 (0)	1 (100)	0	
Total=136			72 (52.29)							12 (8.82)

Confirmed *PKS* Positive = *clbA* (+), *clbB* (+) and *clbQ* (+)
Potential *PKS* = *clbA* (+) or *clbB* (+) or *clbQ* (+) or two of them; n = number of isolates.

OQ116747, OQ116748, OQ116749 and OQ116750, respectively.

One of the most common pathogens associated with UTIs was *E. coli* (Gajdács *et al.*, 2019). The results showed that the prevalence of *E. coli* among UTI patients was slightly low compared with other international results which reported that the prevalence of *E. coli* was 50 to 57.5% (Kabugo *et al.*, 2017) but much higher than the prevalence rate found in Kurdistan Region in Iraq (21.1%). The variation of the prevalence rate could be due the differences in the sample size, different population, or the improvement in management of urinary tract infections. However according to phylogenetic analysis, *E. coli* strains could be divided into four groups and that group B2 and, to a lesser extent, group D were the main virulent extra-intestinal strains, while the majority of commensal strains fell into group A (Rangama *et al.,* 2022). This congruent with our finding, as most of the isolates associated with urinary tract infections fell in B2 phylogenic group. However, in contrast with other studies, a marked number of UPEC fell into group A. Furthermore, most of the commensal strains fell into group B2. The distribution of phylogenetic types and virulence genes may vary between different countries, for example, the majority of UPEC in China and Russia belonged to group A (Gatya Al-Mayahie *et al.,* 2022), present results can be clarified by geographical variation. The present study demonstrated that all tested strains (100%) were resistant to Norfloxacin. These results were much higher than the rate (14%) reported by Shukla *et al.* (2022) and the rate found in Rural Medical College of Maharashtra (13.3%; More *et al.*, 2017). Also, the present study demonstrated that 100% of the tested isolates were resistant to Ceftizoxime which were significantly higher than in University of Sarajevo (6.33%; Abduzaimovic *et al.*, 2016).

The results revealed high resistance to Piperacillin (95.57%), which was adjacent to the results (100%) stated by Al-Dulaimi (2016) and higher than that 35.5% obtained by Bazaid *et al.* (2021). In general, the present results concluded that the Norfloxacin resistance *E. coli* was other β-lactam antibiotics resistant. The resistance differed from one antibiotic to another that may be due to the type of antibiotic. This could also be due to how much of this antibiotic was used among patients in Iraqi community. In addition to that the resistance against any antibiotic depends on the amount of β-lactamase enzyme and its type or how many of PBB2 a produced by each strain of bacteria.

All these reasons could create variations in the rate of resistance. The tested *E. coli* isolates showed high resistance to Cefsulodin (100%) which was markedly higher than that obtained in another study in Nepal (40.8%) by Kushwaha *et al.* (2021). The tested *E. coli* isolates showed high resistance to Ticarcillin (94.68%) which was comparable to a study carried out in Hillah City, Iraq (100%) by Al-Dulaimi (2016). Additionally, the tested *E. coli* isolates showed high resistance to Tetracycline (100%) which was similar to a study (100%) carried by Sharma *et al.* (2016).

The results showed high resistance to *E. coli* against Trimethoprim (91.13%) which agreed with study obtained by Balkhi *et al.* (2018). This may be due to vast uses of antibiotics in Iraq. Comparing with 1970's, in less than 10% of the population *E. coli* isolates were resistant to trimethoprim. Reports from the 1980s showed an increasing frequency; the rates of resistance reaching 15-20% (Frimodt-Moller *et al.,* 2023). It is noticeable from the results obtained in this study that the UPEC developed resistance to the usually used antibiotics. Based on the sensitivity profile obtained in the present study, ceftazidime/avibactam, amikacin, gentamycin and imipenem can be considered as a first therapeutic choice in treatment of urinary tract infections in Basra city.

Colibactin is a bacterial toxin encoded by *pks* island which is composed of *clbA-S* genes (Chen *et al.,* 2022). This toxin is considered to be involved in creating DNA interstrand crosslinks causing DNA damage which can result in gene mutations. Recently many studies reported the association of colibactin-producing *E. coli* with colorectal and bladder cancer and indicating that *pks* island can play a role as carcinogenic risk factor and could be used as a good predictive biomarker for cancer development (Sarshar *et al.*, 2017). In this study, the prevalence *pks*+UPEC among patients suffering from urinary tract infections and bladder cancer were investigated. *cblB* gene as representative marker of the whole *pks* island was selected and confirmed by the presence of *clbA* and *clbQ* which were located

close to 5' and 3' ends, respectively. UPEC as confirmed *pks*+strains was counted when they were positive to *cblB, clbA* and *clbQ* genes. Any other strain harbored single or double *pks* gene considered as a potential *pks*+UPEC needs to be confirmed by further study.

The rate of the confirmed pks+UPEC (positive for all three genes) was 8.82% among the tested isolates, of which 75% were obtained from UTIs and 25% from bladder cancer and most of them (75%) belonged to phylogenic group B2. Furthermore, most of these isolates (75%) were multidrug resistant strains and positive for cytotoxic necrotizing factor 1 encoding gene. The present results indicated the presence of whole pks islands which might enable UPEC to synthesize functional colibactin. The results represent first epidemiological data on pks island carring *E. coli* from Iraq. The prevalence rate of pks+ strains obtained in this study was comparable to that reported by Suresh *et al.* (2018) who found that out of 462 extra intestinal *E. coli*, 35 (7.6%) were *pks*+ and most of them (97%) belonged to pathogenic phylogroup B2. In contrast, the results were much less than that reported by other studies (43. 51%) by Shimpoh *et al.* (2017). Iyadorai *et al.* (2020) found 16.7 and 4.3% of the *E. coli* were *pks* positive obtained from colorectal cancer (CRC) and healthy controls, respectively. Such variation in the results could be due to that many of the previous studies based on presence of one genetic marker which may not tell the real results or using samples different sample sizes and specimen sources. Additionally, the obtained prevalence rate of *pks*+strains in this study could be increased to 61.76% by adding the potential *pks*+strains. However, this needs to be confirmed through further studies by using a different set of primers to detect more regions in *pks* island cluster genes.

Several previous studies demonstrated that most of *pks* positive *E. coli* belonged to phylogenic group B2 which was concordant with our results (Taieb *et al.,* 2016; Sarshar *et al.,* 2017). In this study, the prevalence of colibactin producing *E. coli* was found to be sensible among clinical *E. coli* isolates. These isolates carrying combined virulence genes demonstrated multiple drug resistance. These findings provide essential baseline data which would contribute to understand facts of the epidemiology of possibly genotoxic phenotypes of *E. coli* and their clinical consequence. It is hoped to expand this result on genomic and landscape scales to have further understanding into the evolution and dissemination of such isolates at community and clinical levels.

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