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The Prevalence and Sequencing of the Pathogenic Genotoxic Gene (*clbA*) in Bacteria *E.coli* from Patients with Bladder Cancer and UTI in Baghdad Province

[1]Hayjaa M. AL-Hamadani, [2]Ashwak B. AL-Hashimy

[1]Department of Medical Laboratory Techniques, College of Medical Technology, Al-Farahidi University
[2]Institute of Genetic Engineering and Biotechnology for Postgraduate Studies, University of Baghdad

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Abstract: Bladder cancer is among the most prevalent cancers worldwide, with 549,393 new cases reported in 2018. Approximately 3% of all new cancer was diagnoses and 2.1% of all cancer deaths are due to urinary bladder cancer. Carcinoma of the urinary bladder is one of the most prevalent cancers in Iraq. This study aims to collect 50 blood and urine samples from patients suffering from urinary tract infections and bladder cancer, and 50 urine and blood samples from healthy people considered to be control, with age ranged from 30-85 years old, from different hospitals (ALyarmouk hospital, Medicine city and Royal hospital) in Baghdad/Iraq between the period from October 2022 until August 2023. Our study is intended for most likely the first time in an Iraqi series, study the relationship between *E.coli* bacteria and its gene colibactin with bladder cancer. The isolation and identification of bacteria *E.coli* from patients by traditional and molecular methods was done, and an estimate of the virulence gene (*clbA),* the result found that (4 samples) 8% of the 50 patients case were having the colibactin gene. And finally nucleotide sequencing of the PCR products of the *clbA* gene has been performed for 4 positive *clbA* gene samples.

Keywords: Colibactin, Infection, Cancer, Bladder.

Corresponding author: (hijaa.mozher1100a@ige.uobaghdad.edu.iq).

Introduction

Bladder cancer is the ninth most commonly diagnosed cancer worldwide, with more than 150,000 deaths per year, and an estimated male-female ratio of 3.8:1.0. Incidence of bladder cancer in Iraq on constant rise, with (80%) were males and (20%) were female. Urothelial cells, which are specialized transitional epithelial cells that line the urinary bladder and urinary tract, accommodate the volume of urine generated by flattening under pressure. Additionally, the bladder is lined with smooth muscle that may relax to accept larger amounts and tighten to evacuate urine through the urethra (1). The urothelial cells that line the bladder and urinary tract are always exposed to environmental chemicals that could cause mutations. The kidneys filter these chemicals out of the urine. It is not surprising that these urothelial cells, mostly found in the bladder, cause the majority of cancer cases, particularly in the developed world. Most bladder cancers may be dated directly to exposure to environmental and

occupational toxins, with tobacco smoke being the most prevalent. Men's higher exposure to cigarette smoke and occupational hazards may help explain the fourfold gender disparity in bladder cancer incidence. Following tobacco use, the likelihood of bladder cancer is next only to the risk of lung cancer (2).

Urinary tract infections (UTIs) are one of the most common bacterial infections, affecting approximately 150 million individuals each year. UTIs occur most frequently in women, with more than 60% of females diagnosed with a UTI during their lifetime. The severity of these infections ranges from asymptomatic bacteriuria and cystitis, *i.e*. infections localized to the bladder, to urosepsis, which can be fatal. Recurrences are very frequent, since approximately 30% of women experience a new UTI episode after resolution of the initial infection (3). *Escherichia coli* strains, termed uropathogenic *E. coli* (UPEC) cause approximately 80% of all UTIs. UPEC strains produce a large number of virulence factors. In particular, several toxins have long been associated with UPEC pathogenicity, such as α-hemolysin and CNF1 toxins. More recently, a large proportion of UPEC strains which carry *pks* pathogenicity island encoding the genotoxin colibactin have been described. The *pks* pathogenicity island, composed of *clbA-S* genes, encodes a polyketide- non-ribosomal -peptide (PK-NRP) biosynthesis machinery (4). Colibactin is first synthesised as an inactive prodrug by ClbN followed by the sequential interventions of multiple Clb enzymes. The ClbP peptidase subsequently cleaves the C14-Asparagine (C14-Asn) motif thereby releasing the mature, active form of colibactin with its twin warheads. The genotoxin alkylates adenine residues on both strands of DNA, producing DNA interstrand cross-links. These highly toxic DNA lesions initiate a DNA damage response, by phosphorylating replication protein A (pRPA) and phosphorylating the H2AX histone variant (pH2AX) (5, 6).

Incomplete repair of this DNA damage can result in gene mutations. *E. coli* strains carrying *Pks Island* have been shown to promote colon carcinogenesis in different mouse models (7), (8). In epidemiological studies, *pk*s+ *E. coli* strains are more prevalent in the gut microbiota of patients with colorectal cancer and a distinct mutational signature in human cancer genomes, predominantly colorectal tumours, was recently associated with colibactin genotoxic activity, further implicating an involvement of colibactin-producing *E. coli* in tumorigenesis (9), (10). This mutational signature has also been identified in tumours of the urinary tract (11, 12).

Materials and methods

Samples collection

Seventy urine samples collected from Patients suffering from UTI and BC also, with age ranged from (30-85 years old), from different hospitals in Baghdad province (AL-Yarmouk, Medicine city, and Royal hospitals) between the period from October 2022 until August 2023 (13).

All urine samples were cultured on different media (Blood, MacConkey, and EMB agar). The isolated colony were identified depending on morphological characteristics and Vitek-2 system (14).

Molecular detection

The ABIOpure extraction process was utilized to extract DNA from an activated pure culture of *E. coli* bacterium. DNA band detection by means of Agarose gel electrophoresis. The study then employed conventional

PCR using the specific primers listed in Table (1) to identify the presence of the specific detection gene of *E.coli* bacteria genes (*UidA)* and a virulence gene (*clbA*) that have linked to developing bladder cancer.

Table (1): The optimum condition of the detection of genes (*UidA* and *clbA*).

Genes	primer Sequences	Size (bp)	Condition	Ref.
UidA	F:CATTACGGCAAAGTGTGGGTCAAT R: CCATCAGCACGTTATCGAATCCTT	658	95°C 5min 95°C 30sec 55°C 30sec. 72°C 30sec. 72°C 7min.	(15)
ClbA	F: CTAGAT TAT CCG TGG CGA TTC R: CAG ATA CAC AGA TAC CAT TCA	1002	95°C 5min 95°C 30sec 54°C 30sec. 72°C 30sec. 72°C 7min.	(16)

Quantitation of DNA

The A Quantus Fluorometer was used to measure the amount of extracted DNA in order to assess the quality of the samples for future applications. One µl of DNA was mixed with 200 µl of diluted Quantifluor Dye. Readings of DNA content were discovered following a 5-minute room-temperature incubation time.

Primer preparation

The Macrogen Company provided these primers when lyophilized. Lyophilized primers were dissolved to a final concentration of 100 pmol/l in nuclease-free water to create a stock solution. 10 µl of primer stock solution, maintained at -20 C in the freezer, was mixed with 90 µl of nuclease-free water to form a working primer solution containing 10 pmol/l of these primers (Table 2).

Table (2): Lists the elements of each gene's PCR combination.

Component	Volume	Final Concentration
FIREPol® Master Mix , 5X	**10 µl**	**1X**
Forward primer	**1 µl**	**0.5 µM**
Reverse primer	**1 µl**	**0.5 Mm**
DNA template	**3**	**15 ng**
Nuclease free water	**5 µl**	
Final volume	**20 µl**	

Statistical analysis

The IBM SPSS was the program used to analyze the data. Cronbach alpha was used to assess the authenticity and dependability of the data. Using SPSS, the frequencies were computed, the Student's t-test, chi-square, and ANOVA were performed to the sociodemographic and colibactin gene presence data, and findings are considered significant if $p>0.05$. ANOVA and the Student's t-test were utilized to determine the significant differences between cancer patients and healthy controls.

Sequencing of the *clbA* gene and sequencing analysis

Nucleotide sequencing of the PCR products of the *clbA* gene has been performed for 4 positive *clbA* gene, selected strains (HYJ-IRQ1_ HYJ-IRQ4) including these 4 isolates from bladder cancer patients with UTI. These were sent to Macrogen Inc., South Korea public biotechnology company for sequencing by sanger method to identified the single nucleotide sequence gene in both directions.

Results and discussion

Out of 70 urine samples collected from patients suffering from BC and UTI, 50 sample were analyzed for *E.coli* identification. The occurrence of *E.coli* in the patients is shown in the (Table 3).

Table (3): The significant difference in the detection of *E.coli* between the patients and the healthy ones l.

		No. %		Total	Chi-square
		Patients	Control		
Bacteria	No *E.coli*	0	50	50	0.001**
	E. coli	50	0	50	
Total		50	50	100	

The present study revealed that out of 70 urine sample collected from patients with BC and UTI only 50 sample was detected as *E.coli* bacteria (71, 4%), while the other 20 samples were belongs to other bacteria type implicated in UTI developing (Table 4).

Table (4): The prevalence of *E.coli* bacteria from 70 urine sample.

Gender		Age	Types of bacteria
Male	Female		
(46)	(4)	Age between (30_85)	*E.coli*
(13)	(1)		*Klebsiella pneumonia*
(2)	(No)		*Staphylococcus aureus*
(2)	(No)		*Staphylococcus saprophyticus*
(1)	(No)		*Proteus miribalis*
(No)	(1)		*Enterococcus fecalis*

A research introduced by Mlugu, *et al*., (17) was also shows that the results is somewhat agreed with our study, *E. coli* was the most predominant bacteria (47%; 66/141). According to two further studies conducted in Lebanon (18, 19), *E. coli* accounted for 60.64 percent of all isolates during the ten-year period and was the most common type. Next in line were *Streptococcus agalactiae, Pseudomonas aeruginosa, Enterococcus sp., Klebsiella pneumoniae, and Proteus sp.*

In the table (Table 5), our research showed that the incidence of the bladder cancer increased with age.

Mlugu *et al*., (13) reported that the prevalence of UTIs was 41% (141/344) and elders (>=60 years) had five times higher odds of having UTI as compared to adolescents ($p < 0.001$). Age-associated changes in immune function, exposure to nosocomial pathogens and an increasing number of comorbidities put the elderly at an increased risk for developing infection.

Table (5): The distribution of patients group according to age.

		No. Patients	Total	p-value
Age	≤50	6	6	0.001**
	>50	44	44	
Total		50	50	

Table (6), shows the relation between cigarate smoking and the happening of bladder cancer. This result is supported by Zheng, *et al.* (20) who have shown in them results, that studied BC cases had higher smoking index with mean of 7.77±3.76 compared to controls 3.08±1.88 (P=<0.001). Abdulwahab, *et al*., (21) proved that their study are consistent with the findings of the previous epidemiological studies and confirm that smoking is a major risk factor for urinary bladder cancer and preventive strategies should be directed toward smoking as risk factor for urinary bladder cancer in Basrah.

The risk is greater because the chemicals in tobacco smoke can end up in your urine. These harmful chemicals can damage your bladder's lining and increase your risk for bladder cancer. Urothelial carcinoma, which can be found in the cells that line the inside of the bladder, is the most common type of bladder cancer.

Table (6): The effect of smoking on the number of cases of bladder cancer.

		No. Patients	Total
Smoker	Non smoker	17	17
	Smoker	33	33
Total		50	50

Further molecular diagnosis for *E.coli* bacteria by using a specific diagnostic gene of *E.coli (UidA)* using a specific primer, and the results were detected by electrophoresis on 2% agarose and exposed to U.V light (Figure 1).

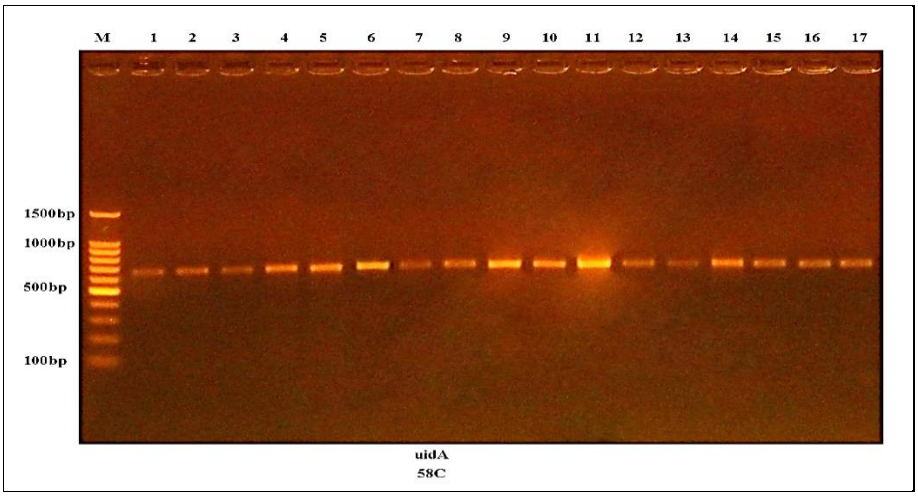

Figure (1): Agarose gel electrophoresis (1,5 gm agarose, 75 V for 1:45 hour) of *Uid A* gene PCR products (658bp) codify for *UidA* of *E.coli* isolates

The pks pathogenicity island encodes the polyketide/nonribosomal peptide combination known as colibactin toxin. It has been demonstrated that pks+ *E. coli* infection of eukaryotic cell lines results in DNA double-strand breaks (DSBs), which produce megalocytosis and cell cycle arrest (22) (Figure 2).

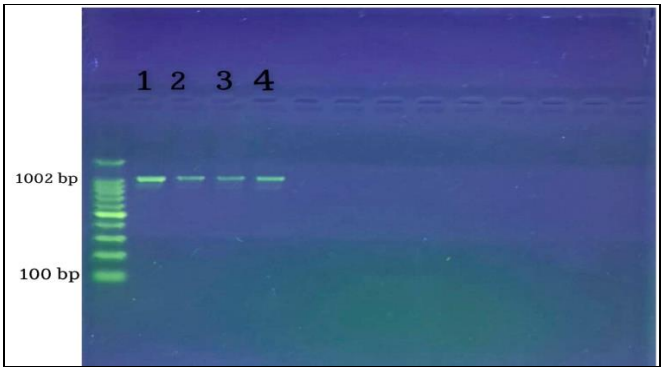

Figure (2): Agarose gel electrophoresis (2% agarose, 75 V for 1:45 hour) of *clbA* gene PCR products (1002bp) codify for colibactin gene of *E.coli* isolates.

In our project, we found that the colibactin gene was found in four samples from people whose data indicated that they were not smokers, but they suffered from urinary tract infections and bladder cancer. This may strengthen the relationship that says that this gene's presence in bacteria, especially when they are not treated and repeated infections with them, can lead to the developing of bladder cancer. To test the capability of Sanger sequencing to confirm the diversity of microbiota in urine specimen, the PCR product was sent to Macrogen company for Sanger sequencing. The results of sequencing related to *clbA* genes (23) (24). The sequencing result of *clbA* gene shows that *E.coli* isolates having 100% similarity to the sequence of *clbA* gene that was available in Gene bank and according to the accession numbers (CP122448.1).

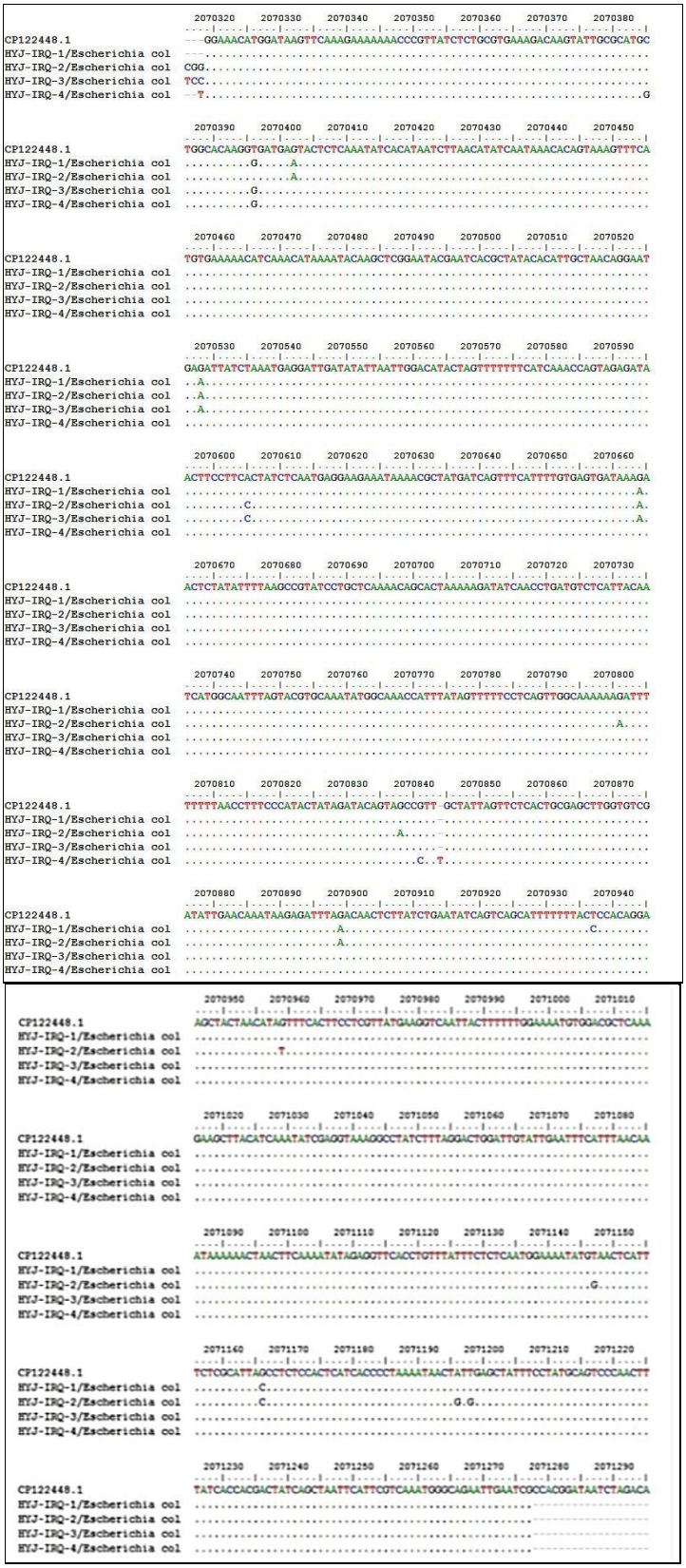

Figure (3): Allignment of partial *clbA* gene seq. for 4 iraqi *E.coli* isolates, including (*E.coli* accession no. CP122448.1).

Table (4): Diversity of *clbA* gene mutations in a selected four strain associated with *E. coli* isolated from cancer patients with UTI and BC (CP122448.1).

No. of isolate and accession no.	Type of mutation	Location	Change of bases	Identity
HYJ-IRQ-1	G/T	2070396	Substitution (transition)	98%
	A/G	2070402	Substitution (transition)	
	A/G	2070528	Substitution (transition)	
	A/G	2070664	Substitution (transition)	
	A/G	2070899	Substitution (transition)	
	C/T	2070937	Substitution (transition)	
	C/G	2070166	Substitution (transition)	
HYJ-IRQ-2	A/G	2070402	Substitution (transition)	96%
	A/G	2070528	Substitution (transition)	
	C/A	2070605	Substitution (transition)	
	A/G	2060664	Substitution (transition)	
	A/G	2070801	Substitution (transition)	
	A/G	2070838	Substitution (transition)	
	A/G	2070899	Substitution (transition)	
	T/G	2070959	Substitution (transition)	
	G/T	2071147	Substitution (transition)	
	C/G	2071166	Substitution (transition)	
	G/A	2071196	Substitution (transition)	
	G/T	2071198	Substitution (transition)	
HYJ-IRQ-3	G/T	2070396	Substitution (transition)	98%
	A/G	2070598	Substitution (transition)	
	C/A	2070605	Substitution (transition)	
	A/G	2070664	Substitution (transition)	
HYJ-IRQ-4	G/C	2070385	Substitution (transition)	97%
	G/T	2070396	Substitution (transition)	
	C/G	2070841	Substitution (transition)	
	- /T	2070844	Insertion	

As we seen in table (3-6), we notice a diversity of mutation in gene *clbA* recorded in the four isolated strain of *E.coli*, which deposited in NCBI GeneBank database under serial accession no. This mutation prevalence between substitution and insertion, and compared with ref sequence (*Escherichia coli* (CP122448.1)).

Conclusion

We can conclude that recurrent urinary tract infections by *E.coli* that are left untreated can lead to the formation of bladder cancer and on the other hand, the colibactin gene may play an important role due to its ability to destroy DNA. We suggest that more samples should be collected, the scope of sample collection expanded, and the colibactin gene foundation in furthermore samples to prove this more.

References

1. Yaseen, N..; Aziz, R. ; AL-Shawk, R.; Saleem, M.; Al-Azzawi, I. and AL-biaty, H.(2016). Urinary marker (MMP-9) and bladder cancer. Iraqi Journal of Cancer and Medical Genetics, (9): 1.
2. Halaseh, S.; Halaseh, S.; Alali, Y.; Ashour, M. ; Alharayzah, M. and Alharayzeh, M. (2022). A review of the etiology and epidemiology of bladder cancer: All you need to know. ***Cureus***, 14(7): e27330.
3. Mohamed, I. and Al-Azawi, A. (2022). Evaluation of antibacterial activity of laurus nobilis leaves extract against *Escherichia coli* isolates. Iraqi Journal of Biotechnology, 21(2): 623-631.
4. Sung, H.; Ferlay, J.; Siegel, R. L.; Laversanne, M.; Soerjomataram, I.; Jemal, A., *et al*. (2021). Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA: a cancer journal for clinicians, 71(3): 209–249.
5. AL-Jubouri, S. and Shami, A. (2022). Iraq cancer archive. Journal of Clinical and Translational Oncology Iraq cancer Archive, 2(1): 1-5.
6. Usama, R.; Hameed and Laith A. Yaaqoob (2022).Antibacterial Activity of Cobalt and Titanium (Co: Tio2) Core-Shell Nanoparticles against E. coli Isolated From Urinary Tract Infections. Iraqi Journal of Biotechnology, V (21) 2, Pages 341-357.
7. Chagneau, C.; Massip, C.; Bossuet-Greif, N.; Fremez, C.; Motta, J. ; Shima, A., *et al*. (2021). Uropathogenic *E. coli* induces DNA damage in the bladder. ***PLoS pathogens***, 17(2): e1009310.
8. Mahdi, S. ; Al-Jobori K. and Mohsen, W. (2022). Identification of *Escherichia coli* isolated from cow, cow workers, farms, shared farm environments in Karbala governorate and evaluation of its antibiotic resistance. Iraqi Journal of Biotechnology, 21(2): 300-307.
9. Luma, M. (2019). The Effect of Alcoholic Extracts of Zingiberofficinale Anti-*E.coli* Isolates Isolated from Urinary Tract Infection. Iraqi Journal of Science, V(60)10, Pages 2136-2140.
10. Almasaudi, H.; Alaubydi, M. and Jawad, S. (2018). Detection of cnf1 gene in the local human uropathogenic *E. coli* isolate and study some immunological and histopathological effects in vivo. Iraqi Journal of Biotechnology (17)1, Pages 27-36.
11. Jiang, Y.; Stornetta, A.; Villalta, P.; Wilson, M. ; Boudreau, P. D.; Zha, L., *et al*. (2019). Reactivity of an unusual amidase may explain colibactin's DNA cross-linking activity. Journal of the American Chemical Society, 141(29): 11489-11496.
12. Ayoub, N.; Abdallah, K. and AL-Khazraji, S.(2014). Bacterial profile of urinary tract infections in Diabetic postmenopausal women. Journal of the Faculty of Medicine Baghdad**,** 56(1): 79-84.
13. Pleguezuelos-Manzano, C.; Puschhof, J.; Rosendahl Huber, A.; van Hoeck, A.; Wood, H.; Nomburg, J., *et al*. (2020). Mutational signature in colorectal cancer caused by genotoxic pks+ *E. coli*. ***Nature***, 580(7802): 269-273.
14. Al-Hamdani, H. and Al-Hashimy, A. (2020). Molecular detection of UREC, HPMA, RSBA AND MRPA genes of Proteus Mirabilis urinary tract infection in patient with rheumatoid arthritis. The Iraqi Journal of Agricultural Science, 51, 245-251.
15. AL-Jubouri, S. S. and Shami, A. M. (2022). Molecular Detection of Cephalosporin Resistance Genes in

Escherichia coli Isolated from Urinary Tract Infections in Baghdad Hospitals. Iraqi Journal of Biotechnology, 21(2): 145-152.

16. Adamus-Bialek, W.; Wojtasik, A.; Majchrzak, M.; Sosnowski, M. and Parniewski, P. (2009). (CGG)4-based PCR as a novel tool for discrimination of uropathogenic *Escherichia coli* strains: comparison with enterobacterial repetitive intergenic consensus-PCR. Journal of clinical microbiology, 47(12): 3937–3944.
17. Dubois, D.; Delmas, J.; Cady, A.; Robin, F.; Sivignon, A.; Oswald, E., *et al*. (2010). Cyclomodulins in urosepsis strains of *Escherichia coli*. Journal of clinical microbiology, 48(6): 2122–2129.
18. Mlugu, E., Mohamedi, J., Sangeda, R. *et al.* Prevalence of urinary tract infection and antimicrobial resistance patterns of uropathogens with biofilm forming capacity among outpatients in morogoro, Tanzania: a cross-sectional study. BMC Infect Dis 23, 660 (2023).
19. Daoud, Z. and Afif, C. (2011). *Escherichia coli* isolated from urinary tract infections of Lebanese patients between 2000 and 2009: epidemiology and profiles of resistance. Chemotherapy Research and Practice, 2011.
20. Auda, J. and Khalifa, M. (2019). Cloning and expression of a lipase gene from Pseudomonas Aeruginosa into E. Coli. Iraqi Journal of Agricultural Sciences, 50(3): 768-775.
21. Zheng, Y.; Amr, S.; Saleh, D.; Dash, C.; Ezzat, S.; Mikhail, N. *et al*. (2012). Urinary bladder cancer risk factors in Egypt: a multicenter case–control study. Cancer epidemiology, biomarkers and prevention, 21(3): 537-546.
22. Abdulwahab Al-Shakour, A.; Al-Naama, L. and Ajeel, N. (2014). Smoking and urinary bladder cancer: A case-control study in Basrah. The Medical Journal of Basrah University, 32(1): 1-7.
23. Iftekhar, A.; Berger, H.; Bouznad, N.; Heuberger, J.; Boccellato, F.; Dobrindt, U., *et al*. (2021). Genomic aberrations after short-term exposure to colibactin-producing *E. coli* transform primary colon epithelial cells. Nature communications, 12(1): 1003.
24. Ashwak B. Al-Hashimy and Weam K. Al-Musawy 2020. Molecular Study and Antibiotic susceptibility patterns of some Extended Spectrum Beta-Lactamase Genes (ESBL) of Klebsiella pneumpniae in Urinary Tract Infections. Journal Phys.: Conf.Ser. 1660 012017.