Detection Of Escherichia Coli Virulence Genes (Pap , Afa , Iha Ipr2 And Hly) Isolated From Patients With Urinary Tract Infections In Babylon Province, Iraq

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KEYWORDS
Escherichia coli; UTIs; virulence genes;, pap ; afa;, iha ; ipr2 ; hly

ABSTRACT
Abstract. The current study included a collection of 110 urine samples of both sexes of different ages for patients with urinary tract infections, who consulted Al-Hilla Teaching Hospital and the Children and Maternity Hospital in Al-Hilla city, through the period from May 2020 to December 2020, to investigate some virulence factors associated with Escherichia coli bacterium. The results of the biochemical tests revealed that 74 (67.3%) isolates were belonging to E. coli that was isolated from the urine samples. The results also indicated that the females were more infected 47 (63.5 %) with urinary tract infections than males 27 (36.4 %). Some virulence factor genes have also been investigated using the polymerase chain reaction (PCR) technique, which are (pap, afa, iha, ipr2 and hly genes). The PCR results for E.coli genes revealed that, 11 isolates contain pap gene (7 (14.8 %) and 4 (14.8 %)), while 9 isolates possesses afa gene (6 (12.7 %) and 3 (11.1 %)), whereas 5 isolates have gene iha (3 (6.3 %) and 2 (7.4 %)) , In addition to 30 isolates have irp2 gene (21 (44.6 %) and 9 (33.3 %)) and 27 isolates contain hly gene (19 (40.4 %) and 8 (29.6 %)). These percentages are for females and males respectively. Conclusions: E.coli is the bacterium that dominant the UTIs. Females are more likely to develop UTIs than males. The irp2 gene responsible for taking iron is the most genetically studied genome that causes UTIs, followed by the gene hly responsible for producing α-hemolysin enzyme second among the gene of the virulence factors under study.

1. Introduction

Urinary tract infections are one of the most common bacterial infections in patients [Pourakbari et al., 2019; Wnorowska et al., 2019], affecting people of all ages [Malekzadegan et al., 2018; Lee et al., 2015], and are associated with a high morbidity rate that affects people all over the world [Katongole et al., 2020; Dehbanipour et al., 2016; Mukherjee et al., 2013]. Extraintestinal pathogenic Escherichia coli (ExPEC) has been shown to cause a wide range of infections, including urinary tract infections, sepsis, pneumonia, meningitis, and skin and soft tissue infections [Dehbanipour et al., 2016]. The ability of UPEC to enter, colonize, climb, and persist in the uroepithelium is linked to its ability to create biofilms and possess various virulence factors, according to the majority of studies [Katongole et al., 2020]. The bacteria have a wide range of genetic variation, especially among the genes that confer virulence [Hutton et al., 2018]. virulence factors found in UPEC isolates that aid bacterial invasion [Malekzadegan et al., 2018; Dehbanipour et al., 2016; Sintsova et al., 2019].

The adhesions by which UPEC usually binds to the urinary tract epithelial cell related to a-fimbrial adhesion (afa), P fimbrial adhesion (pap), and iha adhesion siderophore genes. In addition to adhesions, hemolytic factors are important virulence factors. The cytolytic effect of alpha-haemolysin (HlyA) encoded by the a-hly gene is responsible for bacterial penetration through the epithelial barrier [Katongole et al., 2020]. Restricted epidemiological research on virulence genes have been reported among the UPEC isolated from symptomatic patients with UTIs in Iraq, Therefore, this research aimed to determine the genetic diversity among E. coli strains isolated from patients with UTIs, who were admitted to two main hospitals in AL- Hilla city (Hospital for Maternal and Pediatrics and Al-Hilla Teaching Hospital) in Babylon province, Iraq.

2. Methodology

Collection of Specimens

110 urine specimens were obtained from patient clinically diagnosed with urinary tract infections. Those patients attended the two main hospitals in AL- Hilla city (Hospital for Maternal and Pediatrics and Al-Hilla Teaching Hospital). The samples were taken from both sexes and all age

groups from infants to elder during the period from December 2019 to March 2020. To investigate the virulence genes (*pap, afa, iha, ipr2* and *hly* genes) of *Escherichia coli*. 5 ml urine of each patient (the specimen was collected from midstream urine) was taken and placed in sterile tube [Walker *et al*., 2015].

Bacterial Identification

A loopful of each specimen were taken and cultured on MacConkey, blood, and eosin methylene blue agar, on petri dishes, the plates were then incubated for 18 - 24 h at 37 °C to observe the morphology of colonies. Gram stain and conducting biochemical tests such as catalase, oxidase, indole, methyl red/ voges-proskauer, citrate tests, along with fermentation of glucose, lactose, maltose, sucrose, motility and gas production were all carried out for all grown colonies. For bacterial preservation, a screw capped tubes containing nutrient agar medium as slanted were inoculated with the bacterium and incubated at 37 ° C for 24 hours, then stored at 4 ° C. For long storage, the isolates were stored in brain heart infusion broth supplemented with 15% glycerol at -20 ° C [Walker *et al*., 2015].

DNA extraction

LB broth was used as enrichment media to subcultured bacterial strains as an overnight incubation period and genomic DNA was extracted by using DNA extraction kit (Geneaid, UK) according to manufacturer's instruction.

Detection of uro-virulence genes

Polymerase chain reaction (PCR) were achieved in a final volume of 25 µl. Then DNA amplification was carried out with the thermal cycler. The primers which were used in PCR in this study with their sequences and PCR condition are listed in table 1. The PCR was carried out with the thermal cycling including addition of 2.5ul downstream and upstream primer, 2.5µl of free nuclease water, 5µl of genomic DNA and 12.5µl of the master mix. The PCR amplified products were pictured through ethidium bromide staining after gel electrophoresis of 10 µL of the final reaction mixture in 1.5% agarose gels run for 45 minute at 70 volt. The size of the amplicons was determined through comparison to the 100-1500 bp, allelic ladder (Promega, USA).

3. Results and discussion

Isolation and Identification

In the current study, 74 E. coli isolates (67.3%) were isolated from 110 samples collected from patients with UTIs. The growing colonies appeared on the MacConkey Agar pink, rounded shape with regular rims, small and diameters range from (1-2) mm. While small colonies emerged in a bright gray color on the medium of the blood agar, surrounded by a halo type β hemolysis with diameters ranging from 1-2 mm. Microscopic examination showed Gram negative, rod-shaped bacterium, not composed of spores. The growing isolates on the selective medium were diagnosed based on biochemical tests, as the results in Table 2 showed, all isolates tested positive to the catalase test, maltose, lactose, methyl red and indole test. While all isolates tested negative to the oxidase, urease, H2S production, Voges- proskauer and Simon citrate tests. The results indicated that the females were more infected 47 (63.5 %) with UTIs than the males 27 (36.4 %).

Detection of virulence genes by using PCR technique

The PCR technique was used to investigate some of the genes responsible for the virulence factors in E.coli by using primers for the virulence genes (pap ,afa , iha , irp2 and hly) .Some virulence factors were investigated for 74 bacterial isolates, as the genes responsible for the virulence factors, which are pap gene responsible for coding to form a fimbriae, afa gene responsible for the fimbrial adhesion , the iha gene responsible for the adhesions and synthesis of capsule , irp2 gene responsible for iron uptake and hly gene responsible for the α- heamolysin toxin. The results demonstrated that only 11 bacterial isolates (7 (14.8 %) female and 4 (14.8 %) male) had the pap

gene, also 9 bacterial isolates (6 (12.7 %) female and 3 (11.1 %) male) had the afa gene, in addition to 5 bacterial isolates showed 3 (6.3 %) female and 2 (7.4 %) male of the bacterial isolates had a gene iha, while 30 bacterial isolates (21 female (44.6 %) and 9 male (33.3 %) showed that they had the irp2 gene and 27 bacterial isolates (19 female (40.4 %) and 8 male (29.6%) showed that they had the gene hly. as shown in Table 3.

Table 1. Primers and PCR stipulations utilized to amplify genes encoding pap, afa, iha,

Genes	Primer sequence (5′-3′)	Size of product bp	PCR condition	Reference
***Pap* F 1x**	GCAACAGCAACGCTGGTTGCATCAT	336bp	95 ºC 5min 94ºC 1min 51ºC 5min 68 ºC 8min 65 ºC 10min	[Yamamoto *et al.*, 1995]
R 30X	AGAGAGAGCCACTCTTATACGGACA			
***Afa* F 1x**	GCTGGGCAGCAAACTGATAACTCTC	750bp		[LeBouguenec *et al.*, 1992]
R 30X	CATCAAGCTGTTTGTTCGTCCGCCG			
***Iha* F 1x**	CTGGCGGAGGCTCTGAGATCA	827bp		[Johnson *et al.*, 2000]
R 30X	TCCTTAAGCTCCCGCGGCTGA			
***irp2* F 1x**	AAGGATTCGCTGTTACCGGAC	413bp		[Ewers *et al.*, 2005]
R 30X	AACTCCTGATACAGGTGGC			
***Hly* F 1x**	AACAAGGATAAGCACTGTTCTGGCT	13bp		[Yamamoto *et al.*, 1995]
R 30X	ACCATATAAGCGGTCATTCCCGTCA			

Our results showed that irp2 gene was the most predominant Uro virulence gene (77.9%) followed by hly (70%), pap (29.6%), afa (23.8%) and iha (13.7%). Our results showed that irp2 gene had the highest level and iha gene had the lowest rate of virulence genes in E. coli isolated from patients with UTIs. The gel electrophoresis showed that the molecular weight of pap gene was 336 bp as shown in Fig. 1, 750 bp of afa gene as exposed in Fig. 2, 827 bp of iha gene as in Fig. 3, 413 bp of irp2 gene as shown in Fig. 4 and 113 bp of hly gene as revealed in Fig. 5.

Table 2. The biochemical tests of E. coli

Biochemical tests	Results
Maltose	+
Lactose	+
Urea's	-
Gas production	+
H2S production	-
Kligler iron agar	A/A
Simons citate	-
Voges- proskauer	-
Methyl Red	+
Indole	+
Eosin-Methlene blue	+
Catalase	+
Oxidase	–
Motility	+

(+) positive (-) negative (A) acid

Table 3. Numbers and percentages of genes of virulence factors in E. coli isolates using PCR technique.

Virulence genes	Number of *E. coli* isolates	Number of isolates from female (%)	Number isolates from male (%)	Total percentage %
pap	11	7 (14.6)		
afa	9	6 (12.7)		
iha	5	3 (6.3)		
irp2	30	21 (44.6)		
hly	25	19 (40.4)		

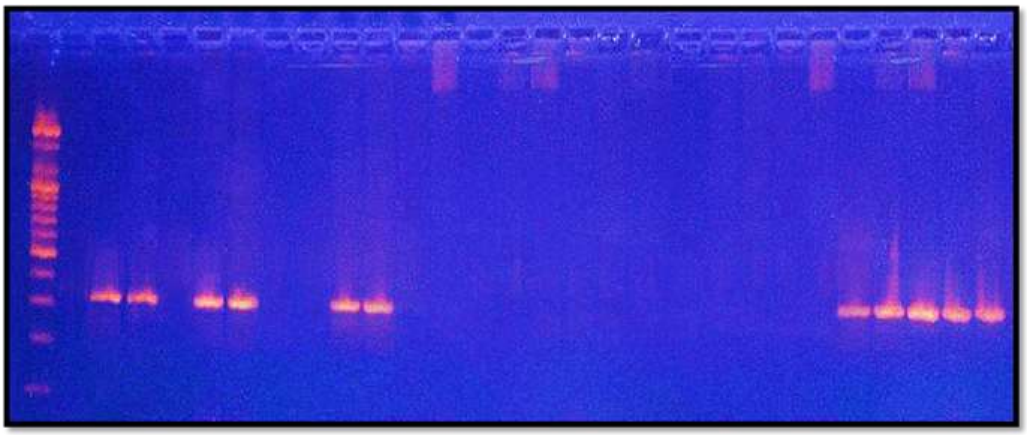

Figure 1. The gel electrophoresis of PCR amplified of pap gene with 336 bp on 1.5% agarose gel at 70 volt for 45 min visualized under UV after staining with ethidium bromid. Lane (M): DNA marker Ladder 100 -1500 bp ladder. Lanes: No.(2,3,5,6,9,10,24) (seven) E. coli isolates from urine show positive results from females ,and (25,26,27,28) (four) E. coli isolates from urine show positive results from males.

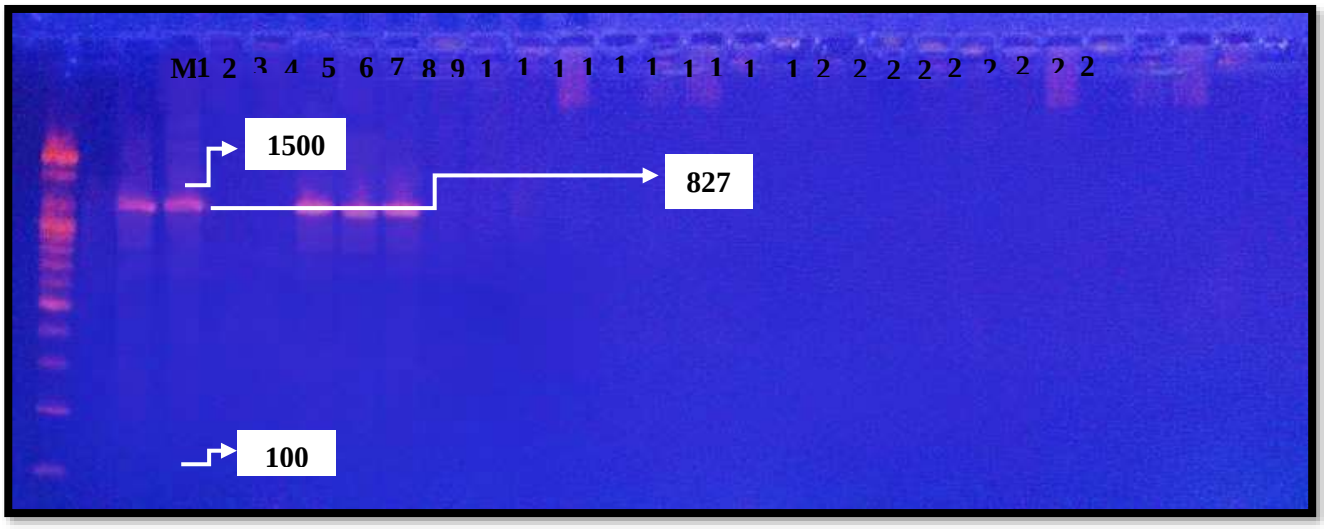

Figure 3. The gel electrophoresis of PCR amplified of *iha* gene with 827 bp on 1.5% agarose gel at 70 volts for 45min visualized under UV after staining with ethidium bromid. Lane (M): DNA marker

Ladder 100 -1500 bp ladder. Lanes: No.(2,3,6) (three) *E. coli* isolates from urine show positive results from females and (7,8) (two) *E. coli isolates* from urine show positive results from males.

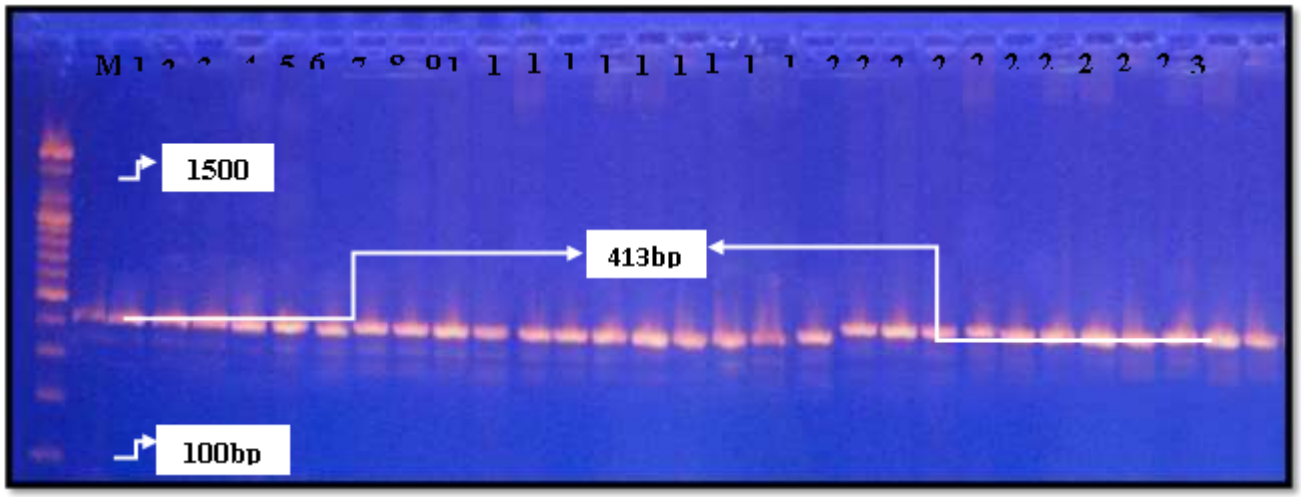

Figure 4. The gel electrophoresis of PCR amplified of irp2 gene with 413 bp on 1.5% agarose gel at 70 volt. for 45min visualized under UV after staining with ethidium bromide. Lane (M): DNA marker Ladder 100 -1500 bp ladder. Lanes: No. (1,2,3,4,5,6,9,10, 11,12,13,14,15,16,17,18,19,20,21, 23,24) (twenty one) E. coli isolates from urine show positive results from females and (7,8,22,25,26,27,28,29,30) (nine) E. coli isolates from urine show positive results from males.

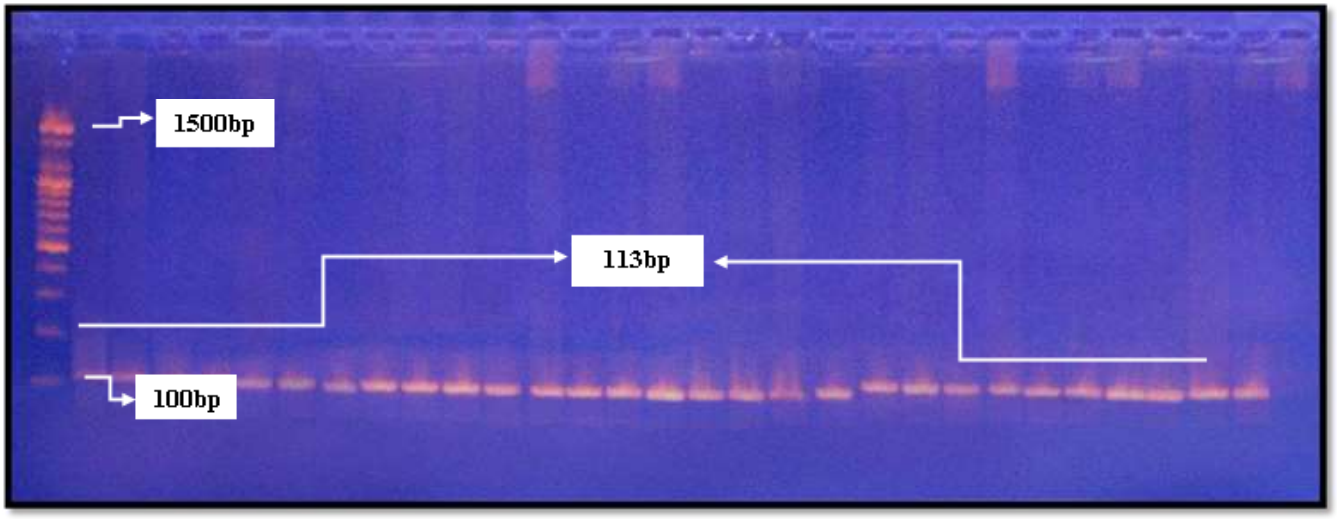

Figure 5. The gel electrophoresis of PCR amplified of hly gene with 113 bp on 1.5% agarose gel at 70 volts, for 45min visualized under UV after staining with ethidium bromide. Lane (M): DNA marker Ladder 100 -1500 bp ladder. Lanes: No. (1,2,3,4,5,6,9,10,11 ,12,13,14,15,16,17,18,19,20,21) (nineteen) E. coli isolates from urine show positive results from females and (7,8,22,25, 26,27,28,29) (eight) E. coli isolates from urine show positive results from males

Discussion

In the present study, urine specimens were gained from patients with UTIs. The bacterial isolates under study were diagnosed primarily through the study of cultural, microscopic characteristic and biochemical tests. E. coli was detected in 74 (67.3%) isolates from urine specimens. Our results of isolating E. coli showed differences with other studies [Isberg et al., 2019; Goudarzi et al., 2017]. While other studies indicated a higher isolation rate of E. coli from UTIs than our results ranging between 80-85% [Katongole et al., 2020; Czaja et al., 2009]. Also the bacteria E. coli was detected in

(36%) isolates from urine specimens [Mukherjee et al., 2013]. While Milovanovic et al., [Milovanovic et al., 2019] showed the isolation rate of E. coli is only 9.2%, which is a lower rate when compared with our results.

Our results also showed that the rate of isolation E. coli 47(63.5 %) of the patients were female and 27(36.4 %) were male. These results were close to study approved by Funfostuk. et al, [Funfstuck et al., 1999]. According to an assessment, virtually 40-50% of women practice UTIs once in a lifespan [Dehbanipour et al., 2016]. UTIs are the most commonly reported infection in adults and have a high rate of recurrence in women [Sintsova et al., 2019]. Also Pourakbari,et al [Pourakbari et al., 2019] reported that the rate of isolation E. coli 83% of the patients were female and 17% were male.

The present study showed that 11 isolates (29.6%) have the gene for pap, and this gene is significant for the virulence of E. coli due to association of gene pap with fimbriae that help bacteria adhere to endothelial cells and cause disease, especially in the urinary tract [Knobl et al., 2004]. Also our results provided 9 isolates (23.8%) contain the afa gene. This gene is responsible for the formation of cilia that help bacteria to adhere and thus invade the endothelial tissue of the urinary tract and cause infection [Wult and Connell, 2000]. While only 5 isolates (13.7%) revealed that they possess the iha gene, the iha gene is the gene adherence factor responsible for the formation of the capsule that helps bacteria adhere to the epithelial wall of urinary tract tissue as well as the capsule has a great importance in the bacteria's resistance to antibiotics and the phagocytic process by leukocytes [Karimian et al., 2001].

Also 30 isolates (77.9%) from the 74 isolates showed that they had the irp2 gene. The irp2 gene has a great contribution in helping bacteria to take iron and store it for use in anaerobic conditions as iron is necessary to multiply the bacteria numbers and is an important step for colonization [Iivermore , 2001]. The results showed that there were 27 isolates (70 %) from the 74 isolates contain hly gene, the hly gene (α-hemolysin) is a virulence factor for E. coli that causes tissue damage and a lack of local immune defenses. It also lyses red blood cells, triggering urinary tract infection [Karimian et al., 2001]. Our results are inconsistent with those of another study that indicates the occurrence of pap, afa and hly genes in Uro-pathogenic E. coli was; 21%, 8% and 0% respectively [Katongole et al., 2020]. While another study indicated that pap (35.7 %), afa (45.2%) and hly (23.8%) genes were more detected in the cystitis isolates [Malekzadegan et al., 2018]. Also, Karimian et a l., [Karimian et al., 2001] indicated that the incidence of hly, pap, afa, iha, and irp2 virulence genes were 50.4, 50.4, 8.13, 17.88 and 11.38%, respectively.

In comparison with a study by Bahalo et al. [Bahalo et al., 2013], isolation of afa gene it reached (34.3%), and the rate of pap gene reached by (30.0%), which identical with our results, also results the rate of iha gene 13.8% which agree with our results. While the same study showed higher isolation rates for the two genes 96.2% for irp2 gene and (92.3%) for hly gene. Another study showed that E. coli (21%) were haemolytic [Kausar et al., 2013]. The prevalence of genes, pyelonephritis associated pili (pap gene), hemolysin (hly gene), and among the isolated strains was (27.1%) and (13.5%), respectively, according to studies on children with UTIs. This research also revealed that pyelonephritis was more common in cases of positive virulence genes [Farshad and Emamghorashi, 2009].

The virulence characteristics of E. coli isolates that infect the urinary system improve their ability to induce disease in this organ system [8]. In order to colonize the host urinary tract effectively, UPEC strains transfer a variety of virulence factors. Flagella’s induce bacteria and adhesions to migrate to the uroepithelium and bind to it, allowing resistance to the contagion. Iron acquisition systems (e.g. siderophores) allow UPEC to acquire iron to guarantee its development [Schwab et al., 2013]. UPECs colonize the bladder and cause cystitis at first. Then, in an ascending direction, it would most likely reach the kidney, causing acute pyelonephritis or spreading to the blood, causing urosepsis [Malekzadegan et al., 2018] . Indeed, it's been suggested that UPEC isolates have the highest number of islands associated with pathogenicity (PAIs), which encode a variety of virulence determinants

involved in host defense adhesion, invasion, and bacterial tolerance, and hence affect the pathogenicity of symptomatic or complicated UTIs. Our findings have shown, amid previous research, that the UPEC strains in Iraq have a distinct virulence profile. These differences in the occurrence of UPEC virulence genes have shown that the virulence properties of UPEC strains are highly dependent on the geographical area as well as the local environmental conditions. UPEC strains isolated from UTI patients in Iraq have a distinct epidemiology and prevalence of virulence factors. There are potentially great rules for the occurrence of virulence genes in UPEC strains in traditions, dietary intakes, levels of public health, patient health and even methods of sampling.

Conflict of interest

There is no conflict of interest

Reference

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