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BACTERIOLOGICAL AND GENETIC STUDY FOR DETECTION OF UROPATHOGENIC ESCHERICHIA COLI ISOLATED FROM CASES OF URINARY TRACT INFECTION

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INTRODUCTION

Escherichia coli is one of the most important bacteria that colonized in the intestinal tract of human. It was isolated from the feces of an infant in 1885 by Theodor Escherich (Croxen *et al*., 2013) and originally called "*Bacterium coli* commune" due to the fact that it's found in the colon. UPEC is most frequently associated with UTIs that bacteria colonizes the urinary tract and may ascend towards the bladder to cause cystitis. In untreated cases bacteria may ascend to the ureters and kidney to establish a secondary infection, acute pyelonephritis with the possibility of causing irreversible kidney damage leading to kidney failure and death (Bien *et al*., 2012).

Urinary tract infections affect people in varying incidences depending on age and gender. Women are at greatest risk, nearly half of them have a UTI in their lifetime and annually over 15 million women suffer from UTIs in the United States with each episode causing serious deterioration in the quality of life (Foxman, 2002; Hannan *et al*., 2012).Young sexually active women are particularly prone to UTI with an incidence of approximately 0.5 episodes person per year. This is not the case in men younger than 50 years of age, where UTI is rare and is generally secondary to urologic abnormalities (Wiles *et al*., 2008). In older people in general, UTI is also the most common bacterial infection that is often asymptomatic. In the community, approximately 10% of older men and 20% of older women have asymptomatic bacteriuria (Rowe and Juthani-Mehta, 2014).

In hospitals Catheter acquired urinary tract infection is one of the most common health care acquired infections in which 80% of these infections are attributable to use of an indwelling urethral catheter. Recent surveys report a urinary catheter is the most common indwelling

device, with 17.5% of patients in European hospitals having a catheter (Nicolle, 2014) and 23.6% in US hospitals (Magill *et al*., 2014).

Bacterial adherence to host urethral and bladder cells is widely accepted as a pivotal step in developing and establishment of a stable UTI (Mahbub *et al*., 2011; Jahandeh *et al*., 2015).

To resist elimination by the shear forces caused by urine flow, UPEC has a variety of fimbrial and non-fimbrial adhesins that facilitate attachment of the bacterium to bladder cells to act as invasions (Spurbeck and Mobley, 2013).Type 1 pili are considered one of the most important virulence factors involved in the establishment of a UTI. *Fim*H is encoding for adhesin protein located at the distal end of the pilus that functions as the ligand for monomannose and trimannose containing glycoprotein receptors. Receptors for type 1 fimbriae are present on erythrocytes, buccal epithelial cells, intestinal cells, vaginal cells and uroepithelial cells (Erjavec and Žgur-Bertok, 2011). Interestingly, the *Fim*H adhesin mediates both bacterial adherence and invasion of host cells, also contributes to the formation of intracellular bacterial biofilms by UPEC (Wright *et al*., 2007).

MATERIAL AND METHODS

Sample collection

A total of (102) urine samples were collected from patients with urinary tract infection from AL-Imamein kadhimein medical city, Al-Yarmouk teaching and Al-Kadhimiya pediatric hospitals in Baghdad during the period from 1/10/2016 to 1/1/2017. Urine samples were taken from both male and female using a sterile containers. The samples were directly streaked on nutrient and MacConkey agar, incubated at 37° C for 24 hrs.

Isolation of Uropathogenic *E. coli* (Forbes *et al.*, 2007)

After incubation, a loopfull of lactose fermented colonies from MacConkey agar were sub-cultured on EMB agar for further identification and incubated at 37°C for 24 hrs, then colonies were subjected to other identifications tests.

Identification of Uropathogenic E. coli

Morphological characteristics (Atlas *et al*., 1995)

Colonies that able to grow on selective media were further identified by studying their morphological characteristics beginning by staining with Gram stain and study their

characteristics under microscope (size, colour, shape, arrangement, edge of colonies, spore formation).

Biochemical tests

These biochemical tests were achieved for further identification of selected isolated colonies and as follows:

A. Indole Production Test (Collee *et al*., 1996)

Test tubes containing peptone water broth were inoculated with fresh culture of each suspected colonies and incubated at 37°C for 24 hours. After that 0.5 ml of Kovac's reagent was added, the presence of red ring on the surface of the medium indicates a positive result.

B. Methyl Red Test (Atlas *et al*., 1995)

Test tubes containing MR-VP broth were inoculated with fresh culture of each suspected colonies and incubated at 37°C for 24 hours. Then five drops of methyl red reagent were added, mixed and readings immediately, the presence of bright red color indicates a positive reaction.

C. Voges-Proskauer Test (Atlas *et al*., 1995)

Test tubes containing MR-VP broth were inoculated with fresh culture of each suspected colonies and incubated at 37°C for 24 hours. Then 1ml of VP1 and 3ml of VP2 were added to 5ml of bacterial culture and shaked for 30 seconds. The formation of pink to red color indicates a positive result.

D. Citrate Utilization Test (Atlas *et al*., 1995)

Simmon's citrate slants were inoculated by stabbing into the buttom of the slant with fresh culture of each suspected isolate then incubated at 37°C for 24 hours. Changing the color of the medium from green to blue indicates a positive result.

E. Triple Sugar Iron Test (Atlas *et al*., 1995)

Test tubes containing triple sugar iron slants were inoculated by stabbing into the buttom of the slant with fresh culture of each suspected isolate and incubated at 37°C for 24 hrs, then the results were recorded as follows:

Slant /Bottom	**Color**
Alkaline / Acid	Red / Yellow
Acid / Acid	Yellow / Yellow
Alkaline / Alkaline	Red / Red

The formation of black precipitation indicates the production of H2S while pushing agar to the top indicates CO2 formation.

F. Oxidase test (Garrity, 2001)

Selected isolated colonies was transferred by wooden stick on a filter paper contain few drops of oxidase reagent and mix together. Then formation a deep purple colour on filter paper within 30 sec indicate a positive result.

G. Catalase test (Warren *et al*., 2000)

Selected isolated colony were transfer by a wooden stick on a clean slide, then few drops of catalase reagent (3%) H_2O_2 were added and mix together. The appearance of gas bubbles indicate a positive results.

Identification of Uropathogenic *E. coli* by API 20 E system

API 20E system strip was used to identify enteric gram negative bacteria. Identification of the isolates was carried out by sub-culturing suspected colonies from MacConkey Agar plates on API 20E microtubes system. This system was designed to contain 20 performance standard biochemical tests. Each test in this system is preformed within a sterile plastic microtube which contains the appropriate substrates that fixed to an impermeable plastic strip (gallery). Each gallery contains 20 microtubes (each of which consist of a tube and a couple selection).

Preparation of the Galleries

Aliquots of 5ml of tap water was dispensed in to the incubation tray to provide a humid atmosphere during incubation.

Preparation of Bacterial Suspension

Single colony from MacConkey agar was picked, and suspended in 5ml of saline by rubbing against the side of the tube and mixed thoroughly.

Inoculation of the Galleries

With a sterile Pasteur pipette, the twenty microtubes were inoculated according to the manufactures instruction, both the tubes and couple section of CIT, VP and GEL microtubes were filled to the end of the tube, also couple section of the ADH, LDC, ODC, H2S and URE micro tubes were completely filled with sterile mineral oil.

Incubation of the Galleries

After inoculation, the plastic lid was placed on the tray and the galleries were incubated for 18-24 hours at 37°C.

Reading the Galleries

All the reactions that not requiring reagents were recorded first, then the following reagents were added to their corresponding microtubes:

A. One drop of 3.4% ferric chloride added to the TDA microtube.

B. One drop of Kavoc's reagent added to the IND microtube.

C. One drop of Voges-Proskauer reagent added to VP microtube.

Identification of the Isolates

Identification of the isolates using the analytical profile index (Numerical Coding) for rapid identification of species and biotype level that were done as supplied by the manufacturer. To use the index, the biochemical profile obtained must be transformed into a numerical profile then compare it with those listed in the index by transforming all 20 biochemical results into a seven-figure numerical profile (seven-digit number) by placing them in to groups of three then consigning a specific value for each positive result. Each positive result is given a value equal to 1, 2 or 4 according to the position of the test in its group. The sum of these three values was given the corresponding figure (zero is given for negative reaction). The seven digits numerical profile is then looked up in the index and identification of the isolates.

Genetic analysis

Extraction of genomic DNA

Genomic DNA was extracted according to the Promega kit protocol and as follows:

3 ml of LB broth that was inoculated with 2-4 colonies from MacConkey agar then incubated at 37°C for 24hrs with shaking. 1 ml of an overnight bacterial culture were put in eppendorf tube and centrifuged at 13000-16000 rpm for 2 minutes to pellet the bacterial cells, the supernatant was removed. Added 100 µl of freshly prepared lysozyme solution to the pellet, chilled for 30 min on ice, then centrifuged and removed the supernatant. Washed the pellet with D.W and centrifuged again. Added 600µl of nucleic lysis solution, mix gently by inverting and incubated for 5 min at 80°C, then cooled to room temperature. Added 3µl of RNase Solution and mixed well, incubated at 37°C for 30 minutes, then cooled to room temperature. 200µl of Protein Precipitation Solution were added to the DNA from residual proteins, mixed by vortex and incubated on ice for 5 minutes. Then Centrifuged at 13,000–

16,000 rpm for 3 minutes. The supernatant was transferred to a clean eppendorf tube contains 600µl of room temperature isopropanol, mixed by inversion, centrifuged and decant the supernatant. 600µl of 70% ethanol at room temperature were added to the pellet and mixed, Centrifuged for 2min. Then aspirated the ethanol and air-drying the pellet for 10–15 minutes. Rehydrated the DNA pellet in 100µl of Rehydration Solution for 1 hour at 65°C.

PCR Principle

To carry out a PCR experiment, four fundamental components are required. These components include:

1. DNA template containing the region of the DNA fragment to be amplified.
2. DNA polymerase used to synthesize a DNA copy of the region to be amplified.
3. A pair of oligonucleotide primers complementary to the DNA regions at the 5` and 3` ends of the DNA region that is to be amplified.
4. A supply of deoxy nucleotide triphosphates (dNTPs) from which the DNA polymerase builds the new DNA.

PCR amplification of DNA involves three stages with alternating temperatures. In the first stage, at 94°C, DNA is completely denatured to single-stranded DNA. The second stage, at 40-70°C, involves annealing of the target DNA by primers complementary to the 3' ends of opposite strands of the DNA. In the third stage, at 72°C, DNA polymerase extends each primer, duplicating the target sequence. These three stages are repeated 30-40 times yielding new duplicates every cycle, resulting in many copies of the wanted DNA fragment, which are the PCR products. In this study all (50) Uropathogenic *E.coli* bacterial isolates were screening for the presence of *fim*H virulence gene by amplification used specific primer.

Table 1: Primer of *fim*H virulence gene.

Gene	Primer sequence	Size of prodct (bp)	Refrences
fim H	**F:**TGCAGAACGGATAAGCCGTGG **R:**GCAGTCACCTGCCCTCCGGTA	508	(Yun *et al*., 2014)

Table 2: Optimal parameters of PCR amplification for *fim*H virulence gene.

Step	No. of cycle	Temperature –time
Initial Denaturation	1 cycle	94^0C for 5 minute
Denaturation	35cycle	94^0C for 30 second
Annealing		62^0C for 30 second
Extension		72^0C for 30 second
Final Extension	1 cycle	72^0 C for 10 min.

RESULTS AND DISCUSSION

Isolation and identification of uropathogenic *E. coli*

A total of 102 urine samples were collected from patients suffering from urinary tract infections in (3) hospitals in Baghdad, AL-Imamein kadhimein Medical city, Al-Yarmouk teaching and Al-Kadhimiya pediatric hospitals during the period from 1/10/2016 to 1/1/2017.

Results have shown that among the total of 102 urine samples that were collected from cases of UTIs, only 76 isolates (74.5%) were gave typical morphological characteristics and biochemical tests that related to *Escherichia Coli*, while the rest 26 isolates (25.5%) were belong to pathogenic bacteria from different genera like *Staphylococcus spp*., followed by *Klebsiella spp*., *Proteus spp*., *Pseudomonas spp*., *Enterobacter spp*. and *Citrobacter spp*. respectively as shown in table (3), therefore members of *Enterobacteriacea* family and specially uropathogenic *E. coli* are considered as the main causative agents of UTIs.

Table 3: The ratio of isolation of uropathogenic *E. coli* from UTI cases in different hospitals in Baghdad.

Hospitals	No. of UPEC	%	No. of others bacteria	%	Total
Al-Kadhimiya pediatric	15	14.7	5	4.9	20
Al-Yarmouk teaching	21	20.6	9	8.8	30
Al-Imamein kadhimein	40	39.2	12	11.8	52
Total No.	76	74.5	26	25.5	102

Results of isolation in this study has been agreed with Vranic *et al*., (2017) who collected Out of 122 urine samples from female outpatients, UPEC was isolated from 82 urine samples, making it the most common cause of UTIs (67.21%), followed by *Proteus spp*. (9.83%) and *Enterococcus faecalis* (7.37%). *Enterobacteriaceae* family are considered as the most important human pathogens that caused UTIs. They comprise approximately 50% of all isolates identified in hospital laboratories in the United States (Karlowsky *et al*., 2003).

In Iraq a study at AL-Yarmouk Teaching Hospital in Baghdad revealed that from a total of 129 urine samples collected from patients suffering from UTIs, 43 uropathogenic *E. coli* isolates were detected. UPEC strains was regarded as one of the major causative agents of UTIs in Iraq (Abdul-Ghaffar and Abu-Risha, 2017). Another study in Baghdad city reported by Khalil, 2016 include isolation a total of 143 urine samples from UTIs patients in diabetes center of AL-Yarmouk hospital revealed that *E. coli* was the most common cause of UTI in

diabetic patients (28.6%) Followed by *Klebsiella spp.* (20%) and *Staphylococcus aureus* (16.7%).

During this study, from a total of 76 isolates that diagnosed as UPEC in patients with UTIs, 50 isolates (65.7%) were isolated from female and 10 isolates (13.2%) were from male, their ages are between (18-50) years for both gender. Also results revealed that 16 isolates (21.1%) were isolated from children between (1-10) years, the incidence of infection with UPEC in young girls was higher than boys.

In a study reported by Tarchouna *et al*., (2013), 90 UPEC strains were isolated from patients with UTI for the period from 2008 to 2009. The ratio of male to female was 2:5 and the average age was 49 years. The high ratio of UPEC that isolated from females has been agreed with Neamati *et al*., (2015) who collected out of 507 urine samples from both gender, The most common isolated pathogens was UPEC (82.6%), 64.5% of the isolates were from females while 35.5% of them were from males. In another study in Zakho city of Iraq indicated that among the total of 205 positive UPEC strains that were isolated from cases of UTIs, (57%) of isolates were from females and (43%) of them were from males. The percentage of UPEC isolates was high in people older than 18 years and younger than 60 years of age (Polse *et al*., 2016). Also a study in a Al-Karkh Surgery Hospital in Baghdad city by Kareem and Rasheed, (2011) revealed that out of 311 urine samples that were collected from patients suffering from UTIs, (68.75%) of the isolates were returned to UPEC, 100 isolates were from females and 25 isolates were from males.

The higher incidence of UTIs in women is related to contamination of perineum with pathogenic *E. coli* that ascending to the urethra and regarded as one of the important route for UTIs in women (Dielubanza and Schaeffer, 2011). Also Shorter length of urethra that nearly to the excreta passage is regarded as an important source for pathogens (McLaughlin and Carson, 2004). Studies also revealed that drier urethral meatus in women and lack of antibacterial properties provided by prostatic fluid in men may be regarded as predisposition factor for UTIs in women (Sood and Gupta, 2012.

Asymptomatic bacteruria (ASB) can affects about (4-7%) of pregnant women, ASB acquired early in pregnancy and can evolved to pyelonephritis later in 13.27% of cases in women, also infection by diabetes increased the incidence of infection by ASB that can detected in 26% of diabetic women compared with 6% in women without diabetes (Parvin, 2009).

Studies indicated that sexually active women are prone to UTIs at incidence 0.5 episode per women every year due to sexual intercourse when bacteria enter in female urinary tract and cause UTIs (Wiles et al., 2008), also use of diaphragm for birth control increases the risk of infection with UPEC because the diaphragm location doesn't permit for full empty of the bladder that allow for the bacteria and urine to collect together (Ahmed, 2015).

Urinary tract infection is less common in men than in women because the male urethra is long, making its difficult for the bacteria to spread to the bladder (Sheerin, 2011). Risk factors for UTIs in young men may include sexual transmission by an infected female partner, anal intercourse and preputial obliteration or may be associated with a kidney stone (Johnson and Delavari, 2002). UTIs usually occurs in older men who have an enlarged prostate or when a catheter is used to drain the bladder. Also bacteria can be spread from another site of the body and reach to the urinary tract by the bloodstream (Abrams *et al*., 2009).

In children, the high incidence of infection with UTI has been agreed with Wojnicz, 2007 who isolated out of 66 strain of UPEC from children with pyelonephritis, the prevalence of UTIs in girls is 6.5% compared with 3.3% in boys. Also another study in pediatrics hospital in Tehran was revealed that a from a total of 100 UPEC isolates detected in urine samples of children suffering from UTIs, 75% of them were from girls and 25% of them were from boys. The higher percentage of girls infection by UPEC may be due to a poor hygiene condition or error in management made by clean of the perineum forwards from the anus to the vulva (Dormanesh *et al*., 2014). In Iraq a study reported by saeed *et al*., (2015) at Pediatric hospital in Irbil city, UPEC was the most common isolated pathogen (33.8%), 70 isolates were from females and 30 isolates were from males. The highest percentage of infection was occurred at age between (6-10) years with a females predominance.

Diagnosis of uropathogenic *E. coli* that caused UTIs is depends upon isolation and laboratories identification of the bacteria. The sample were directly streaked on MacConky agar and incubated at 37°C for 24 hrs. Colonies was appeared as deep purple colour due to lactose fermentation. Also it's appeared as circular, flat and moist with entire margin on this medium. MacConky agar is a selective plating medium used for isolation of *Enterobacteriaceae* and related gram negative rods. Lactose fermenting bacteria produce colonies that have varying shades of red due to conversion of neutral red indicator dye below

pH 6.8 (Atlas *et al*., 1995). *E. coli* is a chemoheterotroph m.o that isolated on chemically defined medium contain a source of energy and organic growth factors (McEwen *et al*., 2010.

For confirmative diagnosis a loopfull of lactose fermented colonies were further streaked on (EMB) agar plates and incubated at 37°C for 24 hrs. EMB agar is a selective medium used for isolation and detection of *Enterobacteriaceae* from mixed specimens. The aniline dyes (eosin and methylene blue) in this medium combine to form a precipitate of green metallic sheen at acidic pH serving as indicators for acid production from lactose (Leininger *et al*., 2001).

Microscopic examination

Identification of the suspected isolated colonies by gram stain is depend upon morphological characteristics (size, shape, arrangement). All strain were appear as pink rods, slender, or oval shaped organisms, short to medium length, straight or slightly curved, non-sporulating occurring as singles or in pairs (McEwen *et al*., 2010).

Biochemical tests

According to the results of morphological and microscopic characteristics, bacterial isolates were subject first to number of biochemical tests that they were gave positive results in catalase test, but its give negative results in oxidase test. Catalase is an enzyme that catalyzes the decomposition of hydrogen peroxide (H_2O_2) to water and gaseous oxygen, its prevent the accumulation of toxic metabolites (warren *et al*., 2000).

E. coli isolates that grow on TSI medium has a A/A profile with a gas production. No H_2S production on TSI was appeared, change the colour of medium is due to fermentation of sugar in the presence of acids that permits for differentiation of fermentative bacteria (Atlas *et al*., 1995). *E. coli* isolates were gave positive results in indole production test due to the ability of bacteria to hydrolyze tryptophan to indole by production of tryptophanase enzyme. Also its gave positive results in methyl red. But its gave negative results in simmon citrate utilization test and Voges-proskauer test. Also the bacteria appear as motile rods in motility test as show in table (4).

Table 4: Results of biochemical tests for UPEC isolated from UTIs.

Biochemical test	Indole	Methy red	Voges-Proskauer	Citrate	Oxidase	Catalase	Motility	TSI
UPEC	+	+	-	-	-	+	+	+

API 20 E system

In Api 20 E system, *Escherichia Coli* isolates were subjected to further identifications. It's give negative results in Arginine Dihydrolase test (ADH), Citrate Utilization test (CIT), H_2S production test, urease production test (URE), Tryptophan Deaminase test (TDA), Voges-Proskauer test (VP), Gelatin Liquefaction test (GEL) tests as shown in figure (1).

Isolates were gave positive results in Indole Production test (IND), beta- lactamase test test (ONPG), Lysine Decarboxylase test (LDC), Ornithine Decarboxylase test (ODC), glucose, mannitol, sorbitol, rhaminose, melibiose fermentation tests but give negative results in sucrose, inositol, amylase fermentation tests as shown in table (5).

Figure 1: identification of uropathogenic *E. Coli* by API 20 E system.

Table 5: Results of biochemical tests for uropathogenic *E. Coli* by API 20 E system.

Biochemical test	ONPG	ADH	LDC	ODC	CIT	H_2S	URE	TDA	IND	VP	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA
UPEC	+	-	+	+	-	-	-	-	+	-	-	+	+	-	+	+	-	+	-	+

The results mentioned above were in agreement with those mentioned by (Talaro, 2008). The results of Api 20 E system in this study revealed that only 76 isolates (74.5%) from a total of 102 isolates were identified as uropathogenic *E. Coli*.

Detection of virulence genes by PCR technique

***fimH* gene**

All 50 DNA samples of UPEC were subjected to molecular detection through PCR amplification of the *fim*H gene by using specific primer, 49 isolates of them (99%) were gave a positive results of sharp amplified bands with molecular size 508bp as shown in figure (2).

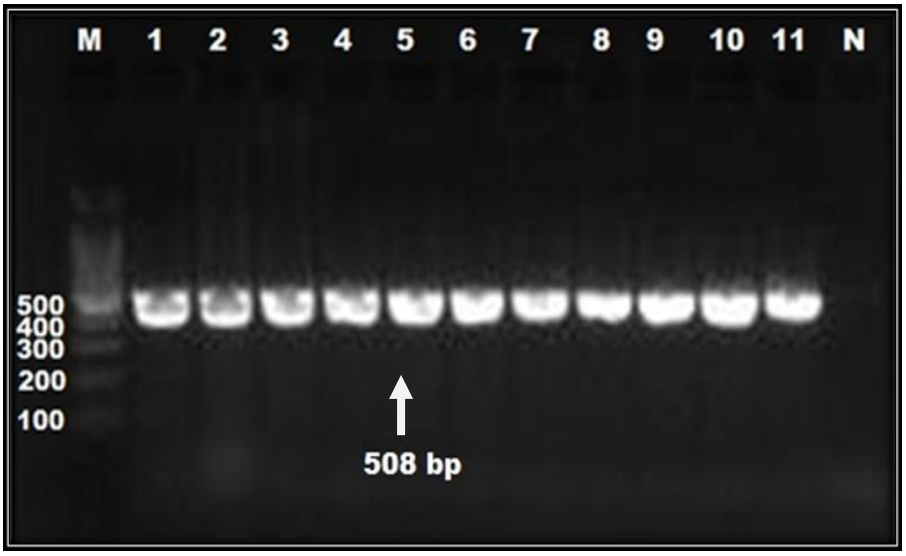

Figure (2): Gel electrophoresis for PCR product of *fim*H gene. (Agarose 2%, 10min. at 100 voltage and then lowered to 70 volts, 60min.).Visualized under U.V light after staining with ethidium bromide. Line M: DNA marker (100-1000bp), Line 1-11: represented positive results of *E.coli* isolates that gave amplified product (508 bp), Line N : negative control.

Binding of UPEC to the mannose containing receptor of host cells is mediated by an adhesin encoded by the *fimH* gene. The *fim* gene cluster are present on the chromosome of pathogenic strains of *E. coli.* Nine genes have been identified within the *fim* operon which encodes the genes necessary for synthesis, assembly and regulation of type 1 pili which are short filamentous organelle encoded by a contiguous DNA segment, (Hung *et al*., 2002; Le Trong *et al*., 2010).

The results of a study by Hojati *et al*. (2015) in Iran indicated that more than 90% of uropathogenic *E. coli* isolates harbored the *fim*H gene, so the high binding ability of *fim*H may lead to increased pathogenicity of *E. coli strains.* thus, *fim*H could be used as a possible diagnostic marker. Also another study published in china for dignosed the virulence genes of UPEC showed that 92% of UPEC isolates posses a *fim*H gene (Zhao *et al*., 2009). A study by

Esam *et al*. (2015) for determine the occurrence of virulence genes expressing fimbriae revealed that among a 112 UPEC isolates obtained from outpatients suffering from UTIs in seven Iraqi hospitals, *fim*H gene were detected in 91.07% of UPEC isolates.

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