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Molecular detection of Hemolycin in *Escherichia coli* and attempt to inhibition by using the Probiotics

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Abstract

The current study included of (60) urine samples were collected from patients suffering from urinary tract infection in Tikrit Hospital during the period from April 2017 to June 2017 The results of identification showed that among (49) at percentage (81.6 %) positive samples were found (24) of samples with percentage (48.97 %) were *Escherichia coli* isolates .

The susceptibility of isolates to avariety of antibiotics has been investigated, the results revealed that the isolates were (100%) resistant to (Erythromycin and Nalidixic acid), also these isolates have the highest percentage of resistant to Ceftriaxone (91.6%), Cephalothin (95.8%), Ampicillin sulbactam (91.6%), Nitrofurantoin (87.5%), where as isolates have shown lowest percentage of resistant to Chloramphenicol (12.5), Trimethoprime (20.83%). antagonistic activity of four types of probiotics bacteria against (24) isolates *Escheriachia coli* were performed. Results showed that all probiotics types have inhibition activity against *E.coli* isolates and the diameter of inhibition zone was highest (20.8 mm) when probiotic *L.casei* used ,while *Bifido.bifidum* showed less inhibitory activity against *E.coli* isolates with (14.5mm)of inhibition zone

In this study the Polymerase chain reaction technique (PCR) were used for detection of hemolysin gene (hly A). Two primers (hlyA) with 561 bp and (hlyA) with 1177 bp were used for this purpose. The results were shown that only (62.5%) and (50%) of 24 *E.coli* isolates were carried the genes hlyA (561bp), hly A (1177bp) respectively. Furthermore the investigate of molecular influence of probiotics on hemolysin genes were done by using PCR. The results showed some of them lost hly A gene after treating with probiotics. In conclusion, hemolysin toxin gene is important virulence factor for uropathogenic *E.coli* and using PCR technique was appeared highly specific,very sensitive method, more than it serves as asuitable molecular diagnostic tool for detection UPEC producing hemolysin toxin .

Introduction

Urinary tract infection (UTI) considered among of the most common bacterial diseases, that inffect many of the world's people [1]. It is one of the most commonly occurring medical problems, causing significant morbidity rate and healthcare costs [2]. The term "urinary tract infection" may be defined as the presence of multiplying microorganisms in any site of the urinary tract, that which includes the bladder, kidneys and collecting systems [3].

UTI usually starts when infect bladder "cystitis" and then developed to infect kidney (pyelonephritis), lastly resulting renal failure [4].The evolution of UTIs disease depends on several factors ,the integrity of host defense mechanisms, and the virulence of the organisms pathogen [5]. Urinary tract infections (UTI) are a major public health concern in developing countries. It is caused by a group of pathogens uropathogenic *Escherichia coli* "UPEC" are the most popular etiological agent, Most UTIs are caused by *E. coli*, accounting for up to 90% of community-acquired UTIs [6].The *E. coli* that successfully invade the urinary tract harbour specific factors that enables

them to survive. These strains of *E. coli* are commonly named uropathogenic *E.coli* or UPEC.
The pathogenesis of UPEC were documented and it is mediated by the production diversity of Virulence Factors (VFs)[7].Virulence factors of *E. coli* (UPEC) gave resistance against the effects of host defense, Moreover, virulent bacteria are cabable to produce molecules that actively inhibit the immune response of the host, thereby enhancing bacterial persistence and tissue damage. The genes encoding virulence factors of UPEC are localized to chromosomal gene clusters called "pathogenicity islands" [8].
Virulence factors (VFs) associated with UPEC include adhesins (P fimbriae, type 1 fimbriae, S and F1C fimbriae, afimbrial adhesin), toxins (hemolysin, and cytotoxic necrotizing factor), siderophores (the aerobactin system) and polysaccharide coatings [9]. one of the most important secretory virulence factor of *E.coli* is alipoprotein called α-haemolysin (HlyA) [10], The toxin alfa-hemolysin (HlyA) is capable to lysis erythrocytes and cells of the hosts, this a process can facilitate the passing of mucosal barriers, damage effector immune cells and release host nutrients and iron stores [11] , this nutrients utilized by UPEC for growth and/or survival [12].The expression of α-hemolysin was shown to increase the clinical severity of urinary tract infections [13]. Development of antibiotic resistance among pathogen is one of major drawback to the use of antibiotics [14]. The increasing prevalence of antibiotic resistant bacteria, high costs of antibiotic drug and unsatisfactory therapeutic alternatives have stimulated an interest in novel, non-antibiotic based methods for preventing and controlling UTIs [15]. The World Health Organization (WHO) [16]discuss the increase in resistance to antibiotics, which today is "essential public health problem in both "developed and developing countries" in the world. "In this meeting, the WHO induce global programmers to reduce the use of antibiotics and increase efforts to prevent disease through the development of newer, more effective and safer therapies .
Lactic acid bacteria (LAB) and bifidobacteria are the majority of microorganisms that used as probiotics. LAB "lactobacilli species" are the most commonly utilized group of microorganisms due to their potential beneficiary properties as probiotics. The antagonistic activities of these bacteria are known to inhibit a large number of enteric and urinary pathogenic bacteria [17]. The role of Lactobacillus to prevent and treatment of some infection have documented. Lactobacillus strains have commensally in the human body [18]. the beneficial influence associated to its ability to inhibit the growth of pathogens, there by the secretion of antibacterial substances including "lactic acid, hydrogen peroxide "[19]. Probiotics are being considered as non-pharmaceutical and very safe potential alternatives to prevent, treatment diversity of pathologies inclusive urinary tract infections [20]. Because members of Lactobacillus genus are most commonly given safe or generally recognized as safe (GRAS) status, species of this genus are added to food as probiotics[21], probiotics were defined as "live microorganisms which, when administered in adequate amounts cofer ahealth benefit on the host "[22].

Aims of the study

●-Isolation and identification of *E.coli* from patients with urinary tract infections.
●-determination the bacterial susceptibility of *E.coli* isolates to different antibiotics.
●-Assessment of antagonism activity of some probiotics against E.coli isolates in vitro.
●-Detection the virulence genes, Hemolysin genes (hly A) from uropathogen *E.coli* by using PCR technique.

Material and methods

Samples collection: Atotal (60) samples of urine were collected from outpatients with asymptomatic U.T.I that attending Tikrit hospital, since this study was conducted from period at the beginning of April 2017 to the end of June 2017.
Mid – stream urine (MSU) sample was collected (30 ml of urine) in sterile screw capped tubes after instructed the patients to clean the perineum and to prevent contact between urine and skin . Samples of urine were collected and transported to the laboratory with 30 minutes for microbiological study **[23]**.If there was adelay for any reason, the sample was refrigerated at 4c°**[24].**

Culturing

The urine specimen was mixed thoroughly and the calibrated loop inserted vertically, then the urine samples were cultured on blood agar base and Mac Conkey agar, EMB agar (for initial isolation of Enterobacteriaceae members) and incubated at 37c° for 24 h **[25]**.

Bacterial isolation and identification :

Bacterial isolates were identified by using conventional methods and biochemical analysis (included oxidase, catalase, indole test, methyl red, voges proskauer, citrate utilization, etc….) as compared with identification scheme described by **[26]**. Identification of bacterial isolates was carried out by observing main features of colony morphology (shape, color, size, edges, texture, etc …), on surface of MacConkey plates.
Then single pink colonies which were lactose fermenters on MacConkey plates and those which gave metallic green color on EMB agar were picked off and were transferred to new MacConkey agar plates for further purification by dilution streaking to obtain single isolated colonies , then used for further diagnosis .

Hemolysin production test .

The production of hemolysin was tested on blood agar plates. Tested bacteria were examined by streaking on plate of blood agar, after 18-24h of

incubation at 37c° , the appearance of aclear zone around the colony indicates hemolysis **[27].**

Antibiotic susceptibility testing

Antimicrobial susceptibility tests for all *E.coli* isolates were performed by the modified Kirby – bauer disk diffusion method [28], against apanel of antimicrobial standards as follow, Chloramphenicol (C 10mcg), Tobramycin (Nitrofurantoin (F 100 mcg), Nalidixic acid (NA 30 mcg), Tetracycline (TE 10 mcg), Erythromycin (E 10 mcg), Ciprofloxacin (CIP 10 mcg),Gentamycin (CN 10 mcg), Ceftriaxone (CTR 30 mcg), Cefepime (CPM 30 mcg), Cephalothin (KF 30 mcg), Amoxicillin / Clavulanic acid (SAM mcg), Ampicillin – Sulbactam (SAM 20mcg), Trimethoprime (TMP 10). The selection of antibiotics disc was performed according to the "clinical and laboratory standard institute" [29]. Results were recorded and compared with the standard levels to know whether isolates were Sensitive, Intermediate or Resistant to the concerned antibiotics. The interpretation of inhibition zones of test culture was done according to**[30].**

Probiotics isolates

Strains of probiotics (*Lactobacillus. casie, Lactobacillus. acidophilus, Lactobacillus. plantarum, Bifidobacterium .bifidum*) were obtained from college of Agriculture of Tikrit university.

Preparation of cell free supernatant (cfs)

Each Probiotic lactobacillus was cultivated in MRS broth(De Man Rogosa Sharp) to 24h at 37c° under micro - aerophilic by using candle Jar. cfs was obtained by centrifuging the culture, (10,000 rpm, 10 min), followed by filteration of the supernatant through a 0.2 µm pore size filter **[31].**

Antagonism activity of probiotics

The antimicrobial activity of the cell –free culture supernatant of isolated (against the *E.coli* isolates was determined by the agar well diffusion assay according to the method by [32]. An overnight culture of *E.coli* isolates in Nutrient broth was prepared .These bacteria (approximately $1.5x10^8$ bacteria/ml, turbidity compared against MacFarland) were inoculated by streaking the swab over the surface of Muller hinton agar, wells sized (6 mm) were cut into the agar plate and 50 µl of each cell – free culture supernatant (CFS) was placed into each well. The plates were incubated for 24 h at 37c° microaerophilic by using candle Jar and inhibition of growth was examined by clear zone surrounding each well. The diameter of the growth inhibition zones were measured and recorded in millimetre (mm).

Genomic DNA extraction

E.coli strains (24 isolates) were grown in Lauria Bertoni (LB) broth medium over night at 37c° and Genomic DNA were extracted according to the method mentioned by **[33].**

DNA was extracted as follow : 1.5 ml of a saturated culture was harvested with centrifugation for 3 min at 12,000 rpm. The cell pellet was resuspended and lysed in 200 µl of lysis buffer (40 Mm Tris-acetate Ph 7.8, 20 Mm sodium-acetate, 1 Mm EDTA, 1% SDS) by vigorous pipetting. To remove most proteins and cell debris, 66 µl of 5M NaCl solution was added and mixed well, and then the viscous mixture was centrifuged at 12,000 rpm for 10 min at 4°C. After transferring the clear supernatant into a new vial, an equal volume of chloroform was added, and the tube was gently inverted at least 50 times when a milky solution was completely fonned. Following centrifugating at 12,000 rpm for 3 min, the extracted supematant was transferred to another vial and the DNA was precipitated with 100% EtOH, washed twice with 70% EtOH, dried in speed-vac, and redissolved in 50 µl 1 x TE buffer.

DNA concentration and purity were determined by measuring the absorbance of diluted DNA solution at 260 and 280 nm using Nano Drop (Thermo scientific, Germany) which is used according to the protocol described by [34]**.** The quality of the DNA was determined using 1% agarose gel electrophoresis stained with ethidium bromide, samples were stored at ˉ20c° .Then the DNA samples of *E.coli* isolated were universe concentration about 50 ng / µl.

Primers

Two primer (forward "F" and Reverse "R") were used, they are provided by (Midland certified Reagent Company, USA), in alyophilized form which can be re – dissolved with deionized sterile water to give100 pmol / µl as afinal concentration .

PCR

PCR assay was performed by using specific primer for detection hemolysin toxin genes (hly A). details of primer sequences, predicted sizes of the amplified products and annealing temperature for each primer are illustrated in Table (1):

Table 1: Primers pairs sequences and Amplicon size of hemolysin toxin gene (hly) of *E.coli*

Virulence factor	Primers	5'-sequences-3'		Amplicon size (bp)	References
Hemolysin	hly A	F	GTCTGCAAAGCAATCCGCTGCAAATAA	561	**[35]**
		R	CTGTGTCCACGAGTTGGTTGATTA		
	hly A	F	AACAAGGATAAGCACTGTTCTGGCT	1177	**[36]**
		R	ACCATATAAGCGGTCATTCCCGTCA		

The PCR Premix was prepared by using (Accupower ® PCR Premix kit. Bioneer, Korea). The PCR tube contains freeze – dried pellet of (*Taq* DNA polymerase 1U, dNTPS 250Mm Reaction buffer with 1.5 Mm $MgCl_2$ 1x, Stabilizer and Trackingdge).

PCR Protocols

Several experiments were carried out for optimization and to arrive to optimum conditions. the PCR amplification was prepared according to Kit instructions in 20 µl total volume by added 2 µl

(25ng) of purified Genomic DNA, 1 µl of each primer (F,R) 10 (Pmol / µl) and 15 µl of nuclease – free water. The amplification procedure performed with thermal-cycler were specific for each primer pair, as follows:

Primer name	Thermocycling condition					
Hly A (556 bp)	Stage 1(1 cycle)		Stage 2 (30 cycle)		Stage 3 (1 cycle)	
	94	2 min	94	1 min		
			60	1 min	72	5 min
			72	1 min		
Hly A (1177bp)	Stage 1(1 cycle)		Stage 3 (25 cycle)		Stage 3(1 cycle)	
	94	10 min	94	1 min	72	7 min
			64	1 min		
			68	3 min		

The ampilified DNA products was visulated by ethidium bromide staining after standard gel electrophoresis of 10 µl of the final reaction mixture in 2% agarose or by agarose gel electrophoresis stained by red save dye solution (20,000 x), finally the gel was photographed by gel documentation system. The size of the PCR products were estimated by comparing them with 100 bp DNA ladder.

Effect of probiotics on *E.coli* Hemolysin Genes

The inhibitory effect of Probiotics (*Lactobacillus casei, Lactobacillus acidophilus, Lactobacillus plantarum, Bifidobacterium.bifidum*) against *E.coli* which carried the Hemolysin Genes was conducted as follows;

1-Each coli isolates which carried the genes were cultured on Brain heart infusion broth at 37c° for 24 hr, and then the growths were compared with McFarland standard.

2- Amount 10 ml MRS broth culture for each probiotics containing approximately $1.5X10^8$ bacteria/ml were added to each *E.coli* isolates (separately), then each tubes were incubated overnight at 37c° microaerophilic by using candle Jar.

3- loopfull of each tube above were cultured on MacConkey agar and incubation overnight at 37c°.

4- single coloy from MacConkey agar was cultured again on LB broth at 37c° for 24 hr., then detection of hemolysin virulence genes were carried by PCR technique .

Results and Discussion

Atotal of 60 urine samples from patients suspected of urinary tract infection were collected for the study .All *E.coli* isolated on MacConkey agar medium were reidentified according to morphological identification properties on MacConkey agar and EMB agar; microscopic (gram stain, shape and arrangement of the cells), further identification of *E.coli* was also achieved by some biochemical characteristics such as (catalase, oxidase, IMVIC ,..) according to[37]

Out of 60 urine samples that cultured, 49 (81.6 %) were positive for culture of uropathogen, while (11/18.4%) were negative. from the 49 positive samples, (24/48.97%) samples infected with *E.coli* and (25/51.03%) samples infected with other UTI pathogen These results were near to those reported by [38,39], who found that the percentage positive cultures of urine samples were (83%), (77.3%) respectively.

Similar studies done in different area but such results were in disagreement with those, [40] when found lower percentage (39%) than the results in this study. The over all results shows *E.coli* has the highest isolation rate in different studies with percentage (55.5%), (31.5%) [41]. *Escherichia coli* is one of the major causes of human infectious diseases and is also the most common cause of urinary tract infection (UTI) [42] The severity of the infection depends both on the virulence of the infecting bacteria and on the susceptibility of the host.

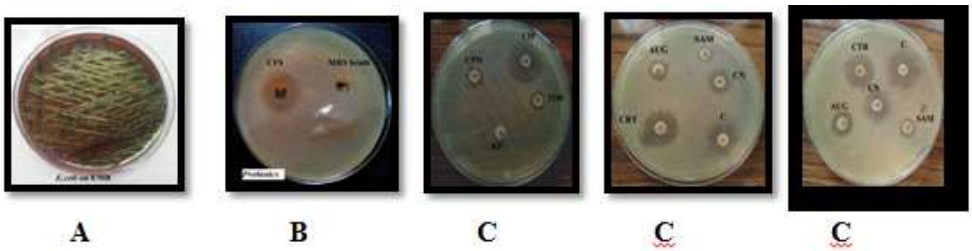

Fig (1): A- *E.coli* grown on Eosine methylene blue (EMB), B- The inhibitory effect of cell-free culture supernatant (CFS) of Probiotics against *E.coli* isolates, C- The susceptibility test of antibiotics against *E. coli* isolates

Antibiotic susceptibility test

All 24 E.coli isolates were tested for antibiotic susceptibility against (14) different standard antibiotics disc by using disk diffusion method [28] and were interpreted according to clinical laboratory standard Institute Guidelines [30]as sensitive , Intermediate , Resistant . The isolate of *E.coli* showed different susceptibility towards antibiotics as shown in Table (2), Fig (1). Antibiotic susceptibility testing of isolates revealed that all isolates of *E.coli* are multi

- resistant to more than one antibiotics, the highest rate of resistance is seen with Erythromycin, Nalidixic acid at percentage (100%), followed by Cephalothin (95.8%), Ceftriaxone (91.6%), Nitrofutantoin (87.5%), Ampicillin (91.6%), Results also found that *E.coli* isolates showed moderately Resistance against to Cefepime (62.5%), Ciprofloxacin (70.83%), Tobramycin (66.7%). while E.coli isolates showed remarkable sensitive towards Chloramphenicol (87.5%) and Trimethoprime (79.17%) .

The percentage resistance of Amoxicillin and Ampicillin described in the present study nearly with those reported by other research as[43], found the prevalence of resistance to Amoxicillin was (81.7%), also this study finding agrees with the results reported by [44] with percentage (90%) resist to Ampicillin. The high rate resistance to third generation ceftriaxone (91.6%) and cephalothin (95.8) were higher than former results obtained by [45] who found that the resistance to ceftriaxone (78%), but percentage to cefepime (67%) was nearly to our study (62.5%). Gram-negative bacteria display resistance to β-lactam antibiotics there by Production of β-lactamases [66]. (ESBLs) are a group of enzymes that are capable to hydrolyze a variety of β- lactams including penicillin, cephalosporins like ceftazidime, cefotaxime, ceftriaxone and monobactams but do not hydrolyze cefoxitin. [47]

Table (2) antibiotics susceptibility tests for *E.coli* isolates.

No	antibiotic	Conc. µg/disc	No.of Resistant strains (100%)	No.of Intermediate sensitive strains (100%)	No.of Sensitive strains (100%)
1	Amoxicillin /Clavulanic acid(AUG)	30	20(83.4%)	-	4(16.6%)
2	Ampicillin sulbactam (SAM)	20	22(91.6%)	-	2(8.4%)
3	Cefepime (CPM)	30	15(62.5%)	1(4.2%)	8(33.3%)
4	Ceftriaxone (CTR)	30	22(91.6%)	-	2(8.4%)
5	Cephalothin (KF)	30	23(95.8%)	1(4.2%)	-
6	Chloramphenicol (C)	10	3 (12.5%)	-	21(87.5%)
7	Ciprofloxacin (CIP)	10	17(70.83%)	-	7(29.17%)
8	Erythromycin (E)	10	24(100%)	-	-
9	Gentamicin (CN)	10	12(50%)	-	12(50%)
10	Nalidixic acid (NA)	30	24(100%)	-	-
11	Nitrofurantoin (F)	100	21 (87.5%)	2(8.4%)	1(4.1%)
12	Tetracycline (TE)	15	20(83.4%)	4(16.6%)	
13	Tobramycin (TOB)	10	16(66.7%)	-	8(33.3%)
14	Trimethoprime (TMP)	10	5(20.83%)	-	19(79.17%)

Amultidrug resistance strains that are resistant to more than of antibiotics tested which reflected the fact that ampicillin and tetracycline were the most commonly prescribed antibiotics in the hospital even before the results of urine analyses so they are the most easily available in the market without prescription and because they were also very cheap in terms of cost, [48]. The resistance percentage of *E.coli* to Tobramycin (79.17%) was in agreement with the study of [43], who found the prevalence of resistance was (87%) to Tobramycin, this resistance of *E.coli* bacteria has been occurred because the reduced penetration of antibiotics inside cell and its low affinity for the bacterial ribosome or drug deactivation by microbial cell enzyme. [49], also the percentage of resistance to ciprofloxacin described in present study agree with those reported by [2], and also agree with the study of [50] which found *E.coli* isolates moderately resistant to ciprofloxacin (64%), and the results disagrees with the results reported by [51] who found that *E.coli* isolates highly sensitive (100%) to ciprofloxacin, adecline in the activity of ciprofloxacin would be especially problematic in view of the ability of gram negative bacilli acquired resistance to all the classes of antimicrobials [52] .

Parvin (2009) have demonstrated that *E.coli* isolates are high resistance to Nalidixic acid as percentage (90.5%) and to Tertracycline (85.7%), and this result agreed with result obtained in the present study, also [53] have reported that chloramphenicol resistance at (44%) and resistance to Trimethoprime at (27%) . this finding disagrees with current study at resistance percentage (12.5%), (79.17%) respectively, while [54] found that (66.7%) of *E.coli* isolates were sensitive to chloramphenicol, it was still lower than those found in the present study. as shown in table (1).

The widespread emergence of antibiotic resistance, particularly multidrug resistance, among bacterial pathogens has become one of the most serious challenges in clinical therapy [55]. Antibiotic resistance is aconsiderable clinical problem especially in treatment of infections by *E. coli.* These resistance has increased during the previous years and normal microbial flora of intestinal has became areservoir for resistant genes [56]. This may be due to an inevitable genetic response to the strong selective pressure imposed by antimicrobial chemotherapy, which plays a vital role in the evolution of antibiotic resistance among bacteria. The plasmid of these

bacteria (which contain resistant gene) transport to another cells of bacteria and species [57].

Drug resistance became is acritical medical problem, because of very fast arise and amutant strains of pathogen that are insusceptible to medical treatment are spread . Microorganisms use varied mechanisms to acquire drug resistance viz. horizontal gene transfer (plasmids, transposons and bacteriophages), recombination of foreign DNA in bacterial chromosome and mutations in different chromosomal locus [58].

Antagonism activity of probiotics

The antagonism activity of cell free supernatant (CFS) of probiotics against *E.coli* isolates was evaluated using well diffusion method. The results of inhibition zone on *E.coli* are reported in Table (3), Fig (1).

Table (3) inhibitory effect of probiotics on E.coli.

Probiotic	average Inhibition zone diameter (mm)
Lactobacillus. casei	20.8
Lactobacillus. acidophilus	17
Lactobacillus.plantarum	16.4
Bifidobacterium.bifidum	14.5

Despite that all probiotics bacteria exhibit serious inhibitory effect on *E.coli* isolates, it was found that the isolates of *L.casei* has the strongest inhibitory effect with inhibition zone diameter at average (20.8)mm against growth of E.coli, the lowest inhibitory effect was noticed for probiotic *bifidi.bifidum* towards all *E.coli* isolates (14.5) mm, *while L.acidophilus , L.plantarum* gave (17) mm, and (16.4)mm inhibition zone against *E.coli* (respectively).

In comparison of this study with others ,the results of [59] revealed that *L plantarum* showed inhibitory effect against *E.coli* islates after 24 h incubation, also the inhibition zones of *bifido.bifidum* as compare with the inhibition zones resulted from [60], while these results were lower than former results obtained by [61], who found the highest inhibitory effect of probiotics bacteria (*L.acidophilus, L.casei, L.plantarum*) against *E.coli* isolates with inhibition zone diameter estimated as (48.83, 27.33, 25) respectively. on the other hand the the results of other research showed more decreased in inhibition zone diameter of two types of probiotics (*L.casei, L .plantarum*) against E.coli isolates with diameter ranged between (7.5-10)mm [62], However, the results of current study was supported with the findings published by [63], who found that Lactic acid bacteria has strongest inhibitory effect against different enteropathogens especially *E.coli. Lactic acid* bacteria and Bifidobacteria are the majority of microorganism which used as "probiotics", these bacteria have antagonistic activities that inhibit many of urinary pathogens [64]. The antimicrobial effect of probiotics attributes to organic acids cause reduction of PH causes acididification of the cell cytoplasm while undissociated acid lipophilic can diffuse passively across the membrane and alert the cell membrane permeability which results in disruption of substance transport system. [65], also lactic acid bacteria produce lactic acids and H_2O_2, which can prevent the overgrowth of other microorganisms including *E.coli* [66].This condition will create an unfavorable environment by secretion bacteriocin, NO_2, H_2O_2, this will attenuate other pathogen by removing essential nutrition and secretion acetic acid, lactic acid, fatty acid which decrease PH and also this probiotics interact with the toxin that secreted from other pathogenic bacteria [67]. This metabolites produced during the fermentation process [68], also the decrease of PH convert organic acid to lipid soluble and diffuse into cytoplasm through the cell membrane [69]. As many reported found that many of LAB bacteria are synthesized bacteriocins were synthetic ribosomaay, this peptides are more effective against many gram negative bacteria [70] .

Hemolysin production

To assay the capability of *E.coli* isolates to production hemolysin, all E.coli isolates (24) were grown on blood agar plate , the clearing was observed .Results of this study showed (11/ 45.83%) from 24 *E.coli* isolates were able to produce hemolysin on blood agar , while (13/ 54.17%) of *E.coli* isolates are not hemolytic .

This results agreed with the results obtained by [71], which found that 12(40%) out of (30) isolates of *E.coli* were able to produce hemolysin on blood agar , while other isolates 18(60%) had no ability to produce hemolysin , also these study in accordance with previous report by[72] , who found that among 220 isolates of E.coli from UTI, (41,36%) are hemolytic, but these results do not resemble the results obtained by [73] who found that high percentage of *E.coli* capable to produce hemolysin when grown on blood agar . Hemolytic activity of isolated revealed that these isolates were pathogenic due to production of hemolysin, which binds with the hemolysin receptor present on the surface of RBC , that favor hemoylsis . [74]

PCR amplification and expression of hemolysin genes (hly A).

The genes encoding virulence factors of UPEC are localized on chromosomal gene clusters — pathogenicity islands [75]. Bacterial virulence factors play a vital role in determining whether an organism will invade the urinary tract and the level of infection acquired**.** The PCR amplification for genomic DNA were used to detect presence of hemolysin genes (hly A) in *E.coli* strains from patients with UTI, two specific primer were used in this study, PCR amplification of hly A gene in positive samples were shown clear PCR product band on agarose gel electrophoresis at (561bp) for the first primer and 1177bp for the second .

Atotal of 24 UPEC strains were used to identify the virulence hemolysin gene (hly A), the results found that the frequency of hly A (encoded hemolysin with

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561bp) was (15/24%) isolates with percentage (62.5%) as shown in Fig (2). In other study demonstrated that (35%) of E.coli isolates were contain hemolysin genes hly A (561bp) [76], this results were lower than our study .

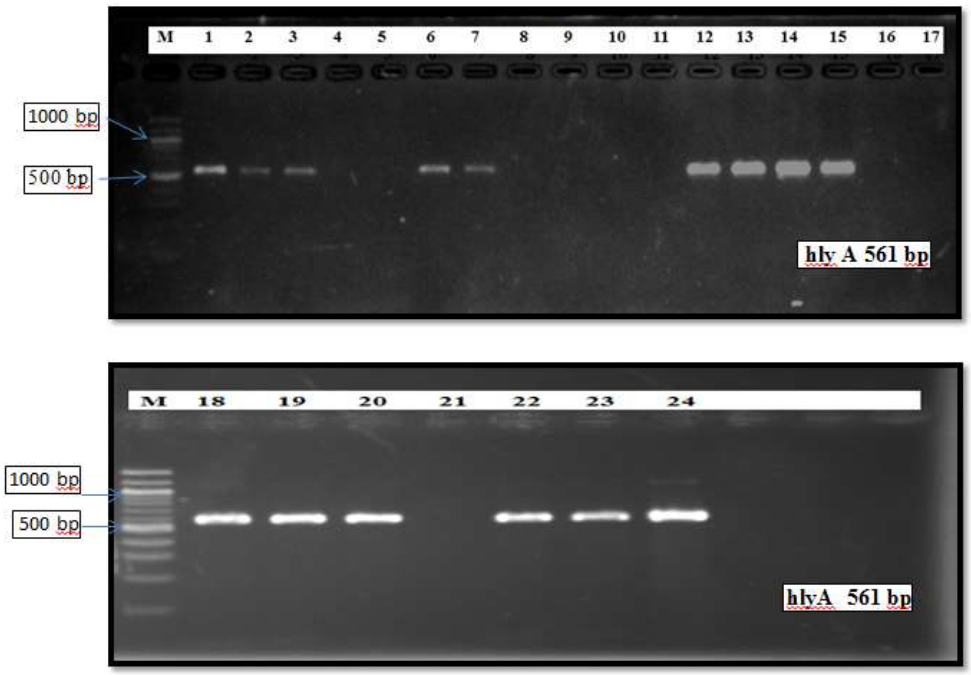

Fig (2): Gel electrophoresis of PCR products (561 bp) of *E.coli* hemolysin gene (hly A) on (2%) agarose before treated with probiotics .M : Marker 100 bP , Lane (1,2,3,6,7 ,12,13,14 ,15,18,19,20,22,23,24) positive samples for hemolysin toxin hly A at 561bp PCR product , Lane (4,5, 8,9,10,11,21) negative samples for hemolysin toxin hly A at 561bp.

screening (hly A) gene with (1177)bp for *E.coli* isolates were showed (12/50%) isolate of bacteria carried the gene hly A, also the Results show atotal of (12) isolates carried both genes of hemolysin, as shown in figure (3).

The prevalence of hemolysin gene (1177 bp) observed in this study (50%) was marginally lower compare with the studies [77] which reported the prevalence of hly A (1177bp) was (90%) and (76%) respectively. on the other hand ,the prevalence of gene was higher compared with previous study by [78], who found that from atotal (161) *E.coli* strains isolated from children with UTI, (9.94%) were positive for the prescence of hlyA, also another study showed from atotal 200 of *E.coli* isolates (21%) only were hemolytic [79], also another study indicated that hly A were present in (23%) of all E.coli isolates, which are still lower than our finding [80].

α-haemolysin (hlyA) is a lipoprotein considered as one of the most important virulence factor secreted by UPEC *E. coli* [81]. Hemolysin has a number of effects on the host, largely due to the formation of unregulated pores for ion transmission across the membranes of a variety of cell [82], Hemolysin molecules insert into lipid-containing membranes producing cation-selective channels of large conductance with a diameter of 2 nm [83].

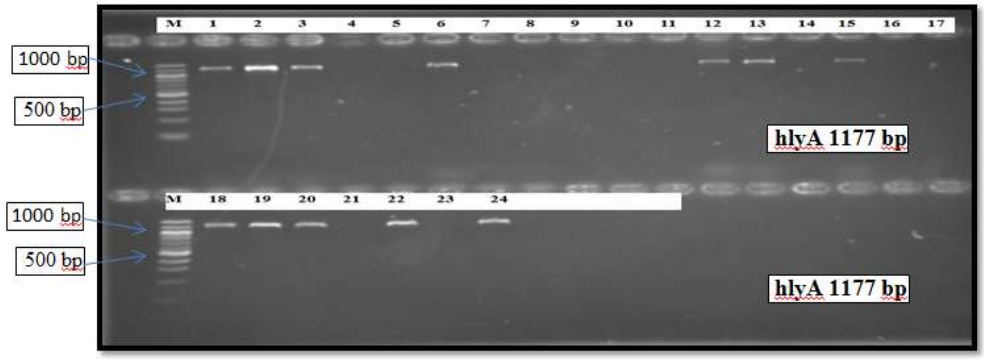

Fig (3) : Gel electrophoresis of PCR products (1177 bp) of *E.coli* hemolysin gene (hly A) on (2%) agarose before treated with probiotics. M: Marker 100 bP ,Lane (1,2,3,6,12, 13,15,18,19,20,22,23,24) positive samples for hemolysin toxin hly A at 561bp PCR product , Lane (4,5, 7,8,9,10,11,14,16,17,21) negative samples for hemolysin toxin hly A at 1177 bp.

The absence of hemolytic phenotype in the presence of hly A in *E.coli* isolates have been characterized and may be due to defects in the hly BCD genes or to defects in the transcriptional activator rfaH [84], where the hly operon required for synthesis and extracellular secretion of *E. coli* hemolysin contain four structural genes arranged in the order hly C, hly A, hly B, and hly D [85], secretion of the Escherichia coli toxin hemolysin A (hly A), requires three accessory proteins hly B, hly D and Tol C. where it was found, in the absence of hly C, the hly A proteins is secreted but in lower amounts and also this product is hemolytically inactive [86].

After E.coli isolates (which contain hly A genes) were treated with mixed of probiotics (*L. acidophilus, L.casei, L.plantarum, Bifido.bifidum*), DNA extraction of this isolates were done, and PCR was carried to determined the hemolysin genes .

In general, the results of this test showed that (13) from (15) isolates stay carring the hly A genes with (561bp), and (2) of isolates lost the hemolysin synthesis and the gene coding to hemolysin, as shown in figure (4), while (7) of (12) isolates just still carrying the hemolysin genes with (1177bp), where more than (5) of isolates lost this genes, after exposed to probiotics aweak band appeared, however this cases also positive because the weak bands appear in the same level of strong bands, this reason of this phenomenon is either of the weakness of the gen hly A of *E.coli* after exposed to probiotics or the concentration of DNA samples are little.

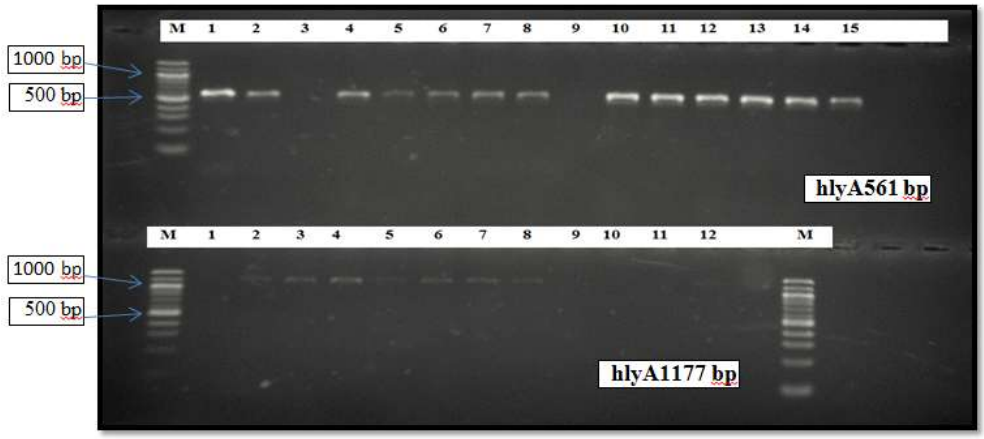

Fig (4) : Gel electrophoresis of PCR products of *E.coli* hemolysin gene (hly A) on (2%) agarose after treated with probiotics.M : Marker 100 bP ., Lane (1,2,4,5,6,7,8,10, 11,12, 13,14,15) positive samples for hemolysin toxin hly Aat 561bp PCR product ,Lane (2,3, 4,5,6,7,8) positive samples for hemolysin toxin hly A at 1177 bp Lane (3,9,1,9,10, 11,12) negative samples for hemolysin toxin hly A for each primer

The advances in molecular technology have facilitated apprehensive studies regarding uro pathogenic *E. coli* [87]. The rapid assessment of virulence determinants detected by polymerase chain reaction (PCR) may be useful for diagnosis and therapeutic strategies [88].

The purpose of this study was to find asafety method to reduce pathogenicity of high virulence pathogenic bacteria responsible for UTI by using natural material and attempt to find asafety method to solve the problem of multi-drug resistance pathogen.

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الكشف الجزيئي عن الهيمولايسين في بكتريا *E.coli* ومحاولة التثبيط بأستخدام المعززات الحيوية

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الملخص

تضمنت الدراسة الحالية جمع (60) عينة ادرار من المرضى المصابين بألتهاب المجاري البولية في مستشفى تكريت وللفترة من شهر نيسان 2017م ولغاية شهر حزيران 2017 م، وقد أظهرت نتائج التشخيص أن من بين (49) عينة بنسبة (81.6%) وجدت عزلات بكتريا *Escherichia coli* في (24) عينة بنسبة (48.97%). وقد تم التحري عن حساسية العزلات تجاه أنواع مختلفة من المضادات الحيوية، وقد كشفت النتائج مقاومة العزلات بنسبة (100%) تجاه المضادين (Erythromycin , Nalidixic acid)، وقد امتلكت العزلات نسبة مقاومة عالية تجاه المضادات Ceftriaxone (91.6%), Cephalothin (95.8%), Ampicillin sulbactam (91.6%), Nitrofurantoin (87.5%). بينما أظهرت العزلات نسبة مقاومة واطئة تجاه Trimethoprime (20.83%), Chloramphenicol (12.5%)، كما تم أختبار الفعالية التضادية لأربعة أنواع من المعززات الحيوية تجاه (24) عزلة من بكتريا *E.coli*، وقد أظهرت النتائج أمتلاك جميع المعززات الحيوية للفعالية التضادية تجاه عزلات *E.coli*، وقد أعطى أعلى قطر تثبيط (20.8) ملم عند أستخدام بكتريا *Lactobacillus casei* ، بينما أظهرت بكتريا *Bifidobacterium bifidum* أنها أقل فعالية تثبيطية تجاه عزلات *E.coli* مع قطر تثبيط (14.5) ملم.

أستخدمت في هذه الدراسة تقنية ال PCR للكشف عن جين الهيمولايسين (hly A)، وقد أستخدم أثنين من هذه البادئات النوعية (hly A (561 bp، hly A (1177bp)، وقد أظهرت النتائج أن (62.5%) و (50%) من مجموع (24) عزلة من بكتريا *E.coli* تمتلك جين hly A(561bp)، hly A (1177bp) بالتتابع. أضافة الى ذلك، تم التحري عن التأثير الجزيئي للمعززات الحيوية على جين الهيمولايسين بأستعمال PCR. وقد أظهرت النتائج أن بعض من هذه العزلات قد فقدت الجين hly A بعد المعاملة بالمعززات الحيوية، يستنتج من ذلك أن جين ذيفان الهيمولايسين هو عامل ضراوة مهم للمرض البولي *E.coli* وأن أستعمال تقنية ال PCR تعد طريقة جدا حساسة وذات خصوصية عالية، وأكثر من ذلك أنها تعد أداة تشخيصية جزيئية مناسبة للكشف عن ذيفان الهيمولايسين الذي ينتجه الممرض البولي.