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Virulence Characteristics of Multiple Drug Resistant Uropathogenic Escherichia coli from patients in some clinics of Sebha

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Abstract Urinary tract infections are the most common health problems in many countries of the world. Although *E. coli* is normal intestinal flora but considered as the main prominent opportunistic active uropathogen because of its pathogenicity which referred to it's different virulence factors like hemolysin, biofilm, enterotoxins and others. In this study, 18 negative bacterial species were isolated from 30 urine samples for outpatients of some clinics in Sebha city, where *Escherichia coli* was the most common bacterial species in the patients of both sexes (19.15%). Virulent strains were identified based on its resistance to antibiotics, hemolysis, cell surface hydrophobicity as well as the formation of biofilm. The sensitivity test of Uropathogenic *E. coli* (UPEC) strains showed that all isolates were multi drug resistant as 100% of the strains were resistant to antibiotics like Oxacillin, Penicillin, Ampicillin, Amoxicillin while only (13%) of the strains were resistant to Nitrofurantion, Ciprofloxacin, Imipenem. The results showed that all isolates had not the ability to produce hemolysin, but they differed in their ability to form biofilm, where (33.3%) of the strains formed a thick biofilm, while) 40%(formed moderate biofilm and only (26.7%) of the strains formed weak biofilm, Additionally, we found that (66.6%) of the strains carried hydrophibicity markers while (33.3%) of the UPEC strains did not have those markers. Occurrence of virulence factors in UPEC strains confirms the association of UPEC with urinary pathogenicity and their resistance to antibiotics.
Keywords: Multi drug resistance, Urinary tract infections, Uropathogenic *Escherichia coli*, Virulence factors.

خصائص الفوعة (دراسة العوامل) الممرضة لبكتيريا uropathogenic *Escherichia coli*

المقاومة للمضادات الحيوية في المرضي المترددين على بعض مصحات مدينة سبها

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الملخص التهابات المجاري البولية هي إصابة مرضية للمجاري البولية وتعتبر من أكثر المشاكل الصحية شيوعا في كثير من دول العالم؛ بالرغم من أن بكتيريا *Escherichia coli* هي ساكن طبيعي في الأمعاء إلا أنها تعتبر من أكثر الميكروبات الانتهازية أمراضية للجهاز البولي؛ وذلك لمقاومتها للمضادات الحيوية ولما تمتلكه من عوامل ضراوة متنوعة مسؤولة عن امراضيتها مثل: الهيمولايسين، الغشاء الحيوي، السموم الخارجية وغيرها. في هذه الدراسة تم عزل 18 نوع من البكتيريا العصوية السالبة من 30 عينة بول للمرضى المترددين على بعض مصحات مدينة سبها، حيث كانت بكتيريا *E. coli* من أكثر الأنواع البكتيرية تكراراً في الحالات المرضية من الجنسين بنسبة 19.15%. سلالات Uropathogenic *E. coli* (UPEC) تم تعريفها على أنها ممرضة على أساس مقاومتها للمضادات الحيوية و قدرتها على تحليل كريات الدم الحمراء، قدرتها على تكوين الغشاء الحيوي وأيضا امتلاكها لخاصية كره الماء، بين اختبار حساسية سلالات Uropathogenic *E. coli* للمضادات الحيوية أن كل السلالات المعزولة (100%) كانت مقاومة للعديد من المضادات الحيوية مثل: Oxacillin, Penicillin, Ampicillin and Amoxicillin، بينما 13% فقط من السلالات مقاومة للمضادات Nitrofurantion, Ciprofloxacin and Imipenem، كما أبرزت النتائج أن كل السلالات المعزولة لم تكن لها القدرة على تحليل كريات الدم الحمراء ولكن اختلفت في قدرتها على تكوين الغشاء الحيوي، 33.3% من السلالات كان لها القدرة على تكوين غشاء حيوي قوي السمك، 40% كونت غشاء متوسط و 26.7% كونت غشاء ضعيف، كما تبين أن أكثر سلالات Uropathogenic *E. coli* المعزولة 66.7% كانت تمتلك خاصية كره الماء، بينما 33.3% منها كانت لا تمتلك هذه الخاصية، أن امتلاك سلالات UPEC للعوامل الممرضة قد زاد من امراضيتها و مقاومتها للمضادات الحيوية.

الكلمات المفتاحية: التهابات المسالك البولية، العوامل الممرضة، بكتيريا اشريشيا كولاي، مقاومة العديد من المضادات الحيوية.

Introduction

Urinary tract infections (UTIs) are one of the inflammatory diseases resulted from high multiplication of many pathogens in the urinary apparatus, causing alterations in the perfect function of the urinary tract and kidneys, one of such pathogens is the bacteria [1].Urinary Tract Infection (UTI) involving the presence of bacteria in the urinary tract (UT) which is naturally sterile If symptoms, such as painful or frequent urination or blood in the urine, are present [2]. However, many of these individuals experience no symptoms, this condition is termed asymptomatic bacteriuria (ABU) [3]. Cases of symptomatic bacteriuria are classified either as cystitis when infection is limited to bladder or pyelonephritis when the kidney is infected [3]. Global records on the disease report that among children, the infection is more prevalence in young girls, except in the neonatal age group where boys predominate [4]. It is also estimated that about 20% of women develop an UTI during their lifetime; Furthermore, UTIs account for approximately 23% of all hospital-acquired infections [4]. A study by [5] found that over a 12 month period UTIs had the highest incidence (35 %) of all nosocomial infections in a district general hospital, and the majority of patients were over 60 years of age. UTIs are also the most common infection in long-term care facilities, where they account for 20–60% of all antibiotic prescription use [6]. Microbes might cause symptomatic bacteriuria has been reported for many Gram-negative Species, such as *Escherichia coli*, *Klebsiella*, *Proteus* and *Pseudomonas* and rarely, by Gram-positive organisms such as haemolytic *Streptococci* and *Staphylococcus saprophyticus* [4]. However, The main aetiological agent of UTIs is well documented as *Escherichia coli* [7]. *Escherichia coli* are a very diverse species of bacteria found naturally in the intestinal tract of all humans and many other animal species [7]. A subset of E. Coli are capable of causing diarrhoeal disease, and a different subset cause extra-intestinal disease, including urinary tract infection (UTI), *E. coli* accounts for as many as 90% of all UTIs seen among ambulatory populations [3],[6]. The ability of uropathogenic *E. coli* (UPEC) to cause symptomatic UTI is associated with the expression of a variety of virulence factors, including adhesins (e.g., type 1 and P fimbriae) and toxins (e.g., hemolysin) [8],[9]. Toxins are important virulence factors in a variety of *E. coli* mediated diseases. Production of toxins by colonized *E. coli* may cause an inflammatory response, a possible pathway for UTI symptoms. Two toxins associated with uropathogenic *E. coli* are a-hemolysin (HlyA) and cytotoxic necrotizing factor 1 (CNF1) [3]. Hemolysin is a protein can induce osmotic lysis of erythrocyte because of its boreforming activity and cytotoxic to several types of human cell. *E. coli* can produce several types of hemolysin including extracellular protein (α-hemolysin), cell bound protein (β- hemolysin) and a hemolysin produced by nalidexic acid resistant mutant (γ-hemolysin) [9]. The difficulty in eradicating a chronic infection adherent to each other and to surfaces or interfaces associated with micro colony and biofilm formation [10]. They are embedded in a matrix of extracellular polymeric substances they have produced, and exhibit an varied phenotype with respect to growth rate and gene transcription[10]. High antimicrobial concentrations are required to inactivate organisms growing in a biofilm, as antibiotic resistance can increase 1,000 fold. According to a publication by the National Institutes of Health, more than 80% of all infections involve biofilms [11]. The importance of cell surface hydrophobicity as a virulence factor (that facilitates bacterial adherence to mammalian cells) is known for nearly a century now [9], [11]. It is an important factor assisting *E. coli* to adhere to various surfaces for colonization [3]. Fluoroquinolone are preferred as initial agents for empiric therapy of UTI for their high bacteriological and clinical cure rates [11&12]. Abuse and improper prescribing policy of antibiotics causes remarkable increase of antibiotic resistance pattern among the *E. coli* isolates from UTI, these types of resistance associated with genetic mutation and intra or inter species transfer of resistance gene through plasmid [12],[13]. Considering the majority of UTI cases caused by *E. coli* and increasing use of antibiotics followed by growing resistance in bacteria, the present study was conducted to identify UPEC strains and also investigate the above mentioned virulence factories and the drug resistance pattern of those *E. coli* strains collected from outpatients in some clinics in Sebha.

Materials and methods

Study area

Clean catch urine samples were collected from 30 out patients "males and females" attending the medical reference laboratory "Alkorda" ,the Sebha medical center laboratory and the laboratory of sugar clinic in Gaded district. The study duration was six months in 2016"from April to September" and three months in 2017 "from January to March". The study was carried out in microbiology laboratory of the department of botany, faculty of science, Sebha University.

Sample collection

Clean catch midstream urine was collected from each patient into 20ml sterile container, the specimen was labeled, transported and analyzed within an hour after collection.

Sample processing

Microbiological culture method

Serial dilutions of 10^{-1}, 10^{-2} in addition to the raw sample 10^{0} were prepared of the 30 urine samples, with 1ml of each solution added to 5ml of MacConkey broth (Oxoid) in triplicate and incubated at 40°C for 48 hours. A positive sample for *E. coli* presence was observed by color change from pink to yellow. About 1ml of positive test tubes contents were transferred into 5ml tryptophan broth (1%) and incubated at 37°C for 24 hours. Positive test tubes were also plated using Eosin Methylene blue agar, MacConkey agar and incubated at 37°C for 24 hours. Three drops of Kovac's reagent were added to the test tubes. A

positive test for *E. coli* indicated by the formation of a reddish ring at the surface of the broth. The presence of *E. coli* on the plates were confirmed by identification of rose colonies on MacConkey agar and bright green colonies on Eosin Methylene blue agar. The pink and green colonies on selective media were subjected to microscopical and appreciate biochemical test for proper identification. Identification of bacterial isolates was done by standard microbiological procedures as identified in [14] (Gram stain- colonial morphology- oxidase reaction and biochemical tests (APi20E test strips).

Antibiotic susceptibility testing of isolated *E. coli* strains

The antibiotic susceptibility testing was carried out on Mueller Hinton agar by disc diffusion method using the following antibiotic. Imipenem (10µg), Amoxicillin (25µg), Penicillin (10iu), Gentamicin (10µg), Oxacillin (1µg), Tetracyclin (30µg), Ciprofloxacin (5µg), Chloramphenicol (30µg), Nitrofuration (300µg), Streptomycin (10µg) and Ampicillin (10µg) [15]. Bacterial suspension was prepared from an overnight culture of UPEC strains at 10^5cfu/ ml(compared with the fifth tube of MacFarland standard), 0.5ml of each strain was placed on the Mueller Hinton agar (in triplicate) and it was spread using sterile swap, Then the antibiotic discs were placed on the surface of culture media. The MHA plates were then incubated at37°C for 24 hours. After 24h incubation, the presence of an inhibition zone around the disk was considered as an evidence of the antibiotic effect on the tested strain. While in the absence at such area, the test was considered negative and the strain was resistant to the antibiotic. The result of sensitivity test of UPEC strains were compared with the guidelines for antimicrobial susceptibility testing to evaluate whether the isolated UPEC strains are resistant or sensitive to the tested antibiotics in this study. Multi-drug resistant (MDR) strains were defined as those which showed resistance to three or more of the tested antibiotics [16].

Phenotypic assay to define virulence factors

a) Hemolysin production: UPEC strains were tested for hemolysin using Blood human (5%) agar. The Petri dishes containing this medium were inoculated with 24hours UPEC strains and they incubated at 37°C for 24 hours. The ability of UPEC strains to produce hemolysin was determined by the presence of hemolytic halo around the colony [16].

b) Biofilm formation: Method described by [17], was used to determine the UPEC strains ability for biofilm formation. In this method a loop full of UPEC strains was inoculated in test tube contained 10ml of trypticase Soy broth with 1% Glucose. The tubes were incubated at 37°C for 24 hours. After incubation the contents of the tubes were removed and washed with phosphate buffer saline (PBS) (pH7.3) and they were dried ,then the crystal violet stain (0.1%) were added to each tube for 15 minutes and then the stain was washed with deionized water and the tubes were dried. The results was read by observing the formation of biofilm as a layer at the internal wall tubes by naked eye and comprise with tub negative control (tube contains TSB medium without inoculation). Thickness and color of layer consider an evidence of bacterial ability for biofilm formation.

c) Cell surface hydrophobicity: UPEC strains were tested for their hydrophobic property by using different concentration of ammonium sulphate, bacterial suspension was prepared in PBS at 10^5 cfu\ml then 20µl of the bacterial suspension was mixed with 20µl of 1M, 1.5M and 2M ammonium sulphate on glass slide. Clumps were observed by naked eye. Strains were considered hydrophobic, if they aggregated in the PBS concentration of ≤ 1.5 [16].

Results and discussion

Distribution of negative bacilli bacteria in relationship with gender

The results of the present study showed that negative rods bacteria associated with urinary tract infections were more frequent in females than males. 14 bacterial species were isolated from females while 10 species were associated with males urinary tract infections **Fig. 1 A,B .** *E. coli* was the most frequent isolated bacteria from both males and females by 4 isolates (25%) and 5 isolate(16.15%) respectively **Fig. 1 A,B** .The second common isolated bacteria was *Providencia stuartii* by 18.75% of males and 16.13% of females **Fig. 1 A, B**. The prevalence of *E. coli* followed by *Pseudomonas aeruginosa* in urinary tract infections was mentioned in many studies [18],[19]. *Klebsiella pneumoniae* and *Brucella spp* were isolated by 6.5% of females and 6.25% of males. The current study revealed that some bacterial species were associated with urinary tract infections in females without males including *Klebsiella oxytoca, K.pneum.ozanae, proteus vulgaris, Shigiella spp, Sallmonella spp,Serratia ficara and Serratia liquefaciens* **Fig. 1 B**. By contrast, *Pseudomonas fluorescens, Aerobacter hydrophila, Pseudomonas luteola and Providencia rettg*eri isolated of males only. Some of the isolated bacterial species in this study have been registered as pathogens associated with urinary tract infections for the first time like *Ochrobactrum anthropi, Aerobacter hydrophila and Brucella spp* **Fig. 1A**. *E. coli* was isolated more frequent of females than males, the same results were confirmed by [18] where *E. coli* strains were isolated by 20.16% of females urinary tract and only by 9.9% of males urinary tract. The prevalence of urinary tract infections in females than males was interpreted due to the anatomical structure of males and females genital tract. The males urethra nature protects it of the fecal contamination in addition to the antibacterial property of prostate secretions [20],[21].

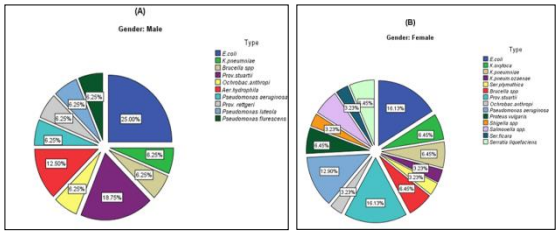

Fig 1. Distribution of Gram negative bacteria causing urinary tract infection (A) in males, (B) in females.

E. coli was the most common bacteria in males and females followed by *Prov. stuartii* and *Pseudomonas aeruginosa*. In the current study Fifteen *E. coli* strains were isolated from the clinical urine samples, these isolates were tested for their antibiotics sensitivity and some of their virulence characteristics were studied.

Antimicrobial susceptibility profiles of UPEC

Recently antibiotic resistance of uropathogenic *E. coli* became serious threat for human health. In this study all UPEC isolates 100% were multi drug resistant (MDR) as all of them were resistant to more than three antibiotics **Fig.2**. In this study antibiotic resistance profile revealed that 100% of UPEC isolates were resistant to Oxacillin, Penicillin and Ampicillin, 87% were resistant to Amoxicillin while 60% of UPEC isolates were resistant to Streptomycin, 47% to Chloramphenicol and 33% to tetracycline and Imipenem while only 13% of strains were resistant to Gentamicin, Ciprofloxacin and Nitrofurantion **Fig.2**. The previous studies reported similar results as they found that 100% of UPEC isolates were resistant to Amoxicillin ,Oxacillin and Penicillin [12]. The resistant of UPEC strains to antibiotics especially Penicillins can be explained by their ability to produce β- lactamase compounds which have the ability to hydrolyze β-lactams loop found in the four most important antimicrobial substances [22]. In contrast, Ciprofloxacin was one of the most effective antimicrobial agents against UPEC isolates, it is considered to be one of the most effective antibiotics for urinary tract complications. On the other hand, one study reported that UPEC isolates were resistance to Ciprofloxacin [23]. Nitrofurantion and Impinem were also effective antimicrobial agents against most of UPEC isolates the same findings were reported by [18]. Furthermore, most of the strains were sensitive to Gentamicin and tetracycline making them suitable agents for the treatment of urinary tract infections.

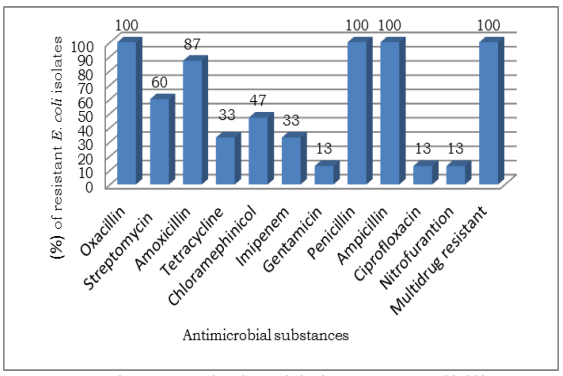

Fig 2. The antimicrobial susceptibility and resistance pattern of 15 UPEC isolates. Ciprofloxacin, Nitrofurantoin and Gentamicin were the most active antimicrobials as only (13%) of isolates were resistant to them ,these were followed by Imipenem and tetracycline (33%) , but sensitivity to amoxicillin, was found to be low (87%). While all the strains were resistant to penicillins (100%).

Virulence Characteristics of UPEC isolates

a) Hemolysin production: The present study showed that UPEC isolates were unable to produce hemolysin, on the other hand, study reported that 60% of UPEC isolates were able to hydrolyze blood partially (α- hemolysin), whereas α- hemolysin was observed only in 2% of fecal *E. coli* isolates [16]. Hemolysin is a toxin responsible for blood hemolysin and it is the most common pathogen associated with cystitis [24].

b) Biofilm formation: To determine the ability of UPEC strains to form the biofilm, the test tube method was used. The current study found that all UPEC isolates were able to produce biofilm with varying degree of thickness. Strong biofilm production was caused by 33.3% of the isolates, while most of the isolates were able to form moderate biofilm 40% and only 26.7% of UPEC isolates formed weak biofilm **Fig.3, 4.In** the present study the association between the ability of strains to form biofilm and their resistance to antibiotics was confirmed, as all UPEC isolates were multi drug resistant and produced layers of biofilm with different thickness. This is due to the ability of biofilm to inhibit and limit the entry of antibiotics therefore, antibiotics fails to penetrate the membrane which surrounded by a complex of proteins and sugar [25], similar results reported by [11].

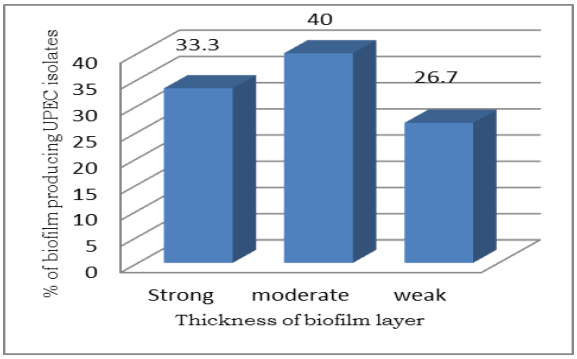

Fig 3. Biofilm-forming abilities of uropathogen-ic *Escherichia coli* isolates.

All isolates had the ability to form biofilm, most of them formed moderate biofilm (40%). 33.3% formed strong biofilm, only 26.7% formed weak biofilm layer.

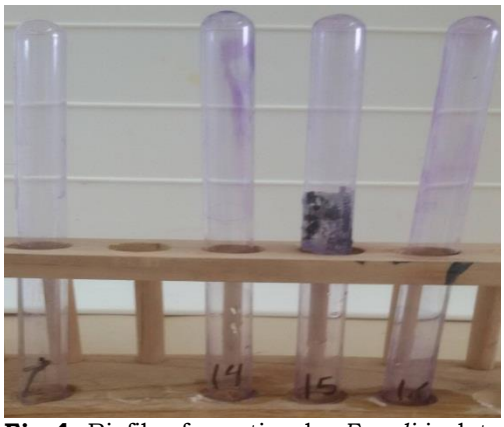

Fig 4. Biofilm formation by *E. coli* isolates in test tubes method.1. Negative control (TSB only), 14. Strong biofilm formed by UPEC isolate,15. Weak biofilm formed by UPEC isolate, 16. Moderate biofilm formed by UPEC isolate.

c) Cell surface hydrophibicity property. Our study revealed that most of the UPEC strains 66.7% were hydrophibic as they formed clumps with ammonium sulphate at concentrations of 1M, 1.5M.While cell surface hydrophibicity markers were absence in 33.3% of UPEC isolates **Fig.5**. Cell surface hydrophibicity markers have a prominent role in the pathogenicity of *E. coli* in the urinary tract as they facilitate UPEC adherence through the mammalian cells [16]. The present study confirmed that the occurrence of virulence factors in UPEC strains increase their resistance to antibiotics and facilitate their adherence and invasion in the urinary tract.

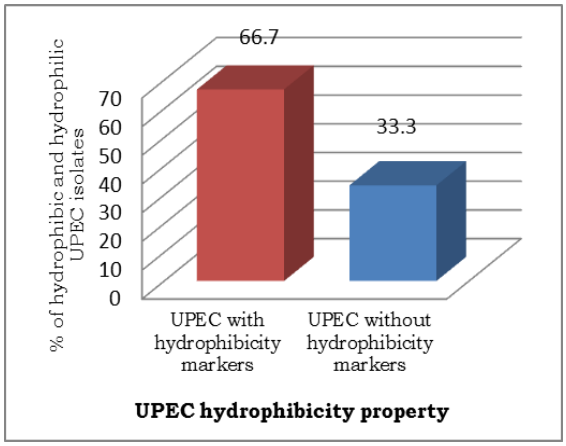

Fig 5. Cell surface hydrophibicity property of UPEC strains.

(66%) of the isolates were hydrophibic while in 33.3% of UPEC strains hydrophibicity markers were absent.

Conclusion

High level of antibiotic resistance pattern was found among Uropathogenic *E. coli* which were isolated in this study. It is quite alarming to note that almost all of the isolates included in this study were found resistant to more than three antibiotics. Antibiotic resistance is becoming serious problem for the individuals admitted to health care centres. However, we recommended that, for empiric treatment of UTIs Sebha locality, Nitrofurantoin, Ciprofloxacin, and Gentamicin are the first agents of choice. Acquisition of UPEC strains for virulence factors such as biofilm and cell surface hydrophibicity markers has increased their pathogenicity. As a further work we recommend testing the effect of plant extracts and probiotics on UPEC strains to detect their potential use as alternatives of urinary tract infections treatment.

Abbreviations

E. coli: *Escherichia coli*
UTI: Urinary tract infection.
ABU: Asymptomatic bacteriuria**.**
MDR: Multi drug resistant**.**
Cfu\ml: Colony forming unit per milliliter.
MHA: Mueller Hinton Agar**.**
TSB: Trypticase soy broth**.**
PBS: Phosphate buffer saline.

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