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Study of Some Virulence Factors of Bacteria Isolated from Urine among the Patients of Type 2 Diabetes Mellitus in Baghdad

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Abstract

The study included 110 urine samples from patients with type 2 diabetes mellitus from patients attending the Specialized Center for Endocrinology and Diabetes and Al-Nu'man Hospital at period from 9/1/2020 to 10/11/2020. 75 bacterial isolates were obtained and distributed as follows, 20(26.6%) isolates of *E. coli*, 20(26.6%) isolates of *S. aureus*, 15(20%) isolates of *Klebsiella pneumonia*. while, *Pseudomonas aeroginosa*, *Protues mirabilis*, *Staphylococcus warneri* and *Micrococcus spp.* reported 5(6.7%) isolates for each species and there were high significant ($P<0.01$) differences between the percentages of bacterial isolates. Samples were cultured on MaConkey and blood agar for initial detection of bacteria, and then the biochemical tests were performed on all isolates. In addition, using the API 20 E diagnostic system to diagnose the Gram-negative bacteria and API Staph to diagnose Gram-negative bacteria. Some virulence factors produced from bacterial species, including Hemagglutination, were also investigated. The results showed that the highest rate of agglutination in bacterial isolates of K. pneumonia and E. coli was 100% and 90%, respectively, while the percentages of the remaining isolates under study ranged from 20. -60%.

Keywords: Diabetes mellitus; Urinary tract infection; Virulence factors.

Introduction

Diabetes mellitus is one of the most common chronic metabolic disorders at the present time, and the disease is characterized by the inability of the body to produce or use insulin properly, as insulin decrease affects the metabolism of carbohydrates, proteins, fats, and the movement of water and ions [1]. Type 2 diabetes or the so-called non-insulin dependent diabetes mellitus (NIDDM) or adult diabetes is a disorder characterized by high blood glucose caused by the inability of pancreas to produce enough insulin or the cells' resistance to the insulin that produced by pancreas, It usually occurs due to the influence of overlapping factors or upon advancing age [2]. Therefore, the current study was designed to focus on type 2 diabetes, which is the most common among other types of diabetes, as it represents

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90-95% of all cases [3]. Infections of the urinary tract and the formation of stones in human are important infections that cause high health damages, and Gram negative bacteria due to its virulence factors, especially bacteria of the intestinal family, are among the main causes of these infections [4]. Urinary tract infections have become one of the most common diseases that require treatment by specialist doctors and it has an effect on people of all ages, from newborns to the elderly, and one of the most common bacterial species responsible for urinary tract infection and originating in the intestine is from the Enterobacteriaceae family such as (Proterus) (5) and several types of bacteria Klebsiella, Staphylococci, Enterobacter, Proteus , Pseudomona, and these types of bacteria are often isolated from people with diabetes and those with urinary tract infections, but most of them are E. coli and rarely to anaerobic organisms [6]. In recent times, urinary tract infections have increased due to the indiscriminate use of antibiotics, which led to the emergence of new strains resistant to it, and thus an increase in the rates of these infections and that some of these factors are able to transfer from one strain to another with wide ranges, especially in some types of normal flora, which are naturally present in the gastrointestinal tract, which is one of the main sources of urinary tract infections [7]. In addition, bacteria containing virulence factors that increase its pathogenicity such as the attachment to the epithelial cells of urinary tract [8]. So, the aim of the present study is to investigate the virulence factors of bacteria isolated from urine of patients with urinary tract infection and diabetes mellitus.

Materials & Methods

75 isolates of urine samples of both sexes and all age groups were collected from several hospitals in Baghdad, including Al-Nu`man Hospital, the Specialized Center for Endocrinology and Diabetes, for the period from 9/1/2020 until 10/11/2020, in which sterile plastic bottles were used to collect the sample For one time, the patient was recommended to leave the initial flow of urine and collect the mid-stream urine. The plates were incubated at 37 ° C and 24 hours for the initial isolation. After that, a number of diagnostic, morphological and biochemical tests were performed.

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Morphological and culture characteristics of bacterial isolates

Colonies were prepared for each species on media by the Loop conveyor and fixed by passing them on the flame and staining them with a Gram stain to diagnose the colony shape and the interaction with stain, and examined under a light microscope to distinguish the positive and negative bacteria for the Gram stain [9]. The isolates were diagnosed based on the morphological characteristics of the colonies according to Its color, smell and its size on MaConkey and blood agar, in addition to its ability to analyzed the red blood cells on blood agar and then perform several tests, including the biochemical tests using the API 20 E system and the API staph in addition to the Vitek-2 test.

Detection of bacterial virulence factor

Hemagglutination [10]

A- Preparing human red blood cells

Human blood type O + obtained was used. The sample was centrifuged in a central centrifuge at a speed of 1500-2000 rpm / minute for five minutes using test tubes to obtain sediment of red blood cells. The cells were washed with normal solution four times, where the suspension is discarded at the same speed, so that the blood suspension is ready for testing.

B- Test perform

The Slide agglutination method was used to perform the examination, by mixing a drop of 0.05 ml of bacterial suspension at the age of 24 hours with a drop of red blood cells, and then the two drops were mixed using a light microscope. A control experiment was conducted with each isolate by mixing a drop of bacterial suspension with a drop of normal saline on a clean glass slide.

Testing the susceptibility of isolates to adhesion to epithelial cells [10]

A- Preparation of human epithelial cells

The epithelial cells were obtained from sediment of urine samples of uninfected females. The cells were washed after sedimentation with normal saline four times by

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repeating centrifugation each time for 5 minutes at a speed of 1500-2000 rpm and then re-suspending the cells with normal saline.

B- Test perform

1. 0.5 ml of a 24-hour-old bacterial culture was taken, and 0.5 ml of the epithelial cell suspension was added to it.
2. The mixture incubates at a temperature of 37 ° C for an hour with stirring for 10 minutes.
3. The cells were washed four times with a normal saline, with centrifuge, for 5 minutes, at a speed of 1500-2000 rpm each time.
4. A drop of the final suspension was taken on a glass slide and left to dry at room temperature, then fixed with the heat of flame and the slide was stained with Gram stain.
5. - The results of adhesion are observed under an optical microscope using an oily lens, as the positive result appears by sticking bacteria individually or in clusters on the surface of the epithelial cells.
6. Normal saline with epithelial cells without bacterial attachment used as a negative control.

Investigating for lipolytic enzyme production:

The petri dishes method was used to investigate the isolates producing the lipase enzyme as mentioned in [11] inoculation with Tween agar prepared in a circular swab by transferring part of the bacterial culture. The petri dishes were incubated at 37 ° C, and the petri dishes were observed daily for a week. The positive result was recorded by the appeared of dark spot around the colony.

Investigating for DNase production [11]

- ❖ The examination was carried out by inoculating (DNA agar) medium prepared with the bacterial isolates under study by the planning method.
- ❖ The plates were incubated at a temperature of 37 m for a period of 24 hours.
- ❖ Then cover the surface of the plate with 2 ml of the prepared toolidin blue dye for 10 minutes.
- ❖ Wash the dish with distilled water more than once until the blue color runs off the petri dishes.

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- The positive result was observed when the petri dishes appeared blue, while the colonies and the surrounding area were colored pink to purple.

Results & Discussion

Sample collection and bacterial identification

75 isolates were collected from urine samples of both sexes for patients with type 2 diabetes, from several hospitals in Baghdad, including Al-Nu`man Hospital / Specialized Center for Endocrinology and Diabetes, for the period from 9/1/2020 until 10/11/2020. The patient was recommended to leave the primary diuresis and collect the mid-steam urine and record the patient's information. The urine samples were initially cultured on blood agar and MaConkey agar, by sterile loop, and by planning method to obtain separate colonies. The petri dishes were incubated at 37 ° C in the incubator for a period of 24 hours for the initial isolation. After that, a number of morphological and biochemical diagnostic tests were conducted for the bacteria involved in the study, where the diagnosis of bacteria was confirmed by API system, as 75 bacterial isolates were obtained, distributed as follows, 20(26.6%) isolates of *E. coli*, 20(26.6%) isolates of *S. aureus*, 15(20%) isolates of *Klebsiella pneumonia*. while, *Pseudomonas aeroginosa*, *Protues mirabilis*, *Staphylococcus warneri* and *Micrococcus spp.* reported 5(6.7%) for each isolate respectively, and there were high significant ($P < 0.01$) differences between the percentages of bacterial isolates as shown in table (1) and figure (1). The current study showed that E. coli was the highest (26.6%) among the bacterial genera that were isolated from diabetics, and this is consistent with [12] and [13] where they also found that E. coli was the largest percentage of infections UTI in patients with or without diabetes mellitus. Most studies indicate an increase in the incidence of urinary tract infection caused by Staphylococcus aureus ، and this is consistent with the results of our current study. Where [14] indicated that S. aureus comes second in causing urinary infections. Staphylococcus is a very successful pathogen as a result of its possession of many virulence factors that enable it to cause infection and invade the local tissues of humans, such as some of them possessing the capsular and producing enzymes such as the protease enzyme, in addition to the produce various toxins as hemolysin [15].

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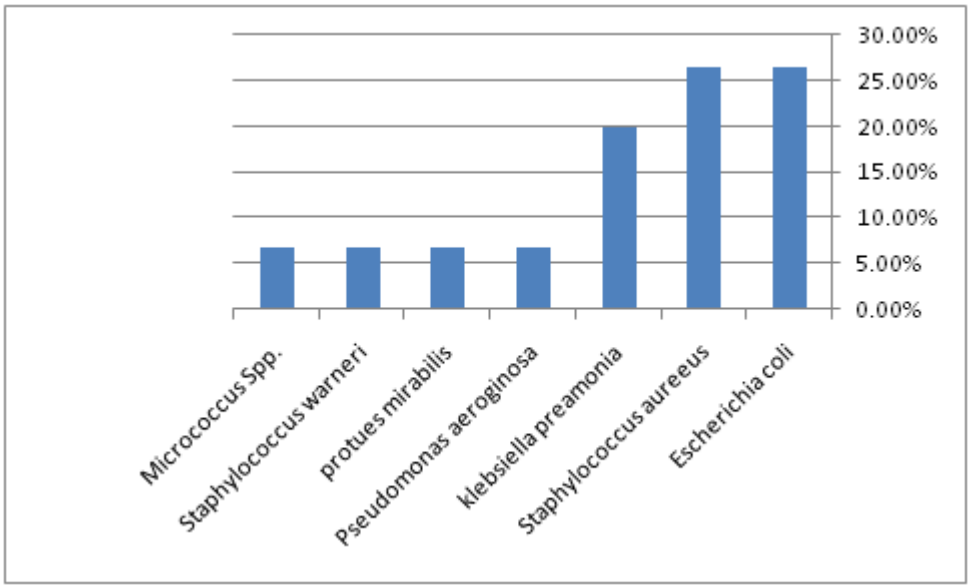
Table (1): species of isolates from the diuretic of patients with type II diabetes

Isolates	Codes	No.	Percentage
Escherichia coli	E1.....E20	20	%26.6
Staphylococcus aureus	S1.....S20	20	%26.6
klebsiella pneumonia	K1.....K15	15	%20
Pseudomonas aeroginosa	Ps1 .....Ps5	5	%6.7
protues mirabilis	Pr1.....Pr5	5	%6.7
Staphylococcus warneri	Sw1.....Sw5	5	%6.7
Micrococcus Spp.	M1.....M5	5	%6.7
Chi-square value x^2		---	**38.9****
Significant at P<0.01**			

Figure (1): species of isolates from the urine of patients with type II diabetes



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Detection of bacterial virulence factor

Hemagglutination [10]

The results of the adhesion are observed by using an optical microscope, as the positive result shows the adhesion of bacteria alone or in clusters on the surface of the epithelial cells. Use normal saline with epithelial cells only as a negative control assay. The results show the percentages of the bacterial isolates with or without hemagglutination, where the highest rate of hemagglutination was observed in the K. pneumonia and E. coli by 100 and 90%, respectively, while the percentages of the remaining isolates ranged from 20-60% as shown in table (2). Where there were significant differences between isolates belonging to the same bacteria on the one hand and isolates belonging to different types of bacteria. The study of [16 & 17] confirmed that the explanation for the effectiveness of hemagglutination carried out by many bacterial species is their possession of plasters represented by the protein structures on the outer surface of the cell. It is necessary in the development of the pathogenesis. In addition to the ability of these structures, including cilia, to cause hemagglutination, they can attach to other cells such as epithelial cells. The results of our current study regarding agglutination of E. coli and K. pneumonia bacteria agreed with what they found [18], as it stated that the highest agglutination rate was in these types of bacteria.

Table (2): Investigation of bacterial isolates that form hemagglutination

Isolates	**%-**	**%+**	**Chi-square value**
Staphylococcus aureus **20**	-14 S3,S4,S8,S9,S11,S12,S13,S14, S15,S16,S17, S18,S19,S20	+6 S1,S2,S5,S6,S7, S10	
Percentage	%70	%30	**16
klebsiella pneumonia **15**	-	15+ K1…K15	
		%100	
Escherichia coli	2-	+18	

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20	E5,E11	E1,E2,E3,E4,… E10,E12,..E20	
	%10	%90	****64**
Pseudomonas aeruginosa **5**	3- P2,Ps4,Ps5	2+ PS1,PS3	
	%60	40%	***4**
proteus mirabilis **5**	2- Pr1,pr5	3+ ,pr2,pr3, Pr4	
	%40	%60	***4**
Staphylococcus warneri **5**	Sw1,Sw3,Sw4,Sw5	1+ Sw2	
	%80	%20	****36**
Micrococcous Spp. **5**	4- M1,M2,M4,M5	1+ M3	
	%80	%20	****36**
Chi-square value	****65.9**	****126.1**	
***Significant at P< 0.05. Significant at** P<0.01.**			

Testing the ability of isolates to adhere to epithelial cells

K. pneumonia showed the highest rate of adhesion to epithelial cells, with 100%, followed by Escherichia coli isolates, which recorded 90% the consistency of epithelial cells compared with the rest of the isolates and the differences were statistically significant and highly significant between the different bacterial isolates in terms of adhesion to epithelial cells, as shown in table (3). It is also noted in the same table that the number of positively tested Gram-negative bacteria is greater than that of positive bacteria especially *K. pneumoniae*, which aid its adhesion [16]. The results of our current study are in agreement with previous studies, including [18-19] which showed that K. pneumonia has the ability to adhere to epithelial cells and also

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agreed with [20], the study [21] indicated that the adhesion of microbes to the epithelial surfaces is an initial and basic stage of infection. The [22] also mentioned that the adhesion factors are of great importance in the settlement of inflammatory bacteria in the tissues of the body, as the cilia adhere to the L-layer (Glycolipid) present in the epithelial cells, and this is related to pyelonephritis. Recent studies indicate that filamentous structures or outer membrane proteins (OMPs), as well as lipopolysaccharides (LPs) and glycoproteins, act as adhesions that facilitate fixation of the microorganism in host tissues [23-24].

Table (3): investigation of bacterial isolates (attachment to epithelial cells)

Isolates	**%-**	**%+**	**Chi-square value**
***Escherichia coli* 20**	E3,E4	E1,E2,E5,E6,E7,E8 E9,E10,E11,E12, E13,E14,E15,E16 E17,E18,E19	
Percentage	%10	90%	**16
***Staphylococcus aureus* 20**	S3,S4,S6,S7,S8,S9,S11, S12,S13,S14,S15,S16,S17, S18,S19,S20	S1,S2,S5,S10	
	%80	%20	
***klebsiella pneumonia* 15**	-	K1,K2,K3,K4,K5,K6 K7,K8,K9,K10,K11 K12,K13,K14,K15	
		%100	**64**
***Pseudomonas aeruginosa* 5**	P2,P4,P5	PS1,PS3	
	60%	40%	*4

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proteus mirabilis **5**	Pr2, ,Pr5	Pr1,Pr3, Pr4	
	%40	%60	***4**
Staphylococcus warneri **5**	Sw1,Sw2,Sw3,Sw4,Sw5	-	
	%100		****36**
Micrococcous Spp. **5**	M2,M3,M4,M5	M1	
	%80	%20	****36**
Chi-square value	****85.68**	****108.2**	
***Significant at P< 0.05. Significant at** P<0.01.**			

Lipase production investigation

The method of investigating the production of the lipase enzyme depends on one basis, which is the splitting of the molecule of the base material for the action of the enzyme represented by (80Tween) into free fatty acids and alcohol (25). The dish method was used to investigate the production of the enzyme by the isolated bacteria under study. The fatty acids formed interact with the calcium present in the medium of (80Tween) to form the fatty acid salts that appear in a precipitate form, which is manifested by the formation of an opaque zone around the bacterial colony producing the enzyme Lipase. High statistical significance compared to the rest of the other isolates, as shown in table (4). The results of current study are in agreement with the study [26] in terms of *Staphylococcus spp.* and *P. mirabilis* producing the lipase enzyme, as well as current study agreed with [18, 27-28]. The study [29] indicated that the production of the lipase enzyme in S. aureus is a chromosomal dominant that plays a role in the colonization process of bacteria on the skin. The study [30] referred that the lipase enzyme is ideal at pH = 6, and it needs calcium as a catalyst to stimulate the enzyme's activity, so calcium chloride salts are added to the medium, which highlights the positive result. The study [25] indicated that the ability of

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bacteria to degrade the host cell fats is an important factor in the spread of bacterial pathology.

Table (4): investigation of the production of bacterial isolates for Lipase enzyme

Isolates	Enzyme production	Without enzyme production	Chi-square value
***Escherichia coli* 20**	3+ E3,E5,E8	-17 E1,E2,E4, E6,E7, E9,E10,E11, E12,E13,E14, E15,E16,E17,E18,E19, E20	
Percentage	15%	%85	****49**
***Staphylococcus aureus* 20**	18+ S1,S2,S3,S4,S5,S6,S7,S10, S11,S12,S13,S14,S15,S16,S17,S18 S19,S20	2- S8,S9	
	90%	%10	****64**
***klebsiella pneumonia* 15**	5+ K1,K2,K6,K7,K8	-10 K3,K4,K5,K9,K10,K11,K12, K13,K14,K15	
	%33.3	%66.6	****11.56**
***Pseudomonas aeruginosa* 5**	+5 Pr1……..Pr5	-	
	100%		
***proteus mirabilis* 5**	4+ Ps1,Ps2,Ps3,Ps4	1- Ps5	

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	80%	20%	
Staphylococcus warneri **5**	Sw1….Sw5	-	
	100%		****36**
Micrococcous Spp. **5**	+4 M1,M2,M3,M4	1- M3	
	80%	%20	****36**
Chi-square value	****95.4**	****110.2**	
***Significant at P< 0.05. Significant at** P<0.01.**			

Screening for DNase production (Cowan, 1986)

The examination was performed by inoculating (DNA agar) with the bacterial isolates under study by planning method. The dishes were incubated at 37 m for a period of 24 hours. Then, cover the surface of the plate with 2 ml of blue toluene dye for 10 minutes. Wash the dish with distilled water more than once until the blue color is finished flowing from the dish. The positive result was observed when the plate appeared blue while the colonies and the surrounding area were colored pink to purple. The results related to the production of the under study bacterial isolates of the DNA enzyme (DNase) showed that the bacterial isolates of Staphylococcus aureus, proteus mirabilis and Staphylococcus warneri showed the highest percentage of enzyme production by 80.60 and 60%, respectively, while the rest of the bacterial isolates under study did not produce this. The enzyme, as shown in table (5). The results of our current study are in agreement with the study of Jumaili (2005), which showed that the proportion of DNA production of Staphylococcus aureus is 80%, while the rest of the isolates gave a negative result for the test, and also the results of our current study are in agreement with Study [31] as it showed that the production of the DNA enzyme from proteus mirabilis was close to the results of our current study with a production rate of (62.2%). The production of the enzyme DNase increases the pathogenicity of S. aureus bacteria and is one of the important virulence factors for it

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[25]. S. aureus, present in diuresis, increases the incidence of complications in transplant patients.

Isolates	**Production**	**Without production**	**Chi-square value**
***Escherichia coli* 20**		E1……..E20	
Percentage		100%	-
Staphylococcus aureus **20**	+16 S1,S2,S3,S4,S9,S10,S11, S12,S14,S15,S16,S17,S18,S19,S20	4- S5,S6,S7,S8	
	80%	20%	****36**
klebsiella pneumonia **15**		K- K1,…,K15	
		%100	
Pseudomonas aeruginosa **5**	3+ Pr1,Pr2,Pr3	-2 Pr4,Pr5	
	60%	40%	
proteus mirabilis **5**		Ps1,…….Ps5	
		%100	***4**
Staphylococcus warneri **5**	+3 Sw1,Sw2,Sw3	2- Sw4,Sw5	
	%60	40%	***4**
Micrococcous Spp. **5**		M1….M5	

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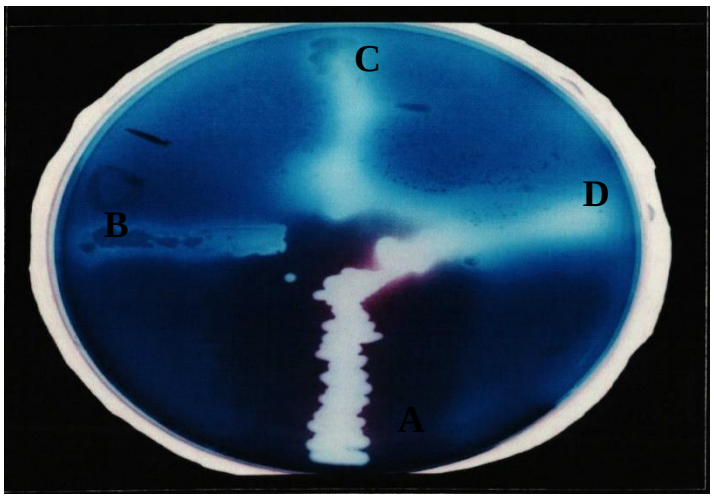
		100%	**36
Chi-square value	**4NS**	**110	
***Significant at P< 0.05. Significant at** P<0.01.**			



- Staph aureus is positive for A / A
- P. mirabilis / positive B.
- E. coli is a / negative result-C
- K. pneumonia with negative bacteria / D.

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