Reactive Oxygen Species Induced by Enterobacteriaceae in Human Uroepithelial Cells

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Abstract

Three hundred mid-stream urine specimens were collected from 300 pateints with Urinary Tract Infections (UTI). One hundred and thirty isolates were obtained from mid-stream urines specimens included: Escherichia coli (63.84%), Klebsiella spp.(23.07%), (K. oxytoca 16.15%, K. planticola 6.92%). Enterobacter aerogenes (6.19%) and Proteus spp. (6.92%) (P. vulgaris 4.61%, P. mirabilis 2.31%). They were identified according to the cultural and biochemical properties. Patients were divided into five groups (A, B, C, D and E) according to the pus cells level in their urine specimens. Moreover, the type and prevalence of bacterial infection in pus cells in each studied group were detected. E. coli performed the highest percentage (61.36%) in all studied groups, particularly group A. Also the study includes the assessment of ROS inducing uropathogens which in measured by using malonadialdehyde (MDA) method. The results showed that the level of ROS was significantly (P<0.05) increased according to the level of pus cells. Thus, group E showed high level of ROS (9.08 nmol/l) in comparison with other groups in this study. On the other hand, the ability of uropathogens to induce ROS was determined. E. coli isolates particularly E. coli19 showed a putative efficiency for induction of ROS (11.62 nmol/l). In contrast, K.planticola4 exhibited the lowest level of ROS (3.14 nmol/l).

INTRODUCTION

Urinary tract is one of the most common sites of bacterial infection, in general urinary tract infections represent a major health problem in many areas of the world, and it is the most frequently encountered infection in daily practices (1). Many bacteria has ability to cause UTI, such as *E.coli, Klebsiella spp., Entrobacter areogenes,* and *Proteus spp.,*many studies reported that *E.coli* gave the highest percentage in patients beside other species, which found in low percentage (2).

Reactive oxygen species (ROS) are deleterious in excess. They are naturally produced by aerobic metabolism and are a permanent threat to living organisms (3). All organisms have developed ways of protecting themselves against ROS, including specific defenses and global responses that enable cells to survive periods of oxidative stress. Both types of protection are regulated and responded to the environment-associated oxidative threat (4).

ROS instability and inability to permeate lipid membranes usually provide an effective shield against propagating damage. However, through reactions with polyunsaturated fatty acids, they generate lipid hydroperoxides and unsaturated aldehydes, which are highly electrophilic, stable, readily propagating between cellular compartments, and capable of reacting with proteins and nucleic acids (4 , 5).

This chain reaction of lipid peroxidation accounts for the role played by ROS in the pathogenesis of atherosclerosis, ischemia, reperfusion injury, and other diseases (5).

The influence of reactive oxygen species (ROS) on cells becomes of increasing interest and the cells damage caused by an excessive production of free radicals or reactive oxygen species (ROS) has been extensively studied. In patients with UTI, an elevated production of ROS in urine and increased ROS-mediated damage of epithelial cells membranes have been detected (6).

It is now well established that mitochondria is the main site of the generation of oxygen radicals, such as, superoxide anion, hydroxyl radical, singlet oxygen and hydrogen peroxide (7). It is estimated that 1–4% of oxygen reacting with the respiratory chain leads to the formation of superoxide radicals ($O^{2.-}$). The other sources of reactive oxygen species include radiation, cytotoxic chemicals and antibiotics (4 , 7).

Malondialdehyde (MDA) is an indicator of lipid peroxidation which increases in various diseases. This increase is reflected in enhanced excretion of several MDA derivatives in the urine (8).

The aims of this study were:

Determination of induced ROS by uropathogens in uroepithelial cells of patients with UTI.

MATERIALS AND METHODS

Patients and urine samples

A total of 300 of midstream urine specimens from patients with UTI only were collected in 5 ml of sterile container.

Those patients were referred to Al-Yarmouk Hospital during the period from 1st of Jan. 2009 to 1st of April 2009, with symptoms suggesting acute UTI. Inclusion criteria were dysuria, frequency, urgency, and abdominal flank pain with or without fever. Patients receiving antibiotic therapy were excluded from the study.

Culture

A loopful of undiluted urine sample was spreaded on MacConkey and Blood agar. The plates were incubated at 37°C for 18h. After incubation, the growth was observed as well as the ability to ferment lactose. Non-fermentable

colonies were re-incubated on blood agar and incubated at 37°C for 18h (9).

Identification of Bacteria

Small part of selected colony of positive culture was transferred and fixed on a microscopic slide, then stained with gram stain to examine cell's shape, grouping and spore forming then biochemical test we used to complete identification (10).

Assay of MDA

Measuring the malondialdehyde (MDA) by thiobarbituric acid (TBA) reactivity is the most widely used method for assessing lipid peroxidation. Malondialdehyde was estimated according to the modified method by Hunter 1985 (11). The pink color which produced in this method is due to the formation of an adduct between the thiobarbituric acid (TBA) and malondialdehyde under acidic conditions (12).

MDA levels were measured by a spectrophotometer. The reaction mixture contained 0.1ml urine sample, 0.2ml of 8.1% SDS (sodium dodecyl sulfate), 1.5ml of 20% acetic acid, and 1.5ml of 0.8% aqueous solution of thiobarbituric acid. The mixture pH was adjusted to 3.5 and the volume was finally made up to 4.0ml with distilled water and 5.0ml of the mixture of n-butanol was added. The mixture was shaken vigorously. After centrifugation at 4000 rpm for 10 minutes, the absorbance of the organic layer was measured at 532 nm. MDA level was expressed as nmol/l (11).

Result and Discussion

Isolation and identification

Results of 300 mid-stream urine samples collected from patients suffering from symptoms referred as urinary tract infection showed that 130 (52.0%) specimens have growth on MacConkey and blood agar.

Examination of suspected isolates showed that 63.84% (n=83) of isolates belonged to *E.coli* according to the cultural and microscopical properties. Whereas, 23.07% (n=30) of positive cultures were belonged to the *Klebsiella spp*.

In addition 6.15% (n=8) of isolates were belonged to *Enterobacter spp.* Furthermore, 6.92% (n=9) of isolates belonged to *Proteus* spp., the colonies appeared a special phenomenon called swarming (table 1).

Biochemical properties of isolates

Table (2) showed the biochemical characteristics of uropathogens isolated from UTI patients.

It was indicated that 63.84% (n=83) of isolates gave clearly positive result for Indole, methyl red, and orthinine, but they were negative for Vogas-proskauer, urease production, Simmon citrate, and oxidase test. On the other hand, these isolates fermented sugar and produced gas on TSI medium but no H_2S was noticed. Thus, these properties were represented *E.coli* (13).

While 23.07% (n=30) of isolates gave clearly positive result on Indole medium,Vogas-proskauer test, produced urea on urea agar, and Simmon citrate medium, but they were negative to methyl red assay (except *Klebsiella planticola* gave positive of this test), orthinine test, and oxidase test. On the other hand, these isolates fermented sugar and produced CO_2 without production H_2S on TSI medium. Therefore, it can be concluded that these isolates represented *Klebsiella oxytoca* and *Klebsiella planticola* according to (13). Notably, *Klebsiella oxytoca* formed 16.15% (n=21) whereas *Klebsiella planticola* formed 6.92% (n=9).

In addition, 6.15% (n=8) of isolates gave clearly positive result for Vogas-proskauer, Simmon citrate, and orthinine assays, and were

negative to oxidase, Indole, methyl red, and urease production test. On the other hand, these isolates able to ferment sugar and produce CO_2 without producing H_2S on TSI medium. These properties related to *Entrobacter aerogenes* (13).

As well as 4.61% (n=6) of isolates were clearly positive to Indole, methyl red, urease production, and Simmon citrate assays, but they were negative for Vogas-proskauer, and oxidase test. These isolates were fermented sugars, produced gas, and H_2S on TSI medium. Thus, these properties were represented *Proteus vulgaris* (13).

In contrast, *Proteus mirabilis* that formed 2.31% (n=3) of isolates was showed negative result to indole test and positive result to orthinine assay.

In general, these isolates considered as uropathogens of urinary tract infection, they have virulent factors such as adherence factors, and endotoxin (13).

Level of pus cells in UTI patients

The specimens were divided into 5 groups according to the level of Pus cells as shown in table (3).

The pus cells give an indication to the severity of urinary tract infections (15).

According to WHO standards, less than 6 pus cells in a urine specimen, it will consider as a healthy. the result indicated that group A has a level of pus cells ranged between (6-10 c.mm) no less no more because if pus cells were less than 6 it considered as healthy group (16).

Moreover, this group was formed (33.80%) of 130 specimen, and number of patient in group A was appeared with significance differences in comparison with all groups at (P<0.05).

In regard to group B, the result showed increasing in the total number of patients, 48 (36.90%). With significance differences in number of patients when compared with groups A, C, D, and E respectively at (P<0.05).

In the group C the number of patient was significantly decreased ,(13.10%) when compared with the above groups, and appeared with significant differences in number of patients when compared with groups A, B, D, and E, respectively at (P<0.05).

Group D showed significant decreasing in the incidence (7.7 %),and gave significant differences in number of patients in comparison with groups A, B, and C, respectively, but not significant with E group at (P<0.05).

While group E was considered the highest group that exhibited high level of pus cells (over 40 c.mm) with low percentage in incidence among UTI cases 8.50% (n=11).Group E was showed significant differences in number of patients in comparison with groups A, B, and C, respectively, but not with group E (P<0.05).

MDA associated with level of pus cells

Table (4) showed the level of MDA, in each studied group of UTI, associated with level of pus cells. Group A revealed low level of MDA, it was 4.75 nmol/l. Thereafter, the level of MDA was increased significantly due to increasing pus cells level, thus group E represent the highest value of MDA concentration (9.08nmol/l) in comparison with other groups.

Although the level of MDA in group A was low, it achieved a significant difference with other groups and control at p<0.05.

In Group B, the level of MDA was significantly (p<0.05) increased (5.76 nmol/l) when compared with other groups and control (2.76 nmol/l).

Obviously, the level of MDA was increased in group C, D and E (7.21, 8.03, and 9.08 nmol/l, respectively). This increasing was significant in comparison with other groups and control at $p<0.05$.

The efficient of bacterial isolates for the induction it MDA of patients with UTI

Figure 1 showed the ability of *E.coli* isolates to induce ROS in UTI patients. Markedly, the isolate *E.coli*19 had high efficacy for induction of ROS in comparison with other isolates; the concentration of MDA was11.62 nmol/l. In contrast, *E. coli3* exhibited less efficiency for induction MDA, the concentration of MDA was 3.51 nmol/l.

Figure (2) showed the ability of *E.aerogenes* isolates to induce MDA in UTI patients. Obviously the isolate E.*aerogenes*4 the highest efficiency for the induction MDA in comparison with other isolates; the MDA was 7.55 (nmol/l). In contrast, *E.aerogenes*1 induced low level of MDA, ration of MDA.

Figure 3 showed the ability of *K.oxytocato* induce ROS in UTI patients. The isolates *K. oxytoca*19 and *K. oxytoca*11 expressed high level of MDA (8.21, and 8.12 nmol/l, respectively).On the contrary the isolate *K. oxytoca*2 induced low level of ROS; (4.35 nmol/l).

In addition Figure 4 shows ability of *K.planticola* to induce ROS in UTI patients. The isolates *K.planticola*8 showed the highest level of MDA (6.86 nmol/l), while the isolate *K.planticola*4 expressed the lowest level of MDA (3.14 nmol/l).

Figure (5) shows the ability of *P. mirabilis* to induce ROS in UTI patients. The isolate *P. mirabilis3* revealed high level of MDA (7.32 nmol/l), while the *P. mirabilis*2 isolate shows low level of MDA was (5.51 nmol/l).

Figure 6 showed the efficiency of *P.vulgaris* for the induction of ROS in epithelial cells in patients with UTIs. It was indicated that the isolate *P. vulgaris*5 exposed the highest level of MDA; the concentration of lipid peroxidation was 7.97 nmol/l. The next was isolate *P. vulgaris*4 which showed higher level of MDA than *P. vulgaris*2; the concentration of lipid peroxidation was 7.62 and 6.19 nmol/l, respectively. Whereas the isolates *P. vulgaris*6, *P. vulgaris*3 and *P. vulgaris*1 revealed low level of MDA, it was 5.63, 5.42 and 4.03 nmol/l respectively.

In general, the results showed the reactive oxygen species (ROS) in UTI patients was higher than healthy person haven't any bacterial infection, also in this study showed the healthy groups haven't high level of ROS, for this reasons agreed with previous study (17,18).

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Table (1) the cultural and morphological characteristics of uropathogen isolated from patients with UTI

Bacterial Isolate	No. of Isolates	Percentage of total isolates %	Cultural Properties on		Morphological Characteristics	Motility
			MacConkey agar	Blood agar		
E.coli	83	63.84	Pink colony	White colony	Coccobacilli or Bacilli	+
Klebsiella spp.	30	23.07	Pink colony mucoid	White colony	Bacilli	-
Enterobacter Spp.	8	6.15	Pink colony	White colony	Bacilli	+
Proteus spp.	9	6.92	Pale colony	Swarming	Coccobacilli	+

+Positive (motile)

-Negative(Non-motile)

Table (2) Biochemical properties of Uropathogens isolates from UTIs patients

Bacterial Isolates / Tests	*E.coli*	*Klebsiella oxytoca*	*Klebsiella planticola*	*Enterobacter aeorgenes*	*Proteus mirabilis*	*Proteus vulgaris*
Oxidase	-	-	-	-	-	-
Indole	+	+	-	-	-	+
Methyl Red	+	-	+	-	+	+
Vogas Proskawer	-	+	+	+	-	-
Simmon Citrate	-	+	+	+	+	+
Urease	-	+	+	-	+	+
TSI	A/A+-	A/A+-	A/A+-	A/A+-	A/A++	A/A++
Ornithine	+	-	-	+	+	-

(+) Positiveresult

(-) Negative result

A\A ++ (yellow slant (acid), yellow buttacid, and no gas or H_2S production).

A\A + - (yellow slant (acid), yellowacidbutt, gas production,and no H_2S formation).

Table (3) Grouping of pus cells in urine sample from patients with UTI

Level of Pus cell in specimens			
Groups	**Pus Level c.mm**	**Number of patients**	**Percentage %**
A	**(6-10)**	**44 a**	**33.80%**
B	**(11-20)**	**48 b**	**36.90%**
C	**(21-30)**	**17 c**	**13.10%**
D	**(31-40)**	**10 d**	**7.70%**
E	**(Over 40)**	**11 d**	**8.50%**
Total		**130**	**100%**

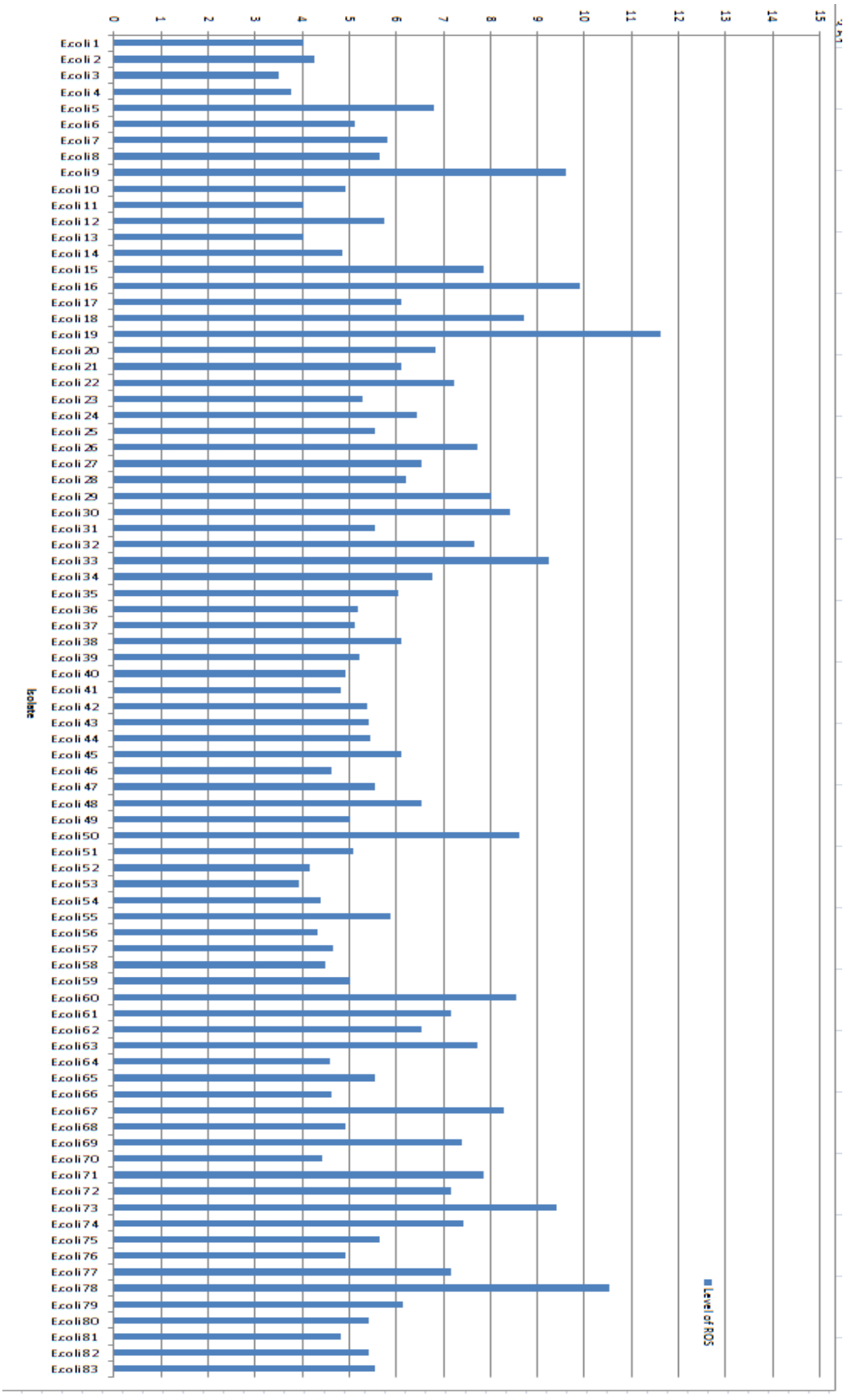
- The identical small letters refer to non- significant differences between number of patientsin each row at p<0.05 level.

Table (4) the level of MDA in UTI groups

Groups	Mean MDA of concentration (nmol/l) ± SD
A	**4.75 ±0.60 a**
B	**5.76 ±0.59 b**
C	**7.21 ±0.54 c**
D	**8.03 ±0.53 d**
E	**9.08 ±0.30 e**
Controls	**2.76 ±0.17 f**

-The identical small letters refer to non- significant differences between mean of MDA and controls in each row at p<0.05 level.

± SD: Standard Deviation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Level of ROS

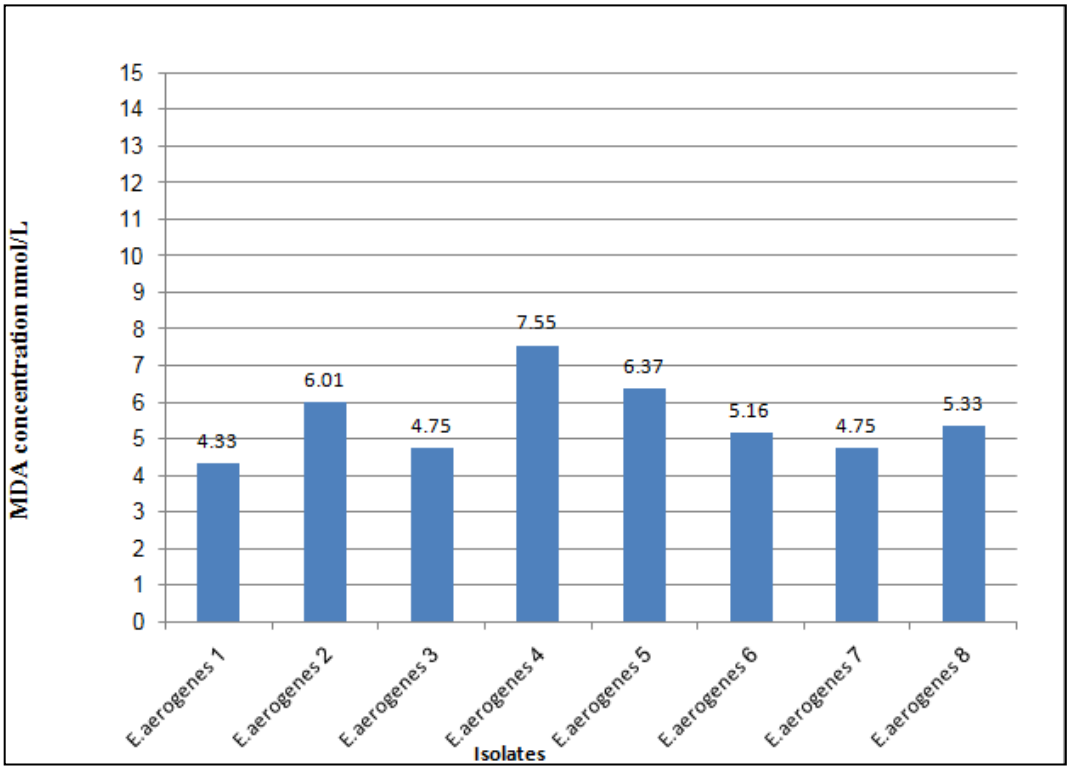

Figure (2) MDA level induced by *Entrobacter aerogenes* isolates in UTI patients

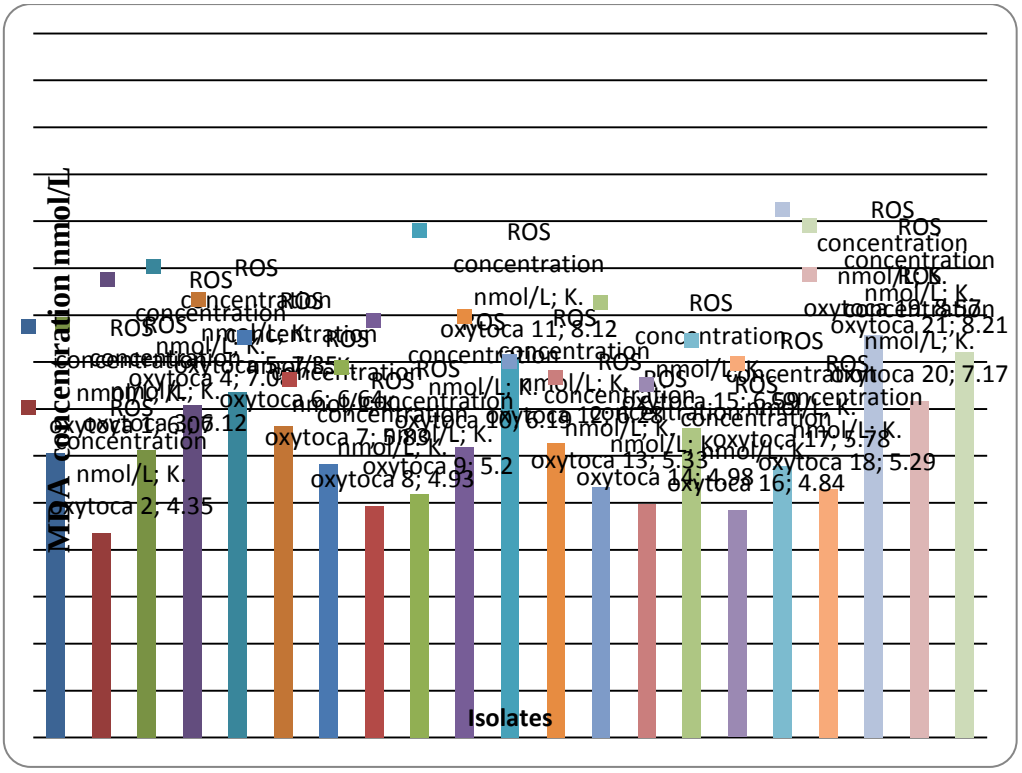

Figure (3) MDA level induced by *Klebsiella oxytoca* isolates in UTI patients

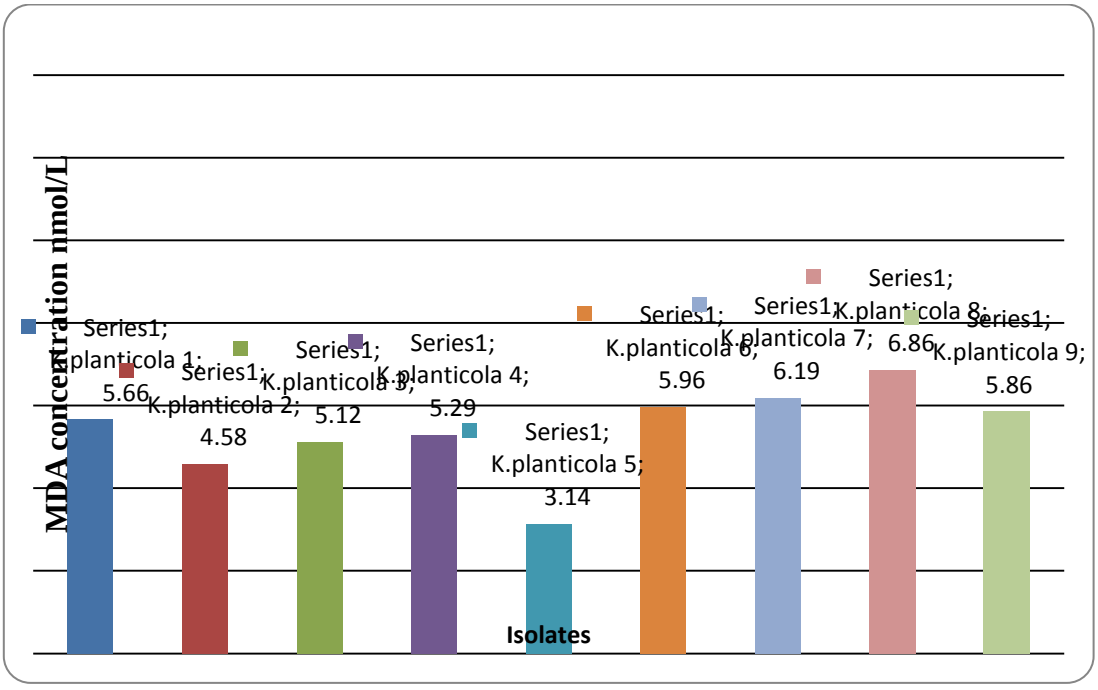

Figure (4) MDA level induced by *Klebsiella planticola* isolates in UTI patients

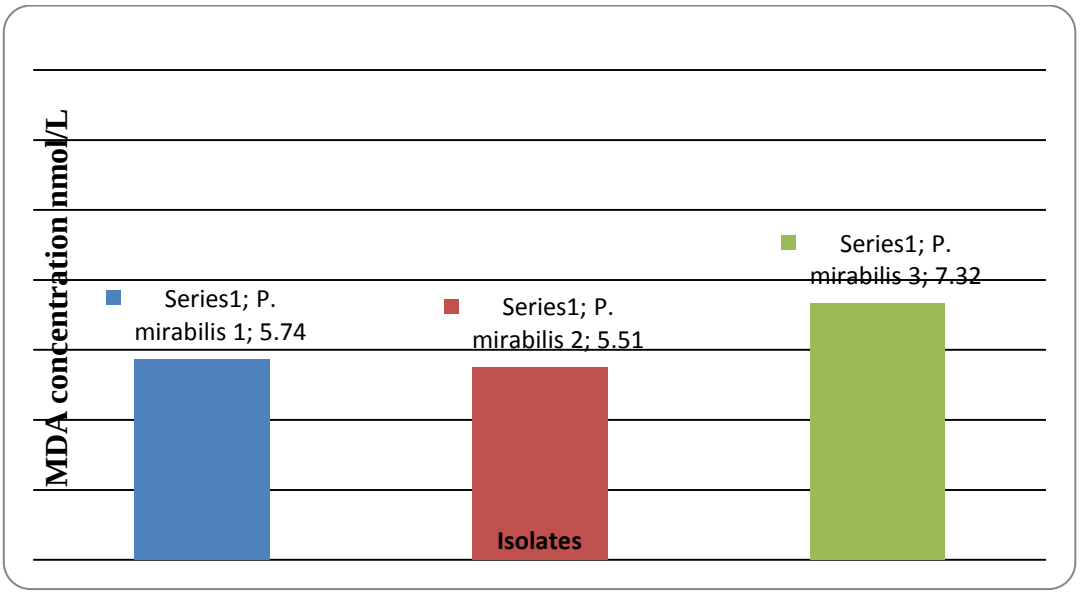

Figure (5) MDA level induced by *Proteus mirabilis* isolates in UTI patients

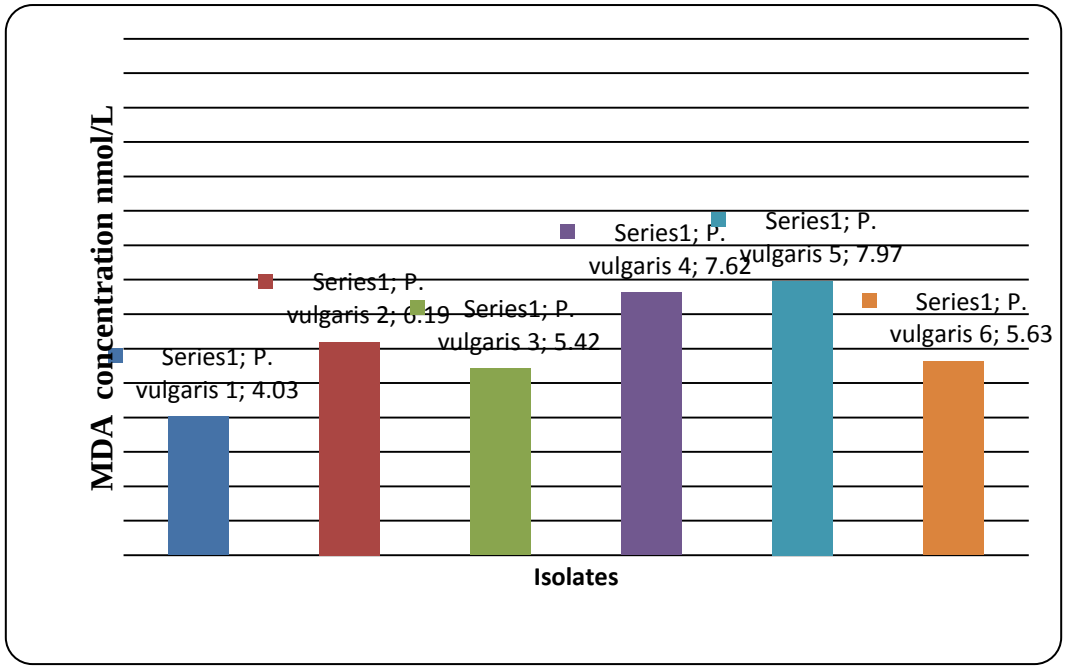

Figure (6) MDA level induced by *Proteus vulgaris* isolates in UTI patients

الخلاصة

جمعت ثلاثمئة عينة ادرار من مرضى يشكون من اخماج المجاري البولية. تم الحصول على مئة وثلاثون عزلة من عينات الادرار وتضمنت : *Escherichia.coli* بنسبة 63.84% و بكتريا *Klebsiella spp.* 23.07% (*K.Planticola* 6.92% و *K.oxytoca* 16.15%) وبكتريا *Entrobacter aerogenes* بنسبة 6.15% واخيراً بكتريا *Proteus* spp بنسبة 6.92 (*P.vulgaris* 4.61% و *P.mirabilis* 2.31%). شخصت العزلات بالاعتماد على الصفات الزرعية المظهرية البايوكيميائية. تم تقسيم المرضى الى خمس مجموعـات (A,B,C,D,E) اعتمادا على اعداد الخلايا الالتهابية الموجوده في عينات الادرار. تم التحري عن نوع ونسب تواجد البكتريا الممرضة في الخلايا الالتهابية، لوحظ ان بكتريا *E. coli* قد شكلت النسبة المئوية العالية في كل المجاميع المدروسة وخصوصاً المجموعة A (التي اعطت 61.36%)؛ فضلاً عن ذلك، شملت الدراسة على تقييم قابلية الممرضات البولية في حث ROS والذي قيس بالاعتماد على طريقة Malonadialdehyde (MDA). وقد اكدت النتائج ان مستوى MDA قد ازداد معنوياً (بمستوى $P<0.05$) طبقاً لزيادة الخلايا الالتهابية، لذا لوحظ ان المجموعة E قد اظهرت زيادة في MDA (9.08 نانومول \ مل) مقارنةً بالمجاميع المدروسة من جانب اخر، تم تحديد قابلية الممرضات البولية في حث ROS. وكانت عزلات *E. coli* الاكفأ في حث ROS وخصوصاُ العزلة 19 *E. coli* التي اظهرت تركيزاً عالياً من MDA وبلغ 11.62 (نانومول\مل)، على العكس فقد سجلت العزلة *K.planticola* مستوى منخفض من MDA بلغ 3.14 (نانومول\مل).