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COMPARATIVE STUDY BETWEEN *E.COLI* AND *K.PNEUMONIAE* ISOLATED FROM THE SAME CLINICAL SOURCE (UTI) OF BIOFILMS FORMATION AND ANTIBIOTICS RESISTANCE

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ABSTRACT
One hundred urine samples were collected from urinary tract infection patients. Ages were between two months to 72 years old. UTI infections were more common in female than male and the percentage was 76% female,24% in *E.coli* while 88% female,22% male in *K.pneumoniae*. Both species in planktonic state showed resistance to more than six antibiotics, Ciprofloxacin was the most effective antibiotic against both species. Biofilm production was carried on by two methods CRA and TCP, In CRA method 65% of *E.coli* and 58% of *K.pneumoniae* were biofilm former, 23% of *E.coli* and 6% of *K.pneumoniae* were non-biofilm former while 12% of *E.coli* and 36% of *K.pneumoniae* gave non identified results. TCP method showed that 69% of E.coli and 72% of K.pneumoniae were strong biofilm producers,6% of *E.coli* and 12% of *K.pneumoniae* were moderate producers while 25% of *E.coli* and 16% of *K.pneumoniae* were non biofilm producers. Adhesion average to epithial cells of *E.coli* and *K.pneumoniae* was 42.9% and 44% respectively. The stronger biofilm producers isolates from both species were chosen to study the antibiotic susceptibility of these isolates in biofilm state against Ciprofloxacin, Gentamycin and Amikacin in different concentrations. **Conclusion:** It's thought that the clinical source of isolation play a role in the ability of biofilms production and resistance to antibiotics.

KEYWORDS: Biofilms, Adhesion, UTI, E.coli, K.pneumoniae.

INTRODUCTION
The name Enterobacteriaceae was first proposed by Rahn (1937). Enterobacteriaceae comprise a large group of genetically and biochemically related bacteria.[1] Bacteria belonging to the family *Enterobacteriaceae* are facultative anaerobic, non-spore forming rod-shaped bacilli. Members of this heterogeneous group of bacteria do not only form part of the normal flora of humans and animals, but are also widely distributed in various environments such as water, soil and plants.[2] Most of urinary tract infections are caused by gram-negative bacteria like, *E.Coli, Klebsiella species, Proteus mirabilis*, *Pseudomonas aeruginosa*, *Acinetobacter*, and *Serratia*. 90% of UTI cases are caused by gram-negative bacteria while only 10% of the cases are caused by gram positive bacteria. Gram-positive bacteria while only 10% of the cases are caused by gram positive bacteria.[3] Biofilm plays a major role in virulence of bacteria. The possible relationship between bacteria persistence in the urinary tract and the presence of virulence factors (VFs) lead to biofilm formation like adhesins, toxins, lipopolysaccharides, iron acquisition, presence of capsule and serum resistance.[4,5] Biofilm formation enables single cell organisms to assume a temporary multicellular lifestyle, in which group behavior facilitates survival in adverse environments. There are several advantages for microorganisms to form biofilms. They provide enclosed surface space which is occupied and can provide a degree of stability in the growth environment. They might have catalytic functions through the localizing cells in close proximity.[6] Bacteria within the biofilm are protected from antibiotics and immune system to a great extent may be due to the reduction in penetration of antibiotics, nutrients limitation & slow growth or by adaptation.[7] The presence of other bacteria in the hospital environment that have a high antibiotic resistance properties may facilitate the transfer of resistance genes between them, in addition to these isolates may be able to produce an enzymes (e.g.: β-lactamases) which cause degradation of antibiotic.[7] Major portion of biofilm consists of exopolysaccharide and small amount of other organic compounds; this slime layer provides safe environment for the growth of microorganisms, inside biofilm bacteria become expansive and are capable to interact with each other via intracellular communication as result can tolerate the fluctuating environment, formation if biofilm is complicated phenomenon in which genetic

mechanisms and various other factors are involved, among these factors characteristics of substratum and surfaces of bacterial cells are most important.[8] The initial step in biofilm formation involves reversible attachment of planktonic (freely moving individual cell) bacteria to a surface (colonization) by using adhesins.[9] The adhesion process is also affected by organism physiological state, on the other hand in some organisms attachment is high in log phase, while in others attachment is high in stationary phase, the bacteria are still susceptible to antibiotics at this stage.[10] The next step in formation of biofilm is turning the initial reversible binding of organisms to a surface into irreversible binding, followed by multiplication of the bacteria resulting in microcolonies formation, after which production of a polymer matrix around the microcolonies converts it into a mature biofilm architecture varies from flat homogeneous layer of cells, to organize mushroom-like or tower-like structures, this maturation stage is controlled by quorum sensing (QS).[9] Mature biofilm containing cavities that serve as transport channels of water and planktonic cells throughout the biofilm community, and also provides a unique environment for optimum nutrient absorption and waste disposal.[11]

METHODS

Bacterial isolates

One hundred urine sample were collected during October 2016 to January 2016 from some hospitals in Baghdad city; Ibn-El Balady hospital, Al-Kandy teaching hospital and Teaching laboratories in medical city, Fatima Al-Zahraa 50 isolates of *K.pneumoniae* and 65 of *E.coli* were obtained from urinary tract infection patients age were from 2 months to 72 years. Diagnosis were carried out using traditional methods such as microscopic examination cultural chrercteristics (macConkey agar, Eosin methylene blue agar and Chromoagar), and biochemical test (catalase and oxidase) furthermore using vitek 2 compact system for confirm the diagnosis.

Antimicrbial susceptibility test

All isolates were tested for antimicrobial susceptibility depending on the[12] criteria by using agar diffusion method. The antibiotic used in this study were 16 antibiotics which are: gentamicin, Ampicillin, Ciprofloxacin, sulfamethoxazole/trimethoprim, imipenem, ceftriaxone, Azithromycin, piperacillin, Aztreonam, Colistin, Amikacin, Ticarcillin, Chloramphenicol, Cefoxitin, Erythromycin, Nalidixic acid.

Biofilm production

Congo red test

Plates were inoculated in Congo red agar by puring single isolated colony and incubated aerobically for (24-48) hr. at 37^0C, Positive result was indicated by black colonies with a dry crystalline consistency. The weak slime producers usually remained pink, though an occasional darkening at the centers of the colonies was observed. A darkening of the colonies, with the absence of a dry crystalline colonial morphology indicated an indeterminate result.[13]

Tissue Culture Plate Method

The assay was performed in triplicate using 96-well flat-bottomed cell culture plates (Nunc,New York, NY, USA) as described previously.[14] Breifly 10 ml of Trypticase soy broth with 1% glucose was inoculated with a loopful of test organism from overnight culture on nutrient agar.

The culture was further diluted 1:100 with fresh medium. Flat bottom wells tissue culture plates were filled with 0.2ml of diluted cultures individually. Similarly control organisms were also diluted and incubated. After incubation at 37^0C for 24 hours, gentle tapping of the plates was done. The wells were washed with 0.2 ml of phosphate buffer saline (Ph 7.2) four times to remove free floating bacteria. Biofilms which remained adherent to the walls and the bottoms of the wells were stained with 0.1% crystal violet. Excess stain was washed with deionized water and plates were dried properly. Optical densities (O.D) with a micro ELISA auto reader at wave length 570 nm.

Ability of *K.pneumoniae* and *E.coli* to adhere to epithelial cells

This test was done according to[15]

- (0.5) ml of both *K.pneumoniae* and *E.coli* suspension were transferred to test tubes separately and these tubes each of them contain (0.5) ml of epithelial cell suspension.
- The tubes were incubated at a temperature 37°C min by using water bath in a shaking range 70 r.p.m for 60 min.
- The tubes were centrifuged at 3000 r.p.m for 10 m and the sediment was washed with phosphate buffer saline and this process was repeated 3 to 4 times to get ride of un adhered cells.
- The sediment was suspended with a drop of phosphate buffer saline and this suspension was transferred to glass slide by Pasteur pipete and left to dry in the air then the slides were stained by crystal violet to detect the adhesion average to epithelial cells.
- The slides were tested by using the oil lenses and the adhered cells were counted by using equation:

Adhesion average

The number of epithelial cells with bacteria adhered was divided by the total number of epithelial cells observed by microscope.

Antibiotic susceptibility assay on abiotic surfaces

Biofilm allowed to form as above, after incubation 24hr, two antibiotics were prepared in different concentration from stock using this formula:-

$$C1V1 = C2V2$$

Which: C= concentration, V= volume

These antibiotic were dissolved in brain heart infusion broth and then 180 µl from different concentration of antibiotics were added to 20 µl of bacteria in microtiter plate and incubated 24hr at 37^0C, After incubation, gentle tapping of the plates was done.

The wells were washed with 0.2 ml of phosphate buffer saline (pH 7.2) four times to remove free floating bacteria. Biofilms which remained adherent to the walls and the bottoms of the wells were fixed with 2% sodium acetate and stained with 0.1% crystal violet. Excess stain was washed with distilled water and plates were dried properly. Optical densities (OD) of stained adherent biofilm were obtained with a micro ELISA auto reader at wave length 570nm.[16]

RESULT AND DISCUSSION

The urinary tract infections are an important worldwide health problem And the spread of these infections in hospitals environment in some Baghdad hospitals is the main reasons made the causative pathogens to be multidrug resistant. In this study showed a higher proportion of UTI in females more than males. *E.coli* isolated from females in percentage 76% while isolated from males in percentage 24%, also *k.pneumoniae* isolated from females in percentage 88% while isolated from males in percentage 22%. This is understandable due to the anatomy and is a consistent trend worldwide. The study is agreement with.[17] The antibiotic susceptibility test was done by agar diffusion method for both species against 16 antibiotics , *E.coli* under study showed resistence as follow:

53% for Nalidixic acid, 37% for sulfamethoxazole/trimethoprim, 66% for gentamicin, 17% for Colistin, 86% for Ampicillin, 12% for Ciprofloxacin, 43% for Amikacin, 96% for Chloramphinicol, 8% for imipenim, 26% for Azithromycin, 91% for Erythromycin, 77% for piperacillin. In addition to some isolates that had shown intermediate resistance against some antimicrobials such as Nalidixic acid 13%, sulfamethoxazole/trimethoprim 3%, gentamicin 2%, Ampicillin 2%, Ciprofloxacin 2%, Amikacin 29%, imipenem 23%, Azithromycin 12%, Erythromycin 9%, piperacillin 11%. Shrooq[18] showed that E.coli from different clinical source including the urine were highly resistant to Ampicillin, Naildixic acid while it was sensitive to ciprofloxacin and amikacin While, K.pneumoniae result were as follow

58% for Nalidixic acid, 72% for sulfamethoxazole/trimethoprim, 68% for gentamicin, 92% for Colistin, 88% for Ampicillin, 14% for Ciprofloxacin, 56% for Amikicin, 96% for Chloramphinicol, 64% for ceftriaxone, 48% for Cefoxitin, 94% for Ticracillin, 58% for Aztreonam.

In addition some isolate had shown intermediate resistance against some antimicrobials such as Nalidixic acid 12%, sulfamethoxazole/trimethoprim 2%, Ampicillin 10%, Ciprofloxacin 2%, ceftriaxone 8%, Cefoxitin 12%, Ticracillin 2%, Azteronam 10%. Ravichitra *et al*.[19] have been reported that *k.pneumoniae* which isolated from urine were revealed high level of resistance towards third generation cephalosporins. The ability of biofilm production result in CRA method showed that 65% of *E.coli* isolates and 58% of *K.pneumoniae* were biofilm producers, 23% of *E.coli*, 6% of k.pneumoniae isolates were non-biofilm producers while 12% of *E.coli*, 36% of *K.pneumoniae* were non-identified figure(1).

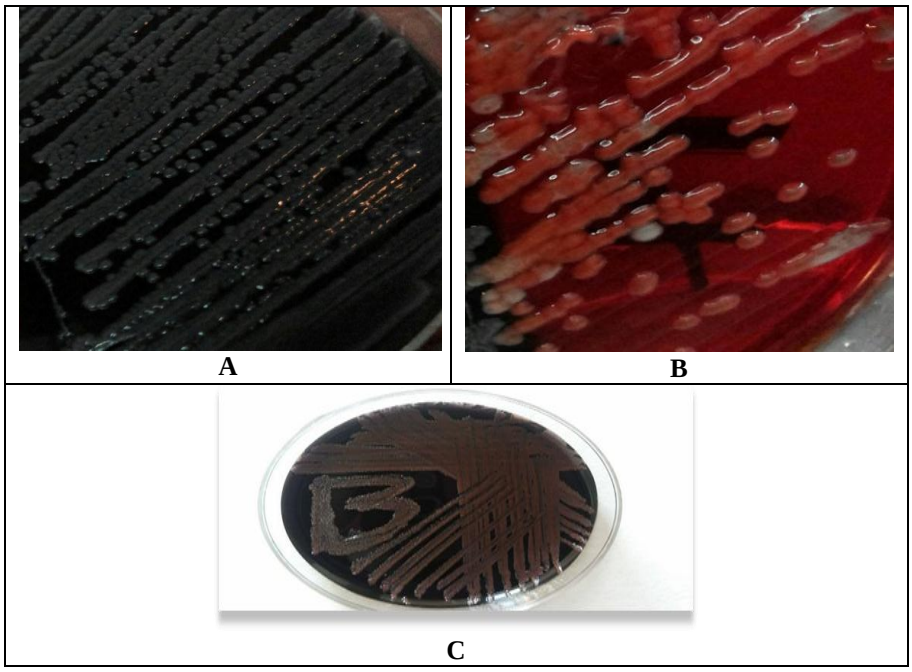


Figure (1) Biofilm production on congored agar (A=Biofilm production=Non-biofilm production, C=Indeterminate result)

The results of *E.coli* approach to Deepika and Meena[20], who indicate that the 73% of *E.coli* isolated from UTI patients were biofilm producer and gave the black colour colonies on congo red agar plate while 27% isolates gave pink colour colonies indicating non biofilm production while, The results of k.pneumoniae approach to Niveditha *et al*[13],who indicate that 63%of isolates gave the black colour colonies on CRA while 37% of isolates gave the pink colour that indicate non biofilm production Table (1).

Table (3) shows *E.coli* and *k.pneumoniae* ability to produce biofilm in CRA method

NO. of isolates	Congo-red method	
	E.coli	***K.pneumoniae***
1	Positive	Positive
2	Negative	Positive
3	Non-identified	Non-identified
4	Negative	Non-identified
5	Positive	Positive
6	Negative	Negative
7	Non-identified	Positive
8	Positive	Positive
9	Positive	Positive
10	Negative	Positive
11	Positive	Positive
12	Non-identified	Positive
13	Positive	Non-identified
14	Non-identified	Non-identified
15	Positive	Non-identified
16	Positive	Non-identified
17	Positive	Non-identified
18	Non-identified	Positive
19	Positive	Positive
20	Negative	Positive
21	Positive	Positive
22	Negative	Positive
23	Positive	Positive
24	Positive	Positive
25	Positive	Non-identified
26	Positive	Positive
27	Positive	Positive
28	Positive	Positive
29	Positive	Non-identified
30	Negative	Positive
31	Positive	Positive
32	Positive	Non-identified
33	Positive	Negative
34	Negative	Non-identified
35	Positive	Non-identified
36	Positive	Non-identified
37	Positive	Positive
38	Positive	Negative
39	Negative	Positive
40	Negative	Positive
41	Positive	Positive
42	Positive	Positive
43	Positive	Non-identified
44	Positive	Non-identified
45	Positive	Non-identified
46	Non-identified	Non-identified
47	Negative	Positive
48	Negative	Positive
49	Positive	Positive

50	Negative	Non-identified
51	Non-identified	
52	Positive	
53	Positive	
54	Positive	
55	Positive	
56	Positive	
57	Positive	
58	Positive	
59	Positive	
60	Negative	
61	Negative	
62	Positive	
63	Positive	
64	Non-identified	
65	Positive	

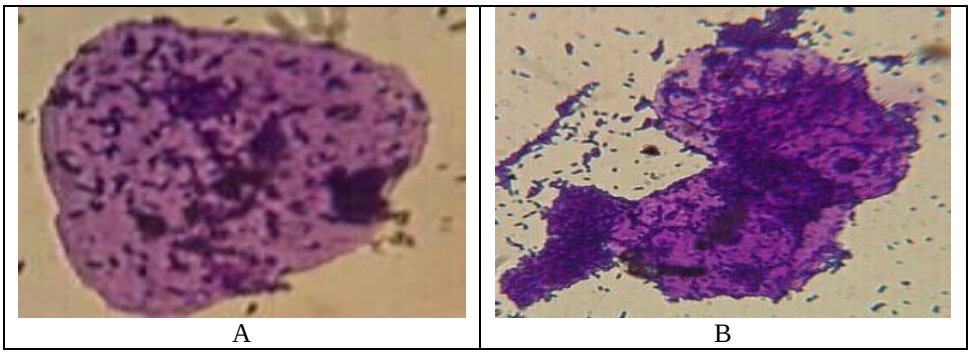
In TCP method 69% of *E.coli*, 72% of *K.pneumoniae* isolates were strongly biofilm former due to strong adherence, 6% of *E.coli*, 12% of *K.pneumoniae* isolates were moderate biofilm former finally 12% of *E.coli*, 16% of *K.pneumoniae* were non biofilm former Table (2).

Table (2) shows *E.coli* and *k.pneumoniae* ability to produce biofilm in TCP method

NO. of isolates	TCP	
	E. coli	***k.pneumoniae***
1	Strong	Strong
2	Non-adherent	Strong
3	Strong	Strong
4	Non-adherent	Strong
5	Strong	Strong
6	Non-adherent	Weak
7	Strong	Weak
8	Strong	Strong
9	Strong	Strong
10	Non-adherent	Strong
11	Strong	Strong
12	Strong	Non-adherent
13	Strong	Non-adherent
14	Strong	Strong
15	Strong	Strong
16	Strong	Strong
17	Strong	Strong
18	Weak	Strong
19	Strong	Strong
20	Non-adherent	Strong
21	Strong	Strong
22	Non-adherent	Strong
23	Strong	Strong
24	Strong	Strong
25	Strong	Strong
26	Strong	Strong
27	Strong	Strong
28	Strong	Strong
29	Strong	Strong
30	Non-adherent	Strong
31	Strong	Strong
32	Strong	Non-adherent
33	Strong	Non-adherent
34	Non-adherent	Non-adherent
35	Strong	Weak

36	Strong	Non-adherent
37	Strong	Weak
38	Strong	Non-adherent
39	Non-adherent	Strong
40	Non-adherent	Strong
41	Strong	Strong
42	Strong	Strong
43	Weak	Strong
44	Strong	Non-adherent
45	Strong	Strong
46	Strong	Strong
47	Non-adherent	Strong
48	Non-adherent	Weak
49	Weak	Weak
50	Non-adherent	Strong
51	Weak	
52	Strong	
53	Strong	
54	Strong	
56	Strong	
57	Strong	
58	Strong	
59	Strong	
60	Non-adherent	
61	Non-adherent	
62	Strong	
63	Strong	
64	Non-adherent	
65	Strong	

The results of *E.coli* partially agree with Sarojgolia *et al* (21) revealed that 69% of *E.coli* isolated from UTIs patients were higher biofilm producer while The results of *K.pneumoniae* partially agree with Sanhez *et al*. (22) revealed that 76% of isolates were determined to be positive for biofilm formation while 24% of isolates were to be negative for biofilm formation. The adhesion process is very important in causing the urinary tract infections, The present study showed that adhesion average of *E.coli* was 42.9% while the adhesion average of *K.pneumoniae* was 44% figure (2).



A B

Figure (2) shows the ability of two species to adhere to epithelial cells (A=*E.coli*, B= *K.pneumoniae*)

Rita[23] investigated that adhesion average to uroepithelial cells for both *E.coli* and *K.pneumoniae* was 44.2,39.3 respectively.

Bacteria in biofilms form exhibit higher antibiotic tolerance than planktonic form, so MIC value for bacterial biofilms can be up to 1000 higher than for their relative planktonic bacteria.[9] Bacteria in biofilm communities behave differently in comparison to planktonic form.[24] we chosed 8 isolates of *E.coli* and 8 isolates of *K.pneumoniae* because they were higher biofilm producers and they were multidrug-resistance in planktonic state. The Antibiotic Susceptibility Assay That explain the importance of biofilm in increasing antibiotic resistance by both *K.pneumoniae* and *E.coli* against three antibiotics in different concentration table.[3]

Table (3) Antibiotic susceptibility Assay of *E.coli* and *K.pneumoniae*

Bacteria	Ciprofloxacin (Mg/L)		Amikacin (Mg/L)		Gentamycin (Mg/L)	
	Antibiotic resistance	Biofilm susceptibility	Antibiotic resistance	Biofilm susceptibility	Antibiotic Resistance	Biofilm susceptibility
EC46	5Mg	5Mg	30Mg	30Mg	10Mg	16Mg
EC24	5Mg	5Mg	30Mg	30Mg	10Mg	12Mg
EC45	5Mg	5Mg	30Mg	40Mg	10Mg	20Mg
EC62	5Mg	5Mg	30Mg	40Mg	10Mg	20Mg
EC23	5Mg	5Mg	30Mg	30Mg	10Mg	16Mg
EC17	5Mg	5Mg	30Mg	40Mg	10Mg	16Mg
EC42	5Mg	5Mg	30Mg	30Mg	10Mg	20Mg
EC33	5Mg	5Mg	30Mg	40Mg	10Mg	16Mg
Kp26	5Mg	5Mg	30Mg	30Mg	10Mg	16Mg
Kp31	5Mg	5Mg	30Mg	40Mg	10Mg	12Mg
Kp25	5Mg	5Mg	30Mg	30Mg	10Mg	12Mg
Kp14	5Mg	5Mg	30Mg	30Mg	10Mg	15Mg
Kp39	5Mg	5Mg	30Mg	30Mg	10Mg	16Mg
Kp45	5Mg	5Mg	30Mg	40Mg	10Mg	12Mg
Kp20	5Mg	5Mg	30Mg	40Mg	10Mg	20Mg
Kp30	5Mg	5Mg	30Mg	30Mg	10Mg	12Mg

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