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Quorum sensing and Biofilm formation by Bacterial Isolates from Hemodialysis Patients

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In this study, blood and urine samples were collected from (60) patients with hemodialysis with age range between (12-82) years old from both sexes. All samples were subjected to identify the bacterial aerobic cultivation. Different types of bacteria were isolated that caused septicemia or urinary tract infection. The predominant bacteria were *E. coli* for Gram negative, and *S. aureus* for gram positive. Quorum sensing were studied for *E. coli* and *K. pneumoniae* and the results shown that all tested bacteria show an aggregation of the bacterial cells in the presence of homoserine lactone and the best interval for accumulation of homoserine lactone was after 4 hours in *E. coli*, 2 hours in *K. pneumoniae*. biofilm formation were studied for all bacterial isolates and the results shown that the all bacteria can form biofilm, *S. epidermidis* (87.5%), *S. aureus* (85.7%) as strong where *E. coli* and *Proteus mirabilis* show moderate biofilm formation.

Key words : Hemodialysis, Quorum sensing, Biofilm formation

Introduction:

Hemodialysis is a method that used to achieve the extracorporeal removal of waste product such as creatinine and urea and free water from the blood when the kidneys are in a state of renal failure. Hemodialysis is one of three renal replacement therapies (the other two being renal transplant and peritoneal dialysis). An alternative method for extracorporeal separation of blood components such as plasma or cells is apheresis. Hemodialysis is the most common method use to treat advanced and permanent kidney failure (NKUD, 2011). Quorum sensing (QS) is a mechanism through which bacteria regulate gene expression by cell density. The bacteria produce hormone-like compounds called auto inducers that interact with regulatory proteins after they reach a certain threshold concentration. Several quorum-sensing systems are also extremely important to human health, as they regulate virulence determinant as in bacterial pathogens (Dong *et al*., 2007). Auto inducers can be thought of pheromones chemicals produced by an individual that can be sensed, and interpreted as a specific piece of information, by other individuals within a population. It is released by cells, diffuse through the extracellular environment, and is (detected) by neighboring cells, often resulting in concentration-dependent changes in gene expression. However, when sufficient bacteria are present, auto inducer concentrations reach a threshold level that allows the bacteria to sense a critical cell mass and, in response, to activate or repress target genes (Viana *et al*., 2009). Biofilm are complex communities of surface attached aggregates of microorganisms embedded in a self-secreted extracellular polysaccharide matrix, or slim (Stoodley *et al*., 2002).Once formed, biofilm act as efficient barriers against antimicrobial agents and the host immune system, resulting in persistent colonization and/or infection at the site of biofilm formation (Edwards and

Harding, 2004). Biofilm organisms are significantly more resistant to environmental stresses or microbial deleterious substances (such as antibiotic and biocides) than planktonic cells. Biofilm cells present on infected tissues or medical devices are less susceptible to host immune responses than planktonic cells (Donlan, 2002; Stoodley *et al.,* 2002). There are many advantages to the growth pattern of biofilms: First, bacteria are protected from the inhibitory effects of antimicrobial compounds, biocides, chemical stress (such as pH and oxygen),and physical stresses(like pressure, heat, and freezing). Second, the polymeric matrix increases the binding of water and leads to a decreased chance of dehydration of the bacterial cells-a stress that planktonic cells are subject to, and third, close proximity of the microorganisms in biofilms allows nutrients, metabolites, and genetic material to be readily exchanged (Davey and o'Toole 2000; Donlan, 2002). Bacteria within a biofilm typically undergo a phenotypic change whereby microbial virulence factor production is altered and metabolic rate and motility are reduced (Edwards and Harding, 2004). Channels formed within the protective environment of the biofilm facilitate the transport of nutrients and microbial waste products (Harrison-Balestra *et al*, 2003). Intracellular signaling molecules produced by bacteria within the biofilm are able to traverse these channels and influence the overall growth pattern and behavior of the biofilm in response to various host and environmental factors (Mack *et al*, 2008).
The aim of study is isolation and identification of bacteria from hemodialysis patients, and study of quorum sensing in gram negative bacteria and biofilm formation for all bacterial isolates.

Materials and Methods:
Collection of specimens: Blood and urine samples were collected from 60 patients suffering from renal faultier from both sexes who are admitted to hemodialysis department. The period from September 2012 through March 2013. The bacterial isolates were identified after staining with gram stain, specific biochemical tests were done to reach the final identification according to (McFadden, 2000 and Forbes *et al*., 2007).

Quorum sensing: Detection of quorum sensing in *E. coli* and *K. pneumonia:* Luria broth media were prepared and supplemented with 1% aspartic acid and distributed in six flasks. After incubation with *E. coli* or *K. pneumonia*, the flasks were incubated at 37^{o}C for intervals (2, 3, 4, 5, 6, 24 hrs.). The quorum sensing test was done on slide through mixing one drop of supernatant and one drop of fresh bacterial growth, and then stained with gram stain and the slide was examined under microscope. The positive result was scored due to the presence of aggregation bacterial cells

Biofilm formation: Tissue culture plate method (TCP): For biofilm detection the Tissue Culture Plate (TCP) assay (also called semi quantitative microliter plate test) described by Christensen *et al*., (1985) was used for detection of biofilm formation with some modification as follow: Isolation from fresh agar plates were inoculated in TSB containing 1% glucose and incubated for 18 hours at 37°C and then diluted 1:100 with fresh TSB. Individual wells of sterile, polystyrene, 96 well-flat bottom tissue culture plates' wells were filled with 150µl aliquots of the diluted cultures and only broth served as control to check non-specific binding of media. Each isolate was inoculated in triplicate. The tissue culture plate were incubated for 24 hours at 37°C. After incubation content of each well was gently removed by tapping the plates. The wells were washed four times with phosphate buffer saline (PBS pH 7.2) to remove free-fluting 'planktonic' bacteria. Biofilms formed by adherent 'sessile' organisms in plate were fixed by placing in oven at 37°C for 30 min. All wells stained with crystal violet (0.1% w/v). Excess stain was rinsed off by thorough washing with deionized water and plate was kept for drying. Addition of 150µl of acetone/ethanol (20:80v/v)

mixture to dissolve bounded crystal violet. Read the optical density (O.D) at 630 nm no interpreted according to the following Table:

Table (1): Classification of bacterial adherence and biofilm formation by TCP method

Mean of OD value at 630nm	Adherence	Biofilm formation
<0.120	non	Non
>0.120-0.240	Moderately	Moderate
>0.240	Strong	High

Results and Discussion:

Isolation of Bacteria: Blood and urine samples collected from (60) patients with hemodialysis patients were subjected for culturing on bacterial culture media. The results shown in Table (2) revealed that 37 (61.6%) samples gave positive bacterial culture whereas 23 (38.4%) showed no bacterial growth. Regarding urine samples, 14(37.8%) were positive bacterial cultures. Meanwhile, bacterial growth was found in 23(62.2%) of blood samples culture. These results agree with that obtained by (Otto, 2008) who found that (65%) samples were positive for bacterial growth. Also, (Vuong *et al.*, 2004) reported that negative bacterial growth was found in approximately (18%) of the cultures of dialysis patients. Iqbal *et al.,* (2008) found that 41% of cases were Gram positive while Gram –negative bacteria isolated in 52%. The presence of different types of bacteria in hemodialysis patients may be attributed to many patients under hemodialysis presence of diabetes; presence of gastroenteritis; urinary tract infection or intra-abdominal pathology or patients under chemotherapy; and prior antibiotic use or prophylaxis.

Table(2) Number and percentage of bacterial isolated from hemodialysis patients

Results	Source of culture		
	Blood N0. (%)	Urine N0. (%)	Total of samples N0. (%)
Positive culture	23 (38%)	14 (23.4)	37 (61.6)
Negative culture	37 (61%)	46 (76.6)	23 (38.4)
Total	60 (100%)	60 (100%)	60 (100%)

The organisms responsible for hemodialysis related are Gram-positive in two-thirds of cases; predominantly *S. epidermidis* and S. *aurous* (Nassar and Ayus; 2001). Causative Gram-negative organisms have included *Enterobacter cloacae*, *Serratia* species, *Klebsiella pneumonia, Proteus mirabilis* and *E. coli* (Mokrzycki, 2009).

Types of Bacterial isolates: It is shown in Table (3) and Table(4) the frequency of bacteria in blood and urine samples, it is clear from the total number of isolates that Gram-negative bacteria are more frequent than Gram-positive in urine samples. This agree with (Nassar and Ayus, 2001) who found that Gram- negative bacteria represent about (65%) of micro-organisms that isolated from hemodialysis urine samples and that this type of bacteria has assumed a primary lethal role among the cases of hemodialysis patients infection and septicemia. The predominance of Gram negative bacteria is clear from the high frequency of *E. coli* in urine samples. This agree with (Katneni and Hedayati, 2007) who found the most commonly isolated organisms from urine samples in hemodialysis patients were *E. coli*

followed by *Enterobacter cloacae* and *K. pneumoniae* and these results are also in accordance with other studies (Summers, 2005).

Table(3): Frequency of Gram-negative bacteria

Bacterial isolates	Blood	Urine	Total%
	Frequency	Frequency	
E. coli	0	8	8
K. pneumoniae	0	3	3
P. mirabilis	1	1	2
Serretia spp.	0	1	1
Total	1	13	14(37.8)

In this study the more frequent Gram positive bacteria isolated from the blood is *S. aureus* followed by *S. epidermidis*, β-hemolytic *Streptococcus pneumonia.* These results were approximately near with that of Katini and hedayati, (2007) who showed that the most common bacteria isolated from blood culture were *S. aureus* followed by *S. epidermidis*. It is clear that the patients under hemodialysis can be contaminated by microorganisms that migrate from the gastrointestinal, urinary and respiratory tracts (Duran *et al* 2009). This indicates the idea of autoinfection that hemodialysis patients suffer from in addition to the infection acquired from the burn unit itself.

Table 4: Frequency of Gram positive bacteria

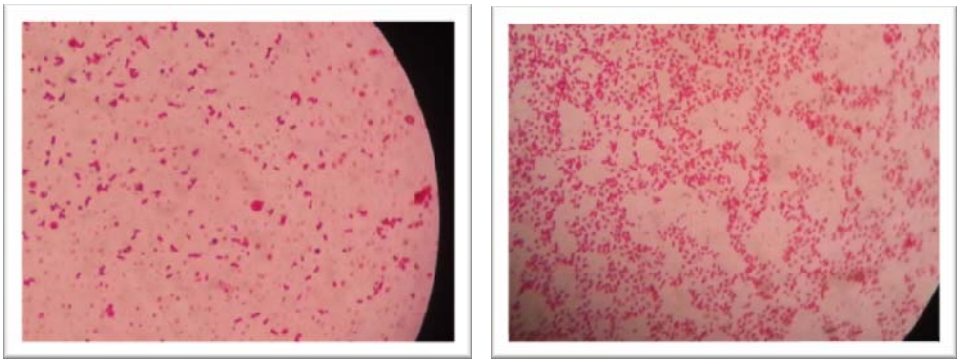
Bacterial isolates	Blood	Urine	Total
	Frequency	Frequency	
S.aureus	14	0	14
S.epidermidis	8	0	8
St.pnumoniae	1	0	1
Total	23	0	23(62.2%)

Bacterial infections represent a common and important health problem for patients with end-stage renal disease who undergo maintenance hemodialysis.

Quorum sensing in *E. coli* and *K. pneumoniae*: To study quorum sensing in *E. coli* and *K. pneumoniae*, aspartic acid was used as the main focal metabolites for homoserine synthesis. It was observed that homoserine was accumulated in culture media after the addition of KCN in which the later will inhibit threonine synthesis, through its effect on thereonine synthase enzyme as shown in Figure (1) and Figure (2).

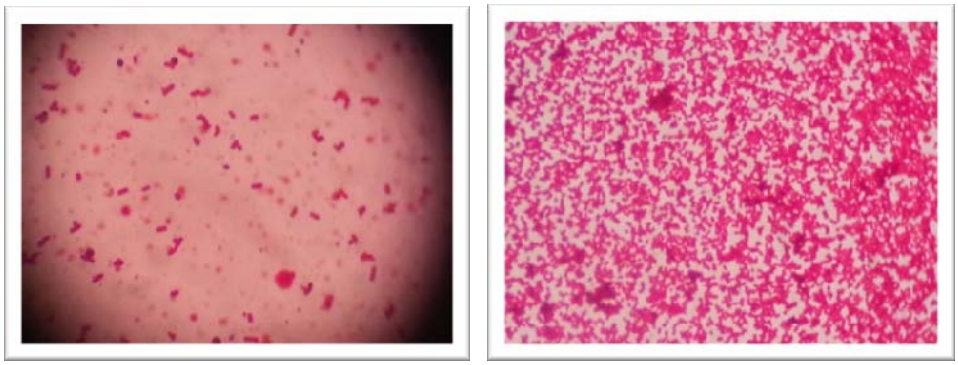
Homoserine lactone production was also checked by using (sodium nitroprusside test) in order to ensure that quorum sensing occurs as a result of homoserine lactone synthesis. The presence of aggregation of *E. coli* and *K. pneumoniae* as in Figure (2) was considered a positive result versus the negative results in the absence of homoserine lactone. The best interval for accumulation of homoserine lactone was after 4 hours for *E. coli* while 2 hours was the best time for accumulation of homoserin lactone for *K. pneumoniae* to become

at maximum concentration. However, under certain condition the bacteria can form homocystein as a result of production of homocystein synthase, and the later may be used as indicator for homoserine lactone synthesis. Quorum sensing may play an important role in enhance assess to nutrients and more favorable environmental niches, and they enhance action against competing bacteria and environmental stresses. Cellular processes modulated by quorum sensing are symbiosis, transfer of conjugative plasmids, sporulation, antimicrobial peptide synthesis, regulation of virulence, and biofilm formation (Atkinson *et al*., 2006). AHL-mediated cell-to-cell signaling has been observed in many natural environments (Whitehead *et al*., 2001) and has been shown to play a role in diverse function such as pathogenesis. AHLs produced by bacteria could serve as potential biomarkers in the management of bacterial disease and, thus, monitoring them in biological samples may be a significant analytical tool for the investigation of such disease (Kumari *et al*., 2008).



E. coli control *E. coli* QS positive results

Figure (1) Detection of quorum sensing in *E. coli*



Klebsiella pneumonia control *Klebsiella pneumonia* QS positive results

Figure (2): Detection of quorum sensing in *Klebsiella pneumonia*

Homoserine production was checked through the separation of supernatant from culture media, and then the supernatant was dialyzed verses Luria–Bertoni broth free of KCN. After 24 hours the dialyzed homoserine containing media were inoculated by *E. coli* or *K. pneumoniae* separately again for 24 hours and then sodium nitroprusside test was to detect methionine or homocystein synthesis through conversion of homoserine to homocystein (Surette and Bassler, 1998). A variety of different molecules can be used as signals, oligopeptides in Gram-positive bacteria, N-Acyle Homoserine Lactones (AHL) IN Gram-negative bacteria. Bacteria that use QS constantly produce and secret these signaling

molecules (auto inducer). They also have a receptor that can specifically detect the inducer. When the inducer binds the receptor, it activates transcription of certain genes, including those for inducer synthesis. When only a few of the same kind are in the vicinity, diffusion reduces the concentration of the inducer in the surrounding medium to almost zero, so the bacteria produce little inducer. However, as the population grows the concentration of the inducer passes a threshold, causing more inducer to be synthesized. This forms a positive feedback loop, and the receptor becomes fully activated. Activation of the receptor induces the up regulation of other specific genes, causing all of the cells to begin transcription at approximately the same time (Melissa and Bassler, 2001).

Biofilm formation: Bacterial biofilm cause chronic diseases that are difficult to control and in present study, differentiation of bacteria as biofilm producers and non-biofilm producers using (ELISA) was based on OD570 measurements. Total of (37) isolates from different types of bacteria were tested for their ability to produce biofilm. From these isolates (19),(15) were form strong and moderate biofilm respectively(91.9%) and (3) isolates(8.1%) were non biofilm formation as shown in Table(5),(6).

Table(4): Production of biofilm in Gram-positive bacteria

Bacterial isolates (NO.)	BIOFILM			
	Strong	Moderate	% of biofilm formation	Weak
S.aureus(14)	10	2	85.7%	2(14.3%)
S.epidermidis(8)	5	2	87.5%	1(12.5%)
St.pneumoniae(1)	1	0	100%	0(0%)

Table(5):L- production of biofilm in Gram-negative bacteria

Bacterial isolates (NO.)	BIOFILIM			
	Strong	Moderate	% of biofilm formation	Weak
E.coli(8)	1	7	100	0(0%)
K.pneumoniae(3)	2	1	100	0 (0%)
P.mirabilis(2)	0	2	100	0(0%)
Serratia spp.(1)	0	1	100	0(0%)

85.7%(12/14) of *S. aureus* and 87.5%(7/8) *S. epidermidis* were formed biofilm whereas 14.3 (2/14) and 12.5%(1/8) from both species respectively were not have biofilm, this result approximately agree with Otto, (2008), who found that 89% from *S. aureus* were formed biofilm after incubation to 24 hours. *S. aureus*is recognized as the most frequent causes of biofilm-associated infections, it is especially capable of adhering to a large variety of matrix components to initiate colonization this adherence is frequently mediated by protein adhesions of the family known as MSCRAMM (microbial surface components recognizing adhesive matrix molecules). The collagen-binding protein, fibronectin-binding proteins, and fibrinogen-binding proteins belong to this family (Gotz, 2002). *S. epidermidis* is known as an opportunistic pathogen because it predominantly causes infection in immune compromised individuals such as patients with intravascular catheter. The inherent capacity of this

organism to cause infection derives primarily from its ability to form mucoid biofilm on the inert synthetic surfaces of indwelling medical devices (Vadyvaloo and Otto, 2005).On the other hand (100%) of *St. pyogenes* were formed biofilm. These results agree with (Davey and O,Toole, 2000). Regarding (100%) from all Gram –negative bacteria were formed biofilm as showed in Table (5). *E. coli* were formed biofilm and these results similar with the results obtained by (Beloin *et al*., 2008), who observed that *E. coli* formed biofilm because it encodes a peptide methionine sulphoxide reductase that responsible for biofilm formation in these bacteria. *K. pneumoniae* is often involved in biofilm-related infections, Madam *et al.,* (2008) found that 62.5% of *K. pneumoniae* produce biofilm and this less than the results obtained in this study, the reason may be the small sample size (N=3) may account for this. On the other hand Macleod and Stickler, (2007), found that 335 from *proteus* spp. were formed biofilm. Jones *et al.,* (2007) suggest that the formation of swimmer cells could represent a method by which *P. mirabilis* can disperse from the mature biofilm to seed new surfaces and blocked catheter more quickly than the wild-type strain in the catheterized bladder model. *Serratia spp*. were formed biofilm (100%) these result nearly compatible with Labbate *et al.,* (2004) who found that *Serratia* formed biofilm in medical devices and responsible for 1.4% of nosocomial bacteremia.

Conclusions

Quorum sensing was carried out through the production of homoserine lactone by Gram-negative bacteria. Most bacterial isolates form biofilm by TCP method.

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