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Effect of iron and zinc on biofilm formation in *proteus mirabilis* isolated from urinary tract infections

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Proteus mirabilis is one of the most common pathogens and has the ability to form biofilm especially on catheters. Bacterial biofilms play an important role in urinary tract infections (UTIs) caused by these bacteria. The aim of this study was to analyze the role of some of divalent cations (Iron and zinc) on biofilm formation by *P. mirabilis* and comparison of results with the growth rate and exopolysaccharide production for this species. A total of 42 *P. mirabilis* isolates recovered from patients with UTI from Baghdad hospitals, Iraq, and identified by microscopic examination, biochemical tests and API 20E system. It was found that all the isolates had the ability to form biofilm by using microtiter plate method. The concentrations of Iron (10, 25, 50 and 75 µg/ml) exhibited increasing effect on biofilm formation while the higher concentrations gave an inhibition activity, the results of exopolysaccharide production, by using phenol-sulphuric acid method, were similar to biofilm formation, where the growth rate was not affected by the most of iron concentrations except 150 and 200 µg/ml. In the case of zinc, the results revealed that the most of zinc concentrations used caused inhibition for *P. mirabilis* growth, exopolysaccharide production and biofilm formation. The current study revealed the importance of iron in the formation of biofilm in *P. mirabilis* and the components of exopolysaccharide, in contrast with Zinc which act as antibacterial and antibiofilm agent. These findings suggest the possible utilization of zinc as a new tool to fight biofilms especially in UTI.

Keywords: *Proteus mirabilis*, biofilm, Iron, Zinc.

INTRODUCTION

P. mirabilis strains are more commonly associated with urinary tract infections; particularly in patients undergoing urinary catheterization, leading to serious medical problems. Biofilm formation, swarming motility, and ureolytic activity are virulence factors characteristic of *P. mirabilis* strains (Jacobsen and Shirtliff, 2011). A biofilm is a formation of communicating microorganisms that adhere to certain surfaces and to neighboring cells and are covered with an extracellular matrix. It is often formed on catheters and surgical implants (Kokare et al. 2009). Biofilm formation by *P. mirabilis* is an important factor in the establishment of infections, which leads to a significant challenge to cure the biofilm-associated infections (Jones et al. 2005). *P. mirabilis* adheres to living tissue or nonliving surfaces by forming a biofilm. The biofilm protects these bacteria from the host defense system and from antibiotics; often leading to repeated infection (Jacobsen and Shirtliff, 2011). Iron is an essential nutrient for bacterial growth and is crucial for bacterial energy production, nucleotide synthesis, and regulation of gene expression (Ratledge and Dover.,2000). Iron regulation of biofilm formation has been detected in many bacterial species. It was found that iron is required for biofilm formation in *Escherichia coli*

(Wu and Outten, 2009). In the bacteria, Zn has a role in protein stabilization and catalysis, this metal is important for a broad range of cellular processes in which Zn-binding proteins are involved such as in Zinc-metalloproteases (Finney and O'halloran, 2003). The composition and quantity of exopolymers in the biofilm correlate with bacterial species and the environmental conditions such as carbon source and balance between the elements such as nitrogen, potassium and phosphorus (Vu et al. 2009). The availability of minerals and trace elements affects host-pathogen interactions, especially pathogen survival, host physiology and the expression of virulence traits (Kierek and Watnick, 2003).

The aim of current study was investigate the effect of different concentrations of divalent cations such as Iron and Zinc on the growth, biofilm formation and exopolysaccharide production in the pathogenic bacteria *Proteus mirabilis* isolated from urinary tract infections and suggestion its role in adhesion of these bacteria.

MATERIALS AND METHODS

Bacterial isolates and identification

A total of 42 *Proteus mirabilis* clinical isolates were obtained from 110 urine samples of hospitalized infected patients with Urinary Tract Infections (UTI) in Baghdad hospitals, Iraq. Identification of bacterial isolates was carried out by the phenotypic methods including cultural characteristics, swarming appearance and pleomorphic Gram-negative. Biochemical reactions including indole test and other biochemical reactions related to *Proteus mirabilis*. were also verified (Crichton, 1996). The identification also confirmed by using API 20E system.

Biofilm formation assay and quantification

The ability of *Proteus mirabilis* to form biofilms were assayed by using sterile 96-well tissue culture plates containing 50 µl of Mueller–Hinton broth per well, a 50 µl of fresh bacterial suspension (1.0 McFarland) was added. After incubation at 37 °C for 24 hours, the content of each well was gently removed by tapping the plates. The wells were washed with 200 µl of sterile saline to remove free-floating bacteria. Biofilms formed by adherent cells in plate were stained with 0.1% crystal violet and incubated at the room temperature for 20 minutes. Excess stain was rinsed off by thorough washing with deionized water and plates were fixed with 200 µl of 96% ethanol. Optical densities (OD) of stained adherent bacteria were measured at 570 nm using an ELISA microplate reader. All tests were performed in triplicate (O'toole and Kolter, 1998).

Evaluation of divalent metals on planktonic viability

Proteus mirabilis was suspended in sterile Mueller–Hinton broth and mixed for 5 min. The suspension was added to Mueller–Hinton broth supplemented with FeCl3 and ZnCl2 added in increasing amounts (10, 25, 50, 75, 100, 200 µg/ml) at a targeted absorbance (600 nm). The broth was added to a 96- well microtiter plate at a final test volume of 200 µl. Each plate row contained a minimum of 3 replicates per test condition. The plates were incubated in a shaker incubator at 37 °C and 250 rpm for 24 hours. After the incubation period, the plate was read using a microplate reader at 600 nm to evaluate the effect of metals on planktonic viability via absorbance. Viability was confirmed by monitoring bacterial growth in the presence of the metal, by quantifying the increase in absorbance every 2 h over a period of 24 hours (Vega et al. 2014).

Effect of metal ions on biofilm formation

A modified crystal violet assay was employed to test the effect of Iron and Zinc on biofilm formation. Different concentrations (10, 25, 50, 75, 100, 200 µg/ml) of each ion was in sterile 96-well tissue culture plates containing 50 µl of Mueller–Hinton broth per well. A 50 µl of fresh bacterial suspension (1.0 McFarland) was added to each well. Growth control (cells + broth), media control (only broth) and blank control (broth + metal) were included. After incubation at 37 °C for 24 hours, the biofilm biomass was assayed using the crystal violet staining assay as described above and the reaction mixture was read spectrophotometrically at 570 nm (Chaieb et al. 2011).

EPS (Exopolysaccharide) extraction

The EPS was extracted according to Smitinont et al. (1999). The overnight culture of bacteria was taken into vials and centrifuged at 10,000 rpm for 20 min at 4°C to remove bacterial cells. The obtained supernatant was collected into a fresh vial and precipitated with two volumes of absolute chilled ethanol by incubating the mixture at 4°C for overnight. The precipitated EPS was collected by centrifugation at 10,000 rpm for 20 min at 4°C and the supernatant was decanted. The pellet containing EPS was dried at room temperature. The total carbohydrate content in the EPS was

estimated by phenol-sulphuric acid method (Kodali and Sen, 2011.

RESULTS AND DISCUSSION

Isolation and identification of isolates

During the period from March to May, 2016, 110 urine samples were collected from patients with urinary tract infection from Baghdad hospitals, Iraq. The age of patients was ranged from 9 months to 61 years, they were predominantly female (69 %). From results of biochemical tests and API 20E System, it was found that *Proteus mirabilis* isolated from 42 patient (38.2%) and all the isolates were positive for biofilm formation. Biofilm development on urinary catheters is a problem in patients with UTI and this factor promotes urinary tract infection, which leads to high mortality rates (Dohnt et al. 2011). *P. mirabilis* was often associated with Urinary Tract Infections (UTI) in patients carried mechanical devices such as catheter and the ability of *P. mirabilis* to attach in the hospital devices is due to biofilm formation in these bacteria. It was found that biofilms produced by *P. mirabilis* is increasing source of catheter associated UTI infection in the hospitals (Stickler, 2008).

Effect of Iron on biofilm formation

The effect of different Iron concentrations on biofilm formation in *P.mirabilis* was summarized in Table (1) by using Microtitre plate assay as showed in Figure (1)

Table: 1. Effect of different Iron concentrations on planktonic viability, biofilm formation and EPS (Exopolysaccharide) production in *Proteus mirabilis* (24 hours incubation).

Iron concentration (µg/ml)	Planktonic viability (Absorbance 600 nm)	Biofilm (Absorbance 570 nm)	EPS (µg/ml)
0	0.98	1.08	3.20
10	1.20	1.12	3.80
25	1.13	1.40	4.51
50	1.08	1.21	4.11
75	0.93	1.19	3.32
100	0.85	0.92	2.81
150	0.64	0.43	1.03
200	0.51	0.39	0.88

The current study revealed that all the isolates (42) of P.mirabilis had the ability to form biofilm.

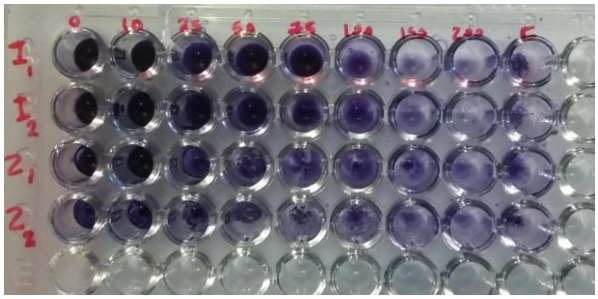

Figure: 1. Microtitre plate assay of biofilm formation for *P. mirabilis*, using different concentrations of Iron and Zinc and control (Biofilms formed by adherent cells in plate were stained with 0.1% crystal violet).

Planktonic viability was conducted after 24 hours by measuring absorbance at 600 nm. *P.mirabilis* grew from 0.98 to about 0.85 during 24 hours with Iron concentrations ranging from 0 (control) to 100 µg/ml, indicating that the tested Iron concentrations were below lethal levels. With the exception of the wells containing 150 and 200 µg/ml of Iron, all concentrations resulted in the same growth pattern with 24 hours period. Also it was found that the Iron concentration 25 µg/ml gave relative increasing of absorbance to 1.13 while the concentrations 150 and 200 µg/ml of Iron showed growth inhibition. In contrast to the planktonic cell results, attachment (biofilm formation) increased from an absorbance of 1.08 (control) to 1.40 (at 25 µg/ml). It was demonstrated that biofilm formation enhanced with increasing Iron concentrations, while the decreasing in attachment was detected at the concentrations 150 µg/ml and 200 µg/ml.

This study was aimed to understand whether the biofilm production by the isolated microorganism was Iron dependent or not. The results showed that the EPS production was significant at the concentrations 25 and 50 µg/ml and there was no significant EPS production was observed at high concentrations 150 and 200 µg/ml . EPS production was maximum at the concentration 25 µg/ml. From these results, it was concluded that the low concentrations of Iron in the medium of growth enhanced the biofilm formation and increasing the EPS production and the maximum values showed at the concentration 25 µg/ml, while the high concentrations of Iron (150 and 200 µg/ml) caused inhibition of growth, biofilm formation and EPS production.

Several studies indicated to the role of Iron in the growth and the attachment of pathogenic bacteria. The deficient of Iron in the growth medium cause a decrease in the bacterium

surface hydrophobicity and major alterations in the surface protein composition (Conte et al. 1996). Pathogenic bacteria require various iron acquisition mechanisms to obtain iron from host tissues in order to establish infection in the human body, therefore microorganisms may obtain iron by invading host cells or by releasing siderophores, which are low-molecular-weight compounds with high affinity to chelate iron from iron-binding proteins (Miethke and Marahiel, 2007). Siderophores dissolve these Fe^{+3} ions for microbial interactions, biofilm formation and microbial survival, as soluble Fe^{+3} complexes that can be taken up by active transport mechanisms (Banin et al. 2005). However, in *Legionella pneumophila* iron prevents biofilm formation (Hindre et al. 2008). Several studies demonstrated that iron positively regulates biofilm formation in *Staphylococcus* and it was found that the biological iron chelators inhibit adhesion of *S. aureus* and *S. epidermidis* to polystyrene, polyurethane and silicone surfaces (Ardehali et al. 2002). Bacterial EPS play an important role in biofilm development, e.g. they participate in the adhesion process, where they intensify the cells binding to solid surfaces (Vu et al. 2009). *A. ferrooxidans* strains that exhibited higher levels of activity were found to possess greater concentrations of ferric ions complexed within their EPS. Since the presence of ferric ions is important for attachment to pyrite, cells with more iron were found to adhere more rapidly than cells possessing lower iron concentrations (Kinzler et al. 2003).

Effect of Zinc on biofilm formation

The effect of different Zinc concentrations on biofilm formation in *P.mirabilis* was summarized in Table (2) and Figure (1).

At the concentrations of Zinc 10 and 25 µg/ml in the growth medium for *P.mirabilis*, it was found that the growth rate was normal, as in the control, and these tested Zinc concentrations were below lethal levels. Planktonic viability of *P.mirabilis* was decreased above the concentration 50, hence this ion gave inhibition effect for these bacteria and this effect increased gradually with the increasing of ion concentration. Biofilm formation results (Table 2 and Figure 1) demonstrated that the maximum biofilm formation (1.2) was at the concentration 10 µg/ml in contrast with the control (1.08). The inhibition of biofilm formation was obvious at the Zinc concentrations above 50 µg/ml, however the concentration 200 µg/ml exhibited the highest inhibition (0.09).

Table: 2. Effect of different zinc concentrations on planktonic viability, biofilm formation and EPS (Exopolysaccharide) production in *Proteus mirabilis* (24 hours incubation).

Zinc concentration (µg/ml)	Planktonic viability (Absorbance 600 nm)	Biofilm (Absorbance 570 nm)	EPS (µg/ml)
0	0.98	1.08	3.20
10	1.01	1.20	3.42
25	0.92	1.05	3.04
50	0.78	0.98	2.54
75	0.56	0.64	2.27
100	0.51	0.51	1.97
150	0.48	0.32	1.30
200	0.05	0.09	0.85

Exopolysaccharide production in the present of different concentrations by using phenol-sulphuric acid method was summarized in Table (2), the results indicated that affecting on EPS production by Zinc ion similar to affecting on biofilm formation, hence the high concentrations of this ion had inhibitory effect on EPS production while the maximum production of EPS was at the concentration 10 µg/ml. The effect of Zn in biofilm formation is correlate with the production of exopolysaccharides and adhesins production (Cobine et al. 2013).

Zinc used at nonbactericidal concentrations can inhibit biofilm formation by several Gram-negative and Gram-positive bacterial swine pathogens and the mechanism of antibiofilm activity of may be due to interact with the components of matrix (Wu et al. 2013). Zinc may also affect on cellular mechanisms such as signalling and gene regulation, and may be bind to the ferric uptake regulator and effect on iron homoeostasis (Klemm et al. 2010).

The study of Faiz et al. (2011) demonstrated that Zn completely inhibited the growth of *Salmonellae,* enteropathogenic *Escherichia coli*, *Shigellae* and *Vibrio cholerae* isolated from diarrhoeal stool specimens and most of them were inhibited at a concentration of 0.06 mg/ml to 0.5 mg/ml of Zn. Zinc could be used with other disinfectant to control environmental biofilms which important in the persistence of bacterial pathogens, such as *E. coli* and *Vibrio cholerae* (Shikuma and Hadfield, 2010). In *Streptococcus* the expression of the adhesion protein laminin is repressed under high concentrations of Zn, and this is mediated by AdcR, the Zn transcriptional regulator in this bacterial group (Shafeeq et al. 2011

CONCLUSION

In conclusion, the present findings suggest that Iron can support biofilm formation in *P.mirabilis* in contrast with the antibiofilm activity of zinc, this study may establish a new mechanism to reduce the pathogenicity caused by biofilm.

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