The Role of Lipopolysaccharide and O-Antigen of Proteus Mirabilis in Urinary Stones Formation, In-Vitro Study

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Abstract:
The ability to induce stone formation *in-vitro* of Proteus mirabilis isolates was investigated using whole bacterial cell, lipopolysaccharide, and O-antigen of these isolates. The results showed that all three parts have the ability to form crystallization in artificial urine solution, which based on the differences in urease activity and chemical structure of LPS and O-antigen. The whole bacterial cell of isolate No. 6 (*P. mirabilis* O18) revealed significant differences ($P < 0.05$) in the ability to bind with calcium (251.5 µg/ml) and magnesium (75.4 µg/ml) compared with the whole bacterial cell of isolate No. 14 (*P. mirabilis* O3) and isolate No. 3 (*P. mirabilis* O16), where the calcium concentrations were 238.77 µg/ml and 227.12 µg/ml, respectively; and magnesium concentrations were 53.34 µg/ml and 37.68 µg/ml for isolates No. 14 and 3, respectively. In contrast, LPS and O-antigen of isolates No. 14 and 3 showed significantly differences ($P < 0.05$) in the ability to metal binding with calcium (25.77 µg/ml and 25.06 µg/ml), respectively, and magnesium (6.6 µg/ml and 7.5 µg/ml), respectively, compared with LPS and O-antigen of isolate No. 6, where the calcium and magnesium concentration were 18.03 µg/ml and 3.16 µg/ml, respectively.

Introduction

Proteus species are motile, Gram-negative bacteria within the Enterobacteriaceae that cause urinary tract infections, primarily in patients with long-term urinary catheters in place or structural abnormalities of the urinary tract (O'hara *et al*., 2000 and Al-Marzoqi *et al*., 2013). *Proteus* infections are known to be frequently persistent and difficult to treat and can lead to several complications such as acute or chronic pyelonephritis. Additionally, *Proteus* species are the most common bacilli associated with the formation of bacteria-induced bladder and kidney stones (about 70%of all bacteria isolated from such urinary calculi) (Coker *et al*., 2000 and Ali H. Al-Marzoqi, 2008).

Urease is the essential virulence factor of these bacteria involved in stone formation. Ammonia, produced by the enzymic hydrolysis of urea, elevates urine pH, causing supersaturation and crystallization of magnesium and calcium ions as struvite ($MgNH_4PO_4.6H_2O$) and carbonate apatite [$Ca_{10}(PO_4)_6.CO_3$], respectively (Kramer et al., 2000).

It has been found that, in addition to urease activity, bacterial exopolysaccharides contribute to stone formation. Polysaccharide produced by bacteria may aggregate precipitated urine components to form a stone. Proteus bacilli have capsular polysaccharide (CPS) and lipopolysaccharide (LPS, endotoxin) on their surfaces. CPS is the most external surface component of these bacteria, but detailed studies have shown that only a few strains can synthesize a capsule antigen, and its structure is identical to the O-specific chain of their LPS (Rozalski et al., 2012).

LPS is the main component of the outer membrane and one of the major virulence factors of these bacteria. It consists of a polysaccharide part, containing an O-specific chain (O-antigen, O-PS), and a core region, as well as a lipophilic region, termed lipid A, which anchors the LPS to the bacterial outer membrane. It has been well documented that Proteus is an antigenically heterogeneous genus, principally because of structural differences in the O-specific polysaccharide chain of LPS. In most Proteus strains, O-specific polysaccharides have been found to be acidic due to the presence of uronic acids and various non-carbohydrate acidic components, including phosphate groups (Manos and Belas, 2006 and Hawraa and Al-Marzoqi, 2014).

The role of acidic polysaccharides in the pathogenicity of Proteus, especially in urinary tract infections, is controversial. The negatively charged polysaccharides are important barriers against the bactericidal action of the complement system (Kaca et al., 2000).

The acidic character of Proteus extracellular polysaccharides may play a crucial role in stone formation within the urinary tract. It has been documented that the anionic groups found on bacterial polysaccharides influence struvite and carbonate apatite formation because they enable these macromolecules to bind cations (Ca^{+2}, Mg^{+2}) via electrostatic interactions that accelerate supersaturation and crystallization of these ions (Clapham et al., 1990 and Ali H. Al-Marzoqi et al., 2009). Dumanski et al. (1994) speculated that the structure and anionic character of Proteus mirabilis O6 CPS enhances struvite formation by weakly concentrating Mg^{+2} ions during struvite crystal formation. As mentioned before, O-specific polysaccharides also reveal a partially

anionic character and, unlike CPS, they are located on the surface of each *Proteus* strain. Hence, the goal of this study was to establish the role of lipopolysaccharide and O-antigen in urinary stones formation using crystallization experiments in vitro compared with the whole bacterial cell.

Materials and Methods:
Identification and biochemical tests of bacterial isolates
A total of 25 *Proteus mirabilis* isolates were obtained from 125 specimens, which belonged to patients suffering from urinary stones infections. The *P. mirabilis* isolates were previously identified using macro-microscopic, biochemical test, Vitek 2 system and polymerase chain reaction (PCR) technique by using target gene 16S *rRNA*. Also, the urease activity and other virulence factors of bacterial isolates were previously investigated.

LPS extraction and purification:
The LPS of 10 *P. mirabilis* isolates were extracted and purified by using Tri-reagent method as described by Yi and Hackett (2000). LPS extracts were electrophoresed on SDS-PAGE according to Sambrook and Rusell (2001). Finally, LPS in gel was stained by silver nitrate stain according to Chevallet *et al*. (2006).

O antigen isolation and purification:
The O antigen of 10 *P. mirabilis* isolates was isolated and purified by LPS acid degradation method described by Perepelov *et al*. (2005).

A 50 mg of LPS was hydrolyzed in aqueous 2% HOAc at 100 ^{o}C for 2 hr. until lipid A part precipitation. The lipid A part of LPS was removed by centrifugation 10000 rpm for 20 min., and then washed twice with D.W. and dried. The supernatant containing OPS was purified by gel permeation chromatography using Sephadex G-50 on a column (56 x 2.6 cm) with 0.05 M pyridinum acetate buffer pH 4.5 as eluent. The organic polysaccharide (OPS) was eluted after the void volume as single peak. Fractions containing a high molecular mass polysaccharide were pooled, concentrated, centrifuged, and freeze-dried; the polysaccharide yield was 53% of the LPS weight.

***In vitro* urinary stone formation:**
Crystallization of urine mineral was tested using *P. mirabilis* isolate number 6, 14, and 3 (whole bacterial cell, LPS and O-antigen for each isolate) because of the differences among these isolates in virulence factors activity as well as they revealed differences in chemical structure of LPS O-specific parts. The experiment was carried out according to Clapham *et al*. (1990).

Measurement of calcium and magnesium concentration:
The metal binding of calcium and magnesium was measured before and after crystallization.

Before crystallization, whole bacterial cell, LPS, and O-polysaccharide (O-antigen) were suspended in synthetic urine and incubated at room temperature for 1 hr to allow binding of cations. Samples were then placed in dialysis tubing and unbound metals were removed through dialysis against water, and the bound calcium and magnesium ions were measured.

After crystallization, the calcium and magnesium concentration were measured using the formation stones of each samples (whole bacterial cell, LPS, and O-polysaccharide). The formations stones were grinded and suspended with D.W, and then the calcium and magnesium ions were measured.

The calcium concentration was determined by using colorimetric method as described by Curhan *et al*. (1998) with commercially a variable kit (Cromatest kit, Spain), while magnesium concentration was determined according to the Titan yellow method described by Heaton (1960).

Statistical Analysis:
Statistical analysis was performed using statistical software package (IBM, SPSS V.20). Data were statistically described in terms of frequencies (number of cases) and relative frequencies (percentages). For descriptive statistics, (mean valve ± standard error) were given. The paired sample T test was performed to examine the difference between calcium and magnesium concentration before and after in vitro crystallization ($P< 0.05$). The last differences significance (LSD) was calculated for calcium and magnesium concentration ($P< 0.05$).

Results and Discussion:
LPS extraction and purification
LPS extraction and purification as well as O antigen acid degradation were investigated for 10 isolates of *P. mirabilis*. The LPS of these isolates was visualized by silver nitrate staining as shown in the figure (1). The LPS of *P. mirabilis* strains produced dark "stair case" pattern of bands due to carbohydrate chain length variation of O-antigen portion. Mainly two types of band pattern were observed. Some intense and highly concentrated bands were observed at upper gel layer and some intense bands, which were not clearly distinguished, were observed at lower surface. The bands at bottom were supposed to have short chain O-polysaccharide and those at the top have long chain of O-polysaccharide.

The current study used Tri-Reagent method for LPS extraction and purification, which was based on the using of guanidinium thiocyanate reagent. This method was in agreement with Yi and Hackett (2000) they

showed a fast, convenient extraction method for lipopolysaccharide (LPS), using a commercial RNA isolating reagent, allows the isolation of LPS or lipid A from low milligram (dry weight) quantities of bacterial cells. The method avoids the use of specialized equipment and has been used for processing relatively large numbers of samples. The major components of the commercial RNA isolating reagent, Tri-Reagent, are phenol and guanidinium thiocyanate in aqueous solution. The bacterial cell membranes were disrupted with guanidinium thiocyanate, which eliminates the need for mechanical cell disruption or heating. The LPS isolated using the Tri-Reagent procedure has been observed to be cleaner, containing fewer contaminants and degradation products, than that isolated using the conventional hot phenol water method.
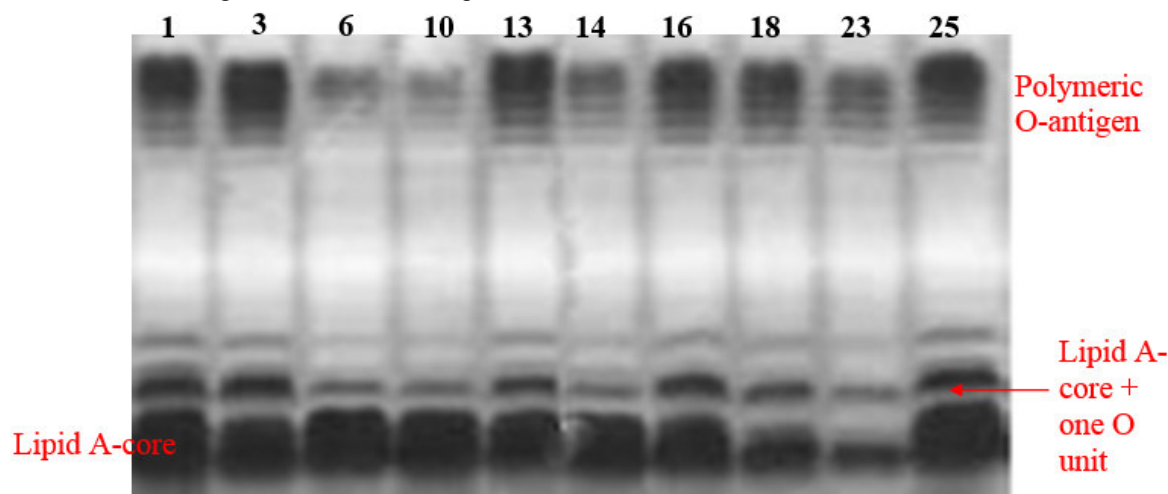
Figure (1): SDS-PAGE analysis of LPS of *P. mirabilis* isolated from urinary stone patients by using silver nitrate staining. Numbers at the top indicate the strains number of *P. mirabilis* isolates.

***In-vitro* urinary stone formation**
The results in the table 1, 2 and 3 showed there were significant differences $P< 0.05$ in the metal binding to calcium and magnesium ions for each whole bacterial cell, LPS, and O antigen of the tested *P. mirabilis* isolates before and after crystallization, as well as there were significant differences between whole bacterial cell, LPS, and O antigen of tested *P. mirabilis* isolates in the ability to bind with calcium and magnesium as shown in the figure 2 and 3, respectively.

The whole bacterial cell of the isolate number 6 showed the highest binding to Ca^{+2} and Mg^{+2} (251.5 and 75.4 µg/ml), respectively compared with isolates number 6 and 3, while the whole bacterial cell of isolate number 3 showed the lowest binding to Ca^{+2} and Mg^{+2} (227.12 and 37.68 µg/ml) respectively compared with isolate number 14 (238.77 and 53.34 µg/ml) respectively. This was attributed to the highest urease activity of the isolates No. 6 (129 u/ml) compared with isolates No. 14 (84 U/ml) and isolate No. 3 (59 U/ml) (data not shown). This result was agree with the result of Torzewska et al. (2003) who found that P. vulgaris O47 revealed the highest urease activity and thus showed the high significance difference $P< 0.05$ in metal binding to calcium and magnesium ions compared with P. vulgaris O12 which revealed the lowest urease activity and thus bounded significantly smaller $P< 0.05$ amount of calcium and magnesium ions.

Magana-Plaza et al. (1971) who study the biochemical characteristics of urease from P. rettgeri and stated that the P. rettgeri, which has the highest ability to split urea was considered the most active pathogen that able to form infection stones.

Table (1): Ca^{+2} and Mg^{+2} concentrations (µg/ml) of whole bacterial cell of *P. mirabilis* before and after crystallization.

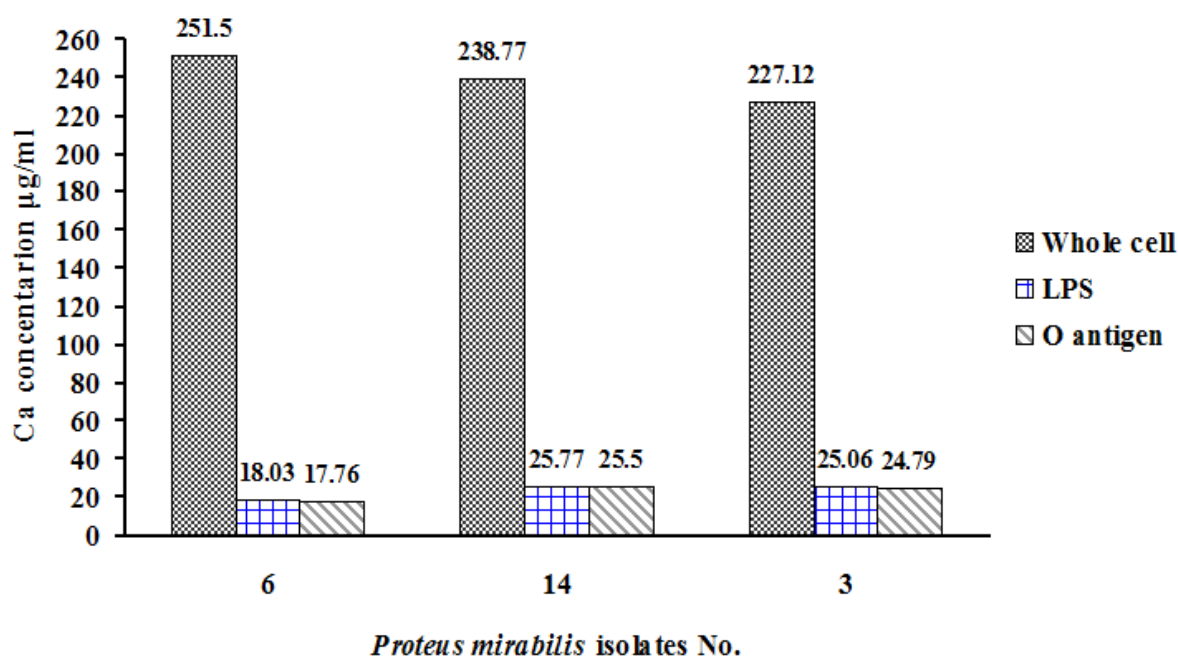| Isolate No.	Before crystallization	After crystallization	t- Value	Sig. (2-talied)
	Mean(±) standard error of Ca^{+2} concentration (µg/ml)			
6	7.5 ± 0.40	251.5 ± 1.90	132.72	0.000
14	2.09 ± 0.04	238.77 ± 1.53	160.42	0.000
3	0.92 ± 0.07	227.12 ± 2.81	71.062	0.000
	Mean(±) standard error of Mg^{+2} concentration (µg/ml)			
6	2.4 ± 0.41	75.4 ± 1.47	39.805	0.001
14	2.01 ± 0.06	37.7 ± 6.72	5.745	0.029
3	1.06 ± 0.12	53.34 ± 1.54	31.942	0.001

Table (2): Ca^{+2} and Mg^{+2} concentrations (µg/ml) of LPS of *P. mirabilis* before and after crystallization.

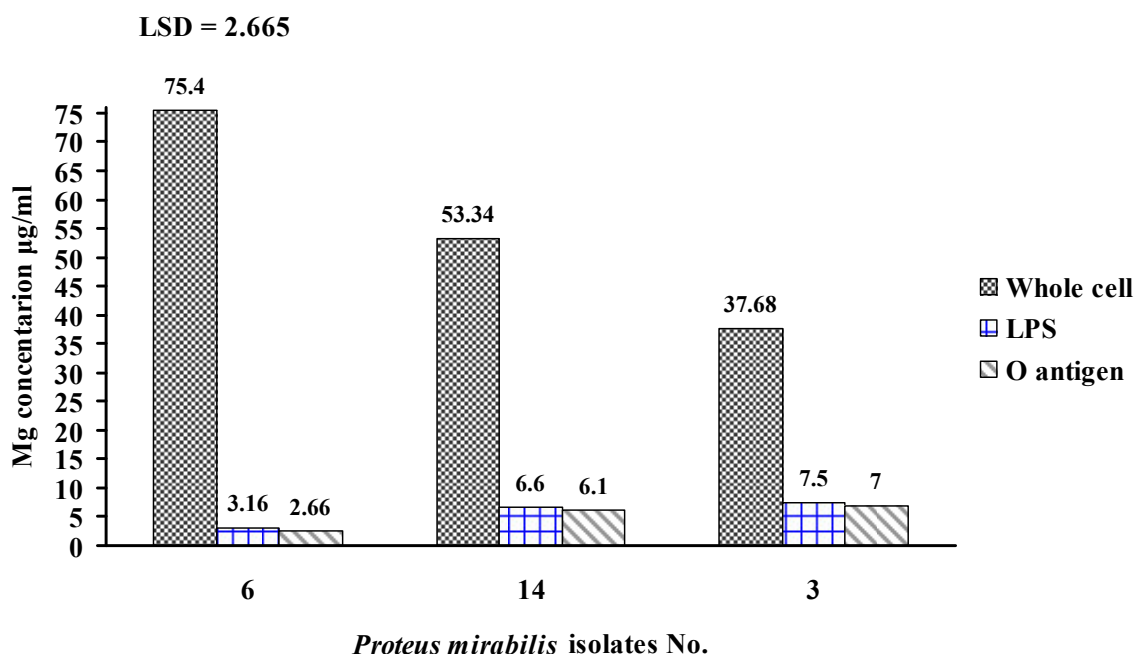| Isolate No.	Before crystallization	After crystallization	t- Value	Sig. (2-talied)
	Mean(±) standard error of Ca^{+2} concentration (µg/ml)			
6	2.5 ± 0.61	18.03 ± 1.08	31.818	0.001
14	2.22 ± 0.30	25.77 ± 4.15	4.909	0.039
3	1.25 ± 0.27	25.06 ± 1.20	25.446	0.002
	Mean(±) standard error of Mg^{+2} concentration (µg/ml)			
6	1.6 ± 0.26	3.16 ± 0.19	3.801	0.063
14	1.54 ± 0.27	6.6 ± 0.32	33.055	0.001
3	0.46 ± 0.06	7.5 ± 0.15	36.900	0.001

Table (3): Ca^{+2} and Mg^{+2} concentrations (µg/ml) of O antigen of *P. mirabilis* before and after crystallization.

Isolate No.	Before crystallization	After crystallization	t- Value	Sig. (2-talied)
	Mean(±) standard error of Ca^{+2} concentration (µg/ml)			
6	2.1 ± 0.23	17.76 ± 1.36	12.973	0.006
14	1.82 ± 0.16	25.5 ± 1.01	20.915	0.002
3	0.85 ± 0.14	24.79 ± 1.33	16.979	0.003
	Mean(±) standard error of Mg^{+2} concentration (µg/ml)			
6	1.5 ± 0.12	2.66 ± 0.08	9.278	0.011
14	1.44 ± 0.35	6.1 ± 0.30	16.056	0.004
3	0.36 ± 0.05	7.0 ± 0.32	18.727	0.003

LSD = 1.875

Figure (2): Mean of Ca^{+2} concentrations (µg/ml) of *P. mirabilis* after crystallization.

LSD = 2.665

Figure (3): Mean of Mg^{+2} concentration (µg/ml) of *P. mirabilis* after crystallization.

On the other hand, the study showed that the LPS of the isolates 14 and 3 showed significantly difference ($P< 0.05$) for binding to Ca^{+2} and Mg^{+2} compared with isolate number 6 at the same time there were no significance difference between isolates number 14 and 3. This was attributed to the differences in the chemical structure of LPS and O antigen as reported by Torzewska *et al*. (2003) who showed that the polysaccharide *P. vulgaris* isolates revealed weak and strong ability for binding to cations (calcium and magnesium) and also he concluded that the polysaccharide part of *Proteus* LPS may either enhance or inhibit the process of crystal formation, depending on the chemical structure composition of the molecule and its affinity for cations.

Dumanski *et al*. (1994) showed that there were significantly differences in the metal binding of isolated *P. mirabilis* capsule (CPS) to magnesium ions and he attributed that to the differences in chemical structure of the CPS polymers and the chemical solution, which used in crystallization experiment.

One has to bear in mind that, in the host, bacterial endotoxin is present not only as a component of glycocalyx in biofilms, but also as free molecules originating from dead cells or released from urinary stones during their surgical removal, which causes serious health problems, hence, it is possible that the same polysaccharide, through its cation affinity, may increase or inhibit the process of crystallization, depending on its location. Free endotoxin molecules with a high affinity for cations may act as crystallization inhibitors, since cations bound to such macromolecules would be washed out by the flow of urine. Conversely, in the biofilm, local accumulation of ions by anchored endotoxin would lead to stimulation of the crystallization process (McAleer *et al*., 2002; Boelke *et al*., 2001 and Hawraa and Al-Marzoqi, 2014).

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