www.jmscr.igmpublication.org
Impact Factor 3.79
ISSN (e)-2347-176x
crossref DOI: http://dx.doi.org/10.18535/jmscr/v3i8.25

IGM Publication
Journal Of Medical Science And Clinical Research
An Official Publication Of IGM Publication

The Role of Purified Urease from *Proteus Mirabilis* PMS17 in Stones Formation

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ABSTRACT

Introduction: *Injuries and Pyelonephritis have attention of researches in the medical field, because it represents a problem of the important problems, it's also one of the important disease frequent occurrences in human. The ability of P.mirabilis (Proteus mirabilis)* PMS17 *to cause these infections due to the possession of many virulence factors such as urease, hemolytic, outer membrane protein, in addition to its ability to invade cells and adhesion by pili.*

Material and Methods*: This study was included the isolation of 67 isolates of Proteus spp. From 350 samples of urinary tract infection (U.T.I) patients from different hospitals in Baghdad city, purification of Proteus urease by three steps which included , precipitation by ammonium sulfate 25-50% saturation , ion exchange and gel filtration chromatogrophies and study behavior of the resistance stone formation in animals .*

Results: *Results indicated that 190 samples (54, 28%) were positive results of bacteriological culture. Proteus mirabilis urease of local isolate number 17 was extracted and purified 167.35 fold with yield of 19.72% and specific activity 441.80 units / mg protein by DEAE-Sepharose and Sephacryl S-200 chromatographies.*

The result indicated persistent of Proteus infection in bladder and kidney of preimmunized and non-immunized rabbits. No infection with Proteus was recorded in bladder and kidney of the flurofamide treated animals. All the preimmunized and non-immunized infected animals were showed with alkaline urine, whereas the flurofamide treated animals were showed acidurea. Urinary bladder and kidney stones were found in 50 % and 40 % respectively of the infected non-immunized animals, whereas in infected immunized animals these stones are found 40 % and 30 % respectively. Urinary stones were composed mainly of struvite and a small amount of apatite carbonate and few mixed stones.

Introduction

Injuries and Pyelonephritis have attention of researches in the medical field, because it represents a problem of the important problems, it's also one of the important disease frequent occurrences in human [1,2,3]. The ability of *P.mirabilis* to cause these infections due to the possession of many virulence factors such as urease, hemolytic, outer membrane protein, in

addition to its ability to invade cells and adhesion by pili [4,5].

Urease of the most important virulence factors, as it leads to damage and necrosis kidney tissue [6, 7, and 8]. Many methods have been used to get rid of urinary stones whether surgical or interference by fragmentation mediated by sound waves or X-laser or using chemicals and each has its disadvantages and side effects in addition, the stones reappearing in the kidney patients, it has been observed that immunization against urease helps to heal the colon infection and also prevent colitis [9, 10, 11,12]. The aim of this study to isolation of *P .mirabilis* from urinary tract infections from Baghdad city, purification of urease, immunological laboratory study of behavior of the resistance stone formation in animals.

Materials and methods

Bacterial sample:

Samples of this study were collected from urinary tract infection patients .All samples were collected from patients who attended Al-Kadhimiya teaching hospital, Baghdad teaching hospital, during the period February 2013 to November 2014 and were investigated for Proteus infection by many tests, and observed swarming phenomena on Blood agar [13, 4].

Enzyme extraction:

It has been transferred a single colony from the isolate colony under study to lauria broth medium supplement with 0.1% urea incubated in shaking incubator until reached the absorbance 1.5 nm [14]. The resulting suspension was centrifuged at 1000xg for 20 min the supernatant was removed and precipitation was washed by phosphate buffer and centrifuged as previous step , enzyme extracted by frizzing and thawing method according to [15] , the urease activity and protein concentration were determinate .

Estimation of protein:

The dye binding method of Bradford [16] was used in determination the protein content of the crude extract, ammonium sulfate fraction and the concentrated Sepharose that showed urease activity.

Enzyme unit:

Urease activity was estimated according to the [17], the amount of enzyme needed to convert 1µmol of urea to ammonium in one minute at 37^{O}C.

Urease purification:

1. **First step (Ammonium sulfate):** 2.95 gm of ammonium sulfate were added to a 22 ml aqueous extract with constant stirring to a concentration of 25-50% saturation, the precipitate were removed by centrifugation at 9000xg for 20 min. the clear supernatant was then adjusts with constant stirring and kept overnight at 4^{0}C, the precipitate thus formed was collected by centrifugation as above, the pellets were resuspended in distilled water , extensively dialyzed against distilled water using 2000 Da cut-off dialysis tubing (sigma chemical company).
2. **Second step (Ion exchange chromatography):** By using DEAE – Sepharose column (pharmacia company).The concentrated crude applied to a column of DEAE – Sepharose (1.5 × 15cm)was equilibrated with 20 mM phosphate buffer ,the column of DEAE-sepharose washed with the same buffer and the enzyme was eluted with linear gradient in the same buffer .
3. **Third step (Gel filtration chromatography):** The active concentrated urease was placed on sephacryl S-200 column (2×75 cm) which had been equilibrated with 20 mM phosphate buffer (PH7.5) and washed with the same solvent, fraction of 3ml were collected.

Infection of urinary tract by *P.mirabilis PMS17*:

Used in current study, forty of white rabbits, weight ranged between 250-350gm, rabbits were divided into groups as follows:

First group (control): Included 10 rabbits, their bladder were injected through urethra with 1 ml sterilizes saline.

Second group: Injected their bladder through urethra with bacterial inoculums *Proteus mirabilis* (PMS17) which contained (2×10^8cell).

Third group: Injected their bladder through urethra by bacterial inoculums which contained (2×10^8 cell), each rabbit was given before 15 mg /kg urease inhibitor (flourofamid) daily through the mouth.

Four groups: Bladder of each rabbit was injected through urethra with bacterium inoculums which contained (2×10^8cell). Each rabbit was given before serum rabbit (serum separated from rabbit after injected by mixing cofactor and enzyme at rate 1:10 over forty days and average of three injections.

Investigation of *P.mirabilis* in the urine and kidney tissues:

The study of the content of each the urine and kidney tissue of *P.mirabilis* as follows:

One animal were scarified from each group /week ,each kidney was removed and divided into two halves ,one half was used for morphological study and the other half for bacteriological study , urine was collected at the time of scarified for bacteriological and pH determination .

The chemical composition of urinary stones:

Methods were used to detected these ions (CO_2^{3-}, $Po4^{3-}$, NH^{4+}, Ca^{2+}, Mg^{2+}) depending on [18,19].

Results:

Table (1) showed urease purification from *Proteus mirabilis* PMS17 by three steps which included precipitation by ammonium sulphate 25-50% saturation, DEAE –Sepharose exchange chromatography and gel filtration on Sephcryle S-200 column, the obtained purification fold and recovery were 167.35 and 19.72% respectively.

Table (1): Urease purification from *P.mirabilis* (PMS17).

Purification step	Volume (ml)	Activity unit (unit/ml)	Protein (mg/ml)	Specific activity (unit/mg)	Total activity(unit)	Yield (%)	Folds
Crude extract	150	6775.1	2564.5	2.64	1016265	100	1
Ammonium sulfate (25-50%)	60	9318.57	1467.49	6.35	559114.2	55.02	2.41
DEAE – Sepharose	50	7033.76	38.33	183.51	351688	34.61	59.51
Gel filtration Second part	40	5010	11.34	441.80	200400	19.72	167.35

The results of the current study showed absence of any macroscopic abnormal changes on both the bladder and kidney animals group first (control group) , and free Urine and kidney from the presence of *P.mirabilis* PMS17 .(Table 1).

Table (2): Data from animals of the first group (control group) during the experiment period.

Week	PH	Viable cells count (CFU)			Macroscopically Changes		Stones presence	
		Urine	Right kidney	Left Kidney	Bladder	Kidney	Bladder	Kidney
First	6.5	0	0	0	-	_	_	_
Second	6.7	0	0	0	_	_	_	_
Third	6.5	0	0	0	_	_	_	_
Fourth	7	0	0	0	_	_	_	_
Fifth	6.8	0	0	0	_	_	_	_
Sixth	6.6	0	0	0	-	-	-	_
Seventh	6.5	0	0	0	-	-	-	-
Eighth	6.7	0	0	0	-	-	-	-
Ninth	6.9	0	0	0	-	-	-	-
Tenth	6.7	0	0	0	-	-	-	-

Table (3, 4, 5) illustrated the number of viable cells of *P.mirabilis* PMS17 after bladder injected by (2×10^8viable cells) in second group, bladder rabbit was injected with 2×10^8 viable cells and in the third group given before urease inhibitor (flurofamid), the last group (fourth group) illustrated the number of *P.mirabilis* which given before extracted rabbit serum.

Table (3): Illustrate the number of viable cells of *P.mirabilis* PMS17 after bladder injected by (2×10^8viable cells) (second group).

Week	PH	Viable cells count (CFU)			Macroscopically Changes		Stones presence	
		Urine	Right kidney	Left Kidney	Bladder	Kidney	Bladder	Kidney
First	8.5	6.8×10^6 (6.83)	0	0	-	_	_	_
Second	8.5	7.2×10^6 (6.86)	0	0	_	_	_	_
Third	8.7	7.4×10^6 (6.87)	0	0	_	_	_	_
Fourth	9	1.2×10^7 (7.08)	3.0×10^6 (6.48)	0	_	_	_	_
Fifth	9.2	2.0×10^7 (7.30)	5.0×10^6 (6.70)	2.0×10^6 (6.30)	_	_	_	_
Sixth	9.3	3.0×10^7 (7.48)	7.1×10^6 (6.85)	4.0×10^6 (6.60)	◊		+	_
Seventh	9.4	7.2×10^6 (6.86)	3.1×10^6 (6.49)	2.2×10^6 (6.34)	◊		+	+
Eighth	9.1	6.3×10^6 (6.80)	2.8×10^6 (6.45)	2.1×10^6 (6.32)	◊		+	+
Ninth	9.2	5.7×10^6 (6.76)	2.1×10^6 (6.32)	1.3×10^6 (6.11)	◊	□	+	+
Tenth	9	5.3×10^6 (6.72)	2.2×10^6 (6.34)	1.1×10^6 (6.04)	◊	□	+	+

◊ : Bladder wall redden □ : Pus in kidney

Table (4): Illustrate the viable cells of *P.mirabilis* after bladder injected by 2×10^8 viable cell , which given before urease inhibitor (flurofamid) (third group).

Week	PH	Viable cells count (CFU)			Macroscopically changes		Stones presence	
		Urine	Right kidney	Left Kidney	Bladder	Kidney	Bladder	Kidney
First	6.6	6.2×10^6 (6.79)	0	0			–	–
Second	6.5	6.4×10^6 (6.81)	0	0			–	–
Third	6.7	6.8×10^6 (6.83)	0	0			–	–
Fourth	6.9	1.5×10^7 (7.18)	2.5×10^6 (6.40)	0			–	–
Fifth	7.0	1.8×10^7 (7.26)	3.5×10^6 (6.54)	1.5×10^6 (6.18)			–	–
Sixth	6.7	2.0×10^7 (7.30)	5.0×10^6 (6.70)	2.5×10^6 (6.40)			–	–
Seventh	6.8	2.5×10^7 (7.40)	5.5×10^6 (6.74)	2.5×10^6 (6.40)			–	–
Eighth	6.7	2.5×10^7 (7.40)	5.4×10^6 (6.73)	3.7×10^6 (6.57)			–	–
Ninth	6.6	2.2×10^7 (7.34)	4.4×10^6 (6.64)	3.1×10^6 (6.49)			–	–
Tenth	6.7	1.9×10^7 (7.28)	3.1×10^6 (6.49)	2.6×10^6 (6.41)			–	–

Table (5); illustrate the viable cells of *P.mirabilis* after bladder injected with 2×10^8 viable cells , which given before serum rabbit (fourth group).

Week	PH	Viable cells count (CFU)			Macroscopically changes		Stones presence	
		Urine	Right kidney	Left Kidney	Bladder	Kidney	Bladder	Kidney
First	8.3	6.4×10^6 (6.81)	0	0	-		–	–
Second	8.5	$6{,}8\times10^6$ (6.83)	0	0	–		–	–
Third	8.8	7.1×10^6 (6.85)	0	0	–		–	–
Fourth	9.0	2×10^6 (7.30)	0	1.5×10^6 (6.18)	–		–	–
Fifth	9.0	2.1×10^6 (7.32)	4.1×10^6 (6.61)	2.5×10^6 (6.40)	–		–	–
Sixth	9.2	2.5×10^6 (7.40)	5.5×10^6 (6.74)	2.5×10^6 (6.40)	–		–	
Seventh	9.3	6.5×10^6 (6.81)	3.5×10^6 (6.54)	2.5×10^6 (6.40)	⬯		+	+
Eighth	9.1	6.1×10^6 (6.79)	3.1×10^6 (6.49)	2.2×10^6 (6.34)	⬯	▯	+	+
Ninth	8.8	5.5×10^6 (6.74)	2.9×10^6 (6.46)	2.3×10^6 (6.36)	⬯	▯	+	+
Tenth	8.1	5.1×10^6 (5.71)	2.6×10^6 (3.41)	1.9×10^6 (3.28)	⬯		+	–

⬯ : Bladder wall reddens ▯ : Pus in kidney

Results in table (6) showed there are three types of stones at different ratio, stuvite, apatite carbonate and mixed stones. And the current study showed that the stones extracted from kidney of second animals group, presence of two types of stones included struvite and apatite carbonate.

Results in table (7) showed that there are three types of stones from bladder of fourth group while kidney stones from the same group included two types struvite and apatite carbonate

Table (6): weight, the position of stones formation from bladder and kidney second group

group	week	Stones number			Stone weight (mg)			Stones composition		
		Bladder	Right kidney	Left kidney	Bladder	Right Kidney	Left Kidney	Bladder	Right kidney	Left kidney
Second	Sixth	1			61.3			NH_4^+ Mg^{2+} PO_4^{3-}		
	seventh	5	1		547.9	85.2		NH_4^+ Mg^{2+} Ca^{2+} PO_4^{3-} CO_3^{2-}	NH_4^+ Mg^{2+} PO_4^{3-}	
					123.7			Ca^{2+} PO_4^{3-} CO_3^{2}		
					268.6			NH_4^+ Mg^{2+} PO_4^{3-}		
					345.7			NH_4^+ Mg^{2+} PO_4^{3-}		
					379.3			NH_4^+ Mg^{2+} PO_4^{3-}		
	eighth	2	1	1	114.8	83.7	64.1	Ca^{2+} PO_4^{3-} CO_3^{2}	NH_4^+ Mg^{2+} PO_4^{3-}	NH_4^+ Mg^{2+} PO_4^{3-}
					98.2			Ca^{2+} PO_4^{3-} CO_3^{2}		
	ninth	2	1		77.4	869.9		Ca^{2+} PO_4^{3-} CO_3^{2}	NH_4^+ Mg^{2+} PO_4^{3-}	
					192.5			NH_4^+ Mg^{2+} PO_4^{3-}		
	tenth	1	1		69.1	54.4		NH_4^+ Mg^{2+} PO_4^{3-}	Ca^{2+} PO_4^{3-} CO_3^{2}	
								NH_4^+ Mg^{2+} PO_4^{3-}		

Table (7): Composition, weight, position of stone formation from bladder and kidney fourth group rabbits.

Group	week	Stones number			Stone weight (mg)			Stones composition		
		bladder	Right kidney	Left kidney	Bladder	Right Kidney	Left kidney	Bladder	Right kidney	Left kidney
Fourth	Seventh	3		1	126.8		59.2	NH_4^+ Mg^{2+} PO_4^{3-}		Ca^{2+} PO_4^{3-} CO_3^{2-}
					201.4			NH_4^+ Mg^{2+} PO_4^{3-}		
					133			NH_4^+ Mg^{2+} PO_4^{3-}		
	Eighth	4	1		108.6	81.0		Ca^{2+} PO_4^{3-} CO_3^{2-}	NH_4^+ Mg^{2+} PO_4^{3-}	
					393.7			NH_4^+ Mg^{2+} Ca^{2+} PO_4^{3-} CO_3^{2}		
								NH_4^+ Mg^{2+} PO_4^{3-}		
					271.4			NH_4^+ Mg^{2+} PO_4^{3-}		
					306.4					
	Ninth	1	1	1	99.8	82.2	60.3	NH_4^+ Mg^{2+} PO_4^{3-}	NH_4^+ Mg^{2+} PO_4^{3-}	NH_4^+ Mg^{2+} PO_4^{3-}
	Tenth	1				49.5		Ca^{2+} PO_4^{3-} CO_3^{2}		

Discussion

This study was included the isolation of 75 isolate of *proteus* spp from 350 samples of UTI patients from different hospitals in Baghdad city .Results indicated that 190 samples (54,28%) were positive results of bacteriological culture .Diagnostic results indicated that 67 (89.33 %) isolates were be *P.mirabilis* while the remaining 8 (10.66 %) isolates were *P.vulgaris*.

While [20] noted in his study about urinary tract infection caused by *Proteus* spp, that the incidence of *P.mirabilis* were 85.14% compared to *P.vulgaris* were 14.86%, and from this study and other studies its clear the high percentage of urinary tract infection by *P.mirabilis*, due to its ability to cause this infection more than other *proteus* spp. so it was selected in a subsequent experiments.

Results indicated that all local isolates were urease production, isolate number 17 was chosen as the best urease producer in the presence of 0.1% urea. *P.mirabilis* urease of local isolate number 17 was extracted and purified 167.35 fold with yield of 19.72% and specific activity 441.80 u/mg protein by DEAE –Sepharose and Sephacryl S-200 chromatography's (Table1).

Urease has been purified from a number of bacteria [21], purified urease from *Bacillus pasteurii* by using ammonium sulfate and acetone. [22] studied *P.rettgeri* urease and purified it 24.8 fold with recovery 2.2% using Sephadex G-200 Hydroxyaptite and DEAE-Sephadex .

Purified *P.mirabilis* urease 145.23 fold with a yield 23.96% using DEAE –Cellulose and Sephacryl S-200 [23].

Table (2), revealed the absence of any macroscopic abnormal changes in both bladder and kidney for control group (first group) which were injected normal saline.

Bacterial inoculums was prepared from the isolate 17 and used to inoculate the bladder of 30 male rabbits in three infected groups, each group of ten animals, one group was preimmunized with unease whereas the other group was given orally a dose of flurofamid.

One animal were sacrificed from each group /week.

From Table (3) it was noted the absence of any abnormal macroscopic changes on both bladder and kidney in second group, while increase in PH value about 8.5, 8.5. 8.7 Respectively, and the number of *P.mirabilis* in the urine about 6.8×10^6 ($6.83 \log_{10}$), 7.2×10^6 ($6.86 \log_{10}$), 7.4×10^6 ($6.87 \log_{10}$), viable cell for first third weeks respectively, there is no stones but observed increase in turbidity through fourth and fifth weeks and the presence of a simple salt deposits, and the PH about 9 , 9.2 in the fourth and fifth week respectively, in the sixth week , it's the beginning of stone formation ,the PH was about 9.3 and observed redness in bladder wall , and bladder stones was bigger than kidney stones in the seventh week which filled in the Right kidney rabbit , it was noted the presence faster on the outer surface of the kidney , as well as in the pulp area and rupture in the tissue of the pelvis and pulp this is because of the presence of big stone.

No infection of Proteus were recorded in bladder and kidney of the flurofamid treated animals of third group (Table 4) . It was noted the absence of any abnormal macroscopic changes on both the bladder and kidneys, while have been observed the number of *P.mirabilis* in the urine about 6.2×10^6 (Log_{10} 6.79) , 6.4×10^6 (Log_{10} 6.81) , 6.8×10^6 (Log_{10} 6.83) for the first three weeks respectively. It was also noted that the PH of the urine within normal levels 6.6, 6.5, 6.7 respectively ,also did not notice any stones during period experimental.

From Table (5) It was observed the presence of the *P.mirabilis* in the urine and kidney rabbit forth group which was given before serum rabbit , the number of viable cells reached approximately 2.0×10^6 ($\log_{10}$ 7.30) ,2.1×10^7($\log_{10}$ 7.32) in the forth and fifth week respectively ,while viable cell reached 1.5×10^6 ($\log_{10}$ 6.18) cell in left kidney while the right kidney showed free of bacteria in forth week .The PH was about 9.0 in the urine of forth and fifth week , no good configuration stones , but observed increase in turbidity through forth and fifth week and the presence of a simple salt deposits .

The result of the current study, showed a continuation infection by *P.mirabilis* in the kidney and bladder during the period of the experimental in the second and fourth groups (Table 3, 5), and the use of flurofamid prevent the formation of urinary stones through inhibition of urease activity and no bacterial effect has been observed in rabbit urine and kidney in third group (Table 4).

Millner and his group [24] pointed that the flurofamid had effect in the prevention of bladder stones and they noted that a single dose of flurofamid 15mg /kg or three doses 5 mg/kg by orally prevent bladder stones.

Uroliths composed as a result of urinary tract infection with *P.mirabilis* urease which decomposed urea to ammonia which raises PH urine, leading to crystallization of potassium and magnesium ions which are deposited composed magnesium and ammium phosphate stones called struvite and calcium phosphate called apatite [25, 2 and 26].

The results of this study showed there is no relationship between the *P.mirabilis* infection and stones formation in rabbit urine which already injected with serum extracted from rabbit, and bladder and kidney stones were found in 50% and

40% respectively of the infected non-immunized animals in the second group Table (3), whereas in infected immunized animals these stones are found 40% and 30% respectively Table (5). In addition to the close similarity in the ratio formation stones between second and forth groups animals, the results showed continuation of infection caused by *P.mirabilis* in the rabbit urine groups throughout the study period.

All the preimmununized and non-immunized infected rabbits were showed with alkaline urine (Table 3, 5); whereas the flurofamid treated rabbits were showed acid urea (Table 4).

Table (6) indicated the qualitative chemical analysis from animals bladder , that the stones which extracted from animals seventh week were composed from PO^{3-}_4 , NH^{+}_4 and Mg^{2+} which indicates that stone was struvite , while animals eighth week , analysis showed that the first stone composed of Ca^{2+} ,PO^{3-}_4 and Ca^{2+} which shows that it kind of apatite carbonate , and the second stone was mixture from PO^{3-}_4 ,NH^{+}_4, Ca^{2+} and Mg^{2+} Ions and the third and fourth stones were struvite because it composed of PO^{3-}_4 , NH^{+}_4 , Mg^{2+} ions Also qualitative chemical analysis showed the components of stones extracted from the bladder ninth week showed the presence of one of the stones apatite carbonate , while stone from tenth week it was from struvite .from this concluded three types of stones at different ratio , struvite (54.54%), apatite carbonate (36.36%) and mixed stones (9.09%) .

The current study showed that the stones extracted from kidney of second animals group , presence of two types of stones included struvite (80%) and apatite carbonate (20%).

Results in table (7) showed that there are three types of stones from bladder of fourth group included struvite (66.67%), apatite carbonate (22.22%), and mixed stones (11.11%) .While kidney stones from the same group included two types struvite (75%) and apatite carbonate (25%). Urinary bladder and kidney stones were found in 50% and 40% respectively of the infected non-immunized rabbits whereas in infected immunized rabbits these stones are found 40% and 30% respectively.

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