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RESEARCH ARTICLE

Flagellin Immunization against Urinary Tract Infection Caused by p. Mirabilis

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Abstract

Objective *Proteus mirabilis* is dimorphic a (gram negative bacteria that alternates between a rod – shaped swimmer stage and multinucleate, highly elongated swarming stage. Methods Urine Samples collected from three hospitals in Baghdad city from adult and child patients Suffering from urinary tract infections for 6 months .The samples get reached 1152 distributed among the patients old between few days to 80 years most patients living in hospital with urinary tract catheter , 56 samples of *Proteus mirabilis* were identified after isolation and diagnosis of bacterial species in different methods because there different complications in UTIs Patients specially Stone formation , catheter obstruction , and renal damage. Results these bacteria were diagnosis in api 20 E and vitek2 system and one isolate of *Proteus mirabilis* was selected for the study according to distance movement by measurement (swarming motility). Flagellin which is the flagella protein was extracted and predicated, followed by SDS-polyacrylamide gel electrophoresis for protein molecular weight estimation and concentration of protein counted by Bradford method ,then immunization protocol were tested in Albino rabbits in different concentrations 10,20,40 microgram from flagella protein then immunoglobulin antibody IgG ,IgM in serum were tested by single radial immunoglobulin diffusion plate , positive results showed raised the concentrations of these antibodies with high significant differences .Immunized females rabbits was tested to ascending urinary tract infection challenge by inoculation 5×10^7 cfu from *P.mirabilis* and tested in 1 , 3 , 7 , 14 days , any females rabbits didn't show urinary tract infection by *P.mirabilis* when urine culture tested and, the concentration of IgG , IgM antibody in balance also showing white blood cells in normal range .While rabbits females that inoculated by 5×10^7 cfu in positive control group showed urinary tract infection by *P. mirabilis* after 7 days post inoculation the tested urine culture showed raised in IgG , IgM and white blood cells concentration. Conclusions In the present study, flagellin protein immunization in rabbit showed that there are rising in IgM and IgG titers. After challenging by ascending urinary tract infection, we showed neutralization of IgG and IgM and white blood cells.

Keywords: *Flagellin, P. Mirabilis, UTI, Vaccine.*

Introduction

Proteus mirabilis is dimorphic a (gram negative bacteria that alternates between a rod-shaped swimmer stage and multinucleate, highly elongated swarming stage [1, 2].Swarming behavior is characterized by the development of concentric rings of growth consist from cyclic events of swarmer cell.

This cycle growth formed striking bull's eye pattern colony often associated with growth of *P. mirabilis* bacteria [3, 4]. It caused many different diseases. In Urinary tract such as cystitis and acute pyelonephritis [5, 9] and kidney stones [10, 12] especially in patients with functional or anatomy abnormally in urinary tract and long catheterized patients [13, 15].*Proteus mirabilis* contributed in bacteriemia most frequently due to UTI or CAUTIs compared to other sources of infection *Proteus mirabilis* is linked with CAUTIs where it can be due to complications including crystalline biofilms, urinary stones, pyelonephritis and septicemia [2, 4, 16]. *Proteus mirabilis* distinctive dimorphic

nature correlated to its motility [17] in the vegetative state and during growth in liquid media *Proteus* bacteria occur as short motile rods (1 to 2 µm in length), with 6 to 10 peritrichous flagella and swimming behavior [9] . *Proteus mirabilis* flagella are generally similar to flagella produced by other bacteria, there are two unusual features of *Proteus mirabilis* flagella.

First all genes encoding flagellar components, including the Class I flagellar master regulatory genes flh DC (P.M 1171-72), are found within a single 54 kb locus in the chromosome (PM11617-72) This is in contrast to most other flagella-producing bacteria which including have flagellar operons in disparate loci second, *Proteus mirabilis* encodes two flagellins , flaA (PM11620 and FlaB .

(PM11619) also known named as flic1 and flic2 which comprise the filament whip structure of the flagellum the flagellar master regulator (Flh DC) , and agene contribute in regulation of flh DC (Umo A) were known as fitness factors for renal colonization, Molecular mass for *P. mirabilis* flagellin between (36.7KDa) [18] to (41KDa) [19] .

When used SDS PAGE electrophoresis analyses. Flagellin is the flagella filament protein which is considered an important bacterial structure that is recognized by the innate immune system [20, 22]. It is sensed by the host via Toll-like receptor 5 and NoD – like receptors also triggers pro inflammatory response S. [23, 24]. Installation of purified *P. mirabilis* Flagellin in into bladder showed elicits leukocyte infiltration, histological changes in tissue of bladder and elevated raised Fxcll , Cxclo and 1L 6 m-RNA [23].

Methods

Collection Urine Samples

One Thousand Hundred Fifty Tow urine samples were collected from three hospitals included in the study (Martyr Ghazi AL-Hariri, Child protection, Educational laboratories). In Baghdad city between January 2017 to June 2017. From patients suffer from UTIs (adult, child) especially catheterized patients that living in hospital, mid stream urine samples were collected in sterile containers by using clean and sterile catch methods recommended [25]. All samples cultured on Blood agar and MacConkey agar plates, using sterile standard loop then incubated at 37℃ for 24 hours. Fifty six samples of *proteus miraibils* was identify by 1.API20 E system 2.VITEK 2 systems [26, 27].

Isolation and Purification of Flagella

Flagella were prepared by many differential centrifugations as described by Bahrani et al. with some modification. An overnight culture 300ml of *P.mirabilis* was used to inoculate 60 Luria Britine agar plates (large size plate)for 48 h at 37 ℃, harvested by glass rod in 10 Mm potassium phosphate (pH 7.0), centrifuged (5,000 × g, 15 min , 4℃) washed , and resuspended in in phosphate buffer (100 ml /6g wet weight)of cells.

Then the suspension was blended in 45 sec at setting 4 and then centrifuged (16000 × g, 15 min 4℃). The supernatant was centrifuged (30000 × g, 3h, 4℃), and pellet was washed twice in phosphate buffer and centrifuged (30000 × g, 2h, 4℃).Then lipholized until used [19].

Swarming Behavior Assay

The swarming migration distance assay was prepared as described by Liaw et al (2001) [28] . An overnight bacterial culture 5 microliters was inoculated centrally on to the dry Luria Britine agar plate then incubated at 37℃ the swarming migration every 60 minutes for 8 hours and after 24 hours.

SDS-PAGE) SDS-Polyacrylamide Gel Electrophoresis)

A flagella sample was prepared by adding 4X SDS sample loading buffer to protein samples followed by heating at 90-95℃ for 2 min in order to denature proteins.

Native Polyacrylamide Gel Electrophoresis

The procedure used was the same for SDS-PAGE except that the gels and the running buffer was prepared without SDS furthermore, the sample was not heated and , SDS and reducing agent (β-mercaptoethanol) was omitted when preparing 4X sample buffer [29] .

Estimation of Protein Concentration

Bradford method used to estimated protein concentration was prepared by dissolved 1 mg of protein powder per 1ml PBS then 50

microleiter was take with 20 microleiter from sample with 1 ml from Bradford Coomasie Brilint blue with 50 microleiter NaoH and 20 microleiter from protein sample [30].

Immunization in Animals Laboratories

Four groups of rabbits used in the study, three groups for three different concentrations of flagella 10, 20, 40 microgram with incomplete Fruends adjuvant and the last group for control. All rabbits used in the same age and weight immunization protocol were done by subcutaneous in first, eighth days then give three boosters IV injection every week [31]

Quantitative Evaluation Of Immunoglobulin (Igg,Igm) In Rabbits Serum

Determination of the IgG , IgM protein by radial immune diffusion plate , the plate removed from its envelope and left for a few minutes in room temperature to stand , then filled every wells with 5microliters of serum , after closed the plate placed in moist chamber for (72 ,96 hr) IgG , IgM respectively.

Challenged the Rabbits That Immunized By Flagella

Challenged was prepared in nine rabbits was divided in three groups first group with three rabbits negative control ,second group was contain three immunized rabbits females which tested for challenged ,it was inoculated with 5×10^7 cells from *P. mirabilis* by transurethral injection method then these rabbits tested for IgG , IgM , WBC and urine culture in (1,3,7,14) days post inoculation . The third group with three females tested for positive control to ascending urinary tract infection also inoculation with 5×10^7 cells from *P. mirabilis* also tested to IgG, IgM, and WBC and urine culture in 7 days post inoculation [32].

Statistical Analysis

The statistical analysis system – SAS (2012) program was used to effect of difference factors in study parameters. Chi – square test was used to significant compare between percentage and least significant difference – LSD test (ANOVA) was used to significant compare between means in this study [33].

Result

Fifty six samples were isolation and diagnosis by API 20 E and Vitek2 system as show in Figure (1) and (2) [26, 27].

Figure 1: Show diagnosis of *p. mirabilis*

Swarming Behavior Assay

From 56 samples were selected 20 give heavy swarming in plate after cultured then Five strains of *p. mirabilis* isolated from UTI give the largest distance of swarming behavior after tested every one hour for 8 hour and after 24 hour in the 8 hour the distance between 85 mm to 101 mm, while in 24 hour 113 mm to 135 mm as show in Table (1) and Figure (3) [34].

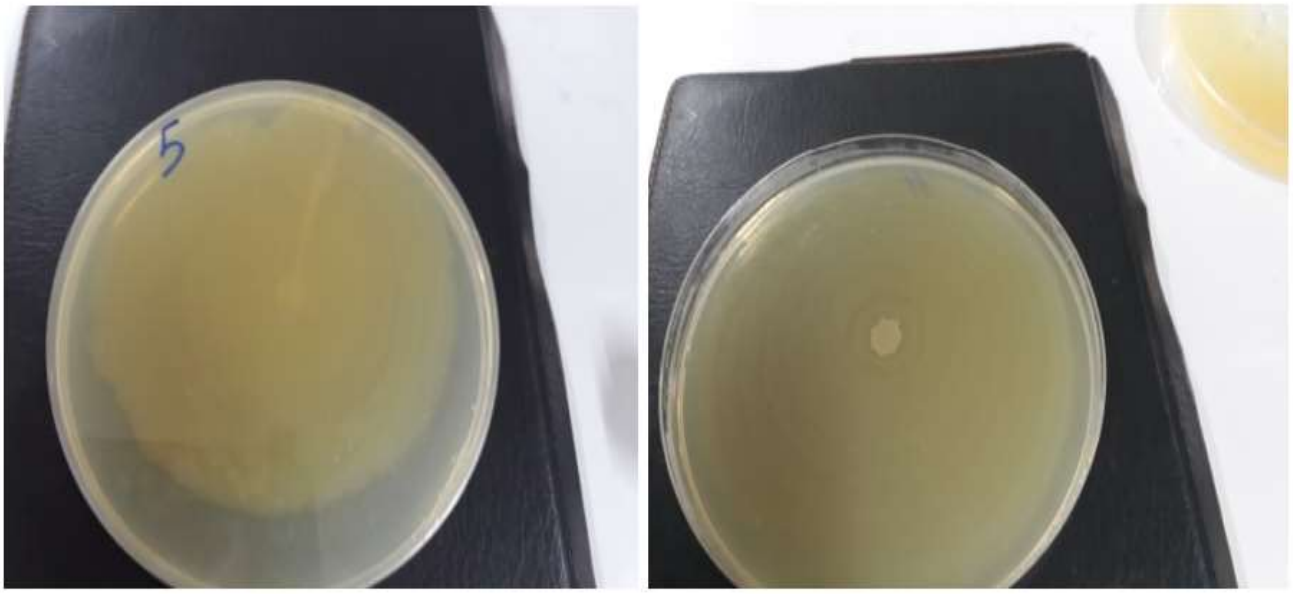

Figure 2: a swarming in 8hr b swarming in 24hr

Table 1: show distance of swarming behavior assay for 20 *P. mirabilis* strain

samples	1 hr	2 hr	3 hr	4 hr	5 hr	6 hr	7 hr	8 hr	24 hr
1	0	0	10	14	15	20	22	25	115
2	0	0	15	20	24	46	71	89	135
3	0	0	10	12	25	56	78	85	135
4	0	0	8	10	22	44	70	78	115
5	0	0	10	12	21	35	62	70	110
6	0	0	6	10	20	31	40	52	122
7	0	0	7	13	24	35	53	60	128
8	0	0	10	12	18	25	57	62	111
9	0	0	8	10	17	22	47	61	114
10	0	0	17	20	28	50	95	101	135
11	0	0	15	20	26	25	70	83	118
12	0	0	11	15	20	50	75	90	130
13	0	0	10	15	20	57	90	95	113
14	0	0	10	14	15	50	68	75	128
15	0	0	8	12	15	35	44	73	100
16	0	0	10	11	17	40	53	70	119
17	0	0	10	13	20	47	56	66	113
18	0	0	7	15	20	25	40	77	117
19	0	0	7	11	20	23	43	73	128
20	0	0	8	12	20	25	47	78	118

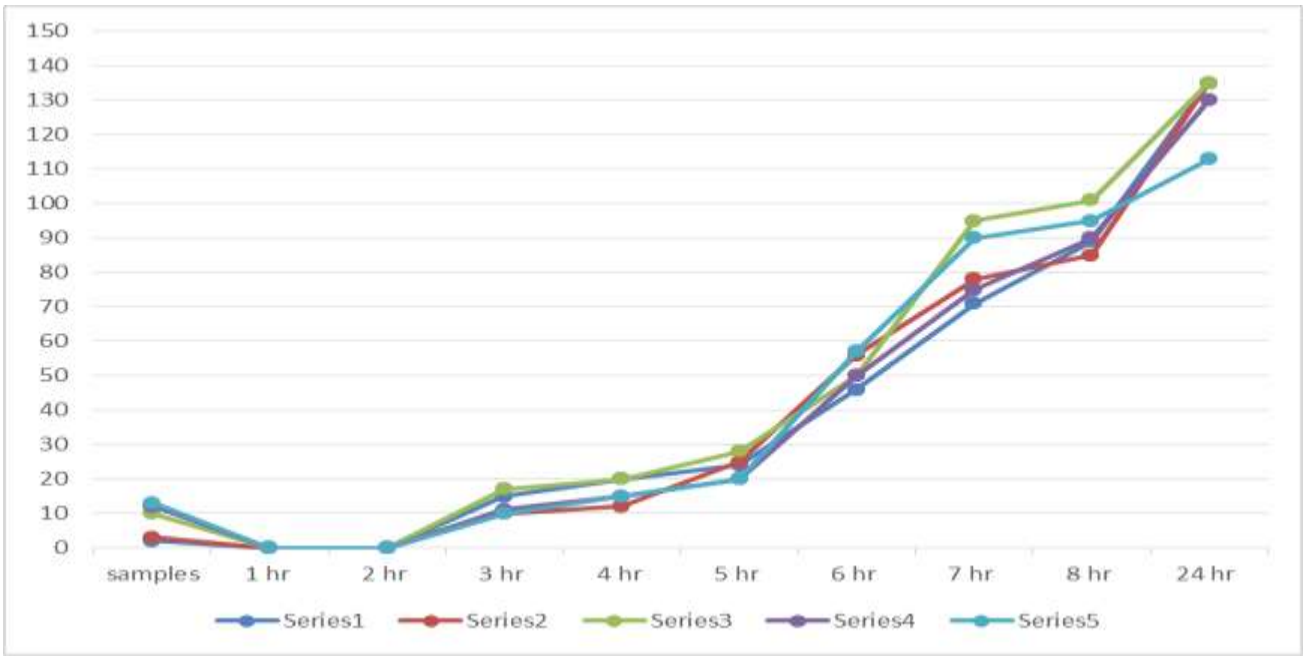

Figure 3: swarming behavior assay for 5 *p. mirabilis* strain isolated from UTI

Isolation and Purification Flagella

One strain was selected from the five strains that given largest distance in swarming after 8 hour from culture then used to isolation and purification of flagella by differential centrifugation under cool conditions [19]. Flagella protein was storage in -20℃ until used as show in Figure (4).

Figure 4: *P. mirabilis* flagellin

SDS-PAGE) SDS-Polyacrylamide gel electrophoresis)

Electrophoretic analyses showed apparent molecular masses between 41 kDa for flagellin, and the native polyacrylamide gel electrophoresis showed one thick largest bands in area between 40 kDa to 50 kDa [19]. As shown in Figure (5).

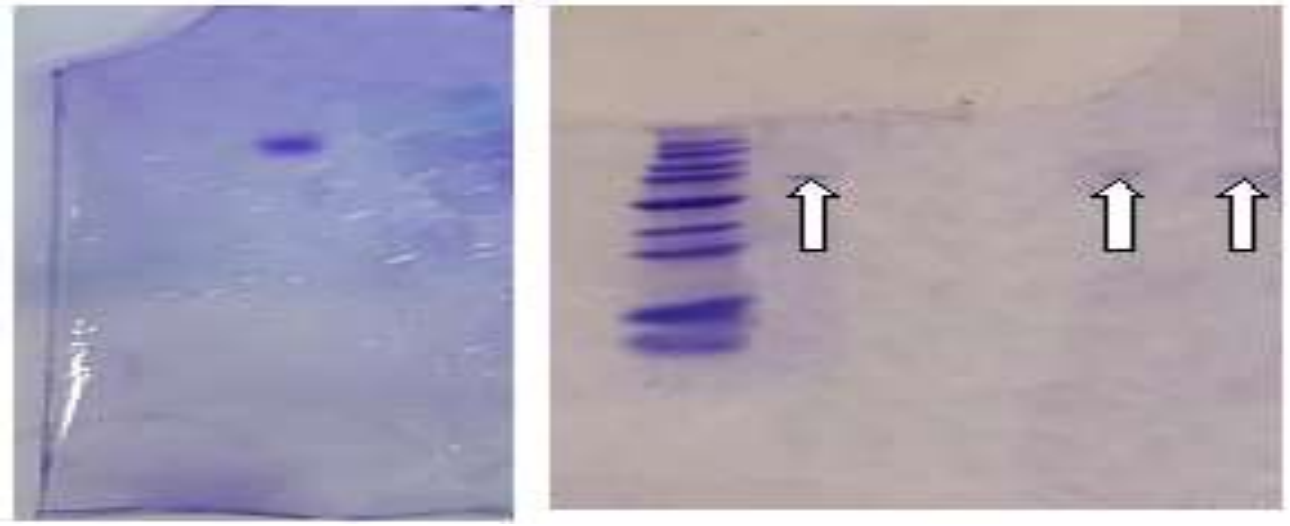

Figure 5: a. show electrophoresis for flagellin without SDS b. show SDS- electrophoresis for flagellin

Estimation of Protein Concentration

When protein estimated by Bradford method result showed in each 1mg of prepared powder concentration of protein 75 microgram, the total weight 2775 microgram.

Quantitative Evaluation Of Immunoglobulin (Igg , Igm) In Rabbits Serum

Results of rabbit's immunization by subcutaneous route showed redness and fever after 24hr for inoculation as show in Figure (6). And IgG and IgM antibodies showed elevation in the titer of them and different significant value especially for IgG antibody in different duration of immunization protocol when immunized by different flagella protein concentrations.

As show in Table 2, 3 and Figure 7, 8, 9, 10 that agreement with Campodonico et al, [31].

Figure 6: a. Rabbits before immunization b. Rabbits after immunization

Table 2: Compare between difference groups in results of IgM after immunization

Groups of Rabbits (Conc. of protein)	Mean ± SE			
	Zero day IgM	IgM after 2 subcutaneous inj. 2 weeks	IgM after 2 booster I.V. inj. 4 weeks	IgM after 3 booster I.V. inj. 6 weeks
G1: 10 µg	72.43 ± 20.11	139.70 ± 44.02 a	335.46 ± 37.93	276.80 ± 48.25
G2: 20 µg	86.87 ± 18.74	110.83 ± 17.44 ab	303.16 ± 14.03	245.96 ± 15.99
G3: 40 µg	103.56 ± 10.16	138.10 ± 9.77 a	384.26 ± 9.30	330.43 ± 3.48
G4: Control inj. adj only	72.30 ± 8.28	94.00 ± 12.27 b	---	---
LSD value	49.678 NS	41.337 *	82.917 NS	101.81 NS
* (P≤0.05). Means having with the different letters in same column differed significantly				

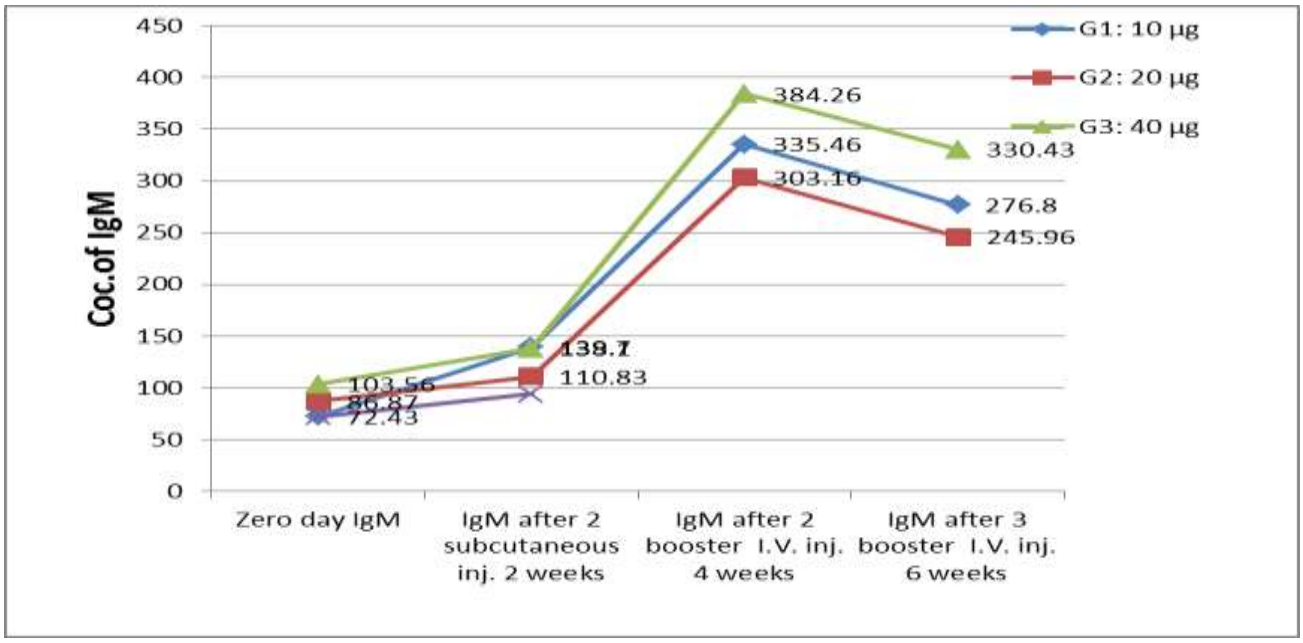

Fig. 7: immune response for IgM after immunization protocol

Fig. 8: IgM estimation by radial immunoglobulin diffusion

Table 3: Compare between difference groups in results of IgG after Immunization

Groups of Rabbits (Conc. of protein)	Mean ± SE			
	Zero day IgG	IgG after 2 subcutaneous inj. 2 weeks	IgG after 2 booster I.V. inj. 4 weeks	IgG after 3 booster I.V. inj. 6 weeks
G1: 10 µg	500.69 ± 23.26	634.79 ± 25.77 ab	1947.07 ± 117.42 b	2023.80 ± 99.47 c
G2: 20 µg	514.76 ± 51.70	648.46 ± 113.50 ab	3033.57 ± 176.51 a	3346.00 ± 169.39 a
G3: 40 µg	559.83 ± 41.82	733.27 ± 39.85 b	2141.60 ± 22.30 b	2765.23 ± 57.63 b
G4: Control inj. adj only	488.14 ± 12.34	470.45 ± 8.34 b	---	---
LSD value	116.63 NS	201.06 *	425.90 *	409.02 *
* (P≤0.05). Means having with the different letters in same column differed significantly				

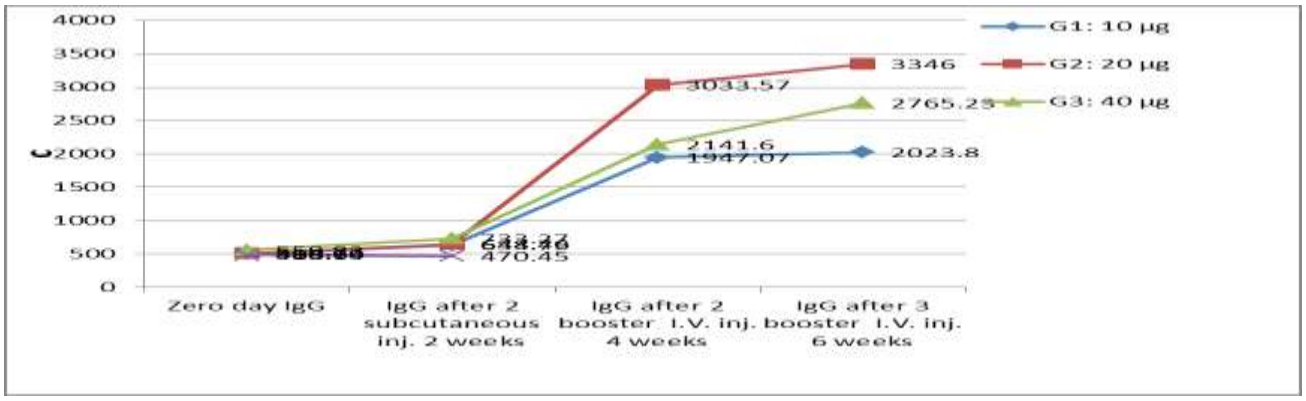

Fig 9: immune response for IgG after immunization protocol

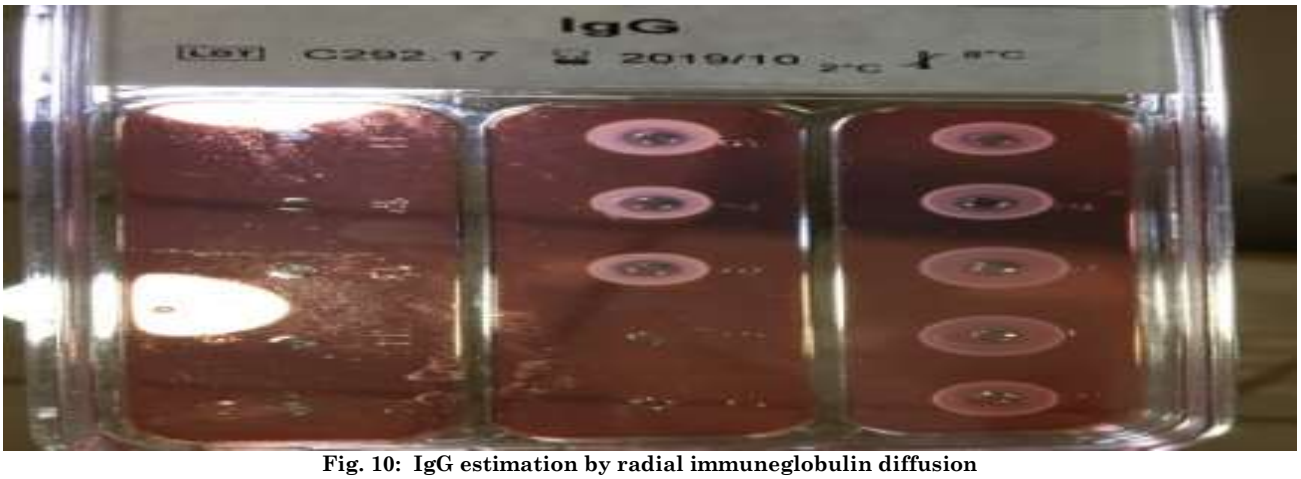

Fig. 10: IgG estimation by radial immuneglobulin diffusion

Challenging of ascending utis by *p. Mirabilis* after Immunization Protocol

Challenging results shown significant different between three rabbits females groups under challenging as shown in Table (4) , group one (negative control) three females rabbits IgM , IgG , and WBCs levels for this 3 parameters with normal value .group two (females challenging) indicated neutralize or balance the levels of IgM and IgG and WBC after immunization protocol when tested the rabbits serum in 1 , 3 , 7 , 14 days and urine culture for this group don't shown found *P.mirabilis* in urine while found *Staphylococcus xylosus* that found as normal flora in animals [32].

As showed in Figure (11). The three group (positive control) shown elevation in 3 parameters IgM , IgG , and WBCs levels when inoculation *P. mirabilis* by ascending urinary tract method , in addition to appearance of *P. mirabilis* bacteria when urine cultured tested that mean induced UTIs in positive control group without immunized post inoculation as shown in Figure 12.

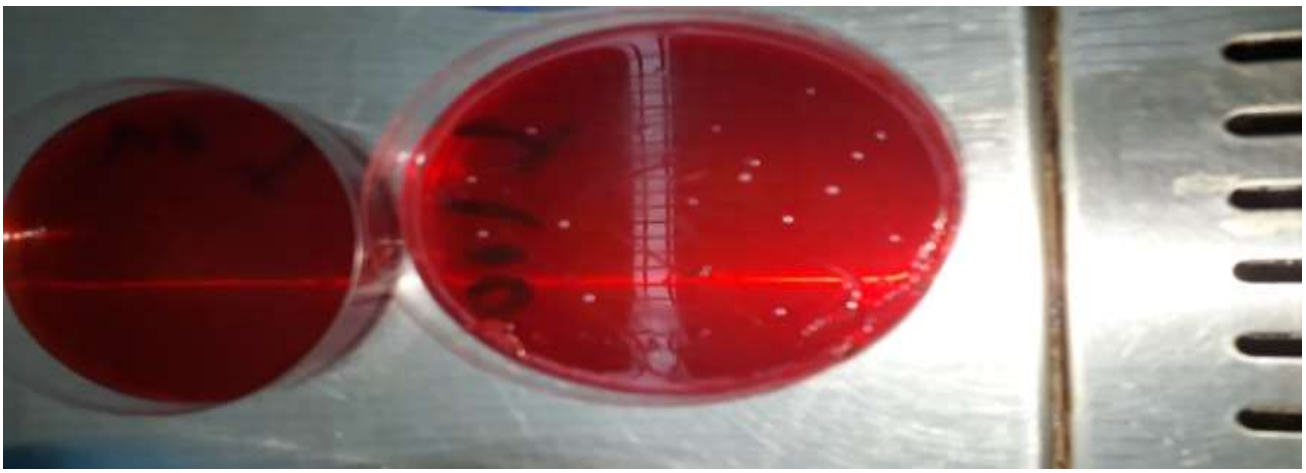

Fig. 11: *Staphylococcus xylosus* isolated from rabbits urine during challenging

Table 4: Compare between difference groups in results of Challenge post Immunization

Groups		Mean ± SE		
		IgG	IgM	WBC
G1: Control	Negative	631 ± 43.44 c	154.73 ± 26.01 c	6.53 ± 0.62 b
G2: Female for Challenge	Post 1 day incubation	702.56 ± 73.83 bc	242.56 ± 31.37 b	6.56 ± 0.26 b
G2: Female for Challenge	Post 3 day incubation	757.33 ± 17.93 bc	207.80 ± 9.39 bc	7.63 ± 1.83 ab
G2: Female for Challenge	Post 7 day incubation	812.33 ± 26.39 b	229.73 ± 35.16 bc	5.43 ± 0.46 b
G2: Female for Challenge	Post 14 day incubation	772.83 ± 42.78 b	215.58 ± 28.53 bc	6.17 ± 0.56 b
G3: Control	Positive	970.76 ± 24.28 a	388.16 ± 2.63 a	9.20 ± 0.15 a
LSD value	---	130.59 *	77.612 *	2.631 *
* (P≤0.05). Means having with the different letters in same column differed significantly				

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G2: Female for Challenge	Post 14 day incubation	772.83 ± 42.78 b	215.58 ± 28.53 bc	6.17 ± 0.56 b
G3: Control	Positive	970.76 ± 24.28 a	388.16 ± 2.63 a	9.20 ± 0.15 a
LSD value	---	130.59 *	77.612 *	2.631 *
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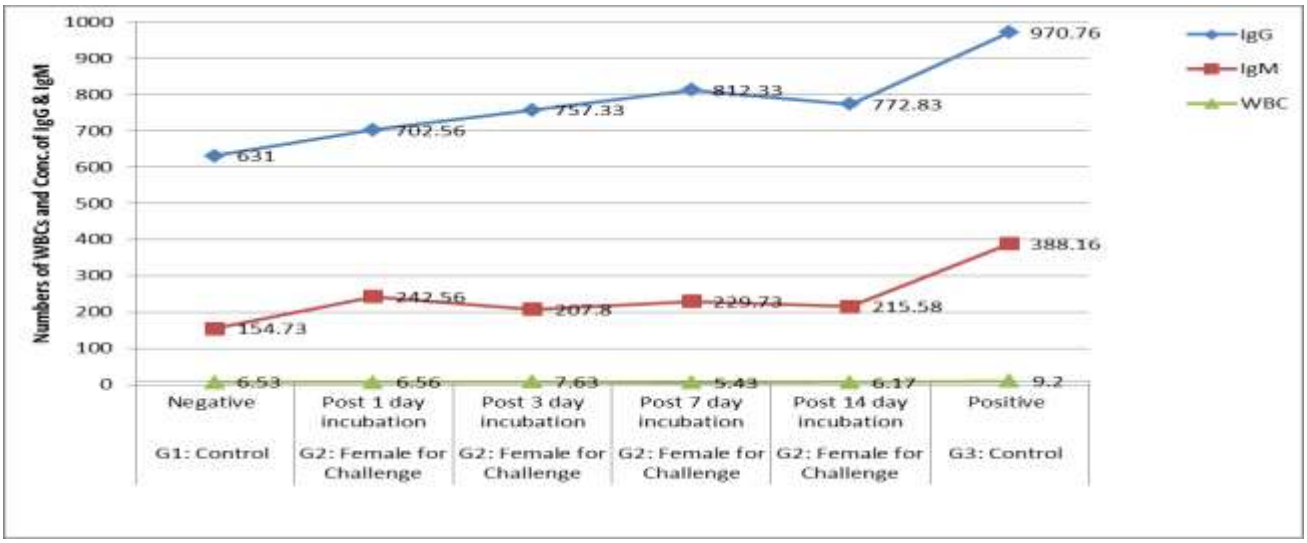

Fig. 12: immune response for IgG, IgM and WBCs after Challenging protocol

Discussion

The high prevalence of *p. mirabilis* specially in catheterized UTI patients and humans hospitalized in the emergency units of hospitals furthermore , the increased frequency of the antibiotics resistance among the antibiotics used for treatment of UTIs is the another challenge that shows to the isolates represent the need for vaccine against UTIs [35].

In last year's many studies addressed and touched vaccine for UTIs, and there are only several animal experiments about evaluation of the vaccine candidates against UTIs caused by *p. mirabilis* that perform the need for more studies to develop effective vaccines against UTIs [36, 37]. Most studies are outstanding with different targets and adjuvants to accelerate the development of vaccines for UTI.

Many different targets used against *p. mirabilis*, MrpH and MrpA fimbrial and MrpA.FliC (fimbria with flagella that induced humoral and cellular responses Th1, Th2) have been the most promising vaccines against *p. mirabilis* [37, 39]. Furthermore, in many previous findings the adjuvant properties of several components of uropathogens are suggested [43, 41]. In the present study flagellin when inoculation in rabbits by two administration methods (subcutaneous and intravenously) immunization protocpol give highly levels of IgG and IgM for 8 weeks and when challenging for ascending UTIs by *p. mirabilis* the raised levels for IgG and IgM neutralized without occur UTIs by *p. mirabilis* after tested in (1 , 3 , 7 , 14) days.

These study result promising vaccine candidates could be valuable in prevention of recurrent UTI caused by *p. mirabilis*. Our study agreements with new study Habibi et al and Asadi et al [39, 42].

Conclusions

In the present study, flagellin protein immunization in rabbit showed that there are raising in IgM and IgG titers. After challenging by ascending urinary tract infection, we showed neutralization of IgG and IgM and white blood cells.

Abbreviations

UTI: urinary tract infection, CAUTI: catheter associated urinary tract infection, *P. mirabilis*: *Proteus mirabilis*.

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