Multidisciplinary

Biochemical test for proteus mirabilis isolatated from urine and wound samples

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Abstract
A total of 150 urine samples were obtained from patients admitted to Al-Hilla Hospital and Al Hussein Training Hospital with urinary tract infections (53 males and 97 females, ages 10-65). Haemolysin, urease, fllagellin, adhesion factors genes (MR/P, MR/K, UCA, and ATF), and beta-lactamase genes were detected in all isolates (100%). Quorum sensing was also researched after Proteus mirabilis was discovered. The bacterial cells aggregated after being added to the supernatant, which was most likely induced by the presence of homoserine lactone.

Keywords: *Proteus mirabilis, Proteus vulgari*, urinary tract infections, molecular vitek system

1. Introduction

One of the most common groups of infections in humans has been affected by either the upper (pyelonephritis) or lower (cystitis) urinary tract and urinary tract infections (UTIs) have been considered. The reservoir from which urinary pathogens exit contains the digestive system. The most important cause of UTIs in all population groups, which accounts for more than 95% of all UTIs, is Enterobacteriaceae. Among the *Escherichia coli* microbes were the most common invasive, producing (90%) UTIs in outpatients and then about 50% in inpatients. One common cause of UTIs in catheter patients and then those with urinary tract abnormalities are *Proteus* species. It has been shown that there is a tendency to the upper urinary tract where it can cause serious kidney damage, acute pyelonephritis, bladder or kidney stones, fever and bacteremia. *E coli* can also be found in soil, water, and then faeces. It was an opportunistic pathogen of humans, when it was known to cause urinary tract infections and then wound infections [1]. Several potential virulence factors, including fimbriae-mediated adherence to the urothelium, urease creation, eukaryotic cell attack, cleavage of IgG and IgA via a proteolytic enzyme, hemolysin production, and then flagella-dependent swarming kinetics, were responsible for the pathogenesis of *P. Mirabilis* [2].

2. Materıals and methods

2.1 Patients

A total of 150 samples (75 urine and 75 wound) were collected from patients with urinary tract infections (53 males and 97 females, aged 10-65 years) who were admitted to Hilla- Hospital and Al Hussein Training Hosptial.

2.2 Preservation of bacterial isolates

Bacterial isolates were maintained on nutrients and agar infusion of the heart 4 °C for monthly rearing on fresh culture media. For a long time in Brain Heart Infusion (BHI) broth added 15% glycerol. Then store at -20 °C for 6-8 months [3].

2.3 Laboratory diagnostics

2.3.1 Bacterial identification assays

According to the diagnostic procedures recommended by (MacFaddin 2000) and (Benson 2001) [7] for *P. mirabilis* associated with the patients under study was isolated and identified as follows. One colony was taken from each baseline positive culture. Its determination depends on the characteristics of morphology (colony size, shape, colour, crowd, odor, transparency, edge, and texture height). Then colonies were examined by gram stain to observe the bacterial cells. Specific biochemical tests were performed to reach the final determination.

2.3.2 Biochemical tests

Catalase test, oxidase test, indole test, methyl-red test, Voges-Proscar test, citrate usage test, gelatinase test, motion test and Kligler iron agar exams were performed to identify *P. mirabilis* isolates from other species as biochemical assays. These examinations were conducted as per sample [4].

2.3.3 Virulence factors assay

2.3.3.1 Test of hemolysin

Most of the hemolytic bacteria were evaluated by culturing on 5% sheep blood agar at 37 °C for 24 h. Blood agar is a differential means of hemolysis, based on the ability to break down hemoglobin or red blood cells, 3 groups of microorganisms can be described; Alpha hemolysis (α-hemolysis) green to light brown halo was seen around colonies, beta hemolysis (ß-hemolysis) clearing area around colonies, and gamma hemolysis (γ-hemolysis) no hemolysis

was observed [5].

2.3.3.2 Analysis of urea

In this assay, we inoculated urea-slants from the bacterial suspension by laying streaks over the entire slanted surface, and the tubes were incubated with lids diluted at 37 °C, and then the color change of the medium was examined after the 16 h incubation period. Urea production was indicated by a medium color change to pink [6].

2.3.3.3 Production of extracellular proteases

M9 media supplemented with 2% agar was used for protease detection. After sterilization in an autoclave and cooling at 50 °C, 0.25 g/L glucose (filtration sterilized) was added, then 1% gelatin was added to the medium. After these media were inoculated with bacterial isolates and incubated at 37°C for 24 h; 3 mL (5%) trichloroacetic acid was added to precipitate the protein. The formation of a transparent area around the colony indicates a positive result [7].

Aggregation inhibition by resveratrol, p-nitrophenylglycerin, lipid acids, urea, sodium azide and ethanol

2.3.3.4 Swarming behavior assay

The migration distance test was performed as previously described [8, 9] Briefly, an overnight culture of *P. mirabilis* (5 µL) was centrally inoculated onto LB-swarmed agar plates (2% w/v) with different concentrations of anti-swarming agents: (0, 10, 20, 30, 40, 50 and 60 µg/mL) of resveratrol, (0, 50, 100, 150 and 200 µg/mL) of p-nitrophenyl glycerol, (0.00125, 0.0025, 0.005 and 0.01 w/v) fatty acids (palmitic acid), (0, 0.5, 1, 1.5, 2, 2.5 and 3 v/v) of urea, (0, 0.01, and of sodium azide, and another plate to which two drops of 90% ethanol are added to its cover. The plates were then incubated at 37 °C and the swarm migration distance was determined by measuring the swarm interfaces of bacterial cells after inoculation.

2.3.3.5 Measuring growth rates

P. mirabilis nocturnal culture was diluted 1:100 in fresh LB broth containing various concentrations of resveratrol (0, 10, 20, 30, 40, 50 and 60 µg/mL) and (0, 50, 100, 150, 200 µg/mL) of P-nitrophenyl glycerol. We also used other high concentrations of resveratrol (0.5, 1, 1.5, 2, 2.5 and 3 mg/mL) to determine the effect on the growth inhibition of *P. mirabilis*. The growth rate was monitored as OD 600 at 1-h intervals [10].

2.3.3.6 Measurement of cell length

Cell length measurement was performed as described in (Liaw *et al.* 2004, Tegos *et al.* 2006). Briefly, 150 µL of fixed-phase LB cultures were spread onto LB agar plates without or with appropriate resveratrol and p-nitrophenylglycerol, and then incubated at 37 °C for different times. After incubation, cells were harvested from the entire surface of the agar plates by washing in 5 mL phosphate-buffered saline (PBS). Bacterial cells were fixed and exposed to gram stain examined by optical microscopy at 1000X magnification, and converted into digital images using a digital camera. The lengths of 100 cells in each sample were determined, and the average was calculated.

2.4 Quorum Sensing Detection in *P. mirabilis*

2.4.1 Procedure

Quorum sensitivity was detected according to (Abdel-Latif 2012, Sabri 2011).

2.4.2 Chemical detection of homoserine lactone

Homoserine production was examined by separating the filter from the culture media, and then the supernatant was washed with KCN-free LB. After 24 h the media containing hydrolyzed homocysteine was inoculated by *P.mirabilis* again for 24 h and then the trademarked assay was used to detect methionine or homocysteine synthesis by converting homocysteine to homocysteine [11].

2.4.3 Brand test

The interaction of sodium nitroprusside with sulfhydryl components (eg cysteine, homocysteine) results in pink or red-purple combined products [12].

2.5 Statistical analysis

Frequencies (number of cases) and relative frequencies (percentages) was described the data statistically. All statistical calculations were performed using Microsoft Excel 2013 (Microsoft Corporation, New York, USA) or Quick Calcs Online for Scientists (Chartboard Software Inc., USA).

3. Results and discussion

3.1 Isolate volatile common

Obtaining a total of 150 amples (75 urine and 75 wound) from patients (of both sexes) with urinary tract infection, other bacteria (76.7%) were isolated from urine samples of patients who were used with catheters and non-catheters, followed by *P. vulgaris* (13.3%), *P. mirabilis* (7.3%) and *P. penneri* (2.7%). as shown in Table 1.

Table 1: Distribution of bacterial isolates from specimens

Noun isolates	Number isolates		% Singles
	Resources isolates		
	Urine	Wounds	
P. vulgaris	20	-	20 (13.3%)
P. mirabilis	11	-	11 (7.3%)
P. penneri	4	-	4 (2.7%)
bacteria Other	40	75	115 (76.7%)
Sum	75	75	150

However, there are no isolated varicose types of catheter patients. The reason for this result may be that other bacteria such as *E. coli* cause more than 75% of community-acquired UTIs in all age groups. *S. saprophyticus* accounts for about 10%. In hospitalized patients, *E. coli* accounts for about 50% of cases. Gram-negative species *Klebsiella spp*., *Proteus spp*., *Enterobacter spp.* and *Serratia spp*. account for about 40%,

while Gram-negative bacterial cocci *E. faecalis*, *S. saprophyticus*, and *S. aureus* account for the remainder (Steven and Maxine 2009, Stewart 2010). Our study is identical to that obtained by Juma *et al.* (2011), Tai (2002), and Laftaa (2001) who isolated *Proteus spp*. from urine samples at a rate of 33.33%, 38% and 37.33% respectively. Al-Grawy (1999) isolated *Proteus* from urine in only 6% and their study is not agree with our results.

3.1.1 Urea production

Urease is the hallmark of infection with *Proteus spp*. It is considered as one of the most important factors of virulence in *Proteus*. Therefore, in this study, specific primers to amplify the urease gene (ureABC) from *P. mirabilis* isolates by PCR. The results showed that all isolates gave the ureABC amplicon product with a size of 489 bp where the urease gene and hly gene were detected at the same time because they had the same conditions in PCR. Confirming these results further by detecting urease in those isolates outwardly, as it was found that all (100%) gave a positive result for extracellular urease. Our study is in agreement with the findings of Mobley and Chippendle (1990) [14, 15], who found (100%) of *Proteus* isolates showed its potency produce urease. It produced a high amount of urease compared to other bacteria. The main complicating factor associated with UTIs caused by *Proteus* species is urolithiasis, or the formation of stones within the urinary tract. Producing the stone is about the action of the bacterial enzyme urease. As a result of increased pH, precipitation naturally soluble minerals, namely magnesium, ammonium and calcium phosphate, which lead to the formation of struvite and apatite crystals [16].

3.1.2 Flagellin

We used specific primers for amplification of the fla gene by PCR and this gene has been detected with an amplicon size of 247 bp in all. Flagellin is encoded by flaC, and the flaD gene encodes the flagellated filament covering protein (Belas 1994). Studies indicate that the major flagellin protein of *Proteus* undergoes antigenic variation through homologous recombination as three copies of the flagellin specific gene (flaA, flaB, flaC) located on the volatile genome with only one copy being actively expressed [16]. One of the most surprising results is that all the genes associated with the skin located together in one place, which is very unusual. Another interesting feature is a transcript of multiple genes encoding flagellin, flaA and flaB, which are located in close proximity to each other. Normally, the flaA allele is expressed while flaB is silent. However, these genes can combine, resulting in the formation of antigenically distinct flagella (Manos and Bellas 2004). Given that flagellin is a strong antigen, it has been hypothesized that this recombination could contribute to immune evasion during infection. Therefore, flagellar gene rearrangement is a mechanism of host immune evasion by *Proteus* and is highly relevant to *Proteus* infection since flagella are highly immunogenic [13].

3.1.3 Swarming behaviour

In this study, all *P. mirabilis* isolates showed swarming movement as 100%, when grown on agar plates. The results of the current study are consistent with those obtained by Iwalokun *et al.* (2004), Al-Baghdadi *et al.* (2009) [4] and Friday *et al.* (2011) who found all *Proteus* isolates showed swarming activity as 100%.

3.1.3.1 Inhibition of *P. mirabilis* aggregation by resveratrol

Crowding behavior of *P. mirabilis* includes on LB agar on different. The swarming behavior was noticeable. It was inhibited at concentrations as low as 20 µg/mL and completely inhibited at 60 µg/mL (Fig. 1). The inhibitory effect of resveratrol on aggregation may arise from a toxic effect on bacteria. To test this possibility, a nocturnal culture of *P. mirabilis* was inoculated in LB broth containing different concentrations of resveratrol and the bacterial growth rate was monitored as shown in Figure 1. The growth rate of *P. mirabilis* was slightly inhibited. But not significantly because it grew in all tubes little care of whether or not resveratrol was present, indicating that resveratrol could inhibit aggregation but not growth of *P. Mirabilis.*

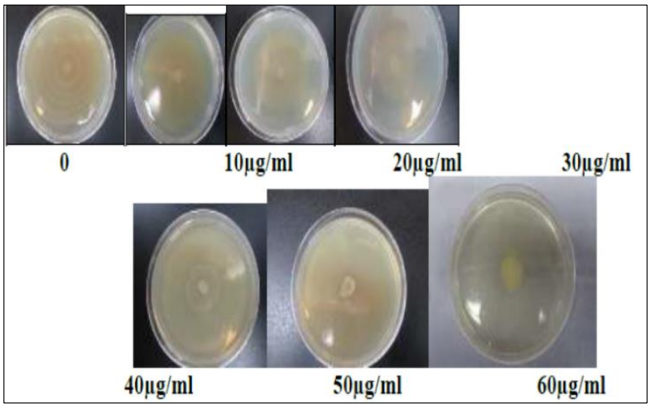

Fig 1: Halo images of swarming plates containing different concentrations of resveratrol (0, 10, 20, 30, 40, 50, 60 µg/mL) at 16 h immunization

Monitoring of cell morphology after inoculation of a nocturnal culture of *P. mirabilis* on LB plates containing different concentrations of resveratrol. As shown in Figure 2, in the absence of resveratrol, swarmed cells are longer than bacterial cells in the presence of resveratrol at a concentration of 60 µg/mL, indicating that swarm differentiation was inhibited. Inhibition of differentiation began to be observed at a concentration of 20 µg/mL resveratrol. Very few elongated swarming cells were observed at a resveratrol concentration of 40 µg/mL. With an increase in the concentration of resveratrol to 60 µg/mL, only short vegetative cells were observed. These results indicate that stromal differentiation of *P. mirabilis* was indeed inhibited by high concentrations of resveratrol. To study whether production of virulence factors (hemolysin and urease) was also affected by resveratrol, hemolysin and urease production were determined in *P. vulgaris* taken from LB agar plates containing different concentrations of resveratrol.

The emergence of bacterial strains make up that show resistance to various antibiotics is a major threat to public health. As a consequence, there is a renewed interest in antibacterial targets which, by attenuating virulence, disrupt the ability of pathogenic bacteria to cause infection (Hinkelmann and Kempthorne 2008). The purpose of this study was to find out the effect of plant extracts (Resveratrol) against the urinary pathogenic *P. mirabilis*.

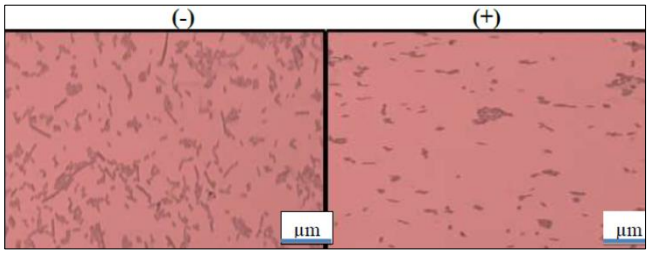

Fig 2: Microscopic observation of *P. mirabilis* cells isolated from LB plates without resveratrol (−) with resveratrol 60 µg/mL (+) shown at 1000X magnification

In this study, we found that resveratrol has the ability to significantly inhibit aggregation of *P. mirabilis* at a concentration as low as 20 µg/mL and completely inhibit aggregation at 60 µg/mL (Figure 2). Also, it had the ability to suppress the production of virulence factors (hemolysin and urease) at concentrations of 50 and 60 µg/mL (Figure 4) but it did not significantly affect bacterial growth at concentrations up to 60 µg/mL (Figure 2).

Resveratrol affects the viability of *P. mirabilis* at a concentration of 3 mg/mL (data not shown). This means that resveratrol can only inhibit swarming and virulence factor production without significantly inhibiting the growth of *P. mirabilis*. Based on this result, we concluded that the aggregating ability of *P. mirabilis* correlated with its ability to express virulence factors and these results were similar to those reported by Allison *et al.* (1992) [2] and Wang *et al.* (2010). Whereas, troop differentiation of *P. mirabilis* and expression of virulence factors, such as urease, hemolysin and proteases, have been shown to be coordinately regulated in *P. vulgaris*.

3.1.3.2 Inhibition of *P. mirabilis* swarming by PNPG

In this study, we found that PNPG has the ability to inhibit crowd migration of *P. mirabilis* also in a dose-dependent manner (Figure 3). Crowding behavior was significantly inhibited at concentrations of 50 µg/mL and completely blocked at 150 and 200 µg/mL (Figure 3). It tested the inhibitory effect of PNPG on bacterial growth in addition to inoculation of a nocturnal farm of *P. mirabilis* in LB contains on different concentrations of PNPG, the growth rate of bacteria was monitored as shown in Figure 3. The growth rate of *P. mirabilis* was not inhibited by PNPG. At 16 h after inoculation, bacteria grew approximately to similar densities, regardless of the presence of PNPG. We concluded that the inhibitory effect of PNPG on crowding is not likely to be due to the inhibition of cell growth.

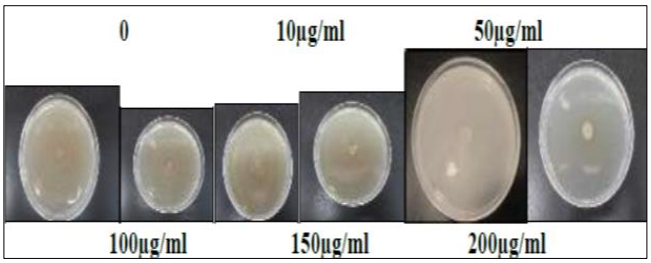

Fig 3: Histograms of swarming plates containing different concentrations of PNPG (0, 10, 50, 100, 150, and 200 µg/mL) at 18 h after inoculation Inhibition of cell length and virulence factor production by *P. mirabilis*

Cell morphology observed after inoculation of an overnight culture from *P. mirabilis* on LB swarm plates containing different concentrations of PNPG. As shown in Figure 4, in the absence of PNPG, swarmed cells were longer than bacterial cells in the presence of PNPG at a concentration of 200 µg/mL, indicating that swarm differentiation was suppressed. Differentiation inhibition should be observed at a PNPG concentration of 50 µg/mL. With increasing concentration of PNPG to 150 and 200 µg/mL, only shortened cells were observed. These results indicate that crowd differentiation of *P. mirabilis* was highly inhibited to find out whether the production of virulence factors (hemolysin and urease) was also affected by PNPG, the production of hemolysin and urease at *P. mirabilis* obtained from LB agar plates containing different species. As shown in Figure 5, the file did not product of virulence factors is significantly affected in PNPG concentrations (0-100 µg/mL) for urease and (0-50 µg/mL) for hemolysin but inhibited in the presence of increasing concentrations (150 and the 200 µg/mL).

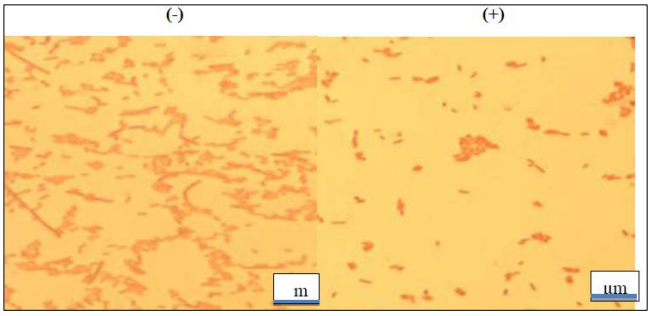

Fig 4: Microscopic observation of *P. mirabilis* isolated from LB plates without PNPG (−) and with 200 µg/mL PNPG (+) is shown at 1000X magnification

It is well established that motility and virulence factor expression are coordinately regulated in several pathogens including *P. mirabilis* and *Pseudomonas aeruginosa*, which is often implicated in persistent UTIs (Ronald 2003, Wang 2006, Nashkar *et al.* 2011). PNPG effectively prevents swarming and produce the virulence factor of *P. mirabilis* (Liaw *et al.* 2001). The mechanism which is underlying inhibition is unclear. Also, it the ability to suppress the production of the virulence factor; hemolysin. It was inhibition at concentrations (100, 150 and 200 µg/mL) while urease suppressed at (150 and 200 µg/mL) (Figure 5). But

didn't effect significantly inhibits bacterial growth even at high concentrations (200 µg/mL) (Figure. 5). This means that PNPG has the ability to inhibit swarming and production of virulence factor without significant inhibition of *P. mirabilis* growth. Accordingly, we concluded that the linked *P. mirabilis* ability to express virulence factors and these results.

It is now known that several bacterial functions including aggregation, biofilm formation and secretion of virulence factors important for successful and recurrent bacterial infection are related to cell density-mediated gene expression termed QS (Whitehead 2001, Fuqua and Greenberg 2002, Krishnan *et al*. 2012). Regulation of multicellular bacterial behavior such as swarming is a complex process. In *Proteus*, several genes involved in flagellar locomotion and QS were found to play a regulatory role in this process (Wei *et al.* 2004). In this study, we showed that PNPG has a negative regulatory effect on swarming behaviour, since bacterial motility is essential for swarming behaviour, it is possible that PNPG inhibits motility and thus swarming. Also, it has been suggested that disruption of the pathogen's QS system can lead to a significant reduction in virulence factor production (Schauder and Bassler 2001, Lyon and Muir 2003, Mihalik *et al*. 2008). Therefore, the possible mechanism by which PNPG could inhibit *P. mirabilis* mobilization and virulence factor expression may be due to its action as a bacterial QS inhibitory compound. Some articles have reported that PNPG may have an inhibitory effect on the gene RsbA (the regulator of swarming behavior) that regulates swarming of proteins and leads to expression of the common virulence factor and aggregation may be due to its action as a bacterial quorum inhibitory complex. Some articles have reported that PNPG may have an inhibitory effect on the gene RsbA (the regulator of swarming behavior) that regulates swarming of proteins and leads to expression of the common virulence factor and aggregation may be due to its action as a bacterial quorum inhibitory complex.

3.1.3.3 Regulation of *P. mirabilis* swarming with fatty acids

In this study, we tested the effect of a series of fatty acids including lauric acid, myristic acid, palmitic acid, stearic acid, and oleic acid, on *P. mirabilis* moles on LB (2%) agar plates at 37 °C. The concentration of these externally added fatty acids was 0.01% (µg/mL). We found that while oleic acid is aggregation-promoter, lauric acid, myristic acid, palmitic acid, and stearic acid significantly inhibit aggregation (Figure. 5).

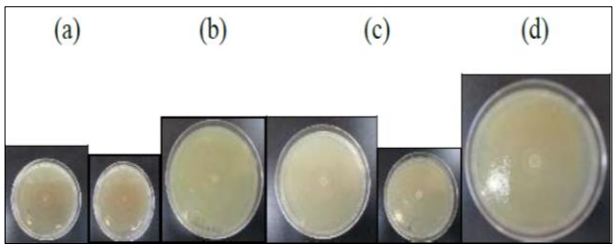

Fig 5: Halo images of swarmed plates containing different fatty acids. Representative panels are depicted in A. a, containing no fatty acids; b, oleic acid. c, stearic acid. d, palmitic acid

This inhibitory effect is dose-dependent, myristic acid at 0.00125%, 0.0025%, 0.005% and 0.01% (µg/mL) were added to LB swarming agar plates, followed by swarming assay. As shown in Figure 6 swarming of *P. mirabilis* was inhibited by myristic acid in a dose-dependent manner. A similar dose-dependent inhibitory effect on the crowds was also observed for lauric acid, palmitic acid and stearic acid (data not shown). Our results were similar to those reported by Liaw *et al.* (2004), Lai *et al.* (2005), and Inoue *et al.* (2008), in which some SCFAs, including lauric, myristic, palmitic, and stearic acids, have been reported to have colony-suppressed spread over swarm panels in *P. mirabilis*, *Serratia marcescens*, and *P. aeruginosa*. There is sample evidence to suggest that fatty acids or their derivatives may be involved in the regulation of gene expression to modulate swarming and virulence (Liaw *et al.* 2004).

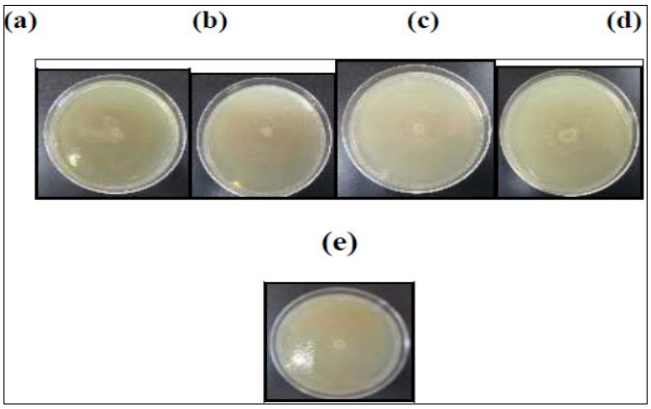

Fig 6: Halo images of swarm plates containing different concentrations from myristic acid. Representative panels are depicted in A. a, containing no fatty acids; b, 0.00125%; c, 0.0025%; d, 0.005% and e, 0.01% (w/v)

Swarm cells and swarming behavior differentiation are the result of complex sensory transmission and global control mechanisms. *Proteus* requires swarming sensing and integration of a variety of environmental, cell-to-cell, and intracellular signals and involves the regulated expression of gene networks that lead to morphological and physiological changes (Liaw *et al.* 2004). Although a great deal of information has accumulated regarding the mechanisms of swarming in *Proteus*, the signals that regulate swarming and signal transduction pathways are still poorly understood. In this study, we present that fatty acids act as environmental indicators to regulate *P. mirabilis* reproduction. Specifically, while oleic acid enhanced crowding, some saturated fatty acids, such as myristic acid, lauric acid, palmitic acid, and stearic acid, prevented crowding. The effect of fatty acids on *Proteus* swarm may be due to the presence of the gene RsbA (a regulator of swarming behavior), which may be a phosphoteric transporter containing the two-component bacterial signaling system, that could act as an inhibitor of swarm differentiation in *Proteus* (Takeda *et al*. 2001). It is possible that, upon uptake, these fatty acids or their derivatives act as signals to regulate aggregation through a RsbA-dependent or RsbA-independent pathway. RsbA, is

homologous to the transmembrane sensor histidine kinases of the two-component family of regulatory proteins, suggesting that RsbA may function as a sensor of the environmental conditions required to initiate swarm migration (Belas *et al.* 1998). Therefore, our data demonstrate that fatty acids can act as extracellular signals to regulate swarming in *P. mirabilis*.

4. Conclusion and recommendation

Conclusions

Proteus was prevalent among urological patients. It was confirmed that all *P. mirabilis* isolates have the ability to possess more than one virulence gene such as hemolysin, extracellular proteases, urease, adhesion factors, crowding activity and multidrug resistance genes. It was confirmed that there are many factors that affect the activity of crowding such as resveratrol, PNPG, fatty acids, urea and ethanol. Our results from this study indicate that resveratrol and PNPG have the potential to be an antimicrobial agent against *P. mirabilis* infection.

References

1. Abbott SL. *Klebsiella*, *Enterobacter*, *Citrobacter*, *Serratia*, *Plesiomonas*, and other *Enterobacteriaceae*, In Manual of Clinical Microbiology. Murray, P. R., Abraham, E. J., Shin, S., J. and Malaviya, R. (eds). ASM Press, 2007, p698-711.
2. Allison C, Lai HC, Hughes C. Co-ordinate expression of virulence genes during swarm-cell differentiation and population migration of *Proteus mirabilis*. Mol Microbiol. 1992;6:1583-1591.
3. Abdellatif M. Differential expression of microRNAs in different disease states. Circulation Research. 2012;110(4):638-650.
4. Al-Baghdadi O, Ewies AAA. Topical estrogen therapy in the management of postmenopausal vaginal atrophy: an up-to-date overview. Climacteric. 2009;12(2):91-105.
5. Aneas MA, Portaro FC, Lebrun I, Juliano L, Palma MS, Fernandes BL. *ZapA*, a possible virulence factor from *Proteus mirabilis* exhibits broad protease substrate specificity. Braz. J Med. Biol. Res. 2001;34:1397-1403.
6. Alamuri P, Eaton KA, Himpsl SD, Smith SN, Mobley HLT. Vaccination with *Proteus* toxic agglutinin, a hemolysinindependent cytotoxin *in vivo*, protects against *Proteus mirabilis* urinary tract infection. Infect Immun. 2009;77:632-641.
7. Benson HJ. Microbiological application, laboratory manual in general microbiology. 8th ed. W.b. Mcgraw Hill, 2001.
8. Belas R, Manos J, Suvanasuthi R. *Proteus mirabilis* ZapA metalloprotease degrades a broad spectrum of substrates, including antimicrobial peptides. Infect Immun. 2004;72(9):5159-5167.
9. Cai HY, Archambault M, Gyles CL, Prescott JF. Molecular genetic methods in veterinary clinical bacteriology laboratory: current usage and future applications. Animal Health Res Rev. 2003;4:73-93.
10. Colly JG, Fraser AG, Marmion BP, Simmon A. Makic and McCartncy medical microbiology. 14th. the Churchill living stone. Inc. USA, 1996.
11. Chalmeau J, Monina N, Shin J, Vieu C, Noireaux V. α-Hemolysin pore formation into a supported phospholipid bilayer using cell-free expression. Biochim. Biophys. Acta. 2011;1808:271-278.
12. Coker C, Poore A, Li X, Mobley HLT. Pathogenesis of *Proteus mirabilis* urinary tract infections. Microbes Infect. 2000;2:1497-1505.
13. Dolchak, Kirk. Molecular cloning of *Proteus mirabilis* uroepithelial cell adherence (*uca*) genes. Infect. Immun. 2005;63:2082-2086.
14. Kim BN, Kim NJ, Kim MN, Kim YS, Woo JH, Ryu J. Bacteraemia due to tribe *Proteeae*: a review of 132 cases during a decade (1991-2000). Scandinavian Journal of Infectious Diseases. 2003;35(2):98-103.
15. Krishnan T, Yin WF, Chan KG. Inhibition of Quorum sensing controlled virulence factor production in *Pseudomonas aeruginosa* PAO1 by ayurveda spice clove (*Syzygium aromati*cum) bud extract. Sensors. 2012;12(4):4016-4030.
16. Griffith DP, Musher DM, Itin C. Urease. The primary cause of infection-induced urinary stones. Invest. Urol. 1976;13(5):346-50.