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The impact some of nutrients on swarming phenomenon and detection the responsible gene *RsbA* in clinical isolates of *Proteus mirabilis*

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ABSTRACT

The effect of ten different chemical compounds (comprised of carbohydrates, nutrients and nanoparticles) were tested against the swarming phenomenon of clinical isolates that belong to Proteus spp. The carbohydrates used in the experiment were starch, sucrose, lactose and glucose. The largest swarming diameter of the test culture occurred in the presence of glucose due to its stimulatory effect. However, sucrose and lactose showed an inhibitory effect on swarming of the test culture. On the other hand, starch had different effects according to its concentration; it showed an inhibitory effect at 1% and 2%, while it stimulated the swarming at 5 % concentration. Comparatively, when test culture grown on MacConkey agar, swarming did not occur due to the inhibitory role of bile salt and crystal violate. The yeast extract and indomie additives were stimulators for swarming with increased concentration, while Peptone showed moderate effects and red pepper powder had an inhibitory effect on swarming. This study was aimed to isolate and investigate the effect of some chemical and nutrient compounds on swarming phenomenon of P. mirabilis that allows the microorganism to invade human urinary tract and cause infection. We can concluded that Red pepper as natural alternative of antibiotics could be employed in the treatment of patients diagnosed with UTI caused by P. mirabilis due to its inhibitory role on the swarming action. However, consuming indomie noodles with its additives may have a stimulatory effect on swarming which may allow P. mirabilis to reach and colonize other sites of urinary tract and cause infection.

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INTRODUCTION

Proteus mirabilis is a clinically important pathogen of the urinary tract and in patients with indwelling urinary catheters (Warren *et al.*, 1982). These patients often develop bacteriuria, cystitis, kidney and bladder stones, catheter obstruction (due to stone encrustation), and acute pyelonephritis (Burall *et al.*, 2004). This bacterium has the ability to express many virulence factors, including urease, metalloprotease, hemolysin, Lipopolysaccharide, Outer-membrane proteins (OMPs), and lecithinase (Dattelbaum *et al.*, 2003; Al-Isawi, 2011). In order to invade human urothelial cells, *P. Mirabilis*

needs to coordinate the regulation of these virulence factors with swarming motility (Lai *et al.*, 2001). The swarming phenomenon helps to distinguishes *Proteus* ssp. from other members of the Enterobacteriaceae family (Mobley and Belas, 1995). The presence of flagella on the surface of pathogenic and opportunistic bacteria have been thought to facilitate the colonization and dissemination from the initial site (Liaw, 2003), description of swarming- faulty or defective *Proteus* transposon mutants has indicated that a fundamental several proteins are share in regulation of swarming, such as FlhD2C2, FlhA, Umo, Lrp, RsbA, RsmA, SpeB, and others, these are implicated in regulation of swarming and virulence factors expression (Fraser and Hughes, 1999; Hay *et al.*, 1997). Among the mentioned above regulator proteins, RsbA containing phosphor-transmitter of the bacterial two- combination signaling system (Takeda *et al.*, 2001; Liaw, 2003), it works as negative regulator of swarming segregation and virulence factor expression in *P. mirabilis* (Liaw *et al.*, 2005), but protein rsmA is consider a universal regulator protein, it's widely distributed among different type of bacteria (Liaw, 2003). The combination of the RsbA- and RsmA-dependent pathways with other signal pathways to set swarming and other virulence factors expression is not known currently. *P. mirabilis* swarming calls for the sensing and blend of a different environmental, cell-to-cell, and intracellular signals. These signs may involve those transmitted by high population density, peptides and amino acids, surface contact, and intracellular cations (Fraser and Hughes, 1999; Lai *et al.*, 1998). Induced of the flhDC operon begin swarms cell differentiation, which includes the evolution of characteristic features like cell hyper-flagellation, elongation, and multi-nucleation (Eberl *et al.*, 1996). The major surface molecule of Gram-negative bacteria interacts with the host and, depending on the dose, induces an inflammatory response (Brandenburg and Wiese, 2004). The ability to invade cultured human urinary epithelial cells is associated with differentiated swarmer cells and not with undifferentiated swimmer cells (Allison *et al.*, 1994). Many authors observed different materials had an effect on the swarming phenomenon. Twenty amino acids were investigated on the swarming phenomenon of clinical *Proteus* strains; serine, glutamine, and methionine were found to enhance swarming, while the other 17 amino acids had shown inhibitory effect (Iwalokun and Akinwumi, 2002). However, It was found that the expression of numerous virulence factors such as hemolysis, urease, protease, flagellin, and swarming of *P. mirabilis* curb when treated by (PNPG) P-nitrophenylglycerol (Liaw *et al.*, 2000). Ghaidaa and his colleagues monitored the impact of resveratrol on *Proteus Vulgaris*; resveratrol (3, 5, 4'-trihydroxy-Trans-stilbene) is a natural phenol produced of the roots of the Japanese Knotweed, it has antioxidant and anti-inflammatory activities and has effectiveness against *P. Vulgaris*, an important pathogenic bacteria that cause urinary tract infection. It showed an inhibitory effect on the production of hemolysin and urease associated with inhibition of swarming (Ghaidaa *et al.*, 2014).

MATERIALS AND METHODS

Bacterial strain, culture conditions, and identification

Fifteen clinical *Proteus* strains were obtained from the Central Public Health Laboratory-quality control unit and from post-graduate students of the Department of Biology, College of Science, Mustansiryah University. These isolates were cultured on brain heart infusion broth at 37°C and on MacConkey agar plate (Oxoid). Pale non-lactose fermenter with fishy odor colonies were selected and streaked on blood agar plates (blood agar base –Oxoid supplemented with 5% of human blood) to observe swarming motility. These isolates were identified by biochemical tests (Atlas and Snyder, 2006). A Vitek-2 compact system was employed for the confirmation of identification. Bacterial strains were maintained on deep Nutrient agar slant (Oxoid) for 8-10 weeks with periodic subculture and nutrient broth (Oxoid) with 35 % glycerol at -20°C. The molecular manner was employed to re-confirmed the diagnosis of the slant cultured by Thermo cycle PCR, this technique needs particular primers for 16srRNA gene, it's include sequence, R 5-' TCTTTTGCAACCCACTCCCAT -3′ , F 5-'CACGCAGGCGGTCAATTAAG-3′ and; *rsbA* gene F 5-' CTATACCTACCGCACCATGT -3′ and R 5-' GAAGTCCCATCCGTTGATAC -3′ In suitable PCR tube, it was contain 12 μl of master mix; 2 μl of template DNA mixed with 1 μl each set of primers, the rest of total volume was achieved to 25 μl by sterile deionized D.W, the mixture vortexing well. The PCR programmed for the 16srRNA gene were involve, primary denaturation step, denaturation, annealing, and flowed by extension, at 94^{o}C for 4minutes, 25-30 cycles at 94 ^{o}C for 45 seconds, 58 ^{o}C for 25 seconds and then 72 ^{o}C for 60 seconds respectively. The final extension step was done at 72 ^{o}C for about 8-10 minutes; finely, the reaction mixture was holded at 4 ^{o}C until used (Mokhtar *et al.*, 2016).

Compounds

Ten chemical compounds in this study comprising carbohydrates (starch, sucrose, lactose, and glu-

Table 1: Percentage and distribution of routine antibiotics disc against *Proteus mirabilis*

Antibiotic item	Range of inhibition zone diameter mm	Susceptibility
Ciprofloxacin (CIP)	19-23	100 %Sensitive
Imipenem (IPM)	20-21	100 %Sensitive
Amoxicillin +Clavulanic (AUG)	19-24	100 %Sensitive
Gentamycin (GEN)	20-25	100 %Sensitive
Ceftriaxone	20-23	100 %Sensitive
Cefixime (CEF)	19-25	100 %Sensitive
Norfloxacin	21-25	100 %Sensitive
Tetracycline	20-24	60 % Sensitive
	10-13	20 % Resistance
	15-17	20 % Intermediate
Amikacin	21-25	60 % Sensitive
	12-14	40 Resistance

cose), nutrients (peptone, yeast extract, indomie additives, and red pepper), Antibiotic discs were obtained from Oxoid Ltd. But, the indomie additives and red pepper obtained from local markets in Baghdad.

Antibiotic susceptibility test

All the antibiotics disks in this study include Imipenem 10μg, Tetracycline 30μg, Amikacin 30μg, Norfloxacin, Cefixime 5μg, ceftriaxone, Gentamycin 10μg, Amoxicillin Clavulanic acid 30 μg, and Ciprofloxacin 5μg. The antibiotic sensitivity report was performed according to Kirby - Bauer disc diffusion fashion on Mueller-Hinton agar (Morello *et al.*, 2006). It Briefly, the investigated isolates allowed to multiplication for overnight at 37ºC in BHI broth referred to 0.5 McFarland turbidity standard equal to 10^8 cfu/ml (Mcfarland, 1907) , the MH agar plates were fully spreading with 0.1ml of growth suspension and then fixed antibiotics disks on the surface. The applied plats left for 10-15 minutes and then incubated for 24 h at 37ºC as a standard cultural condition. The fixed antibiotics were classified as sensitive (S), Intermediate (I), or resistant (R) according to diameters of halo zone in millimeters (mm) around the individual disk, the results were compared with clarifying list of (CLSI, 2018).

Anti-swarming effect

The aqueous solutions of the Carbohydrates and nutrients were prepared at different gradient concentration (1, 2, 3, 4 and 5 %) by dissolving suitable required grams in total volume (10 mL) of distilled water and heated at boiling degree 100 °C., then distributed in separated test tubes. Glucose, Sucrose, Starch, Lactose were sterilized by filtration, while Peptone, Yeast extract, indomie additives, red pepper powders were sterilized by autoclave. A tube contains 10 mL distilled water only was considered as control. All above tubes were inoculated with 100 μL of overnight culture of *P. mirabilis* and incubated at 37C°for 24 hrs, and then, 5 μL of the culture vaccinated on the center of blood agar plates and incubated for 24 hrs at 37C° The outer diameter of swarming (movement waves) zone from the start point of inoculation was recorded in millimeter and compared with control. The chemical compounds that enhanced a colony growth region wider than the control was considered and listed as a stimulating compound, while it was assorted as inhibitory at any rate the outer diameter was miner than the control point (Laftaah, 2012).

RESULTS AND DISCUSSION

The clinical isolates in this study were diagnosed by vitak 2 system, and the results of the amplification 16sRNA gene were shown clear band with size 857 bp on gel agrose electrophoresis, which affirms these isolates Proteus spp (Figure 1). All isolates were harbor RsbA gene that related with swarming (Figure 2). However, differ in their ability to express of swarming ranged (17 -40mm) from initiate spot site. The effects of various compounds on swarming of investigated Proteus spp are shown in (Figure 3 and Figure 4). The highest swarming occurred in the participation of glucose. Comparatively, no swarming occurred on MacConkey agar due to the inhibitory role of bile salt and crystal violate on the phenomenon. While sucrose and lactose showed an inhibitory effect on swarming. Starch showed varied behavior, it developed a partial inhibitory effect at 1% and 2%, while it stimulated the swarming at 4% and 5% concentration. Yeast extract and indomie additives induced swarming with increased

concentration. However, Peptone and red pepper powder inhibited the phenomenon. The red pepper was considered as an adequate antibacterial agent because of its remarkable ability to arrest swarming with increased concentrations. The antibiotic sensitivity story clarified that all isolates of Proteus spp. were shown a sensitive pattern (100 %) to Ciprofloxacin, Imipenem, Amoxicillin +Clavulanic, Gentamycin, Cefixime, Ceftriaxone, and Norfloxacin. While appeared sensitivity (60%) toward Tetracycline and (60%) go for Amikacin, respectively (Table 1).

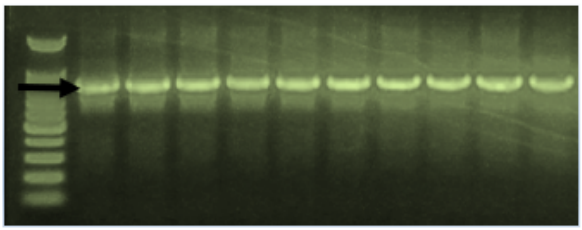

Figure 1: Agrose Gel electrophoresis (1%), TBE buffer (1x) stained with safety dye M: DNA ladder (100 bp); pointed bands1-10 shown positive for 16SrRNA gene (857 bp size)

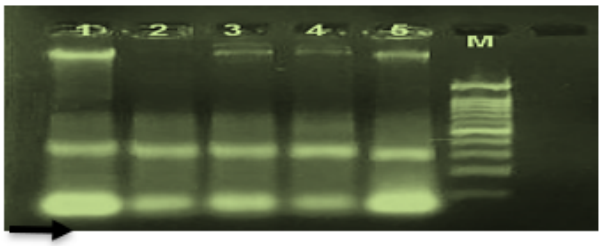

Figure 2: Agrose Gel electrophoresis (1%), TBE buffer (1x)stained with safety dye M: DNA ladder (100 bp); pointed bands 1-10 shown positive RsbA gene (310 bp size)

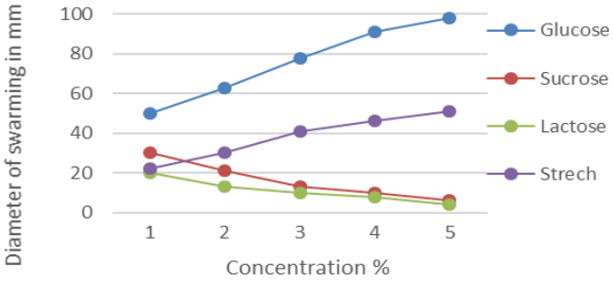

Figure 3: Effect of carbohydrates on swarming diameter

The clinical isolates in this study were diagnosed by vitak 2 system and molecular technique. Many authors have used 16srRNA gene in their reports

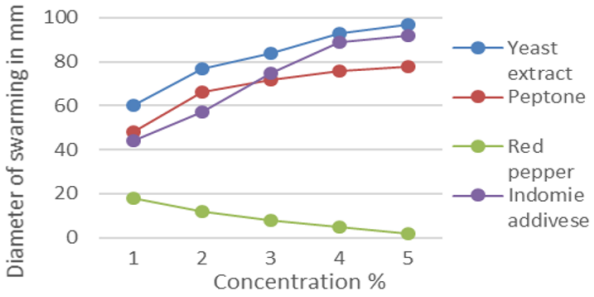

Figure 4: Effect of diffrent Nutrients on swarming diameter

for confirmation of *P. mirabilis* (Kadhum and Khalaf, 2009; Adnanp *et al.*, 2014) the molecular technique for bacterial identification has preference vs. ordinary and commune biochemical tests for many reasons: it is highly protected and conserved in types or among species in the same genus, actually it present in all microorganisms as structural gene because it has not changed (Patel, 2001). Also, the PCR technique is easy, reliably, and accurate, and rapid procedure to diagnosis (Barbut *et al.*, 2011). In this study all isolates were harbor *RsbA* gene that related with bacterial movement (Figure 2) and showing variation in swarming expression by *P. mirabilis* which might be due to different reasons related to the strains themselves (e.g. strain variation, their origin etc) or growth and incubation conditions such as incubation period, pH, temperature, nutrition ingredients in media or expression of certain related swarming genes (Liaw, 2003). This explanation corresponds with Senior's experiment when he observed a strong correlation between the ability of *P. mirabilis* to swarm growth and the ability to produce protease (Senior, 1999). *P. mirabilis* has the ability to promote infection of a host during swarming and highly motile because the swarming cells could migrate through the urinary tract and cause many infections. The swarming phenomena has been studied in different genera *Serratia* spp., *Salmonella* spp., *Aeromonas* spp., *Bacillus* spp., *Yersinia* spp., *Pseudomonas* spp., *Vibrio* spp., *E. coli* and *P. mirabilis* (Stickler and Hughes, 1999). Swarm cell discrimination and swarming style are the results of congregation sensory transduction and universal control mechanisms. *P. mirabilis* swarming oblige the sensing and integration of a different of environmental, cell-to-cell, and intracellular signals and share regulated expression of gene networks leading to occurred in physiological and morphological changes (Fraser and Hughes, 1999). The signals regulating swarming and the pathways for signal transduction are still understood. In this

study, we attend clue that chemical compounds avail as environmental cues to affect *P. mirabilis* swarming. Specifically, carbohydrates such as glucose support swarming motility with increased concentration, the diameter of swarming reached at a maximum value of 98 mm at 5% concentration (Figure 3). This role may attribute to an enhanced growth rate of swarm cells and utilized glucose as a carbon source. The inhibitory effect of sucrose and lactose on swarming may be assigned to that *P. mirabilis* is incapable to ferment them. Moreover, *P. mirabilis* lacks the ability to express or produce amylase that used to degrade complex carbohydrates such as starch. There is no report that refers to the effect of any carbohydrates on *Proteus spp*. swarming (Figure 3). Yeast extract and peptone are among other nutrients that form basic ingredients in culture media also has support action lead to enhanced swarming with increased concentration. The maximum swarming diameter was 76 and 95 mm, with 5 % of yeast extract and peptone, respectively (Figure 4). Yeast extract and peptone are good sources of vitamins that has a stimulatory effect on the bacterial growth rate. Indomie additives also have a role enhancing swarming with increased concentration, and the maximum swarming diameter was reached up to 89 mm with 5 % concentration (Figure 4). Indomie Co. claims that its noodles product is made from high-quality flour supplied by Bogasari flour mills meeting international standards, and fortified with vitamin A, B1, B6, B12, iron, folic acid and niacin. There is no available reports on the effect of folic acid, iron, and vitamins on swarming of *P. mirabilis*, but the addition of exogenous iron leads to restore the biofilm formation and swarming motility of *P. aeruginosa* in the presence of the spent medium of Streptomyces sp. BFI 230 (Kim *et al.*, 2012) and determines swarming initiation and biofilm formation in *Serratia marcescens* as a model (Lin *et al.*, 2016). Red pepper powder had shown an inhibitory effect on swarming, and the diameters recorded were 18, 10, 6 mm, when increased concentration to 1%, 2%, 4%, and 5%, respectively (Figure 4). However, most of the anti-swarming effects of red pepper are linked to other compounds like Chrysoeriol, which has very powerful antimicrobial action. Though many phenolic combinations such as capsaicin have many activity roles such as anti-inflammatory, antimicrobial, antioxidant, antitumor activities, bacteriostatic activity versus some Gram-negative bacteria. It also shown an inhibitory role for the formation of biofilm, which is base for bacteria cohesion. Capsaicin inhibits *Porphyromonas gingivalis* growth, gingivomucosal inflammatory cytokine secretion, and in vitro osteoclastogene and has a role as antioxidant, antimicrobial, anti-inflammatory and antitumor activities and protection against degenerative diseases (Zhou *et al.*, 2014; Arimboor *et al.*, 2015). Iwalokun and his co-workers found that some of the amino acids, such as glutamine, serine, and methionine enhanced swarming motility, while the other 17 amino acids have an inhibitory effect on swarming (Iwalokun and Akinwumi, 2002). Fatty acids represented as signals to regulate swarming in *P. mirabilis*. The swarming was increased with Oleic acid treatment while inhibited swarming was shown when P. mirabilis was treated with myristic acids, lauric acid, stearic acid, and palmitic acid (Liaw *et al.*, 2005). P- Nitrophenylglycerol (PNPG) also can inhibit not only swarming but also the ability of *P. mirabilis* to express other virulence factors such as urease and hemolysin which coupled to swarming and prevent its invasion to human urothelial cells (Allison *et al.*, 1994; Liaw *et al.*, 2000). Ag Nanoparticles and TiO2 Nanoparticles lead to down regulation in fliL Gene Expression that related with swarming movement in Proteus spp (Saleh *et al.*, 2019). There are several other theories that have been suggested to explain the mechanism of swarming of bacteria. The negative chemotaxis (Smith, 1975), accumulation of secondary metabolites in the colony vicinity, impairment of flagellation (Boer *et al.*, 1975), enhanced growth rate (Jones and Park, 1967) were some of the reasons suggested explaining the swarming of *Proteus* and *Vibrio* species. But irrespective of the nature of flagellation, the formation of lateral flagella is requisite for swarming and is aided by signals that trigger the change (Manson, 1992). The chemical compounds that inhibit swarming may be attributed to complex with flagellar proteins of swarming cells and cause its disintegration (Williams and Schwarzhoff, 1978) or impairs formation of flagella and motility (Kopp *et al.*, 1966). The effect of all the compounds tested in this study might be due to the following; these compounds may acts as extracellular signals or intracellular signals, may serve as cell-cell communication signals that interact with some of membrane sensor proteins, or may affect membrane fluidity. Also, these compounds may interact with the activity of RcsC-RsbA proteins (through either an RsbA-dependent or RsbA-independent pathway) to regulate swarming and virulence factors expression in *P. mirabilis.* Other reason might be that these compounds may have an inhibitory effect on rsb A gene that regulated *P. mirabilis* swarming (Liaw, 2003; Liaw *et al.*, 2005).

The antibiotic sensitivity story was to clarify that all isolates of *P. mirabilis* were shown in (Table 1). Pel-

grift and his colleagues were mention in them report the swarmer cells of Proteus spp were appeared variety in their style, increased in their resistance to different antibiotics, but may come back to planktonic cells when transported in to the broth media, and then showing renew their antibiotic susceptibility (Pelgrift and Friedman, 2013). Our results disagreement with other studies were showed 100% of Proteus spp resistant to cephalothin and about (80%) were sensitive for gentamicin, but against amikacin, the results were appeared (35 %) of isolate resist (Kadhum and Khalaf, 2009). The microbe acquires resistance to different antibiotic or chemical compounds via a plasmid or by chromosomal beta-lactamase expression (Song *et al.*, 2011). Also, Decreasing their permeability, effluex pumps, transposons, it reasons for bacterial resistance to win (Karlowsky *et al.*, 1998). The variety in the antibiotic susceptibility tests may be attributed to the main ingredients in a cytoplasmic membrane such as lipopolysaccharides, lipoproteins, and polysaccharides; on another hand misuse and abuse of antimicrobial agents may lead to spreading antibiotic resistance (Mordi and Momoh, 2009; Enabulele *et al.*, 2006).

CONCLUSIONS

This study demonstrated that red pepper induced swarming inhibition of uro-pathogenic *P. mirabilis* in vitro and could be employed in the treatment of patients that suffer from UTI caused by *P. mirabilis.* Also, it is recommended, according to this study's findings, to avoid the consumption of indomie with its additives due to their stimulatory action on swarming of *P. mirabilis*, which may give allow *the microorganism* to reach and colonize other sites of the urinary tract causing infections. We suggest using swarming inhibitors (shown in Figures 3 and 4) in culture media to help in the diagnosis of *P. mirabilis.* Also, we suggest using these inhibitors with antimicrobial agents (Antibiotics) to get a more bacteriostatic or bactericidal effect on dangerous opportunistic *Proteus* spp.

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