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The Effect of Sodium Azide on Swimming and Swarming Phenomena of *Proteus mirabilis*

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Abstract : This study aimed to detect the effect of sodium azide on swimming and swarming motilityof *P. mirabilis*. A total of 450 mid-stream urine samples have been collected from patients with urinary tract infection whom admitted to the hospitals in Annajaf Al-Ashraf province for consultancy during the period from October 2015 to February 2016.The results showed that out of 150 bacterial isolates only 36isolates were belong to *P. mirabilis* according to conventional methods (depending on microscopic and culturing examination as well as biochemical test) whereas molecular technique using *16SrRNA* gene by PCR technique confirmed that 29 isolates were belong to *P. mirabilis*.As a typical *P. mirabilis* all of bacterial isolates were able to swimming and swarming motility when cultured on semi-solid and solid media respectively. The addition of sodium azide with 0.005% and 0.01% concentrationscause inhibition of swimming and swarming phenomena.
Key words : *P. mirabilis*, *fumC*, swarming, mutation, pathogenicity.

Introduction:

Proteus is Gram negative, anaerobic rods belong to the family Enterobacteriaceae, It has more than four species most of them known to cause disease in humans are associated with opportunistic infections[1].*P. mirabilis* expresses several virulence factors involved in uro-pathogenesis like adhesions, swarming motility, urease, hemolysin, proteases and LPS endotoxins [2].

Swarming phenomenon is one of the important virulence factors that aid in the pathogenicity which allows *P. mirabilis* to migrate from one site to another and also helps in its colonization [3]. Swarming is a multicellular differentiation phenomenon that allows a bacterial population to move on a solid surface in a coordinate manner. It involves cell to-cell signaling and multicellular interactions and is related with the variation of morphological characterization of bacteria depending on growth media [4,5]. Swarming of *P. mirabilis* involved four stages: 1- surface induced swarmer cell, 2- a large period prior to swarming migration, 3- active swarming migration, 4- and a consolidation phase [6]. Initiation of swarming requires a signal induced by putrescin[7,8].Swarmer cells differ from vegetative cells as it has cell wall permeability, antibiotic sensitivity, lipopolysaccharide composition, enzymatic activity, and response to amino acid attractants [9].

Many factors may be involved in swarming of *P. mirabilis* across solid surface such as polysaccharides (including LPS) and extracellular component [10,11] and fatty acid [12]. The outer membrane (OM) of swarmer cells reveals higher fluidity than does the OM of the swimmer cells. Differences are also found in some cellular proteins levels and in the expression of some enzymes such as tryptophanase, phenylalanine deaminase, and urease, as well as *HpmA* hemolysin[13].

A number of theories have been suggested to explain the cyclic nature of swarming and the onset consolidation. According to Lominski and Lendrum [14], the central colony that are actively growing and dividing produce and excrete a cell toxic metabolites that disperses and spreads into the medium and establishes a gradient of decreasing concentration away from the colony and when the metabolites concentration reaches a dangerous level it will trigger the transformation of normal short cells to swarm cells by inhibiting the cell-division and stimulating the synthesis of flagella. The highly motile long forms (swarm cells) once formed is able to detect the toxic product and swarm away from the center of colony (down the concentration gradient) in a negative chemotactic response. When the swarmer cells reaches an area of agar with a low level of metabolites, movement stops and the swarm cells (long bacilli) fragment to form a shorter cells and start multiply, and when the concentration of metabolites increase critical concentration the swarm cell will form again and the process is then repeated in cycle forming the concentric circles pattern that appear on several agar media. Other theories demonstrate the cyclic nature of swarming is a results of many changes in gene expression and mathematical models involving change in population density or water activity at the periphery of expanding cells [15, 16, 17].

However, various substances exhibit an anti-swarm characteristics on solid media such as nitrophenyl glycerin, urea, ethanol, sodium azide , barbitone and active charcoal [18,19].Because of the important role of fimbriae in the pathogenicity of *P. mirabilis,* this research was aimed at investigating the effect of sodium azide on swimming and swarming phenomenon.

Materials and Methods

Isolation and identification of *P. mirabilis*: 450 mid-stream urine specimens were collected from patients suffering from urinary tract infection at different age groups from both sexes. All samples were cultured on MacConkey and Blood Agar Base for primary isolation of bacterial isolates. Microscopic examination, bacterial colonies characteristic on selective and deferential culture media and biochemical test (conventional methods) as well as molecular technique (using *16SrRNA* gene by PCR technique) were used for identification of *P. mirabilis*[20].

Detection of swimming and swarming phenomena: A method described by Girgis[21]was carried out. Briefly, semi-solid Luria Bertani medium(with3 g/l of agar) for swimming and solid Luria-Bertani medium(with 8g/l) for swarming were inoculated with a spot of 0.5µl of *P. mirabilis* suspension (bacterial density equal to McFarland standard tube NO. 0.5)and incubated aerobically at 37ºC for 24 hr. to detect swimming and swarming phenomenon. Three replicates were used for each isolates.

Detect the effect of sodium azide on swimming and swarming phenomena. Two concentration of sodium azide (0.005% and 0.01%) were used to evaluate its effect on swimming and swarming phenomenon according to the method that described by Kearns (2010). As described above semi-solid and solid Luria Bertani medium were supplemented with 0.005% and 0.01% of sodium azide separately to each one.A spot of 0.5µl of *P. mirabilis* suspension (bacterial density equal to McFarland standard tube NO. 0.5)was cultured on the media and incubated aerobically at 37ºC for 24 hr.

Molecular Experiment

Extraction of bacterial DNA:

Boiling method as described by Sambrook [22], was carried out to extract the template DNA from *P. mirabilis* isolates. Briefly, 10 ml of a fresh brain heart infusion culture were centrifuged at 6000 rpm/10 min and the pellet was washed twice with STE buffer (0.058gm of NaCl, 0.015gm of Tris base and 0.004gm of EDTA in 10ml of D.W.), then 200µl of SET bufferwas added; mixed well and heated to boiling for 10 min,then transferred to a water bath at 80Cº for 4minutes. Later ona lysate wasincubated on ice bath for 5 min. The mixture was centrifuge for 30 min at 15000 rpm. The supernatant was transferred to new Eppendorf tube and mixed with 0.7 v:v of isopropanol and incubated at -4ºC overnight. The precipitated nucleic acid was recovered by centrifugation at 10000 rpm/15min and the pellet was washed with70% ethanol and drained off till no trace of ethanol was seen,then 200µl of TE Buffer was added to re-suspend the DNA and it was preserved at -20ºC till use.

Amplification of target gene:

Monoplex PCR technique has been carried out to amplify *16SrRNA* using F -GAGTTTGATCCTGGCTCAG- and R -GGTTACCTTGTTACGACTT-to confirmed identification of *P. mirabilis*[23],and *fumC* using F-ATCCACGCGCTTGCTTTAAC- and R-TACGCTCACGGTTTGGTTCA-to detect fumerase encoding gene(Designed in this study). For both genes PCR mixture was used with a final volume of 20µl consist of 5µl of master mix (2.5U-iTag DNA polymerase,2.5mM dNTPs,1X reaction buffer and 1X Gel loading buffer), 2µl of each forward and reverse, 5µl of DNA template and 6µl nuclease free water.Gradient PCR was performed in PCR thermocycler (Biometra, USA) and the most proper conditions were explained in Figure (1). The resulted amplicon was electrophoresis on 1% agarose gel stained with 0.5µg/ml of ethidium bromide at 80V. for 1.5hr. and the gel was visualized by UV Transilluminator unit and photographed[24].

Gene Name	Primary Denaturation	30X PCR cycles			Final Extension
		Denaturation	Annealing	Extension	
16S rRNA	94.0° 2.00min	94.0°C 1.00min	50.2°C 1.00min	72.0°C 1.00min	72.0°C 5.00min
fumC	94.0° 2.00min	94.0°C 1.00min	53.0°C 1.00min	72.0°C 1.00min	72.0°C 5.00min

Figure (1): PCR thermocycler conditions.

Results and Discussion:

Identification of *P. mirabilis*

The initial diagnosis that based on microscopic examination and characteristic of bacterial colonies on selective and differential culture media as well as biochemical test showed that out of 150 bacterial isolates only 36 isolates were belong to *P. mirabilis*.

Genotypic identification of 36 isolates showed that only 29 isolates were belong to *P. mirabilis*by appearance of amplicon with molecular weight 1500bp (figure 2).

Proteus species are normal flora of the human intestinal tract [25]. It is an opportunistic pathogen which under favorable conditions causes urinary tract infection and It is commonly associated with complicated urinary tract infection and they are important causative agents in community acquired and nosocomial urinary tract infection(UTI) [26.]

Molecular study using *16SrRNA* was carried out to confirm the identification of *P. mirabilis*. The results of gel electrophoresis of amplicone resulted from amplification of *16SrRNA* gene showed that 29 isolates (19.33%) out of 36 suspected isolate belonged to *P. mirabilis* as the amplicone appeared with molecular weight 1500bp as shown in figure (2).

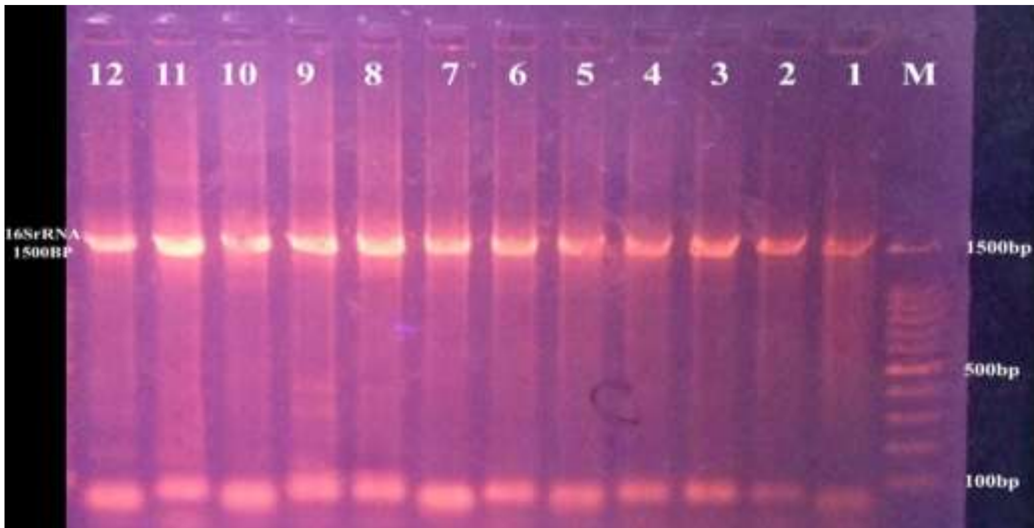

Figure (2): Gel electrophoresis of PCR product of *16SrRNA* of *P. mirabilis* (amplicon with 1500bp). Lane M: DNA marker(100bp) ; Lane 1,2,3,4,5,6,7,8,9,10,11,12: amplicon of *16SrRNA* of *P. mirabilis*. (1% agarose, 80Volt for 90Min).

P. mirabilis is one of the most common causative agents of several opportunistic hospital acquired infection such as nose, respiratory tract, skin, ear, gastroenteritis, wounds and burns [27]. Usually they affect the upper part of urinary tract causing infections such as cystitis, urolithiasis (kidney or bladder stones), and acute pyelonephritis and occasional cases of neonates or infants meningitis, bacteremia, wound infections, septicemia, and rheumatoid arthritis [28].

Detect the effect of sodium azide on swimming and swarming of *P. mirabilis*

The results of this study showed that all of *P. mirabilis* isolates form swarmer and swimming cells during their growth on solid and simi-solid media as well as a consolidation phase appeared(Figuer3). To evaluate the effect of sodium azide the results showed that sodium azide (a poison of aerobic respiration) cause inhibition of swimming and swarming phenomenon when used in both 0.01% and 0.005% concentrations as shown in figure (3).

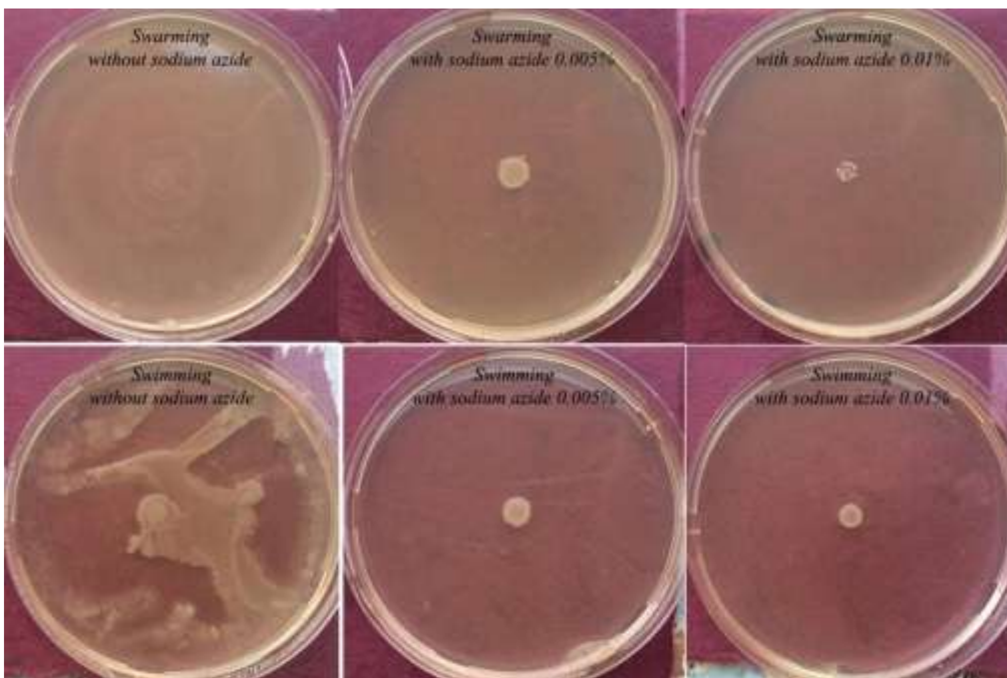

Figure (3): Swimming and swarming phenomenon of *P. mirabilis* with or without sodium azide.

Swarming proses of *P. mirabilis* involved differentiation of bacterial cell into a large (750um), a multi-nucleated highly motile, hyper flagellated cell [29]. Many factors are necessary for swarming phenomenon including flagella and chemotaxis [30,31,32]. The appearance of consolidation phase revealed a high regulation of metabolic activity of *P. mirabilis*. Several studies improved that during consolidation phase (a term called on swarmer cells that slow down or cease movement and differentiate into shorter rod shaped cells) about 541 genes were up regulated. Members of these genes were associated with metabolic activities of bacterial cells [33].

On the other hand initiation of swarming requires a signal that induced by putrescine [34] and glutamine [35], such signals were induced by regulation of gene expression. Many studies referred to many factors that may be an important role in movement of *P. mirabilis* on solid surfaces such as polysaccharides (as well as LPS) fatty acids and extracellular matrix [36].Such factors may be regulated by at least 9 genes as [37] as he confirmed that during swarming, only 9 genes were up regulated. Both swimming and swarming motility require functional flagella. Genes that involved in biosynthesis of flagellum are clustered in several operons that make up regulation of flagella [38].

Because of this phenomenon, isolation of *P. mirabilis* from clinical samples was very difficult or makes it impossible sometimes so that many attempts were carried out to develop an anti-swarming agent.

Several studies referred to using some chemical compounds for inhibition of swarming cell [39]. Showing that inhibition of motility may be carried out by using phenyl-ethanol whereas sodium azide may inhibit the growth of bacterial cells, while [40] noted that 0.125mg of triclosan lead to inhibition of 90% of *P. mirabilis* isolates.

Christopher[41] found that wild type strain of *P. mirabilis* lost swarming motility when grow on agar containing sodium azide which poisons aerobic respiration. Inhibition of aerobic respiration lead to inhibition of TCA cycle by disrupting of genes encoding TCA cycle enzymes (succinate dehydrogenase and fumerase) causing aberrant swarming phenomenon as concluded [42,43].

Molecular Detection of *fumC gene:*

fumC gene was detected in all *P. mirabilis* isolates by PCR technique and the results showed that all of the isolates were possess this gene by appearance of amplicon with molecular weight 1060bp on agarose gel (figure 4).

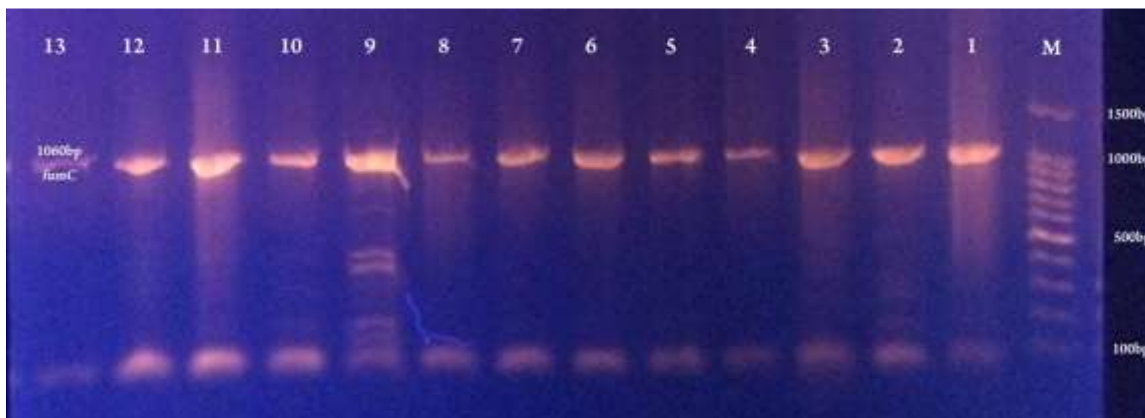

Figure (4): Gel electrophoresis of PCR product of *fumC* of *P. mirabilis* (amplicon with 1060bp). Lane M: DNA marker(100bp) ; Lane 1,2,3,4,5,6,7,8,9,10,11,12,13: amplicon of *fumC* of *P. mirabilis.* (1% agarose, 80Volt for 90Min.).

During the complete oxidation of TCA cycle, Fumarase enzyme which encoded by *fumC* catalyze the oxidation of fumerate to malate, while TCA cycle under anaerobic respiration operate in reduction pathway by reduce malate to fumerate [44].

fumC play an important role in swimming phenomenon of *p. mirabilis*, this is due to the fact that swarming differentiation relies on a complete oxidation of TCA cycle because of succinate dehydrogenase is part of the aerobic pathway and addition of fumerate was not effect on fumerase mutant *P. mirabilis*[45].

Swarming phenomenon requires a proton motive force that results in motion of flagella. The activity of thousands of coordinated flagella during the swarming phase and consolidation phase requires a considerable energy and up-regulation of genes involved in nutrition uptake (amino acids and peptic uptake system) to replenish energy reserves [46].

Several studies improved that a mutation that disrupts genes encoding enzymes of TCA cycle such as succinate hydrogenase and fumerase cause an aberrant swarming phenomenon [47,48].

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