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Phenotyping and Molecular Characterization of *Proteus vulgaris* Isolated from Patients with Urinary Tract Infections

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By

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Summary

In this study, a total of 150 urine specimens, only 43 isolates of *Proteus* species are isolated and from these only 28 isolates were identified as *Proteus vulgaris*. It was showed that all isolates (100%) possess haemolysin

gene, urease gene, fllagellin gene, adhesive factors genes (MR/P, MR/K, UCA and ATF), and Beta-lactamase gene. Whereas, 90% of the isolates gave positive result for metalloprotease *ZapA* gene and 50% for quinolones resistant gene.

The swarming activity was detected in all isolates(28) and the anti-swarming agents (resveratrol, p-nitrophenyl glycerol, fatty acids, urea ,sodium azide and ethanol) were used to show their ability to overcome this activity .It was found that there was diminishing in swarming activity when these agents are added to the culture media separately. Also it has been investigated the ability of resveratrol and p-nitrophenyl glycerol to inhibit production of haemolysin and urease in addition to swarming inhibition.

Furthermore, the susceptibility of *Proteus vulgaris* isolates to a variety of antibiotics has been investigated. It has been found that all the isolates were resistance (100%) to ampicillin, amoxicillin, cephalothin, chloramphenicol and ceftazidime, and some isolates have shown lower frequencies of resistance to ceftriaxone (89%), ciprofloxacin (82%) and cotrimazole (92.8%).Whereas, all the isolates were sensitive 100% to amikacin, gentamycin, nitrofurantion, imipenem, meropenem, and norfloxacin.

The detection of quorum sensing produced by *Proteus vulgaris* was also studied. It was found that there was an aggregation of the bacterial cells after addition of the supernatant that likely occurs as a result of the presence of homoserine lactone. The best interval for accumulation of homoserine lactone was after 4 hours of incubation in which the homoserine lactone reached at maximum concentration.

Proteus isolates were subjected for genotyping by using specific primers for genes (16SrRNA and *rpoB*). The examination of *Proteus* isolates by 16SrRNA sequencing showed between ٣ -٨% nucleotide differences and it was shown from the constructed phylogenetic tree that *Proteus penneri* and *Proteus mirabilis* have related one to another where both of them have the same branch in the phylogeny tree. Whereas, *Proteus vulgaris* was found to be located in another branch of the tree. In contrast to *rpoB gene* sequencing which was showed between 5 and 13% nucleotide differences and it was found that clinical strains were generally closely related to their respective type and type strain of *Proteus penneri* were close somewhat to *Proteus vulgaris* in phylogeny tree than to *Proteus mirabilis.*

1.1. Introduction

Urinary tract infections (UTIs) are considered as one of the most common groups of infections in humans and affect either the upper (kidneys-pyelonephritis) or the lower (bladder-cystitis) part of the urinary tract. The gastrointestinal tract is a reservoir from which uropathogens emerge. Reflecting this, *Enterobacteriaceae* are the most important cause of UTI in all population groups, accounting for more than 95% of all UTIs. Among these microbes, *E. coli* is by far the most common invader, causing some 90% of UTIs in outpatients and approximately 50% in hospitalized patients. *Proteus species* is a common cause of urinary tract infection (UTI) in catheterized patients and those with urinary tract abnormalities. It shows a predilection for the upper urinary tract where it can cause serious kidney damage, acute pyelonephritis, bladder or renal stones, fever and bacteraemia.

Proteus vulgaris belongs to family *Enterobacteriaceae* and is a rod-shaped, Gram negative bacterium that inhabits the intestinal tracts of humans and animals. It can also be found in soil, water and fecal matter. It is an opportunistic pathogen of human, it is known to cause urinary tract infections and wound infections. Several potential virulence factors, including adherence to the uroepithelium mediated by fimbriae, urease production, invasion of eukaryotic cells, cleavage of IgG and IgA by a proteolytic enzyme, haemolysin production, and swarming motility dependent on flagella may be responsible for the pathogenicity of *P. vulgaris*.

The adaptation of microorganisms to antibiotics causes proliferation and persistence of drug resistance, currently a major public health problem, therefore it is urgent to discover new drugs endowed with antimicrobial activity. In recent years, an increasing interest has been biologically active compounds including antioxidants from plants and other natural sources.

Molecular phylogenetic is the branch of phylogeny that analyses hereditary molecular differences, mainly in DNA sequences, to gain information on an organism's evolutionary relationships. The sequence of the 16SrRNA gene has been widely used as a molecular clock to estimate relationships among bacteria (phylogeny), but more recently it has also become important as a means to identify an unknown bacterium up to the genus or species level. Advances have been made in automating and minimizing the detection times using biochemical methods, however, biochemical identification is not accurate for determining the genotypic differences of microorganisms. The16SrRNA has properties, which predestine it as a universal phylogenetic marker. There are regions on the

16SrRNA that are quite conserved and others, which are variable. Comparing the differences in the base sequence of this 16SrRNA gene is, therefore, an excellent means to study evolutionary changes and phylogenetic relatedness of organisms.

The gene coding for the Beta subunit of the RNA polymerase, *rpo*B has been proposed as an alternative biomarker for microbial community studies. This gene is described as possessing the same key attributes as 16SrRNA, in that it is common to all bacteria and is a mosaic of conserved as well as variable sequence domains. Most importantly, the *rpo*B gene exists as a single copy in bacterial genomes. The *rpo*B gene, has emerged as a core gene candidate for phylogenetic analyses and identification of bacteria, especially when studying closely related isolates. Sequencing of *rpo*B enables efficient estimation of bacterial G+C% content, DNA–DNA hybridization value and average nucleotide identity (percentage of the total genomic sequence shared between two strains) when taxonomic relationships have been firmly established. Together with the 16S rRNA gene, *rpo*B has helped to delineate new bacterial species and refine bacterial community analysis.

The aim of this study:

To characterize the clinical isolates of *Proteus vulgaris* in terms of the molecular and phenotypic.

This study has been carried out to achieve the following objectives:

1-Collection of urine samples from patients with UTI and indwelling materials.

2-Isolation and identification of *Proteus vulgaris* by biochemical tests.

3-Detection of some virulence factors genes.

4-Detection of adhesive factors genes such as *mrp*, *mrkA*, *uca and atf* fimbrial genes using genetic markers.

5-Study the effect of some chemicals on *P. vulgaris* swarming and some virulence factors.

6-Study the effect of some antibiotics on *P. vulgaris* isolates.

7-Detection of quorum sensing in *P. vulgaris* isolates.

8-Genotyping of *Proteus* strains by PCR using genetic markers such as: 16SrRNA and *rpoB*.

1.2. Literatures review

1.2.1. Urinary tract infections

Urinary tract infections (UTIs) are among the most frequently occurring human bacterial infections, accounting for about 20% of all infections acquired outside the hospital. Almost 90% of UTIs are ascending, with bacteria gaining access to the urinary tract via the urethra to the bladder and then to the upper part of the urinary tract (Negut and Buiuc, 2008). The

organism causing a UTI usually originates from the patient's own bowel flora. The most frequent etiological agents causing UTIs are gram-negative bacteria belonging to the *Enterobacteriaceae* family (Gupta *et al*., 2001 ; Stickler *et al.,* 2003). *Proteus* bacilli play a particularly important role in urinary tract infections (UTI), which can be subdivided into two categories: hematogenous infections and ascending infections (Abraham *et al*., 2001). The second type of UTI is more common to *Proteus* strains. *Proteus mirabilis* is one of the most common causes of UTIs in individuals with long-term indwelling catheters or complicated UTIs and of bacteremia among the elderly. UTI are also caused by the two other species, *P. vulgaris* and *P. penneri* (Stickler *et al*., 2003 ; Sosa and Zunino, 2009; Stickler and Feneley, 2010).

1.2.2. Classification and environmental distribution of *Proteus* spp.

The genus *Proteus*, which was described for the first time by Hauser in 1885, belongs to the *Enterobacteriaceae* family. In this family it is placed in the tribe *Proteeae,* together with the genera *Morganella* and *Providencia* (Rozalski and Staczek, 2011). *Proteus* spp. consist of gram-negative, motile, aerobic rod-shaped bacilli generally range from 0.3 to 1.0 µm in width and 0.6 to 6.0 µm in length (Abbott , 2007). *Proteus* species are distinguishable from most other genera by their ability to swarm across agar surfaces of solid media. The genus *Proteus* currently consists of five species: *P. mirabilis*, *P. vulgaris*, *P. penneri*, *P. hauseri* and *P. myxofaciens*, as well as three unnamed *Proteus* genomospecies. *Proteus myxofaciens* is the only *Proteus* species without any significance in the pathogenicity of humans, it has been isolated from living and dead larvae of the gypsy moth *Porteria dispar* (O'hara *et al.,* 2000; Janda *et al.,* 2006). *Proteus* microorganisms are

widely distributed in the natural environment, including polluted water, soil, and manure. Due to their proteolytic activity, the ability to hydrolyze urea to ammonia and carbon dioxide, as well as the oxidative deamination of amino acids, these bacteria are involved in the decomposing of the organic matter of the animal origin. They are also present in the intestines of humans and animals. *P. mirabilis* was most frequently isolated from dogs, cows and birds, whereas *P. vulgaris* was most frequently isolated from pigs and cold blooded vertebrates (Manos and Belas, 2006).

Proteus species are normal flora of the human intestinal tract, along with *Escherichia coli* and *Klebsiella* species, of which *E. coli* is the predominant resident (Struble *et al*., 2009). *Proteus* spp. are important causative agents in community-acquired and nosocomial UTIs; within Europe and North America, 4 to 6% of *Proteus* infections are community-acquired and 3 to 6% are nosocomial (Abbott, 2007). They are generally considered pathogenic for young individuals and opportunistic pathogens for the elderly (Coker *et al*., 2000). The rate of infection is highest among the elderly, particularly those with indwelling catheters (long-term catheterization) or under frequent antibiotic therapy (Ronald, 2002; Kim *et al*., 2003). Other target groups include prepubescent males and females, with higher rate of infection reported among uncircumcised males (Abbott, 2007).

1.2.3. Mode of transmission:

Proteus spp. are part of the human intestinal flora and can cause infection upon leaving this location. They may also be transmitted through contaminated catheters (particularly urinary catheters) or by accidental

parenteral inoculation. The specific mode of transmission, however, has not been identified (Abbott, 2007).

1.2.4. Pathogenicity of *Proteus spp.*

Proteus rods are opportunistic bacterial pathogens which under favorable conditions cause urinary tract infections (UTIs), commonly associated with complicated urinary tract infections (Abbott, 2007). They generally affect the upper urinary tract (common site of infection), causing infections such as urolithiasis (stone formation in kidney or bladder), cystitis, and acute pyelonephritis. Rare cases of bacteraemia, associated with UTIs, with *Proteus* spp. have also been reported. Other infections include septicaemia and wound infections, meningitis in neonates or infants and rheumatoid arthritis (O'hara *et al*., 2000; Ronald, 2002).

Janda *et al*. (2006) and Kalra *et al*. (2011) reviewed endocarditis due to *Proteus* species and Okimoto *et al*. (2010) reported *P. mirabilis* pneumonia. Brain abscesses during *P. vulgaris* bacteremia were described by Bloch *et al*. (2010). However, it should be stressed that *Proteus* bacteria cause UTIs with higher frequency. This type of infections is classified as uncomplicated or complicated. Uncomplicated infections occur in patients, who are otherwise considered healthy, whereas complicated infections usually take place in patients with a urinary catheter in place or with structural and/or functional abnormalities in the urinary tract, suffering from another illness, immunocompromised, as well as after surgical intervention in the urogenital system. It was found that *Escherichia coli* is a common cause of uncomplicated infections. Complicated UTIs might be polymicrobial and are usually caused by Gram-negative bacteria *Proteus* spp., *Providencia stuartii*, *Morganella morganii*, *E. coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia* as well as some Gram-positive bacteria (Nielubowicz and

Mobley, 2010). *Proteus* species can cause hematogenous infections and ascending infections, however, the latter are more common for these microorganisms (Jacobsen *et al*., 2008).

1.2.5. Virulence factors of *Proteus spp.*

1.2.5.1. Adhesion factors

Bacterial adhesion onto mucosal or urothelial cells is an important phenomenon determining bacterial virulence. Infection in the urinary tract is related in part to the ability of bacteria to adhere and colonize the gut, perineum, urethra, bladder, renal pelvicalyceal system, and renal interstitium (Oelschlaeger *et al*., 2002). Adhesion is particularly important when infection occurs in an anatomically normal urinary tract, but it is also important in recurrent cystitis and infection complicating indwelling bladder catheters (Nielubowicz and Mobley, 2010).

Adherence is the key of virulence property of *Proteus spp*. This organism attaches to and swarms across the surface of urinary catheters to gain a foothold in the urinary tract (Jones *et al*., 2005). Once within the bladder, bacteria counteract the flushing motion of urine flow by attaching to epithelial cells (Jansen *et al*., 2004). Adherence factors may also play a role in the ability of this organism to colonize the kidneys or cross the renal barrier into the bloodstream. To establish a CAUTI, *Proteus* spp. must first gain access to the urinary tract. Although the catheter provides a direct route to the bladder, *Proteus* spp. needs to adhere tightly to the catheter to resist the flow of urine and must traverse this surface or disrupt the flow of urine to gain entry to the bladder. *Proteus spp.* readily colonizes the lumen and external surface of all current catheter types, and adherence is enhanced in

the presence of urine (Stickler *et al*., 2006; Williams and Stickler, 2008). Five fimbrial types have been characterized and reviewed for *Proteus* rods, but little is known about the contribution of fimbriae to catheter adherence and colonization (Rocha *et al*., 2007).

A-Mannose resistant *Proteus* like (MR/P) fimbriae

The mannose-resistant *Proteus*-like (MR/P) fimbria are perhaps the best-understood fimbriae expressed by *Proteus* strains during UTIs. These fimbriae are thick channeled (7 to 8 nm) and are classified as mannose-resistant hemagglutination. These fimbriae assemble through the chaperone-usher pathway (O'May *et al*., 2008). The genes required for the expression of MR/P fimbriae on the cell surface are encoded on the *Proteus* chromosome on two divergent transcripts, *mrpABCDEFGHJ*- (designated the *mrp* operon) and *mrpI*. Some of the proteins encoded by the *mrp* operon include the fimbrial structural subunit *MrpA*; the terminator for fimbrial assembly, *MrpB*; the minor fimbrial subunit MrpG; the tip adhesin *MrpH*; and the repressor of flagellin synthesis, *MrpJ* (Jansen *et al*., 2004).Promoter sequences have only been found upstream of *mrpI* and *mrpA*, suggesting that genes *mrpABCDEFGHJ* are located on one transcript. The intergenic region between *mrpI* and *mrpA* contains an invertible element flanked by inverted repeats (Nielubowicz, 2010).This invertible element contains a canonical σ70 promoter that, when in the proper orientation, drives expression of *mrpA*. The *mrpI* gene encodes a site-specific recombinase that reverses the orientation of the 251-bp invertible element that precedes the *mrp* operon. Expression of MR/P fimbriae correlates with the orientation of this invertible element (Jacobsen *et al*., 2008). MrpI orients the invertible

element in either an “on” position, allowing the expression of the MR/P fimbria, or an “off” position, in which the promoter is in the opposite orientation and is thus unable to drive transcription (Chelsie and Harry, 2012).

B-Mannose resistant *Klebsiella* like (MR/K) fimbriae

The expression of type 3 fimbriae has been described from many Gram-negative pathogens. Type 3 fimbriae are 2-4 μm wide and 0.5-2 μm long surface organelles that are characterized by their ability to mediate agglutination of tannic acid-treated human RBC (Mannose-resistant *Klebsiella*-like agglutination). Several studies have clearly demonstrated a role for type 3 fimbriae in biofilm formation (Burmolle *et al*., 2008; Ong *et al*., 2009). Type 3 fimbriae also mediate various adherence functions such as binding to epithelial cells (from the respiratory and urinary tracts) and extracellular matrix proteins (e.g. collagen V) (Ong *et al*., 2010).

On the other hand, MR/K fimbriae have been linked with the attachment of organisms to catheter surfaces and with the persistence of catheter-associated bacteriuria (O ’May *et al*., 2008). Expression of these thin non-channeled mannose-resistant fimbriae enables *Proteus* to attach tightly to the Bowman's capsule of the host kidney glomeruli and to the tubular basement membranes (Jacobsen *et al*., 2008).

Type 3 fimbriae belong to the chaperone-usher class of fimbriae and are encoded by five genes (*mrkABCDF*) arranged in the same transcriptional orientation. The *mrk* gene cluster is similar to other fimbrial operons of the chaperone-usher class in that it contains genes encoding major (*mrkA*) and minor (*mrkF*) subunit proteins as well as chaperone- (*mrkB*), usher- (*mrkC*)

and adhesin- (*mrkD*) encoding genes(Huang *et al*.,2009). A putative regulatory gene (*mrkE*) located upstream of *mrkA* has been described previously in *Klebsiella pneumonia* (Ong *et al*., 2010). The *mrk* genes have been shown to reside at multiple genomic locations, including the chromosome (Struve *et al*., 2009), on conjugative plasmids and within a composite transposon (Norman *et al*., 2008).

C. *Proteus mirabilis* Fimbriae (PMF)

Proteus mirabilis fimbriae (PMF) were identified from a crude fimbrial preparation of *P. mirabilis* strain HI4320. The PMF were isolated and their operon nucleotide sequence was determined by Massad *et al.* (1994). The genetic organization of the *pmf* operon revealed the presence of five genes that appear to be contained in one operon: *pmfA* (the major structural subunit), *pmfC* (a putative usher), *pmfD* (a putative chaperone), *pmfE* (a putative minor subunit), and *pmfF* (the putative tip adhesin).Such genetic organization resembles that of uropathogenic *E. coli* P fimbriae (Zunino *et al*., 2003; Jacobsen *et al.*, 2008).

The role of PMF in the virulence of *P. mirabilis* was determined by two studies. In the first, an isogenic *pmfA* fimbrial mutant colonized the bladders of transurethrally challenged CBA mice in numbers 83-fold lower than those of the wild-type strain. However, the mutant colonized the kidneys in numbers similar to those of the wild-type strain. The authors suggested that the role of PMF was just in colonization of the bladder but not in the kidney tissue. The second study also evaluated a *pmfA* mutant and the role of PMF in colonization, and virulence in UTI was assessed using a co-challenge ascending UTI model in CD-1 mice. After 7 days of infection, the mutant

and the wild-type strains were enumerated on non-swarming Luria–Bertani (LB) agar. The number of viable bacteria counted from bladder and kidneys showed that the mutant strain was significantly out-competed by the wild-type strain in colonizing the bladders and kidneys. This suggests that PMF play a role in localization of uropathogenic *P. mirabilis* to the bladder and kidney (Rocha *et al*., 2007).

D- Uroepithelial cell adhesion (UCA)

Uroepithelial cell adhesin (UCA) was initially discovered in an uropathogenic isolate of *P. mirabilis* (strain HU1069) during a screen designed to identify outer membrane proteins that facilitated binding to uroepithelial cells (Rocha *et al*., 2007). The identified UCA protein, the major structural subunit later designated *ucaA* was purified to homogeneity for characterization; the purified protein retained the ability to bind to uroepithelial cells and, additionally, organized into long, flexible filaments with a diameter of 4 to 6 nm, consistent with the appearance of fimbriae. N-terminal sequencing was performed on purified UCA. The sequence of the first 25 amino acids of the UCA protein displayed more similarity to the K99 pilus of *E. coli* (which mediates binding to intestinal epithelium) than to adhesins from uropathogenic *E. coli*. Subsequent determination of the sequence of the *ucaA* gene revealed that *ucaA* has the highest similarity to F17 and F111 fimbriae from bovine enterotoxigenic *E. coli* (Nielubowicz, 2010). These findings led to postulation that UCA may function as a primary adhesin for *Proteus* in the intestinal tract, although this hypothesis has not been experimentally tested (Cook *et al*., 1995). UCA fimbriae have since been renamed non-agglutinating fimbriae (NAF) to distinguish them from other *P. mirabilis* fimbriae that contribute to adherence (O'May *et al.*, 2008). Tolson *et al*. (1995) confirmed that what they designated the NAF subunit

was identical to the previously identified UCA subunit based on N-terminal sequencing. Pre-incubation with monoclonal antibodies specific for NAF significantly reduced binding of *P. mirabilis* to HEp-2 and uroepithelial cells in vitro, providing additional evidence of a role for NAF in adherence to host cells (Latta *et al.*, 1998; Rocha *et al*., 2007).

E- Ambient-temperature fimbriae (ATF)

Ambient-temperature fimbriae (ATF) were classified as a new fimbrial type, as examined by electron microscopy and immunogold labeling (Nielubowicz, 2010). The purified fimbria revealed a subunit with an apparent molecular size of 24 kDa, where the *N*-terminal amino acid sequence does not demonstrate similarity to that of any other fimbriae (Rocha *et al*., 2007). These fimbriae are produced in abundance at 25°C and are also synthesized to a lesser degree at 30 and 37°C. The genes responsible for the production of ATF are organized in the *atf* gene cluster and encode a 19-kDa major-subunit AtfA (Nielubowicz, 2010). Atf sequences represent a minimal fimbrial gene cluster. The *atf* gene cluster, composed of three genes, *atfA*, *atfB*, and *atfC*, appears to carry the oretical minimum number of genes necessary to encode atypical enteric fimbria. A structural subunit (*AtfA*), a chaperone (*AtfB*), and a molecular usher (*AtfC*) are encoded by contiguous genes. No putative regulatory or minor pilin genes are evident. *Proteus* were shown to express *AtfA*, as observed by Western blot analysis, it is suggested that these fimbriae are involved in the colonization of *Proteus* in the environment and are most likely not involved in CAUTIs (Zunino *et al*., 2000).

1.2.5.2. Motility

There are conflicting results concerning the importance of flagella in the infection. Since flagella mediate motility of bacteria, they seem to be required for virulence of uropathogens, particularly during ascending infection (Shirtliff and Leid, 2009).In general, flagella on the surface of bacterial pathogens are thought to assist in host colonization and dissemination, initial attachment, and sensing of the extracellular environment. For *Proteus* species, these surface structures are important in the process known as swarming, a distinct characteristic of these organisms. Swarming may play a role in the migration of *Proteus* strains on catheter materials. Flagella play a role of H antigen and are strongly immunogenic. Bacteria can avoid immune response of human organism due to the antigenic variation process. This phenomenon is based on flagellin genes rearrangement. Flagellar antigenic variations allow bacteria to evade the action of secretory IgA antibodies directed against these organelles (Belas and Suvanasuthi, 2005).

Swarming cell differentiation is important for the virulence of *Proteus* strains during UTIs since several virulence factors, including flagellin, urease, hemolysin *HmpA*, and IgA metalloprotease *ZapA*, are upregulated in the differentiated swarmer vs. swimmer cells (Fraser *et al*., 2002). Swarming is a multicellular differentiation phenomenon that allows a population of bacteria to migrate on a solid surface in a coordinate manner. It is important in movement of *Proteus* species to new locations and most probably helps them in the colonization of macroorganisms. It involves cell to-cell signaling and multicellular interactions and is connected with the possibility of morphological differentiation of bacteria depending on growth media. *Proteus* are dimorphic bacteria, which in liquid media are motile, peritrichously flagellated short rods (1.0 to 2.0 μm in length with 6–10

flagella).These bacteria are called swimmer cells. However, when transferred onto solid media these short rods change into elongated (20–80μm in length), hyperflagellated, multinucleated, nonseptated swarmer cells. The latter migrate out from the inoculation site as long as the population of swarmer cells is reduced on solid surfaces. Then, the consolidation process takes place. In this period of swarming growth, the long rods disintegrate to short bacteria. The processes of differentiation and dedifferentiation of *Proteus* bacteria are cyclic. It results in the formation of characteristic rings of bacterial growth on the agar plate (Verstraeten *et al*., 2008; Morgenstein *et al*., 2010; Rozalski and Staczek, 2010).

1.2.5.3. Biofilm formation

During catheters associated urinary tract infections (CAUTIs), after initial colonization of the catheter surface, *Proteus* species, as with other uropathogens, form distinctive crystalline biofilm structures. Biofilm assists in persistence of *Proteus* in the urinary tract by protecting these organisms from antibiotics and the host immune response and obviously contributes to adhesion to surfaces (Shirtliff and Leid, 2008). Urinary stone formation during *Proteus* mediated UTI is characteristic of this type of infection and is critical for the development of crystalline biofilms. Bacterial-derived stones account for up to 30% of all urinary tract stones worldwide and account for ~ 75% of those urinary stones classified as staghorn calculi (Hochreiter *et al*., 2003) . Crystalline biofilms are especially problematic during CAUTIs since catheters become blocked because of encrustation caused by the formation of these structure (Shirtliff and Leid, 2008).

1.2.5.4. Urease production

Urease has an EC number: (3.5.1.5).It is a metalloenzyme, which catalyzes the hydrolysis of urea to ammonia and carbonate, then digested to carbon dioxide and second molecule of ammonia. This process results in elevation of pH and non-physiological alkalization of urine, which in turn induces the precipitation of magnesium and calcium ions, normally soluble in slightly acidic urine, and the formation of urinary stones, containing struvite and carbonate apatite (Jacobsen *et al.*, 2008; Nielubowicz and Mobley, 2010).

The three structural subunits of urease, α (60-76KD), β (8-21KD), and γ (6-14KD), are encoded by the *ureC, ureB,* and *ureA* genes, respectively (Morou-Bermudez and Burne, 2000). Four additional proteins, encoded by *ureD, -E, -F,* and-*G,* catalyze the incorporation of Ni^{2+} into the active site of urease, a process that is required for the production of a catalytically active urease (Hausinger *et al.*,2001;Koper *et al*.,2004). In bacteria, the enzyme subunit and accessory genes are usually arranged in operons. Some bacterial urease clusters contain additional genes, such as *ureR*, which is involved in the regulation of urease expression in members of the family *Enterobacteriaceae* (Poore and Mobley, 2003).

The potent urease of *Proteus strains* results in the urinary tract containing an abundance of ammonia that would not be present during a single-species infection by a urease-negative organism such as uropathogenic *E. coli* (UPEC), so other species might benefit from the generation of this preferred nitrogen source during a polymicrobial infection. For UPEC, this hypothesis is supported by the finding that expression of *glnA*, which is induced by nitrogen limitation, is upregulated fourfold in bacteria taken from the urinary tract (Snyder *et al.,* 2004). Co-infection with

Proteus may alleviate the nitrogen-limited conditions for UPEC, thereby enhancing the growth or persistence of this urease-negative organism (Chelsie and Harry, 2012).

1.2.5.5. Protein toxins

Proteus rods produce three types of cytolysins, *HpmA* and HlyA hemolysins and *Pta* cytotoxic agglutinin. HpmA is the 166 kD protein, calcium-independent cell-bound hemolysin secreted by the bacteria and activated in the process mediated by *HpmB*. This hemolysin belongs to the pore forming toxins family and it is cytotoxic against a number of cells. *HlyA* is produced by *P. vulgaris*, *P. penneri* and *P. hauseri* strains. It is a strongly cytotoxic, calcium dependent 110 kDa extracellular toxin belonging to the RTX family of proteins (*RTX* - repeat in toxin) (Rozalski and Staczek, 2011; Rozalski *et al*., 2012).

The *hly* operon required for synthesis and extracellular secretion of hemolysin contains four structural genes arranged in the order *hlyC*, *hlyA*, *hlyB*, and *hlyD* (Koronakis *et al*., 2003). Gene *hlyA* encodes the 110-kDa hemolysin protein (pro-HlyA) which represents an inactive precursor of the mature toxin. The conversion of pro-HlyA to the hemolytically active hemolysin (*HlyA*) takes place in the cytoplasm of *bacteria* and is mediated by *HlyC*. The product of HlyB and *HlyD* together with *TolC* is involved in the secretion of the hemolysin through the bacterial cell wall (Holland *et al.*, 2005). The *hlyC* gene product is believed to have dual functions of (i) activation and (ii) transport of hemolysin through the cytoplasmic membrane to the periplasm (Patrick *et al.*, 2010).

Haemolysin was proposed as a virulence factor in *Proteus* because strains with high haemolysin production are more lethal than strains with

low haemolysin production, and the cytotoxicity of *Proteus* rods to human renal epithelial cells is largely due to haemolysin (Chelsie and Harry, 2012). Haemolysin is also thought to facilitate bacterial spread within the kidney and development of pyelonephritis during ascending UTIs (Coker *et al*., 2000).

Proteus toxic agglutinin (Pta), encoded by ICE*Pm1*, is a bi-functional outer-membrane auto transporter that mediates cell–cell aggregation and also contains a catalytically active α-domain (a subtilisin-like alkaline protease domain) capable of lysing kidney and bladder cells (Flannery *et al*., 2009). This unusual adhesin–toxin was first identified as an outer-membrane surface-expressed protein that is recognized by the mouse immune system, and loss of *pta* results in a significant colonization defect in the bladder, kidneys and spleen, as well as reduced pathology(Alamuri and Mobley ,2008 ; Alamuri *et al*.,2009).

1.2.5.6. Persistence and immune evasion

Once *Proteus* rods gain access to the urinary tract, it has a remarkable ability to persist despite antibiotic treatment and catheter changes (O ’May *et al*., 2008). To persist within the host, bacteria must successfully evade innate and adaptive immune responses. One mechanism of immune evasion for many species is to vary the expression or composition of antigenic structures, such as outer-membrane proteins or fimbriae. In *P. mirabilis*, the MRP fimbriae are phase variable (Chelsie and Harry, 2012). Flagellin is also thought to contribute to immune evasion via antigenic variation (Jacobsen *et al*., 2008).

Protease represents the class of enzymes which occupy a pivotal position with respect to their physiological and pathophysiological roles. They perform both degradative and synthetic functions. Protease is divided into exo- and endo-peptidases based on their action at or away from the termini, respectively. They are also classified as serine protease, aspartic protease, cysteine protease, and metalloprotease depending on the nature of the functional group at the active site. Many strains of *Proteus* produce this protease, also referred to as an IgA protease because of its activity on both serum and secretory forms of (IgA1 and IgA2, as well as IgG (Belas *et al*., 2004).

Proteus encodes a metalloproteinase (EC 3.4.24), serralysin (*ZapA*), that cleaves serum and secretory immune-globulin A1 (IgA1), IgA2 and IgG, thereby providing protection from the mucosal immune response. *ZapA* might also cleave complement components C1q and C3, cell matrix components such as collagen, fibronectin and laminin, cytoskeletal proteins such as actin and tubulin, and certain antimicrobial peptides. The importance of *ZapA* is underscored by the finding that mutation of *zapA* results in a dramatic decrease in the recovery of bacteria from the urine, bladder and kidneys (Belas *et al*., 2004; Chelsie and Harry, 2012).

1.2.٥.7. Lipopolysaccharide (LPS)

Lipopolysaccharide is composed of three genetically and structurally distinct regions: O-specific chain (O-antigen, O-specific polysaccharide), the core oligosaccharide and lipid A, which anchors the LPS molecule to the bacterial outer membrane (Raetiz and Whitfield, 2002). LPS containing all these three regions is produced by smooth forms of bacteria. Rough strains

synthesize LPS containing lipid A and the whole core region or only its part. All three regions of *Proteus* LPS have been studied (Rozalski, 2002, 2004 and 2008; Rozalski *et al*., 2002).

A- O-antigens

The differences in the structure of O-antigens serve as a basis for the serological classification of *Proteus* strains. The serological classification scheme currently consists of 78 serogroups (Drazewiecka *et al*., 2008 and 2010; Knirel *et al*., 2011). The prevalence of particular *Proteus* O-serogroups among clinical isolates has been changing over the time. Previously, clinical isolates were found to belong to serogroups O3, O10, O11, O13, O23, O27 and O30. Recently, Wang *et al*. (2010) and Kaca *et al*. (2011) identified five putative *Proteus* O-antigen gene clusters and *Proteus* specific genes of strains classified to five serogroups. The O specific polysaccharide chain exposed outside bacteria is involved in glycocalyx formation. Glycocalyx enables bacteria to grow in a form of biofilm on a solid surface. Bacterial biofilm is defined as a matrix enclosed bacterial population adhering to the surfaces. Bacteria enclosed in a glycocalyx capsule are protected against the action of antibodies, as well as against other immune mechanisms (Donlan and Costeerton, 2002; Stickler and Fenly, 2010).

B- Core oligosaccharide

The core region of *Proteus* lipopolysaccharides was studied by use of rough mutants or smooth forms classified into different serogroups. The structural diversity of the core is characteristic for *Proteus* spp. and makes it different from *E. coli* and *Salmonella* (Vinogradov *et al*., 2002). The *Proteus* core region is composed of two parts – an inner part, common for several number of strains and second, an outer part, which is characterized by

structural variability from strain to strain. The common part is not identical in all *Proteus* strains and is into three forms known as glycoforms I-III. The outer part of the core region (outer core) contains an oligosaccharide characteristic for particular *Proteus* strains (Rozalski, 2008).

C- Lipid A

LPS, released from pathogenic bacteria during infection induces a spectrum of biological activities. Therefore, LPS is also known as an endotoxin. The mechanism of biological action of the endotoxin is common to most bacteria. Its biological domain is lipid A (Łukasiewicz and Ługowski, 2003). *Proteus* lipid A contains glucosamine disaccharide substituted with phosphate residues and fatty acids. It also contains 4-amino-4-deoxy-L-arabinopyranose (L-Ar*ap*4N), which quantitatively substitutes the ester-linked phosphate residue of the glucosamine backbone. LPS of S-forms of bacteria contributes to their resistance against bactericidal action of the serum (Kwil, 2003; Mielnik *et al*., 2004; Kaca *et al*., 2009).

1.2.5.^. Resistance to antibiotics

The etiology of UTI and the antibiotic resistance of uropathogens have been changing over the past years, both in community and nosocomial infection (Manges *et al*., 2006; Ghaly *et al*., 2009). *Proteus spp*. are generally susceptible to broad–spectrum cephalosporins, aminoglycosides, and imipenem (Abbott, 2007). Otherwise, *P. mirabilis* is also susceptible to trimethoprime-sulfamethoxazole, ampicillin, amoxicillin and pipercillin, *P.vulgaris* and *P. penneri* are also susceptible to cefoxitin, cefepime, and aztreonam. *P.mirabilis* is resistant to nitrofurantion. *P.vulgaris* and *P.penneri* are resistant to piperacillin, amoxicillin, ampicillin, cefoperazone, cefuroxime, and cefazolin. Resistance to β-lactam among *Proteus* is emerging. Several mechanisms explain the emergence of drug resistance

have been discovered in the past decades the best known being beta-lactamase and quinolone resistance mechanisms (Karlowsky *et al*., 2002).

A- Beta-lactamases

Beta-lactamases (EC 3.5.2.6) is a type of enzyme produced by some bacteria that is responsible for their resistance to beta-lactam antibiotics like penicillins, cephalosporins, cephamycins and carbapenems. These antibiotics have a common element in their molecular structure: a four-atom ring known as a beta-lactam. The lactamase enzyme breaks that ring open, deactivating the molecule's antibacterial properties. Beta-lactamases are divided into four molecular classes (A, B, C, and D) (Karlowsky *et al*., 2002).Their molecular homology is diverse, and they do not seem to have one common ancestor. The presence of a serine residue in the active center is typical of A, C, and D classes, while in class B beta-latamases, zinc ions are required .Beta-lactamases are encoded by either chromosomes or plasmids. The highly mobile nature of beta-lactamase genes remains an important problem in UTI treatment (Pagani *et al*., 2002; Garcia-Rodriguez and Jones, 2002; Khan and Musharraf, 2004).

A variety of transferable genes encoding b-lactamase activity have been described in clinical environments including *bla*CTX-M, *bla*GES, *bla*OXA, *bla*OXY, *bla*SHV, *bla*SPM, *bla*VEB, *bla*VIM, and ampC alleles. Among the most common *bla* genes is the *bla*TEM-1 gene, the first described *bla* gene and a representative of the *bla*TEM group that now consists of almost 150 different alleles, all encoding different amino acid polymorphisms that extend their substrate range. The newer variants of the *bla*TEM alleles have only been found in clinical isolates and are likely emerging as a result of point mutations and directional selection (Lachmayr *et al*., 2009).

B-Quinolone resistant Mechanisms

Quinolones are a class of molecules that are used extensively in the treatment of many infections. Their availability and use have increased in recent years, especially in developing countries (Bouchakour *et al*., 2010).

For more than 30 years, the only known mechanisms of resistance to quinolones were chromosome borne. The two main mechanisms known to account for quinolone resistance are alteration of drug permeation (*i.e.*, decreased uptake mediated by mutations in the structural or regulating gene of porins, active efflux mediated by mutations in active expulsion pumps and target alteration (*i.e.*, mutation in the quinolone-resistance determining regions (QRDRs) of *gyrA-gyrB* or *parC-parE* encoding topoisomerase II (Hooper, 2003). Recently, plasmid-mediated resistance mechanisms have been described. The first plasmid-mediated resistance to quinolones was discovered in 1998 (Bouchakour *et al*., 2010), in a clinical isolate of *Klebsiella pneumoniae* that could transfer low-level resistance to quinolone to *Escherichia coli* or other Gram-negative bacteria. The plasmid-mediated quinolone resistance gene was named "*qnr*". This gene encoded a 218 amino-acid protein *Qnr* (later named *QnrA*), belonging to the pentapeptide-repeat family. More recently, four other markers (*QnrB* and *QnrS*, *QnrC* and *QnrD*) have been identified in several enterobacterial species (Hata *et al*., 2005; Jacoby *et al*., 2006; Wang *et al*., 2009). These markers interact with quinolones, the topoisomerases, and DNA, thus limiting the binding of the quinolones to their target (Cavaco *et al*., 2009).

The main distinction of *qnr* genes is carried on several integrons (Tran and Jacoby, 2002). These determinants can be easily transferred, accelerating the spread of quinolone resistance through gene transfer

mechanisms. In addition, the described integrons can carry genes which encode for resistance to third-generation cephalosporins (ESBL or ESC or derepressed cephalosporinase) (Bouchakour *et al*., 2010).

1.2.6. Anti–swarmer agents:

Many methods and addition of many chemical substances to the agar medium are recommended in order to inhibit the swarming. For example, the use of very dry plates, MacConkey medium containing bile salts, addition of urea and ethanol (AL-Kaebi and Matrood ,2011), resveratrol (Wang *et al*.,2006), *p*-nitrophenyl glycerin (Liaw *et al*.,2000), sodium azide, barbiton, activated charcoal and sulfonamide (Hernandez *et al*., 1999 ;Iwalokun *et al*.,2004).

A-Resveratrol

Resveratrol is (3,5,4'-trihydroxy-*trans*-stilbene) is a stilbenoid, a type of natural phenol, and a phytoalexin produced naturally by several plants especially the roots of the Japanese Knotweed (*Polygonum cuspidatum*), from which it is extracted commercially when under attack by pathogens such as bacteria or fungi (Wang *et al*.,2006). Phytoalexins are low molecular weight compounds which have been shown to possess biological activity against a wide range of plant and human pathogens (Paulo *et al*., 2010).

Resveratrol is found in the skin of red grapes and in other fruits. It also has been produced by chemical synthesis and by biotechnological synthesis (metabolic engineered microorganisms) (Farina *et al*., 2006; Trantas *et al*., 2009).It has a wide range of biological activities and consequently it has many different targets and mechanisms of action (Tennen *et al*., 2012). Resveratrol can prevent or slow the progression of several diseases,

including cardiovascular disease, carcinogenic disease and neurodegenerative disease (Zamin *et al*., 2006). It also prevents many aging processes and increases longevity. Moreover, resveratrol has anti-inflammatory (Leiro *et al*., 2010), antioxidant and antimicrobial properties (Docherty *et al*., 2007). Recent studies have indicated that resveratrol has growth-inhibitory effects on some bacterial pathogens (Tegos *et al*., 2006). In the course of studying the effect of resveratrol on human pathogens, Wang *et al*. (2006) found that resveratrol could inhibit swarming and virulence factor expression in *P. mirabilis*.

B-p-nitrophenylglycerol (PNPG)

P-nitrophenylglycerol or 1-(4-Nitrophenyl-β-Dglucuronicacid) (PNPG) is a chromogenic β-glucuronidase substrate (Sartory and Watkins, 1999).The anti-swarming agent PNPG has long been used to aid the isolation of small numbers of many different pathogenic bacteria from specimens contaminated with swarming strains of *Proteus* spp. In addition, PNPG has little effect on the results of a variety of identification tests performed directly on colonies from media containing PNPG (Jun *et al.*, 2004). It is relatively cheap, nontoxic and doesn't affect red blood cells; even fastidious pathogens will grow well and with characteristic colony morphology in its presence. Its heat stability and long 'shelf -life' make it convenient to use in the preparation of media (Liaw *et al*., 2000).

C-Fatty acids

The antibacterial activity of long-chain unsaturated fatty acids have been well known for many years. Fatty acids function as the key ingredients of antimicrobial food additives which inhibit the growth of unwanted microorganisms. Linoleic and oleic acids are antibacterial components in the herbs (*Helichrysum pedunculatum* and *Schotia brachypetala*). Besides

normal fatty acids, fatty acid derivatives showing potent antimicrobial activities exist in nature. These are mainly found in microorganisms, algae, or plants, which may mediate chemical defense against microorganisms (Zheng *et al*., 2005). Additionally, long-chain unsaturated fatty acids are bactericidal to important pathogenic microorganisms, including Methicillin-resistant *Staphylococcus aureus*, *Helicobacter pylori* and *Mycobacteria*. These antibacterial actions of fatty acids are usually attributed to long-chain unsaturated fatty acids including oleic acid, linoleic acid, and linolenic acid, while long-chain saturated fatty acids, including palmitic acid and stearic acid, are less active. However, their primary molecular target still remains unknown (Sun *et al*., 2003).

Fatty acids are one of the most ubiquitous components of bacterial cell membranes. Interestingly, it has been shown that exogenously added fatty acids modulate various bacterial activities, including motility, virulence, cell growth, and differentiation (Inoue *et al*., 2008). Swarming growth is also influenced by fatty acids. Oleic acid stimulates, whereas lauric and myristic acids inhibit this phenomenon, respectively. Straight-chain saturated fatty acids (SCFAs) repress swarming motility and hemolysin production in *Proteus mirabilis* and *Serratia marcescens* (Liaw *et al*., 2004; Lai *et al*., 2005).

D-Urea

Urea or carbamide is an organic compound with the chemical formula $CO(NH_2)$ (Welch,2007). It serves an important role in the metabolism of nitrogen-containing compounds by animals and is the main nitrogen-containing substance in the urine of mammals. It is a colorless, odorless solid, highly soluble in water and practically non-toxic (LD_{50} is 15 g/kg for rat), it is neither acidic nor alkaline (Meessen, 2010).

Urea has experimentally been demonstrated to possess anti-swarming properties and recommended for routine laboratory usage (Fons *et al*., 1999). It is commonly used in culture media designed for the identification of pathogens of UTIs including *Proteus* spp. However, reports have been silent on *Proteus* swarming prevention possibilities. Urea is primarily used in selective and composite media to identify urease producing microorganisms. In recent times, the possibilities of exploiting the anti-swarming property of urea to aid isolation and identification of single colonies on solid media are been confirmed (Iwalokun *et al*., 2004).

E. Ethanol

Ethanol also called ethyl alcohol, pure alcohol, grain alcohol, is a volatile, flammable, colorless liquid with the structural formula CH_3CH_2OH, often abbreviated as C_2H_5OH or C_2H_6O. It has bactericidal activity and is used often as a topical disinfectant (Knoll *et al*., 2009).

Ethanol at 90% added to the medium at a 5% concentration is also a very effective anti-swarm agent. It allows an easier isolation of gram-positive cocci and members of the families *Enterobacteriaceae* and *Pseudomonaceae.* However, when ethanol is used in blood agar medium, hemolytic reactions cannot be reliably determined. In some cases, the addition of chemical agents such as ethanol can interfere with the growth of other bacteria (Hernandez *et al*., 1999; Difco, 2009).

F. Sodium Azide

Sodium azide is the inorganic compound with the formula NaN_3. It is used for the preparation of other azide compounds. It is an ionic substance, is highly soluble in water, and is very acutely toxic (Betterton, 2003).It is a potent bacteriostatic that is frequently used to protect a diverse array of stock

solutions (e.g., antibodies) and samples (e.g., milk, fecal specimens) from prokaryotic contaminants. NaN_3 binds to heme-iron (cytochrome oxidase, catalase) leading to chemical asphyxiation of affected cells. However, the bacteriostatic effects of NaN_3 appear to be limited to Gram-negative *Bacteria*, whereas Gram-positive *Bacteria* are largely resistant to the compound (Kerros *et al*., 2012).

The addition of sodium azide to blood agar media was reported to abolish the swarming of *Proteus* without affecting the isolation of clinically important *Staphylococci* and *Streptococci*, but blood agar media containing azide are not widely used in the clinical laboratory because azide turns out to be a poor anti-swarm agent and, it shows growth inhibition of certain *Streptococci* (Hernandez *et al*., 1999; Iwalokun *et al*., 2004).

1.2.7. Quorum sensing:

Numerous species of bacteria employ a mechanism of quorum sensing (QS). This signaling process allows the cells comprising a bacterial colony to coordinate their gene expression in a cell-density dependent manner(Galloway *et al*.,2011).Quorum sensing is mediated by small diffusible molecules termed auto inducers that are synthesized intracellularly (throughout the growth of the bacteria) and released into the surrounding milieu. As the number of cells in a bacterial colony increases, so does the extracellular concentration of the auto inducer. Once a threshold concentration is reached (at which point the population is considered to be "quorate"), productive binding of the auto-inducer to cognate receptors within the bacterial cells occurs, triggering a signal transduction cascade that results in population wide changes in gene expression (Ng and Bassler, 2009; Atkinson and Williams, 2009). Thus, quorum sensing enables the cells within a bacterial colony to act cooperatively, facilitating population-

dependent adaptive behavior (Galloway *et al*., 2011). QS controls genes that direct activities that are beneficial when performed by groups of bacteria acting in synchrony. Processes controlled by QS include bioluminescence, sporulation, competence, antibiotic production, biofilm formation, and virulence factor secretion (Novick and Geisinger, 2008; Ng and Bassler, 2009; Rutherford and Bassler,2012).

Three major quorum-sensing circuits have been described: one used primarily by Gram-negative bacteria, one used primarily by Gram-positive bacteria, and one that has been proposed to be universal (Readind *et al*., 2005). Most Gram negative bacteria use a certain molecule called an acyl homoserine lactone´ or AHL´ that is used as the signaling molecule, whose production depends on *S*-adenosylmethionine (SAM) as a substrate (Wei *et al*., 2011). Gram positive bacteria use oligopeptides as their signaling molecule, which have been cut from a large polypeptide synthesized in the cytoplasm. These two systems are species-specific, and each Gram negative or Gram positive bacteria produce their own specific auto-inducer. Thus, these molecules enable intra-species communication. These are private, secret conversations (Bassler and Waters, 2010).

1.2.8. Genotypic analysis

Various genetic methods have been developed for genotyping of bacteria, since the late 1980 (Cai *et al*., 2003; İbrahim *et al*., 2011). These methods have become frequently used in bacterial identification due to their high resolution. The identified genetic profile of any bacteria by a specific genotyping method can be as unique as fingerprint (Li *et al*., 2009).

Current bacterial genotyping methods can be categorized into three categories: DNA banding pattern, DNA sequencing, and DNA hybridization.

In DNA banding method, DNA bands can be directly generated by digestion of Restriction Endonucleases (REs) or by amplification of known or unknown region of the genome or by a combination of amplification and digestion with restriction enzymes. In DNA sequencing based method, discrimination among the bacterial strains performed after the determination and comparing of a known gene sequence. In DNA hybridization based methods, discrimination of bacteria are carried out by analyzing the hybridization of known probes. DNA macroarray and microarray systems have also been developed to get accurate and faster results in description of bacteria (Versalovic and Lupski, 2002; Li *et al*., 2009; İbrahim *et al*., 2011).

16SrRNA gene sequence analysis is by far the most widely used method for the molecular identification and differentiation of bacterial species (Acinas *et al.,* 2004). The small subunit ribosomal RNA gene is the recognized gold standard for estimating the phylogenetic diversity in microbial communities (Jill, 2004 and Vos *et al.,* 2012). This marker gene is universally present and has the advantage of containing both highly conserved fragments, facilitating the design of PCR primers targeting all members of a community, and more variable regions that allow for the discrimination of different microbial taxa. However, the 16SrRNA gene is not without potential drawbacks, where members of the family *Enterobacteriaceae* have not been subjected to extensive phylogenetic 16SrRNA gene sequence analysis because the high degree of conservation in closely related species leaves many taxonomic problems unresolved. In particular, the genetic relationships between closely related species *Proteus vulgaris and Proteus penneri* could not be clearly resolved by this method (Cao *et al*., 2009). So, the use of alternative markers has been proposed, including the beta subunit of RNA polymerase, *rpoB.*

The use of the *rpoB* gene offers various potential advantages over standard 16SrRNA gene-based approaches. First, since most bacterial genomes contain multiple copies of the 16SrRNA gene, and copy number varies per species, extrapolation of relative abundances from gene recovery frequencies is seriously impaired. This is further complicated by the fact that sequence variation between the different 16SrRNA gene copies present exists in some genomes ,*rpoB* typically occurs in a single copy(Walsh *et al*., 2004; Pei *et al*., 2010). Second, the *rpoB* gene is a highly conserved housekeeping gene and one copy is present in all bacteria because of its essential role in cellular metabolism. Primers and probes targeting the *rpoB* gene have been used for the specific detection and phylogenetic analysis of several bacterial groups belonging to the family *Enterobacteriaceae,* the high level of conservation across 16SrRNA genes can obscure most intraspecific, and sometimes interspecific variation. In contrast, the higher resolution *rpoB* marker is capable of revealing molecular variation down to the population level (Merhej *et al*., 2008; Stoop *et al*., 2009). Third, genetic divergence of *rpoB* correlates better with overall genomic divergence and provides better bootstrap support for phylogenetic reconstruction. Fourth, given the fact that *rpoB* is a protein-encoding gene, the data generated from this marker is more readily interpreted in an evolutionary framework (Adekambi *et al.*, 2009).

2. Materials and Methods:

2.1. Patients:

A total of 150 urine specimens were collected from patients with urinary tract infections (53 male and 97 female with age ranged between 10-65 years old) who were admitted to five hospitals: Babylon Hospital for Maternal and Pediatrics, Al-Hilla Surgical Teaching Hospital, Merjan Hospital, Al-

Hashymia Hospital and Al-Qasim Hospital, during the period from 4/2012 to 1/2013. The types and the numbers of urine samples taken during this study are shown in table (2-1).

Table(2-1):Types and numbers of urine specimens

Samples type	No.
Urine from non-catheterized patient	125
Urine from catheterized patient	25
Total	**150**

2.2 Materials:

The laboratory instruments and equipment, chemical materials, culture media, antibiotics discs and commercial kits which used in this study are illustrated in table (2-2), (2-3),(2-4),(2-5) and (2-6) respectively.

Table (2-2) Laboratory Instruments and Equipment

No.	Instruments& Materials	Company	Country
1.	Incubator	Memmert VWR	Germany USA
2.	***Oven, Autoclave, Benson burner, Water bath***	Memmert	Germany
3.	Vitek system, Vitek glass (polystyrene) test tubes, Densichek meter, Vitek Gram-negative ID plus card (GNI+) Kit	BioMerieux	USA
4.	***Eppendorf 5415 D micro Eppendorf 5417 R centrifuge, Micro centrifuge***	eBay Inc	USA

5.	***Eppendorf 5430D micro centrifuge***	Thomas Scientific	USA
6.	Eppendrof tubes, tips	Sterellin Ltd.	UK
7.	High speed cold centrifuge, PCR tubes, DNA extraction tubes	Eppendorf	Germany
8.	Light microscope	Olympus Carl Zeiss	Japan Germany
9.	Micro imaging microscope	Carl Zeiss	Germany
10.	Micropipette 0.5-10,1-20µl, 5-50 µl,10-200 µl,100-1000µl	CYAN VWR	Belgium USA
11.	Platinum wire loop	Himedia Thomas Scientific	India USA
12.	Wooden sticks	Supreme	China
13.	Refrigerator	Concord	Italy
14.	Stirrer /Hot Plate	Labtech Corning	Korea USA
15.	Plastic test tubes , Millipore filter(0.45µm)	VWR	USA
16.	PCR System/Conventional Thermo cycler and Multiplex PCR	MJ Reasearch Bioneer	USA Korea
17.	Gel Electrophoresis	Shando, scientific co. BIO-RAD	UK USA
18.	Hood	Labogene	Denmark
19.	Ultraviolet transilluminator	Dahan San. Gabriel	Korea CA
20.	Mini vortex	CYAN Fisher Scientific	Belgium USA
21.	Microwave	Kenmore	USA
22.	Digital dry bath(42 C̊,50C̊,100 C̊)	Corning LSE	USA
23.	Rocker ,Rocker incubator	Boucker Scientific	USA

24.	Thermospectronic	Rochester	USA
25.	Sensitive electric balance(mg, g)	Sartorius Fisher Scientific	Germany USA
26.	Spectrophotometers	Bioate	USA
27.	Gel documentation system with a digital camera, Microputser	Bio Rad	USA
28.	ABI 3730 Genetic analyzer	Applied Biosystems	USA
29.	Camera	Canon	Japan
30.	Disposable petri dishes	Al-Hani company	Lebenon
31.	Sterilized cotton swabs, Disposable syringe 10 ml,5ml and 3ml	Sterile EO.	China

Table (2-3) Chemical Materials

N0.	Chemicals	Company/country
1.	NaCl, KOH, Na_2HPO_4 , KH_2PO_4 , K_2 HPO_4 , NH_4Cl, $MgSO_4$, $CaCl_2$, NaH_2 PO_4, H_2O_2, KCN, Trypton, Yeast extract, HCl	Merk Darmstadt-Germany
2.	Alpha-nephthol, P-Dimethyl aminobenzaldehyde, Peptone, Methyl red, Glycerol, Isoprobanol	BDH - England
3.	99% , 95% and 70% alcohol (Ethanol)	BDH –England & Flukachemika- Switzerland
4.	Urea solution (40%)	Flukachemika- Switzerland
5.	Aspartic acid	Vegetadrog -France
6.	Tannic acid, Lauric acid, Myristic acid, palmitic acid, Stearic acid, Oleic acid, P-nitrophenylglycerol, Sodium azide, Urea solution 40%	Fisher Scientific-USA
7.	Gram stain set	Ward's Natural Science -USA

8.	Resveratrol	Thomas Scientific-USA
9.	D-Mannose reagent , Sodium nitroprusside, Potassium cyanide	VWR-USA
10.	Gelatin, Trichloroacidic acid (5%)	Sigma-Germany
11.	Ethidium Bromide	Bio Basic –USA& Alfa Aesar-USA
12.	Big Dye Xterminator	Applied Biosystems- USA

Table (2-4) Culture Media

No.	Media	Company/country
1.	MacConkey agar, Blood agar base, MR-VP broth, Peptone broth, Muller Hinton agar	Himedia - Accumax India
2.	Urea agar base , Simmons citrate agar	Diffco – Michigan
3-	Agarose	Difco –USA & VWR-USA
4-	Nutreint broth, Nutreint agar, Tryptic soy broth, Luria bertani broth &agar	Ward's Natural Science-USA VWR-USA

The antibiotic disks which used in this study are equipped by Bioanalyse company /Turkey:-

Table (2-5): Antibiotic disks

Antibiotic Discs	Disc potency (µg)	Symbol	Producer Organism
Amikacin	30	AK	*Streptomyces* species and *Micromonospora* species Semi-synthetic aminoglycosides
Amoxicillin	25	AX	*Penicillium chrysogenum* Semi-synthetic antibiotic

Ampicillin	10	AMP	*Penicillium chrysogenum* Semi-synthetic antibiotic
Cephalothin	30	CEP	*Cephalosporium acremonium*
Ceftazidime	30	CAZ	*Cephalosporium acremonium*
Ciprofloxacin	5	CIP	*Streptomyces*
Cotrimazole	25	COT	A synthetic antibiotic
Gentamycin	10	GEN	*Actinomycetes* *Micromonospora purpurea*
Imipenem	10	IPM	*Streptomyces cattleya*
Meropenem	10	MRP	*Streptomyces cattleya*
Nitrofurantion	100	F	A synthetic antibiotic
Norfloxacin	10	NX	*Streptomyces*

Table (2-6) Commercial kits

No.	Type of kits	Company/country
1.	DNA extraction kit contents 1-Lyses cells solutions:- -Nuclei lyses solution -RNase solution	Promega-USA
	2-Protein precipitation solution	
	3-DNA precipitation alcohols:- -Isopropanol alcohol -Ethanol alcohol	
	4-DNA rehydration solution	
2.	Illustra bacteria genomicPrep Mini Spin kit contents - Proteinase K - Lysis buffer type 2 -Lysis buffer type 3	VWR-USA

	-Lysis buffer type 4 -Wash buffer type 6 -Elution buffer type 5 -Illustra mini column -Collection tubes	
3.	Accustart ™ Taq PCR SuperMix AccuPower^{TM} PCR PreMix kit contents 1-Taq DNA polymerase enzyme 2-dNTPs (400 µm dATP, 400 µm d GTP, 400 µm dCTP, 400 µm dTTP) 3-$MgCl_2$ (3mM) 4-reaction buffer (pH 8.3) 5- Stabilizer and Tracking dye	VWR-USA Bioneer- Korea
4.	DNA ladder (100bp, 1000bp, 2000bp, 1kb) 1-Ladder consist of 11 double-stranded DNA with size 100-1500bp 2-Loading dye has a composition (15% Ficoll, 0.03% bromophenol blue, 0.03% xylene cyanol, 0.4% orange G, 10mM Tris-HCl(pH 7.5) and 50mM EDTA)	Fisher Bioreagents-USA
5.	Gel loading dye blue	Promega -USA Biolabs-USA
6.	Primers of virulence factors , antibiotics resistance and fimbrial genes	Bioneer-Korea
7.	Primers for molecular typing by 16SrRNA and *rpoB*	IDT-USA
8.	QIAquick gel extraction Kit contents -QIAquick Spin Columns -Buffer QG -Buffer PE -Buffer EB -Collection Tubes	Qiagen-USA

2.3 Preparation of Reagents and Solutions:

2.3.1. Reagents:

●Kovac's reagent:

It was prepared by dissolving 5 gm of P-Dimethyl amino Benz aldehyde (DMAB) in 75ml amyl alcohol. Afterwards, 25 ml of concentrated HCL was gradually added to this mixture. It is used for the diagnostical indole test, to determine the ability of the organism to split indole from the amino acid tryptophan. The indole produced yields a red complex with para-dimethylaminobenz aldehyde under the given conditions (Baron *et al.*, 1994).

●Methyl red reagent:

Methyl red reagent was prepared by dissolving 0.1 gm of methyl red in 300 ml of (95%) ethanol. The volume was then completed to 500 ml by adding distilled water. It was used to detect complete glucose hydrolysis (Holt, 1994).

●Vogues – Proskauer reagent (Barrett's reagent):

It was composed of two solutions as follows:

A. Five gm of alpha nephthol dissolved in 100 ml of 99% ethanol then stored in a dark container away from light.

B. Forty gm of KOH dissolved in 100 ml of distilled water (MacFaddin, 2000).

●Oxidase reagent:

It was prepared freshly in a dark bottle by dissolving 1gm of tetramethyl para phenylene diaminedihydro chloride in 100 ml distilled water (Forbes *et al*., 2007).

●Catalase reagent:

Hydrogen peroxide (3%) was prepared from stock solution in a dark bottle (Forbes *et al*., 2007).

2.3.2 Solutions:

●Normal saline solution:

It was prepared by dissolving 8.5g of NaCl in a small volume of distilled water, then completed to 1000 ml, pH was fixed at 7.2 and sterilized in autoclave at 121^{o}C for 15 minutes, then kept at 4^{o}C, it was used for preparing bacterial suspension that used in haemagglutination test (Sambrook and Rusell, 2001).

●Phosphate buffer solution:

Eighty gram of NaCl, 0.34gm of $KH_2 PO_4$, and 1.12gm of $K_2 HPO_4$ were all dissolved in 1000 ml of distilled water. The pH was adjusted to 7.3, then the solution was sterilized in autoclave (Sambrook and Rusell, 2001).

●Tris EDTA Buffer solution (TE buffer):

The buffer was prepared by dissolving 0.05M of Tris-OH and 0.001M of EDTA in 800ml distilled water. The pH was adjusted to 8 then completed to one litter, autoclaved at 121^{o}C for 15 min, and stored at 4^{o}C until used (Stellwagen and Stellwagen, 2002).

●Tris Borate-EDTA-buffer solution (TBE):

The buffer was prepared by dissolving Tris-OH 0.08M, boric acid 0.05M, EDTA 0.02M in 500ml distilled water. The pH was adjusted to 8, autoclaved at 121^{o}C for 15 min, and stored at 4^{o}C (Sambrook and Rusell, 2001).

●Ethidium bromide solution:

It was prepared by dissolving 0.05g of ethidium bromide in 10ml distilled water and stored in a dark reagent bottle (Sabnis, 2010).

●Trichloroacidic acid (TCA) solution (5%):

Trichloroacidic acid solution was prepared by dissolving 5g of TCA crystals in small volume of distilled water and was completed up to 100 ml of distilled water and kept in the dark. It is used in the extracellular protease production test (Sambrook *et al*., 2001).

2.4 Preparation of culture media:

A culture media were prepared according to the instructions of the company and sterilized by autoclaving at 121ºC for 15 minutes as follow:

●MacConkey agar medium:

MacConkey agar medium was prepared according to the method recommended by the manufacturing company. It was used for the primary isolation of most Gram-negative bacteria and to differentiate lactose fermenters from non-lactose fermenters (Luis *et al*., 2004).

●Nutrient agar medium:

Nutrient agar medium was prepared according to the method suggested by the manufacturing company. It is used to cultivate and activate bacterial isolates when it is necessary (MacFaddin, 2000).

●Nutrient broth:

This medium was used to grow and preserve the bacterial isolates supplemented with 15% glycerol (MacFaddin, 2000).

●Brain heart infusion medium:

Enrichment medium prepared by suspension of 52g Brain heart infusion agar/liter or 37g Brain heart broth/liter, autoclaved (15 min at 121 °C). It is used to grow and preserve the bacterial isolates supplemented with 15% glycerol if the broth is used (MacFaddin, 2000).

●Blood agar medium:

Blood agar medium was prepared by dissolving 40 gm blood agar base in 1000ml distal water. It was autoclaved at 121^{o}C for 15 minute, and then cooled to 50^{o}C. (5%) of fresh human blood was added. This medium was used to cultivate bacterial isolates and determine the ability of bacteria to hemolysis blood cell (Madigan and Martinko, 2005).

- **Tryptic Soy broth:**

This medium was prepared by dissolving 30 g of dehydrated medium in 1 L of deionized water, then sterilized by autoclaving at 121^{o}C for 15 minutes. This medium was used as enrichment medium for cultivation of the bacterial isolates. (Hill *et al*., 1998).

- **Peptone water medium:**

This medium was prepared by dissolving 8g peptone in 1000 ml of distilled water, then distributed into test tubes, and autoclaved. It was used for the demonstration of the bacterial ability to decompose the amino acid tryptophan to indole (Colwell, 1996).

- **MR-VP medium:**

MR-VP medium has been prepared and used to detect the partial and complete hydrolysis of glucose according to MacFaddin (2000).

- **Simmons' citrate medium:**

Simmon's citrate medium has been used for determining the ability of bacteria to utilize citrate as the sole carbon source (MacFaddin, 2000).

- **Kligler Iron agar medium:**

Kligler Iron agar was used for determining glucose and lactose fermentation and possible hydrogen sulfide (H2S) production as a first step in the identification of Gram- negative bacilli (MacFaddin, 2000).

- **Motility medium**:

This medium was prepared by adding 4 gm of agar agar to 100 ml of nutrient broth and completed with 1000 ml distilled water. It was then sterilized by autoclave at 121ºC for 15 minutes. It was distributed in tubes. This medium was used to detect bacterial motility (MacFaddin, 2000).

●Urea agar medium:

This medium was prepared by adding 15 ml of urea solution sterilized by filtration (0.22µm in diameter), to 100 ml of urea agar base sterilized by autoclaving at 121ºC for 15 minutes, and cooled at 50ºC. The pH was adjusted to 7.1, and then the medium distributed into sterilized test tubes and allowed to solidify in a slant form. This medium was used to test the ability of bacteria to produce urease enzyme (Mahon and Manuselis, 2007).

●M_9 medium:

A six g of Na_2HPO_4, 3g of KH_2PO4, 0.5g of NaCl, 1g of NH_4CL were dissolved in 950 ml of distilled water with 2% agar and then sterilized in an autoclave at 121ºC for 15 min. After cooled the mixture at 50ºC, 2 ml of 1M of $MgSO_4$, 10 ml of 20% glucose and 0.1 ml of 1M of $CaCl_2$ (all of which were separately sterilized by filtration) were added to the mixture. The volume was then completed to 1000 ml by adding distilled water. After the inoculation of this media with bacterial strain and incubation for 24 hr at 37ºC, 3ml of (5%) Trichloroacetic acid was added. The formation of transparent area around the colony indicated the positive result. This medium was used to detect the proteolytic activity of bacteria (Karen and Roger, 2002).

●Gelatin liquefaction medium:

It has been prepared by adding 120 grams of gelatin in 1000 ml of warm (50°C) nutrient broth, heated to boiling to dissolve the medium completely and then dispensed into test tubes. Sterilized by autoclaving at 15 lbs

pressure (121°C) for 15 minutes. Allow the tubed medium to cool in an upright position. This medium was used for testing the ability of bacteria for gelatin liquefaction (Isenberg and Garcia, 2004).

●Luria-Bertani broth (LB):

This medium was prepared by dissolving 10g of trypton, 5g yeast extract, 10gm NaCl in 1000ml distilled water, the pH was adjusted to 7.2 then sterilized by autoclave at 121ºC for 15 minutes. This medium was used to cultivate bacterial isolates (Bertani, 2004).

●Luria-Bertani agar (LB):

Prepare LB medium as above for LB broth, but add 10 g/L agar before autoclaving, also it is used for cultivate bacterial strains (Bertani, 2004).

●Muller-Hinton agar medium:

Suspended 38 grams in 1000 ml distilled water, heated to boiling to dissolve the medium completely. Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes, it is used in anti-microbial susceptibility testing (Wood and Washington, 1995).

2.5. Collection of specimens:

The specimens were collected in proper ways to avoid any possible contamination (Collee *et al.,* 1996). Urine samples were generally collected from patients suffering from UTIs (catheterized and non-catheterized patients). Mid-stream urine samples were collected in sterilized screw-cap containers, then the urine samples were inoculated on culture media and incubated aerobically at 37°C for 24h.

2.6. Preservation of bacterial isolates:

The bacterial isolates were preserved on nutrient and brain heart infusion agar slants at 4ºC for month culturing on new culture media. The bacterial isolates

also were preserved for long time in BHI broth supplemented with 15% glycerol then stored at -20ºC for 6-8 months (Collee *et al.*, 1996).

2.7. Laboratory diagnosis:

2.7.1. Bacterial identification assays:-

According to the diagnostic procedures recommended by MacFaddin (2000), and Benson (2001), the isolation and identification of *P. vulgaris* associated with patients under study were performed as follows:

- **Colonial morphology and microscopic examination:**

A single colony was taken from each primary positive culture. Its identification depended on the morphology properties (colony size, shape, color, swarming, odor, translucency, edge, and elevation of texture). The colonies were then investigated by Gram stain to observe bacterial cells. Specific biochemical tests were done to reach the final identification.

2.7.2. Biochemical tests:

The following biochemical tests were performed for the identification of *P. vulgaris* isolates from others. These tests were carried out according to (MacFaddin, 2000) includes:-

Catalase test, Oxidase test, Indol test, Methyl–red test, Vogues –Proskauer test, Citrate utilization test, Gelatinase test, Motility test, Kligler's Iron Agar test.

2.7.3. Virulence factors assay:

- **Haemolysin production:**

Most haemolytic bacteria were assayed by culturing on 5% sheep blood agar at 37°C for 24h. Blood Agar considered as differential medium for hemolysis, based on the ability to break down hemoglobin or red blood cells, 3 groups of microorganisms can be described; alpha-hemolysis (α-hemolysis) a green to light-brown halo is seen around the colonies, Beta-hemolysis (ß- hemolysis) a clearing zone is seen around the colonies, and Gamma-hemolysis (γ- hemolysis) no hemolysis is observed (Dulczak and Kirk, 2005).

●Urease production

In this test we inoculated the urea slant from bacterial suspension by streaking the entire slant surface, incubated the tubes with loosened caps at 37^{o}C then color change of medium was examined after 16h incubation. Urease production was indicated by changed medium color into pink (Winn *et al*., 2006).

●Extracellular protease production:

M_9 media supplemented with 2% agar was used for detecting protease enzyme. After sterilization in autoclave and cooling at 50^{o}C, 0.25gm\L glucose (sterilized by filtration) was added, and the medium was then supplemented by 1% gelatin. After the inoculation of this media with bacterial isolates and incubation at 37^{o}C for 24 hr; 3ml of (5%) trichloroacetic acid was added to precipitate the protein. The formation of transparent area around the colony indicated a positive result (Benson, 1998).

2.7.4. Antibiotic sensitivity test:

The antibiotic sensitivity test of the isolates were determined, using Bauer Kirby disc diffusion method on Mueller-Hinton agar, in this test small filter paper disks (6 mm) impregnated with a standard amount of antibiotic are placed onto an agar plate to which bacteria (The inoculate was prepared directly from an overnight agar plates adjusted to 0.5 McFarland standard of National Committee for Clinical Laboratory Standards (NCCLS) (Watts and Lindeman, 2006) have been swabbed. Then the plates are incubated overnight at 37^{o}C, and the zone of inhibition of bacterial growth is used as a measure of susceptibility, where large zones of inhibition indicate that the organism is susceptible, while small or no zone of inhibition indicate resistance. An interpretation of intermediate is given for zones which fall between the accepted cutoffs for the other interpretations (Alan* **et al., 2006).***

2.7.5. Identification of bacteria by Vitek system

Identification of microorganisms is also accomplished by biochemical methods in Vitek system **(**Pincus, 1998).

●Suspension Preparation

A sterile swab or applicator stick is used to transfer a sufficient number of colonies of a pure culture and to suspend the microorganism in 3.0 ml of sterile saline (aqueous 0.45% to 0.50% NaCl, pH 4.5 to 7.0) in a 12 x 75 mm clear glass (polystyrene) test tube. The turbidity of suspension is adjusted according to McFarland turbidity range for Gram negative (0.50-0.63) and measured using a turbidity meter called the Densichek.

● **Inoculation**

A turbidometrically controlled suspension of pure colonies in saline is inoculated into identification cards. These cards contain different bio-chemical broths in reaction cells and one negative control cell to assess growth and viability of the suspension. Identification cards are inoculated with microorganism suspensions using an integrated vacuum apparatus. A test tube containing the microorganism suspension is placed into a special rack (cassette) and the identification card is placed in the neighboring slot while inserting the transfer tube into the corresponding suspension tube. The filled cassette is placed manually into a vacuum chamber station. After the vacuum is applied and air is re-introduced into the station, the organism suspension is forced through the transfer tube into micro-channels.

Conventional catalase, coagulase, and oxidase tests (where appropriate) and the results of a Gram stain are required before inoculation of cards.

● **Card Sealing and Incubation**

Inoculated cards are passed by a mechanism, which cuts off the transfer tube and seals the card prior to loading into the carousel incubator. All card types are incubated on-line at 35.5 $\pm$ 1.0ºC. Each card is removed from the carousel incubator once every 15 minutes, transported to the optical system for reaction readings, and then returned to the incubator until the next read time. Data are collected at 15-minute intervals during the entire incubation period.

Incubation times vary from two to 15hours depending on the growth rate of the organism. The Vitek programmed computer determines whether each well is positive or negative by measuring light attenuation with an optical scanner. When the incubation period is completed, the reactions are

analysed automatically and the identification is printed.

2.7.6. Mannose-resistant haemagglutination assay (MRHA)

The haemagglutination was detected by clumping of erythrocytes by fimbriae of bacteria in the presence of D-mannose. This test was carried out as per the direct bacterial haemagglutination test - slide method and mannose-resistant haemagglutination tests (Old and Adegbola, 1985).The strains of *P.vulgaris* were inoculated into 1% nutrient broth and incubated at 37°C for 48 hours for full fimbriation. A panel of red blood cells was selected by obtaining blood from human (blood group 'O'). The red blood cells which were then washed thrice in normal saline and made up to a 3% suspension in fresh saline. They were used immediately or within a week when stored at 3-5 °C. One drop of the RBC suspension was added to a drop of the broth culture and slide was rocked to and fro at room temperature for 5 minutes. Mannose-sensitive haemagglutination was detected by the absence of haemagglutination in a parallel set of test in which a drop of 2% w/v d-mannose was added to the red cells and a drop of broth culture. Mannose-resistant haemagglutination was detected by the presence of haemagglutination of 3% 'O' group human RBC in the presence of 2% mannose (Vagarali *et al*., 2008).

Bacterial agglutination of tannic acid treated human erythrocytes (MR/K agglutination) was performed as previously described by Ong *et al*. (2010) to detect the expression of the expression of Type 3 fimbriae.

2.7.7. Inhibition of swarming by (Resveratrol, p-nitrophenylglycerol, Fatty acids, Urea, Sodium azide, and Ethanol):

● **Swarming behavior assay**

The swarming migration distance assay was performed as described previously (Liaw *et al.*, 2001; wang *et al*., 2006). Briefly, an overnight *P. vulgaris* culture (5µl) was inoculated centrally onto LB swarming agar plates (2 %w/v) with different concentrations of anti-swarming agents:(0, 10, 20, 30, 40, 50, and 60µg/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) of fatty acids (Myristic acid); (0 , 0.5 ,1 ,1.5 ,2 , 2.5 and 3 v/v) of urea ;(0, 0.01, and 0.1) of sodium azide ,and other plate adding to its cover 2 drops of ethanol 90%. Then the plates were incubated at 37ºC and the swarming migration distance was determined by measuring the swarm fronts of the bacterial cells after inoculation.

● **Measurement of growth rates**

Overnight *P. vulgaris* culture was diluted 1:100 in fresh LB broth containing different concentrations of resveratrol (0, 10, 20, 30, 40, 50, and 60µg/ml) and (0, 50, 100, 150, and 200 µg/ml) of P-nitrophenyl glycerol. We also used other high concentrations of resveratrol (0.5, 1, 1.5, 2, and 2.5, 3 mg/ml) to determine the effect on the growth inhibition of *P. vulgaris*. The growth rate was monitored as OD_{600} at 1h intervals (Wang *et al*., 2006).

● **Measurement of cell length**

Measurement of cell length was performed as described by (Liaw *et al.*, 2004; Tegos *et al.*, 2006). Briefly, 150µl of stationary-phase LB cultures were spread onto LB agar plates without or with appropriate resveratrol and p-nitrophenylglycerol, then incubated at 37°C for various times. After incubation, cells from the entire surface of agar plates were harvested by washing into 5 ml of phosphate buffer saline (PBS). Bacterial cells were

fixed and subjected to Gram stain examined by light microscopy at a magnification of 1000X, and photo digitalized using a digital camera. The lengths of 100 cells in each sample were determined, and the average was calculated.

2.8. Detection of quorum sensing in *P.vulgaris*:-

●Procedure:

The detection of quorum sensing was carried out according to (Sabri, 2011; Abdul-Lateef, 2011).

1- LB medium was prepared and supplemented with 1% aspartic acid and distributed in six flask.

2- After inoculation with *P. vulgaris*, the flasks were incubated at 37°C for intervals (2, 3, 4, 5, 6, and 24hr).

3- At the end of each interval, KCN (0.01%) was added and then after 18hr the medium was filtered by millipore filter (0.4µm) and then the filtrate was used for quorum sensing detection.

3- The quorum sensing test was done on slide through mixing one drop of filtrate and one drop of fresh bacterial growth , and then stained with gram stain and the slide was examined by microscope.

4- The positive result was scored due to the presence of aggregation of bacterial cells.

●Chemical detection of homoserine lactone:

Homoserine production was checked through the separation of filtrate from culture media, and then the supernatant was dialyzed versus LB free of KCN. After 24 hours the dialyzed homoserine containing media were inoculated by *P.vulgaris* again for 24 hour and then Brands test was used to detect methionine or homocystein synthesis through conversion of homoserine to homocysteine (Sabri, 2011; Abdul-Lateef, 2012).

●**Brands test:-**

Reaction of sodium nitroprusside with sulfhydryl componud (e.g cysteine, homocysteine) yeilds rose or purple red complex products (Navaza *et al.,* 1989).

2.9. Genotyping assays:

2.9.1 DNA extraction from Gram's negative bacteria:

This method was made according to the genomic DNA purification Kit supplemented by the manufacturing company (Promega, USA).

- One ml of an overnight culture was added to 1.5ml micro centrifuge tube.
- The tube was centrifuged at 13.000rpm for 2 min to pellet the cells and then remove the supernatant.
- A 600µl of nuclei lysis solution was added, gently pipette until the cells are re-suspended.
- To lyse the cells the tubes incubated at 80ºC for 5 min., and then cool to room temperature.
- Three µl of RNase solution was added to the cell lysate. Inverted the tube 2-5 times to mix.
- The tube incubated at 37ºC for 15-60 min. and the sample cooled to room temperature.

- A 200µl of protein precipitation solution was added to the RNase. Cell treated lysate. Vortex vigorously at high speed for 20 seconds to mix the protein precipitation solution with the cell lysate.
- The sample was incubated on ice for 5 min.
- The tube was centrifuged at 13.000rpm for 3min.
- The supernatant containing the DNA was transferred to a clean 1.5 ml micro centrifuge tube containing 600µl of room temperature isopropanol.
- Gently mixed by inversion until the thread-like strands of DNA form a visible mass.
- The tube was centrifuged at 13.000rpm for 2min.
- The supernatant were poured off carefully and the tube drained on clean absorbent paper. Added 600µl of room temperature 70% ethanol and gently inverted the tube several times to wash the DNA pellet.
- The tube was centrifuged at 13.000rpm for 2min. carefully aspirated the ethanol.
- The tube was drained on clean absorbent paper and allowed the pellet to air-dry for 10-15min.
- One hundred µl of DNA rehydration solution was added to the tube and rehydrated the DNA by incubation at 65^{o}C for 1hrs.
- The DNA was stored at 2-8^{o}C.

Protocol of Illustra bacteria genomicPrep Mini Spin Kit for purification of genomic DNA from Gram-negative bacteria:

1. Harvesting of Bacterial Culture

a. Transferred 1 ml overnight bacterial culture (A 600 $\leq$ 4.0) to a 1.5 ml micro centrifuge tube.

b. Spun for 30 seconds at full speed (16000 ×g) in a microcentrifuge. Removed as much of the supernatant as possible by aspiration without disturbing the cell pellet.

2. Lysis

a. Added 40µl of Lysis buffer type 2 and immediately mixed by vortexing.

b. Added 10 µl of Proteinase K (20 mg/ml) to the sample and mixed by vortexing for 10 seconds.

c. Added 10µl of Lysis buffer type 3 to the sample and mixed by vortexing for 10 seconds.

d. Spun 5 seconds at 1000 ×g to collect sample in bottom of tube.

e. Incubated the sample for 7 minutes at 55°C.

f. Vortex and spun 5 seconds at 1000 ×g to collect sample in bottom of tube. Continue incubation for a further 8 minutes.

g. Spun 5 seconds 1000 × g to collect sample in bottom of tube.

3. **RNA Removal**

a. Added 5µl of RNaseA (20 mg/ml) to the sample and mixed by vortexing for 10 seconds. Spun 5 seconds at 1000 ×g to collect sample in bottom of tube.

b. Incubated at 15 minutes at room temperature.

4. Purification

a. Added 500µl of lysis buffer type 4 to the sample and mixed by vortexing 10 seconds.

b. Incubated the sample for 5 minutes at room temperature.

c. Vortex and spun for 5 seconds at 1000 ×g to collect sample in bottom of tube. Continued incubation for a further 5 minutes.

d. For each purification that is to be performed, placed one bacteria mini column inside one collection tube.

e. Applied each sample to a separate column.

f. Spun for 1 minute at 11 000 ×g.

g. Discarded the flow through by emptying the Collection tube. Placed the column back inside the collection tube.

5. Wash & Dry

a. Added 500 µl of Lysis buffer type 4 to the column.
b. Spun for 1 minute at 11000 ×g.
c. Discarded the flow through by emptying the collection tube. Placed the column back inside the collection tube.

d. Added 500µl of Wash buffer type 6 to the column.
e. Centrifuged for 3 minutes at 16 000 ×g.
f. Discarded the collection tube and transferred the column to a fresh DNase free 1.5 ml micro-centrifuge tube.

6. Elution

a. Applied 200 µl of pre-heated Elution buffer type 5 directly to the top of glass fiber matrix in the column.

b. Incubated the sample for 1 minute at room temperature.
c. Spun for 1 minute at 11000 ×g to recover the purified genomic DNA as flow through.

d. Proceeded to downstream application. Stored purified genomic DNA at -20°C.

2.9.2 Detection of some virulence factors and antibiotics resistance genes by PCR:-

The PCR amplification mixture has been prepared according to the manufacturer's instructions, by the following steps.

A. Primers

The *Proteus vulgaris* virulence factors and antibiotics resistance genes primers were designed in this study using NCBI GenBank and MP Primer design online. The GenBank: (*ureABC* gene: X51816.1)(Moersdorf and Kaltwasser,1990) (*hly* gene: X12571.1) (Koronakis and Hughes,1988) (*fla* gene: AF221596.1) (Belas and Flaherty,1994) (*ZapA*: U25950) (Wassif *et*

al.,1995), (*bla*TEM gene: GQ983321.1) (Amador *et al*., 2011), and (*qnr* gene: EF488761.1) (Chen *et al*., 2008).

Table (2-7): Primers used in PCR to amplify virulence genes and antibiotics resistance genes

Target gene	Sequence(5′-3′)		Product size
ureABC gene	F*	5'-TGCGGGAATGGCGGTTCGTT-3'	489bp
	R*	5'-GCCAGCAACGACTTCAGTGCCA-3'	
hly gene	F	5'-TCCATTGGAGGTCCTTGGGCAT-3'	120bp
	R	5'-GCATATTGGTTAGCTCGTATTGCAGGT-3'	
fla gene	F	5'-AGGTTGTATCTGGGGTGCCGA-3'	247bp
	R	5'-GCTTGACCCGCTGCATCGTCTT-3'	
ZapA gene	F	5'-TGATCCCAATGGCACATTTCAGGC-3'	308bp
	R	5'-CTTGGAAGTGGGCGCCTGTT-3'	
BlaTEM gene	F	5'-AGAGCAACTCGGTCGCCGCATA-3'	310bp
	R	5'-GCGCAACGTTGTTGCCATTGCT-3'	
qnr gene	F	5'-ACGCCAGGATTTGAGCGACAGC-3'	410bp
	R	5'-CGCTGAGGTTGGCATTGCTCCA-3'	

***F: Forward Primer, R: Reverse Primer**

B- The reaction mixture:

Amplification of DNA was carried out in a final volume of 25µl as indicate in table (2-8).

Table (2-8) Contents of the reaction mixture

No.	Contents of reaction	Volume
1.	Accustart ™ Taq PCR SuperMix	12.5 µl
2.	Upstream primer (10pmole)	1µl
3.	Downstream primer (1opmole)	1 µl
4.	DNA template (50-500 ng)	2 µl
5.	Nuclease free water(ddH2O)	8.5 µl
Total volume		**25µl**

C- Thermal cycling conditions:

The reaction was performed in a PCR thermal cycler apparatus, and after several trials, and according to the manufacture's guide the following program was adopted:-

PCR consisted of a preheating at 95ºC after this initial denaturation step, the mixture was subjected to 30 amplification cycles as shown in table(2-9):-

Table (2-9):- PCR conditions used to amplify genes encoding virulence genes in *Proteus*.

Steps	Temperature	Time	No. of cycles
Initial denaturation	95ºC	5 min	1
Denaturation	95ºC	45 sec	30
Annealing	62 ºC	30 sec	
Extension	72ºC	45sec	
Final extension	72ºC	7 min	1

2.9.3 Detection of fimbrial genes by PCR:

The primers used in this study were synthesized by Bioneer Company and are described in table (2-10). Primers used to amplify *mrp* were derived from the published *mrp* fimbrial operon sequence from strain HI4320; GenBank accession no. Z32686 (Bahrani and Mobley, 1994; Sosa *et al*., 2006). Primers used to amplify *uca* were derived from the published *uca* sequence from strain HU1069; GenBank accession no. U28420 (Cook *et al*., 1995; Sosa *et al*., 2006). *Atf* Primers were derived from the published sequence of the *Atf* fimbrial operon from strain HI4320, accession number z78535 (Massad *et al*., 1996; Zunino *et al*., 2000). Whereas, *mrkA* primers derived from an internal region of *mrkA* (416bp) gene fragments which deposited in GenBank under the accession numbers: FJ96754, FJ96756-FJ96774, and FJ96777-FJ96789 (Jeanmougin *et al*.,1998; Ong *et al*.,2010). The primers and PCR conditions used to amplify genes encoding fimbriae with PCR are listed in table (2-10).

Table (2-10):- Primers sequences and PCR conditions used to amplify fimbrial genes of *Proteus*.

Genes	Primer sequence (5′-3′)		Size of product bp	PCR condition
mrp	F R	5′ -ATTTCAGGAAACAAAAGATG-3′ 5′ -TTCTTACTGATAAGACATTG- 3′	565bp	94ºC 3min 1x 94ºC 30 s 40ºC 30 s 30x 72ºC 30 s 72ºC 7min 1x
mrkA	F R	5′ -AGAGTTTGATCMTGGCTCAG-3′ 5′-AAGGAGGTTATCCANCCRCA-3′	416bp	94ºC 3min 1x 94ºC 30 S 45ºC 30 S 30x 72ºC 30 S 72ºC 7min 1x
atf	F	5′-	382bp	94ºC 3min 1x

	R	CATAATTTCTAGACCTGCCCTAGCA-3′ 5′ -CTGCTTGGATCCGTAATTTTTAACG-3′		94ºC 1min 50ºC 1min 30x 72ºC 1min 72ºC 5min 1x
uca	F R	5′ -AACCAGTTCCGCGTTGGCCTGG-3′ 5′ -CGGAACGGCCTGACGTTGCAT-3′	580bp	94ºC 3min 1x 94ºC 30 s 50ºC 30 s 30x 72ºC 30 s in 72ºC 7min 1x

***F: Forward Primer, R: Reverse Primer**

2.9.4. Genotyping of the genus *Proteus* by 16SrRNA and *rpoB* sequence analysis

Genomic DNA was extracted by a commercial nucleic acid extraction kit (Illustra bacteria genomic Prep. Mini Spin Kit) according to the manufacturer's instructions. PCR amplification was performed in a 25µl reaction volume as shown in Table (2-11).

Table (2-11) clarifies the primers sequences and PCR conditions which used to amplify a portion of the coding region of 16SrRNA and the *rpoB* gene.

Table (2-11):- Primers sequences and PCR conditions for 16SrRNA and *rpoB genes* for *P. mirabilis* and *P. penneri*.

Genes	Primer sequence (5′-3′)		Size of product bp	PCR condition	Reference
16SrRNA	27 F 1492R	5′-AGAGTTTGATCMTGGCTCAG-3′ 5′- ACG GCT ACC TTG TTA CGAC TT-3′	1400	94ºC 5min 1x 94ºC 1min 53ºC 1min 35x 72ºC 1:30min 72ºC 10min 1x	Rongrong *et al*.,2008

16SrRNA	27 F 1522R	5′-AGAGTTTGATCMTGGCTCAG-3′ 5′-AAGGAGGTTATCCANCCRCA-3′	1382	94ºC 5min 1x 94ºC 1min 57ºC 1min 35x 72ºC 1:30min 72ºC 10min 1x	Lee *et al*., 2003 and Nur,2012
rpoB	CM7 CM31bR	5′- AACCAGTTCCGCGTTGGCCTG G-3′ 5′ -CCTGAACAACACGCTCGGA-3′	1088	94ºC 5min 1x 94ºC 1min 46ºC 1min 40x 72ºC 1:30min 72ºC 10min 1x	Mollet *et al*.,1997
rpoB	CM7F CM32bR	5′ -AACCAGTTCCGCGTTG GC CT GG-3′ 5′- CGGAACGGCCTGACGTTGCAT-3′	930	94ºC 5min 1x 94ºC 1min 53ºC 1min 40x 72ºC 1:30min 72ºC 10min 1x	Mollet *et al*.,1997

***F: Forward Primer, R: Reverse Primer**

New primers (Table 2-12) have been designed to amplify *P. vulgaris* 16SrRNA gene on the basis of *P. mirabilis* and *P. penneri* 16SrRNA gene alignment by manual searching for areas of sequence conservation across two species. Given that no regions of sufficient length with 100% complementarity exist in the alignment. Primers were designed with melting temperatures between 50 and 60°C (Baker *et al.*, 2003).

Table (2-12):- Designed primers sequences and PCR conditions for *P. vulgaris* 16SrRNA gene

Genes	Primer sequence (5′-3′)		Size of product bp	PCR condition
16SrRNA	F1 R1	**5′-** CCG AAG GTT AAG CTA CCT AC-**3′** **5′** -CCA TGT GTA GCG GTG AAA TG-**3′**	1400	94ºC 5min 1x 94ºC 1min 47ºC 1min 35x 72ºC 1:30min 72ºC 10min 1x
16SrRNA	F1	**5′-** CTA GCG ATT CCG ACT TCA	800	94ºC 5min 1x

	R1	TG-**3′** **5′** -CGG TCG ATT TAA CGC GTT AG-**3′**		94ºC 1min 48ºC 1min 35x 72ºC 1:30min 72ºC 10min 1x

***F: Forward Primer, R: Reverse Primer**

2.9.5 Detection of amplified products by agarose gel electrophoresis:

Successful PCR amplification was confirmed by agarose gel electrophoresis (Sambrook and Russell, 2001).The PCR products were assessed by electrophoresis in 1% agarose gel with 0.5% ethidium bromide. Agarose gel was prepared by dissolving 0.45gm of agarose powder in 40ml of TBE buffer (pH: 8) in Microwave, allowed to cool to 50ºC and then ethidium bromide at the concentration of 0.5mg/ml was added .

The comb was fixed at one end of the tray for making wells used for loading DNA samples. The agarose was poured gently into the tray, and allowed to solidify at room temperature for 30 min. The comb was then removed gently from the tray. The tray was fixed in an electrophoresis chamber filled with TBE buffer that covered the surface of the gel, 5µl of DNA sample mixed with Gel Loading Dye Blue was transferred into the wells in agarose gel, and in one well we put 1µl of DNA ladder.

The electric current was allowed at 110 volt for 60min. UV Transilluminater was used for the observation of DNA bands, and then gel was photographed using a Gel Documentation system with a digital camera.

2.9.6 DNA extraction from the gel:

This method was carried out according to QIAquick Spin Handbook protocol supplemented by the manufacturing company of QIAquick Gel Extraction Kit (Qiagen-, USA).

1. Excised the DNA fragment from the agarose gel with a clean, sharp scalpel.

Minimized the size of the gel slice by removing extra agarose.

2. Weigh the gel slice in a colorless tube. Added 3 volumes of Buffer QG to 1 volume of gel.

3. Incubated at 50°C for 10 min (or until the gel slice has completely dissolved). To help dissolve gel, mixed by vortexing the tube every 2–3 min during the incubation.

4. After the gel slice has dissolved completely, checked that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose).

5. Added 1 gel volume of isopropanol to the sample and mixed.

6. Placed a QIAquick spin column in a provided 2 ml collection tube.

7. To bind DNA, applied the sample to the QIAquick column, and centrifuged at ≥10,000 xg (~13,000 rpm) for 1 min.

8. Discarded flow-through and placed QIAquick column back in the same collection tube.

9. Added 0.5 ml of Buffer QG to QIAquick column and centrifuged for 1 min. This step will remove all traces of agarose. It is only required when the DNA will subsequently be used for direct sequencing, in vitro transcription or microinjection.

10. To wash, added 0.75 ml of Buffer PE to QIAquick column and centrifuged at ≥10,000 x g for 1 min (let the column stand 2–5 min after addition of Buffer PE, before centrifuging).

11. Discarded the flow-through and centrifuge the QIAquick column for an additional 1 min at ≥10,000 x g. Residual ethanol from Buffer PE will not be

completely removed unless the flow-through is discarded before this additional centrifugation.

12. Placed QIAquick column into a clean 1.5 ml microcentrifuge tube.

13. To elute DNA, added 50 µl of Buffer EB (10 mM Tris. Cl, pH 8.5) or H2O to the center of the QIAquick membrane and centrifuge the column for 1 min at maximum speed.

2.9.7 Sequencing and phylogenetic analysis by 16SrRNA and *rpoB* genes

Partial 16SrRNA (740-1294bp) and *rpoB* (400-641bp) gene nucleotide sequences were obtained from the PCR amplicons generated with the same primers mentioned above in table (2-11) for *P. mirabilis and P. penneri* and table (2-12) for *P. vulgaris* after diluting them to make a 3.2pmol/µl working solution .The PCR products of 16SrRNA and *rpoB* gene were sequenced by using the standard Big Dye Terminator v3.1 protocol. We used Big Dye Xterminator for clean-up and the reactions were run on the ABI 3730 Genetic Analyzer. DNA sequencing compared with those available in the GenBank database by using basic alignment search tool, BLAST of the NCBI to identify whether they aligned with closely related organisms (Lee *et al*., 2003; Adiguzel *et al*., 2009). Percentage nucleotide identity and pairwise uncorrected (p-distances were calculated for 16SrRNA and *rpoB* gene sequences of clinical strains of the *Proteus* examined by using Sequence alignments Explorer CLUSTAL (Felsenstein, 2006). Phylogenetic analysis was carried out using the software MEGA version 4.1(Tamura *et al*., 2007) using the neighbour-joining method to reconstruct the phylogenetic tree with the option of complete deletion of gaps (Felsenstein, 2006; Tamura *et al*., 2007).

2.10. Statistical Analysis

Data were statistically described in terms of frequencies (number of cases) and relative frequencies (percentages) .All statistical calculations were done using Microsoft Excel 2013 (Microsoft Corporation, New York, USA) or Quick Calcs Online calculators for scientists (Graph pad software Inc., San Diego, CA, USA).

The data obtained from testing the effect of anti-swarming agents were expressed as means and means ± standard deviation, and the data analyzed by one-way analysis of variance with $P < 0.05$ being significant, calculated using the GraphPad Prism 5 statistical software.

Percentage of similarities for partial *rpoB* and 16SrRNA sequences obtained in this study were calculated by the FASTA method. Multi alignments were obtained with alignments Explorer CLUSTAL (Felsenstein, 2006).

Phylogenetic analysis was carried out using the software MEGA version 4.1(Tamura *et al*., 2007).

The statistical significance of the *rpoB* phylogenies inferred was estimated by bootstrap analysis with Bootstrap (500 replicates; seed=31332).

3.1. Isolation of *Proteus vulgaris*:

A total of 150 urine specimens obtained from patients (of both gender) with UTIs, Only 28 isolates (18.7%) were identified as *P. vulgaris*, whereas the rest 122 isolates (81.3%) belong to other type of bacteria as shown in table (3-1).

Table (3-1): Distribution of bacterial isolates from urine specimens

Name of Isolates		

	No. of isolates		Isolates %
	Sources of isolates		
	Urine from Non –Catheterized Patients	Urine from Catheterized Patients	
Proteus vulgaris	28		28(18.7%)
P. mirabilis	11		11(7.3%)
P. penneri	4		4(2.7%)
Other bacteria	82	25	107(71.3%)
Total	**125**	**25**	**150(100%)**

Data from table (3-1) indicates that other bacteria (71.3%) were isolated from urine specimens of catheterized and non-catheterized patients, followed by *P. vulgaris* (18.7%), *P. mirabilis* (7.3%) and then *P. penneri* (2.7%). However, there is no *Proteus species* isolated from catheterized patients. The reason of this finding may be due to that other bacteria such as *E. coli* causes > 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. The gram-negative species *Klebsiella*, *Proteus*, *Enterobacter*, and *Serratia* account for about 40%, and the gram-positive bacterial cocci *E. faecalis* , *S. saprophyticus* and *S. aureus* account for the remainder (Stephen and Maxine,2009; Stewart, 2010).

The results of this study are identical with those obtained by AL- Jumaa *et al*. (2011) who have isolated *Proteus* spp. from urine samples at rate of (33.33%) and by AL-Ta'ee, (2002) who has succeeded to isolate *Proteus* from urine sample at rate reaching (38%).

Laftaa, (2001) has also isolated *Proteus* from patients with urinary tract infection at a rate of (37.33%), but AL-Grawy, (1999) has isolated *Proteus* from urine at rate of (6%) only.

3.2. Virulence genes of the bacterial isolates:

3.2.1. Haemolysin production

Because iron acquisition is essential to the survival of invasive strains of *P. vulgaris*, the production of iron acquisition system, hemolysin gene was tested in *P. vulgaris* isolated from urine samples.

The region of *hly* gene was amplified by PCR using *hly* primer pairs. *Hly gene* associated with pathogenicity islands was present in all isolates (100%) and the band size of amplicon was 120bp, the results show in figure (3-1).

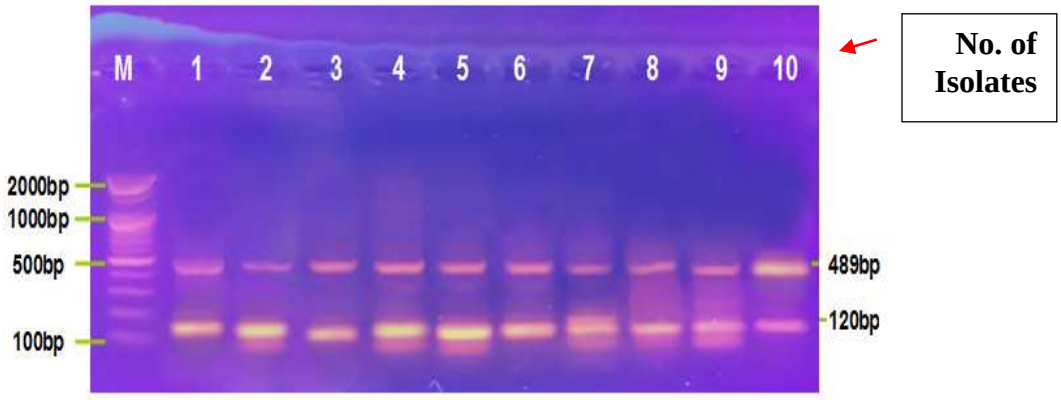


Fig. (3-1):- Gel electrophoresis of PCR of *hly* and *urease (ureABC)* amplicon product. M: Marker; 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10: no. of *P. vulgaris* isolates, *hly* gene amplicon size: 120bp and *ureABC* gene amplicon size: 489bp.

Thermocycling parameters were as follows: an initial denaturation of 95 °C for 5 min, 30 cycles of denaturation at 95 °C for 45s, primer annealing 62 °C for 30s, and extension at 72 °C for 45s.Finally one extension step at 72°C for 7 min. The electrophoresis was carried at 1% agarose gel and the electric current was allowed at 110 volt for 60min.

These positive isolates with *hly* gene were also checked to confirm their ability to produce extracellular hemolysin on blood agar and it was found all of the isolates (100%) have the ability to produce extracellular hemolysin.

These results agree with the results of Bahraini *et al*. (1991), Sosa *et al*. (2006) and AL-Jumaa *et al*. (2011) who demonstrated that all isolates (100%) of *Proteus* isolated from different clinical sources exhibit hemolysis on blood agar plates, but Mishara *et al*. (2001) found that (85.14%) of *Proteus* isolates produce β-hemolysis while other isolates produce α – hemolysis on blood agar plate. Other result reported by Shahla and Farehnaz (2009) referred to all *P. vulgaris* strains have haemolytic activity but at a significantly lower level than the *P. mirabilis* strains. Thus, *P. vulgaris* strains were much less efficient in cell invasion than *P. mirabilis* strains.

Hemolysin is virulence associated factor for many bacterial species including *Proteus* (Shahla and Farehnaz, 2009). The function of hamolysin is to form pores in target host cells (Chalmeau *et al*., 2011). It has been puorposed that hemolytic activity helps *Proteus* spread into the kidneys during infection (Coker *et al*., 2000). This is probably mediated through the increased ability of hemolytic *Proteus* cells to invade host tissue (Morgenstein, 2006).

3.2.2 Urease production

Urease is a hallmark of infections with *Proteus* spp. and it is considered as one of the most important virulence factor of *Proteus*. So, in this study, it has been designed specific primers to amplify urease gene (*ureABC*) from *P. vulgaris* isolates by PCR. The results showed all of the isolates gave *ureABC* amplicon product with size 489bp (fig.3-1), where the detection of urease gene and *hly* gene was carried out at the same time because they have the same conditions in PCR.

These results were more confirmed by detection of urease in those isolates phenotypically, where it was found all of them (100%) gave positive result for extracellular urease.

The results of this study are in agreement with the results of Mobley and Chippendle (1990), Sosa *et al*. (2006), El-Baghdady *et al*. (2009) and Al-Jumaa *et al*. (2011) who found (100%) of *Proteus* isolates showed strong production of urease, also this result was similar to previous reports by Mishara *et al.* (2001) and Gendlina *et al.* (2002) who found that *Proteus* spp. produced high amount of urease compared with other bacteria.

A major complicating factor associated with UTI caused by *Proteus species* is urolithiasis, or the formation of stones within the urinary tract. Stone production is a result of the action of the bacterial enzyme urease. Urease catalyzes the hydrolysis of urea, liberating ammonia and carbon dioxide and increasing the pH of urine. As a result of the pH increase, normally soluble minerals precipitate, namely magnesium, ammonium and calcium phosphate, leading to the formation of struvite and apatite crystals (Nielubowicz, 2010; Griffith *et al*., 2011).

3.2.3 Extracellular protease production

The ability of *P. vulgaris* to produce extracellular protease in M_9 media (supplemented with 20% glucose and 1% gelatin) was investigated, and it was found all of these isolates had the ability to produce extracellular protease after 24 hours of incubation. A clear hallo of transparent area was detected around the colony after the addition of 3 ml of 5% trichloroacetic acid.

Also, it has been tested the presence of *zapA* gene in these isolates that gave positive results for extracellular protease by designed specific primers for protease gene and we have got positive results in 9 isolates (90%) out of 10 selected isolates and the amplicon size of the amplified gene was 308bp ,the results show in figure(3-2).

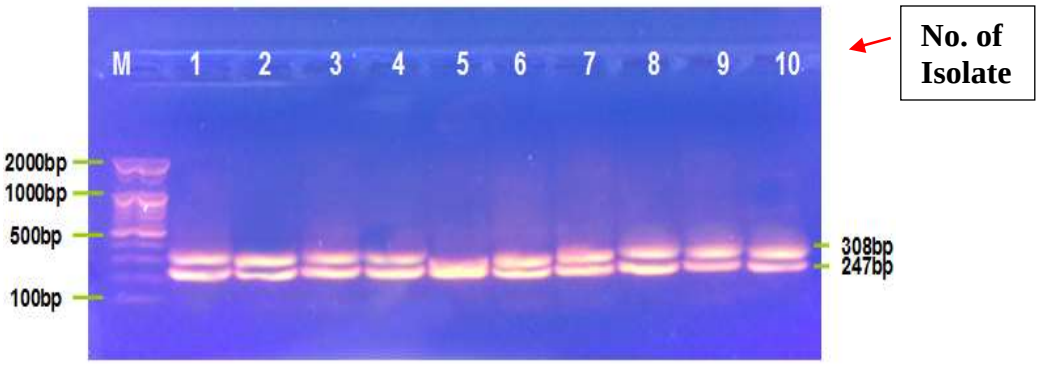


Fig (3-2):- Gel electrophoresis of PCR of *zapA* and *fla* amplicon product. M: Marker; 1, 2, 3, 4, 5, 6, 7, 8, 9, 10: no. of *P. vulgaris* isolates, *zapA* gene amplicon size: 308bp and *fla* gene amplicon size: 247bp.

Thermocycling parameters were as follows: an initial denaturation of 95 °C for 5min, 30 cycles of denaturation at 95 °C for 45 s, primer annealing 62 °C for 30 s, and extension at 72 °C for 45s.Finally one extension step at 72°C for 7 min**.** The electrophoresis was carried at 1% agarose gel and the electric current was allowed at 110 volt for 60min.

As shown in figure (3-2), only one isolate did not give a positive result for *ZapA* although the same isolate gave positive result for extracellular protease production .This means that there are another genes involved in the production of protease.

The results of our study were identical with the results obtained by Coker *et al*. (2000) and Al-Jumaa *et al*. (2011) who referred to that (100%) of *Proteus* species produced protease, but they were not agree with the results obtained by EL-Baghdady *et al*. (2009) who reported that *Proteus* isolates did not show any protease activities.

The protease of *P. vulgaris* produces is a metalloprotease of the serralysin family of zinc proteases, encoded by *zapA*. The C-terminal end of *ZapA* contains a motif indicating it is probably exported by a member of the ABC transporter superfamily of transporters (Ruiz *et al*., 2005). Alkaline pH is optimal for the activity of many of these types of proteases, which is often present due to the urease activity of *P. vulgaris*. Unlike most IgA proteases,

which only cleave IgA at the hinge region, *ZapA* completely degrades IgA (Nielubowicz, 2010). *ZapA* has also been shown to be able to cleave many other proteins found in the urinary tract, including: complement components, cytoskeletal elements, and antimicrobial peptides (Aneas *et al*., 2001).

Similar to other toxins produced by *Proteus*, *zapA* production is increased in swarmer cells (Poore and Mobley, 2003; Alamuri and Mobley, 2008). During infection, *ZapA* is produced and active, causing the degradation of IgA *in vivo*. *ZapA* has also be shown to be needed for infection in the ascending UTI model of infection (Nielubowicz, 2010).

3.2.4 Flagellin

The biosynthesis of flagella is a key process in both motility and swarming and involves numerous genes on the *Proteus* chromosome (Belas and Flaherty 1994; O 'May *et al*., 2008).

It used specific primers to amplify *fla* gene by PCR and it has been detected the presence of this gene with amplicon size of 247bp in all *P. vulgaris* isolates and these results are more clarified in the above figure (3-2).

Flagellin is encoded by *flaC*, and the *flaD* gene encodes for the flagellar filament capping protein (Belas, 1994). Studies suggest that the major flagellin protein for *Proteus* is subject to antigenic variation through homologous recombination as three copies of flagellin-determinant gene (*flaA, flaB, flaC*) that reside on the *Proteus* genome with only one copy that is actively expressed (O' May *et al*., 2008).

One of the most surprising findings was that all flagellum-related genes are located together within a single locus, which is highly unusual. Another interesting feature is the copy of multiple genes encoding flagellin, *flaA* and *flaB*, which are located in direct proximity to each other (Nielubowicz,

2010). Normally, the *flaA* allele is expressed while *flaB* is silent. However, these genes can recombine, resulting in the formation of antigenically distinct flagella (Manos and Belas, 2004). Considering that flagellin is strongly antigenic, it has been postulated that this recombination could contribute to immune evasion during infection. So, flagellin gene rearrangement is a mechanism for host immune system evasion by *Proteus* and is extremely relevant for *Proteus* infections since flagella are highly immunogenic. As a result, any antigenic change could increase the survival of *Proteus* species in the urinary tract through the evasion of sIgA directed toward flagella during colonization in the bladder (Belas 1994; O'May *et al*., 2008).

3.2.5 Adhesive factors genes

Adhesion mediated by fimbriae is a crucial step for successful bacterial colonization of the urinary tract mucosa and has an important role in the pathogenesis of UTI. *Proteus*, a common uropathogen, can express several types of fimbriae simultaneously, including MR/P, MR/K, UCA and ATF fimbriae (Coker *et al*., 2000 ; Zunino *et al*., 2007).

In order to detect *mrp*, *mrkA*, *uca and atf* fimbrial genes, PCR was performed using designed specific primers and genomic DNA from each *P. vulgaris* isolate. PCR amplification of fimbrial genes indicated that all isolates carried those fimbrial genes. In all cases, *P. vulgaris mrp*, *mrkA*, *uca* and *atf* fimbrial genes amplified by PCR exhibited the predicted sizes (565, 416, 580, and 382bp respectively).These results show in figure (3-3), (3-4), (3-5) and (3-6) respectively.

P.vulgaris strains were examined for MR/P and MR/K fimbriae genotypically and also phenotypically by using haemagglutination assay (HA).

Historically, fimbriae of *Proteus spp.* were characterized by their hemagglutination (HA) properties and fell into two classes based on this activity: mannose-resistant/*Proteus*-like (MR/P) and mannose resistant / *Klebsiella*-like (MR/K). As the names imply, HA activity of both types of fimbriae was resistant to mannose (*i.e.*, addition of mannose did not inhibit HA); fimbriae with MR/P activity resulted in agglutination of fresh (but not tannin-treated) erythrocytes, while fimbriae with MR/K activity resulted in agglutination of tannic acid-treated erythrocytes (Nielubowicz, 2010; Rozalsk *et al*., 2012).

The results of haemagglutination assay showed all isolates (100%) agglutinate human red blood cells in the presence of mannose and that means all *P. vulgaris* isolates have *MRP* and *MRK* genes. These results were similar to that results reported by Mishara *et al*. (2001) who found (91) out of (148) (61.49%) *Proteus* isolates gave positive result of CFA in the presence of D- mannose. Also, they were in agreement with results of Sosa *et al*. (2006), who observed that every tested isolate of *Proteus* spp. was able to agglutinate fresh human erythrocyte and this reaction was not inhibited by D-mannose.

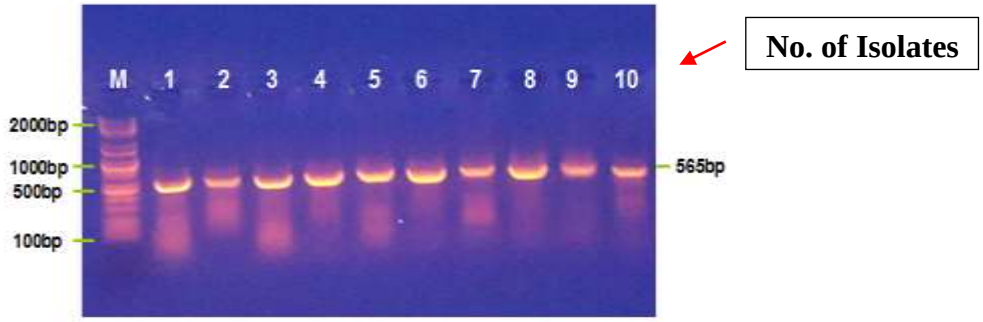


Fig (3-3):- Gel electrophoresis of PCR of *mrp* amplicon product. M: Marker, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10: no. of *P. vulgaris* isolates, *mrp* amplicon size: 565bp.

Thermocycling parameters were as follows: an initial denaturation of 94 °C for 3 min, 30 cycles of denaturation at 94 °C for 30 s, primer annealing 40 °C for 30 s, and extension at 72 °C for 30s.Finally one extension step at 72°C for 7 min. The electrophoresis was carried at 1% agarose gel and the electric current was allowed at 110 volt for 60min.

MR/P fimbriae is encoded by *mrp* operon containing 10 genes located on bacterial chromosome. The fimbrial genes are transcribed and MR/P fimbriae are synthesized when the promoter is in "on" orientation (Li *et al*., 2002; Nielubowicz and Mobley, 2010). In the "off" orientation the production of fimbriae is stopped. Expression of MR/P fimbriae is increased under oxygen limitation (Lane *et al*., 2009). These fimbriae contribute to biofilm formation and facilitate colonization of upper urinary tract and were found more often on bacterial strains which cause pyelonephritis (Jansen *et al*., 2004).

Initial studies revealed that sera from infected mice reacted strongly to MR/P fimbrial preparations, which indicates these fimbriae are expressed *in vivo* (Nielubowicz, 2010). The expression of MR/P fimbriae appears to be highly induced during infection (Li *et al*., 2002).Culture under oxygen limitation *in vitro* induces MR/P expression (Lane *et al*., 2009). Since the urinary tract is proposed to be oxygen-limited (Snyder *et al*., 2005), this condition could contribute to the upregulation of MR/P fimbriae observed *in vivo* (Nielubowicz, 2010).

Other studies have suggested that MR/P fimbriae play a role in the virulence observed during UTIs caused by uropathogenic *P. mirabilis* strains. In the mouse CBA model of ascending UTI, infection with *P. mirabilis* elicited a strong immune response to *mrpA*, the major structural subunit of MR/P fimbria, indicating that MR/P fimbriae were expressed in

vivo (Jacobsen *et al*., 2008). Isogenic mutants incapable of expressing MR/P fimbriae were attenuated when examined in this mouse model. A mutant constitutively expressing MR/P fimbriae outcompeted the wild-type strain in the murine bladder but not the kidneys in a co-challenge experiment, thereby establishing MR/P fimbriae as being an important bladder colonization factor for *Proteus spp.* (Li *et al*., 1999*;* Li *et al*., 2002). Tissue binding studies by Sareneva *et al*. (1990) revealed the propensity of this fimbrial type to adhere specifically to the human renal tubular epithelial cells and to the exfoliated uroepithelial cells of urinary sediment.

Other than MR/P fimbriae are known to a lesser extent MR/K fimbriae. MR/K hemagglutinins were found more frequently on *P. penneri* strains than on *P. vulgaris and P. mirabilis* strains and most probably they facilitated the adherence of bacteria to the urinary catheters (Jacobsen *et al*., 2008).Whereas, in the present study, it has been detected the presence of this type of fimbrial gene (*mrkA*) in all *P. vulgaris* isolates; the results show in figure (3-4).

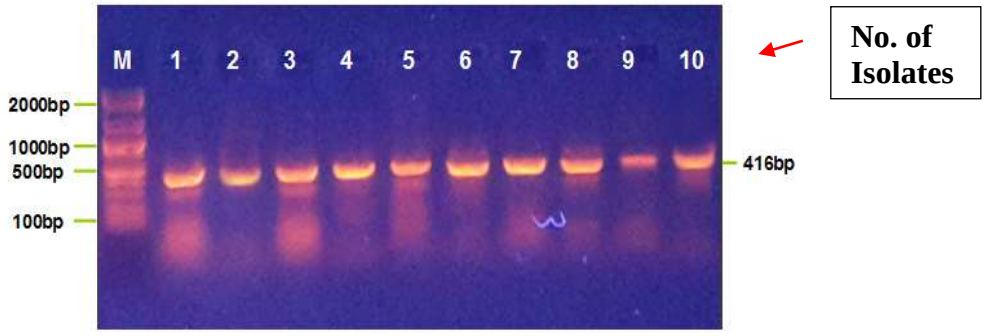


Fig (3-4):- Gel electrophoresis of PCR of *mrkA* amplicon product. M: Marker, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10: no. of *P. vulgaris* isolates, *mrkA* gene amplicon size: 416bp.

Thermocycling parameters were as follows: an initial denaturation of 94 °C for 3min, 30 cycles of denaturation at 94 °C for 30 s, primer annealing 45 °C for 30 s, and extension at 72 °C for 30s.Finally one extension step at 72°C for 7 min. The

electrophoresis was carried at 1% agarose gel and the electric current was allowed at 110 volt for 60min.

Several observations support a role for one of these hemagglutinins, MR/K (type3) fimbriae, in the virulence of uropathogenic *P.mirabilis* within the urinary tract. This species has been shown to adhere in vitro to human uroepithelial cells (Bahrani *et al*., 1993; Rocha *et al*., 2007).

A strain expressing the MR/K hemagglutinin was shown to bind specifically to components of sectioned human kidney, namely, Bowman's capsule of the glomeruli and tubular basement membrane (Sareneva *et al*., 1990 ;Ong *et al*.,2010).Unlike the adherence mediated by the MR/P hemagglutinin, binding to kidney tissue by MR/K-expressing strains was not inhibited by low-molecular-weight components present in urine and thus was suggested to be more likely to play a role *in vivo* than MR/P fimbriae. In addition, Silverblatt (1974) demonstrated that fimbriae morphologically consistent with MR/K fimbriae were expressed in the kidney of an experimentally infected rat. MR/K of other species (i.e. *Klebsiella* and *Providencia* spp.) have also been implicated in adherence to tissues and inert surfaces (Bahrani *et al*., 1993). While these observations support a role of MR/K fimbriae in pathogenesis they by no means constitute proof that this adhesion contributes to virulence where there are, also suggestions that MR/K fimbriae do not play a role in pathogenesis. Silverblatt and Ofek (1978) demonstrated in a rat model of pyelonephritis that MR/K fimbriae were expressed *in vivo* but that, overtime, the bacterial population shifted away from production of this fimbrial type towards the production of MR/P fimbriae. Animals inoculated with bacteria predominantly producing MR/P fimbriae developed more severe lesions in the kidney than animals

inoculated with the bacterial strain cultured in such a fashion so that MR/K fimbriae predominated (Bahrani *et al*., 1993; O'May *et al*., 2008).

Since hemagglutinin was originally characterized in *Klebsiella* strains, the fimbrial adhesin has been referred to as the mannose-resistant, *Klebsiella*-like (MR/K) hemagglutinin. Several studies have clearly demonstrated that type 3 fimbriae also mediates various adherence functions such as binding to epithelial cells and extracellular matrix proteins for instance, collagen V (Tarkkanen *et al*.,1990; Ong *et al*.,2008). A putative regulatory gene (*mrk E*) located upstream of *mrkA* has been described previously in *K. pneumoniae*. These genes have been shown to reside in multiple genomic locations, including the chromosome, conjugative plasmids, and within a composite transposon. Transfer of a *mrk*-containing conjugative plasmid to strains of *Salmonella entrica* serovar *typhimurium, K. pneumoniae,* and *E. aerogenes* species has also been demonstrated (Sonbol *et al*., 2012). Taken together, these data strongly support the spread of the *mrk* genes between Gram-negative pathogens by lateral gene transfer.

Besides MR/P and MR/K fimbriae, the other fimbriae produced by *Proteus* during UTIs are surface adhesins; UCA/NAF and ATF fimbriae which determined not to be involved in the hemagglutination caused by MR/P and MR/K fimbriae (Jacobsen *et al*., 2008).

A small collection of *P. vulgaris* strains revealed that all isolates produced *uca* (Fig. 3-5) .Our results agree with other results obtained by Bijlsma *et al.* (1995) and Cook *et al*. (1995) who found that the 540 bp *ucaA* gene that encodes the major fimbrial subunit of UCA has nucleic acid homology (58%) to the f17a gene of *E. coli*F17 pilin and was identified in all 26 *P. mirabilis* strains tested. Also, somewhat with Tolson *et al*. (1995) results, when they studied the conditions of NAF expression and they found

that seven out of eight strains of *P. mirabilis* clearly produced this fimbrial adhesin in relatively comparable quantities when grown on Luria agar.

Wray *et al*. (1986) characterized UCA, a NAF from *P. mirabilis* HU1069 that was demonstrated to weakly attach to exfoliated human desquamated uroepithelial cells.

These fimbrial subunits were isolated and characterized from a *P. mirabilis* strain 7,570 isolated from a patient with struvite urolithiasis and renamed nonagglutinating fimbriae (NAF) by Tolson *et al*. (1995).

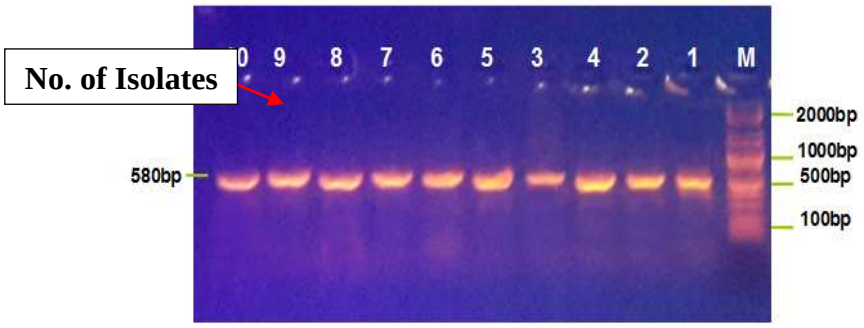


Fig (3-5):- Gel electrophoresis of PCR of *uca* amplicon product. M: Marker, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10: no. of *P. vulgaris* isolates, *uca* gene amplicon size: 580bp.

Thermocycling parameters were as follows: an initial denaturation of 94 °C for 3min, 30 cycles of denaturation at 94 °C for 30 s, primer annealing 50 °C for 30 s, and extension at 72 °C for 30s.Finally one extension step at 72°C for 7 min. The electrophoresis was carried at 1% agarose gel and the electric current was allowed at 110 volt for 60min.

Bacteria expressing NAF adhered strongly to a number of cell lines *in vitro*, including uroepithelial cell (Tolson *et al*. 1997), MDCK (Madin-Darby canine kidney) (Lee *et al*., 2000; Altman *et al*., 2001), and EJ/28 urinary tract tumor cell lines (Latta *et al*., 1998).

Because of its homology to f17a gene of *E. coli* F17 fimbriae that assist in intestinal tract colonization, it is possible that these fimbriae play a role in

the initiation of UTIs by allowing *Proteus* to attach and establish in the intestines and thus form a reservoir of organisms that can potentially cause UTIs. However, there have been no definitive studies examining this possibility (Rozalsk *et al*., 2012).

Other fimbriae gene have been detected in *P. vulgaris* isolates was ambient temperature fimbriae (ATF) .They named ATF because they are expressed optimally during static culture in Luria broth at 23°C. They are also expressed, to a lesser degree, during static and aerated culture in Luria broth at 37°C, but not during culture in minimal medium, on agar plates, or at higher temperatures (such as 42°C) (Nielubowicz, 2010).Expression of ATF does not correlate with hemagglutination (Zunino *et al*.,2000).

P. vulgaris isolates have been screened for production of ATF and all strains tested were positive (Fig.3-6). This finding corresponding with other study about *P. mirabilis* ATF fimbriae by Massad *et al*.(1994) and Rocha *et al*.(2007) who found all examined strains of *P. mirabilis* expressed ATF to varying degrees and as for other enteric fimbriae, the expression of ATF was affected by environmental conditions.

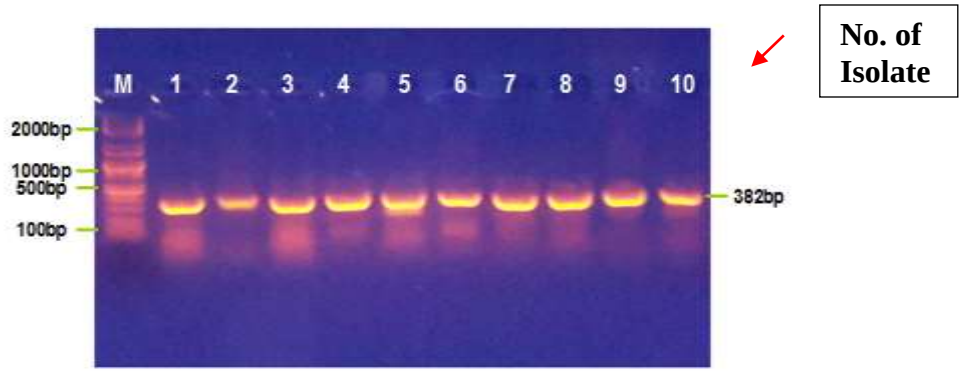


Fig (3-6):- Gel electrophoresis of PCR of *atf* amplicon product. M: Marker, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10: no. of P. *vulgaris* isolates, *atf* gene amplicon size: 382bp.

Thermocycling parameters were as follows: an initial denaturation of 94 °C for 3min, 30 cycles of denaturation at 94 °C for 1 min, primer annealing 50 °C for 1 min, and

extension at 72 °C for 1 min. Finally one extension step at 72°C for 5 min. The electrophoresis was carried at 1% agarose gel and the electric current was allowed at 110 volt for 60min.

Zunino *et al*. (2000) isolated a mutant of *P. mirabilis* that was unable to synthesize ATF. They confirmed that ATF are not required for *P. mirabilis* hemagglutination. This ATF mutant was also used in a mouse ascending UTI model and no significant differences were noted between numbers of infected animals or organs, or between the levels of colonization of kidney or bladders between the wild-type or mutant infections. This indicates that ATF mutagenesis did not affect the infective ability of *Proteus*. Therefore, ATF are more likely to play a role in the survival of *Proteus* in the external environments of a mammalian host due to its optimal expression temperature. So, ATF fimbriae are not important in the pathogenicity of human beings.

Whether ATF play a role in colonization of the urinary tract is difficult to assess. Thus far, no receptor-binding function can be identified. Bacteria expressing ATF do not agglutinate any erythrocyte species. Furthermore, these fimbriae are optimally expressed at 23°C and are less prominent at 37°C. While this suggests that ATF may not contribute significantly to colonization of the mammalian host, we cannot rule out the possibility that these fimbriae are expressed at high levels *in vivo*. For example, *cholera* toxin is optimally expressed by *Vibrio cholera in vitro* at temperatures lower than 37°C, although the toxin is produced during infection (Dirita *et al.*, 1990; Massad *et al*., 1994).

3.2.6 Swarming behavior:

In this study, all *P.vulgaris* isolates (100%) exhibited swarming motility when grown on agar plates. The results of the present study are in agreement

with the results obtained by Iwalokun *et al.* (2004), EL- Baghdadi *et al*. (2009) and Al-Jumaa *et al*. (2011) who found all *Proteus* isolates (100%) exhibit swarming activity.

There is some concerning invasiveness of swarmer cells. Allison *et al*. (1992) showed *in vitro* that this morphotype could invade uroepithelial cells, whereas non-flagellated and non-swarming forms were noninvasive. Other authors (Rozalski *et al*., 1986; Chippendale *et al*., 1994) showed the short rods as invasive forms, rather than swarmer cells. These results were confirmed by Jansen *et al*. (2003) in a mouse model of ascending UTI. These authors have found that the predominant cell type in the urinary tract are short swimmer cells but not elongated swarmer cells. Recently, Fujihara *et al*. (2011) suggested that *Proteus* cells differentiate into hyperflagellated and multinucleated swarmer cells in acidic pH of the host's urine and differentiate back into swimmer forms, when the urinary pH is increased and it is alkaline after urease action.

Also, there is other studies by Zunino *et al.* (1999) and Jansen *et al.* (2003) indicate that this property is not essential in *Proteus* urinary tract infection.

A number of secreted proteins linked with virulence such as hemolysin, urease, and protease produced by swarm cells are responsible for various infections of susceptible hosts (Allison *et al*., 1992; Fraser *et al*., 2002).

The swarm cells were found to be physiologically and biochemically distinct from vegetative cells. During differentiation of vegetative cells into swarm cells, there was increased production of a number of proteins such as

flagellin, urease, hemolysin and protease, which might be related to survival of the organisms. Many enzymatic activities such as tryptophanase, *β*-galactosidase, and alkaline phosphatase are reduced during swarm cell differentiation and their levels again reach to normal level after consolidation (Allison and Hughes, 1991, Pearson *et al*., 2010).

Swarming differentiation correlated with the ability of *Proteus* to invade cells (Allison *et al*., 1992). To improve understanding the consequences of swarming motility inhibition by anti-swarming agents and on virulence even further, we investigated the effect of some anti-swarming agents on some virulence factors production; these substances were observed to cause marked reduction in these parameters. The results are more cleared in the following paragraphs.

3.2.6.1. A-Inhibition of *P. vulgaris* swarming by resveratrol

The swarming behavior of *P. vulgaris* on LB agar containing different concentrations of resveratrol was monitored and we found that resveratrol has the ability to block the swarming migration of *P. vulgaris* in a dose-dependent manner (Fig. 3-7: a). The swarming behavior was significantly inhibited at concentrations as low as 20µg/ml and was suppressed completely at 60 µg/ml (Fig. 3-7:a&b).The inhibitory effect of resveratrol on swarming might arise from a toxic effect on bacteria. To test this possibility, an overnight culture of *P. vulgaris* was inoculated into LB broth containing various concentrations of resveratrol and the growth rate of bacteria was monitored as shown in figure (3-7c).The growth rate of *P. vulgaris* was inhibited slightly but not significantly because it grew in all tubes regardless

of whether resveratrol was present or not, indicating that resveratrol could inhibit swarming but not the growth in *P. vulgaris*.

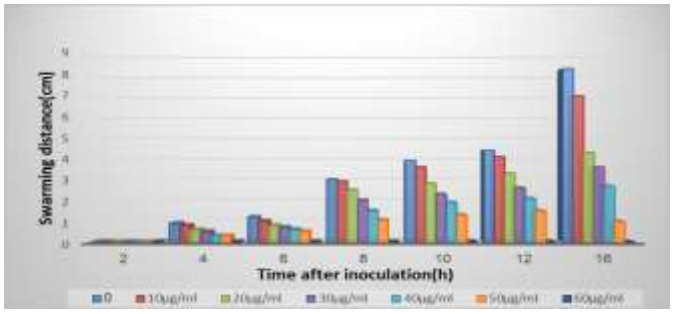
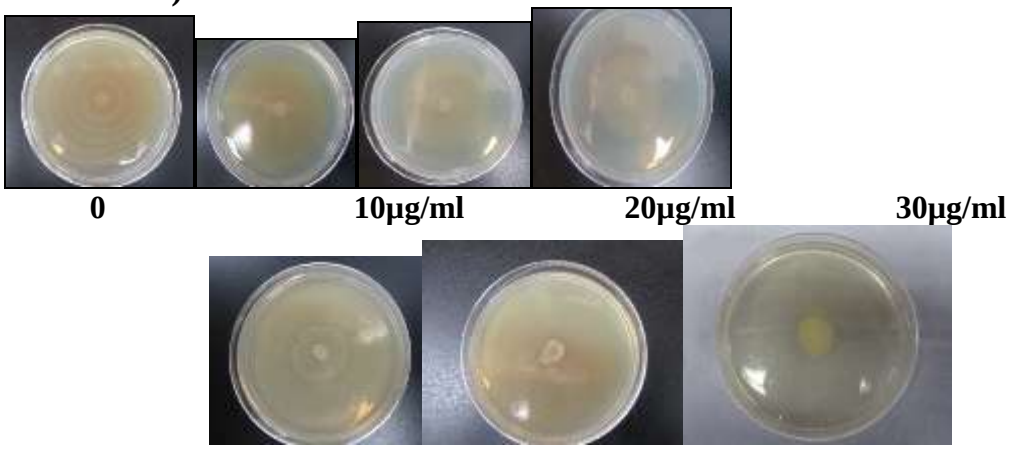


Fig.3-7(a).Effect of resveratrol on the swarming of *P. vulgaris*. The migration distance of *P. vulgaris* in the presence of various concentrations of resveratrol (0, 10, 20, 30, 40, 50, 60 µg/ml). The data represent the mean and SD of three independent experiments and the differences are significant (P value <0.05).



0 **10µg/ml** **20µg/ml** **30µg/ml**

40µg/ml **50µg/ml** **60µg/ml**

Fig.3-7(b). Halo images of swarming plates containing different concentrations of resveratrol (0, 10, 20, 30, 40, 50, 60 µg/ml) at 16h after inoculation.

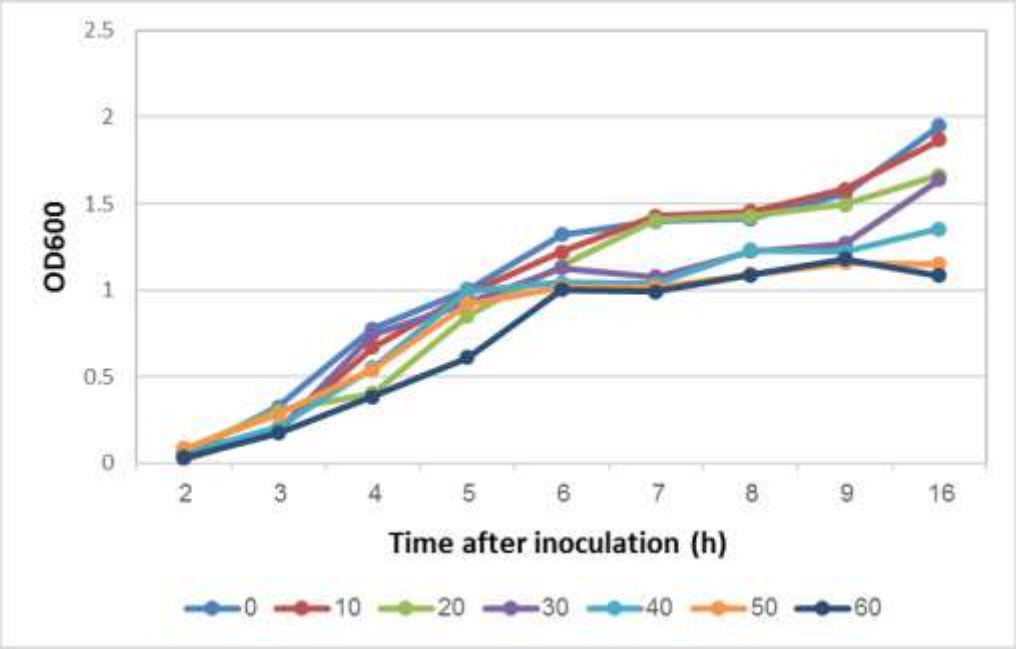


Fig.3-7(c).Effect of resveratrol on the growth of *P.vulgaris*.OD_{600} was measured overtime in the presence of various concentrations of resveratrol (0, 10, 20, 30, 40, 50, and 60 µg/ml). The data represent the mean of three independent experiments, there is no significant difference between concentrations (P value >0.05).

B. Inhibition of cell length and virulence factor production of *P. vulgaris* by resveratrol

Cell morphology was monitored after inoculation of an overnight culture of *P. vulgaris* onto LB plates containing various concentrations of resveratrol. As shown in figure (3-8:a), in the absence of resveratrol, the swarming cells were longer than the bacterial cells in the presence of resveratrol at the concentration 60µg/ml, suggesting that swarming differentiation was inhibited. The inhibition of differentiation started to be observed at a resveratrol concentration of 20µg/ml. Very few elongated swarming cells were observed at a resveratrol concentration of 40µg/ml. As the resveratrol concentration was increased to 60µg/ml, only short vegetative cells were observed. These results indicate that swarming differentiation of *P. vulgaris* was indeed inhibited by high concentrations of resveratrol.

To study whether the production of virulence factors (haemolysin and urease) was also influenced by resveratrol, the production of haemolysin and urease in *P.vulgaris* taken from LB agar plates containing different concentrations of resveratrol was determined. As shown in figure (3-9), the production of virulence factors was affected significantly in the presence of increasing concentrations of resveratrol (50 and 60µg/ml).

The emergence of bacterial strains that exhibit resistance to various antibiotics poses a major threat to public health. As a consequence, there is renewed interest in antibacterial targets which, by attenuating virulence, disrupt the capacity of pathogenic bacteria to cause infection (Hinkelmann and Kempthorne, 2008). The purpose of the present study was to investigate the effect of plant extract (resveratrol) against uropathogenic *P. vulgaris.*

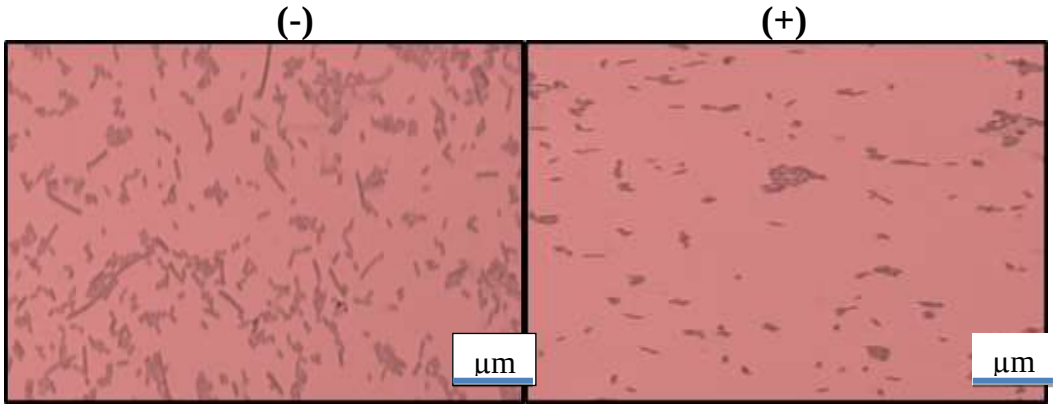


Fig.3-8(a).Microscopic observation of *P. vulgaris* cells isolated from the LB plates without resveratrol (-) shows and with resveratrol 60µg/ml (+) shows at a magnification of 1000X.

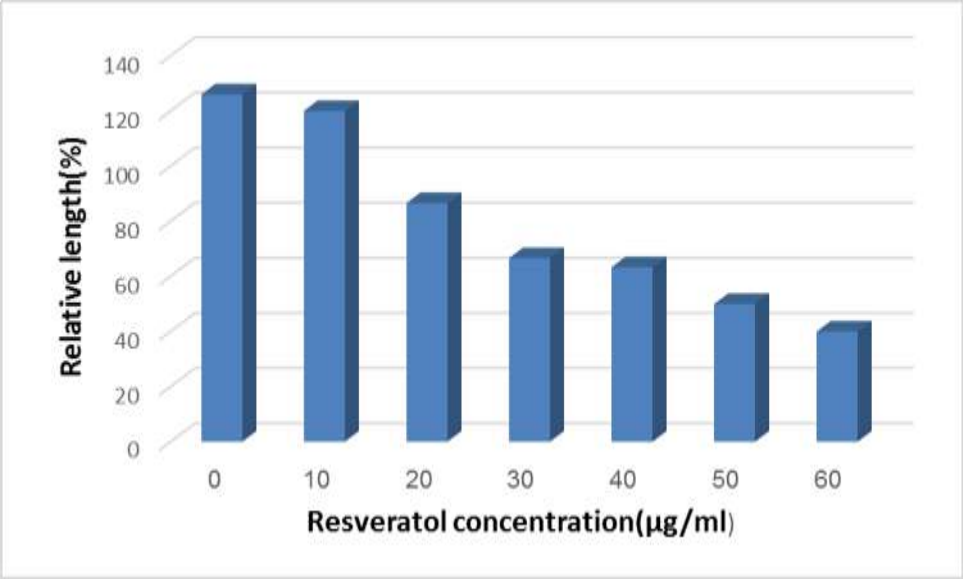


Fig.3-8(b).Effect of resveratrol on the cell length of *P. vulgaris*. The cell length of *P. vulgaris* in the presence of various concentrations of resveratrol (0, 10, 20, 30, 40, 50, 60µg/ml). The lengths of 100 cells in each sample were determined, and the average was calculated. The difference between concentrations is statistically significant (P value <0.05).

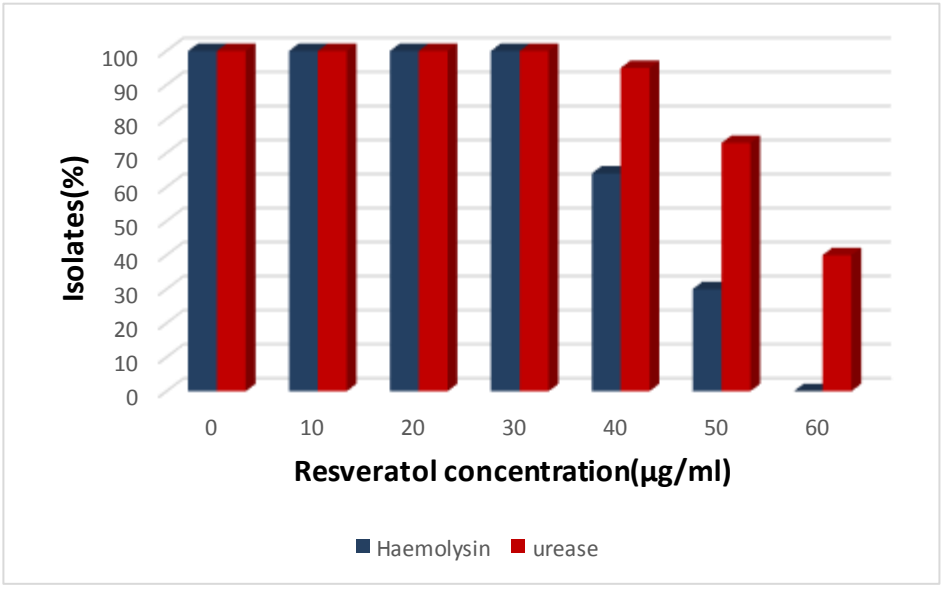


Fig. (3-9).The influence of resveratrol on the production of virulence factors in *P. vulgaris*. The production of haemolysin and urease at different concentrations of

resveratrol (0, 10, 20, 30, 40, 50, 60µg/ml). The data represent the mean of three independent experiments. The difference is statistically significant (P value<0.05).

In this study, we found that resveratrol has the ability to inhibit *P. vulgaris* swarming significantly at a concentration as low as 20 µg/ ml and inhibited swarming completely at 60 µg/ml (Fig.3-7 a & b). Also, it had the ability to suppress the production of virulence factors (haemolysin and urease) at concentrations of 50 and 60µg/ml (Fig.3-9) but it did not significantly affect the growth of the bacteria at concentrations up to 60 µg/ml (Fig. 3-7c). Resveratrol did not affect the viability of *P. vulgaris* at a concentration of 3mg/ml (data not shown). This means resveratrol could only inhibit swarming and virulence factor production without significant growth inhibition of *P. vulgaris*. Based on this finding, we concluded that the swarming ability of *P. vulgaris* is correlated with its ability to express virulence factors and these results were similar to those reported by Allison *et al*. (1992), Wang *et al.* (2006); where it has been shown
that swarming differentiation of *P. mirabilis* and expression of virulence factors, such as urease, haemolysin and protease, are coordinately regulated in *P. mirabilis*.

The possible mechanism by which resveratrol could inhibit *P. vulgaris* swarming and virulence factor expression is by acting as an inhibitor compound for bacterial quorum sensing (QS).Quorum Sensing is the term used for the phenomenon of cell to cell communication in bacteria using secreted chemical signaling molecules called autoinducers. As environmental conditions often change rapidly, bacteria need to respond quickly in order to survive. QS is the regulation of gene expression in response to fluctuations in cell-population density and it enables bacteria to coordinate their behavior. Gram-positive and Gram-negative bacteria use

quorum sensing communication circuits to regulate a diverse array of physiological activities. These processes include symbiosis, virulence, competence, conjugation, antibiotic production, motility, sporulation, and biofilm formation (Dekievit and Iglewski, 2000; Miller *et al*., 2001; Zhang, 2003).So, QS is considered a novel target for antimicrobial therapy.

The continuing emergence of multiple-drug-resistant strains of bacteria has necessitated finding novel strategies for treating bacterial infections and the discovery that a wide spectrum of organisms use quorum sensing to control virulence factor production makes it an attractive target for antimicrobial therapy. Among all the possibilities to inhibit the QS activity, the use of anti-QS compounds could be of great interest to avoid bacterial infections (Adonizio *et al.,* 2006; Truchado *et al*., 2009; Maria *et al.,* 2012). Such antipathogenic compounds, in contrast with antibacterial compounds, do not kill bacteria or stop their growth and are assumed not to lead to the development of resistant strains (Otto, 2004; Fulghesu *et al*., 2007).Different mechanisms have been proposed to explain the interference of QS dependent processes by natural products. Some of these mechanisms are inhibition of signal molecule biosynthesis or acylated homoserine lactone (AHL) QS autoinducers reception (Rasmussen *et al.*, 2005; Vattem *et al.*, 2007), and the enzymatic inactivation and biodegradation of QS molecules (Defoirdt *et al*., 2004). Therefore, we can concluded that the interruption of QS control system has an anti-pathogenic effect and can be used in the treatment of bacterial infections .According to that, *P. vulgaris* swarming and virulence factor expression are generally believed to be regulated through a QS system which requires the sensing and integration of a variety of environmental, cell to cell and intracellular signals (Sturgill and Rather, 2004; Rather, 2005). So,

the environmental changes or the presence of resveratrol in media of *P. vulgaris* has an effect on QS control system.

3.2.6.2. A. Inhibition of *P. vulgaris* swarming by PNPG

In this study, we found that PNPG had the ability to block the swarming migration of *P. vulgaris* also in a dose-dependent manner (Fig. 3-10a). The swarming behavior was significantly inhibited at concentrations 50µg/ml and was blocked completely at 150 and 200µg/ml (Fig. 3-10 a & b).To test the inhibitory effect of PNPG on bacterial growth in addition to swarming, an overnight culture of *P. vulgaris* was inoculated into LB containing various concentrations of PNPG and the growth rate of bacteria was monitored as shown in figure (3-10c).The growth rate of *P. vulgaris* was not inhibited by PNPG. At 16h post-inoculation, the bacteria grew approximately to similar densities, regardless of the presence of PNPG. We concluded that the inhibitory effect of PNPG on swarming was unlikely to be due to inhibit of cell growth.

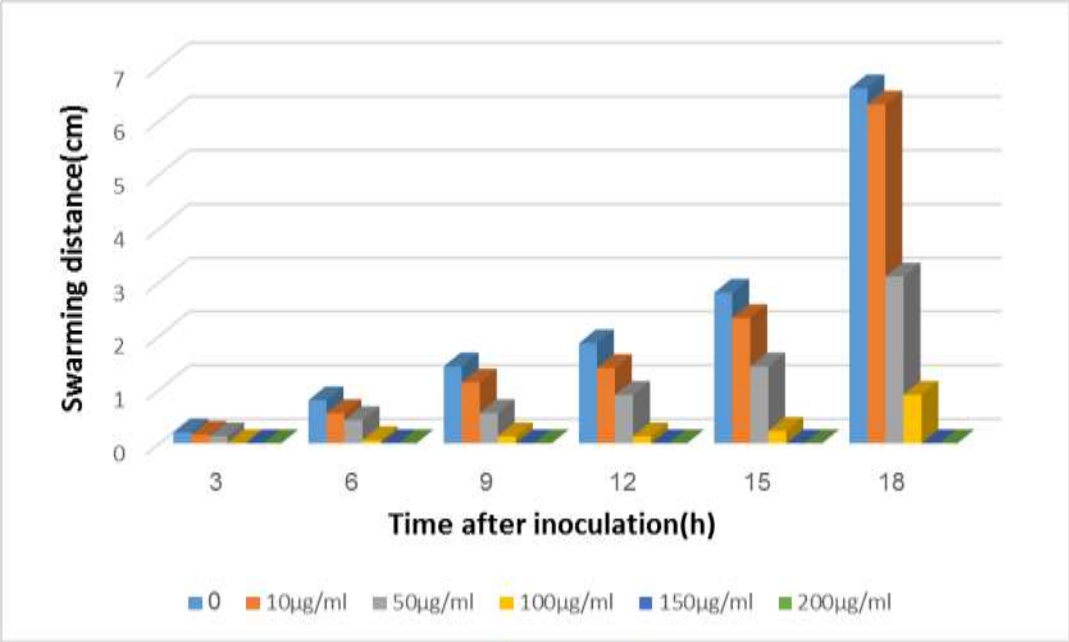


Fig.3-10(a).Effect of PNPG on the swarming of *P. vulgaris*. The migration distance of *P. vulgaris* in the presence of various concentrations of PNPG (0, 10, 50, 100, 150, 200µg/ml). The data represent the mean and SD of three independent experiments and the differences between concentrations are statistically significant (P value <0.05).

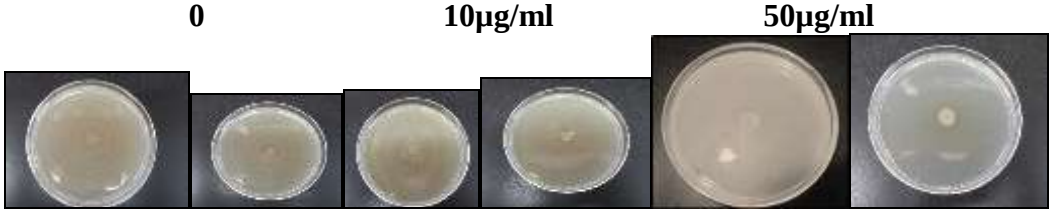


Fig.3-10(b).Halo images of swarming plates containing different concentrations of PNPG (0, 10, 50, 100, 150, and 200 µg/ml) at 18h after inoculation.

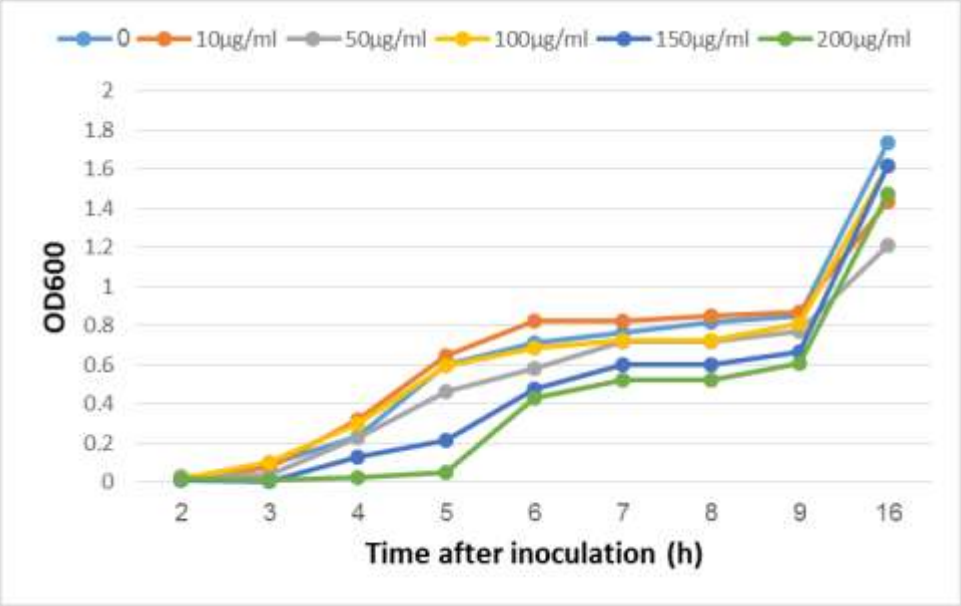


Fig.3-10 (c).Effect of PNPG on the growth of *P.vulgaris*.OD_{600} was measured overtime in the presence of various concentrations of PNPG (0, 10, 50, 100, 150, and 200µg/ml). The data represent the mean of three independent experiments, there is no significant difference between concentrations (P value >0.05).

B-Inhibition of cell length and virulence factor production of *P. vulgaris* by PNPG

Cell morphology was observed after inoculation of an overnight culture of *P. vulgaris* onto LB swarming plates containing various concentrations of PNPG. As shown in figure (3-11a), in the absence of PNPG, the swarming cells were longer than the bacterial cells in the presence of PNPG at the concentration 200µg/ml, suggesting that swarming differentiation was suppressed. The inhibition of differentiation initiated to be observed at PNPG concentration of 50µg/ml. As PNPG concentration was heighten to 150 and 200µg/ml, only shortened cells were observed. These results mention that swarming differentiation of *P. vulgaris* was inhibited by high

concentrations of PNPG. .

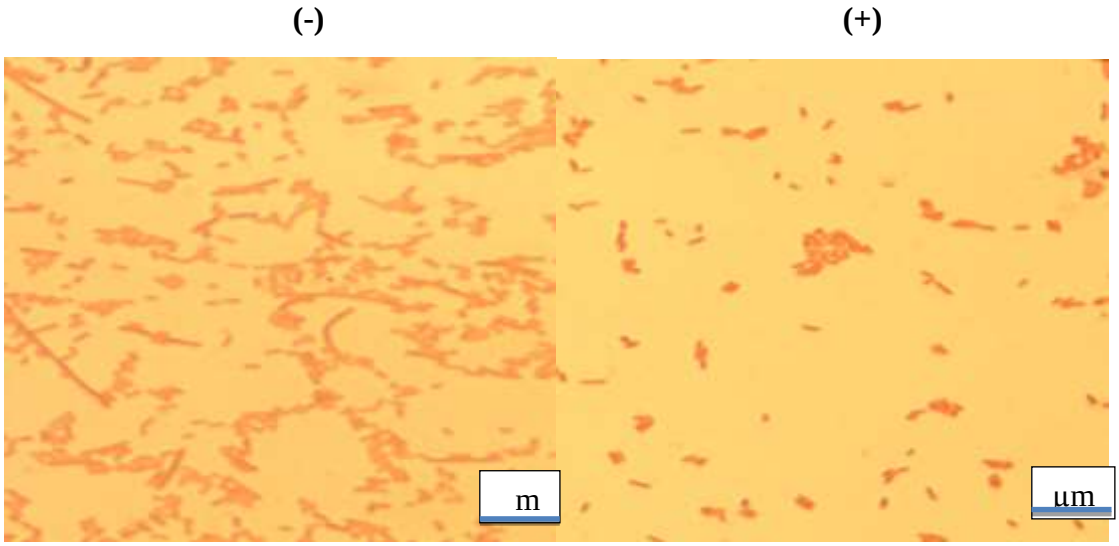


Fig.3-11 (a).Microscopic observation of *P. vulgaris* isolated from the LB plates without PNPG (-) shows and with 200 µg/ml of PNPG (+) shows at a magnification of 1000X. .

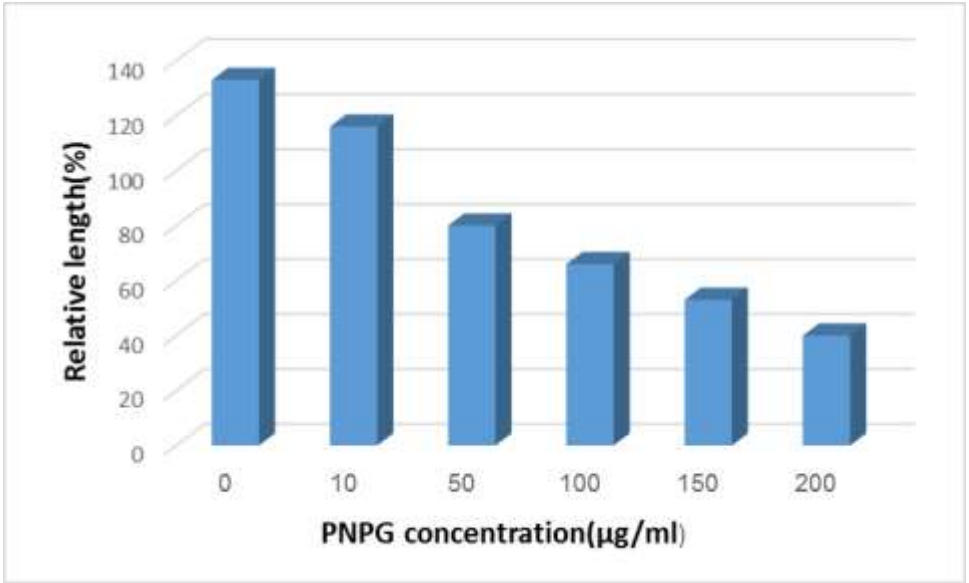


Fig.3-11 (b).Effect of PNPG on the cell length of *P. vulgaris*. The cell length of *P. vulgaris* in the presence of various concentrations of PNPG (0, 10, 50, 100, 150, and

200µg/ml). The lengths of 100 cells in each sample were determined, and the average was calculated. The difference between concentrations is statistically significant (P value<0.05).

To see whether the production of virulence factors (haemolysin and urease) was also affected by PNPG, the production of haemolysin and urease in *P. vulgaris* which taken from LB agar plates containing different concentrations of PNPG was established. As shown in figure (3-12), the production of virulence factors was not impacted significantly at PNPG concentrations (0-100µg/ml) for urease and (0-50 µg/ml) for haemolysin but was inhibited significantly in the presence of increasing concentrations (150 and 200µg/ml).

It has been well demonstrated that swarming motility and virulence factor expression are coordinately regulated in several pathogens including *P. mirabilis* and *Ps. aeruginosa* which are often implicated in persistent UTI (Ronald, 2002; Wang, 2006; Nashikkar *et al*., 2011).

P-nitrophenylglycerol (PNPG) effectively inhibits swarming and virulence factor production of *P. mirabilis* (Liaw *et al*., 2001). The underlying mechanism of inhibition is unclear. Now we have found that PNPG also inhibits swarming and virulence factor production in *P. vulgaris.*

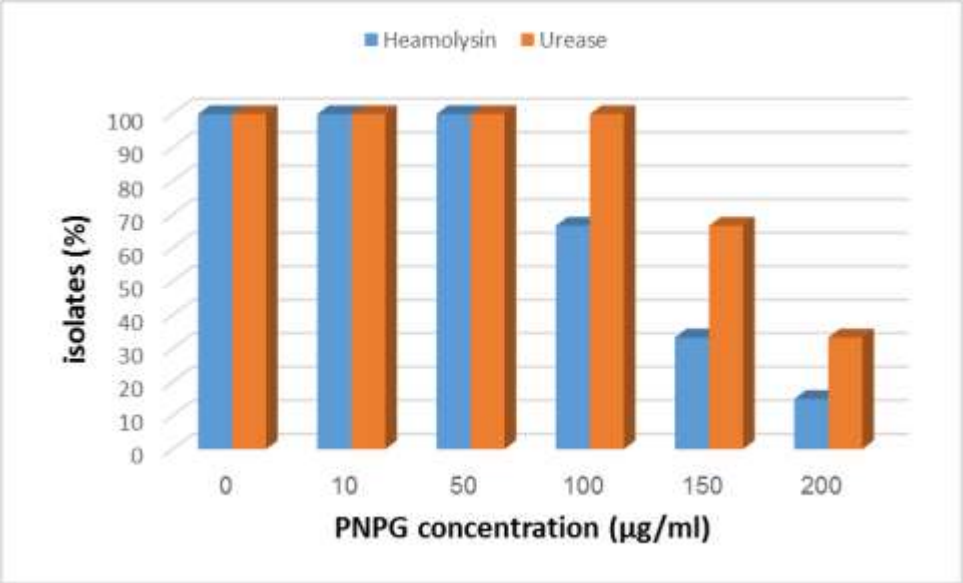


Fig. (3-12).The influence of PNPG on the production of virulence factors in *P. vulgaris*. The production of haemolysin and urease at different concentrations of PNPG (0, 10, 50, 100, 150, and 200µg/ml). The data represent the mean of three independent experiments. The difference is statistically significant (P value < 0. 0 5)

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In this study, we found that PNPG has the capacity to restrain *P. vulgaris* swarming significantly at a concentration as low as 50 µg/ml and inhibited swarming completely at 150 and 200µg/ml (Fig.3-10a & b). Also, it had the ability to suppress the production of virulence factor; haemolysin suppression was at concentrations of (100,150 and 200 µg/ml) whereas urease suppressed at (150 and 200 µg/ml) (Fig.3-12). But it did not significantly affect the growth of the bacteria even at high concentrations (200 µg/ml) (Fig. 3-10c). This implies PNPG has the ability to inhibit swarming and virulence factor production without significant inhibition of *P. vulgaris* growth. According to that, we concluded that the swarming ability of *P. vulgaris* is correlated with its ability to express virulence factors and this results coincides with other results registered by (Liaw *et al.*, 2000; Ronald, 2002; Wang *et al.*, 2006 ; Nashikkar *et al.*, 2011).

It is now well known that many bacterial functions including swarming, biofilm formation, and secretion of virulence factors important to successful and recurrent establishment bacterial infections are related to cell density-mediated gene expression which is termed quorum sensing (Whitehead 2001; Fuqua and Greenberg, 2003; Krishnan *et al*., 2012).The regulation of bacterial multicellular behavior such as swarming is a complex process. In *Proteus*, several of the genes involved in flagellar motility and quorum sensing are found to play a regulatory role in this process (Wei *et al*., 2004). In this study, we have shown that PNPG has a negative regulatory effect on the swarming behavior .As bacterial flageller motility is essential for the swarming behavior, it is possible that PNPG inhibits the motility and thus swarming phenomenon. Also, it has been suggested that inactivating the QS system of a pathogen can result in a significant decrease in virulence factor production (Schauder and Bassler, 2001; Lyon and Muir, 2003; Mihalik *et al.*, 2008). So, the possible mechanism by which PNPG could inhibit *P. vulgaris* swarming and virulence factor expression may be due to its acting as an inhibitor compound for bacterial quorum sensing. Some articles reported that the PNPG may has an inhibitory effect on *rsbA* (regulator of swarming behavior) gene that regulated *Proteus* swarming and lead to a reduction in production of other virulence factors (Liaw *et al.*, 2003; Liaw *et al*., 2004).

3.2.6.3-Regulation of *P. vulgaris* swarming by fatty acids

Fatty acids have been implied as environmental signals for swarming of *P. mirabilis* (Liaw *et al*., 2004). Therefore, we tested the effect of a series of fatty acids, including lauric acid, myristic acid, palmitic acid, stearic acid,

and oleic acid, on swarming of *P. vulgaris* on LB swarming agar (2%) plates at 37°C. The concentration of these exogenously added fatty acids was 0.01% (µg/ml). We found that while oleic acid enhanced swarming, lauric acid, myristic acid, palmitic acid, and stearic acid inhibited swarming significantly (Fig. 3-13 a &b).

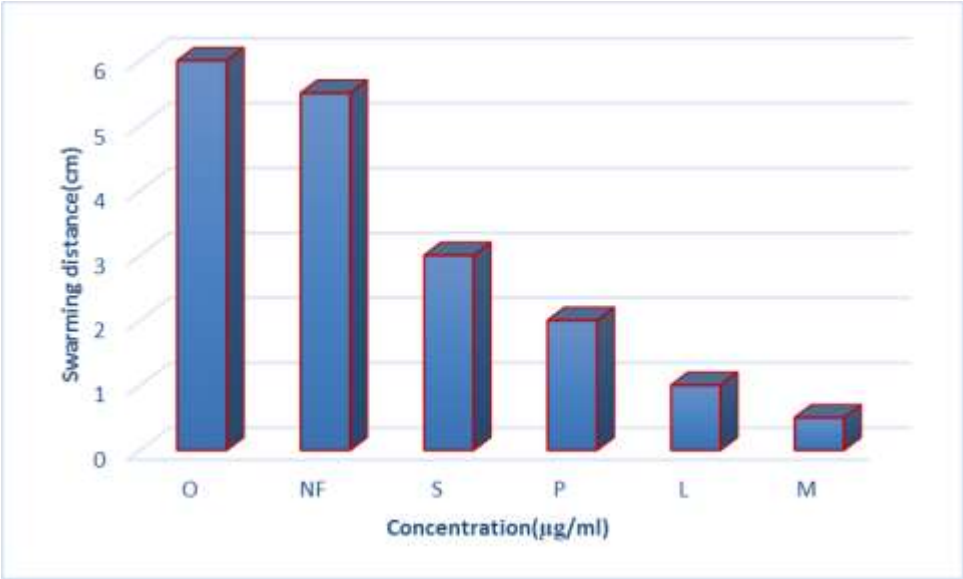
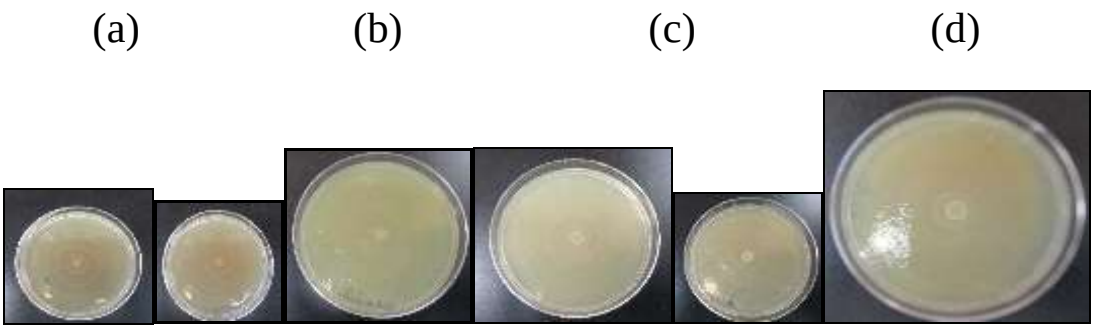


Fig.3-13(a) Swarming migration distance of *P. vulgaris* in the presence of different fatty acids. The concentration of fatty acids added was 0.01% (w/v). The data represent the averages of three independent experiments. O, oleic acid; NF, no fatty acid; S, stearic acid; P, palmitic acid; L, lauric acid; M, myristic acid .



(e) (f)

Fig.3-13 (b) Halo images of swarming plates containing different fatty acids. The representative plates in A were photographed. a, no fatty acid; b, oleic acid; c, stearic acid; d, palmitic acid; e, lauric acid; f, myristic acid.

To test whether this inhibitory effect was dose dependent, myristic acid at 0.00125%, 0.0025%, 0.005% and 0.01% (µg/ml) was added to LB swarming agar plates, followed by the swarming assay. As shown in figure (3-14 a&b), swarming of *P. vulgaris* was inhibited by myristic acid in a dose-dependent manner. A similar dose-dependent inhibitory effect on swarming was also observed for lauric acid, palmitic acid, and stearic acid (data not shown). Our results were similar to that results reported by (Liaw *et al*., 2004; Lai *et al*., 2005 ; Inoue *et al*., 2008), where it has been reported that some SCFAs, including lauric, myristic, palmitic, and stearic acids, repressed colony spreading on swarm plates in *Proteus mirabilis*, *Serratia marcescens* and *Pseudomonas aeruginosa*. There is much evidence indicating that fatty acids or their derivatives can be involved in regulation of gene expression to modulate swarming and virulence (Liaw *et al*., 2004).

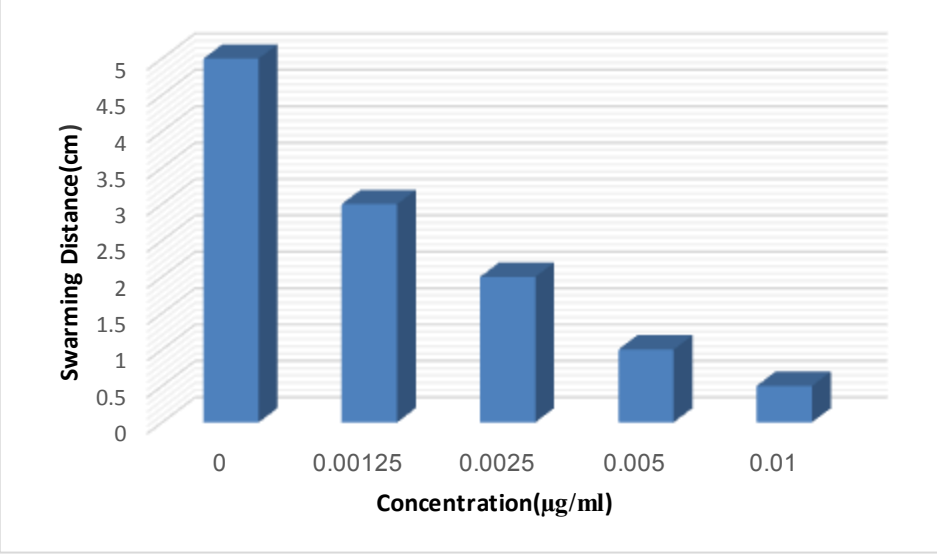


Fig.3-14(a) Swarming migration distance of *P. vulgaris* in the presence of different concentrations of myristic acid. The differences are significant (P value <0.05) .

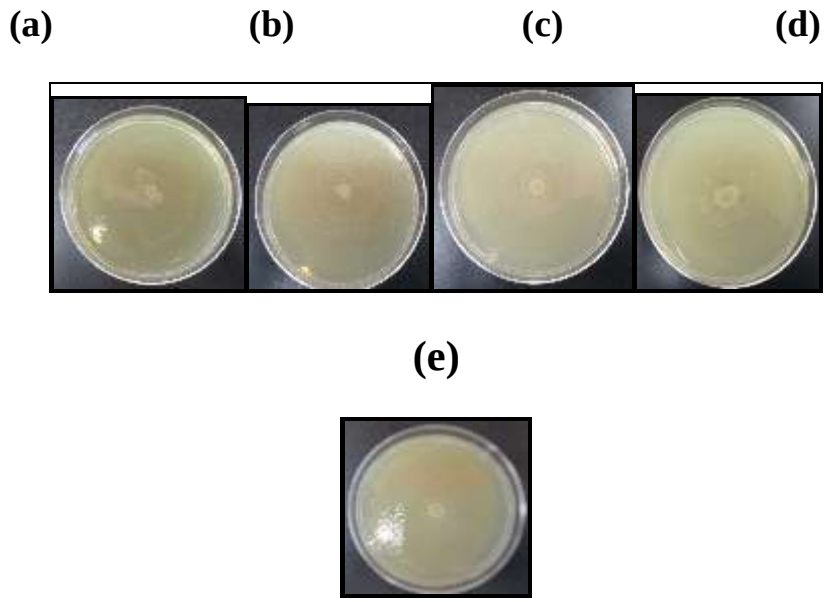


Fig.3-14 (b) Halo images of swarming plates containing different concentrations of myristic acid. The representative plates in A were photographed. a, no fatty acid ;b, 0.00125% ; c, 0.0025% ; d, 0.005% and e, 0.01%(w/v).

Swarm cell differentiation and swarming behavior are the results of complex sensory transduction and global control mechanisms. *Proteus* swarming requires the sensing and integration of a variety of environmental, cell-to-cell, and intracellular signals and involves regulated expression of gene networks leading to morphological and physiological changes (Liaw *et al*., 2004). Although a large body of information concerning swarming mechanisms in *Proteus* has been accumulated, the signals regulating swarming and the pathways for signal transduction are still poorly understood. In this study, we present that fatty acids serve as environmental

cues to regulate *P. vulgaris* swarming. Specifically, while oleic acid enhanced swarming, some SFAs, such as myristic acid, lauric acid, palmitic acid, and stearic acid, inhibited swarming.

The effect of fatty acids on *Proteus* swarming may be due to the presence of *RsbA* gene (regulator of swarming behavior), which may be a His-containing phosphotransmitter of the bacterial two-component signaling system, can act as a repressor of swarming differentiation in *Proteus*(Takeda *et al.*,2001). It is possible that, upon uptake, these fatty acids or their derivatives may act as signals to regulate swarming through either an *RsbA*-dependent or *RsbA*-independent pathway. *RsbA*, is homologous to membrane sensor histidine kinases of the two-component family of regulatory proteins, suggesting that *RsbA* may function as a sensor of environmental conditions required to initiate swarming migration (Belas *et al*., 1998). So, our data demonstrate that fatty acids could act as extracellular signals to regulate swarming in *P. vulgaris*.

3.2.6.4. Inhibition of *P. vulgaris* swarming by Urea

Sterile urea was added to LB agar to a final concentration reaching from 0.0 to 3%. Plates were inoculated in the center with 5µl of a liquid culture of *P. vulgaris* and incubated at 37°C for 16 to 24 h. On agar plates without urea, growth of *P. vulgaris* completely covered the plates; however, the diameter of colonies grown on plates containing urea decreased with increasing urea concentration as mentioned in figure (3-15a &b).

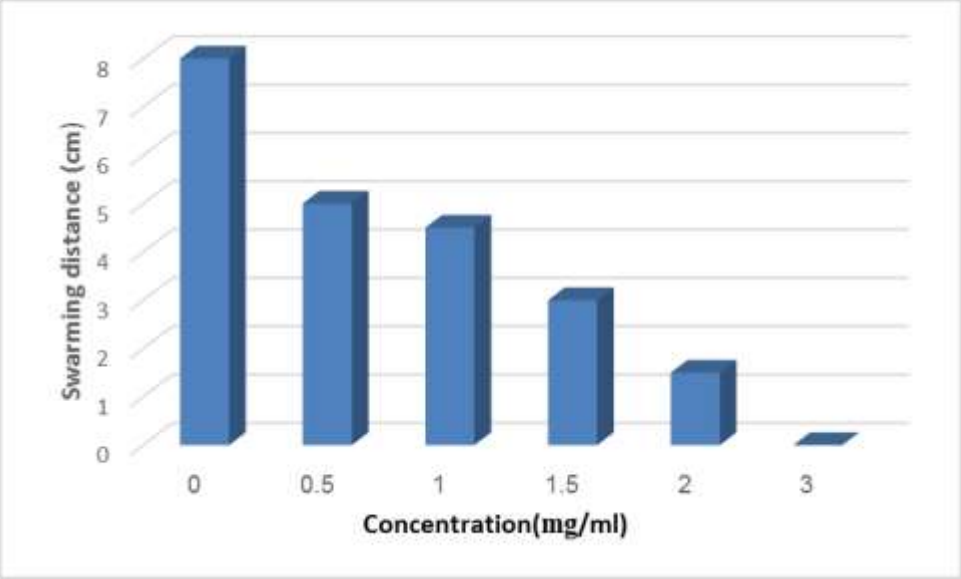


Fig.3-15(a). Swarming migration distance of *P. vulgaris* in the presence of different concentrations of Urea. The differences are significant (P value <0.05).

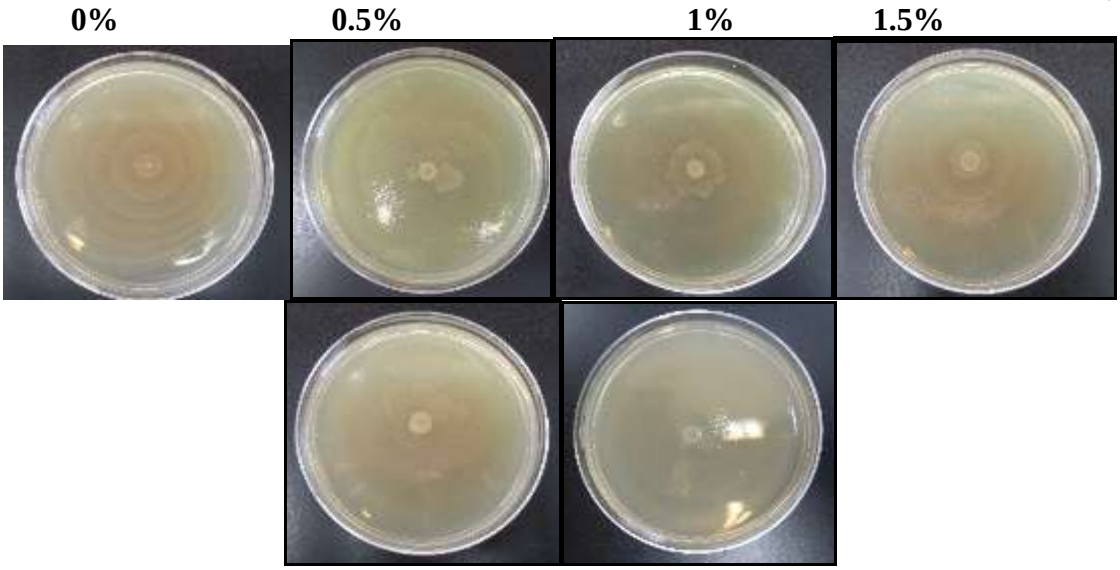


Fig.3-15(b).Halo images of swarming plates containing different concentrations of Urea (Concentration %, w/v).

There is more than one study confirm the anti-swarm property of urea in a solid media, such as study reported by Fons *et al*. (1999) who tested the usefulness of adding urea in isolating single colonies of *Staphylococcus*

intermedius, *Streptococcus* group G spp., and *Pseudomonas aeruginosa* from mixed cultures with *P. mirabilis* by inhibiting *P. mirabilis* swarming, study by Iwalokun *et al*. (2004), recorded the ability of urea at 0.75 - 1.25% to refrain swarming of clinical isolates of *Proteus* that allowed identification of *Klebsiella pneumoniae* and *Staphyloccocus aureus* from mixed growth with *Proteus*. Also, other study by Al-Jumaa *et al*. (2011), Al-Kaebi and Matrood (2011) who showed a significant reduction in the diameter of the swarming of *P. mirabilis* circle in agar plates to which urea has been added.

The inhibitory effect of urea may belong to that urea act as alkaline media and affects the flagella activity. This leads to motility reduction on solid media (Senior, 1999).

Iwalokun *et al*. (2004) demonstrated that the presence of urea on culture media had effects on the expression of plasmid genes that may be involve in swarming motility of *Proteus*.

Urea was observed to cause marked reduction on cellular RNA level and carbohydrate expression, extracellular urease and protease activity, the reduced cellular RNA may be a manifestation to inhibit swarming genes transcription (Rasmussen *et al*., 2000; Iwalokun *et al*., 2004).

3.2.6.5- Inhibition of *P. vulgaris* swarming by ethanol and sodium azide

Other chemicals that have been used to show their ability to inhibit swarming of *P. vulgaris* were ethanol and sodium azide. As shown in figure(3-16), there is a significant reduction in the diameter of the swarming of *P. vulgaris* in plates containing (0.01% and 0.1%) of sodium azide, and no swarming in other plate adding to its cover two drops of (90%) ethanol .

Sodium azide 0.01% Sodium azide 0.1% Ethanol 90%

Fig. (3-16). Halo images of swarming plates containing (0.01% & 0.1%) of sodium azide and plate adding to its cover 2 drops of 90% ethanol.

These results agree with other results reported by Hernandez *et al.* (1999) and Al-Kaebi and Matrood (2011), who demonstrate that ethanol and sodium azide within substances that have anti-swarm properties when they added to a solid media to isolate bacteria in mixed growth with *Proteus strains*; where they consider amongst the materials that effect flagella activity.

The chemical compounds that inhibit the swarming may attributed to complex with flagellar proteins of swarming cells and cause its disintegration or impairs formation of flagella and motility. The enhanced or inhibited swarming of *P. vulgaris* by all chemical compounds tested in this study may attributed to many reasons include; 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, these compounds may interact with

activity of RsbA proteins through either an *RsbA*-dependent or *RsbA*-independent pathway to regulate swarming and virulence factor expression in *Proteus* or these compounds may have an inhibitory effect on *rsbA* gene that regulated *Proteus* swarming (Liaw *et al*.,2004; Laftaah,2012).

3.3. Antibiotic susceptibility test of *P. vulgaris*:

The effect of different antibiotics on *P. vulgaris* isolates was investigated. These isolates showed different susceptibility towards antibiotics used in this study (fig.3-17).

It has been found that the majority of the isolates were multidrug resistant since they were resistant to three antimicrobials agents or more. The highest rate (100%) of resistance recorded with Ampicillin, Amoxicillin, Cephalothin, Chloramphenicol and Ceftazidime, and they are moderately resistant to Ceftriaxone; 25/28(89%), Ciprofloxacin 23/28(82%) and Cotrimazole 26/28 (92.8%).Whereas, all the isolates were sensitive (100%) to Amikacin, Gentamycin, Nitrofurantion, Imipenem, Meropenem, and Norfloxacin.

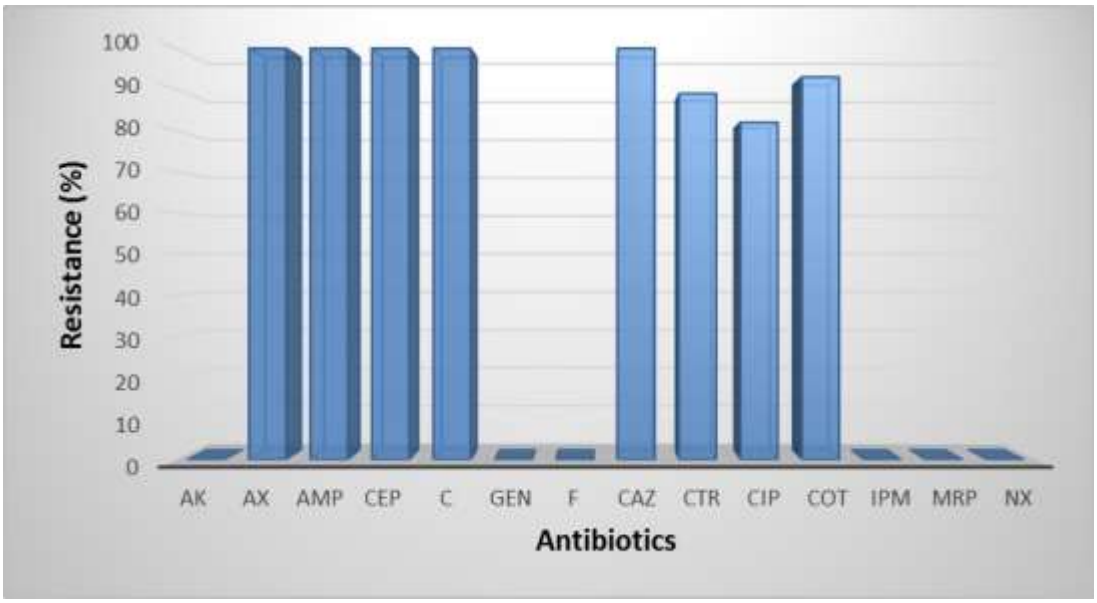


Figure (3-17): Antibiotic susceptibility of *Proteus vulgaris*

AK:Amikacin,AX:Amoxicillin,AMP:Ampicillin,CEP:Cephalothin,C:Chloramphenicol,GEN:Gentamycin, F:Nitrofurnation,CAZ:Ceftazidime,CTR:Ceftriaxone,CIP:Ciprofloxacin,COT: Cotrimazole, IPM: Imipenem, MRP: Meropenem and NX: Norfloxacin.

These results were in accordance with other results reported by other investigators such as results of Feglo *et al*.(2010) who found (84.6%) of *Proteus* isolates recovered from different clinical samples are characterized by multidrug resistant phenotype and Al-Jumaa *et al*. (2011) who have observed most of *Proteus* isolates were resistance to β-lactam group, where they found (80%) of the isolates were resistant to Amoxicillin , (93.3%) of them were resistance to Penicillin and all isolates (100%) were resistant to Cephalothin.

The emergence of multidrug resistance strains which are resistant to most of the antimicrobials agent tested may be due to the fact that ampicillin, amoxicillin, cephalothin considered the most commonly prescribed antibiotics in the hospital even before the results of urine analyses and also the most easily available in the market without prescription and because they were also very cheap in terms of cost. The widespread use and

more often the misuse of antimicrobial drugs has led to a general rise in the emergence of resistant bacteria (Manikandan *et al*., 2011).

Emergence and dissemination of β-lactam resistance in nosocomial *Enterobacteriaceae* became a serious problem worldwide. Especially the increasing resistance to 3rd and 4th generation cephalosporins and carbapenems is of particular concern. Gram-negative bacteria pursue various molecular strategies for development of resistance to these antibiotics: (a) generation of extended-spectrum β-lactamases (ESBL) according to the original definition due to extension of the spectrum of already widely disseminated plasmid-encoded β-lactamases by amino acid substitution; (b) acquisition of genes encoding ESBL from environmental bacteria (c) high-level expression of chromosome-encoded β-lactamase (*bla*) genes as bla_{OXA} or bla_{ampC} genes due to modifications in regulatory genes, mutations of the β-lactamase promoter sequence as well as integration of insertion sequences containing an efficient promoter for intrinsic *bla* genes; (d) mobilization of *bla* genes by incorporation in integrons and horizontal transfer into other Gram-negative species; (e) dissemination of plasmid-mediated carbapenemases as KPC and metallo-β-lactamases, e.g. VIM and IMP; (f) non-expression of porin genes and/or efflux pump-based antibiotic resistance(Pfeifer *et al*.,2010).

A significant increase in resistance of pathogenic strains to Ampicillin and Cephalothin has been found worldwide (Hooton, 2003), but older agents like gentamicin that still show high efficacy against UTI pathogens because of its multiple mechanisms of action seem to have enabled it to retain potent activity against *P. vulgaris* (Manikandan *et al*.,2011).

As shown in figure (3-17) resistant of *P. vulgaris* isolates to Ciprofloxacin was (82%) compared to the results of Al-Jumaa *et al*. (2011) who have reported (53.3%) of *Proteus* isolates are resistant to Ciprofloxacin and Ahmed *et al*. (2007) who have found all isolates of *Proteus* are sensitive to Ciprofloxacin.

Resistance to Ciprofloxacin may be due to one of the three mechanisms of resistance to quinolones which are: mutations that alter the drug targets, mutations that reduce drug accumulation, and plasmid- mediated *qnr* genes that protect cells from the lethal effects of quinolones. These genes are found mainly in *Enterobacteriaceae* and affect the dynamics of development and acquisition of quinolone resistance (Hooper, 2003 and Fonseca *et al*., 2008).

On the other hand, all *P. vulgaris* isolates have shown sensitivity to Amikacin. This result is identical with those obtained by Okesola and Makanjuola, (2009) and Al-Jumaa *et al*. (2011).

All *P. vulgaris* isolates were sensitive to nitrofurantoin. This finding due to nitrofurantoin is bactericidal in urine at therapeutic doses, and its multiple mechanisms of action appear to have enabled it to retain potent activity against *P. vulgaris* (James *et al*., 2002 and Kippax, 2013).

Sensitivity of *P. vulgaris* to norfloxacin was (100%). Resistance to norfloxacin is a rare occurrence and if it occurs. In this case, the cause being spontaneous mutation (range: 10-9 to 10-12 cells). Resistant organisms have emerged during therapy with norfloxacin in less than 1% of patients treated (Padeĭskaia, 2003).

All *P. vulgaris* isolates were sensitive to Imipenem and Meropenem, where carbapenems are antimicrobial drugs effective against gram-negative bacteria (Htoutou *et al*., 2011).

The characterization of various plasmid mediated TEM-type β-lactamase in *Proteus* are evidence of the wide diversity of β-lactamases produced by this species and of its possible role as β-lactamase-encoding plasmid reservoir (Bonnet *et al*., 1999). So, in the present study we selected two important genes that confer resistant to B-lactam and quinolone antibiotics which are *bla* TEM and *qnr* genes.

A-blaTEM gene

β-lactam antibiotics are a broad class of antibiotics that are used on a large-scale. We are studying the enzyme TEM β-lactamase which plays a major role in the development of antibiotic resistance.

All *P. vulgaris* isolates which were resistant for β-lactam antibiotics were having *bla* TEM gene, this results obtained after amplify the DNA of resistant isolates with specific primers for *blaTEM* gene by PCR. Amplicons with predicted size of 310bp were generated and the prevalence rate was (100%) as show in figure (3-18).

The *bla*TEM had a high occurrence (100%), showing its presence in all resistant isolates of *P. vulgaris*. These results are in agreement with previous results reported by Dallenne *et al*. (2010), Tissera and Mae Lee (2013) who found *bla*CTX-M genes with the highest occurrence in clinical *Proteus* isolates in studies conducted in France and in a Chinese urban river. Similarly, *bla*TEM genes were of note-worthy high occurrence too. Where, *blaTEMs* often co-occur with other chromosomal (AmpC) or plasmidic (SHV, OXA, CTX-M) β-lactamases and they are common in commensal bacteria inhabiting the human gut (Salverda *et al*., 2010).

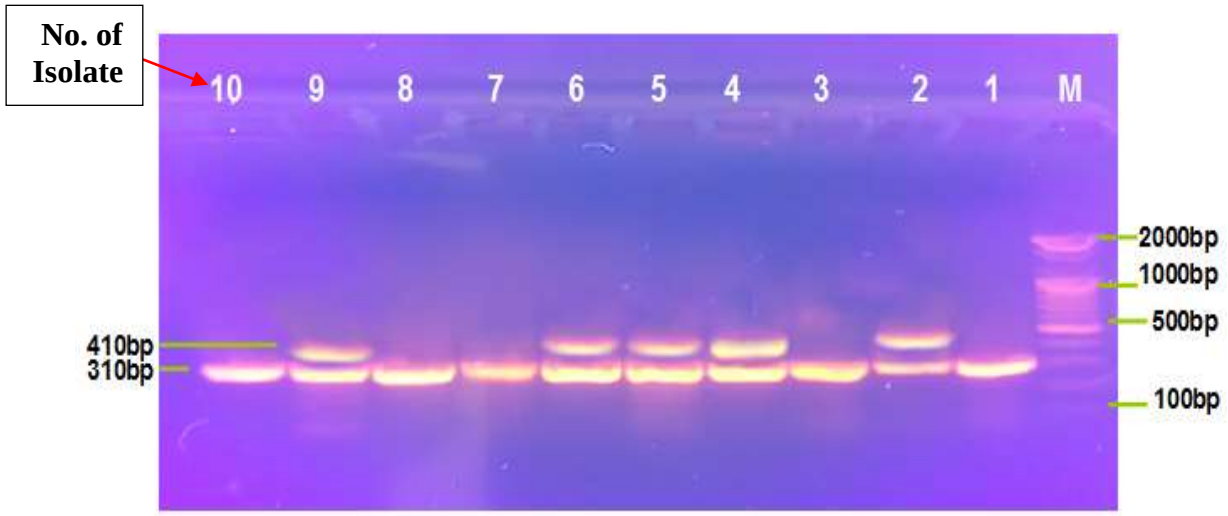


Fig (3-18):- Gel electrophoresis of PCR of *blaTEM* and *qnr* amplicon product. M: Marker; 1, 2, 3, 4, 5, 6, 7, 8, 9, 10: no. of *P. vulgaris* isolates; *blaTEM* gene amplicon size: 310bp and *qnr* amplicon size: 410bp.

Thermocycling parameters were as follows: an initial denaturation of 95 °C for 5min, 30 cycles of denaturation at 95 °C for 45 s, primer annealing 62 °C for 30 s, and extension at 72 °C for 45s.Finally one extension step at 72°C for 7 min. The electrophoresis was carried at 1% agarose gel and the electric current was allowed at 110 volt for 60min.

The increasing variety of β-lactamases produced by isolates of the family *Enterobacteriaceae* raises concern about our dependence on β-lactam drugs and the emergence of pan-resistant species (Qin *et al*., 2008).The TEM β-lactamases are among the best-studied antibiotic resistance enzymes around. They act by hydrolysing the β-lactam ring of penicillins, cephalosporins and related antibiotics and are found at high frequencies in hospitals and clinics around the world (Salverda *et al*., 2010).

Proteus has an intrinsic resistance to ampicillin and cephalosporin due to extended spectrum β-lactamase (Coque *et al*., 2008). Resistance to expanded spectrum cephalosporins may develop through the expression of chromosomally encoded class C beta-lactamases. Horizontal gene transfer mediated by R plasmids, transposons and integrons is largely responsible for

increasing the incidence of antibiotic-resistant infections worldwide (Fam *et al*., 2013).

In terms of danger to human health, previous research highlight that potential extended spectrum β-lactamase (ESBL) species such as *K. pneumonia*e and *E. coli* have a high tendency to possess and transfer *bla* genes (Bailey *et al*., 2011). Transfer may occur by conjugation because the genes are often found on mobile elements like transposons and integrons (Tissera and Mae Lee, 2013). Some of these species may be pathogenic strains that have the potential to cause life-threatening diseases and widespread outbreaks. For instance, *bla*CTX-M and *bla*TEM genes in opportunistically pathogenic *Enterobacteriaceae* have been associated with nosocomial infections (Zhang *et al*., 2008).

B-*qnr* gene

In the present study, PCR was used to detect the prevalence of *qnr* gene in *P. vulgaris* isolates. As it is evident from figure (3-18) that *qnr* gene was detected with (50%) of the isolates. Amplicons with predicted size of 410bp were generated.

Our result corresponds with a previous study by Enabulele *et al*. (2006) who found the average resistance of the Gram negative isolates to the various quinolones ranged from 42.7% to 66.7%. *Klebsiella* were the most resistant isolates with a mean resistance of 66.7% while *Proteus* were the less resistant isolates with a mean resistance of 42.7% and this finding due to the presence of *qnr* gene in these isolates. Also, it was similar to the results obtained by Wallace *et al*. (2000) and *Daini et al*. (2008) who referred to that all Gram negative strains resistant to any antimicrobial agents were also

resistant to ciprofloxacin.

The *qnr* genes were identified also among isolates which were sensitive to quinolones tested. This result has clinical implications since acquisition of the *qnr* genes by quinolone susceptible, ESBL-producing strains could lead to *in vivo* selection of ciprofloxacin and cephalosporin resistant strains, where previous studies showed that *qnr*-positive strains frequently expressed ESBLs and/or ESC (Jacoby *et al*., 2006; Yamane *et al*., 2007).

The resistance mechanism of these drugs was considered to be chromosomally encoded until the discovery of the plasmid-mediated *qnrA* gene in 1998.Thereafter, additional *qnr* genes (*qnrA, qnrB, qnrS, qnrD*) on resistance plasmids were identified worldwide, in various bacterial pathogens(Guo *et al*., 2010). Plasmid-mediated quinolone resistance may facilitate the spread and the increase of the prevalence of quinolone-resistant strains (Bouchakour *et al*., 2010).

Resistance to the quinolones often emerges at low-levels by acquisition of an initial resistance-conferring mutation or gene. acquisition of subsequent mutations leads to higher levels of resistance to the first-generation quinolone, nalidixic acid and a broadening of the resistance spectrum to include second-generation quinolones (first-generation fluoroquinolones) such as ciprofloxacin, followed by newer second- and third-generation fluoroquinolones (Morgan-Linnell and Zechiedrich ,2007).

3.4. Study of quorum sensing in *P. vulgaris:*

To study quorum sensing in *P. vulgaris*, aspartic acid was used as the main focal metabolites for homoserine synthesis. It was observed that homoserine was accumulated in culture media after the addition of KCN in which the later will inhibit threonine synthesis, through its effect on threonine synthase enzyme. Homoserine lactone production was also checked by using brands test in order to ensure that quorum sensing occurs as a result of homoserine lactone synthesis.

Quorum sensing was carried out as mentioned in paragraph (2.7.1). The presence of aggregation of *P. vulgaris* as in figure (3-19) was considered a positive result versus the negative results in the absence of homoserine lactone.

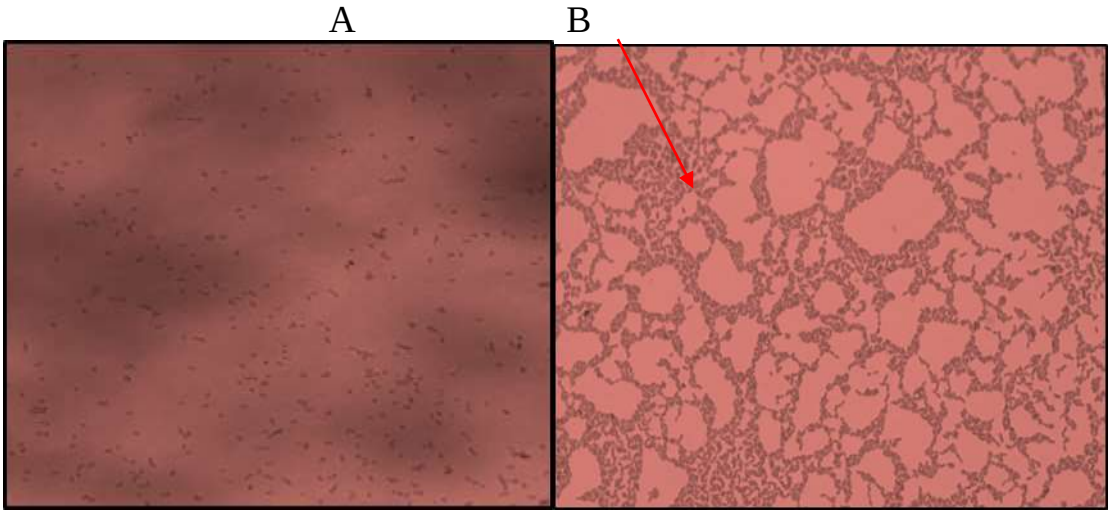


Figure (3-19): Detection of quorum sensing in *P. vulgaris* (100x)

A: control (absence of homoserine); B: positive result (→)

The best interval for accumulation of homoserine lactone was after 4hours of incubation in which the homoserine lactone became at maximum concentration. However, under certain condition the bacteria can form

homocystein as a result of production of homocystein synthase, and the later may be used as indicator for homoserine lactone synthesis.

Bacterial virulence factors are regulated by quorum-sensing molecules which are derivatives of serine substituted by a fatty acid, i.e., acylated homoserine lactones, abbreviated as acyl-HSLs (Soto *et al*.,2002;; Henke and Bassler, 2004; Li *et al*.,2005). The quorum sensing mechanism involves two types of autoinducers: AI-1 based on homoserine lactone and AI-2 based on other molecules. The majority of signal substances in Gram-negative bacteria are substituted by fatty acid derivatives of acyl-HSL (AI-1). There is no evidence that quorum sensing receptors and AI-1 signal molecules are associated with swarming motility in *Proteus* (Belas *et al*.,1998).Whereas, Daniels *et al*. (2004) showed that the QS signal acylhomoserine lactone enhances swarming motility in *Serratia liquefaciens*.

Among the Gram-negative bacteria, the most well studied quorum-sensing system is the LuxR-LuxI homologous system. This quorum-sensing system is widespread among Gram-negative genera and is involved in the regulation of many host-associated phenotypes, including production of virulence factors and secondary metabolites (Lewenza *et al*., 1999; Hentzer and Givskov, 2003).

Two distinct mechanisms of signalling mediated by *N*-AHLs have been described. In most Gram-negative bacteria, the signal is generated by an *N*-AHL synthase of the LuxI family of proteins, and is perceived by an *N*-AHL receptor protein belonging to the LuxR family of transcriptional regulators. The *N*-AHL auto-inducers bind to their cognate LuxR-type proteins only on reaching a critical threshold concentration. Auto-inducer binding controls the transcriptional activity of the LuxR protein in regulating the expression

of target genes, which can include *luxI*. This establishes a positive feedback loop for *N*-AHL synthesis, although it must be noted that positive feedback is not a universal feature of *N*-AHL-mediated quorum-sensing systems. In some Gram-negative bacteria such as *Vibrio* spp., *N*-AHL synthesis is directed by a LuxM synthase (unrelated to LuxI) and perception of the signal involves a cytoplasmic membrane-associated sensor kinase. To date, *N*-AHL-dependent quorum-sensing circuits have been identified in a wide range of Gram-negative bacteria, where they regulate various functions including bioluminescence, plasmid conjugal transfer, biofilm formation, motility, antibiotic biosynthesis, and the production of virulence factors in plant and animal pathogens (Eberl, 1999; Ryan and Dow,2008).

AHLs produced by bacteria could serve as potential biomarkers in the management of bacterial diseases and, thus, monitoring them in biological samples may be a significant analytical tool for the investigation of such diseases (Kumari *et al.,* 2008). Specifically, altered bacteria-host interaction has been implicated in several health imbalances and disorders. For instance, there are several reports referred to the role of bacteria in gastrointestinal disorders such as inflammatory bowel disease and irritable bowel syndrome (Baumgart *et al.,* 2007).

The observation that quorum sensing is linked to virulence factor production and biofilm formation suggests that many virulent Gram-negative organisms could potentially be rendered nonpathogenic by inhibition of their quorum-sensing systems. Research into quorum sensing, and inhibition thereof, may provide a means of treating many common and damaging chronic infections without the use of growth-inhibitory agents, such as antibiotics, preservatives, and disinfectants, that unavoidably select for resistant organisms (Hentzer and Givskov, 2003).

3.5 Genotyping of *Proteus* isolates:

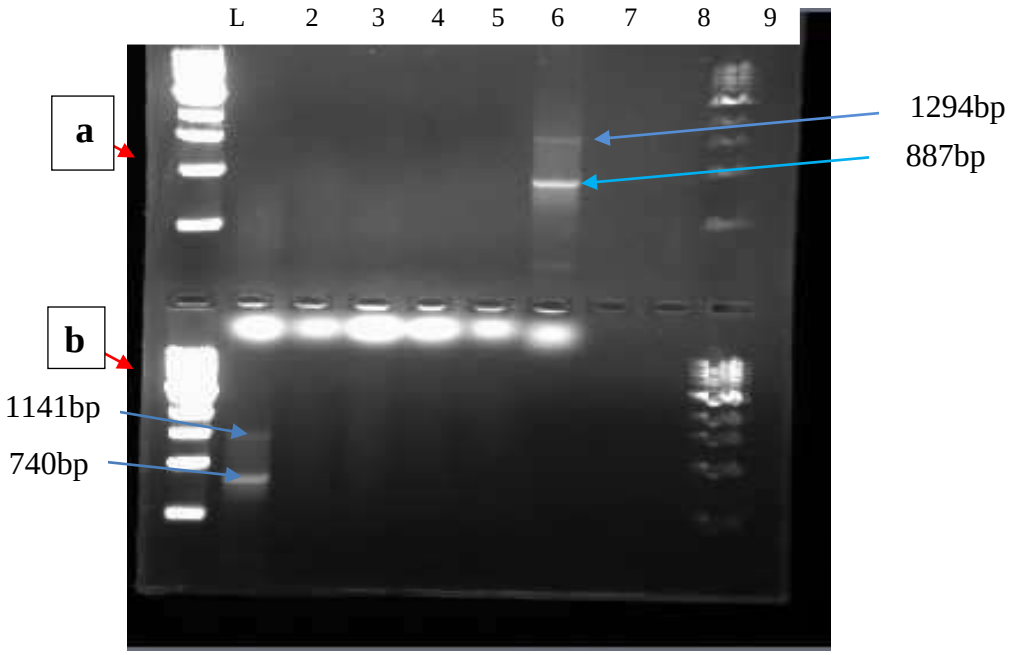
3.5.1 Sequence homology of 16SrRNA gene between *Proteus species*

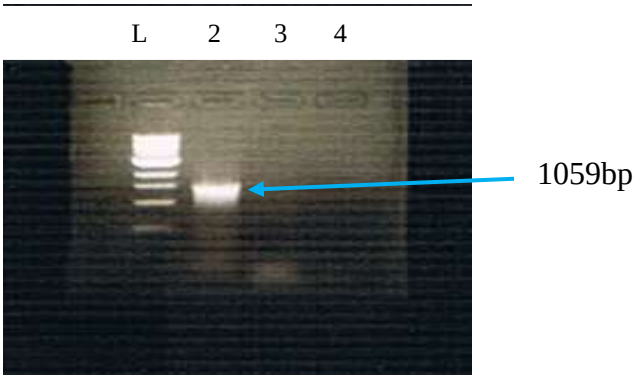
The use of 16SrRNA gene sequences to study bacterial phylogeny and taxonomy has been by far the most common housekeeping genetic marker used for a number of reasons. These reasons include (i) its presence in almost all bacteria, often existing as a multigene family, or operons; (ii) the function of the 16SrRNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time (evolution); and (iii) the 16SrRNA gene (1,500bp) is large enough for informatics purposes (Janda and Abbott, 2007) .

It has been confirmed that sequencing only a part of the 16SrRNA gene can be sufficient to establish phylogenetic relationships (Forney *et al*., 2004, Santos and Ochman, 2004). However, phylogeny estimated from a single gene should be treated with caution (Peixoto *et al*., 2002; Rebecca *et al*., 2007). In this study, the PCR product of 16SrRNA gene from three types of *Proteus* was sequenced. The amplicons size of 16SrRNA gene was 887bp and 1294bp for *P. penneri*, 740bp and 1141bp for *P. vulgaris*, and 1059bp for *P. mirabilis* as shown in figure (3-20).

Percentage nucleotide identity and pairwise uncorrected (p-) distances were calculated for 16SrRNA gene sequences of clinical strains of *Proteus* examined by using alignments Explorer CLUSTAL(Felsenstein, 2006) in Microsoft Mega 4.1 package. The gene sequence of *P. vulgaris* 16SrRNA showed 644/702(92%) homology with the sequence of *P. mirabilis* 16SrRNA and 1065/1096(97%) with *P. penneri*. Whereas, the identity

between *P. mirabilis* and *P. penneri* 16SrRNA was 846/914(93%). The low degree of divergence in the 16SrRNA gene sequences was similar to that reported by Giovanni *et al*.(2011) between closely related members of other genera within the family *Enterobacteriaceae*, such as sequence divergences of 2.4–1.9% were observed when a 978 nt 16SrRNA gene fragment from the type strain of *Proteus myxofaciens* was compared to that of other type strains belonging to the same genus and 16SrRNA phylogenetic analysis could not clearly separate it from the other species of the genus *Proteus* and to other study between *Escherichia coli* and members of the genera *Shigella* and *Salmonella* (95.3–99.6% similarity) or between members of the genera *Enterobacter* and *Klebsiella* (97.2–98.3% similarity) (Fukushima *et al*., 2002). This fact has already suggested the existence of phylogenetically conserved regions among bacteria.





(c)

Figure (3-20). Amplicon product of 16SrRNA of *Proteus* species on 1% agarose gel electrophoresis. L: DNA Ladder (1kb), (a); *P. penneri* (b); *P. vulgaris* and (c); *P. mirabilis*.

Thermocycling parameters for (a)&(c) were as follows: an initial denaturation of 94 °C for 5 min, 35 cycles of denaturation at 94°C for 1min, primer annealing (53°C & 57°C) for 1min, and extension at 72°C for 1:30 min. Finally one extension step at 72°C for 10 min. The same conditions used for (b) only primer annealing was (47°C & 48°C) for 1min. The electric current was allowed at 110 volt for 60min.

Evolutionary tree, based on 16SrRNA gene sequences, were constructed by the neighbour-joining method as shown in figure (3-21). As will be seen in the this figure, *P. penneri* lies in the same branch of the phylogenetic tree with *P. mirabilis* although it has a higher similarity (97%) in 16SrRNA gene sequence with *P. vulgaris* than with *P. mirabilis* (93%).This finding may be due to that the identity of 16SrRNA gene sequence between *P. mirabilis* and *P. vulgaris* was (92%).Whereas, it was (93%) with *P. penneri* according to that *P. mirabilis* being more closer to *P. penneri* than to *P. vulgaris*. So, it is arranged in the same branch of the tree.

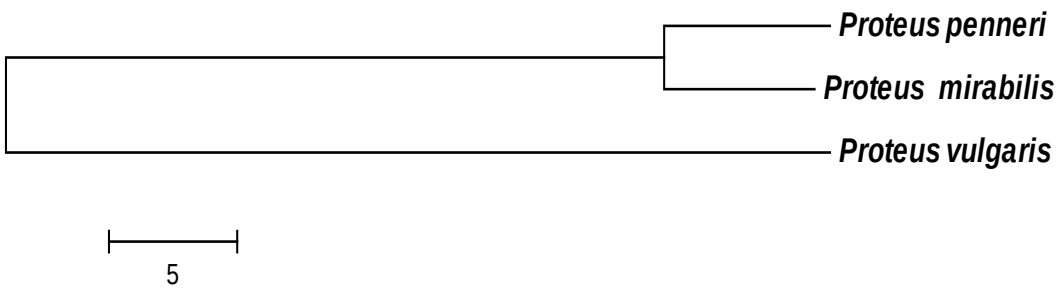


Figure (3-21): Phylogenetic tree of *Proteus species* based on 16SrRNAgene sequence analysis. The tree was constructed with the similarity and neighbor joining by MEGA 4.0.Bar (5) represents substitutions per nucleotide position.

Recently the 16SrRNA gene sequence with the size of 1.5 Kb was considered and widely used in bacterial taxonomey because it contains high conservation region which have variable region in different species (Kox *et al*., 1995). Furthermore, the most importance is that 16SrRNA gene can be sequenced easily. By combining the molecular phylogeny with traditional approaches, such as morphological, physiological and biochemical characteristics, bacteria identification can be carried out more accurately (Mo *et al*., 2003; Li *et al*., 2006; Rongrong *et al*., 2008).

Comparison of polymerase chain reaction (PCR) amplified genomic fragment sequences can be used for the molecular identification of, and differentiation between bacterial species. Of these sequences, that of 16SrRNA encoding gene is by far the most widely used and its usefulness has been greatly enhanced through the establishment of public domain databases, which now contain sequence data derived from more than 5000 species. However, the sensitivity of this approach has been questioned particularly among *Enterobacteriaceae*, which includes many common

human bacterial pathogens because of the high degree of conservation in closely related species (Case *et al*., 2007).

3.5.2 Genotyping of the genus *Proteus* by *rpoB* sequence analysis

Sequence analysis of the RNA polymerase ß subunit encoding gene (*rpoB*) has been proposed as a novel tool for bacterial identification (Giammanco *et al*., 2010). A portion of the coding region of the *rpoB* gene from 3 types of the genera *Proteus* was amplified with specific primers according to the technique described by Mollet *et al*. (1997). The amplicons size generated for the *rpoB* gene of that strains shown in figure (3-22).

The PCR amplicons generated were sequenced with the same primers to obtain partial *rpoB* gene sequences .The *rpoB* sequences examined by nucleotide blast and the percentages of similarity was calculated by FASTA method. The data showed that from 33 local isolates of *Proteus*; 7 isolates have the same *rpoB* gene sequence (99%) of *P. vulgaris* strain ATCC 6380, 12 isolates were (99%) similar to *rpoB* sequence of *P. vulgaris* strain ATCC 6898, and 9 isolates have *rpoB* sequence (99%) identical with *P. vulgaris* strain ATCC 29905. Whereas, 4 isolates were similar (99%) with *P. mirabilis* strain ATCC 56283712, and one strain was similar (99%) to *rpoB* sequence of *P. penneri* ATCC 33915 and these results are more clarified in table (3-2). So, we can named the local strains in the present study according to that name of the standard strains.

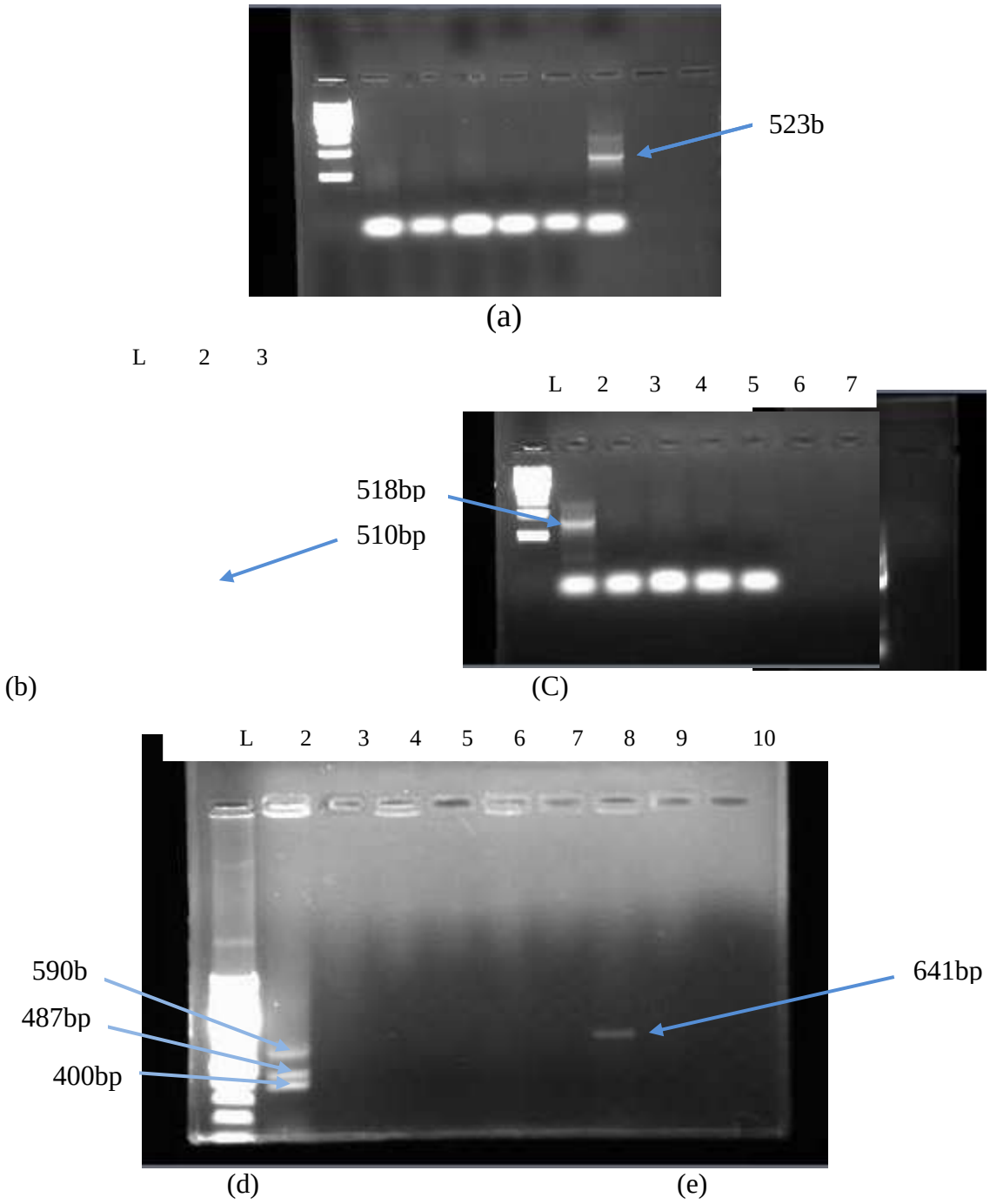


Figure (3-22). Amplicon product of *rpoB* of *Proteus* strains on 1% agarose gel electrophoresis. L:DNA Ladder(1000bp in a, b, c & 100bp in d & e), (a); *P. vulgaris* ATCC 6380 (b); *P. penneri* ATCC 33519 ,(c); *P. mirabilis* ATCC 56283712,(d);*P. vulgaris* ATCC 6898 and (e);*P. vulgaris* ATCC 29905.

Thermocycling parameters for (a) & (c) were as follows: an initial denaturation of 94°C for 5 min, 40 cycles of denaturation at 94°C for 1min, primer annealing (46°C & 53°C) for 1min, and extension at 72°C for 1:30 min. Finally one extension step at 72°C for 10 min. The electric current was allowed at 110 volt for 60min.

Table (3-2): Results of NCBI blast of *rpoB* gene sequences of local isolates

No. of Local Isolates	NCBI Blast Alignment	*rpoB* Sequence Identity (%)
7	*P. vulgaris* strain ATCC 6380	99%
12	*P. vulgaris* strain ATCC 6898	99%
9	*P. vulgaris* strain ATCC 29905	99%
4	*P. mirabilis* strain ATCC 56283712	99%
1	*P. penneri* ATCC 33915	99%

Sequence alignment and phylogenetic analysis were carried out on 500nt fragments using the Alignment Explorer/ CLUSTAL and MEGA software version 4.1(Tamura *et al.,* 2007). To ensure the stability and reliability of phylogenetic relationships among strains used in this study, phylogenetic tree was reconstructed by using the neighbour-joining method, with the option of complete deletion of gaps (Felsenstein, 2006). The statistical significance of the phylogenies inferred was estimated by bootstrap analysis with Bootstrap (500 replicates; seed=31332).

The examination of *Proteus* strains by *rpoB* sequencing showed between 5 and 13% nt differences in the *rpoB* 500 bp region analysed. On the basis of *rpoB* sequence divergence, the type strain of *P. mirabilis* ATCC 56283712 was diverged by 7%, 6%, 5%, and 8% from *P. vulgaris* ATCC 6380, *P. vulgaris* ATCC 29905, *P. vulgaris* ATCC 6898 and *P. penneri* ATCC 33915 respectively. *P. penneri* differed by 6-13% from all *P. vulgaris* strains. Whereas, *P. vulgaris* strains were differing from each other by 2-8% of their nucleotides.

The phylogenetic tree derived from partial *rpoB* gene sequences of clinical strains of 3 species of the genus *Proteus* showed in (Fig. 3-23). As shown in this figure, five *rpoB* groups supported by >50% bootstrap values could be described. The comparison of *rpoB* gene sequence divergences among clinical strains revealed that they were generally closely related to their respective type strain. However, as already shown by the phylogenetic analysis, *P. penneri* strain, and type strain of *P. vulgaris* clustered in two separate groups within the same branch of the tree. This finding may be due to the genetic relationships between closely related species *P. vulgaris* and *P. penneri.*

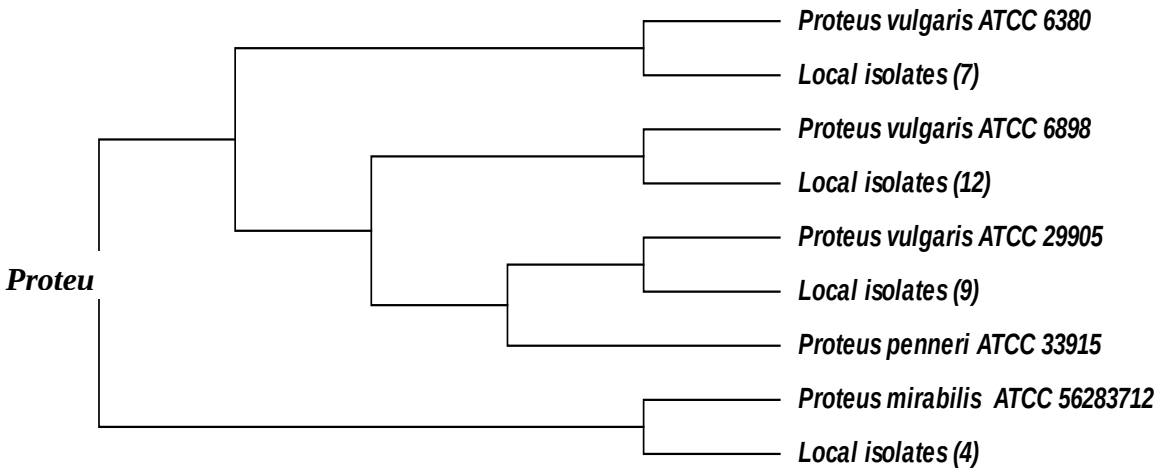


Fig. (3-23). Phylogenetic analysis of partial nucleotide sequences of the *rpoB* gene of type and clinical strains of *Proteus* genus. (7), (12), (9), and (4) represent no. of isolates. The tree was generated using the neighbour-joining method. Bootstrap values > 50%.

Attempts have been made to define a cut-off for *rpoB* gene sequence-based identification of bacteria; Mollet *et al*. (1997) found an intraspecies similarity range of 98–100% when they analysed a 512bp fragment of the *rpoB* gene in clinical isolates of enteric strains. For 600–825bp gene fragments, a *rpoB* sequence similarity of at least 96–97% seems to be the threshold for correct species identification (Ade´kambi *et al*., 2003; Khamis

et al., 2003; La Scola *et al*., 2003). According to these limits, the 8% sequence divergence detected between strains of *P. vulgaris* could be sufficient to suggest possible further taxonomic adjustments within this species.

The *rpoB* gene has been used as a molecular marker for identification, differentiation and phylogenetic analysis of several species of bacteria because

this gene is a highly conserved housekeeping gene with a single copy in the genome that encodes the beta subunit of RNA polymerase, responsible for most catalytic functions of the enzyme (Ferreira-Tonin *et al*., 2012).Since 1997, numerous studies have been developed using the *rpoB* gene, mainly in the clinical and environmental areas(Ade´kambi *et al*., 2008).

Our data indicated that the *rpoB* gene is a suitable and efficient molecular marker for the distinction of *Proteus* species and can be used as an alternative molecular tool for examining phylogenetic relationships of the genus *Proteus* and a powerful tool for the study of different microorganisms.

Conclusions:

The study has arrived at the following conclusions:

1- *Proteus vulgaris* was predominant among patients with UTI.
2- It has been confirmed that all *P. vulgaris* isolates have the ability to possess more than one virulence genes such as hemolysin, extracellular protease, urease, adherence factors, swarming activity and multidrug resistant genes.
3- It has been confirmed there are many factors affecting swarming activity like 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.vulgaris* infection.

4- All isolates are resistant to ampicillin, amoxicillin, cephalothin, chloramphenicol and ceftazidime, and some isolates were resistant to ceftriaxone, ciprofloxacin and cotrimazole. Whereas, all isolates were sensitive to gentamycin, imipenem, meropenem, amikacin, nitrofurantion, and norfloxacin.
5- Quorum sensing was carried out through the production of homoserine lactone by bacterial isolates.
6- Results indicate that 16SrRNA and *rpoB* gene sequence comparison seems to be an appropriate method for inferring genetic relationships within the *Proteus* species and species strains on a molecular basis.

Recommendations:

1- Identifying bacteria isolated in the clinical laboratory by sequence or on molecular basis instead of phenotype which can improve clinical microbiology by better identifying poorly described, rarely isolated, or biochemically aberrant strains.
2- Studying quorum sensing by using other means such as thermones and some signaling proteins.
3- Using biological active compounds from natural sources to inhibit the

pathogenicity of bacteria.

4- The using of new antibiotic should be highly selective and is not used for long time to decrease the chance of emergence of bacteria drug resistant.

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