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DETECTION OF P FIMBRIAE GENES IN *E. COLI* ISOLATED FROM URINARY TRACT INFECTION

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Abstract
A total of 100 clinical isolates of Gram negative bacteria primary identified as *Escherichia.coli* were obtained from different teaching hospitals in Baghdad .The source of these isolates was urinary tract infections(UTI).The collected isolates were initially identified in hospitals as. *E.coli* to confirm preceding diagnostic the isolates were initially identified by culturing on MacConkey agar,Blood agar, chrom agar then identified by some morphological characteries the last step was performed by using the housekeeping gene *16SrRNA*. The results confirm that all the 100 isolates were *Escherichia.coli.* All the isolates showed ability of adherence to uroepithelial cells by using samples from mid-stream urine which was obtained from healthy women .The aggregation of bacterial cells which appeared as a considered as the positive results while the control cell remain clear.purple dotes on the epithelial cell surface after incubation time.Colony adhesion factor (CAFIII) were detected in which agglutination of human red blood cell was used . Results revealed that approximately 62 % of the had the ability to express CAFIII determining by agglutinating human blood cells in the presence of tannic acid.
Polymerase chain reaction (PCR) technique was used to screened *pap* fimbriae genes, these genes *pap E/F, papC/D, papG allel I, pap G allel III* results showed that percentage were 35% , 54% , 13 % , 24 % respectively. In this study it was appeared that only 7 isolates were harbord all the gene (*papE/F* , *papG1* , *papG3* , *papC/D*) while 11 isolates contain 3 genes , 18 isolates contain 2 genes and 29 isolates contain 1 genes while 35 isolates don't contain any genes beside 33 from these negative isolates were negative for colony adhesion factors CAFIII.

Keywords: Uropathogenic Escherichiacoli (UPEC) ; P Fimbriae, polymerase chain reaction

Introduction

Escherichia coli was first described by the German pediatrician Teoder Escherich (1885) from infants stool sample ,the bacterium was first cultured as normal flora after birth, and it was considered as a non- pathogenic (Brooks *et al*., 2010).
After that, it was isolated from the blood of women with urinary tract infection , an incident revealed that this bacteria reach the bladder by the ascending route then delivered to blood (Retrived,2012).In 1945, the researcher Bray diagnosed an isolates of *E.coli* which was the causative agent of diarrhea among children in

England at that time , then it was known as Enterophathogenic *E.coli* (EPEC) (Donnenberg *et al*.,2015). *E.coli* is one of the most intensively studied living species, and has been widely used in the experimental studies of evolution (Blount.,2015). *E.coli* belong to Enterobacteriaceae family, which includes a large number of species with a rapprochement between them (Vila *et al*.,2016). The genus Escherichia contains many species like, *E.blattae, E.hermanii, E.fergusonii and E.vuneris* and *E. coli* which differ from each other in some biochemical reactions , still *E.coli* is the most predominant one and most common type of human pathogenesis (Croxen and Finlay, 2010).

On the other hand, genus *Escherichia* is genetically convergent, especially with the Shigella (Pettengill *et al*.,2016). *E.coli* is naturally endemic the human digestive tract, which makes it possible to infect the human body by opportunistic manner, causing several diseases (Ahmed,2016) .Beside that, it habitat the intestines of many animals thus it has the potential to spread in the environment and contaminates water, food and dairy products, for that their presence in water considered a sign for fecal contamination (Brooks *et al*., 2016).

The most two important diseases caused by this bacteria are urinary tract infection and diarrheal infection. World Health Organization (WHO) reports the higher incidence of world's death rates due to these two diseases, and the most affected age groups for the second infection are children under 5 years of age (Sherchan *et al*,. 2016).

E.coli is the leading cause of about 70% of urinary tract infections especially in women (Bader *et al*.,2017). It comes as the second frequently occurred disease after respiratory infection(in community-related diseases), while *E.coli* infections come first in nosocomial infections beside other genera including *Acinetobacter* and *Pseudomonas* (Wu *et al* ., 2016).

Adherence factors facilitate the attachment to the urinary tract and promote *E.coli* colonization and persistence in the colon or vagina, which may serve as reservoir for ascending infection in the urinary tract (Zhang and Foxman, 2003). Specific adhesion is mediated by bacterial proteins designated adhesions which may or may not be associated with fimbriae. They can be differentiated on the basis on their binding receptor specificity (Schmidt and Hensel, 2004).Various adhesions have been identified such as type 1 fimbriae , P fimbriae , Afmbrial adhesion , S fimbriae, FIC fimbriae, and Dr fimbriae (Antâo *et al.,*2009).Pathogenicity islands located in the genome of bacteria encode adhesions, which mediate the capacity of microbes to attach to specific eukaryotic receptor molecules (Ferdous *et al*.,2015). P-fimbrial type

It is also called the mannose resistant fimbriae, present in some *E.coli* isolates specially these causing infection of the pelvis acute kidney(pyelonephritis) (Khandige *et al*.,2015) .It characterized by their ability to agglutinate the red blood cells ,and this type of agglutination does not inhibit in the presence of mannose sugar therefore described as Mannose-resistance Haemagglutination (MRHA), colony adhesion factor (CAFIII) used as a phenotype detection for P fimbriae (Harvey *et al*., 2007).The adhesion of the P fimbriae is encoded by *papG* and the specific receptor is Gal (α1→4)β Gal-containing glycolipids (Paykoc *et al*.,2018). There are different variants of the P fimbriae that differ in receptor binding properties.The different *PapG* variants (*papGI, papGII* and *papGIII*) or PrsG the *pap*-related sequence G have

different isoreceptors, giving them different specificities for binding (Lane and Mobley, 2007).

Pap operon (Figure 1-3) contains the structural genes *papA*, -*E*, -*F*, - *G*, and -*H*, the usher gene *papC*, the periplasmic chaperone gene *papD*, while *papB* and *papI* genes involved in regulation of pap operon (Stephenson and Brown,2014).UPEC isolates associated with symptomatic disease are more likely to contain multiple P fimbria operons . Expression of P fimbriae is regulated in response to growth and environmental conditions (Khandige *et al*.,2015) .One of the important thing that *pap* operon is subject to phase variation ,which is dependent on a reversible epigenetic switch that controls the initiation of transcription of the *pap* operon genes, resulting in variable on/off expression of the structural subunits .Usually type 1 and P pili are prototypes for understanding the assembly of the chaperone usher pathway(Khandige *et al*.,2015) .

Material and Method

Collection of E.coli isolates: One hundred E. coli isolates were obtained from a patient suffering from urinary tract infection (UTI)in some Baghdad hospitals. They were reunified and diagnosed using the 16s RNA gene.

Phenotypic detection of adherence ability to Uroepithelial cells

According to Svanborg ,(1988), the adherence ability of different bacterial isolates on urinary epithelial cells was described . Samples from mid-stream urine was obtained from healthy women and considered as a source for epithelial cell .It was centrifuge at 3000 rpm for 5 minutes, the sediments containg the epithelial cells was washed 4 times with phosphate buffer saline (paragraph 2.2.2.2.).The sediment cells were suspended by the same buffer, and solutions were used as a source for human epithelial cells to be used later in adhesion of bacteria to epithelial cell (Lomberg *et al*., 1986). Bacteria 10^8 CFU/ml (compared to macferland 0.5×10^8) were added to 10^5 epithelial cells (compared to macferland 10^5).After incubation for 60 minutes at 37 ° C, unconnected bacteria were eliminated by repeated washing with phospate buffer saline .Finally, the cells were fixed and stained by gram stain. Number of adhered bacteria were counted by the directed light microscope. (Eden *et al*., 1988).

Detection of colony adhesion factors (CAFIII) using Hemagglutination method

Phenotypic detection of P fimbriae was determined by agglutinaton of human blood cells A + (paragrah 2.2.7.2.2) in the presence of 0.1 M of Tannic acid (2.2.2.4) (Smyth, 1982). the Test was performed according to Hibberd *et al*.(1991) by mixing in microscope slide one drops of bacterial solution , one drop of red blood cell solution and one drops of phosphate buffer saline with tannic acids and show the heamoagglutination .

Identification of bacteria and P Fimbriae genes: Table 1 and Table 2 represent the primers used in the current study . Genomic DNA was extracted using a DNA extraction kit (Promega-USA) according to the manufacturer's instructions. PCR assay that based on16sRNA housekeeping gene was done described by Salih et al. (2015). It was carried out in 25 µl reaction volumes composed from 12.5 µl of GoTaq®Green Master Mix, template DNA 3µl, forward & reverse primers 1.5 µl for each(0.6 people), and 6.5 µl of Deionized

Nuclease-Free. PCR mixture without template DNA was used as a negative control. PCR was run under the following conditions : primary denaturation step at 94°C for 5 min, 35 repeated cycles start with denaturation step at 94°C for 30 sec, annealing at 58°C for 30 sec, and 1 min at 72°C as extension step followed by final extension step at 72°C for 7 min. The same mixture was used to amplify the studied genes but the annealing temperature and other conditions were running as illustrated in Table 2. The product was running in 1.5% (w/v) agarose (Promega, USA) in 1X TBE buffer (40 mMTris, 20mM boric acid, 1 mM EDTA, pH 8.3), stained with green stain, visualized under UV illumination using a gel image analysis system. Uniplex PCR products for all amplified genes were stored at -20 º, and then nucleotide. Data analyzed using genius software (version 2019 prime) by comparing the result with NCBI control strains.

Table 1. Primer used in the current study with their sequence, molecular size

Primer Name	Forward Primer (5'…….. 3')	Reverse Primer (5'…….. 3')	size (bp)	Reference
16SrRNA	GGAAGAAGCTTGCTTCTTTG CTGAC	AGCCCGGGGATTTCACATCT GACTTA	544	LC 27836
***papC/D* (CU)**	GACGGCTGT ACTGCAGGGTGTGGC G	ATATCCTTTCTGCAGGGATG CAATA	328	CP0325 15
papE/F	GCA ACA GCAACG CTG GTTGCATCA T	AGAGAGAGCCACTCTTATAC GGAC	339	M94076
pap G allel I	TCG TGC TCA GGT CCG GAA TTT	TGG CATCCCCCA ACATTATCG	*258*	AF2374 75
papG allel III	GGC CTGCAATGG ATTTACCTG G	CCACCAAAT GAC CAT GCC AGA	*491*	DQ0103 12.
***afaB/C* (CU)**	GCT GGGCAG CAA ACGATAACTCTC	CATCAAGCTGTT TGTTCGTCCGCC G	750	KR3388 32

Table 2. PCR program application used

Amplified gene	Initial denaturation	No. of cy	Denaturation	annealing	Elongatin	extension
papElF	94 ºC for 5min	40	94 ºC for 30Sec.	63 ºC for 30 sec	72 ºC for 1min	72ºC for 7min
pap C/D	94 ºC for 5min	40	94 ºC for 1min	63 ºC for 30 sec	72 ºC for 1min	72ºC for 7min
pap G	94 ºC for	35	94 ºC for 30	60 ºC for	72 ºC for	72ºC for

allel I	5min		sec	30 sec	1min	7min
pap G allel III	94 ºC for 5min	35	94 ºC for 30 sec	60 ºC for 30 sec	72 ºC for 1min	72ºC for 7min

RESULTS AND DISCUSSION

Identification of E.coli: Genotypic identification using 16s RNA revealed that all the isolated 100% appositive results with amplified size of 544 as compared with DNA ladder. Figure (1-A) illustrated shine bands of positive isolates. It is reported that PCR-based molecular methods have been developed as an alternative method for accurate identification and classification of bacterial species. Especially when the genes amplify the 16s RNA. (Farahaniet al., 2013).16s RNA genes considered one of the basic criteria in the classification because of its regions were highly constant and unable to change over time, also contain areas of highly covariance among types of bacteria so that provides a specific sequence to each type, which means this gene might play important role in diagnosis, when the diagnostic methods is required (Hutchison et al., 2016).The amplified DNA segment for 16s RNA gene was analyzed by Genius Software and the Pairwise identity was 91% which represent the percentage of residues that are identical in the alignment including (figure 1-B). Some differences were recognized between the local isolate and the recorded NCBI strain as clear with gap appeared within the nucleotides.

Adherence to Uroepithelial Cells The results showed the adhesion to epithelial cells occurred between all the isolated (100%).The epithelial cells have many nuts that help them catch bacteria and collect them an around the cell [14].Bacterial adhesion to host cells is an important virulence factor and represents the first step in colonization [15]. The adhesion of bacteriae to uroepithelial cells is used to differentiate between fecal strains and uropathogenic. recommended that uropathogenic E. coli might present a mean of 20 bacteria/ epithelial cell or more, while fecal strains may existing a mean of nearly 7 bacteria/ epithelial cell [16].

Hem agglutinations and expressions of P fimbriae: The results expressed 62 % of the *E.coli* showed their ability to express P Fimbriae determining by agglutinating human blood cells in the presence of Tannic acid . Jacobsen *et, al*. (2008) exhibited the occurrence of P fimbriae as a maximum in urinary tract infection than in fecal isolates of healthy people.

PCR Amplifications for P fimbriae :

Escherichia coli isolates were examined for *pap* fimbriae genotype and specific primers were utilized to detect in genes associated with outer membrane protein usher (*papC*) and periplasmic chaperone (*papD*) **.**Figure (3-14) illustrates the amplified PCR products passing through the two genes as was designed in the current study and size of this amplified region was 328 bp compared with 100 bp DNA ladder. Most of the isolates gave bands of the same amplified size. Polymerase chain reaction showed that the prevalence of Chaperone /Usher *PapC*/D genes was found in 54% of UPEC isolates in the current study . Karimian *et al*. (2012) and Momtaz *et al*.(2013) found the present of *papC* gene were 50.4% , 50.41% respectively .Also Abass *et al*.

(2014) who found that nearly 79% of isolates were positive for *pap*C, suggesting that other adhesion genes were present or *pap* C may be deleted. While this results of the current study not agreed with AL- Ganimi *et al*.(2016) and AL-kubaisy (2013) who mentioned that prevalence of *pap C* gene were only 28.5% and 18.5%, respectively.

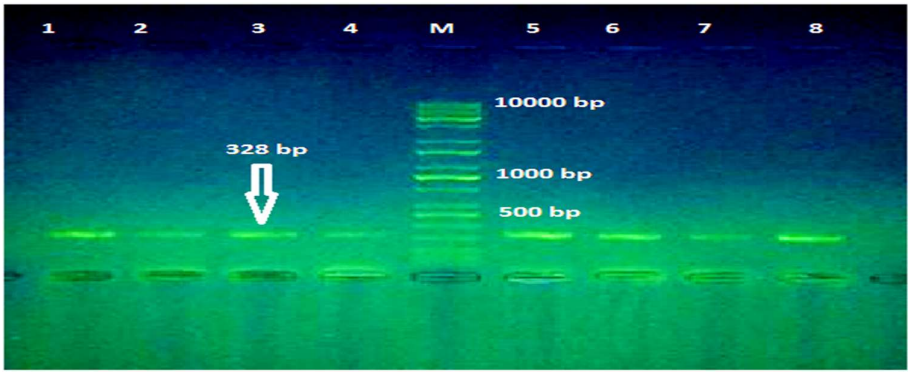
As with other amplified genes, *papC/D* sequence was analyzed then illustrated in figures (3-14B) and (3-14C).The comparison was preformed between this DNA segment and the standered strain CP032515.The proteinID was AYE17289.1while the Pariwise identity was 100% in which niether gaps nor differences in the neuclioted sequence were detected upone these two genes in this study .Blast Hit Length: 309; Interval: 3,479,715-> 3,480,023 from the complete genomic DNA related to an isolated strain from urine.

Wu *et al*.(2008) observed that *pap*C/D assay is the specific and sensitive method in the detection of UPEC strains and the 328 bp sequence may be proved to be valuable for the identification of UPEC strains as the specific molecular marker.

Usually *pap* genes presence had been associated with pyelonephritis ; therefore, higher percentages (over 50%) suggest that the isolates obtained from the UTI have greater capabilities to colonize kidneys and generate pyelonephritis (López-Banda *et al*.,2014) .

P-fimbrial expression is subject to phase variation involving a reversible epigenetic switch between ON and OFF states which responds to regulator proteins encoded within the operon, *pap*I and *pap*B, as well as the global transcription regulators H-NS, cAMP receptor protein (CRP), DNA adenine methylase (Dam) and Leucine responsive protein (LRP) **(**Khandige *et al*.2015).

In 2018, Tseng and his team work demonstrated that P fimbriae involved in pathogenicity and severity of *E. coli* kidney infections in diabetic hosts and there was an increased incidence and severity of kidney infections (Tseng *et al.,*2018) .



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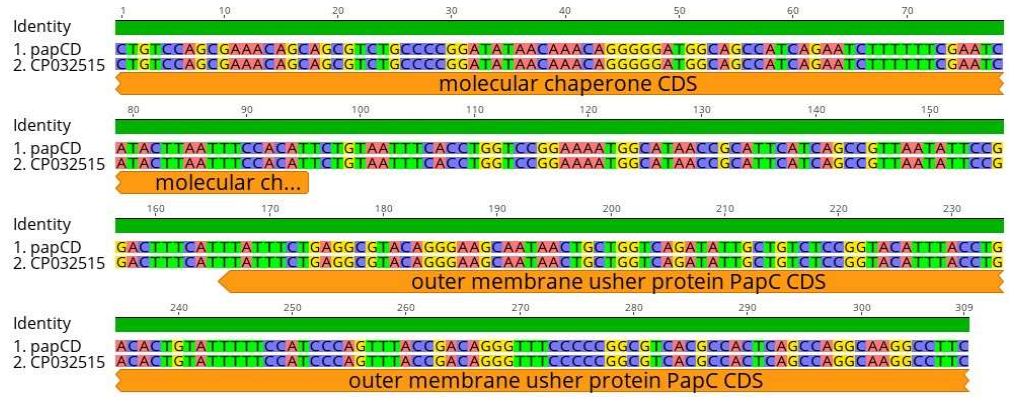

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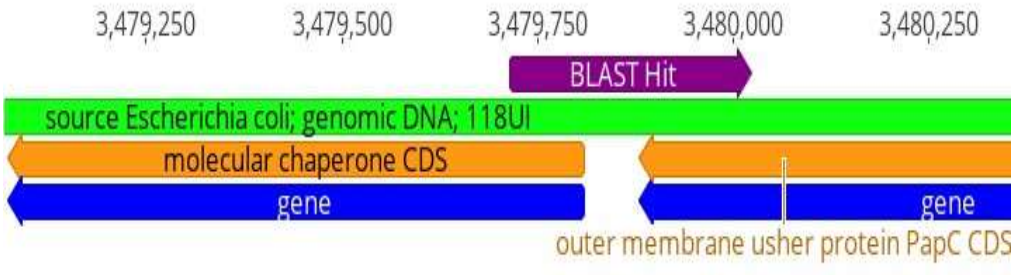


C

Figure (3.14) A:Agarose gel electrophoresis(1% agarose, 7 v/cm2 90 min) using green staining .The amplified size was 328bp .Lane M, molecular size DNA ladder (100 bp DNA Ladder); lanes 1-8 ,Positive PCR bands; B: Pariwise identity and nucleotide sequence for papC/Das compared with the standered strain CP032515 .C: Blast Hit of the amplified gene from the total genomic DNA of the standerd strain

For *pap E\F* (minor structural subunits) the results showed that only 35 % of the isolates harbored this amplified genes segment .Figure (3-15A) illustrates PCR products passing through the two structural *papElF* genes with expected amplified size 339 bp as compared with 100 bp DNA ladder. In this study the low percentage of amplified *pap* gene was noticed although this gene is one of the virulence genes commonly spread in cases of urinary tract infections.

The researchers Tarchouna *et al*.(2013) and Köves (2014) showed that *pap* ratio were 41% and 43% ,respectively, which is higher than the current study and may contribute to the type of UTI infection and source of isolation .

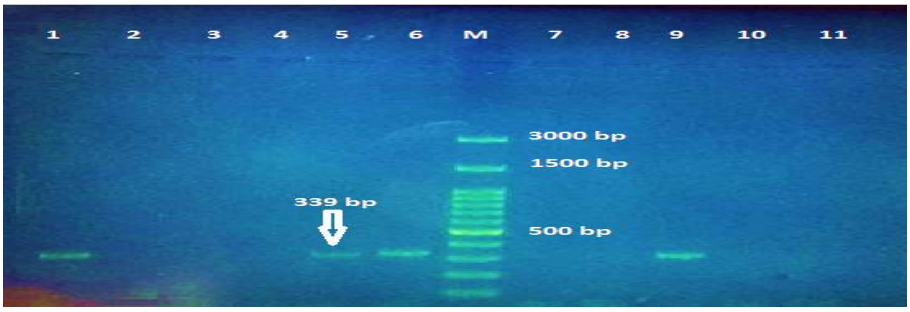
On the other hand , finding of the current study did not agree with Salih (2015) when he noted a high rate of *pap E/F* gene that has been associated with the antibiotics resistance of *E.coli* isolates obtained from UTI. Since the binding to the host cells is a key factor for development of infection , the expression of different types of adhesions, with affinity to distinct specific receptors confers advantages to pathogens (Antao *et al*.,2009).

papE/F gene sequence are demonstrated in figures (3-15B) and (3-15C). The comparison

was done between this DNA segment and the standered strain M94076.The protein ID AAA24283.1,and the Pariwise identity reached 100%. No changes in the entire neucleotide sequence was noticed ,and the Blast Hit Interval: 522 -> 692

The next studded gene in the current study is *papG* which has three alleles .The classes of *papG* genotype differed according to the type of receptors specificity and infection (Hassan *et al* .,2011). The results of the current study revealed that *papGI* was less prevalent than *papGIII* this may due to the site of infection. For *papGI* ,the results showed that only 13% of *E.coli* gave positive result and it could be considered areason able result since *papGI* might have larger prevalence among fecal isolates (Ferreira *et al*.,2018).Figure (3-16) showed the positive gene band for *PapGI* gene at amplified size 258 bp.In Iran, Rahdar *et al*. (2015) reported the frequencies of *pap* genes as 20.5%, 30.2%, 57%, respectively. These differences might be due to the differences in geographical regions. The *pap* gene encodes for P-fimbriae that allows bacteria to adhere to epithelial surfaces and protect them against urine lavage, thus allowing them to ascend further to even cause serious disease (Mohajeri *et al*.,2014).

For *papGIII* gene, 24 % of isolates contained this gene.The gene *papGIII* may be associated with pyelonephritis and bacteremia (Kudinha ,2017).Figure (3-17A) illustrates the amplified PCR products for gene *pap GIII* with molecular size equal to 491 as compared with 100 bp DNA ladder. Simer (2015) in his research reported that the absence of the gene in the isolates may be due to either mutations in the target gene or may result from deletion at the site of the initiator .Al-Mayahie (2013) reported that allele II was found in 32.7% of the urine samples and 3.2% of the stool isolates . Ahmed *et al*. (2016) showed in his study the prevalence of *papG I* and pap*GIII* was 19.4% and 38.8%, respectively. Payko and Turkyilmaz (2018) found in their study the percentage of allele II was (24.8%) followed by allele III (5.3%) and allele I was only 0.8%. A study from Ankara in Turkey done by Arisoy et *al*.(2006) and their results were parallel to the current study, they revealed that almost 23% *E. coli* isolates were positive for *papGIII , papG* allel III gene sequence of *E. coli* was analyzed as esented in figure (3-17 B &C). and the Pariwise identity was 89%. (Figure 3-17B). Many differences were detected in DNA sequence between the local isolates and the standared strain DQ10312 ,while figure (3-17C) represent the Blast Hit for this gene in which the precise amplified segment (purpel color) is clear within the total *pap* operon of the recorded NCBI strain.



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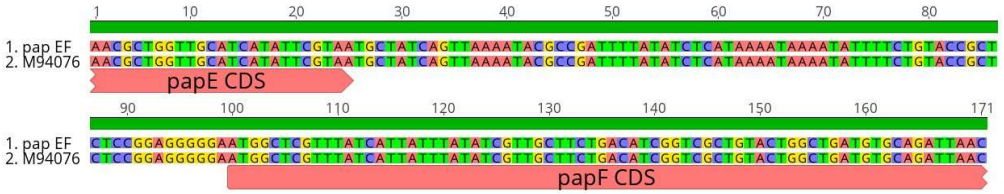

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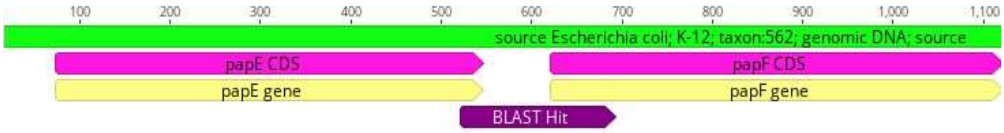


C

Figure (3-15A):Agarose gel electrophoresis of *papE/F* gene (339 bpamplicon). Electrophoresis was run at 1% agarose, 7v/cm^2 for 90 min. lane M: DNA Ladder; lanes 1, 5,6 and 9 represent positive results .B: Pariwise identity and nucleotide sequence for*papE/F*as compared with the standered strain *E.coli* taxon: 562 ; M94076(NCBI).C:Blast Hit of the amplified genes from the total genomic DNA of the standerd strain.

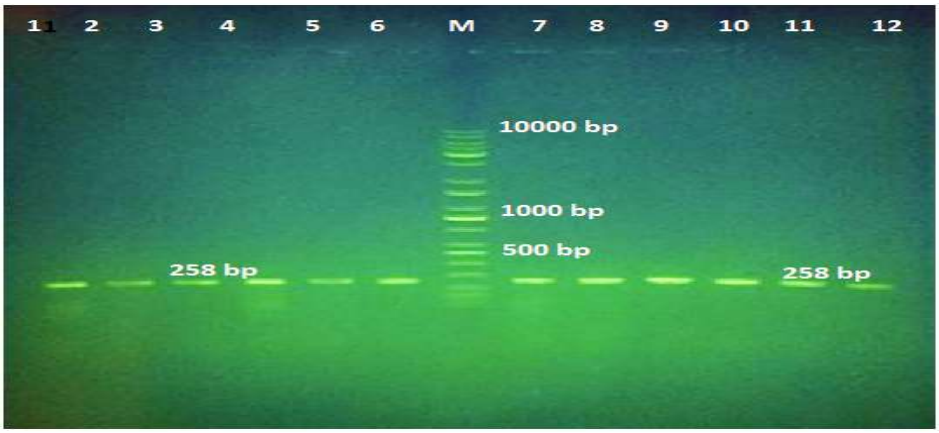
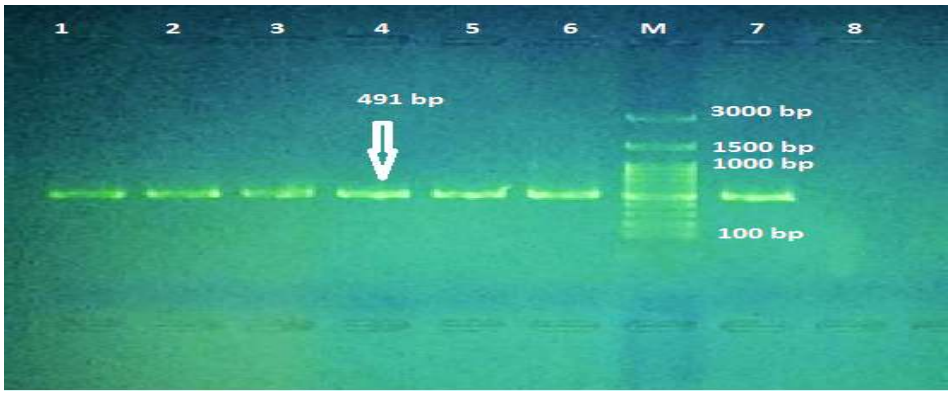


Figure 3.16**:**Agarose gel electrophoresis(1% agarose, 7 v/cm^2 ,90 min) and green staining. to detect *papG1*gene size product (band 258bp).Lane M, molecular size DNA ladder (100 bp DNA Ladder); lanes 1-8, DNAs isolated from *E.coli*samples and all lanes 1-8 showed Positive PCR bands.

In this study it was appeared that only 7 isolates were harbored all the gene (*papE/F,papG1,papG3,papC/D*) while 11 isolates contain 3 genes , 18 isolates contain 2 genes and 29 isolates contain 1 genes while 35 isolates don't contain any genes beside

33 from these negative isolates were negative for colony adhesion factors CAFIII as shown in appendix 1 and appendix 2.Usually pap fimbriae mediates bacterial adhesion on the epithelial cell surfaces by binding with Gal (1-4).There are special receptors on host cell surfaces that are associated with pap fimbria. The acquisition of bacterial isolates of these genes makes them more susceptible to pathogenicity and also increases the speed of bacterial growth. The adhesion of bacteria to the mucous membrane of the bladder is a critical step and dangerous in the disease of *E. coli* and the latest urinary tract infection(Khandige *et al*.,2015).Several properties of uropathogenic *E.coli* isolates are rare or absent in other *E. coli* strains. These properties have been called virulence factors by inference (García-Gutiérrez *et al* .,2015).P fimbriae are expressed by most strains causing acute pyelonephritis ("90%) but by few strains causing asymptomatic bacteriuria (20%) (Li *et al*.,2017)..



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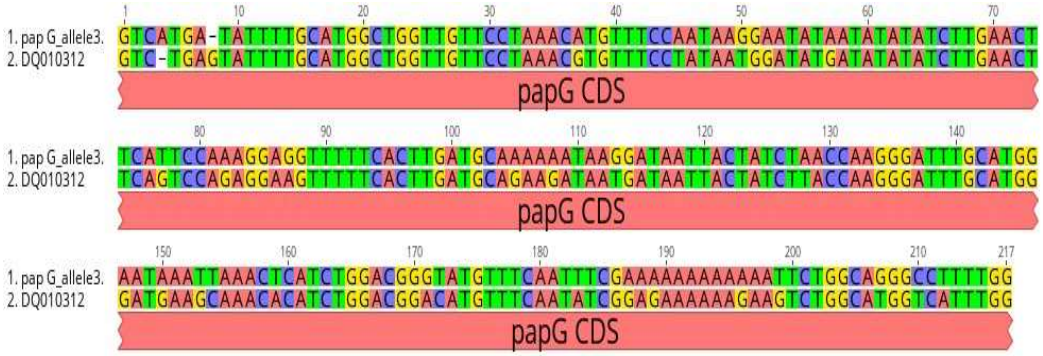

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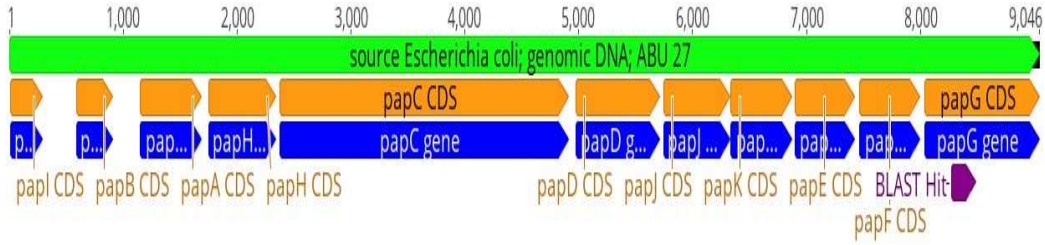

C

Figure (3-17A):Agarose gel electrophoresis of papG III gene (491bp amplicon). Electrophoresis was run at (1% agarose, 7v/cm2 for 90 min) lane M: DNA Ladder; lanes1-7 represent positive results. B: DNA sequencing for papG3 gene illustrating some mutation in E.coliisolates .C: Blast Hit of the amplified segment within the whole pap operon

P fimbriae mediate attachment to urinary tract epithelial cells and enhance cytokine responses *in vitro* and *in vivo* ,mutations in the *pap* gene cluster encoding P fimbriae reduced bacterial persistence in the mouse urinary tract (Flores-Mireles *et al*.,2015).

Mutational inactivation of the *papG* adhesin in a urinary tract pathogen was recently shown to dramatically decrease colonization and inflammation in the kidneys of monkeys (Mousavifar *et al*.,2018). Melican *et al* .(2011) showed that the P and Type 1 fimbriae appeared to act in synergy to promote colonization. P fimbriae enhanced early colonization of the tubular epithelium, while Type 1 fimbriae mediated colonization of the center of the tubule via a mechanism believed to involve inter-bacterial binding and biofilm formation. The heterogeneous bacterial community within the tubule subsequently affected renal filtration leading to total obstruction of the nephron within 8hrs . also results reveal the importance of physiological factors such as filtration in determining bacterial colonization patterns (Bien *et al*.,2012).

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