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Detection of mrkA Gene in Gram Negative Bacteria Isolated from Chronic Rhinosinusitis Patients under Endoscopic Sinus Surgery

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ABSTRACT

Thirty five sinus samples obtained from endoscopic patients who were suffering from chronic sinusitis and admitted to AL-Jumhori, AL-Rabie private, AL-Zahrawi private hospitals in Mosul city from March 2013 to March 2014 were conducted .Samples were cultured and pure isolates were identified to species level using morphological, biochemical and physiological tests. PCR was done using plasmids isolated from species under study by using primers for mrkA gene .mrkA gene was found in four species: *Moraxella* spp., *Enterobacter aerogenes, E. coli* and *Citrobacter freundii*.

Nucleotide sequencing was done for isolated mrkA gene and homology searches were conducted between the sequences of standard gene BLAST program which is available at the National Center for Biotechnology Information (NCBI) and Basic Local Alignment Search program Tool(BLAST). Variations appear as transversion mutation which causes change tryptophan amino acid to leucine amino acid and there was transition mutation which causes change from aspartite amino acid to asparagine.

Keywords: mrkA gene, chronicrhinosinusitis.

التحري عن جين mrkA في الجراثيم السالبة لصبغة كرام المعزولة من المرضى المصابين بالتهاب الجيوب الأنفية المزمن والخاضعين لعملية التنظير الأنفي في الموصل/العراق

الملخص

جمعت 35 عينة من حالات التهاب الجيوب الانفية المزمن والخاضعين لعملية التنظير الانفي خلال الفترة من اذار 2013 ولغاية اذار 2014 من مستشفيات الجمهوري، الربيع الاهلي والزهراوي الاهلي في مدينة الموصل. شخصت العينات اعتمادا على الاختبارات الكيموحيوية والفسلجية. اجري فحص تفاعل البلمرة المتسلسل باستخدام عزل البلازميد من الانواع قيد الدراسة واجري فحص سلسلة تفاعلات البلمرة باستخدام البادئات الخاصة للتحري عن جين mrkA اذ وجد الجين في اربعة انواع : *Moraxella* spp., *Enterobacter aerogenes, E. coli* and *Citrobacter freundii* .

قورنت نتائج تتابعات القواعد النتروجينية للجين mrkA ضمن المركز الدولي لمعلومات التقنات الحياتية National Center for Biotechnology Information (NCBI) وباستخدام برنامج Basic Local Alignment Search program Tool(BLAST) حيث وجد تغاير ظهر بشكل طفرات من نوع استبدال مما ادى الى تبدل الحامض الاميني التربتوفان الى الليوسين كما حدثت طفرة انتقال وتبدل الحامض الاميني الارجنين الى الكلوتامين.

الكلمات الدالة: جين mrkA، التهاب الجيوب الانفية المزمن.

INTRODUCTION

Chronic rhinosinusitis (CRS) is a public health problem that has a significant socio-economic impact (Bachert *et al*., 2014). CRS is characterized by symptoms for nasal irritation anterior and posterior rhinorrhea, and nasal blockage with the concomitant presence of pressure or pain in a sinus distribution that last more than 12 weeks (Payne *et al*., 2011). There are many types of CRS: Allergic fungal sinusitis (AFS), Aspirin exacerbated respiratory disease (AERD), Non eosinophilic sinusitis (NES) and Chronic hyper plastic sinusitis (CHPS)(Desrosiers *et al*., 2011; Glesson *et al*., 2008).

All forms of CRS are associated with the loss of barrier and innate immune functions that would normally prevent infection of healthy sinuses and as such these patients are highly predisposed to frequent and protected bouts of acute sinusitis. Consequently, all forms of CRS are also associated with loss of sterility observed in healthy sinuses and as such are routinely associated with the presence of anaerobic, gram negative organisms, *Staphlococcus aureus*, and other bacteria (Payne *et al*., 2011). Functional endoscopic sinus surgery (FESS) is nowadays regarded as the gold standard for treatment of CRS with or without polyposis refractory to optimal medical treatment. This surgery is based on the principles of improved functions in the side wall of the nose(Basilio *et al*., 2010). FESS is the therapeutic method of choice in surgical therapy of CRS and its considered safe when performed by experienced surgeons (Carceller and sandemetrio,2014).Bacterial biofilms are highly organized structures composed of bacterial communities encased within a protective extracellular matrix, which are resistant to both antibiotic treatment and host defense system. Biofilms are considered a common and important cause of persistent infections. In the field of otolaryngology biofilms have been documented on otitis media with effusion, cholesteatoma tonsillitis and CRS (Chen *et al*., 2012).

Types 3 fimbriae mediate attachment to, and biofilm formation on, extracellular matrix coated surfaces *in vitro* and *in vivo*. This fimbrial type is encoded by a chromosomally borne gene cluster previously shown to be comprised of five genes, these genes include determinants encoding the major fimbrial subunit (MrkA), a chaperone usher system (MrkBC, respectively), the fimbrial tip adhesion (MrkD) and an as-yet uncharacterized structural component (MrkF).(Johnson *et al*., 2011).

Aim:-To detect the mrkA gene variation in gram negative bacteria isolated from CRS patients under FESS.

MATERIAL AND METHODS

Thirty five sinus samples obtained from endoscopic patients who were suffering from chronic sinusitis in Mosul Republic Hospital, Al-Rabeeh and Al-Zahrawi Hospitals in Mosul city from March 2013 to March 2014 were included. Nasal and sinus swabs were collected and transferred by aims transport medium and cultured on MacConkey, blood and chocolate media from HIMEDIA/India then incubated at (37°C) for (24-48h).

The isolates were identified to species level depending on morphological, biochemical and physiological tests and confirmed by Remel RapID^{TM} ONE system and API E20 (Brooks *et al*., 2010).

Plasmid isolation:-

The following steps according to manufacturer's instruction (BIO BASIC INC.), were used for extraction of plasmid in this study:

Add 1.5-5mL overnight culture of studied bacteria in a tube and centrifuge at 12.000rpm for 2 minutes. The liquid was drained completely, then 100µl of solution I was added to the pellet, mixed well, and kept for one minute. 200µl of solution II was added to the mixture, and mixed gently by inverting the tube 4-6 times and then kept at room temperature for 1 minute.

Add 350µl of solution III and mix gently. Incubate at room temperature for 1 minute. Then the tube was centrifuged at 12.000rpm for 5 minutes. The supernatant above was transferred to the EZ-10 column then centrifuged at 10.000rpm for 2 minutes, the wash procedure in previous step

was repeated. The flow-through in the collection tube was discarded, centrifugation was done at 10.000rpm for an additional minute to remove any residual wash solution.

Finally the column was transferred to a clean 1.5ml microfuge tube. 50µl of elution buffer was added into the center part of the column and incubated at room temperature for 2 minute. Centrifugation was done at 10.000rpm for 2 minutes.

Primer:-

Pimer used was from The MIDLAND CERTIFIED REAGENT COMPANY INC.,USA .The sequences of the gene was checked out depending on the Gen Bank Sequence Database (http://www.ncbi.nlm.nih.gov/) as shown in (Table 1).

Table 1: The Primer Sequenced used for detection of mrkA gene.

Primer Name	Sequences	Reference
mrkA	F (5'-GCGGCGGTCAGGTTAATTTC-3') R(5'-TCGCATAGCCGACGAGTAAG-3')	Allen *et al*.,1991 Ong *et al*., 2010 http://www.ncbi.nlm.nih.gov/

Polymerase Chain Reaction (PCR) mixture for genes detection:-

The mixture was prepared like Quick-Load Taq 2X Master Mix, BioLabs

12.5 µl Master Mix.,4.5 µl distilled Water,1 µl Forward Primer,1 µl Reverse Primer and 6 µl Extracted DNA.

Detection of mrkA gene in Some Gram Negative Bacteria:-

The detection was proved like (Ong *et al*., 2010; BurmØlle *et al*., 2008 ; Ong *et al*., 2008). The amplification was carried out as shown in (Table 2). The PCR product was visualized by using UV light box after electrophoresis on a 2% agarose gel.

Table 2: Plasmid Amplification Reaction

Initial Denaturation		35cycles						Final Extension		Cooling	
		Denaturation		Annealing		Extension					
Temperature	Time	Temperature	Time	Temperature	Time	Temperature	Time	Temperature	Time	Temperature	Time
94°C	3M.	94°C	45S.	54°C	1M.	72°C	1M.	72°C	7M.	4°C	2M.

Gene Sequencing:-

Gene Sequencing of PCR product was carried out by microgen company (USA) using an ABI 3730 XL DNA Analyzer, and primer was used in each sequencing reactions. Homology search was conducted between the sequence of standard gene BLAST program (Basic Local Alignment Search Tool) which is available at the National Center Biotechnology Information(NCBI) online at (http://www.ncbi.nlm.nih.gov BLAST/).

RESULTS AND DISCUSSION

Plasmid Isolation:-

The plasmid was extracted from all gram negative species in this study. mrkA gene was found in four isolates:-

Moraxella spp.(2), *Entero.aerogenes*(4), *E. coli*(5), *Citro. Freundii*(6)
As shown in Fig (1).

The length of the gene was different in four isolates may be due to the variation in the gene in each species Ong *et al*., 2010 isolated this gene from plasmid of *E. coli* and *Citro. Freundii*, also (Ong *et al*., 2008) found this gene in *E. coli* plasmid too.

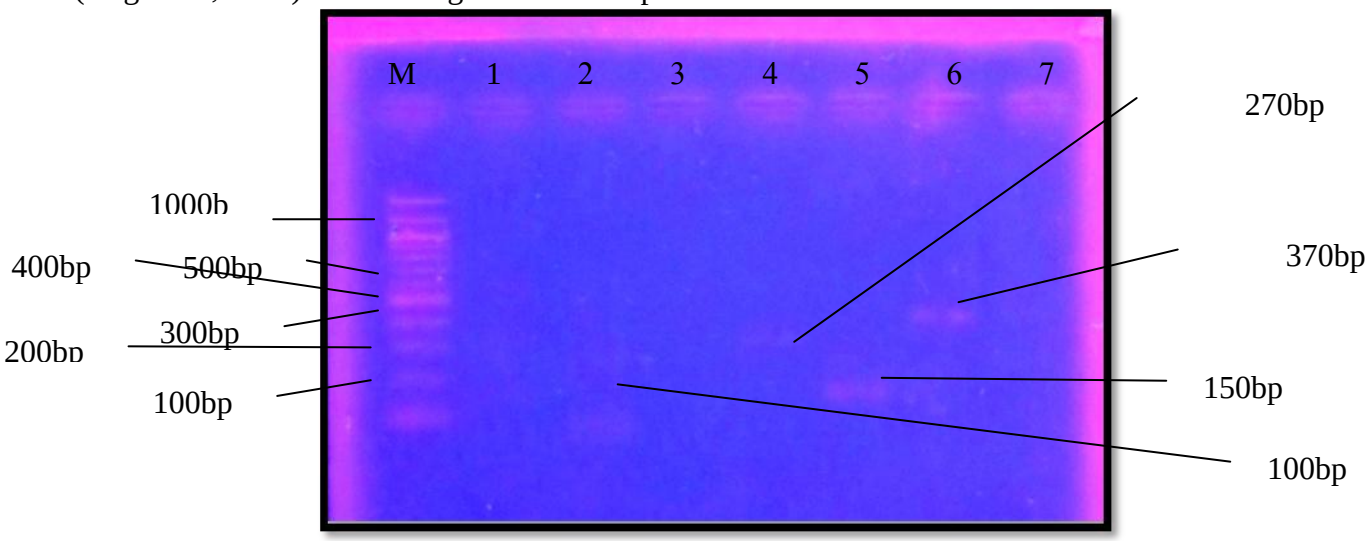

Fig.1: mrkA gene bands for 2(*Moraxella* spp),4 *Entero.aerogenes*,5 *E. coli* and 6(*Citro. freundii*).

Determination of Gene sequencing in *Entero.aerogenes* which have this gene:-

Nucleotide sequencing was done for mrkA genes in *Entero.aerogenes* to confirm the identification of genes and bacterial strains. PCR products of these genes were defined in Microgene company/USA using an ABI 3730X1DNA analyzer. Homology were conducted between the sequence of stranded gene BLSAT program is available at the National Center for Biotechnology Information (NCBI), available at the website (http://www.ncbi.nlm.nih.gov/BLAST/).

Comparison of the sequence of mrkA gene of *E.aerogenes* shows (88%) compatibility with sequence of the standard gene bank as shown in Fig. (2).The nucleotides of gene of *E.aerogenes* were translated to amino acids by using the same program NCBI and it found that these amino acids synthesize hypothetical protein which was reported by Ong *et al*., (2008) who revealed that this gene responsible for encoding a putative major subunit protein and confirmed by Johnson (2011) and Allen *et al*., (1991).

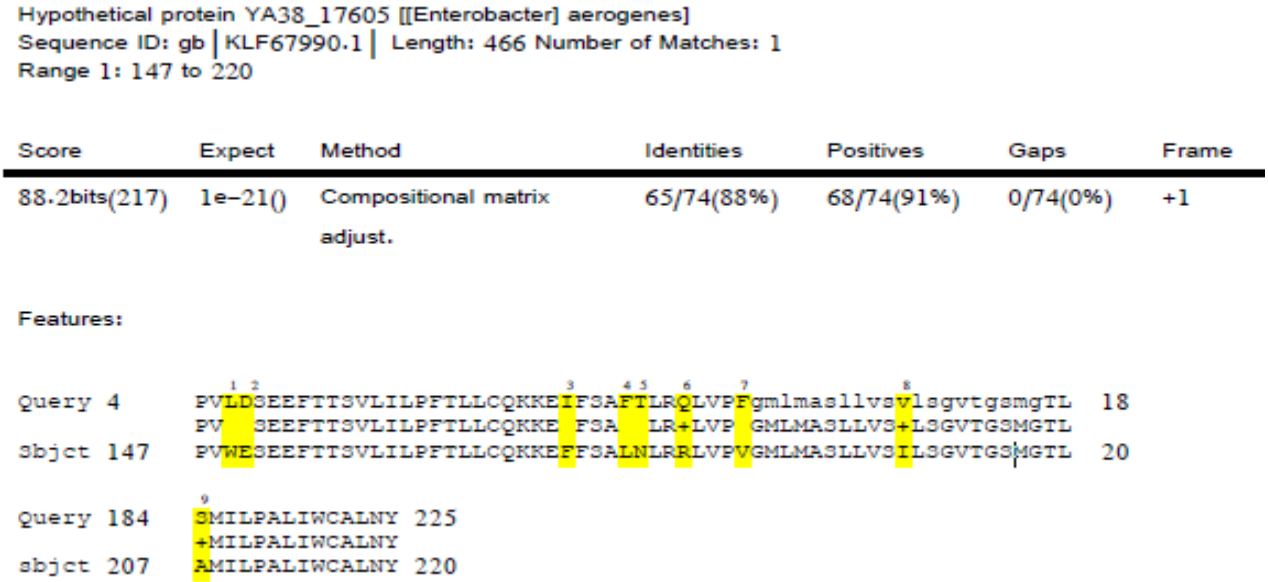

Fig 2: Compatibility with sequence of the standard gene bank.

Nine different deletion,addition and missence mutation were detected in mrkA gene of *E.aerogenes* as shown in (Table 3).

Table 3: mutations in mrkA gene of *Entero.aerogenes*

NO.	Nucleotide change	Amino acid change	Predicated effect	Type of mutation
1	TGG TTG	Tryptophan(W) Leucine(L)	Deletion	Transversion
2	TTT GAT	Phenylalanine(F|) Asparatate(D)	Missense	Transversion
3	TTT ATT	Phenylalanine(F|) Isoleucine(I)	Missense	Transversion
4	TTT TTG	Leucine(L) Phenylalanine(F|)	Missense	Transversion
5	AAC ACC	Asparagine(N) Threonine(T)	Missense	Transversion
6	CGG CAG	Arginine(R) Glutamine(Q)	Addition	Transition
7	GTT TTT	Valine(V) Phenylalanine(F|)	Missense	Transversion
8	ATT GTT	Isoleucine(I) Valine(V)	Addition	Transition
9	GCG TCG	Alanine(A) Serine(S)	Missense	Transversion

CONCLUSION

The gene(mrkA) that was detected in *E.aerogenes* may be responsible for encoding a putative major subunit protein which is considered as a virulence factor and responsible for biofilm formation.

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