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Molecular Study of the *cnf1* and *cnf2* (Cytotoxic Necrotizing Factors) Genes of *Acinetobacter baumannii* Isolated from Different Diseases

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Abstract

Two hundred samples of *Acinetobacter baumannii* have been collected from different types of infections which included 50 samples from blood, 20 samples from urinary tract infections, 30 samples from wound infections, 40 samples from burn infections and 25 samples from stool samples from several hospitals in Baghdad city (Central Children's Hospital, Al Karama Hospital, Karkh General Hospital, Al-Ameen Medical City Hospital, Educational Labs, Baghdad Teaching Hospital, Child Protection Hospital and Burns and Wounds Hospital).These isolates have been grown on MacConkey agar and blood agar and then identified by biochemical tests and also by using API20E and VITEK-2 systems. After identification, 40 isolates have been identified as *Acinetobacter baumannii* (9 isolates from blood, 1 from urinary tract infections, 4 from each of wound infections and respiratory tract infections, 8 from burns and 14 from stool samples). Genotypic detection of some virulence genes of *A. baumannii* which included *cnf1* and *cnf2*, showed the presence of these genes in 10 % and 0%, respectively. Sequencing analysis of *cnf1* gene revealed that it has six genetic mutations affecting the translation of amino acids.

Keywords: Cytotoxic necrotizing factor, *Acinetobacter baumannii*, sequencing.

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دراسة جزيئية لجينات عامل التنخر السمي (*cnf1 , cnf2*) Cytotoxic necrotizing factor لبكتريا *Acinetobacter baumannii* المعزولة من حالات مرضية مختلفة

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الخلاصة

جمعت 200 عينه سريرية تعود لبكتريا *Acinetobacter baumannii* من مصادر سريرية مختلفة شملت: 50 عينه من الدم Blood و 20 عينه من التهاب المسالك البولية Urinary tract infections و 30 عينه من الجروح (Wounds) و 40 عينه من الحروق (Burns) و 35 عينه من التهاب المسالك التنفسية Respiratory tract infection و اخيرا 25 عينه من الخروج Stool من مستشفيات عدة في مدينة بغداد وهي (مستشفى الطفل المركزي التعليمي ومستشفى الكرامة و مستشفى الكرخ العام ومستشفى مدينة الامامين الكاظمين الطبية، المختبرات التعليمية ومستشفى بغداد التعليمي و مستشفى حماية الاطفال ومستشفى الحروق والجروح / مدينة الطب). شخصت العزلات بزراعتها على الاوساط الزرعية (اكار المكونكي MacConkey agar، اكار الدمBlood agar) تم اجراء الفحوصات الكيموحيوية فضلا عن استعمل نظام API 20E والتشخيص النهائي استعمل جهاز الفايتك Vitek-2 بعد تشخيص هذه العزلات تم الحصول على 40 عزلة تعود لبكتريا *A. baumannii* شملت 9 عزلات من الدم و عزلة واحدة من التهاب المسالك البولية و 4 من الجروح و 8 من الحروق و 4 من التهاب المسالك التنفسية و14 عزلة من عينات الخروج. تم التحري عن بعض جينات الضراوة لبكتريا *A. baumannii* وتضمنت هذه الجينات كل من *cnf1* و *cnf2* اظهرت النتائج وجود هذه الجينات بنسبة (10 و 0%) على الترتيب. اما التحليل التتابعي لجين *cnf1* فقد اظهر وجود ستة طفرات وراثية اثرت في ترتيب الاحماض الامينية.

الكلمات المفتاحية: عامل التنخر السمي، *Acinetobacter baumannii*، التحليل التتابعي.

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Introduction

Acinetobacter baumannii is one of the most common sources of infections and more than 80% of infections are related to the duration of hospitalization. These bacteria are opportunistic pathogens and responsible for a large number of hospital infections (Necocomial infection) [1, 2]. These bacteria cause many infections in hospitals such as pneumonia, urinary tract infection, respiratory tract infection, soft tissue infection, meningitis, bacteremia, and skin dermatitis. The incidence of these bacteria depends on many factors such as the age of the patient, the duration of the hospitalization, and the immune deficiency [3].

The disease is caused by a number of virulence factors, including Lipopolysaccharides (LPS), Siderophores, Cytotoxic necrotizing factor, Aerobactin, Colicin V, production of gelatinase, capsule formation, Biofilms, the formation of Pellic formation, production of lipase, Protease, and its susceptibility to adhesion due to the presence of adhesion factors such as cilia and the adhesion of these bacteria to the surface of the host cell [4,5].

The Cytotoxic necrotizing factor (cnf) belongs to the family of necrotizing toxins in the skin, including cnf1, cnf2 and isolated for the first time from *E. coli* and then isolated from other types of bacteria such as *Yersinia pseudotubercolos* and *Bordetella* [6]. The bacteria that produce the cytotoxic necrotizing factor have the potential to grow at the site of infection because of the toxin toxicity factor which has the ability to prevent wound healing and is produced by the bacteria associated with the wounds after surgery. Therefore, the toxicity factor is one of the most important factor of virulence in the bacteria and it has several functions (it increases the Superoxidase generation, and has the ability to adherence to the cells of the epithelium of the human and reduces the phagocytic cells in the body) [7]. The presence of toxic agent cytotoxic necrotizing factor (cnf) in *E. coli* and gram-negative bacteria is due to the presence of genetically-plasmid-dependent genes in several mechanisms, including conjugation and transformation [8]. Studies showed that the toxic agent cytotoxic necrotizing factor (cnf)

was isolated from *E. coli* in 3% of skin dermatitis infections and soft tissue infections and also isolated from intestinal infections and wounds [9,10]. The aim of the present study was to investigate some virulence genes of *A. baumannii* such as *cnf1* and *cnf2*. Sequencing analysis of genes and the detection of mutations that affect the translation of amino acids.

Materials and Methods

Collection of samples

Two hundred samples have been collected from different sources of infections (blood, urinary tract infections, wound infections, burn infections and stool samples) from several hospitals in the of Baghdad Province (Central Children's Hospital, Al Karama Hospital, Karkh General Hospital, Al-Ameen Medical City Hospital, Educational Labs, Baghdad Teaching Hospital, Child Protection Hospital, Burns and Wounds Hospital).

Identification of bacteria

The samples were grown on MacConkey agar, blood agar and then identified by biochemical tests (oxidase and catalase tests), further identification and also by API20E and VITEK-2 systems [11].

Extraction of DNA

DNA kit (Geneaid Biotech kit system, UK) was used to extract the DNA of bacterial isolates according to the manufacturer's instructions.

Primers

They have been prepared according to the instructions of the manufacturer Alpha DNA (Canada) *cnf1* (F: 5'AAGATGGAGTTTCCTATGCAGGAG3') (R: 5' CATTCAGAGTCCTGCCCTCATTATT3')

Gene size (498) bp Yamamoto *et al.* (1995) [12] and *cnf2* (F: 5' AATCTAATTAAAGAGAAC3') (R:5' CATGCTTT GTAT ATCTA 3') Gene size (543) bp Blanco et al. (1996) [13] using sterile distilled deionized water to obtain a concentration of 100 pcomol / microliter. The solution of each initiator was prepared separately at 10 pcomol / microliter by taking 10 microliters of each stock solution and added to 90 microliters of distilled water and mixed well with by vortexing, and kept with stock solutions at 20 ° C until have been used.

Genetic detection of virulence factors using the PCR polymerase reaction mixture, *cnf1* and *cnf2* genes were preparation PCR mixture total size (20 µl) including**:** F-primer (1 µl), R-primer (1 µl), Template DNA (2 µl), Nuclease Deionized nuclease-free water (6 µl) and GO Taq green master mix (10 µl). The contents of the PCR tubes were mixed well using the Vortex and then placed in the PCR thermal cycler, table 1.

Table 1: Optimal conditions for PCR reaction to investigate *cnf1* and *cnf2* genes

Step	Program *cnf1* gene		Program *cnf2* gene	
1	Only one cycle for 4 minutes at a temperature of 95 ° C for the primary DNA denaturation.		Only one cycle for 6 minutes at a temperature of 95 ° C for the primary DNA denaturation.	
2	30 cycle included:		34 cycle included:	
	A	50 sec at 95 ° C for DNA template denaturation.	A	50 sec at 95 ° C for DNA template denaturation.
	B	60 sec at 58 °C for the primers to bind to DNA template annealing at 58 °C for 60 sec.	B	70 sec at 58 °C for the primers to bind to DNA template annealing at 58 °C for 70 sec.
	C	45 sec at 72 ° C for the associated primers to be elongated.	C	55 sec at 72 ° C for the associated primers to be elongated.
3	Only one cycle for8 minute at 72 ° C for the final elongation of the double DNA strip.		Only one cycle for 10 minute at 72 ° C for the final elongation of the double DNA strip.	

Separation of DNA bands

PCR products were separated on a 2% agarose with 5µl Ethidium bromide, at 100 vol. for 80 min. The DNA bands were visualized and photographed under UV light as described previously [14].

Results and Discussion

After identification, 40 isolates have been confirmed to be *Acinetobacter baumannii* (9 isolates from blood, 1 isolate from urinary tract infections, 4 isolates from each wound infections and Respiratory tract Infection, 8 isolates from burns and 14 isolates from stool samples).

Concerning the *cnf1* gene, the results showed that 4 (10%) isolates possessed this gene as shown in Figure (1-A and B). When comparing the bundles with the ladder, the resulting packets were found to have a molecular weight of 498 base pairs.

The results of this study were consistent with the findings of Abd Al-Mahdi *et al.* [15]. Who showed that only two isolates (28%) of *A. baumannii* bacteria had the *cnf1* gene from the total isolates of their study, while the findings of Momtaz *et al.* [16] showed that (35%) of isolates had *cnf1* gene. The results obtained by Daryanavard and Safaei [17] showed that the percentage of possession of *cnf1* gene was 40% of the total isolates.

Cytotoxic necrotizing factor 1 (*Cnf1)* is a virulence factor in *A. baumanniii* which has the ability to adhere to the surface of human epithelial cells, prevents wound healing, and helps bacteria stay at the site of injury after surgery and reduces phagocytic cells in the body [7].
Regarding the *cnf2* gene, the result of the current study revealed that it was not found in any of the obtained isolates of *A. baumannii*, while the results of the studies conducted by Momtaz *et al*. [16] and Tavakol, *et al* [18] showed that the percentage of possession of the *cnf2* gene were 61.25% and 54.54% respectively. Knust and Schmidt [19] reported that the *cnf2* gene was a virulence factor in *E. coli* and also showed the presence of *cnf3* in *E. coli* and *cnfY* gene in the *Yersinia pseudotuberculosis*. The (*cnf1)* gene is the most common causes of adhesion and invasion of *A. baumanni* to the epithelial cells of the human organs [20]. The difference in percentage of the possession of *cnf1* gene between the present study and the other studies may be due to the difference in the type of strain and the area of isolation.

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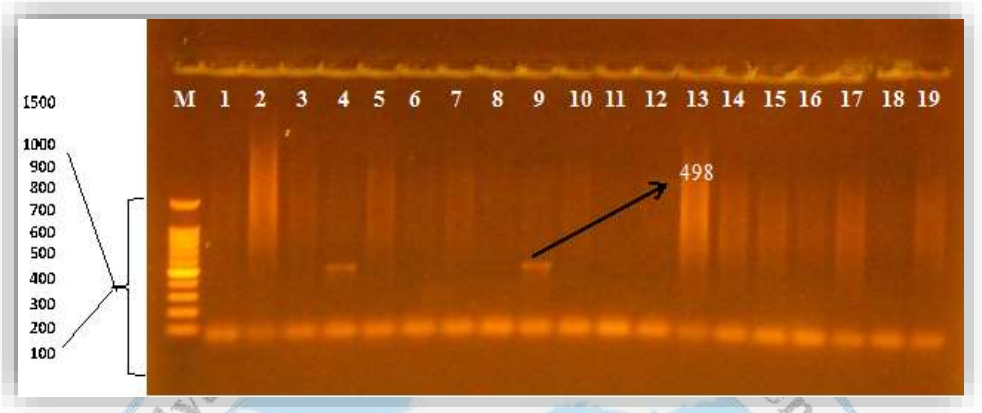

Figure (1-A): Electrophoresis of the PCR products of *cnf1* gene (498 bp) for *A. baumanniii* isolates on 2% agarose and 100 volts for 80 minutes. The M ladder (100-1500 bp), the 4, 9 were positive isolates.

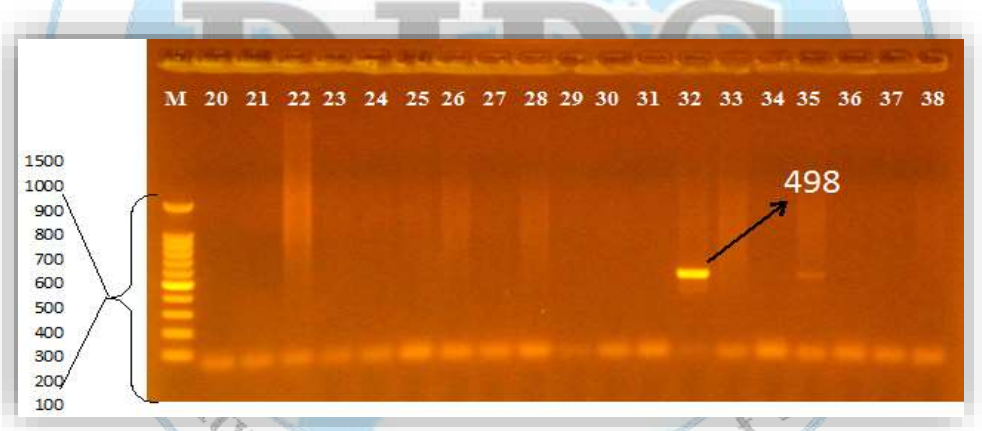

Figure (1-B): Electrophoresis of the PCR products of *cnf1* gene (498 bp) for *A. baumanniii* isolates on 2% agarose and 100 volts for 80 minutes. The M ladder (100-1500 bp), the 32, 35 were positive isolates

DNA sequence analysis of *cnf1* gene

DNA analysis of *cnf1* gene (serial number ACCESSION LC338016) was analyzed in *A. baumanniii*. The results of the analysis showed that there were 6 genetic mutations in isolation A 4 from burns and obtained A9 isolates from stool samples. The Guanine substrate was replaced with the Adenine base at 143 at 4691240 and substituted the Guanine base with the

Thymine nitrogen base at 151 at 4691248 and replaced the Guanine with the Adenine base at 201 at 4691298 and replaced the Guanine with the nitrogen base Adenine at 224 at 4691321. The Thymine base was replaced by the Guanine at 310 at 4691407 and the Guanine substrate was replaced with the Adenine nitrogenous base at 354 at 4691451.The Guanine substrate was replaced by the Thymine base 361 at 4691458. The Guanine substrate was replaced with the adenine base Adenine at 383 at 4691480 and the Thymine base was replaced with the adrenergic base Adenune at 404 at 4691501 as shown in table 5 and figure 2.

When the amino acid translation of the gene was analyzed with the original amino acid results, there was a conversion of the amino acid Serine to the amino acid Isoleucine. Aspartic acid was converted to the amino acid Asparagine and the amino acid Methionine was replaced with the amino acid Arginine and the conversion of the amino acid Glutamic to the amino acid Lysine and replaced the acid Amino acid Arginine with Leucine as shown in table 2.

The results showed silent mutations that did not affect amino acid changes and mutations that led to a change in amino acid in the resulting protein sequence called Missense mutation. The change in the sequence of amino acids was due to the loading of another amino acid instead of the original amino acid. The Missense mutation which effects on the resulting protein depends on the location of the amino acid that has been altered as follows: Acceptable, partially acceptable, or unacceptable, the effect is acceptable for the function of the protein molecule when a change the baseband to the replacement of amino acid with another amino acid with functional aggregates similar to the original amino acid. If the effect of the accepted Missense mutation occurs, then the protein molecule produced from the natural molecule cannot be distinguished. In addition, the partially acceptable effect will produce a protein molecule of partial or abnormal efficacy if the effect of the unacceptable Missense occurs, then the protein molecule cannot function in its turn. When the genetic code changes to one of the three genetic codes: UAA, UAG, or UGA, another mutation developed which is called the Nonsense [21].

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Table 2: Changes in nitrogen bases and their effect on the translation of amino acid for *cnf1* gene for isolation A4 and A9 for *A. baumanniii*

No.	Nitrogen bases	Changes in nitrogen bases	Position	Subject	Amino acid	Changes in amino acid	Type of mutation
1	Guanine	Adenine	143	4691240	Isoleucine	Isoleucine	Silent mutation
2	Guanine	Thymine	151	4691248	Serine	Isoleucine	Missense mutation
3	Guanine	Adenine	201	4691298	Aspartic	Asparagine	Missense mutation
4	Guanine	Adenine	224	4691321	Valine	Valine	Silent mutation
5	Thymine	Guanine	310	4691407	Methionine	Arginine	Missense mutation
6	Guanine	Adenine	354	4691451	Glutamic	Lysine	Missense mutation
7	Guanine	Thymine	361	4691458	Arginine	Leucine	Missense mutation
8	Guanine	Adenine	383	4691480	Leucine	Leucine	Silent mutation
9	Thymine	Adenine	404	4691501	Threonine	Serine	Missense mutation

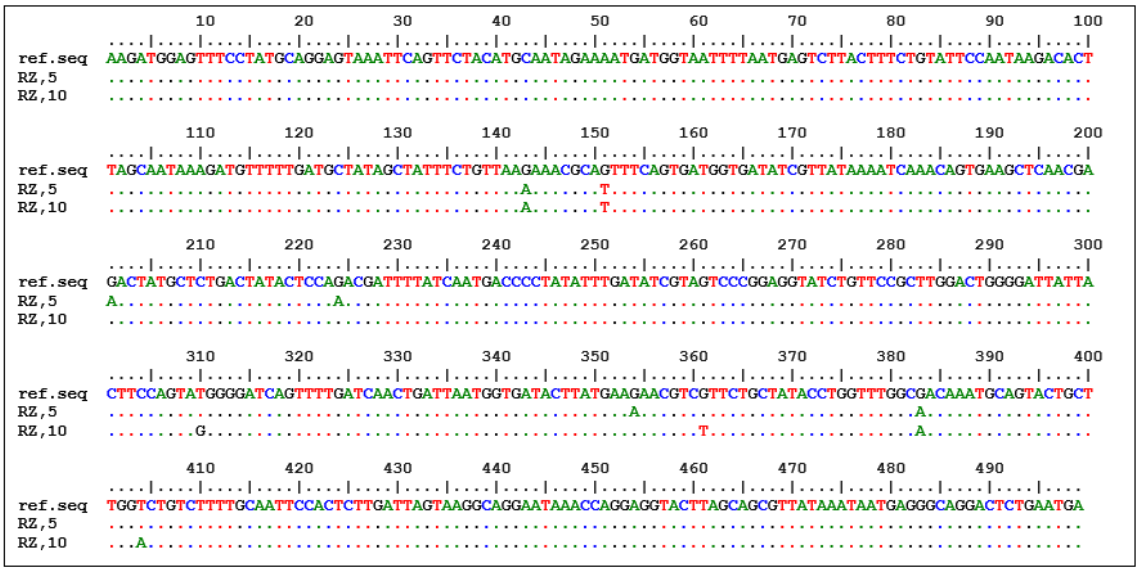

Figure 2: Analysis of the multiple sequences of the reference *cnf1* gene with two changes of isolates A10 and A5 of *A. baumanniii* using the BioEdit Sequence Alignment Editor Software

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