Evaluation of *Citrobacter freundii* as a Heat Labile (LT) Enterotoxin Producer

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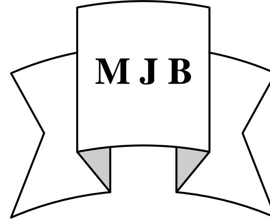

Abstract

This study included the investigation of the ability of *C. freundii* to produce Heat labile enterotoxin by both genotype and phenotype. Only 11 isolates were isolated from 422 clinical sample (282 stool and 140 urine samples), 8 isolates from stool samples and 3 from urine samples. All isolates identified by biochemical tests and confirmed with API 20 E. Molecular detection for gene responsible for heat-labile (*lt, ltI-h* and *ltA*) enterotoxin genes was achieved by PCR technique. The result showed that *ltA* was the heat-labile enterotoxin gene in 5 isolates (45.45%). Rabbit ligated ileal loop assay RIL, was applied to the 11 Isolates, only 4 of the bacterial isolates gave a positive results.

الخلاصة

شملت هذه لدراسة التحري عن قابلية بكتريا *Citrobacter freundii* على إنتاج الذيفان المتغير بالحرارة Heat labile enterotoxin من خلال الطراز الجيني والمظهري. عزل النوع *C. freundii* بواقع ١١ عزلة من مجموع العينات السريرية و البالغة ٤٢٢ عينة (٢٨٢ عينة براز stool و ١٤٢ عينة إدرار urine) والتي شملت ثمان عزلات من عينات البراز و ثلاث عزلات من عينات الإدرار . شخصت العزلات وفق الاختبارات المظهرية والكيموحيوية وباستعمال نظام API 20 E. تم التحري عن الجينات المسؤولة عن إنتاج ذيفان غير ثابت حرارياً Heat labile (LT) (*lt, ltI-h* and *ltA*) باستخدام تقنية سلسلة تفاعل إنزيم البلمرة PCR. حيث بينت النتائج أن *ltA* كان ألجين المسؤول عن إنتاج الذيفان المعوي غير الثابت بالحرارة في خمس عزلات (٤٥,٤٥%). أما عند استخدام Rabbit ligated ileal loop assay (RIL) للتحري عن الطراز المظهري، أعطت أربعة عزلات (٣٦,٣٦%) نتائج موجبة لهذا الاختبار.

Introduction

C. freundii is usually considered a commensal species of the human gut, although some isolates have acquired specific virulence traits that enable them to cause diarrhea. Therefore, virulence factors homologous, and some even identical, to those described in *E. coli* pathotypes were detected in *C. freundii* strains isolated from sporadic cases of infantile diarrhea[10, 12].

Locally, *C. freundii* isolated from different samples include stool, rectal swabs, urine, blood, sputum, cerebrospinal fluid, wounds, ear, nasal and throat swabs in Baghdad and Hilla cities [2, 3]

The LT gene was cloned into *E. coli* and two proteins of molecular

weights 11,500 (B subunit) and 25,500 (A subunits) were produced [5]. The LT A subunit structureal gene (*eltA*) was sequenced and the amino acid sequence deduced. The computed molecular weight of LT A is 29,673Da, The A subunit genes of CT and LT (LT-I) are 78.6% homologous, and the B subunit genes are 78% homologous[16]. The gene of LT-IIa was studied. It is organized in a transcriptional unit similar to those of CT and LT-I. The A subunit gene of LT-IIa was found to be 57% homologous with the A subunit gene of LTh-I and 55% homologous with the A gene of CT. Most of the homology derived from the region of the A gene which encodes the A1 fragment. The B gene of LTIIa was not homologous with the B gene of LTh-I or CT [13].

Materials and Methods

Isolation and identification of *C. freundii*: During the period from July 2010 to October 2010, a total of 422 clinical samples (282 stool and 140urine) were taken from patients with suspected diarrhea and urinary tract infections, 50 healthy individuals (control),samples collected from three hospitals in Najaf (Al-Sadr Teaching, Al-Hakeem, and Al-Zahra Maternity and Children). Identification of Bacterial Isolates were identified to the level of species using the traditional morphological and biochemical tests [11]. All isolates were confirmed identification with API 20 E system. The bacterial isolates were preserved on nutrient agar slant at 4˚C. The isolates were maintained monthly during the study by culturing on new culture media. For long preservation, nutrient broth supplemented with 15% glycerol was used and the isolates were maintained frozen (-20˚C) for long term for several months [4].

Polymerase chain reaction: DNA was extracted by Salting out method [14]. DNA (extracted from bacteria cells) was used as a template in specific PCRs for the detection of enterotoxin genes. A pair of primers for each gene listed in table (1) was used for the amplification of a fragment that covers the entire gene. A single reaction mixture contained 5 µl DNA extract, 12.5 µl Master mix(Master mix 2X 'Promega': Go Tag DNA polymerase is supplied in 2x Green Taq Reaction buffer pH 8.5, 400µm dATP, 400µm dGTP, 400µm dCTP, 400µm dTTP, and 3mM $MgCl_{2)}$, 2µl of 10 pmol/µl of upstream primers specific and, 2µl of 10 pmol/µl of downstream primers specific the volume then completed to 25 µl by nuclease-free water. All the additions were done in laminar flow on ice.

Table 1 Primers Used in PCR (*Bio*Corp)

Primer	Sequence	Amplicon length (bp)	Reference
ltA (F)	5′- GGCGACAGATTATACCGTGC -3′	696	[15]
ltA (R)	5′- CCGAATTCTGTTATATATGTC -3′		
lt(F)	5'-AGCAGGTTTCCCACCGGATCACCA-3'	132	[8]
lt(R)	5'-GTGCTCAGATTCTGGGTCTC-3'		
lt1-h (F)	5'-GCGTTACTATCCTCTCTATG-3'	320	[18]
lt1-h (R)	5'-ATTGGGGGTTTTATTATTCC-3'		

Polymerase chain reaction assays were carried out in a 25 µl reaction volume, and the PCR amplification conditions performed with a thermal cycler were specific to each single primer set depending on their reference procedure, as follows:

Table 2 PCR protocol for genes investigated in this study.

Gene	PCR protocol					
	Initial denaturation temperature (°C) Time (min.)	Thermocycles			Final extension temperature (°C) Time (min.)	No. of thermocycles
		Denaturation temperature (°C) Time (sec.)	Annealing temperature (°C) Time (sec.)	Extension temperature (°C) Time (sec.)		
ltA	94 5	94 30	54 60	72 60	72 10	32
lt	94 5	94 30	47 60	72 90	72 10	25
lt1-h	94 5	94 40	49 60	72 45	72 10	30

Agarose Gel Electrophoresis: The resulting PCR products run in 1.5% agarose gels which prepared by dissolving 1.5 gm of agarose powder in 100 ml of TBE buffer (pH 8) in boiling water bath, allowed to cool to 50˚C, and ethidium bromide at concentration of 0.5 mg/ml was added. Then a tape was placed across the end of the gel tray. The comb was fixed at one end of the tray for making wells used for loading DNA samples. The agarose was poured gently in to the tray, and allowed to solidify at room temperature for 30 minutes. Then the comb was removed gently from the tray and the tap was also removed from the ends of the tray. The latter was fixed in electrophoresis chamber which was filled with TBE buffer has covered the surface of the gel. 5µl of PCR product was loaded into the wells in agarose gel. The electric current was allowed at 70 volt for 2hr.

Electrophoresis Results: The electrophoresis result was detected by using gel documentation. The base pair of DNA bands were measured according to the ladder. The positive results were distinguished when there was DNA band equal to the target product size. Finally, the gel was photographed using gel documentation saving picture.

Preparation of cell-free culture filtrate (Enterotoxin extraction): For the production of crude enterotoxin, *C. freundii* strains were propagated in 20 ml of Tryptone soy broth supplemented with 0.6 % yeast extract. They were incubated at 37 °C with agitation at 120rpm in environmental incubator shaker for 24h. The cultures were then centrifuged in sterile centrifuge tubes at 10000 rpm for 30 min. at 4°C. The resulting supernatant fluids were then filtered through sterile 0.45-µm membrane filters. Cell free filtrates were stored at 4°C for no more than 48h until their use in enterotoxin bioassay (Trower *et al*., 2000).

Rabbit ileal ligated intestinal loop assay (RIL): This test consider efficient for the detection of heat labile enterotoxin. The adult rabbit averaging 1.5 to 2.0 kg in weight used in this test. Animals were fasted for 24 h prior to use. Anesthesia by 0.1ml xyclocaine and 0.2 ketamine, the small bowel was flushed with 10 ml of 0.1 M PBS and ligated segments, usually 10cm long with 5cm intervals, were prepared by employing a single tie of surgical silk between segments. The segment

received 1ml of crude enterotoxin extracted from each isolates and growth medium (negative control), and 1ml of cholera toxin (positive control) after injection of the loops the abdomen was closed. Animals were sacrificed and the abdomen was opened, and the small intestine was excised. After the volume of fluid in each segment was measured (by withdrawal into a syringe of appropriate volume) the lengths of the empty segments were determined, and volumes per length ratios (ml/cm) were recorded. Results were considered valid only if the positive and negative controls gave appropriate responses [6].

Results

Molecular Detection of heat labile enterotoxin of *C. freundii* isolates: DNA was extracted from all isolates in this study and used as a template for PCR assays to heat-labile enterotoxin genes (*lt, lt1-h* and *ltA*) .PCR results showed that *C. freundii* retain *ltA* as a heat-labile enterotoxin gene in 5 isolates(C1,2,3,6 and 10) with percent 45.45%(Fig. 1). Other heat-labile enterotoxin genes showed negative result at least in thermocycles listed in table (2).

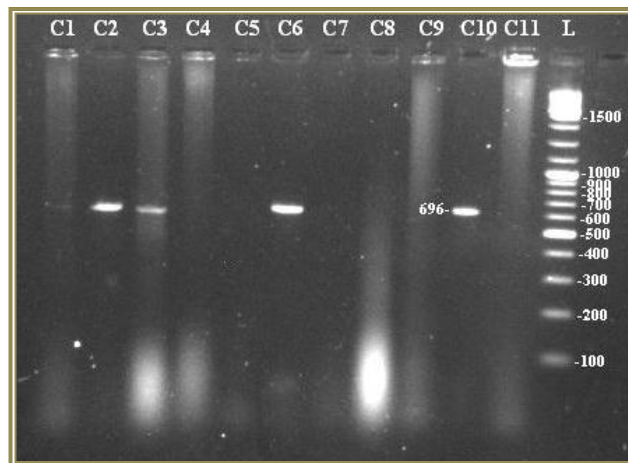

Figure 1 Ethidium bromide-stained agarose gel of PCR amplified products from extracted *C. freundii* DNA amplified with primers *ltA* F and *ltA* R.

- Lanes (C1, 2, 3, 6 and 10) show positive results with *ltA* gene.
- Lanes (C4,5,7,8,9 and 11) show negative results with *ltA* gene
- Lane (L), DNA molecular size marker (100-bp ladder)

Heat-labile enterotoxin bioassay: The Rabbit ligated ileal loop assay RIL (Fig. 2) was applied to the isolates of *C. freundii* isolated in this study. The results showed that only 4 of the bacterial isolates gave a positive results (C1: 0.74, C2: 0.86 C3: 0.76 and C10: 0.76) ml/cm with percent 36.36%, while other bacterial isolates gave a negative result compared with *cholera* toxin as a positive control (1.1 ml/cm) and (TSB+ 6% yeast extract) as a negative control (0.15 ml/cm) as shown in table (3).

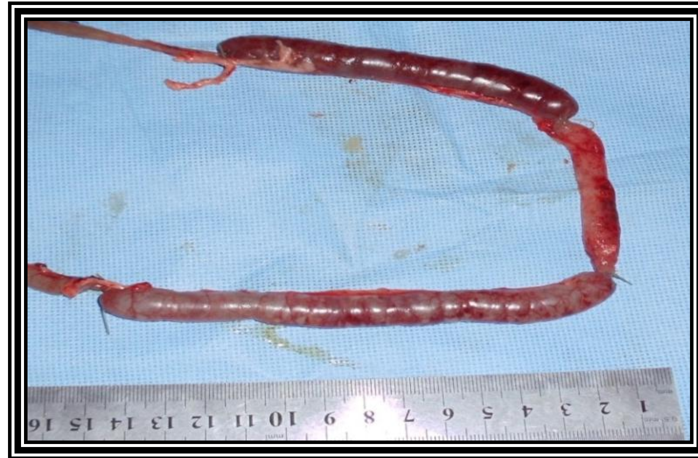

Figure 2 Rabbit ligated ileal loop assay

Table 3 Crude enterotoxin bioassay

Number of isolate	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	Negative control	CT
RIL (ml/cm)	0.74 (+)	0.86 (+)	0.76 (+)	0.32 (–)	0.36 (–)	0.26 (–)	0.32 (–)	0.27 (–)	0.30 (–)	0.76 (+)	0.30 (–)	0.15 (–)	1.1 (+)

(RIL)Rabbit ligated ileal loop assay
(CT) *Cholera* toxin

Discussion

Molecular Detection for heat-labile enterotoxin of C. *freundii* isolates:
From figure (1), *C. freundii* showed positive result for *ltA* (696bp) as genes responsible for heat-labile enterotoxin by PCR. *C. freundii* known to be enterotoxin producer. identification and characterization a gene encoding a homologue of the B subunits of cholera toxin (CTB) and heat-labile enterotoxin (LTB) of *E. coli* from a clinical isolate of *C. freundii* that was found to produce a factor in the culture supernatant that cross-reacted with antibodies to CTB and LTB when assayed by enzyme-linked immunosorbent assay (ELISA) [10]. The gene encoding the ELISA-positive factor, *cfxB*, consisted of 375 nucleotides and was located downstream of an 852-nucleotide open reading frame, *cfxA,* with a 56-nucleotide intergenic space. The cfxB gene was predicted to encode a 125-amino-acid polypeptide. Strains of *C. freundii* isolated from environmental water were identified as heat-labile enterotoxin (LT) producing strains by immunological methods and polymerase chain amplification. A 322 bp amplified fragment was obtained [9].

Enterotoxin bioassay showed in table (3) recorded a marked fluid accumulation in only 4 of the bacterial isolates gave a positive results (C1-3 and C10) ml/cm with percent 36.36% in rabbit ligated ileal loop assay as indicator of heat-labile enterotoxin activity. The ability all strains were capable of producing heat-labile enterotoxin with (100%) by delayed permeability test of rabbit skin and mice paw oedema test taking into account the difference in the toxicity degree among producing strains of this toxin[1]. Strain isolated from urine and stool showed a negative result with RIL[7]. The enterotoxin bioassay has the advantage over cell-culture systems which detect only cytotoxicity [17].

The response of adult rabbit small intestine to the heat-stable (ST) and heat-labile (LT) enterotoxins of *E. coli* were different in a characteristic manner, Fluid accumulation was determined in relation to enterotoxin dose and duration of gut exposure, Maximum volume per length ratios elicited by ST occurred between 4 and 6 h after injection. However, maximum ratios elicited by LT occurred no less than 10 h after injection. Therefore, a 6-h assay time is appropriate for the titration of ST, whereas an 18-h assay is not. The 18-h assay was found more appropriate for LT assay [6]. Detection of STa biological activity in porcine ligated intestinal loop was demonstrated [19].

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