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Evaluation of *Citrobacter freundii* isolated in Najaf governorate as an enterotoxin producer

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Abstract:

This study has been included 422 patients (202 male and 220 female), of which 282 stool samples and 140 urine samples were collected from children under six years of ages and 200 hospital environmental samples as well as 50 apparent healthy as control. Samples collected from three hospitals in Al-Najaf city (Al-Sadr Teaching, Al-Hakeem, and Al-Zahra Maternity and Children) during a period extended from July to October 2010. Only 11 isolates of (*Citrobacter freundii*) with percent 2.60% were isolated from all clinical samples, 8 isolates isolated from stool samples with percent 2.83% and 3 from urine samples with percent 2.14%, whereas no bacteria isolated from both control and hospital environmental samples. The Suckling Mice Assay (SMA) was applied to the 11 isolates of *C. freundii*, only 2 gave positive results. While Rabbit ligated ileal loop assay RIL, only 4 of the bacterial isolates gave a positive results.

Introduction:

Citrobacter 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(Karasawa *et al*., 2002 and Pereira *et al*., 2010).

Epidemiological data suggest that strains which secrete heat-stable toxin (ST), alone or in combination with heat-labile toxin (LT), induce the most severe disease among children (Taxt *et al*., 2010). *C. freundii* complex has been implicated as a cause of gastrointestinal infection and inflammation, acute dysentery, and dyspepsia. Acute symptoms can include profuse, watery diarrhea which is often unaccompanied by abdominal pain, fecal blood, or white blood cells (Guarino *et al*., 1987 and Washington *et al*., 2006).

Materials and methods:

Collection and Handling of Samples: During the period from July 2010 to October 2010, a total of 672 samples collected from three hospitals in Najaf (Al-Sadr Teaching, Al-Hakeem, and Al-Zahra Maternity and Children). The samples distributed into 422 clinical samples (282 stool and 140urine) were taken from patients with suspected diarrhea and urinary tract infections, 50 healthy individuals (control) and 200 hospital environmental samples.

Identification of isolates: *C. freundii* isolates were identified to the level of species using the traditional morphological and biochemical tests, according to the methods of Holt *et al*. (1994), and MacFaddin (2000).all isolates were confirmed identification with API 20 E system. Then 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 (Collee *et al*., 1996).

Experimental animals: New born albino mice (1-4) days obtained from Science College / Kufa University used in Suckling mice assay (SMT). Wild type rabbit (1.5-2) kg used in rabbit ligated intestinal loop assay.

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 24hr. 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).

Enterotoxin Assays (Bioassay):

1-Suckling Mice Assay (SMA): Test using infant mice is a convenient assay for ST enterotoxin. Supernatants of cultures (0.1 ml) injected with hypodermic needle (no. 30) into the milk-filled stomachs of infant mice (1-4) day old and weigh (0.9-1.3) g, the fluid accumulation in the intestine was measured after 4 hr by determining the ratio of intestine to whole body weight. Usually two drops of a 2% solution of food dye were added to each 1 ml of inoculums. A ratio ≥ 0.08 was considered positive for heat stable enterotoxin (Moon *et al*., 1978).

2-Rabbit ileal ligated intestinal loop assay (RIL): This test consider efficient for the detection of heat labile enterotoxin. The adult rabbit averaging weight 1.5 to 2.0 kg used in this test. Animals were fasted for 24 hr 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 (obtained from Science College / Kufa University) as a positive control after injection of the loops the abdomen was closed. Animals were sacrificed, the abdomen was opened, and excised small intestine. 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 (Evans *et al*., 1973).

Results:

Isolation of *C. freundii:* Isolation of bacteria includes investigation of 422clinical samples, 200 hospital environment samples and 50 healthy child as a controle.

Clinical Samples: The results of clinical samples showed 11 samples with percent 2.60% were positive for *C. freundii*, of which 8 isolates (2.83%) from stool and 3 (2.14%) from urine samples, while there was no bacterial isolates from hospital environment and control samples (Table 1). The bacterial isolates abbreviated sequently (C1, C2 …C11).

Table (1): Frequency of *C. freundii* isolated from medical samples.

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Clinical Samples	No. of samples	No. of isolated bacteria	Percent (%)
Stool	282	8	2.83
Urine	140	3	2.14
Total	422	11	2.60
Control	50	0	0

Hospital environment samples: No bacterial isolates has been isolated from hospital environment samples (Table 2).

Table (2): Frequency of *C. freundii* isolated from hospital environment

Hospital environment samples	No. of sample	%	No. of isolates	%
Operating theaters	20	10	0	0
Beds	20	10	0	0
Floors	20	10	0	0
Medical instrument	20	10	0	0
Nasal swabs for medical staff	20	10	0	0
Oral swab for medical staff	20	10	0	0
Ear swab for medical staff	20	10	0	0
Hand swabs for medical staff	20	10	0	0
Doors	20	10	0	0
Infant incubators	20	10	0	0
Total	200	100	0	0

Identification of *C. freundii*: Bacterial identification were performed by streaking of each sample on MacConkey's agar and the suspected colonies of *C. freundii* appear pink (Lactose fermenters)after 24hr incubation period, pale colonies further incubated for 24hr to identify the (late lactose fermenters). The selected colonies were cultured on Xylose lysine deoxycholate agar (XLD), after 24hr the colonies of bacteria appeared yellow, smooth flat and round. Gram stain and microscopical examination showed that 11 isolates were successfully diagnosed as *C. freundii* based on assessment of colony morphology and standard biochemical tests and sets of sugar fermentation besides the API 20E identification system as showed in fig. (1).

Heat-stable enterotoxin bioassay: The Suckling Mice Assay (SMA) was applied to the 11 isolates of *C. freundii*. The test showed that only two of these isolates produce heat-stable enterotoxin (C2: 0.13 and C6: 0.11), with percent 18.18%, while other bacterial isolates gave a negative result compared with (TSB+ 6% yeast extract) as a negative control as shown in table (3).

Heat-labile enterotoxin bioassay: The Rabbit ligated ileal loop assay (RIL) was applied to the 11 isolates of *C. freundii*. The results showed that only 4 (36.36%) of

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the bacterial isolates gave a positive results (C1: 0.74, C2: 0.86 C3: 0.76 and C10: 0.76) ml/cm, 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).

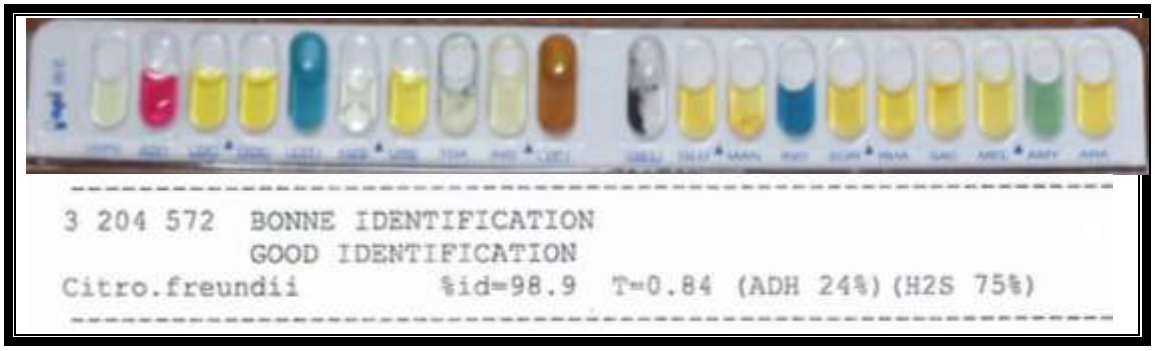

Figure (1): API 20 E identification system for *C. freundii*

Table (3) Crude enterotoxin bioassay

Number of isolate	Enterotoxin score	
	SMA(IW/BW)*	RIL (ml/cm)
C1	0.07 (–)	0.74 (+)
C2	0.13 (+)	0.86 (+)
C3	0.07 (–)	0.76 (+)
C4	0.06 (–)	0.32(–)
C5	0.07 (–)	0.36(–)
C6	0.11 (+)	0.26 (–)
C7	0.07 (–)	0.32 (–)
C8	0.05 (–)	0.27 (–)
C9	0.07 (–)	0.30 (–)
C10	0.05 (–)	0.76(+)
C11	0.05 (–)	0.30 (–)
Negative control	0.06 (–)	0.15 (–)
CT	–	1.1 (+)

*The ratio of intestinal weight to remaining body weight.
(SMA) Suckling mice assay
(RIL)Rabbit ligated ileal loop assay
(CT) Cholera toxin

Discussion:

C. freundii is classically considered as an indigenous intestinal species that is sporadically associated with diarrhea (Pereira *et al*., 2010). Table (1) showed that out of 422 clinical samples, eleven (2.6%) *C. freundii* were isolated, this percent of isolation was convergent with AL-Khafagee (2010) with percent 4.6 % and Al-Muslemawi (2007) with percent 6.6 % while Citrobacter strains were isolated from 46

patients out of 328 with percent 14% by Guarino *et al*. (1987). Isolation percent from diarrheic cases in infant who were under three years old was 2.63% (Al-Hashimi, 2002). The percents of isolation may be varied according to variation in levels of contamination surrounding patients, nutrition of infant, handling and feeding scenarios and environmental factors. All 11 isolates primarily identified as *C. freundii* were subjected to biochemical tests described by MacFaddin, (2000) by culturing the isolates on MacConkey agar which yielded rapid and late lactose fermenters then confirmed by culturing on XLD which yielded a yellow colonies because of fermenting Xylose and failed to decarboxylate lysine (Washington *et al*., 2006 and Samonis *et al*., 2009). Further to conventional biochemical test using API 20E system, there was no difference in the result of manual biochemical tests that carried out according to the MacFaddin, (2000) and that of conventional biochemical test using API 20 E system. According to the API 20E database, by (API Biomerieux SA, Marcy I'Etoile, France) the numerical code yielded (3204572) and related numerical cods represented a "good identification" for *C. freundii* (Lozano-Leon *et al*., 2011).

Enterotoxin bioassay: as shown in table (3) the results marked fluid accumulation in infant mice by the suckling mouse assay in only 2 of the bacterial isolates (C2 and C6), with percent 18.18%, while 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. Al-Hashimi, (2002) showed the ability of (40%) of the strains to produce heat-stable enterotoxin by suckling mice assay and rapid permeability test of the rabbit skin, as well showed that 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. Guarino *et al*. (1987) demonstrate that three strains of *C. freundii*, out of 46 isolates (6.5%) from children with diarrhea, showed a positive response in the SMA, The tests were repeated monthly for 3 months, with repeated positive responses for two strains, whereas the third strain became negative after 2 months, and none of the strain (0%) showed a positive result with RIL.The enterotoxin bioassay by SMA has the advantage over cell-culture systems which detect only cytotoxicity (Trower *et al*., 2000). 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 (Evans *et al*., 1973). Detection of STa biological activity in porcine ligated intestinal loop was demonstrated by Zhang *et al*. (2010).

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الخلاصة:-

جمع خلال هذه الدراسة 422 عينة (202 ذكور و 220 إناث) تمثلت بـ (282 عينة براز stool و 140 عينة إدرار urine) من الأطفال تحت سن السادسة والمصابين بالإسهال والتهاب المجاري البولية، كذلك 200عينة من بيئة المستشفى بالإضافة إلى 50 عينة سيطرة (أطفال أصحاء)، جمعت هذه العينات من ثلاث مستشفيات في مدينة النجف(مستشفى الصدر التعليمي، مستشفى الحكيم و مستشفى الزهراء للأمومة والأطفال) للفترة من تموز ولغاية تشرين الأول 2010. عزل النوع *Citrobacter freundii* بواقع 11 عزلة (2.60%) من مجموع العينات شملت ثمان عزلات من عينات البراز (2.83%) و ثلاث عزلات من عينات الإدرار (2.14%) ولم يعزل من عينات بيئة المستشفى وعينات السيطرة، شخصت العزلات وفق الاختبارات المظهرية والكيموحيوية وباستعمال نظام API 20 E. اجري اختبار الفأر الرضيع Suckling Mice Assay (SMA) لجميع العزلات للتحري عن الذيفان الثابت بالحرارة، فقط عزلتين (18.18%) أعطت نتائج موجبة لهذا الاختبار.في حين عند استخدام Rabbit ligated ileal loop assay (RIL) للتحري عن الذيفان غير الثابت بالحرارة،أعطت أربعة عزلات (36.36%) نتائج موجبة لهذا الاختبار.