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Determination the Pathogenicity of Citrobacter freundii by Using Three Types of Antigens in Najaf City

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Abstract
Background:This study has been included stool and urine samples were collected from children under six years of ages collected from three hospitals in Al-Najaf city (Al-Sadr Teaching, Al-Hakeem, and Al-Zahra Maternity and Children) ,*Citrobacter freundii* isolated from clinical sample. The Suckling Mice Assay (SMA) and Rabbit ligated ileal loop assay (RIL) have been tested to detect production of heat stable and heat labile enterotoxins by bacteria. **Material and Methods:** Three types of antigens were prepared**:** Live Bacteria, Crude Enterotoxin and Partially purified enterotoxin(PPET). LD_{50} for crude and PPET were determined by using white mice (20-25) g, which divided into five groups each group consist of five mice. **Results** LD_{50} of bacterial Suspension, crude enterotoxin and PPET in mice was 2.04×10^7 cell/mouse, 66.6 µg/mouse and 46.2 µg/mouse respectively. **Conclusion***: Citrobacter freundii* considered as a potential pathogen that isolated from clinical samples .Bioassay administrated the ability of this species to produce heat stable enterotoxin by using SMA, and heat labile enterotoxin by RIL.
Aim: The aim of this study was to investigate three types of antigens isolated from *Citrobacter freundii* to determination the pathogencity of this bacteria by LD_{50}.

Introduction

Citrobacter freundii is usually considered as 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).

Material and Methods

The three types of antigens was prepared according to Al-Jamell (2011).
Determination of LD_{50} for Bacterial Suspension: LD_{50} for bacterial suspension was determined by using 25 white mice (20-25) g, which divided into five groups each group consist of five mice. Each group injected intraperitoneally in one of the following bacterial dilution (10^9, 10^8, 10^7, 10^6 and 10^5), and the injected volume was 1ml for each mice. After 5 days LD_{50} was determined according to Reed and Muench (1938).
Determination of LD_{50} for Crude and PPET: LD_{50} for crude and PPET were determined by using 25white mice each (20-25) g, which divided into five groups each group consist of five mice (Banno, 2008). Each group injected intraperitoneally in one of the following concentration from both crude and PPET concentration (100, 80, 60, 40, and 20) µg/ml which prepared in PBS solution and the injected volume was 1ml for each mice. Another group of five mice injected with 1ml of PBS as negative control. After 5 days LD_{50} was determined according to Reed and Muench (1938).

Result

Median Lethal Dose (LD_{50}) for Live Bacteria: According to reed and Munch (1938), LD_{50} dose was evaluated through intraperitoneal route in balb/c mice. Five serial concentrations five mice for each of live bacteria were used and the results showed that the LD_{50} was 2.04×10^7 cell/mouse which equal to 8.16×10^8 cell/kg (Table 1

and fig. 1).

Table (1): LD_{50} of Live Bacteria

Bacterial concentration (cell/ml)	Mortality ratio	Died	Survived	Accumulation values			
				Died (D)	Survived (S)	Mortality ratio $\frac{D}{D+S}$	Percent % $\frac{D}{D+S} \times 100$
10^9	5/5	5	0	11	0	11/11	100
10^8	4/5	4	1	6	1	6/7	85.71
10^7	2/5	2	3	2	4	2/6	33.33
10^6	0/5	0	5	0	9	0/9	0
10^5	0/5	0	5	0	14	0/14	0

$$\text{Proportional distance} = \frac{50 - \text{Mortality below 50 percent}}{\text{Mortality above 50 percent - Mortality below 50 percent}}$$

$$= \frac{50 - 33.33}{85.71 - 33.33} = 0.31$$

$$\text{Negative logarithm of } LD_{50} \text{ titer} = \text{Proportional distance} + \text{Negative logarithm of concentration below 50 percent mortality}$$

$$= 0.31 + 7$$

$$LD_{50} = 10^{7.31} = 10^{0.31} \times 10^7 = 2.04 \times 10^7 \text{ cell/ml}$$

$$= 8.16 \times 10^8 \text{ cell/kg}$$

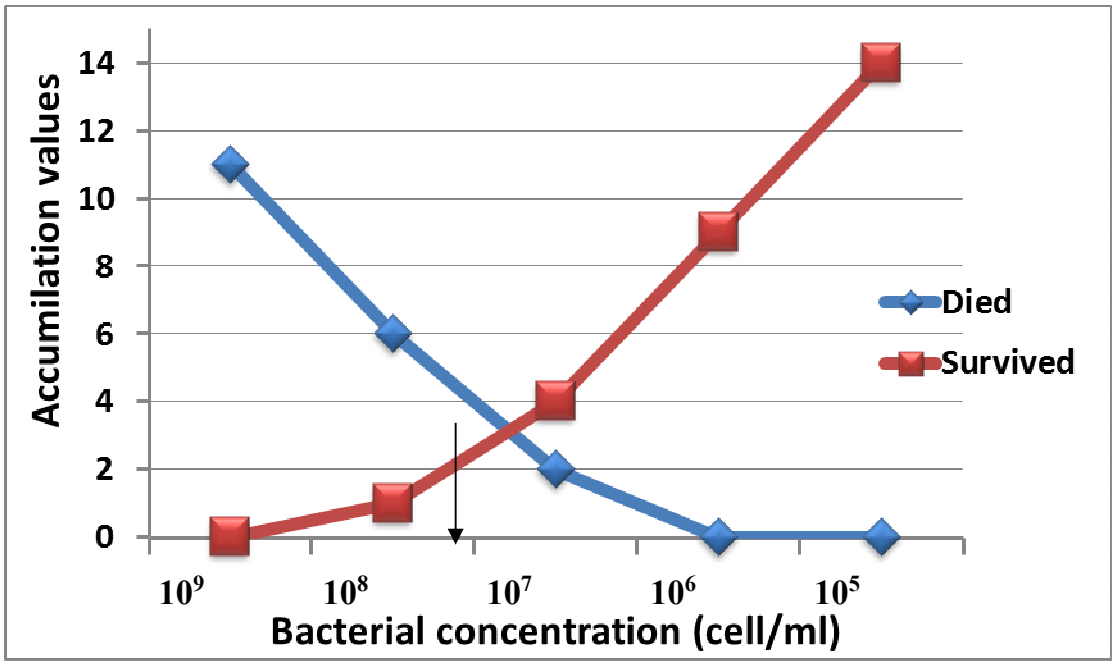

Figure (1): LD_{50} Value of Live Bacteria

Median Lethal Dose (LD_{50}) for Crude Enterotoxin

LD_{50} was evaluated through intraperitoneal route in balb/c mice. Five concentrations of crude enterotoxin were used and the results showed that the LD_{50} was 66.6 µg/ mouse which equal to 2.664 mg/kg (Table 2 and fig. 2).

Table (2): LD_{50} of Crude Enterotoxin

Crude toxin concentration (µg/ml)	Mortality ratio	Died	Survived	Accumulation values			
				Died (*D*)	Survived (*S*)	Mortality ratio $\frac{D}{D+S}$	Percent % $\frac{D}{D+S} \times 100$
100	4/5	4	1	11	1	11/12	91.66
80	3/5	3	2	7	3	7/10	70
60	2/5	2	3	4	6	4/10	40
40	2/5	2	3	2	9	2/11	18.18
20	0/5	0	5	0	14	0/14	0

$$\text{Proportional distance} = \frac{50 - \text{Mortality below 50 percent}}{\text{Mortality above 50 percent - Mortality below 50 percent}}$$

$$= \frac{50 - 40}{70 - 40} = 0.33$$

$$\text{Proportional distance 50 percent} = \text{Proportional distance} \times (\text{Concentration above 50 percent - Concentration below 50 percent})$$

$$= 0.33 \times (80\text{-}60) = 6.6$$

LD_{50} = Proportional distance 50 percent + Concentration below 50 percent

LD_{50} = 6.6 + 60 = 66.6 (µg/ml)

= 2.664 mg/kg

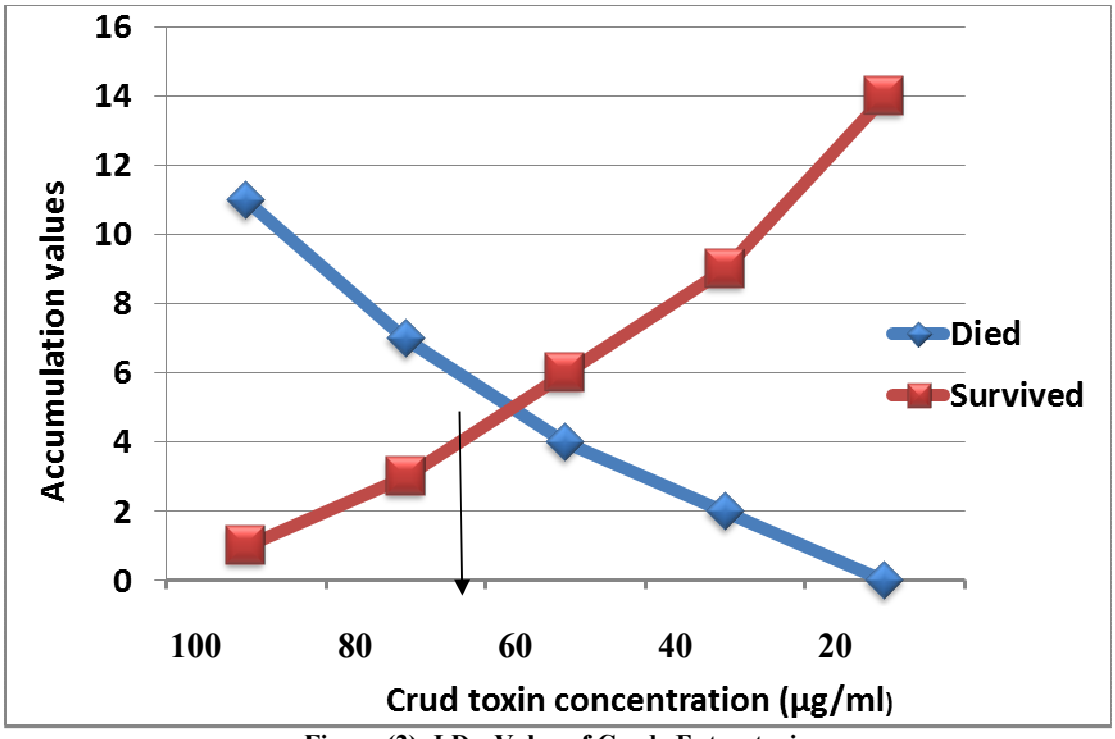

Figure (2): LD_{50} Value of Crude Enterotoxin

Median Lethal Dose (LD_{50}) for PPET

LD50 was evaluated through intraperitoneal route using balb/c mice. Five concentrations of PPET were used and

the results showed that the LD_{50} was 46.2µg/ mouse which equal to 1.848mg/kg (Table 3 and fig. 3).

Table (3): LD_{50} of PPET

Purified toxin concentration (µg/ml)	Mortality ratio	Died	Survived	Accumulation values			
				Died (*D*)	Survived (*S*)	Mortality ratio $\frac{D}{D+S}$	Percent % $\frac{D}{D+S}\times 100$
100	5/5	5	0	16	0	16/16	100
80	5/5	5	0	11	0	11/11	100
60	4/5	4	1	6	1	6/7	85.71
40	2/5	2	3	2	4	2/6	33.33
20	0/5	0	5	0	9	0/9	0

$$\text{Proportional distance} = \frac{50 - \text{Mortality below 50 percent}}{\text{Mortality above 50 percent - Mortality below 50 percent}}$$

$$= \frac{50 - 33.33}{85.71 - 33.33} = 0.31$$

$$\text{Proportional distance 50 percent} = \text{Proportional distance} \times (\text{Concentration above 50 percent - Concentration below 50 percent})$$

$$= 0.31 \times (60-40) = 6.2$$

$$LD_{50} = \text{Proportional distance 50 percent} + \text{Concentration below 50 percent}$$

$$LD_{50} = 6.2 + 40 = 46.2\ (\mu g/ml)$$

$$= 1.848\ mg/kg$$

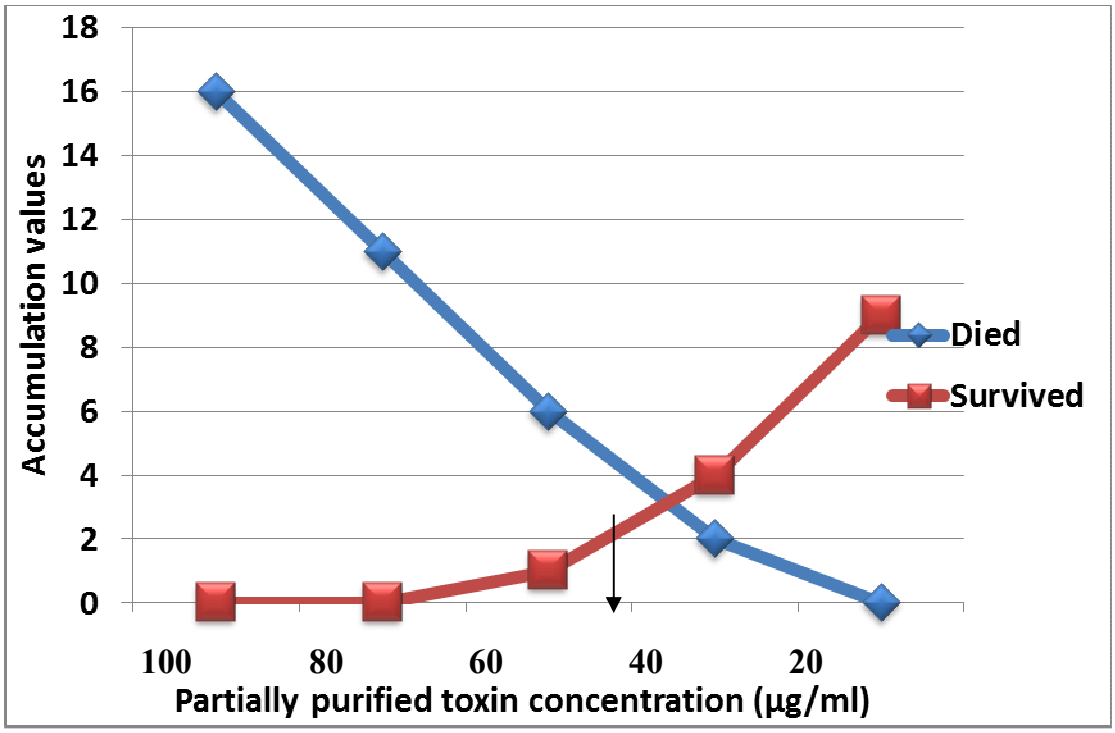

Figure (3): LD_{50} Value of PPET

The crude enterotoxin showed LD_{50} value (2.664 mg/ml) higher than PPET (1.848 mg/ml), while LD_{50} value of *C. freundii* (Live bacteria) 8.16×10^8 cell/kg (Table 4).

Table (4) LD_{50} Values of *C. freundii* (Live bacteria) and its Enterotoxins (Crude & PPET)

Treatment	LD_{50} values	
	Per mouse	Per kg
Live bacteria	2.04×10^7 cell/ml	8.16×10^8 cell/kg
Crude enterotoxin	66.6 µg/ml	2.664 mg/kg
PPET	46.2 µg/ml	1.848mg/kg

Discussion

The LD_{50} of *C. freundii* was about 2.04×10^7 cell/mouse and of crude enterotoxin was about 66.6 µg/mouse and for PPET was 46.2 µg/mouse (table 4-9). Previous study recorded different value for LD_{50} of *C. freundii* suspension were Al-Muslemawi (2007) revealed that the LD_{50} of *C. freundii* was about 3.16×10^6 cell/mouse and Iwahi *et al.*, (1992) estimated 10^5 cell/mouse as LD_{50} for *C. freundii* suspension, the mice die within 2 days. The LD_{50} evaluated by Toranzo *et al.,* (1994) was more than 5×10^7 and describe the *C. freundii* as low virulence.

The difference in the LD_{50} values of bacterial suspension between different studies may be according to different of strains used and the potential virulence factors like enterotoxin, shiga like toxin, outer membrane proteins, LPS and other virulence factor (Al-Muslemawi, 2007).

The study of Banno (2008) estimated the LD_{50} value of *E. coli* PPET (48.75µg/mouse). This value was much related to the present study (46.2 µg/mouse) may be according to resembling of STa enterotoxins of both *E. coli* and *C. freundii*.

Additionally, Pereira *et al*. (2010) identified isolates of *C. freundii* as effective recipient strains for transfer of *E. coli* thermo-stable toxin genes between these species raised considerations about the virulence potential of the bacterial conjugation.

According to Gill (1982), the LD_{50} value of *E.coli* heat stable and heat labile enterotoxin were250µg/kg when the mice injected intravenous, while Abd Al-Hussain (2006) estimate the LD_{50} value of PPET partially purified from *Plesiomonas Shigelloides* (12.5 µg/mouse).

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