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 consist of five mice. Results: LD$_{50}$ of bacterial Suspension, crude enterotoxin and PPET in mice was $2.04 \times 10^7$ 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 pathogenicity 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 (Tast 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 25 white 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 $2.04 \times 10^7$ cell/mouse which equal to 8.16×10$^8$ cell/kg (Table 1...
Table (1): LD$_{50}$ of Live Bacteria

<table>
<thead>
<tr>
<th>Bacterial concentration (cell/ml)</th>
<th>Mortality ratio</th>
<th>Died</th>
<th>Survived</th>
<th>Accumulation values</th>
<th>Mortality ratio</th>
<th>Percent %</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^9$</td>
<td>5/5</td>
<td>5</td>
<td>0</td>
<td>Died (D)</td>
<td>Survived (S)</td>
<td>11/11</td>
</tr>
<tr>
<td>$10^8$</td>
<td>4/5</td>
<td>4</td>
<td>1</td>
<td>6</td>
<td>1</td>
<td>6/7</td>
</tr>
<tr>
<td>$10^7$</td>
<td>2/5</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>4</td>
<td>2/6</td>
</tr>
<tr>
<td>$10^6$</td>
<td>0/5</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>9</td>
<td>0/9</td>
</tr>
<tr>
<td>$10^5$</td>
<td>0/5</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>14</td>
<td>0/14</td>
</tr>
</tbody>
</table>

Proportional distance = 50 - Mortality below 50 percent - Mortality above 50 percent

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

$= 0.31$

Negative logarithm of LD$_{50}$ titer

Proportional distance + Negative logarithm of concentration below 50 percent mortality

$= 0.31 + 7$

LD$_{50}$ = $10^{7.31}$

$= 10^{0.31} \times 10^7$

$= 2.04 \times 10^7$ cell/ml

$= 8.16 \times 10^8$ 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): LD₅₀ of Crude Enterotoxin**

<table>
<thead>
<tr>
<th>Crude toxin concentration (µg/ml)</th>
<th>Mortality ratio</th>
<th>Died</th>
<th>Survived</th>
<th>Accumulation values</th>
<th>Mortality ratio</th>
<th>Percent %</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>4/5</td>
<td>4</td>
<td>1</td>
<td>Died (D) 11</td>
<td>Survived (S) 1</td>
<td>11/12</td>
</tr>
<tr>
<td>80</td>
<td>3/5</td>
<td>3</td>
<td>2</td>
<td>Died (D) 7</td>
<td>Survived (S) 3</td>
<td>7/10</td>
</tr>
<tr>
<td>60</td>
<td>2/5</td>
<td>2</td>
<td>3</td>
<td>Died (D) 4</td>
<td>Survived (S) 6</td>
<td>4/10</td>
</tr>
<tr>
<td>40</td>
<td>2/5</td>
<td>2</td>
<td>3</td>
<td>Died (D) 2</td>
<td>Survived (S) 9</td>
<td>2/11</td>
</tr>
<tr>
<td>20</td>
<td>0/5</td>
<td>0</td>
<td>5</td>
<td>Died (D) 0</td>
<td>Survived (S) 14</td>
<td>0/14</td>
</tr>
</tbody>
</table>

Proportional distance

\[
\text{Proportional distance} = \frac{\text{Mortality above 50 percent} - \text{Mortality below 50 percent}}{50 - 40} = \frac{70 - 40}{50 - 40} = 0.33
\]

50 percent

\[
\text{LD₅₀} = \text{Proportional distance 50 percent} + \text{Concentration below 50 percent}
\]

\[
\text{LD₅₀} = 0.33 \times (80-60) = 6.6
\]

LD₅₀ = 6.6 + 60 = 66.6 (µg/ml)
LD₅₀ = 2.664 mg/kg

**Figure (2): LD₅₀ Value of Crude Enterotoxin**

**Median Lethal Dose (LD₅₀) for PPET**

LD₅₀ was evaluated through intraperitoneal route using balb/c mice. Five concentrations of PPET were used and
the results showed that the LD₅₀ was 46.2µg/ mouse which equal to 1.848mg/kg (Table 3 and fig. 3).

Table (3): LD₅₀ of PPET

<table>
<thead>
<tr>
<th>Purified toxin concentration (µg/ml)</th>
<th>Mortality ratio</th>
<th>Died</th>
<th>Survived</th>
<th>Accumulation values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Died (D)</td>
<td>Survived (S)</td>
<td>Mortonality ratio</td>
</tr>
<tr>
<td>100</td>
<td>5/5</td>
<td>5</td>
<td>0</td>
<td>16/16</td>
</tr>
<tr>
<td>80</td>
<td>5/5</td>
<td>5</td>
<td>0</td>
<td>11/11</td>
</tr>
<tr>
<td>60</td>
<td>4/5</td>
<td>4</td>
<td>1</td>
<td>6/7</td>
</tr>
<tr>
<td>40</td>
<td>2/5</td>
<td>2</td>
<td>3</td>
<td>2/6</td>
</tr>
<tr>
<td>20</td>
<td>0/5</td>
<td>0</td>
<td>5</td>
<td>0/9</td>
</tr>
</tbody>
</table>

Proportional distance = 50 - Mortality below 50 percent

Mortality above 50 percent - Mortality below 50 percent

= 50 – 33.33
= 0.31

Proportional distance 50 percent = Proportional distance × (Concentration above 50 percent - Concentration below 50 percent)

= 0.31 × (60-40)
= 6.2

LD₅₀ = Proportional distance 50 percent + Concentration below 50 percent

LD₅₀ = 6.2 + 40 = 46.2 (µg/ml)

LD₅₀ = 1.848 mg/kg

Figure (3): LD₅₀ Value of PPET

The crude enterotoxin showed LD₅₀ value (2.664 mg/ml) higher than PPET (1.848 mg/ml), while LD₅₀ value of C. freundii (Live bacteria) 8.16 × 10⁸ cell/kg (Table 4).
Table 4: LD$_{50}$ Values of *C. freundii* (Live bacteria) and its Enterotoxins (Crude & PPET)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LD$_{50}$ values</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Per <em>mouse</em></td>
<td>Per <em>kg</em></td>
</tr>
<tr>
<td>Live bacteria</td>
<td>2.04 × 10$^7$</td>
<td>8.16 × 10$^8$</td>
</tr>
<tr>
<td>Crude enterotoxin</td>
<td>66.6 µg/ml</td>
<td>2.664 mg/kg</td>
</tr>
<tr>
<td>PPET</td>
<td>46.2 µg/ml</td>
<td>1.848 mg/kg</td>
</tr>
</tbody>
</table>

**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 (48.75 µg/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 were 250 µg/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).

**References**


