



## Pathogenic Effect of *C. Freundii* in Rats

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### Abstract:

This study was conducted to investigate pathological effect caused by *Citrobacter freundii* in rats, to achieve this goal; faecal samples were collected during the period from December/ 2020 to March/ 2021 different region in Baghdad City. The sample was cultured on different media, biochemical tests, Vitek2 system were applied. A total of 20 rats distributed into 2 group first group received  $1 \times 10^6$  the second group received 1 ml PBS and considered a negative control group after that animals were sacrificed after one week post infection results showed no gross lesions were seen in the examined organs (lung, liver, spleen, intestine and brain) of both groups especially infected group. Histopathological changes of intestine, lung, liver, spleen and brain showed severe histopathological changes in the infected group compared with the negative control groups manifested by massive sloughing of intestinal epithelial lining with severe distension of lamina propria due variable cell types infiltration, severe multifocal vacuolar degeneration of hepatocytes with nuclear pyknosis of adjacent hepatocytes with focal inflammatory cells, white pulp depletion with congestion of red pulp in spleen. The lung shows moderate lymphoid depletion of BALT with necrosis of bronchial mucosa accompanied with polymorphonuclear and few lymphocytes infiltration in mucosa and sub-mucosa.

**Keywords:** *Citrobacter freundii*, Biochemical tests, Vitek2

### المخلص

أجريت هذه الدراسة للتحقق من التأثير المرضي الذي تسببه بكتيريا *Citrobacter freundii* في الجرذان، ولتحقيق هذا الهدف تم جمع عينات من البراز خلال الفترة من كانون الأول 2020 إلى آذار 2021 في مناطق مختلفة في مدينة بغداد. زرعت العينة على أوساط مختلفة واستخدمت الاختبارات الكيموحيوية. تضمنت الدراسة استخدام عشرين جرذاً موزعة على مجموعتين المجموعة الأولى تلقت  $1 \times 10^6$  من البكتيريا المجموعة الثانية تلقت 1 مل من المحلول الملحي الفسيولوجي واعتبرت مجموعة سيطرة سالبة تم التضحية بالحيوانات بعد أسبوع واحد من الإصابة أظهرت النتائج عدم وجود آفات جسيمة عيانية في الأعضاء التي تم فحصها (الرئة، الكبد والطحال والأمعاء والدماغ) من كلا المجموعتين خاصة المجموعة المصابة. أظهرت التغيرات النسيجية المرضية للأمعاء والرئة والكبد والطحال والدماغ تحت المجهر تغيرات نسيجية مرضية شديدة في المجموعة المصابة مقارنة بمجموعات السيطرة السالبة التي تجلت في تقشير كبير للبطانة الظهارية المعوية مع انتفاخ شديد في الصفيحة المخصوصة بسبب ارتشاح أنواع الخلايا المتغيرة، وتكس فجوي شديد متعدد البؤر من خلايا الكبد المصابة بالتضخم النووي للخلايا الكبدية المجاورة مع ارتشاح بؤري للخلايا الالتهابية، ونضوب اللب الأبيض مع احتقان اللب الأحمر في الطحال. الرئة أظهرت استنزاف ليمفاوي طفيف في الأنسجة الليمفاوية المرتبطة بالقصات وتخر في الغشاء المخاطي القصي وارتشاح طفيف في الخلايا متعددة النواة مع عدد قليل من الخلايا اللمفاوية الغشاء المخاطي وتحتة.

الكلمات المفتاحية: سيتروباكتر فروندي، الاختبارات البيوكيميائية، جهاز فيتك

### Introduction

*Citrobacter* is a genus belong to the *Enterobacteriaceae* family, Gram-negative microbes. Species of *Citrobacter* such as *Citrobacter rodentium*, *Citrobacter freundii* was isolated from sewage [1,2], *C. freundii* from gastrointestinal tract of human [3], and from animal intestines.[4] *Citrobacter freundii* is the optional anaerobic Gram-negative coliform bacteria that habitant in soil, water, food and the commensal environment such as hospitals. *C. freundii* have been residents as part of normal flora of the fish intestine and also human, also a provisionally a virulent pathogen able to cause many diseases in people especially the immune-compromised hosts, disease like acute respiratory infection that effect lung, inflammation of the brain membrane, sepsis, bacteraemia, and urinary tract infections.[5-7] *C. freundii* was confirmed by many to cause diarrhoea and by assimilated virulence traits cause diarrhea or food poisoning in human [8,9] and in animals *Citrobacter freundii* also isolated from diarrheal

sample in sheep.[10] In diarrhea-related to *C. freundii* most virulence factors establish were toxins, cholera toxin, Shiga-like toxins, and heat-stable toxins.[8]

### Material and method:-

#### Isolation:-

Fecal sheep gathered from different areas in Baghdad governorate. Then the collected samples were placed in ice and transported to laboratory within period less than two hours. In a test tube 1gram faecal sample mixed with normal saline, mixed well, after that let for one minute, from the normal saline and faecal sample mixture 0.1 ml was inoculated on different media such as (SS) *Salmonella shigella* agar and MacConkey medium, the plates were left to solidify at room temperature, and then are incubated at 37°C for 24-48 hours. Colonies were examined macroscopically concerning their shape, size and colour. After that, they were examined microscopically by Gram-stain and biochemical tests were done including: Urease, Gelatinase, Oxidase and Catalase.[11]

#### C. Freundii Identification by VITEK® 2 System.



The Vitek2 system have been used to identify positive isolates from traditional morphological and biochemical tests. Vitek2 was used to detect the bacterial isolates in accordance with the instructions came with the kit from manufacturer.

#### **Animals and experimental design**

Twenty (20) albino rats were used, 6-8 weeks old, male and female weighting 200-260 gram, distributed in 2 group each group contain 10 rats used for experimental designs of the study. First group rat received a dose ( $1 \times 10^6$  CFU/ml) virulent strains of *Citrobacter freundii* the second group take 1 ml of phosphate puffer saline as negative control after 7days the animals scarified to study the histopathological changes of vital organs (lung, liver, spleen, intestine and brain).

#### **Pathological study**

##### **Macroscopic examination**

The macroscopic appearance of the lesions was recorded to detect presence of any abnormal gross changes in lung, liver, kidney, spleen and intestine including color, size and consistency.

##### **Histopathological examination**

After one week of challenge dose , the samples taken from vital organs of experimental animals included; liver, spleen, lung, intestine and brain., 10% buffer formaldehyde solution were used to fixed the tissues immediately after removal, and left in that solution for 72 hrs. then ,using tab water the specimen was washed and treated regularly with set of upgrading alcoholic from 70% to absolute 100% concentration at two hours for every concentration to eliminate water from tissues after that Xylol used for clearance. semi-liquid paraffin wax used to soaked the specimens (two stages). The blocks of the specimens that made with paraffin wax weren sectioned by rotary microtome at 5 mm. All tissues were stained with Hematoxylin and Eosin (H&E) stain and the histopathological variations were detected with light microscope.<sup>[12]</sup>

#### **Results and discussion**

##### **Identification of *Citrobacter freundii* a. colonial morphology, microscopic examination and biochemical tests:**

Identification of *Citrobacter spp.* was performed primarily through colony shape color and size on *Salmonella Shiglla*, MacConkey and Xylose lysine deoxycholate agar, because they were able to generate H<sub>2</sub>S on S.S. medium within 24 hours, all isolates have a similar colony on SS agar as little or big pale flattened colonies with black centers (Figure 1). The lactose fermentation ability of these bacteria makes the *Citrobacter freundii* isolates showed as

rose colony on MacConkey agar (Figure 2), on XLD *Citrobacter* appeared as yellow colonies (Figure 3). *C. Freundii* isolate were stained red (Gram negative) when staining with Gram stain and appear as bacilli or coco-bacilli. Microscopically, the bacteria appeared under oil immersion lens (100x) as gram negative rods.<sup>[13]</sup> Different biochemical test was done for primary identification of *Citrobacter freundii* isolate, the isolate was positive for catalase and negative for oxidase, urea and gelatinase. Colonial morphology and biochemical test have been counted on for characterization of *Citrobacter spp.* All identification outcome of these parameter for *Citrobacter spp.* are in agreement with Quinn et al.<sup>[11]</sup> who reported that given their capacity to create H<sub>2</sub>S on *Salmonella Shiglla* agar after 24 hours, *C. freundii* emerged as little or big pale flatten colonies with black center, while it appeared as pink colony on MacConkey media and yellowish colony on Xylose lysine deoxycholate agar, this result was closed with what find by Hashim and AlKhafaji,<sup>[13]</sup> who chicken and the colonies on MacConkeys agar was appeared pink due to lactose fermenter, and also on S.S. agar and XLD agar appear as black center and yellow colonies respectively. **b. Vitek two compact kit** The Vitek two compact system have been performed to confirm identification of *Citrobacter spp.* The outcome of the Vitek system revealing the eight bacteria isolated were *Citrobacter freundii* .

Results agreed with Mohammed<sup>[14]</sup> that used the Vitek2 compact system for identification and confirmed the isolated bacteria was *Citrobacter freundii* (98%) also with Hashim and AlKhafaji<sup>[13]</sup> that successfully identified *C. freundii* by Vitek2 compact system, result showed that the isolated bacterium was *Citrobacter freundii* species with probability 97%.

Infectious dose (ID) was approximated by consider the mice group that managed to show sign of infection with no mortality Table (1), represent the results of infectious dose estimation experiment in mice infected with *C. freundii* orally at different doses ranging from ( $1 \times 10^{10}$  -  $1 \times 10^5$ ) Infectious dose was  $1 \times 10^6$ . These results agreed with Mohammed,<sup>[14]</sup> find that infectious dose of *C. Freundii* was  $1 \times 10^6$  while Al-Jamell,<sup>[15]</sup> who stated that the LD<sub>50</sub> of bacterial Suspension of *C. freundii* in mice was  $2.04 \times 10^7$  cell/mouse. These results disagreed with Al-Eqabi and Al-Abedi<sup>[16]</sup> used  $3 \times 10^8$  CFU/ml of the virulent strain (I.P) of *C. freundii*. The results of infectious dose in current study disagreement with Abood *et al.*<sup>[17]</sup> who pointed the LD<sub>50</sub> were  $1 \times 10^6$  cfu/ml and infective dose (ID<sub>50</sub>) were  $1 \times 10^8$  cfu/ml



of typical enteropathogenic *E. coli* (EPEC) in mice when inject intraperitoneally.

#### **Pathological examination**

##### **i. Gross pathological examination**

No gross lesions were seen in the examined organs (lung, liver, spleen, intestine and brain) of all groups especially positive group that were sacrificed after one-week post challenge. This result was inconsistency with Kumar *et al.*<sup>[18]</sup> who clarified that the gross changes appeared after long time of macroscopically alteration of cells

##### **ii. Histopathological examination**

Histopathological examination after 2week post challenge showed that all examined group were affected with variable histopathological changes:

##### **A. Control Positive Group (5<sup>th</sup> group):-**

The results of histopathological examination of rats infected with infectious dose ( $1 \times 10^6$  CFU/ml) of *C. freundii* showed varying degrees of histopathological changes from mild to severe. Generally. The intestine of control positive group showed massive sloughing of intestinal epithelial lining with severe distension of lamina propria due inflammatory variable cell type infiltration (Figure 4). Histopathological section of the spleen of mice shows white pulp depletion with congestion of red pulp (Figure 5). Histopathological section of the liver of rat's vacuolar degeneration of hepatocytes with focal inflammatory cells aggregation (Figure 6). The lung shows severe necrotic of pulmonary parenchyma with congestion of blood vessels (Figure 7), while the brain shows no histopathological lesions (Figure 8). These results of the histopathological study demonstrated the presence of severe histopathological effects in the organs of rats caused by their infection with *C. freundii*, compared to immunized group as mentioned bellow; *C. freundii* have a same functional and structural characteristics with Enteropathogenic *E. coli*, utmost important of them is the capacity to attach closely to the apical superficial of the epithelium of intestine and enhance limited microvillus damage, a distinguishing feature called attaching effacing lesion.<sup>[19]</sup> *C. freundii* contains a type I fimbriae that binds to the end of the glycoprotein on the surface of the host cell, particularly the epithelial cells that border the colon, similar to the enterotoxigenic *E. coli* (ETE).<sup>[20]</sup>

However, *C. freundii* possess many pathogenic factors such as enterotoxins, Shiga-like toxins, cytotoxins, outer membrane proteins, and adhesion factors represented by fimbriae, as well as lipopolysaccharides. Lipidosis on germ cell protection against phagocytosis.<sup>[21]</sup> The thinking of

inter alveolar septa of lung occurred which was related to congestion after spread of bacterial endotoxin in blood and then reach to different tissue including pulmonary tissues, these change in response to increase blood pressure of microcirculation, as well as endotoxin cause subsequent increase capillary permeability.<sup>[22]</sup> The bronchial showed hyperplasia pathological changes as a result to it persist stimulating by bacteria leading to hyperplasia that play a role in decrease the numbers of bacteria in pulmonary tissues.<sup>[23]</sup>

In a study by Al-Eqabi and Al-Abedi.<sup>[16]</sup> Results revealed a different gross pathological effect that included haemorrhage, oedema and congestion of viscera. Moreover, under the microscope pneumonia appear manifested by inflammatory cells permeation, mostly neutrophils, macrophages, plasmacytes and lymphocytes, acute interstitial and intra-alveolar oedema, widespread haemorrhage in lung tissue, emphysema, and atelectasis. The liver tissue shown hepatitis that was observed by a perivascular and peri-portal leukocyte cuffing, noticeable centrilobular along with periportal necrosis, widespread hepatic oedema, and periportal oedema also there was sever fibrosis in interlobular septa and periportal fibrosis with extreme interstitial haemorrhage. Enteritis appeared in the intestine with oedema in the muscle, particularly in lamina propria; furthermore, necrosis of entire villi, epithelial necrosis, mucosal and sub mucosal haemorrhage, and fibrosis were observed, Junior *et al.*<sup>[24]</sup> stated that Concerning histopathology alterations in the liver tissue, kidney and spleen of fish which inoculated I.M. with 100  $\mu$ L of *C. freundii* ( $6.4 \times 10^8$  cfu mL<sup>-1</sup>).

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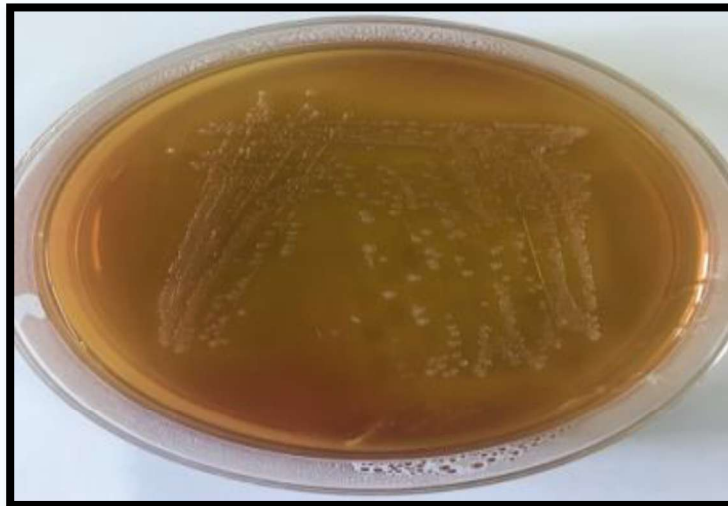
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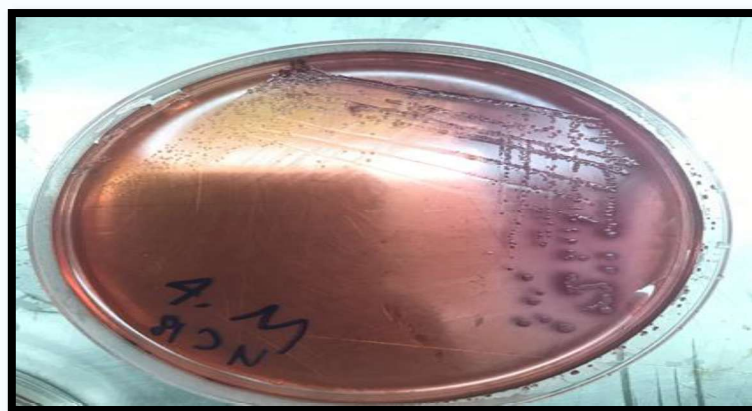
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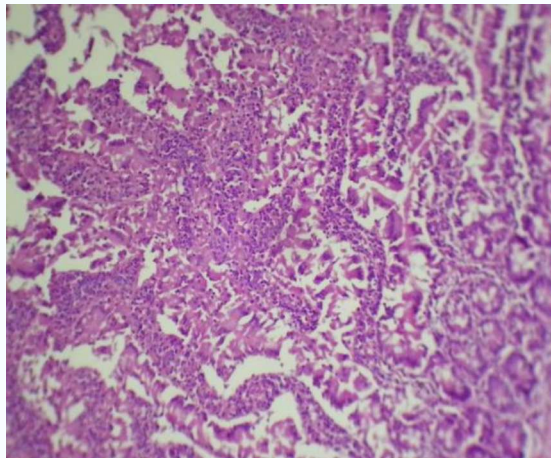
**Figure (1):** *Citrobacter Freundii* on SS agar, shows little or big pale flattened colonies with black centers



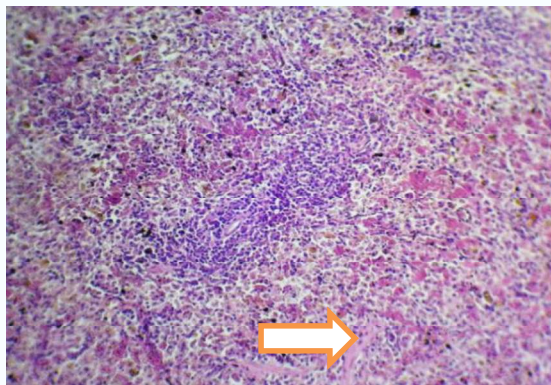
**Figure (2):** *Citrobacter Freundii* on Macconky agar showed as pink colony



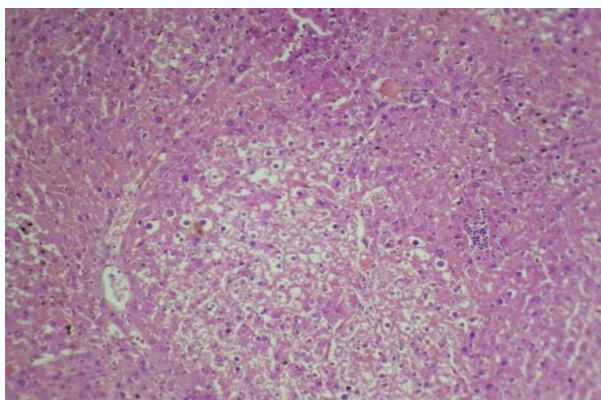
**Figure (3):** Macroscopic appearance of *C.Freundii* on XLD agar, showed the colony with yellow color



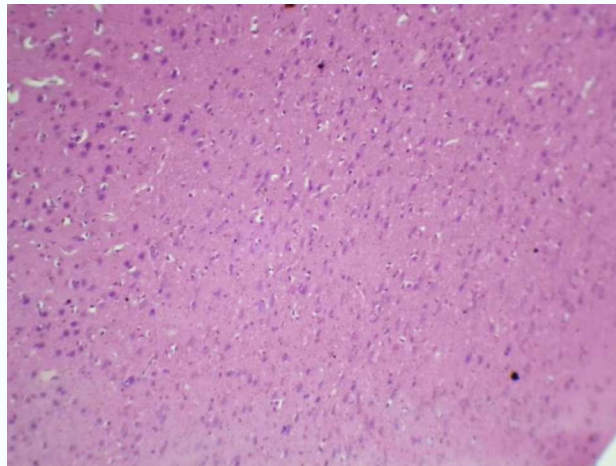
**Figure 4:** Histopathological section of the intestine (control group) shows massive sloughing of intestinal epithelial lining variable inflammatory cell infiltration (  $\longleftrightarrow$  ) (H&E10 $\times$ 10).



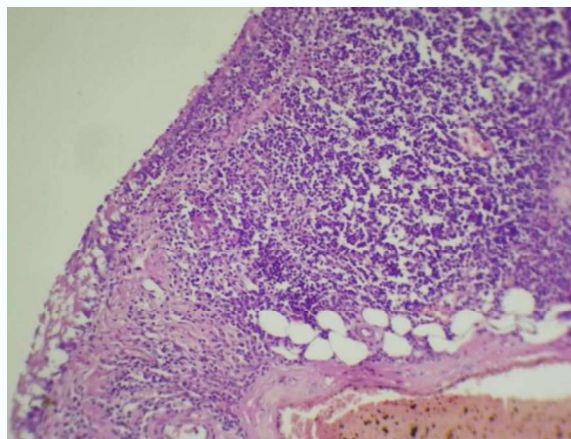
**Figure5:** Histopathological section of the spleen of rats (control group) shows white pulp depletion (  $\rightarrow$  ) with congestion of red pulp (  $\longleftrightarrow$  ) (H&E10 $\times$ 10).



**Figure 6:** Histopathological section of the liver of rats (control group) shows vacuolar degeneration of hepatocytes with focal inflammatory cells aggregation (H&E10 $\times$ 10).



**Figure (7):** Histopathological section of the lung of rats (control group) shows severe necrosis of pulmonary parenchyma (↔) with congestion of blood vessels (→) (H&E10×).



**Figure (8):** Histopathological section of the brain of rats (control group) shows no histopathological lesions (H&E10×).

**Table 1:** Infectious dose estimation of *Citrobacter* isolate in mice.

Groups (n=6)	Bacterial Concentration	Dead	Live	Mortality Percentage % = D/D+L
1	$10^{10}$	6	0	100%
2	$10^9$	5	1	90.9%
3	$10^8$	3	3	62.5%
4	$10^7$	2	4	33.3%
5	$10^6$	0	6	0%
6	$10^5$	0	6	0%