

Extraction and Titration of Leukotoxins from *Fusobacterium necrophorum* Isolates Recovered from Bovine Liver Abscesses in Sulaimaniyah Region

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Summary

This study was conducted to extract and titrate the leukotoxin of *Fusobacterium necrophorum* isolates recovered from 57 abscesses found in 42 livers of slaughtered cattle in Sulaimaniyah region. The culture supernatants of these isolates were subjected to the tetrazolium dye reduction test which revealed that the leukotoxin titer values of 34 *F. necrophorum* subsp. *necrophorum* isolates ranged from 128 to 1024 (with a leukotoxin titer mean of 516±46), whereas the leukotoxin titer values of the 11 *F. necrophorum* subsp. *funduliforme* isolates ranged from 0 to 128 (with a leukotoxin titer mean of 73±12).

Key words: *Fusobacterium necrophorum*; leukotoxin extraction.

استخلاص ومعايرة لوكوتوكسينات جراثيم *Fusobacterium necrophorum* المعزولة من خراجات كبد الابقار في منطقة السليمانية

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الخلاصة

صممت هذه الدراسة لاستخلاص ومعايرة اللوكوتوكسينات لعزلات جراثيم *F. necrophorum* عزلت من 57 خراج وجدت في أكباد 42 بقرة ذبحت في محافظة السليمانية. اخضعت الرواشح الزرعية لهذه العزلات الى فحص اختزال صبغة التترازوليوم والذي اظهر أن قيم معيار اللوكوتوكسين التي كانت تتراوح بين 128 الى 1024 (بمعدل معيار لوكوتوكسين 516±46) لـ 34 عزلة من جراثيم *F. necrophorum* subsp. *necrophorum* بينما كانت تتراوح بين 0 الى 128 (بمعدل معيار لوكوتوكسين 73±12) لـ 11 عزلة من جراثيم *F. necrophorum* subsp. *Funduliforme*.

Introduction

Fusobacterium necrophorum, a gram-negative, anaerobic bacterium and a normal inhabitant of the rumen, is the primary etiologic agent of bovine liver abscesses (1, 2). It is classified into two subspecies, *F. necrophorum* subsp. *necrophorum* and *F. necrophorum* subsp. *funduliforme* (3, 4). The pathogenicity of *F. necrophorum* is attributed mainly to its leukotoxin which seems to be an important virulence factor in the pathogenesis of hepatic and interdigital necrobacillosis and it is indicated to be cytotoxic to leukocytes, macrophages, ruminal epithelial cells, and possibly hepatocytes (5, 6). Although the pathogenicity and virulence factors of *F. necrophorum* have been studied widely for many years, attempts to develop an effective vaccine against liver abscess have not been successful commercially (7). However, previous studies have indicated that antileukotoxin immunity reduced the incidence of hepatic abscesses and interdigital necrobacillosis (5).

The aim of the present study was to extract and titrate the leukotoxins of *F. necrophorum* isolated locally from liver abscesses of slaughtered cattle in Sulaimaniyah region.

Materials and Methods

1. Bacterial isolates

F. necrophorum isolates were recovered from 57 abscesses found in livers of 42 slaughtered cattle in the abattoir of Sulaimaniyah governorate. The isolates were recovered by obtaining swab samples from the inner walls of the liver abscesses which were opened under sterile conditions with a sterile scalpel. The swabs were streaked on brain heart infusion supplemented with 5% horse blood. These media were then incubated in an anaerobic jar with an AnaeroGen gas pack at 39°C (8) for 48-72 hours. Growing colonies that revealed gram negative rod characteristics were re-cultured on brain heart infusion. These media were then incubated aerobically and anaerobically for 48 hours in order to check out the exact growing conditions they need (facultative anaerobe growing condition or strictly anaerobe growing condition). The facultative anaerobic bacteria were excluded.

Strictly anaerobic bacterial colonies were picked up and inoculated into sterilized thioglycollate and brain heart infusion broths. The thioglycollate broth cultures were sealed with rubber stoppers and incubated overnight at 39 °C whereas the brain heart infusion broth cultures were incubated anaerobically using the pyrogallol-carbonate seal according to (9, 10). The overnight cultures of both broths were tested for morphology, staining characteristics (using Gram's stain) and sedimentation of the cultures in the broth.

Following that, the colonies that revealed Gram's negative, rod-shaped bacterial characteristics were re-streaked on brain heart infusion agar supplemented with 5% horse blood and incubated anaerobically in an anaerobic jar with an AnaeroGen gas pack for 48 hours. The recovered colonies were identified according to conventional bacteriological and biochemical procedures according to the instructions of the materials manufacturers and as described by (11-14).

2. Leukotoxin assay

Bacterial isolates identified to be *F. necrophorum* were investigated for their ability of leukotoxin production as follows:

a. Production of leukotoxin

The production of leukotoxin was performed according to (15) in which purified colonies of each isolate were inoculated into 10 ml of a brain heart infusion broth and then incubated for 7 hours at 39°C under an anaerobic condition using pyrogallol-carbonate seal.

b. Extraction of leukotoxin-containing culture supernatant

Extraction of leukotoxin-containing culture supernatant was performed according to (8) in which the culture supernatants of a 7 hours old growth of *F. necrophorum* isolates were obtained by centrifugation at 15000g for 30 minutes at 4°C in a cooled centrifuge then the supernatants were filtered through 0.22µm membrane filter. These samples were stored at -28°C until used for the leukotoxicity assay.

c. Preparation and Determination of the concentration and viability of leukotoxin-target cells

Bovine polymorphonuclear neutrophils (PMN leukocytes) were used as target cells for leukotoxin of *F. necrophorum* organisms. The target cells were prepared according to (16) while the concentration and viability of the PMN leukocytes were determined according to (17) by the trypan blue dye exclusion method.

d. Tetrazolium dye reduction assay

The leukotoxicity assay was performed according to (8) using the tetrazolium dye reduction test. The formazan concentration representing PMN leukocyte viability was determined by measuring the absorbance values in an ELISA reader with dual wavelength (570 nm as test wavelength and 650 nm as reference). The leukotoxicity, expressed in percentage of cell death, and calculated as follows:

$$1 - (\text{absorbance of toxin treated cells} / \text{absorbance of control cells}) \times 100$$

The titer of leukotoxin was calculated as the reciprocal of the higher culture supernatant dilution causing $\geq 10\%$ loss in viability of leukocytes.

Results

1. Bacterial isolates

A total of 110 bacterial isolates were recovered out of the 57 liver abscesses sampled in the current study. Out of these 110, only 45 isolates showed growing and staining characteristics compatible with *F. necrophorum*. Thus, the frequency rate of *F. necrophorum* induced liver abscesses in the current study is 78.9%.

Investigation of the culture and staining characteristics, along with the physiological, biochemical and biological tests (Table 1) of these 45 isolates revealed identification of 2 groups of *F. necrophorum* isolates as follows:

Group 1: This group involved 34 bacterial isolates that showed characteristics compatible with those of *F. necrophorum* subsp. *necrophorum*.

Group 2: This group involved 11 bacterial isolates that revealed characteristics compatible with those of *F. necrophorum* subsp. *funduliforme*.

2. Leukotoxicity titer

The leukotoxin titer values of the 34 *F. necrophorum* subsp. *necrophorum* isolates ranged from 128 to 1024 with a leukotoxin titer mean of 516 ± 46 . *Fusobacterium necrophorum* subsp. *necrophorum* isolates number 15, 16, 21, 27, 33 and 34 showed the highest leukotoxin titer values (1024). On the other hand, the leukotoxin titer values of the 11 *F. necrophorum* subsp. *funduliforme* isolates ranged from 0 to 128 with a leukotoxin titer mean of 73 ± 12 (Table 2).

Table 1: Identification of *Fusobacterium necrophorum* subspecies.

Identification tests	subsp. <i>necrophorum</i>	subsp. <i>funduliforme</i>
Gram's stain	Negative	Negative
Motility test	Non motile	Non motile
Hemolysis test	Wide beta hemolysis	Narrow beta hemolysis
Indole test	+	+
Catalase test	-	-
H ₂ S production	+	+
MR/ VP test	- / -	- / -
Nitrate reduction test	-	-
Gelatin liquefaction	V	V
Fermentation of:		
Glucose	W or -	-
Maltose	-	-
Fructose	-	-
Sucrose	-	-
Lactose	-	-
DNase test	+	-
Phosphatase test	+	-
Lipase test	+	-
Agglutination of chicken erythrocytes	+	-

V: variable reaction W: weak reaction

Table 2: Means of leukotoxin titers in culture supernatants of *F. necrophorum* subspecies *necrophorum* and *F. necrophorum* subspecies *funduliforme* isolates.

Subspecies ^a	Range of leukotoxin titer *	Median of leukotoxin titer	Mean of leukotoxin titer ± SE
<i>F. necrophorum</i> subsp. <i>necrophorum</i>	128-1024	512	516±46
<i>F. necrophorum</i> subsp. <i>funduliforme</i>	0-128	64	73±12

* The range values mentioned in this table refer to the range of leukotoxin titer of 34 isolates of *F. necrophorum* subsp. *necrophorum* and 11 isolates of *F. necrophorum* subspecies *funduliforme* investigated for their ability of leukotoxin production in the current study.

Discussion

Forty five *F. necrophorum* induced liver abscesses out of the 57 cultured liver abscesses (with a frequency rate of 78.9%) have been reported in the current study. Such a higher frequency rate is often attributed to the mechanism by which liver abscess develops in view of the fact that the numbers of *F. necrophorum* bacteria in the rumen are often increased during the conditions of ruminal acidosis that follow sudden change to high-grain diets, because these bacteria use lactate rather than sugars as their major energy source for growth (6). This frequency rate (78.9%) is approximately compatible with that reported by (7) who stated that the incidence of *F. necrophorum* from cultured liver abscesses has ranged from 81 to 100% of abscesses. Both subspecies of *F. necrophorum* were recognized in the present study, however, the most frequently isolated one was *F. necrophorum* subsp. *necrophorum* (34 isolates recovered from 57 liver abscesses) compared to only 11 *F. necrophorum* subsp. *funduliforme*. This finding is not compatible with prevalence values of *F. necrophorum* induced liver abscesses in feedlot cattle reported by (18 and 19). In view of the variation in the type of diet on which the sampled cattle were nourished “cattle involved in the prevalence studies of the authors mentioned above (18 and 19) which are usually kept on high grain diet compared to those involved in the current study which are usually nourished on different types of diet”, this incompatibility in the prevalence values of subspecies *F. necrophorum* bacteria appeared to be realistic because the concentration of both subspecies of *F. necrophorum* bacteria in the rumen is markedly influenced by the type of diet (20).

In this study, bovine polymorphonuclear neutrophils (PMN leukocytes) were used as target cells for leukotoxin of *F. necrophorum* organisms because there is general agreement that the leukotoxin of *F. necrophorum* is a soluble, proteinaceous and heat-labile exotoxin with specificity for ruminant’s neutrophils (6, 8, 21). The leukotoxin titer of *F. necrophorum* subsp. *necrophorum* isolates obtained in the present study ranged from 128 – 1024 (with a leukotoxin titer mean of 516±46), whereas that of *F. necrophorum* subsp. *funduliforme* isolates ranged from 0 – 128 (with a leukotoxin titer mean of 73±12). The difference in leukotoxin production may account for the difference in virulence between the two subspecies (22-25) and it explains why the subspecies *necrophorum* is encountered more frequently in bovine hepatic abscesses than the subspecies *funduliforme* (1, 7, 24). This finding is in agreement with those mentioned by several authors (6, 8, 19, 25) who reported that *Fusobacterium necrophorum* subsp. *necrophorum* produces more leukotoxin than *F. necrophorum* subsp. *funduliforme*.

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