Isolation of Toxigenic Strains of *Clostridium perfringens* from the Soil of Farms in Iran

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Summary

In 158 soil samples collected from certain farms around Razi Institute, 88 strains of Clostridium perfringens *were isolated. Of these, 73 strains were Type A and 15 strains were toxigenic Type D, the causal agent of enterotoxemia of sheep and goats. Of those classified in* Clostridium perfringens *Type A, 63 strains were non-toxic and 10 strains were toxic.*

The technique of isolation, sugar fermentation, toxicity and lecithinase activitity of isolated strains are described in this paper.

Introduction

Clostridium perfringens is one of the organisms which is widely spread in the soil. *Cl. perfringens* is divided into five types: A to E, on the basis of production of major lethal toxins(Table 1).

The isolation and identification of *Cl. perfringens* from the soil and intestinal contents of animals and man has been reported by many workers (1, 2, 3)

The object of this study was, as a preliminary investigation, to isolate and identify the toxigenic strains of *Cl. perfringens* from the soil of farms in Iran.

Materials and methods

The soil samples were collected from certain farms around the Razi Institute. Sample portion, taken from about $\frac{1}{2}$ inch below the surface of the

soil, was transferred into a plastic tube.

Bacterial isolates: A small portion, about the size of a pea, of the dry soil was transferred into a fresh liver broth tube and incubated, anaerobically, at 37°C for 24 h. Smears were prepared on slides and stained by Gram's method. If organisms resembling *Cl. perfringens* were observed, fresh cultures were made by streaking the material onto sheep blood agar plates. The plates were incubated, anaerobically, in Gas-Pak jar at 37°C for 24 h. Two colonies resembling *Cl. perfringens* were picked up and transferred

Table 1. Distribution and characters of the major lethal toxins of different types of *Clostridium perfringens*

Type	Toxin			
	Alpha lethal necrotizing lecithinase	Beta lethal nectorizing	Epsilon lethal necrotizing	Iota lethal necrotizing
A	X	-	-	-
B	X	X	X	-
C	X	X	-	-
D	X	-	X	-
E	X	-	-	X

into fresh liver broth and incubated for 24 h.

Characterisation of isolated strains: The colonies of *Cl. perfringens*, after an overnight incubation, were low convex semiopaque with a entire margine. The colonies were surrounded by narrow zones of complete haemolysis of *theta* toxin. A wider zone of incomplete haemolysis, due to *alpha* toxin, engulfed the narrow zone of complete haemolysis.

1. Fermentation tests: Fermentation tests were carried out in a semisolid medium according to the formula described by Sterne and Batty(4). All isolated strains were tested in the medium containing glucose, maltose, lactose, manitol, sucrose and salicin. Biochemical tests have also been done with nitrate reduction, indole production, gelatin liquifaction, urease production and milk fermentation(5).

Fresh culture of eash isolated strain was inoculated into the above mentioned carbohydrates and biochemical reagents. All cultures were

incubated anaerobically using Gas-Pac jar at 37°C for 24 h. Bromthymol blue solution was used as an indicator for changes of carbohydrates (Table 2).

2. Typing of the isolated strains: The isolated strains of *Cl. perfringens* were typed according to the method described by Sterne and Batty(4). For determination of the major lethal toxins, namely, *alpha*, *beta* and *epsilon* of each isolate, the culture was inocualted into a freshly cooked meat broth in a 500-ml flask and incubated for 5 h. Each culture was centrifuged at 3000 rpm and the supernatant was used for typing of *Cl. perfringens* by the method of neutralisation tests as described by Sterne and Batty(4) and shown in Table 3.

3. Toxicity determination: The fresh culture of each isolated strain was inoculated into a flask of 500 ml medium composed of proteose peptone (Difco) 3%, Na_2HPO_4 1%, NaCl 0.25%, glucose 1%, trace elements and vitamins 0.7% and chopped meat 10% at pH 7.8(6). The six-hour old culture of each flask was centrifuged at 3000 rpm and the supernatant was used for toxicity test of the isolated strains.

The supernatant was divided into 2 portions, one portion was trypsinized by 1% trypsin powder (Difco 1/250) and the other was used untrypsinized. The dilutions were made from supernatant in borate buffer saline (BBS). For determination of MLD of *Cl. perfringens* Type A, dilutions from 1/10 to 1/100 and, for *Cl. perfringens* Type D, dilutions from 1/100 to 1/5000, were made from the supernatant in BBS. Each diluted toxin was injected intravenously into white mice(18-20 g body weight) using 2 mice per dilution. The mice were observed for 3 days and results were recorded(4).

4. Lecithovitellin test: The egg-yolk-plates were prepared for determination of lecithinase production of isolated strains according to the formula described by Sterne and Batty(4). The fresh culture of each isolated strain was streaked on the surface of egg-yolk agar and incubated, anaerobically, at 37°C for 24 h. The results of the test were recorded.

Table 2. Cultural characteristics of *Clostridium Perfringens* strains isolated from the soil

Glucose fermented	*Maltose fermented*	*Lactose fermented*	*Sucrose fermented*	*Salicine fermented*	*Manitol fermented*	*Milk digestion*	*Gelatin*	*Lecithinase*	*Indol*
+	+	+	+	-	-	+	+	+	-

Table 3.Typing of *Clostridium perfringens* by sero-neutralisation in mice

No. ml of filtrate	Antiserum 0.1 ml			ml of diluent	types			
	A	B	C		A	B	C	D
Untrypsinized								
1 0.5	-	-	-	0.3	D	D	D	D
2 0.5	X	-	-	0.2	L	D	D	D
3 0.5	X	X	-	0.1	L	L	L	D
4 0.5	X	X	X	0.0	L	L	L	L
Trypsinized								
5 0.5	-	-	-	0.3	D	D	D	D
6 0.5	X	-	-	0.2	L	D	L	D
7 0.5	X	-	X	0.1	L	L	L	L

L: Lived

D: Died

Results

Fermentation tests, according to the table given by Smith(6), showed that 88 isolated strains were *Cl. perfringens*. The results of typing proved that 73 of strains were Type A and 15 of Type D. Of all *Cl. perfringens* Type A, 63 strains were non-toxic and 10 were toxic for mice. They produced *alpha* toxin. Fifteen isolated strains were toxigenic *Cl. perfringens* Type D, which produced *alpha* and *epsilon* toxins.

All isolated strains produced haemolysis on the surface of blood agar. All isolated *Cl. perfringens* produced colonies on the surface of egg yolk plates. The yellow precipitate surrounding the colonies was indicative of lecithinase activitiy of *alpha* toxin produced by the organism. The titer of lethal toxin produced by Type D varied between 100 to 1500 mouse minimum lethal dose per ml. Type B, C and E could not be isolated.

Discussion

Clostridium perfringens is widely spread in soil(5). This organism is divided into five types, namely, Type A, B, C, D and E. *Cl. perfringens* Type A is more often found in the soil than the other types. *Cl. perfringens* Type A is the causative agent of gas gangrene in man and animals(4). In our investigation on 158 soil samples, collected from farms around the Razi Institute, 88 strains were *Cl. perfringens* Type A, and 15 strains were *Cl. perfringens* Type D, the causative agent of enterotoxemia of sheep and goats. All isolated *Cl. perfringens* Type A produced *alpha* toxin, but *Cl. perfringens* Type D produce *alpha* and *epsilon* toxins. Some of the isolated Type D produce up to 1500 minimum lethal dose toxin per ml.

The isolation of toxigenic *Cl. perfringens* Type D strains from the soil of certain field indicated that this organism may gain access to the digestive tract of animals grazing on such pastures and under optimum conditions produce enterotoxemia. *Cl. perfringens* Type B, C and E have not been isolated in our investigation.

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