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ISOLATION AND TYPING OF *CLOSTRIDIUM* OEDEMATIENS (CL. NOVYI) FROM CASES OF BLACK DISEASE OF SHEEP IN IRAN (*)

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Abstract – *Clostridium oedematiens* types A, B and D have been isolated from liver lesions of 44 cases of black disease of sheep in Iran. The technique of isolation and identification by using fluorescent labelled antibodies is described. The isolates were typed based on their lecithinase, haemolytic, necrotic and lethal activities.

Key words: *Clostridium novyi, Clostridium oedematiens, Clostridium haemolyticum, epidemiology, Iran.*

INTRODUCTION

Clostridium oedematiens is divided in three types, which are pathogenic for man and animals. The classical type A is associated with gas gangrene infections in man and animals, type B is the causal agent of black disease in sheep and occasionally in cattle and type D or *Cl. haemolyticum* is the causal agent of bacillary haemoglobinuria in cattle. According to the investigation published by Oakley *et al.* [1], *Cl. oedematiens* produces six antigenic components which have been designated as alpha, beta, gamma, delta, epsilon and zeta toxins. Alpha toxin is the principal lethal and necrotic component which is produced by type A and B. Beta toxin is necrotic, lethal, lecithinase and haemolytic, and is produced by types B and D. Gamma toxin is produced by type A and is necrotic, haemolytic and lecithinase. Other toxins which are produced by *Cl. oedematiens* are summarised in Table 1.

For type differentiation of *Cl. oedematiens,* alpha, beta and gamma toxins should be detected in culture filtrates of isolated strains, according to the method described by Sterne and Batty [2]. The present communication deals with typing and characterisation of *Cl. oedematiens* strains isolated from liver lesions of sheep suspected of black disease from different parts of Iran.

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MATERIALS AND METHODS

Isolation and culture

For isolation of the extremely fastidious *Cl. oedematiens* organism, materials were taken immediately after death from freshly cut areas of the liver lesions and streaked on a fresh solidified medium [3]. Cultures were then incubated anaerobically for 48 hr at 37°C. To isolate the causative agent in specimens, the materials were suspended in 3 tubes of 10 ml broth and heated in boiling water baths at 90°C for 10,20 and 30 min respectively to destroy the vegetative contaminates and then streaked on freshly solidified medium as above. The typical *Cl. oedematiens* colonies were picked up and cultured to a liver medium and kept as freeze-dried ampoules for further study. A rapid investigation of isolated strains was then made by using fluorescent anti-clostridial globulins*.

Table 1. Toxins produced by *Clostridium oedematiens* [1]

Toxin	Activity	Produced by type A	B	C	D*
Alpha	Necrotising, lethal	+ +	+ +	–	–
Beta	Necrotising, lethal, lecithinolytic, haemolytic	–	+	–	+ +
Gamma	Necrotising, haemolytic, lecithinolytic	+	–	–	–
Delta	Oxygen-labile haemolysin	+	–	–	–
Epsilon	Lipolytic; produces pearly layer	+	–	–	–
Zeta	Haemolytic	–	+	–	–
Eta	Tropomyosinase	–	+	–	+
Theta	Lipase	–	tr	–	+

* *C. haemolyticum.*
+ = produced; + + = produced in lethat amount; – = not produced.

Preparation of toxin

Each strain was inoculated in a flask of 250 ml containing freshly prepared Weinberg's V. F. medium for 24 hr. Each culture was centrifuged at 3000 rev/min for 30 min and the supernatant was divided in two parts, one part being kept in a small screw cup bottle for determination of minimum lethal dose (M.L.D.) and haemolytic tests, the second part being passed through Seitz filters which were used for demonstration of dermonecrotic action in guinea pigs and lecithinase activities.

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Red cell suspension

Blood extracted from horses, cattle, sheep and rabbits was collected in Alsever's solution, washed 3 times in 5 vol of 0.85% NaCl and used as 1% suspension.

Antisera

Clostridium oedematiens types A, B and D antisera used for this investigation were obtained from Burroughs Wellcome (England).

Lecithovitellin and skin tests

Mixtures were prepared containing 0.3 ml of the culture flltrate with 1% of Casamino acid as diluent and 0.1 ml of *Cl. oedematiens* types A, B and D antisera.

The mixture was kept for 30 min at room temperature and injected in 0.2 ml amount into the depilated skin of albino guinea-pigs. 0.5 ml of lecithovitellin was added to the remaining mixture in the tubes and incubated at 37°C for 2 hr. All results in both tests were read and recorded after 24 hr.

The methods of typing of the strains are summarised in Table 2.

Toxicity determination

The supernatant of a 24 hr culture of each strain in V. F. medium was used to determine the minimum lethal dose of the toxin produced by the isolate. The culture was centrifuged at 3000 rev/min for 30 min, then dilutions were made from the supernatant in borate buffer saline (B.B.S.). Each toxin dilution was immediately injected intravenously into White mice (18-20 g), using 3 mice per dilution. The injected mice were observed for 72 hr and the titre of each toxin was recorded.

Haemolytic activity tests

These tests were made in a row of 10 tubes. The toxins of each isolate and saline were mixed to give a final dilution of 1/50, 1/100, 1/200, 1/400 and 1/600 in a total volume of 1 ml. To each row of tubes, 1 ml of 1% suspension of the washed red blood cell from horse, cattle, sheep and rabbit was added. The tubes were kept at room temperature for a further 16 hr.

Table 2. Methods of typing of the strains distinguishing *Cl. novyi (oedematiens)* types A, B and D by the test of their dermonecrotic action in guinea-pigs and of their lecithinase activity [2]

No. of mixture	ml of filtrate	ml of antiserum anti-*cl. novyi*			ml of diluent	Results of lecithinase and skin reactions					
		Type A	Type B	Type D		L	Skin test	L	Skin test	L	Skin test
1	0.3	0	0	0	0.2	+	+	+	+	+	+
2	0.3	0.1	0	0	0.1	−	−	+	−	+	+
3	0.3	0	0.1	0	0.1	+	−	−	−	+	+
4	0.3	0	0	0.1	0.1	+	+	−	−	−	−
5	0.3	0	0.1	0.1	0	+	−	−	−	−	−
	Toxin identified					γ	α	β	α	β	β
	Type of *Clostridium novyi* identified						A		B		D

L lecithinase reaction.

RESULTS

Thirty-three strains of *Clostridium oedematiens* were isolated from sheep liver samples suspected of black disease and received from different parts of the country. These isolates were identified as *Cl. oedematiens* by using fluorescent antibody techniques. Among 33 strains, 27 were type B, 3 type A and 3 type D. Typing was based on the presence or absence of the alpha, beta and gamma toxins in the filtrate of cultures. The strains of type A produced alpha and gamma toxins. The alpha toxin produced yellowish dermonecrotic reactions in the skin of guinea-pigs, and was neutralised by antisera against *Cl. oedematiens* types A, B and B + D but not by *Cl. oedematiens* type D. The gamma toxin of *Cl. oedematiens* type A produced lecithinase which was neutralised only by *Cl. oedematiens* type A antiserum. The strains of type B produced alpha and beta toxins. The alpha toxin produced a necrotising in the skin of guinea-pigs and was neutralised by types A, B and B + D antisera, but not type D antiserum. The beta toxin of type B produced lecithinase which was neutralised by types B, D and B + D antisera.

The titre of the toxin produced by all *Cl. oedematiens* type B varied between 500 and 13,000 minimum lethal dose per ml for mice, *Cl. oedematiens* type A produced toxin with the titre of 100-12,000 M.L.D./ ml and the strains of *Cl. oedematiens* type D did not produce toxin.

The haemolytic activity of the isolates was studied by using red blood cells of rabbits, sheep, cattle and horses. The results indicated that *Cl. oedematiens* type B toxins were more active on red blood cells of rabbits, horses, cattle and sheep respectively.

DISCUSSION

It was previously shown that *Cl. oedematiens* type B was the causal agent of black disease in sheep in this country [4]. Among the 33 strains of *Cl.oedematiens* isolated from different parts of the country, 27 were of type B.

Some were highly toxigenic and have been used in the production of black disease vaccines. Three strains of *Cl. oedematiens* type A were isolated from liver lesions of sheep of black disease and one of them was toxigenic.

Cl. oedematiens type D (or *Cl. haemolyticum)* is known to produce bacillary haemoglobinuria in cattle, but it has rarely been reported as the causal agent of necrotic liver disease in sheep [5]. Three strains of *Cl. oedematiens* type D were isolated from liver necrosis of sheep in this country [6]. The isolated strains were neither pathogenic nor toxicogenic in laboratory animals, the supernatant cultures were strongly haemolysed red blood cells of rabbits, sheep, cattle and horses.

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