Arch. Inst. Razi: 40 37-45 (1989)

CHARACTERIZATION OF CLOSTRIDIUM OEDEMATIENS STRAINS ISOLATED FROM CASES OF BLACK DISEASE OF SHEEP IN IRAN. (*)

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Abstract

Black disease is an acute and fatal disease of sheep and goats in Iran. Fifty one strains of Clostridium oedematiens types A,B, and D have been isolated and typed from liver lesions received from different parts of the country. The technique of isolation and rapid identification by using fluorescent labelled antibodies, typing, sugars fermentation, toxicity and haemolytic activity of the isolated strains are described.

Introduction

Black disease is an acute and fatal disease of sheep and goats in Iran. Sporadic outbreaks of black disease have been observed in some areas where the animals are affected with fluke infestation. The mortality of cases have been found to be associated with immature forms of Fasiola gigantica and Dicrocaelium lanceolatum (1).

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* Presented to the Xx 111 World veterinary Congress, 1987, Montreal, Canada.

Geographical Distribution

Black disease is known to exist in sheep and goats husbandry in many parts of Iran (2). The disease has been diagnosed long time ago but the causative agent was isolated in 1969 from a liver lesion of a sheep belonging to herd of 500 sheep around Razi Institute. Black disease is more prevalence in Isfahan, the central part of Iran.

From 1961 up to 1986 more than 330 suspected livers were received for diagnosis of black disease from different parts of the country. Among them 187 were positive by using fluorescent labelled antibody technique (3).

The table No.1 Shows the in cidence of black disease in different parts of the country

TABLE NO. 1

Cases of black disease of sheep and goats in which Cl. oedematiens was isolated		
No.	**AREA**	**Number of isolated Strains**
1.	Hesssarak (around Razi Institute)	16
2.	Isfahan	20
3.	Hydar-abad (animal husbandry)	2
4.	Taleghan	2
5.	Sirjan	2
6.	Ghazvin	2
7.	Tchalus	3
8.	Brojerd	2
9.	Pashand	2
10.	Zanjan	2

Materials and Methods

Clostridium oedematiens type B is the causative agent of black disease of sheep. The organism is a strict anaerobe and difficult to isolate especially when specimens are not fresh. The materials were taken from freshly cut area of the liver lesions streaked on a freshly solidified medium (4). The smear was taken from liver lesion and the organism identified by Cl. oedematiens fluorescent antibody. Positive liver lesions were streaked on fresh solidified medium and incubated anaerobically for 48 hours at 37°C in the anaerobic Gas-Pak jar. The colonies resembling to Cl. oedematiens were picked up and transferred into fresh liver tube medium and incubated anaerobically. After 48 hrs culture smears were taken and stained by Cl. oedematiens fluorescent antibody. The positive cultures were then freeze dried in ampoules and kept for further studies. From 1964 up to 1985 fifty one strains of Cl. oedematiens were isolated from specimens of the liver lesions of sheep suspected to black disease from different parts of Iran.

Characterisation of the Isolated strains

1. Fermentation tests:

Fermentation tests have been done in a semisolid medium according to the formula described by Sterne and Batty (5). Fermentation tests were carried out in glucose, maltose, lactose, sucrose, salicin and manitol. Biochemical reactions have also been done with nitrate reduction, indole, gelatin liquifaction, urease production and milk fermentation according to the table given by Smith (6). Each strain was inoculated in the mentioned carbohydrates and biochemical reactions. All cultures were incubated anaerobically in Gas-Pak jar at 37°C for 48 hrs. Brom-thymol blue solution was used as an indicator for changing of the pH of the

carbohydrates.

2. Toxicity determination:

Each strain was grown from a tube of liver medium to the flask of 500 ml of medium composed of 5% chopped meat particles 3% proteose peptone and 1% maltose in meat infusion broth at pH 8.0 (7).

The cultures were incubated at 37°C for 72 hrs. A sample was taken from each flask and centrifuged at 3000 r.p.m. for ten minutes.

The supernatant was diluted from 1/500 up to 1/20,000 for determination of minimum lethal dose in white mice each weighing 18 to 20 grams. Each dilution was injected intravenously and the mice were observed for 3 days and the results were recorded.

3. Haemolytic activity test:

Blood of horse, cattle, sheep and rabbit were collected in Alsever's solution and centrifuged two times to obtain washed red cells. Then ten per cent was diluted in saline for the tests. Toxic filtrate was obtained in 48 hours culture in the mentioned medium for M.L.D. titration. Two fold dilution of 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256 and 1/512 of toxin was prepared with saline in a total volume of 1 ml, then 0.5 ml. of one per cent of red blood cell of each of animals mentioned above was mixed to the diluted toxin and incubated at 37°C for two hours. The results of each dilution was recorded.

4. Lecithovitellin and skin tests:

For typing of the isolated strains, the mixtures were prepared containing 0.3 ml of the culture filtrate with one per cent casamino acid as diluent and 0.1 ml. of Cl. oedematiens types A, B, and D antisera were added to the mixtures. The mixtures were kept for 30 minutes at room temperature then 0.2 ml. were injected into the depilated skin of albino guinea pigs, (Sterne and Batty, 1975) (5).

Results and Discussion

Fifty one strains of Cl. oedematiens were isolated from liver lesions of sheep, cattle and goats suspected to black disease received from different parts of the country. Cl. oedematiens, the causative agent of black disease, was isolated and identified by using fluorescent antibody technique.

The results of the cultural characteristic and typing of Cl. oedematiens isolated strains are shown in the table No. 1.

The results of haemolysis of the rabbit, horse, cattle and sheep red cells by isolated strains of Cl. oedematiens toxin are shown in the table No. 2.

The cultural characteristic of isolated strains of Cl. oedematiens were varied in fermentation of carbohydrates. All strains fermented glucose and maltose, some strains fermented sucrose (6), but none of them fermented lactose, salicin and manitol. All strains liquefied gelatin and most of them digested milk, but none of them were able to produce indol and urease. Few strains reduced nitrate to nitrite. Among 51 isolated strains 44 were type B, 4 type A and 3 type D.

The results of titre of Cl. oedematiens type B toxin was between 500 to 13000 M.L.D. per ml in mice. The titre of toxin produced by type A was 100–12000 M.L.D. per ml and one was not toxic. The strains of Cl. oedematiens type D did not produce toxin. All isolated strains were tested for haemolytic activity by red blood cells of rabbit, sheep, cattle and horse. The results proved that Cl. oedematiens toxins were more active on red blood cells of rabbits, horses, cattle and sheep respectively. Black disease is one of the fatal diseases of sheep and goats in Iran. It was previously diagnosed that Cl. oedematiens type B was causal agent of black disease of sheep in this country (8,9).

The strains were isolated and typed from liver lesions of the specimens received from different parts of country and sheep husbandry

around Razi Institute. Among isolated strains 44 were type B and 4 type A 3 type D. The majority of isolated strains were toxigenic in mice and haemolytic on red blood cells of animals. Some toxins were more active on red blood cells of rabbit up to 1/512 of diluted toxin but some were less toxic on the same red blood cells. Some of the strains of type B were highly toxigenic in mice, but some produced less toxin. Two strains of Cl. oedematiens type B isolated from liver lesions of cattle suspected to black disease, one of them was highly toxigenic in mice. There is not any reports in the literature, but three strains of Cl. oedematiens type B were isolated from liver lesions of goats suspected to black disease. Two of the isolated strains were toxigenic.

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Table No. 2

Cultural characteristics of Clostridium oedematien isolated strains

Strain Nos:	Fermentation tests: Glucose	Maltose	Lactose	Sucrose	Salicin	Mannitol	Indol	Nitrate reaction	Gelatin liquefaction	Milk fermentation	M.L.D / ml. titration	Type identified	No. of Type
C. N.											100–12000	A	4
950–1000	++	++	–	+	–	–	–	+	++	++	500 to 1300	B	44
											non - toxic	D	3

++ = Produced by all strains

+ = Produced by some strains

Table No 3

Haemolysis of rabbit, horse, cattle and sheep red cells by Clostridium oedematiens toxin

Clostridium oedematiens toxins	Red Cell type	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024
Strains Nos: 950 – 1000	Rabbit	+++	+++	+++	+++	+++	+++	+++	+++	+	–
	Horse	+++	+++	+++	+++	+++	+++	++	++	–	–
	Cattle	+++	+++	+++	+++	+++	+++	++	+	–	–
	Sheep	+++	+++	+++	+++	+++	++	+	–	–	–

+++ = Complete haemolysis
++ = Partial haemolysis
– = No haemolysis