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ISOLATION OF CLOSTRIDIUM SEPTICUM FROM CASES OF MALIGNANT OEDEMA OF CATTLE IN IRAN

By

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INTRODUCTION

Although different anaerobic bacteria such as Cl. perfringens Type A, Cl. septicum, Cl. oedematiens type A and Cl. histolyticum were found to be the causative agents of malignant oedema of domestic animals (McDonald and Collins, 1947); in cattle, the infection is mostly due to Cl. septicum (Smith 1957, Vawter 1956).

Cl. septicum produces four different toxic substances, namely, Alpha, Beta, Gamma and Delta toxins.

Alpha toxin, the principal lethal toxin of the organism, has lethal, haemolytic and necrotizing activities (Bernheimer, 1944). The activity of this toxin could be demonstrated by intravenous injection into mice or by intracutaneous inoculation of guinea-pigs and rabbits.

Beta toxin is an enzyme (desoxyribonuclease), whose activity may be demonstrated by its ability of destroying the nuclear materials of rabbit leucocytes or by desoxyribonucleic acid (Warrack *et* al; 1951). Gamma toxin is also an enzyme (Hyaluronidase). The necrotizing activity of the Delta toxin could be demonstrated by intracutaneous inoculation of guinea-pigs, while its haemolytic activity is shown on sheep, horse, rabbit and guinea pigs' red blood cells (Nicolle *et* al., 1915).

Recently Cl. septicum was isolated in three cases of malignant oedema of cattle in this laboratory. The following report presents the results of the test performed to characterize the isolates.

MATERIALS AND METHODS

Isolation of organism. The organism was isolated from the specimens (bone) received from the Veterinary Department. The specimens were collected from suspected cases of malignant oedema of cattle in Zahedan, Teheran and Karadje. The samples were cultured aerobically and anaerobically in liver broth. After 24 hours the cultures were subcultured in Weinberg's (Viande-Foie), V.F. deep agar and on freshly prepared blood agar in the usual manner. The isolated colonies in solid medium were transferred into V.F. broth to obtain seed material for further investigations.

Haemolytic activity test. The isolate was inoculated into V.F. medium containing 10 per cent glucose, the culture was centrifuged at 3000 r.p.m. for 10 minutes and the supernatant was used for the test. In a row of ten tubes, saline and toxin were mixed to give final dilution of 1/2 1/5 1/10 1/20 1/50 1/100 1/200 1/500 and 1/1000 in a total volume of 1 ml. 0.5 ml. of a 5 per cent suspension of washed sheep red blood cells in saline was added to each tube. The contents of the tubes were well mixed by inversion of tubes over non absorbent paper. Simultaneously 1 ml. of the toxin was mixed with 0.1 ml. of the specific Burroughs Wellcome diagnostic Cl. septicum antiserum, it was allowed to stand at room temperature for 30 minutes and then mixed with 0.5 ml. of red blood cells suspension in the same manner. The degree of haemolysin was recorded one hour after mixing.

Neutralization test in the rabbit skin. The culture filtrate of the isolate was used for the test, 0.5 ml. of the toxin was mixed with 0.2 ml. of the nutrient broth and 0.1 ml. of the specific Cl. septicum antiserum. As a control, 0.5 ml. of the toxin was mixed with 0.3 ml. of nutrient broth. The mixtures were left at room temparture for 30 minutes and then 0.3 ml. of each mixture was inoculated intradermally into the depilated skin of a rabbit. Serum-toxin mixture was inoculated on the right side and diluted toxin on the left side of the rabbit. The reactions were observed and recorded 24 and 48 hours post inoculation.

Neutralization test in mice. A 24 hour culture of the isolate in V.F. broth containing 10 per cent glucose was filtered and then 0.5 ml. of the obtained toxin was mixed with 0.2 ml. of nutrient broth and 0.1 ml .of specific antiserum. Cl. septicum, Cl. sordelli, Cl. welchii type A and Cl. chauvoei specific diagnostic sera obtained from B.W. were used in this study.

The serum toxin mixtures were kept at room temperature for 30 minutes and then 0.3 ml. of the mixture was intravenously injected into a group of two mice per mixture. The inoculated mice were closely observed for 24 hours.

Sensitivity test with antibiotics and sulfonamides. The technique developed by Chabbert (1953) was followed. A 24 hour culture of the isolate was mixed with preheated agar at 45°C and then the mixture was delivered into plates each containing four different disks of antibiotics or sulfonamides. The plates were then incubated anaerobically in the Brewer anaerobic jar for 24 hours at 37°C. The results were recorded after 24 hours incubation.

Toxicity titration. A 24 hour culture of the isolate in V.F. medium with 5% glucose was used to determine lethal dose of the toxin produced by the isolate (Guillaumie 1941). The culture was centrifuged at 5000 r.p.m. for 10 minutes and then dilutions were made from the supernatant in borate buffer saline (BBS). Each toxin dilution was immediately injected intravenously into white mice, using two mice per dilution. The inoculated mice were observed for 48 hours and titer was calculated.

RESULTS

Three strains were isolated from samples collected in 3 cases of malignant oedema of cattles. The biochemical properties of these strains are summarized in table No. 1.

TABLE NO. 1

Biochemical Properties of the Isolates

Differential Characteristics		Strain No. 902	Strain No. 903	Strain No. 906
Gelatin liquefaction		+	+	+
Indole production		—	—	—
Nitrites produced from nitrates		+	+	+
Hydrogen sulphide production		—	—	—
Safranin		+	+	+
Neutral red		+	+	+
Milk medium		+	+	+
Urease production		—	—	—
Coagulated serum		—	—	—
Sugars	Glucose	+	+	+
	Levulose	+	+	+
	Galactose	+	+	+
	Saccharose	+	+	+
	Mannitol	—	—	—
	Salicin	+	+	+
	Lactose	+	+	+
Pathogenicity to Guinea-pigs.		+	+	+

The toxin produced by strains Nos. 902, 903, 906, under the condition of the test, show the maximum titre 250, 150, 200 M.L.D. per ml. respectively.

The haemolytic activities of the isolates are shown in table No. 2.

The results of neutralization tests of the toxins with specific Cl. septicum antiserum performed in the skin of the rabbit, showed that the toxins mixed with the Cl. septicum antiserum were completely neutralized and the mixtures did not produce any reaction when they were inoculated into the rabbit skin. When the toxins were inoculated in the skin of the same rabbit an area of whitish necrotic lesions with irregular shape haemolysis zone was observed.

TABLE NO. 2

The Haemolytic Activity

Strain No:	HAEMOLYSIS IN DILUTION:						
	1/2	1/5	1/10	1/20	1/30	1/50	1/100
902	+	+	+	+	+	—	—
903	+	+	+	+	—	—	—
906	+	+	+	+	—	—	—

The same results were obtained when the neutralization test was performed by mixing the toxins with Cl. septicum, Cl. sordelli, Cl. welchii type A, and Cl. chauvoei specific diagnostic sera, and then the mixtures tested for the unneutralized toxins in mice. Mice inoculated with mixtures of toxins and Cl. septicum antiserum survived, while the animals inoculated with other serum-toxin mixtures as well as those inoculated with similarly diluted toxin with nutrient broth, died within 24 hours post inoculation.

The sensitivity of the isolated strains were studied to thirteen antibiotics and five sulfonamides. The results summarized in the table No. 3 indicated that the strains were sensitive to five more commonly employed antibiotics, Tetracycline, Aureomycin, Chloromycetin, Furacine and Demethylchlor-tetracycline, and all five used sulfonamides. The organisms were found resistant or slightly sensitive to Neomycine, Dihydrostreptomycin, Nystatin, Oxacillin, Colistin, Vancomycin, and Penicillin.

An attempt was made to reproduce the disease by inoculating a healthy calf with strain No. 902. The animal was injected intramuscularly with 1 ml. of 24 hours culture of the strain in V.F. broth.

The inoculated calf showed a rise in body temperature together with lameness, disinclination to move and the cessation of appetite and rumination within 15 hours of post inoculation. The animal however died of toxemia 36 hours after the appearance of the first symptoms.

On post mortem examination the muscles of the inoculation site were dark red in colour and haemorrhagic but firm and dry on section. There was considerable quantities of exudate. The organism with the same properties of the inoculated strain was microscopically observed in the site of infection and it was isolated from the infected tissues.

TABLE NO. 3

Sensitivity tests to antibiotics and sulfonamides

No. of strains examined	Observation zones	ANTIBIOTICS	SULFONAMIDES
3	sensitive	Tetracyclin	Sulfathiazole
3	"	Chloromycetin	Triple sulfa
3	"	Furacine	Madribon
3	"	Demethylchlor Tetracycline	Sulfadiazine
2	"	Aureomycin	Gantrisin
3	slightly sensitive	Vancomycin	—
3	"	Penicillin	—
1	"	Aureomycin	—
3	resistant	Neomycin	—
3	"	Dihydrostrepto-mycin	—
3	"	Nystatin	—
3	"	Oxacillin	—
3	"	Colistin	—

SUMMARY

Three strains of Cl. septicum were isolated from three cases of malignant oedema of cattle in Iran. The isolates were identified as Cl. septicum based on their biochemical properties, pathogenicity for guinea pigs and cattle. The toxins produced by the isolates were also completely neutralized by specific Cl. septicum antiserum, while retained its toxicity when mixed with Cl. sordelli, Cl. welchii type A, and Cl. chauvoei specific diagnostic sera. Other properties of the toxin were also found similar to those of Cl. septicum.

RÉSUMÉ

Dans la maladie de l'oedeme malin chez les bovins en Iran, trois souches de cl. septicum ont été isolées.

Les caractéres culturaux, biochimiques, hémolytiques, toxicologiques et le pouvoir pathogéniques ont été étudiés. Toutes les souches isolées ont montré le caractére typique de Cl. septicum.

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REFERENCES

1-- Bernheimer, A.W. (1944). J. Exper. Med; 80: 309-20.
2— Chabbert, Y. (1953) Ann. Inst. Pasteur, 84; 545
3— Guillaumie, M. (1941). Ann. Inst. Pasteur, (1941). **67:** 112
4— McDonald, I.W., and Collins, F.V. (1947). Australian Vet. J. 23: 50
5— Smith, L.DS. (1957). Advances in Veterinary Science. P. 496.
6— Vawter, L.R. (1956). Diseases of Cattle. P. 570
7— Warrack, G.H., Bidwell, E.; and Oakley, C.L. (1951). J. Path. and Bact., 63: 293.