

Isolation and Identification of *Closteridium* Strains from Cattle Malignant Edema Cases

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Summary

Out of 38 cases of malignant edema of cattle 19 *Closteridium* strains, *Cl.septicum*, *Cl.chauvoei*, *Cl.novyi* and *Cl.perfringens* were isolated during 1990-1992. Among isolated strains, three strains were *Cl.septicum*, four strains *Cl.chauvoei*, one strain *Cl.novyi* and eleven strains *Cl.perfringens* type A. Identification of strains was carried out by using fluorescent-labeled antibody technique. The hemolytic activity, toxicity and pathogenicity of the isolated strains were studied in this investigation.

Key words: malignant edema, cattle, AFT, *Closteridium* spp.

Introduction

Malignant edema is one of the acute infections in cattle and sheep. Fever, depression, edematous swelling around wounds and a short fatal course characterize the disease. It is caused by anaerobic bacteria residing in intestines, soil and manure. It may cause economic losses result from high mortality, expensive treatments, and costs of preventive programmes. Malignant edema occurs in all breeds, sexes and ages of cattle and sheep following wound contamination (Carlton 1995). The bacteria causing malignant edema include several species of anaerobes *Cl.septicum*, *Cl.chauvoei* commonly, *Cl.perfringens*, *Cl.novyi* and *Cl.sordelli* occasionally (Rue Jensen 1979). The vegetative forms are highly susceptible to prolonged drying, high temperature and chemical disinfections but spores withstand temperature of 120C for 10 min and survive in soil for many years. At the time of or following injury, spores of *Cl.septicum*, *Cl.chauvoei* as well as other anaerobes of clostridia-seeded soil, manure and skin are carried into the wounds. The necrotic alpha toxin of them that are produced during multiplication of organisms in wound infection has important role in pathogenesis of disease (Smith 1984). Strains of *Cl.novyi* type A have been implicated as cause of wound infection in animals. It is lethal and necrotizing which has important role in pathogenesis of malignant edema in cattle. Strains of

Cl.perfringens type A produce alpha toxin, which has important role in myonecrosis (Rood *et al* 1997).

For diagnosis of malignant edema pathological specimens have been submitted from different parts of Iran. The causal agents of malignant edema were studied in this investigation.

Materials and Methods

Isolation of the causative organism. Pathological specimens including muscle, bone and blood were received from different part of Iran. The muscle lesions, bone marrow and blood samples were inoculated into tubes containing fresh liver broth. The tubes were incubated anaerobically for 24-48h at 37C in Gas-Pak jars. If growth appeared in the tubes, the smears were taken to check the organisms if resembling Clostridia. For isolation of the organism fresh blood agar plates were streaked and incubated anaerobically for 24-48h at 37C in Gas-Pak jars. The colonies resembling Clostridia (hemolytic zone around the colonies) were picked up, transferred into tubes of fresh liver broth and incubated anaerobically for 24h. The cultures obtained after 24h were controlled in slant agar and nutrient broth for anaerobic organisms. The smears were also taken for Gram staining and also stained by *Cl.septicum*, *Cl.chauvoei* and *Cl.oedematiens* fluorescent antibody (Batty 1967).

Fermentation tests. The organisms were further confirmed and identified by fermentation tests on glucose, maltose, lactose, sucrose, salicin and mannitol. Biochemical reactions have also been done with nitrate reduction, indole, gelatine liquifaction, urease production and milk fermentation according to the table given by Smith (1986). The positive and pure cultures were freeze dried in ampoules and kept for further studies.

Demonstration of toxin. The special medium used for production of *Cl.perfringens* toxin was as follows:

Proteose peptone (Difco).....	3%
Na ₂ HPO ₄	1%
NaCl.....	0.25%
Chopped meat.....	10%
Glucose.....	1%
Trace elements & vitamins.....	0.7%
pH=7.6	
Autoclaved at 110C for 30 min.	

Proteose peptone, sodium hypophosphate and sodium chloride were dissolved in distilled water and the pH was adjusted to 7.6. It was boiled again and distributed into tubes each containing 25ml of the medium. A piece of chopped meat was added to each tube. The tubes were then autoclaved at 110C for 30 min and kept in refrigerator. Glucose and trace elements and vitamins solution were added at the time of use of the medium.

The special medium used for production of *Cl.septicum* and *Cl.chauvoei* toxin was as follows:

Proteose peptone (Difco).....4%
Glucose0.5%
NaCl.....0.5%
Cysteine hydrochloride.....0.05%
pH=7.5
Autoclaved at 110C for 30 min.

The special medium used for production of *Cl.novyi* toxin was as follows:

Proteose peptone (Difco).....4%
Maltose.....1%
Na₂HPO₄.....0.5%
Chopped meat.....5%
Maceration.....100ml
pH=7.6
Autoclaved at 110C for 30 min.

The method of media preparation for *Cl.septicum*, *Cl.chauvoei* and *Cl.novyi* were the same as that of *Cl.perfringens*. Whenever required, the tubes of special media were boiled, cooled and inoculated with the fresh culture of *Clostridium* spp. They were incubated for 24h at 37C anaerobically in Gas-Pak jar whereas the tubes of *Cl.perfringens* were incubated for 5h. Agar slants and broth were used as control of the cultures. The cultures were centrifuged at 3000 rpm for 10-15 min and the supernatant (filtrate) were collected and used for different purposes such as:

a) *Typing of Cl.perfringens organism.* 10 ml of the obtained toxin was divided equally into two tubes. One tube was treated with 1% trypsin (Difco) and incubated for 1h at 37C. 0.5 ml from each tube was immediately injected intravenously into mice (18-20gr) using 2 mice per tube. The result was read for 3 days. The death was

occurred only in treated mice so the identified organism was *Cl.perfringens* type A. (Stern 1975).

b) *Pathogenicity test.* This test was done in case of *Cl.septicum*, *C.chauvoei* and *C.novyi*. The fresh cultures were made in fresh liver broth. Each culture was injected deep intramuscular to two guinea pigs weighing 300-400gm with 0.25ml and 0.5ml of the culture respectively. The guinea pigs were observed for 3 days and the results of the death of them were recorded.

c) *Haemolytic activity test.* The test was carried out according to Ramachandran (1969). The blood of horse, cow and sheep were collected separately in flasks. 0.85% physiological saline, filtrates of *Cl.perfringens*, *Cl.septicum*, *Cl.chauvoei* and *Cl.novyi* culture, 1% suspensions of the washed RBCs of horse, cow, sheep and rabbit and also Alsever's medium by following composition were prepared:

Dextrose.....	20.5gm
Sodium chloride.....	4.2gm
Sodium citrate.....	8gm
Citric acid	0.55gm
Distilled water.....	1lit

Autoclaved at 120C for 30 min.

Three sterile flasks (300ml) were taken and 100ml of Alsever's medium were added to each flask and 10ml to the Wellcome tube. Then 100ml of horse, cow and sheep's blood was added to each flask respectively, and 10ml of rabbit's blood to a tube. A portion of each flask and a tube was centrifuged and washed three times. Then 1% suspensions of the washed RBCs were made ready.

Toxins and saline were added in 9 sterile tubes of the four rows to get final dilutions of 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256 and 1/512 in total volume of 1ml. To each row of tubes, 0.5ml of 1% suspension of the washed RBCs from each animal was added respectively. The contents of all tubes were mixed well by inverting them over a piece of non-absorbent paper. They were incubated at 37C for 2h. The results of each dilution were recorded. The haemolysis produced by *Cl.chauvoei* strains in the tubes was observed at the end of 16h at 37C.

Results and Discussion

Out of thirty-eight specimens received for identification of the causal agents of malignant edema, nineteen specimens were positive. *Cl.septicum*, *Cl.chauvoei*,

Cl.oedematiens and *Cl.perfringens* were isolated from pathological specimens of cattle. Among isolated strains confirmed by fluorescent antibody, 3 strains were *Cl.septicum*, 1 strain *Cl.novyi*, 4 strains *Cl.chauvoei*. Fermentation tests showed that 11 isolated strains were *Cl.perfringens* and from the typing results all of them were proved to be type A.

All isolated strains were tested for hemolytic activity by red blood cells of rabbit, sheep, cattle and horse. The results are shown in table 1. All isolated strains of *Cl.perfringens* produced toxin and killed the injected mice. All strains of *Cl.septicum*, *Cl.chauvoei* and *Cl.novyi* were pathogenic for guinea pigs. The toxins of all isolated strains also hemolysed the rabbit, horse, cattle and sheep red blood cell.

Table 1. Hemolysis of rabbit, horse, cattle and sheep red blood cells by Clostridial toxins

<i>Clostridium</i> spp.	No.	Rabbit RBCs	Horse RBCs	Cattle RBCs	Sheep RBCs
<i>Cl.perfringens</i>	11	+++	+++	++	+
<i>Cl.septicum</i>	3	+++	+++	+++	++
<i>Cl.chauvoei</i>	4	+++	+++	+++	++
<i>Cl.novyi</i>	1	+++	++	++	+

(+++)=complete haemolysis

(++)=partial haemolysis

(+)=less partial haemolysis

The causative agent of malignant edema in cattle and sheep are widely spread in soil (Smith 1984). Isolation of toxigenic strains of the mentioned clostridia indicated that these organisms may gain access to the traumatic wound and caused malignant edema in cattle and sheep.

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