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Clostridium Difficile

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PREFACE

It is not surprising that **Clostridium difficile** like other nonpathogenic members of the group has received so little attention in the past. In this particular case the literature is extremely limited and the few reports there are about have not been confirmed by many workers. Thus the author has tried not only to check very carefully the characteristics of this organism which is mentioned in the literature, but also to expand to some extent our present knowledge about **Clostridium difficile**.

For the sake of brevity and continuity of the reports I have not attempted to give my findings under a multiplicity of headings, whether I agreed with previous workers or not. For example, under the little "Characteristics of strains received" I have not only compared my findings with the results of previous workers, but I have also included reports of more extended experiments, the application of new methods, as well as a few striking new points in relation to morphology, nutritional requirements, sensitivity to different antibiotics, its rate of survival and its toxicity.

REVIEW OF LITERATURE

It was a notable addition to the current knowledge of anaerobic bacteriology when Hall and O'Toole in 1935 while studying the normal intestinal flora of the nursling isolated and described a new obligatory anaerobic

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bacillus from the stools of new born infants. They named it **bacillus difficilies** because of the unusual difficulty encountered in its isolation and study, owing to its slow growth and few striking physiological properties. This species was isolated in pure culture from 13 specimens obtained from 4 infants under 10 days of age. In 3 of these infants the bacillus was associated with the Kopfschenbakterien; it appeared and apparently disappeared at about the same time and under the same conditions. When both were present, each complicated the isolation of the other. Their description of this organism is: actively motile, heavy-bodied rod with elongated subterminal or nearly terminal spores, producing small amounts of sulphurated hydrogen. Neither gelatin nor Leoffler's blood serum was liquefied in 15 days. No change or only a trace of gas was produced in milk on prolonged incubation; deep agar colonies appeared after the second day of incubation reaching a diameter of 1 mm in about 3 days without production of any gas. Blood agar slant colonies were irregular in form, flat and non-haemolytic. Both acid and gas were produced in dextrose, levulose, mannitol, salicin and xylose, but only traces of gas and no acid were produced in galactose, maltose, saccharose, lactose, raffinose, inulin and glycerol. The most remarkable property of this anaerobe was found to be its pathogenicity for guinea-pigs and rabbits. Subcutaneous inoculation of the crude cultures and culture filtrates of this organism into guinea-pigs showed from moderate to marked oedema with spasms that superficially resembled those of tetanus, except that extensor muscles were more markedly affected and the attacks were more transient than those of tetanus.

Careful search of the literature substantiates Hall and O'Toole's assertion that the species had not been previously described. The early literature recorded organisms morphologically similar, as B. polypiformis of Liborius (1), B. Radiatus of Luderitz (2) and Bacillus 1 of Rodella (3). The former two are to be differentiated from **bacillus difficilis** by their rough colony formation in deep gelation or agar. The feature of skatol production by Rodella's Bacillus 1 is not possessed by **B. difficilis**.

In 1936 cultures of one strain from each of the four babies were turned

over to M. L. Snyder (Colorado University) for further investigation. Snyder isolated additional 17 morphologically and culturally similar strains from 182 stools of infants between 2 weeks and one year of age and tested them. He gave the following description: large Grampositive rods with elongated subterminal to terminal non-bulging spores. In smears the rods were usually found singly, in pairs or in short segments. Coverslip preparations showed sluggish motility in 24-hour peptone broth cultures.

Culturally all strains produced non-haemolytic, greyish, rough colonies on blood agar slants.

Biochemically all strains failed to attack coagulated blood serum, liquified gelatin slowly (a significant difference from the results of Hall and O'Toole), formed only gas in milk, did not produce indole, and fermented glucose, levulose, mannose, xylose, salicin and mannite, but not glactose, lactose, succrose, raffinose, inulin, dextrin and glycerol.

Hall and O'Toole, from their rough studies on broth culture filtrates of this organism suggested that B. difficilis produced a soluble exotoxin, probably a neurotoxin. Later Snyder succeeded in immunising rabbits with increasing doses of 48-hour veal infusion broth cultures given subcutaneously, but owing to the toxicity of his strain he found considerable difficulty in building up a satisfactory titre. The antiserum prepared against one of the strains agglutinated to some extent all the strains tested. The four original strains from Hall and O'Toole were about equally agglutinated with this antiserum and the four pathogenic strains isolated by Snyder himself were agglutinated equally well or to a higher titre than the homologous strain. Furthermore, four non-pathogenic strains were also agglutinated at a 1 in 640 of serum dilution. Some strains were partially agglutinated at 1 in 40; the remaining four strains showed only slight agglutination at 1 in 20. He interpreted these results as showing an antigenic subdivision in this species. Snyder also proved filterability, thermolability, pathogenicity and antigenicity of the filtrate of B. difficilis and thus indicated that **B. difficilis** produced a true exotoxin. The toxin of **B. difficilis** was rather weak and culture-filtrates showed markedly variable toxicity; different filtrates might

be non-toxic or might have a minimum lethal dose of about 0.001 ml. Filtrates lost their toxicity on storage.

The list of susceptible animals was also extended by Snyder to include the cat, rat, dog and pigeon. In immunising rabbits considerable difficulty was encountered because it was easy to kill the animals. In most instances animals could be protected against toxin by the same amount of antiserum if it is given up to four hours after injection of toxin. In repeating his experiments through the series of tests he found one of the antisera protected against all the toxins produced by his pathogenic strains of **B. difficilis** which suggests strongly that all toxigenic strains of **B. difficili** produce the same toxin.

It was suggested by Hall and O'Tooèe that some of the unexplained convulsions in infants might be explained on the basis of sbsorption of the toxin of **B. difficilis** from the intestinal tract. Snyder carried out several sets of experiments to study on the absorption of **B. difficilis** toxin from the intestines of animals. Guinea-pigs and rats given toxin by mouth and dogs in which toxin was injected directly into the small intestine did not show any trace of illness, whereas the control animals given toxin subcutaneously died.

MATERIALS AND METHODS

Three freeze-dried strains of **Clostridium difficile** from the American Type Culture Collection were obtained through the International Centre for Information on and Distribution of Type Cultures. Their specification is given below. It was not possible to obtain **Clostridium difficile** strains from any European collection. These strains have been once examined by Professor Smith, Virginia Polytechnic Institute, who told me what was known about them.

Strain	Abbreviation	Date of Preparation	Received
1. 9689	A1	Unknown	15.11.1974
2. 17857	A2	"	15.11.1974
3. 17858	A3	"	15.11.1974

A further three strains were received with the following specifications from the Laboratory of Hygiene, Ottawa, Canada.

Strain	Abbreviation	Date of Preparation	Received
1. G-173	G1	30.11.1953	10.1.73
2. G-238	G2	13. 7.1954	10.1.73
3. G-311	G3	9.10.1959	10.1.73

A single sample which is called "ONT" in our work was also provided by the Agricultural College, University of Guelph, Ontario, Canada, in February 1974. This strain plus another single strain from Smith (specification 2003, abbreviation S) were primarily subcultured by Dr. T. H. Williets and passed to me for further study. Except for the latter two strains which were already subcultured, the rest of the cultures were rehydrated aseptically by the addition of 0.3 - 0.4 ml of nutrient broth to the ampoules and mixed well; the mixture was then transferred to a boiled and cooled cooked-meat medium and incubated at 37°C. A2 and G2 strains exhibited a prolonged lag period, so that 72 hours of incubation were required before a satisfactory growth was observed. To ensure their viability as well as their purity they were immediately plated on fresh and heated blood agar.

NUTRITIONAL REQUIREMENTS AND CHOICE MEDIUM SELECTION

Selection of an ideal medium for the growth of **Clostridium difficile** led me to try different sorts of media such as glucose broth, cooked meat broth

(Robertson), thioglycollate broth, reinforced Clostridial medium (Hirsch and Grinsted, 1954; Gibbs and Hirsch, 1956) and besides these the following mixture was tried: Yeasterole 0.3g; Peptone 1.0g; Lab. Lemco 1.0g; Mannite 1.0g; Agar (NZ) 2.0g; Neutral Red 1 ml of 10 per cent. solution; Na acetate 0.5g; Cysteine 0.0gg; Neomycin 0.02g; Distilled water 100 ml.

The growth of this organism was also checked on fresh, heated and concentrated blood agar plates. Finally the Wijewanta (19961) three-step process media were tried. This when modified and slightly adjusted, was found to be the most suitable medium for the growth of this fastidious organism. It consists of:

Step I.
Ox Heart Peptic Disest Liver Brotn
Step II
Sorbic Acid Polymxin B Sulphate Thioglycollate Medium
Step III
Yeast Extract Blood Agar Medium with Chloral Hydrate Sodium Azide

RATE OF SURVIVAL OF CLOSTRIDIUM DIFFICILE IN MECONIUM

In order to study the inhibitory effect of meconium on **Cl. difficile** and to check the suitability of this selected media the following experiment was carried out.

Strain A2 was chosen for this experiment. By the use of a standard drop method for the determination of average colony counts of different dilutions on blood agar plates under anaerobic conditions I found that each mililitre of a 18-hour-old culture of this special strain contained approximately 53 million live organisms. A series of ten-fold dilutions from the above culture was prepared (10-1 to 10-9). Two mililitres of each of these dilutions was added to and thoroughly mixed with 0.5 g. of non-sterile meconium and the same amount of each dilution was also added to the same weight of sterilised

meconium. Each of these mixtures was immediately plated on fresh blood agar medium and at the same time inoculated into our selected media (3-step process). After an incubation period of 48 hours under anaerobic conditions the readings were recorded.

A. Growth on direct platings:
   1. A good growth of **Clostridium difficile** was observed on all plates where sterile meconium was used.
   2. In the case of non-sterile meconium even after a prolonged incubation not even a single colony of **Clostridium difficile** was detectable, but instead an abundant growth of anaerobic cocci was observed.

B. Growth on Modified Media. Wijewants. 1961.
   1. A good growth of **Clostridium difficile** was obtained up to the dilution of 10-6 with non-sterile meconium.
   2. In the case of sterile meconium considerable growth was extended to the next dilution (10-7).

RESULTS:

About 106 **Cl. difficile** organisms were needed to give a fairly good growth when mixed with 0.5 g. of non-sterile meconium when passed 3-step culturing process. Only about 10-11 **Cl. diffcile** are required for the same purpose with 0.5 g. of sterile meconium. **Cl. difficile** colonies appear smaller in size when non-sterile meconium is used.

THE TOXIN OF CLOSTRIDIUM DIFFICILE

Several experiments were carried out to find the peak of toxin production of **Cl. difficile** in cooked meat medium. It showed that this varied in case of each strain but usually lying somewhere between the 4th and 6th day of incubation. In this work 5-day-old cultures were used throughout the experiments.

TOXOID PREPARATION

To calculate the right amount of formaldehyde for toxoiding the **Cl. difficile** toxin, the free amino groups of the filtrate were primarily determined by calculation of free amino groups in leucine equivalents and lysine correction value.

Procedure of the experiment:

A formolised toxin sample was tested for lethality at weekly intervals for 3 weeks. Toxoiding was continued for one week after the filtrate ceased to be lethal for mice.

Rabbit injections:

After a negative sterility test it was then inoculated to two rabbits. Both rabbits were bled 10 cc before inoculation. Every other day animals were injected subcutaneously with increasing doses of 0.1-0.2 and 0.3 cc. After three subcutaneous injections and an interval of six days the toxoid was injected intravenously to the animals in the same amount and order as subcutanous injections. After 3 weeks animals were bled again and a booster dose of 0.5 cc was given intramuscularly. Fourty days after the first subcutaneous injection the animals were again bled and the toxin-antitoxin test was performed. Two-fold dilutions of toxin $\frac{1}{5}$ - $\frac{1}{5/2/0}$ was used against animals serum.

None of the **Cl. difficile** strains received from abroad was accompanied by any date sheet, and the only information that was received in this connéction was from Professor Smith, but did not concern any of the above strains. Thus it was not possible to compare the results of this work with studies of **Cl. difficile** by others.

Comparative study of the known strains of **Cl. difficile** showed all the strains, except S snd ONT, to be morphologically and culturally identical,

but divided into sub-groups on the basis of the agglutination test. The best growth of this fastidious organism was obtained when modified enriched medium of Wijewanta (1961) was used. Rate of survival of this organism was high when mixed with sterile and non-sterile meconium. About 10 times as many organisms had to be added to unsterile meconium as to sterile meconium for the organism to be isolated. The low pH value of the enriched medium showed a marked inhibitory effect on this organism. The sensitivity of this organism to antibiotics follow the same pattern as of anaerobic cocci which makes it unfavourable for the easy separation of **Cl: difficile** from meconium.

Colonial appearances of the known **Cl. difficile** strains were studied on simple agar, fresh and heated blood agar, yeast extract blood agar with chloral hydrate sodium azide, solid blood agar, Loeffler tubes and lactose egg-yolk milk agar media. The haemolytic effect of these strains were studies on erythrocytes of different animals such as horse, guineapig, rabbit, sheep and man, (0-and B-groups). 23 carbohydrates were tried to study the fermentation characteristics of this organism. In addition to the above experiments the microscopic examinations including Electron Microscopy, Seanning Microscopy and sectioning revealed the following definition about the received **Cl. difficile** strains: Grampositive spore-forming rods, the size varying from (2 to 9) X (0.3-0.7) um depending on the age of the culture. A Gram variability as well as formation of considerably long chains may occur after prolonged incubation. In fresh cultures the rods appear to have straight walls which slightly curve at the ends and have a vertical axis. Colonies are punctiform on ordinary agar and solid blood agar. On the rest of the plates mentioned previously they show the following characteristics: 1-3 mm in size, greenish, slightly raised, entire or slightly irregular edged, smooth, butyrous, semitransparent tp opaque and non-haemolytic. They do not possess proteolytic and lipolytic activities and also no putrefactive odour is produced. In smears cells are arranged usually in pairs, but also singly and occasionally in short chains. Spores are produced rather easily,

they are subterminal or terminal, oval in shape, and do not distend the organism's body. Free spores are not infrequently seen. They are capsulated and flagellated (most probably holotrichous, shown by electron microscopy). Their motility varies from sluggish to active in different strains. They take up Gram staining unevenly. They have metachromatic granules. They reduce nitrates, do not liquify gelatin, but after 30 days of incubation the setting time for gelatin was one hour greater than the usual time required. Milk and litmus milk media remain unchaged. Most strains produce H2S after 48 hours. Indol is produced by most of the strains. None of the strains lactose, maltose and dulcite. They ferment levulose, xylose, mannose ferment lactose, maltose and dulcite. They ferment levulose, xylose, mannose, mannite, salicine, glactose, trehalose, sorbose, cellobiose, arabinose, aesculine and melezitose vary readily and to a lesser degree they ferment glucose, glycogen, soluble starch, sucrose, raffinose and inositol. Pathogni-city: A study of pathogenic properties revealed that the rate of toxin production in fluid media reasches its peak between the 4th and the 6th day of incubation. The filtrates vary greatly in toxi-city and some are non-toxic, others weakly toxic. Intraperitoneal injection of the crude cultures is lethal for mice and the selected strains in this experiment killed these animals within 6-26 hours. The organism can be easily isolated from whole heart and the peritoneum of the inoculated animals. Intraperitoneal injection of washed cells of the selected **Cl. difficile** strains caused death in mice within 4-12 hours. Filtrate of **Cl. difficile** is also lethal for mice and the selected strains produced death within 8-24 hours. Intraperitoneal injection of the filtrate heated at 50° and 60°C. for five minutes and exposure of the filtrate to light did not cause death in mice which shows that the toxin is thermolabile as well as sensitive to light. Subcutaneous injection of the filtrate of one selected strain produced death in guineapigs shortly after 2 days without any obvious signs of any convulsions. Intramuscular injection of 2 other strains affected the animals only temporarily, but did not cause death. A 6-day-old culture of **Cl. difficile** injected intramuscularly caused death after 24 hours with convulsions localised around the hink limbs. This also proves the

production of toxin after an adequate time of incubation. Only one strain was found to be pathogenic for the guinea-pig and the rest of the strains produced only erythema, fatigue and sensitivity to sound, light and contact, which was overcome in a couple of days. Serologically both flagellar and somatic antigens were tested which agglutinated in varying degrees the homologous strain (A3), which proves an antigenic subdivision in the species.

An effective antiserum was produced in rabbits by injecting the animals with **Cl. difficile** toxoid. Under experiment the degree of precipitation in titrating animals serum against toxins of different strains received from abroad is given in page 7.

All but two of the strains (S and ONT) resembled one another closely, and agreed very well with the descriptions of **Cl. difficile** in the literature particularly in the discriminating characters: failure to liquify gelatin, capsulation, production of indol, reduction of nitrates, and presence of metachromatic granules. The other two strains are very unlike the others and very unlike the descriptions of **Cl. difficile**. They spore easily, digest meat particles in fluid media, produced skatol and were haemolytic, they liquified gelatin, and altered litmus milk, were proteolytic and lipolytic, and fermented many sugars. In all these respects they far more closely resembled **Cl. sporogenes**, but further work will be needed to confirm this identification.

ISOLATION OF CLOSTRIDIUM DIFFICILE

Attempts were made to isolate **Clostridium difficile** from meconium samples collected from the Leeds Maternity Hospital as well as from the laboratory animals faeces from the Department of Bacteriology animal house.

A. Isolation of Clostridium difficile from meconium:

75 specimens were examined, of which 20 were nearly black, 13 were green and 42 were brown. None of the specimens were typical yellow milk

stool. All specimens were examined within half-an-hour after the passage. The pH value ranged from 6 to 6.8 with the mode at 6.5 It was not possible to collect the samples on sterile diapers.

The specimens were cultured in modified Wijewanta media as has been described previously. Bacteria isolated from three specimens showed morphological characteristics resembling that of **Clostridium difficile** and further study was carried out for their identification. These three strains are referred to here as D1, D2, and D3.

B. Isolation of Clostridium difficile from the faeces of Laboratory animals:

45 laboratory animal faeces samples were examined for the above purpose. Animals included albino rabbits, guinea-pigs, white rats and white mice. Organisms resembling **Clostridium difficile** were isolated from 2 samples of rats faeces, and are referred to here as E1 and E2. The remaining samples were negative. The procedure for isolation and identification of these 2 suspected organisms are as described earlier. In the following work the characteristics of the strains isolated from both meconium and animal faeces are mentioned together.

PROCESS OF RECORDING THE CHARACTERISTICS OF THE ISOLATED STRAINS

1. Direct Smear Preparations: Suspending samples in distilled water and examining the slides microscopically stained by Grams method and methylene blue failed to show any traces of organisms resembling **Clostridium difficile** in both new-born and animal faeces.

2. Smears prepared from two-day-old cultures of the suspected organisms in ox heart peptic digest liver broth stained by Grams method showed G × rods measuring (4-8) X (0.5-0.8) um, terminal or subterminal spores that did not distend the sporangism. Cells were arranged singly, in pairs and occasionally in short chains.

3. Cultural characteristics in fluid medium

None of the suspected organisms digested meat particles of the ox heart peptic digest liver broth medium. Minute amounts of gas was produced by E1 and E2 strains after 72 hours of incubation. Neither putrid odour nor à change of colour of the medium was produced by any of the strains. A good growth was obtained in all the cases after an incubation period of 48 hours.

SUMMARY

Seventy-five meconium samples were examined for the presence of **Cl. difficile**; 3 strains were isolated. Additionally 45 laboratory animal faeces specimens were tested for the same purpose, a further 2 cases were isolated. These five suspicious strains were identified as **Cl. difficile** according to the tests mentioned in the previous paragraphs. The organisms isolated here showed the same characteristics as five of the strains received and also as the organisms isolated from the inoculated animals with the crude cultures of **Cl. difficile**. These organisms were variable in size, roughly 2-9 X0.3-0. 8u, Gram positive rods, motile, capsulated, flagellated, most probably peritrichous, possessing non-bulging spores located terminally or subterminally, free spores were rarely detectable. Cell arrangements: singly or in pairs and occasionally in short chains. On longer incubation the organisms slightly shifted to become Gram variable and longer in size. Colonies on ordinary agar and solid blood agar appeared to be punctiform and rough. On the other hand the colony appearance on the rest of the solid media which are mentioned previously are as follows: 1-3 mm in diameter, greenish, smooth, non-haemolytic, entire some showing slight irregularities of their edges. Colonies slightly raised, butyrous and semi opaque to opaque. This organism does not liquify the serum of Loeffler medium and also does not cause any changes of this medium. The metachromatic granules are readily seen by Albert's staining. Neither proteolytic nor lipolytic activities are possessed by this organism. Sensitivity to antibiotics showed the same pattern as mentioned about the strains received.

H2S production was positive after 48 hours. All the strains reduce nitrates. Most of the strains produce Indole and none liquify gelatin and also none produce any changes in Litmus Milk medium. The agglutinating serum prepared in rabbits as mentioned before were tested against heated and formolised suspensions of the strains isolated; serum produced against strain A3 agglutinated to some extent all the strains tested. The D1 and E1 were agglutinated to a greater degree than the homologous strain. The strains D2 and D3 were flocculated equally as the homologous strain. Furthermore E2 strain was also agglutinated at the 1 in 640 serum dilution. An effective antiserum was produced in rabbits by injecting with **Cl. difficile** toxoid.

CONCLUSION

Careful examination of seven strains received from abroad labelled as **Clostridium difficile** as well as three strains of **Clostridium difficile** isolated from 75 meconium samples of new-born infants and two strains of the some organism isolated from 45 samples of laboratory animal faeces by the author, was carried out and provided the basis for the following revised description of this organism.

Clostridium difficile is a Gram-positive, strictly anaerobic sporeforming rod, taking up the Gram stain unevenly, and varying in size from (2 X 0.3) to (9 X 0.7) um. A slight Gram-variability and a noticeable variation in size can occur after prolonged incubation of its cultures. Under such circumstances formation of short chains and filament-like structures as well as club-shaped and curved bodies are not uncommon. In fresh cultures the rods have a vertical axis and the cell walls are straight but become round at both ends. Flagellation is peritrichous, and th re is a thin capsule. The spores are oval and subterminal, or, very rarely, terminal, and never distend vegetative organisms. Free spores are not uncommon in 9-day-old cultures.

This organism grows best in a modified Wijewanta's (1961) threestep media. It grows moderately in cooked-meat broth (Robertson) as well as in thioglycollate broth and reinforced clostridial medium (Gibbs and Hirsch. 1956) and grows poorly in peptone and glucose broths; it does not digest the meat particles in media containing them, or produce any change of colour or special odour. Some strains produce a little gas.

It is neither proteolytic nor lipolytic, and does not haemolyse horse, guinea-pig, rabbit, sheep or human erythrocytes.

Most of the strains produce indole. They all reduce nitrates, but do not liquify gelatin even after several weeks of incubation. Most of the strains produce H2S after 48 hours. The motility varies from sluggish to actively motile in different strains. All strains are granulose positive; none liquifies or blackens the Loeffler medium. None of the strains produces any changes in litmus milk.

The colonial appearance of this organism, as is shown in the charts, differs widely on different media. On the medium containing double strength agar it appears as rough, rhizoid, greyish, flat, opaque and dry colonies varying in diameter from one to two milimeters, whereas on the medium containing a lower agar concentration the colonies are larger (1-3 mm), greenish in colour, slightly convex, smooth and butyrous.

None of the strains ferments lactose, maltose, or dulcite. They all ferment levulose, xylose, mannose, mannitol, salicin, galactose, trehalose, sorbose, cellobiose, arabinose, easculin and melezitose very readily and to a lesser degree they ferment d-glucose, glycogen, soluble starch, sucrose, raffinose and inositol; acid is always produced and sometimes a little gas.

Clostridium difficile is very sensitive to chloromycetin, terramycin and erythromycin. It is sensitive to neomycin and resistant to ponicillin, sulphamethazole and polymixin B sulphate.

Clostridium difficile produces an exotoxin, which is lethal for mice and guinea-pigs. The rate of toxin production in the fluid media reaches its peak between the fourth and the sixth day of incubation. The filtrates vary greatly in toxicity, some are non-toxic, others weakly toxic. This toxin is thermolabile and sensitive to light.

Intrapefitoneal injection of the crude cultures, washed cells and filtrates of **Clostridium difficile** into mice kills the animal within 4 to 24 hours. In the case of the crude culture inoculations this organism can be isolated from the ground-up heart of the injected mice.

Subcutaneous injection of small amounts of the crude cultures of **Clostridium difficile** into guinea-pigs produces erythema of the skin which disappeared in 2 - 3 days. Intramuscular injections of the same culture in large doses into this animal will produce death after 24 hours. Apparent localised convulsions around the site of injection is a dominant feature before the death of the animal.

Both somatic and flagellar antigens are present with noticeable antigenic subdivision in the species. An effective antiserum can be produced by rabbits receiving Cl. **difficile** toxoid.

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