

THE BACTERIA OF THE CLOSTRIDIUM BOTULINUM C AND D TYPES

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E.M. ROBINSON

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INTRODUCTION.

During the last seven years a great deal of information has been obtained about certain bacteria which for the sake of convenience will be called the Clostridium botulinum C and D types. The question as to whether some of the bacteria to be described should be classified as new types or given the general designation of "C" or D type will be discussed later in the course of this thesis. Since the investigations of von Ermengem (1912) in Germany, commenced in 1895, a great deal of research has been carried out by workers in human medicine on the bacteria of the Clostridium botulinum A and B types. This has been very ably summarized by K.F. Meyer (1928) in his article on "Botulismus" in the latest edition of Kolle and Wassermann's "Handbuch der pathogenen Mikroorganismen". To the work of Meyer and his co-workers is due most of our recently acquired knowledge of botulism.

It was not until 1922, however, that it was realized that other types of the Cl. botulinum than the A and B existed. The first organism of the C type to be described was isolated by Bengtson (1922). It was obtained from larvae of *Lucilia caesar* taken from the carcass of a chicken which had died of "limber-neck", the name given by American writers to a disease in poultry in which the chief symptom is that of paralysis. The term "limber-neck" is somewhat loosely used and may embrace a number of conditions in which symptoms due to affection of the nervous system appear, but there is very little doubt that the majority of cases of "limber-neck" are in reality true botulism. The toxin of

this organism, a strict anaerobe, when given per os or by subcutaneous inoculation to small animals such as rabbits, guinea pigs or mice, produced symptoms indistinguishable from those seen in botulism due to toxins of the A or B types. It was found that the toxin was not neutralized by the antitoxins against the latter two types.

Bengtson gave a detailed description of the organism isolated by her, including the effect of the toxin on various types of laboratory animals, and in addition on pigeons, chickens and monkeys. The organism has been classified in Bergey's Determinative Bacteriology as *Clostridium luciliae*, the suitability of which name will be discussed later.

Seddon (1922) published a description of an organism isolated by him from a decomposed bone from an area in Australia, where the so-called "Tasmanian Midland Disease" occurred. This disease is known under other names in different parts of Australia, and is essentially a type of botulism due to eating toxic carcass material. Seddon (1925) was subsequently able to demonstrate the toxin of the same organism in material such as decomposed rabbit carcasses, and to show that such toxic material had a wide distribution. He found that the toxin of his organism, which closely resembled the *Clostr. botulinum* A and B in morphological and some cultural characteristics, was not neutralized by antitoxins against the A and B types. Not being aware at the time of the work of Bengtson, he described the organism as *Bacillus parobotulinus*, considering it to be a new species. This it may eventually prove to be but comparative tests seem to show that it varies but little from the *Cl. botulinum* C in its main features though, as will be seen later, toxin-antitoxin tests show that antitoxin against Seddon's organism may not neutralize *Cl. botulinum* C toxin.

Graham and Boughton (1923) gave a description of an organism isolated by them from fowls affected with the disease previously mentioned as "limber-neck". They mentioned that the type of "limber-neck" associated with the C type of botulinus organism was not so fatal as that associated with the A and B. types as described by Hart (1920) and others in the United States of America. They suggested that for the C type toxin to produce symptoms in poultry, some lowering of the resistance was necessary, such as might be caused by parasitic infection or certain bacterial diseases. The organism was isolated from the intestinal contents of affected fowls and in one case it was actually shown that extracts of the intestinal contents would produce symptoms of botulism in guinea pigs.

From the intestinal contents of a horse and of a steer that had died showing symptoms of botulism Graham and Boughton were able to isolate an organism corresponding morphologically, culturally and by comparative toxin-anti-toxin tests with their Cl. botulinum C.

The Cl. botulinum C of Bengtson and that of Graham and Boughton correspond in most of their characteristics and there is little doubt that they are of the same type and cannot be differentiated.

Theiler (1920) obtained impure toxic cultures of an organism of the botulinus type from carcass material which had produced "lamsiekte" in cattle in South Africa. This disease is a type of botulism causing heavy mortality in cattle in certain parts of South Africa, and is associated with the occurrence of osteophagia due to phosphorus deficiency in the vegetation. When eventually isolated in pure culture (1927) the organism was found to be related to the C type, definitely not the A or B type, of the Cl. botulinum<sup>m</sup>, but comparative toxin-antitoxin tests showed that it did not correspond to either the A, B or C types.

Provisionally the name *Cl. parobotulinum bovis* was given to it but, as suggested by Meyer and Gunnison (1929)<sub>4</sub> and Weinberg (1927), a better name for it would be *Cl. botulinum D*.

This *Cl. botulinum D* resembles the C type more than the A or B judging by its morphological and cultural characteristics and only on toxin-antitoxin tests can it be differentiated from the C type. A further strain of *Cl. botulinum* has been isolated from carcase material from the same farm as the *Cl. botulinum D* was obtained from, but appears not to correspond exactly to the description of that organism. The comparison will be given under the section of this paper dealing with comparative toxin-antitoxin tests.

Theiler and the writer (1927) described an organism isolated from a rat carcase which had caused botulism in mules at the Veterinary Research Laboratory at Onderstepoort Pretoria. The cases were very acute and death occurred in a few hours after symptoms were shown. The food was found to have been contaminated by decomposition products from a rat. Saline extracts from the rat's carcase produced symptoms of botulism in guinea pigs and eventually an organism which produced typical symptoms of botulism as seen in the mules, was isolated from the toxic material. Morphologically and culturally the organism was very similar to the *Cl. botulinum C* and *D*, but could be shown to be definitely not the *D* type as judged by toxin-antitoxin tests. Recently it has been shown by Graham (private communication) and by the writer, that *Cl. parobotulinum equi*, as the organism from the rat carcase was named, cannot be differentiated from *Cl. botulinum C* when compared by toxin-antitoxin tests. As will be seen from the other tests carried out by the writer, the *Cl. parobotulinum equi* may have to be considered as a variant of the C type.

No other pure culture strains of the *Cl. botulinum*

type have been available in this work, but the writer has been fortunate in obtaining a variety of strains in impure culture from various sources. The amount of labour involved has precluded the possibility of the writer with the time at his disposal, attempting to isolate these strains in pure culture. It is, therefore, possible that they may have more than one type of toxin producing anaerobe in them, but the toxin antitoxin tests do not suggest that this is the case though, as will be seen in the discussion of their classification by toxin-antitoxin tests, the results in some cases were not completely uniform.

These impure cultures include three strains from carcase material or intestinal contents of cattle from a farm where lambsiekte was constantly occurring. Three other strains were obtained from the carcasses of rats which had been allowed to decompose in closed fruit jars at this Laboratory. A reference is made by the writer (1929) to these latter three strains in an article to appear in the next report of the Director of Veterinary Services, now in the press. It will be seen from the toxin-antitoxin tests in another part of this article that they are Cl. botulinum D types. Several other strains were encountered. One (No. 628) was obtained from the caecal contents of a horse which died of botulism on a farm near Johannesburg. Two horses actually died, showing very acute symptoms of botulism and it is of interest to note that the owner had been carrying out an intensive campaign for the destruction of rats in his forage store. Another strain (No. 11) was obtained from the carcase of a kitten which had died in some hay which had been associated with an outbreak of botulism affecting several donkeys and a horse in a contractor's stables in Johannesburg. Strain 628 was a C type and 11 apparently a D type. These cases are described in detail in the article by the writer (1929) previously referred to. From some further cases described in the same article three

more strains were obtained.

During December 1928, eighteen water birds, mainly ducks, died in the Pretoria Zoo shortly after a very heavy rainfall. The birds were all in one particular pond, and the symptoms shown were those of "limber-neck". From the coecal contents of several of these birds, toxic cultures were obtained which produced typical symptoms of botulism in guinea pigs.

Early in 1929, carcass material, chiefly decomposed bones, was obtained from sheep which had died of a condition resembling lamsiekte as seen in cattle. These sheep had developed osteophagia as a result of phosphorus deficiency in the vegetation, and the bones were obviously lighter and more brittle than they normally should have been. From these bones, highly toxic cultures containing a toxin of the botulinus type were obtained. The strain will be referred to as Bekker as it was obtained by Mr. J.G. Bekker, Veterinary Research Officer, from Bredasdorp in the Cape Province. The last strain to be mentioned was from an outbreak of a disease in turkeys which caused a heavy mortality and the birds showed symptoms which very closely resembled those of botulism. The writer did not actually see the birds during life and was only able to obtain one dead one for investigation. From the coecum of this bird a highly toxic culture was obtained which produced typical symptoms of botulism in guinea pigs. Extracts of intestinal contents given to the guinea pigs per os did not produce any symptoms.

As emphasized by K.F. Meyer in his article on botulism in the latest edition of Kolle and Wassermann's "Handbuch der pathogenen Mikroorganismen" (1928), to have positive proof that botulinus organisms found in the intestinal contents of animals are associated with botulism in these animals, one must in addition demonstrate actual toxin in the material.



In the writer's cases, it is only claimed that the presence of spores of a botulinus type were present in the intestinal contents of animals or birds which had died with symptoms of botulism.

It is, however, only fair to state that the evidence of the association of organisms of the botulinus C type so far described, with botulism in different animal species has been circumstantial. One refers in particular to the work of Bergtson, Seddon, and Graham. The chain of evidence is rarely complete and in the case of cattle and horses, the demonstration of toxin is difficult when one takes into consideration the enormous bulk of the ingesta.

In comparing the bacteria of the Clostridium botulinum C and D types, it will be convenient to consider them under four headings:

- (1) Comparative morphological and cultural characteristics
- (2) Comparative toxicity for different animal species.
- (3) Comparison by cross agglutination tests.
- (4) Comparison by toxin antitoxin tests.

(1) Comparative morphological and cultural characteristics.

Comparison of the bacteria of the Cl botulinum C and D types by their morphological and cultural characteristics is of very little use in their classification. One is struck by the great similarity of the various types comprising these groups.

All these types appear to be motile under anaerobic conditions but not under aerobic. In all of them the morphology is very similar, the length being 6-8 $\mu$  and width .5 to .8 $\mu$ , though shorter forms are often present. Chain formation is common, but the filaments are rarely long. Most of the bacteria occur singly or in pairs, end to end. In colonies, the organisms are mainly filamentous, whereas in media where gas production occurs such as Hibley or chopped meat, the

filaments are less frequent.

In the writers experience the *Cl. paratubulinum* equi or C. as it should be called, has always appeared larger than the other types and a number of forms resembling the organs of *Cl. chauvoei* have been seen in cultures of this organism. In the case of the C type obtained from the United States of America, it always appeared somewhat smaller than the other types. It is probable that all the types after being kept in culture media for some years become somewhat smaller in appearance.

In all the types the organisms usually only stain positively by gram in young cultures. After about 48 hours incubation, many gram negative forms are present and they become progressively more frequent until in old cultures most of them are gram negative. Spores are not frequent in chopped meat cultures and are very rare in Hibler's medium. In smears made from colonies in deep glucose or liver agar shake cultures, or from cultures in fluid media with a diffusion shell containing particles of meat, the writer has been unable to demonstrate spores microscopically. The spores are usually terminal, less frequently subterminal. Meyer and Gunnison (1928) state that in Kettger's egg medium spores of *Cl. botulinum* D are frequent and are subterminal.

No blackening has been observed in chopped meat medium or Hibler's. There is no actual change to be observed in the meat or brain. In Hibler's medium there is marked gas production and the fluid becomes turbid, the bacteria later settling down on the surface of the meat. In chopped meat medium there is not so marked a production of gas and the fluid portion of the medium becomes only slightly turbid.

Fluid cultures of all the types the writer has dealt with have exhaled a characteristic slightly sour smell which is, however, not unpleasant.

In other fluid media such as glucose, glucose liver, liver and serum broth, the medium first becomes turbid but

the growth rapidly settles out in the course of a few days incubation leaving the fluid clear.

Sugar reactions. According to Meyer (1928) the Cl. botulinum A and B types ferment the following sugars, glucose, lactose, maltose, laevulose, dextrin, glycerol and salicin. The C type ferments galactose and inositol but not dextrin. B. parobotulinus does not ferment sugars.

The writer has carried out sugar tests using a liver broth medium which had been exhausted by coliform bacilli as a basis, with 1% of the sugar added, incubation being in an anaerobic jar. Cl. botulinum D fermented glucose, laevulose, lactose, maltose, glycerine, dulcitol and galactose. Cl. botulinus C (equi) fermented glucose, laevulose, lactose, maltose and glycerine.

In their article on Cl. botulinum D, Meyer and Gunnison (1928) mention that the proteolytic properties are low as they are in the C types but somewhat higher than in the latter.

Isolation. B. Dubowsky and K.F. Meyer (1922) in an article on the methods employed by them in the isolation of the Cl. botulinum A and B types in the United States give much valuable information as to technique and many valuable hints as to short cuts in the pure culture isolation of these particular types. For the isolation of the Cl. botulinum C and D types the deep glucose agar or glucose liver agar shake culture (Vignol<sup>a</sup>) is the most satisfactory method which has so far been devised. The C and D types produce fluffy colonies like pieces of cotton wool teased out. In glucose agar they do not show a dense central portion but in glucose liver agar they do. The latter medium is usually fragmented whereas in glucose agar one may see the colonies simply packed in the tube without any fragmentation.

On the surface of solid media the writer has found great difficulty in getting the various types to colonize

out. In a few cases where colonies were obtained they were of the rhizoid type described by Meyer and Gunnison (1928).

In all the types the writer has collected, non-toxic colonies have been encountered, and often they are frequent. Large quantities of these non-toxic cultures have been inoculated into guinea pigs without producing any symptoms. The writer has tried inoculating guinea pigs with several consecutive doses of non-toxic cultures followed after an interval by a dose of homologous toxin in amounts of about 2 to 5 M.L.D's but there was no evidence of any immunity having developed. One may, therefore, consider that these types have completely lost their toxicogenic power. This formation of non-toxic types probably goes on in nature and may account for the falling off in virulence often seen in the botulinus types subcultured continuously.

The writer has made numerous attempts to reactivate these non-toxic types by growing them with a large assortment of bacterial types, and in a mixture of bacteria of decomposition, but without result. Guinea pigs when dosed with non-toxic botulinus cultures and killed a few hours later did not develop any toxin of the botulinus type in their carcasses when allowed to decompose.

In a short article on the isolation of the C types the writer (1927) described the technique employed by him. He found that commencing with actual decomposed carcass material it was fairly easy to reduce the number of bacterial types to about four or five by boiling the cultures from the carcass material for an hour. The spores of the botulinus C and D types resisted such boiling. Subsequently the isolation of the organisms in pure culture was a question of continuous subculturing in shake cultures and testing the colonies for toxicity. The plate method of Sturges and Kettger (1929) using non-sporulating bacteria such as cocci or coliforms was found of some assistance.