J. Hyg., Camb. (1979), **83**, 1
Printed in Great Britain

Immunofluorescent study of the spore antigens of proteolytic strains of *Clostridium botulinum*

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(*Received* 6 *January* 1978)

SUMMARY

By means of the spore fluorescent antibody technique, 31 strains of *Clostridium botulinum* types A (18 strains), B (10 strains) and F (3 strains) were found to belong to the same homogeneous group irrespective of their toxigenic types. Some strains of this species also cross-reacted with certain strains of *Clostridium sporogenes* types I, II and III and *Clostridium histolyticum* type II. By spore antigenic analysis it was found that *Clostridium parabotulinum* contained two components designated L and M, the former describing species specificity; the latter was the cross-reacting component shared by some strains of *Clostridium sporogenes* and *Clostridium histolyticum*. Following this, a scheme showing the distribution of spore antigenic components among various species of *Clostridium* was given.

INTRODUCTION

Strains of *Clostridium botulinum* are divided into six types (A–F) according to the antigenic specificity of the toxins they produce, whereas their biochemical activity separates them into proteolytic and non-proteolytic groups. Thus, proteolytic types A, B and F and non-proteolytic types B, C, D, E and F of *Cl. botulinum* exist. Sharing of somatic antigens among strains of proteolytic types A, B and F has been reported by Walker & Batty (1964), Solomon, Lynt, Kautter & Lilly (1971) and Lynt, Solomon & Kautter (1971). Partial cross-agglutination of *Cl. sporogenes* with the somatic antisera of the proteolytic group of *Cl. botulinum* has also been observed by Mandia (1955), Solomon *et al.* (1971), and Lynt *et al.* (1972). Except by means of the toxigenicity test it is impossible to distinguish *Cl. botulinum* from *Cl. sporogenes* on the basis of physiological or biochemical characteristics (Lynt *et al.* 1972).

Princewill (1979*a*) divided *Cl. sporogenes* into types by means of the spore antigens. The antisera obtained in that study have been used to determine the immunological relationship between *Cl. sporogenes* and the proteolytic strains of *Cl. botulinum* by the indirect fluorescent antibody test (FAT).

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0022-1724/79/8 0003–1978 $01.00

Table 1. *Strains and sources of* Cl. botulinum *cultures used*

(a, Originally from Dr D. Berkowitz, U.S. Army, Natick, Mass.; b, originally from National Canners Association (NCA), California, U.S.A.; c, originally from National Collection of Type Cultures (NCTC), Colindale, London; d, originally from Dr L. V. Holderman, Virginia Polytechnic Institute, Blacksburg, Virginia.)

Serial number	Toxigenic types	Type and number in original collection		Origin
168	A	CN	751	Wellcome Research Laboratories, Langley Court, Beckenham, Kent
169	A	CN	5008	
170	A	CN	640	
171	A	CN	1354	
172	B	CN	1356	
173	B	CN	5009	
174	A		117	Dr H. Meisel, Serum Research Institute, Warsaw
175	A		190	
176	A		365	
177	B		140	
178	B		366	
179	B		924	
180	A		33a	Dr T. A. Roberts, Meat Research Inst., Langford, Bristol
181	A		62b	
182	A		387c	
183	A		1192b	
184	A		2012c	
185	A		2916c	
186	A		3805c	
187	A		3806c	
188	A		4587c	
189	A		7272c	
190	A		9837c	
191	B		53a	
192	B		213b	
193	B		751c	
194	B		3807c	
195	B		7273c	
196	F		Fd	
197	F		T-15	National Collection of Industrial Bacteriology, Aberdeen
198	F		T-42	

MATERIALS AND METHODS

These were similar to those used in previous reports (Princewill, 1978, 1979*a*, *b*), with the addition of the following:

Cl. botulinum strains. Of the 31 strains (Table 1), 18 were of type A, 10 of type B and 3 of type F; all were proteolytic. Four of the type A were from the Wellcome Research Laboratories, Langley Court, Beckenham, Kent, England; 3 from the late Dr H. Meisel, Serum Research Institute, Warsaw, Poland; and 11 from Dr T. A. Roberts, The Meat Research Institute, Langford, Bristol, England. Two of the type B cultures were from Beckenham, 3 from Warsaw and 5 from Bristol. Of the 3 strains of type F, one was obtained from Bristol and the

Table 2. *Fluorescent antibody (FA) reactions of spores of proteolytic types A, B and F of* Cl. botulinum *with spore antisera* of* Cl. sporogenes

		Number of positive reactions† with *Cl. sporogenes* antisera against strains of:						
		Type I					Type II	Type III
Type	No. of strains tested	2	17	37	75	98	59	60
A	18	5	2	0	2	6	7	5
B	10	1	1	2	1	1	2	4
F	3	0	0	0	0	0	0	0

* Antisera used at 1/100 dilution.
† Fluorescent intensity 3+ or 4+.

other 2 from the National Collection of Industrial Bacteria (NCIB), Torry Research Station, Aberdeen, Scotland.

These strains have been given serial numbers which have been used in this investigation.

RESULTS

Screening with Cl. sporogenes *spore antisera*

To determine the extent of sharing of spore antigens by strains of *Cl. sporogenes* and *Cl. botulinum*, spores were prepared from the 31 strains of *Cl. botulinum* and screened with the spore antisera prepared against strains of *Cl. sporogenes* (Princewill, 1979*a*) by FAT.

The results summarized in Table 2 show that there was no regular pattern of reaction between the spore antisera of *Cl. sporogenes* and the spores of strains of *Cl. botulinum*. Even antisera of the same type of *Cl. sporogenes* did not always react with the same strains of *Cl. botulinum*. Moreover, the cross-reactions among the strains of *Cl. botulinum* did not agree with the toxigenic type; there was no reaction with the 3 strains of type F tested and some of the strains of types A and B.

Cross-fluorescence tests

In the light of the results of the preliminary screening tests, one strain each of *Cl. botulinum* type A (strain 188) and type B (strain 172) was selected for the production of spore antisera. A strain of type F could not be included, because none of the strains sporulated sufficiently to provide spores for immunization. The two spore antisera obtained were then used along with the *Cl. sporogenes* antisera in cross-fluorescence tests.

The results (Table 3) show that both antisera to *Cl. botulinum* types A and B fluoresced to full titre with all the strains of homologous and heterologous types. This confirms that the spore antigens of *Cl. botulinum* are not related to the toxins produced by this organism. The cross-fluorescence of strains of *Cl. sporogenes* was of a low titre and the pattern of reaction did not follow any regular order, some strains of a type reacting whereas others did not.

Table 3. *Fluorescent antibody (FA) titres of spore antisera of* Cl. sporogenes *and* Cl. botulinum

(Antisera 172 and 188 reacted to full titre with spores of the 3 strains of *Cl. parabotulinum* type F; they also reacted partially with spores of *Cl. histolyticum type* II; but they did not react with spores of *Cl. bifermentans*, *Cl. butyricum* and *Cl. histolyticum* type I).

	FA titres, against strains in column 1, of								
	Cl. sporogenes antisera against strains of							*Cl. botulinum* antisera against strains	
	Type I					Type II	Type III		
Strains	2	17	37	75	98	59	60	188A	172B
Cl. sporogenes									
Type I									
2	51200	25600	12800	25600	51200	—	—	—	—
17	25600	25600	25600	25600	12800	—	—	160	160
37	25600	25600	25600	12800	25600	—	—	1000	1000
75	51200	25600	12800	51200	25600	—	—	—	—
98	51200	25600	25600	25600	51200	—	—	1000	160
Type II									
9b	—	—	—	—	—	6400	—	400	400
27	—	—	—	—	—	6400	—	160	—
42	—	—	—	—	—	3200	—	—	160
59	—	—	—	—	—	6400	—	1000	1000
Type III									
60	—	—	—	—	—	—	6400	2000	2000
Cl. botulinum									
Type A									
169	160	—	—	—	160	320	—	10000	10000
171	—	—	—	—	—	320	—	10000	10000
174	320	320	—	320	320	—	—	10000	10000
175	320	320	—	320	320	—	—	10000	10000
176	—	—	—	—	—	—	160	10000	10000
181	—	—	—	—	—	—	160	10000	10000
182	—	—	—	—	—	—	320	20000	10000
183	160	—	—	—	160	320	—	10000	10000
184	—	—	—	—	—	320	—	10000	10000
187	—	—	—	—	—	160	160	10000	10000
188	160	—	—	—	160	320	—	**20000**	10000
189	—	—	—	—	160	160	—	10000	10000
190	—	—	—	—	—	—	160	10000	10000
Type B									
172	—	160	—	—	—	160	—	20000	**20000**
173	—	—	—	—	—	—	160	10000	10000
177	—	—	—	—	—	—	160	10000	10000
178	—	—	320	—	—	—	—	20000	20000
179	320	—	320	320	320	—	—	10000	10000
191	—	—	—	—	—	320	320	20000	20000
194	—	—	—	—	—	—	160	10000	10000

— = No fluorescence at 1/100 dilution of antiserum.

https://doi.org/10.1017/S0022172400025778 Published online by Cambridge University Press

Table 4. *Fluorescent antibody (FA) titres of spore antiserum to* Cl. sporogenes *strain* 2 *cross-absorbed with spores of* Cl. sporogenes *and* Cl. botulinum

	FA titres, of antiserum against spores of *Cl. sporogenes* 2, against:									
	Cl. sporogenes antigens					*Cl. butulinum antigens*				
Absorbing spore	Type I		Type II		Type III	Type A			Type B	
antigen	2	98	9b	59	60	174	175	188	172	179
None	51200	51200	40	40	40	320	320	160	—	320
Cl. sporogenes										
2	—	—	—	—	—	—	—	—	.	—
98	—	—	—	—	—	—	—	—	.	—
9b	1600	1600	—	—	—	—	—	—	.	—
59	3200	1600	—	—	—	—	—	—	.	—
60	800	1600	—	—	—	—	—	—	.	—
Cl. botulinum										
174	12800	12800	—	—	—	—	—	—	.	—
188	6400	6400	—	—	—	—	—	—	.	—
172	6400	12800	—	—	—	—	—	—	.	—
179	12800	12800	—	—	—	—	—	—	.	—

— = less than 10.
. = test not done.

Table 5. *Fluorescent antibody (FA) titres of spore antiserum to* Cl sporogenes *strain* 59 *cross-absorbed with spores of* Cl sporogenes *and* Cl. botulinum

	FA titres, of antiserum against spores of *Cl. sporogenes* 59, against:									
	Cl. sporogenes antigens					*Cl. botulinum* antigens				
	Type I		Type II		Type III	Type A			Type B	
Absorbing strain	2	98	9b	59	60	174	175	188	172	179
None	80	20	6400	6400	40	40	40	320	160	80
Cl. sporogenes										
2	—	—	800	1600	—	—	—	—	—	—
98	—	—	800	800	—	—	—	—	—	—
9b	—	—	—	—	—	—	—	—	—	—
59	—	—	—	—	—	—	—	—	—	—
60	—	—	1600	800		—	—	—	—	—
Cl. butulinum										
174	—	—	3200	3200	—	—	—	—	—	—
188	—	—	1600	3200	—	—	—	—	—	—
172	—	—	1600	1600	—	—	—	—	—	—
179	—	—	3200	1600	—	—	—	—	—	—

— = less than 10.

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Table 6. *Fluorescent antibody (FA) titres of spore antiserum to* Cl. sporogenes *strain* 60 *cross-absorbed with spores of* Cl. sporogenes *and* Cl. botulinum

	FA titres, of antiserum against spores of *Cl. sporogenes* 60, against:									
	Cl. sporogenes antigens					*Cl. botulinum* antigens				
	Type I		Type II		Type III	Type A			Type B	
Absorbing strain	2	98	9b	59	60	174	175	188	172	179
None	20	20	20	40	6400	80	40	160	40	80
Cl. sporogenes										
2	—	—	—	—	1600	—	—	—	—	—
98	—	—	—	—	1600	—	—	—	—	—
9b	—	—	—	—	800	—	—	—	—	—
59	—	—	—	—	800	—	—	—	—	—
60	—	—	—	—	—	—	—	—	—	—
Cl. botulinum										
174	—	—	—	—	3200	—	—	—	—	—
188	—	—	—	—	1600	—	—	—	—	—
172	—	—	—	—	1600	—	—	—	—	—
179	—	—	—	—	1600	—	—	—	—	—

— = less than 10.

Table 7. *Fluorescent antibody (FA) titres of spore antiserum to* Cl. botulinum *strain* 188 *(type A) absorbed with spores of* Cl. sporogenes *and* Cl. botulinum

	FA titres, of antiserum against spores of *Cl. botulinum* 188, against:									
	Cl. sporogenes antigens					*Cl. botulinum* antigens				
	Type I		Type II		Type III	Type A			Type B	
Absorbing strain	2	98	9b	59	60	174	175	188	172	179
None	40	1000	400	1000	2000	10000	10000	20000	20000	10000
Cl. sporogenes										
2	—	—	—	—	—	8000	8000	8000	8000	4000
98	—	—	—	—	—	4000	4000	8000	8000	4000
9b	—	—	—	—	—	8000	8000	4000	8000	4000
59	—	—	—	—	—	8000	8000	8000	4000	8000
60	—	—	—	—	—	8000	8000	8000	8000	4000
Cl. botulinum										
174	—	—	—	—	—	—	—	—	—	—
188	—	—	—	—	—	—	—	—	—	—
172	—	—	—	—	—	—	—	—	—	—
179	—	—	—	—	—	—	—	—	—	—

— = less than 10.

https://doi.org/10.1017/S0022172400025778 Published online by Cambridge University Press

Table 8. *Fluorescent antibody (FA) titres of spore antiserum to* Cl. butulinum *strain* 172 *(type B) absorbed with spores of* Cl. sporogenes *and* Cl. botulinum

	FA titres, of antiserum against spores of *Cl. botulinum* 172, against:									
	Cl. sporogenes antigens					*Cl. botulinum* antigens				
	Type I		Type II		Type III	Type A			Type B	
Absorbing strain	2	98	9b	59	60	174	175	188	172	179
None	20	160	400	1000	2000	10000	10000	10000	20000	10000
Cl. sporogenes										
2	—	—	—	—	—	8000	8000	4000	8000	4000
98	—	—	—	—	—	4000	8000	8000	8000	4000
9b	—	—	—	—	—	4000	8000	4000	4000	4000
59	—	—	—	—	—	4000	8000	8000	4000	8000
60	—	—	—	—	—	4000	8000	8000	8000	8000
Cl. botulinum										
174	—	—	—	—	—	—	—	—	—	—
188	—	—	—	—	—	—	—	—	—	—
172	—	—	—	—	—	—	—	—	—	—
179	—	—	—	—	—	—	—	—	—	—

— = less than 10.

Table 9. *Distribution of spore antigens in species of proteolytic clostridia*

(+, present; −, absent; ±, present in some strains; (+), possibly present.

	Spore antigenic components										
Organism	A	B	C	D	E	F	G	J	K	L	M
Cl. sporogenes											
I	+	−	−	+	−	−	+	−	−	−	±
II	−	+	−	+	−	−	−	−	−	−	±
III	−	−	+	+	−	−	−	−	−	−	±
Cl. histolyticum											
I	−	−	−	−	+	+	−	−	−	−	−
II	−	−	−	−	−	+	+	−	−	−	±
Cl. bifermentans	−	−	−	−	−	−	−	+	−	−	−
Cl. butyricum	−	−	−	−	−	−	−	−	+	−	−
Cl. botulinum											
A	−	−	−	−	−	−	−	−	−	+	+
B	−	−	−	−	−	−	−	−	−	+	+
F	−	−	−	−	−	−	−	−	−	+	(+)

Immunofluorescence cross-absorption tests.

To observe the degree of sharing of spore antigens, cross-absorption tests were conducted on some of the antisera with a few selected strains of both *Cl. sporogenes* and *Cl. botulinum*.

The results (Tables 4–8) show that the cross-reacting antibodies could be removed by absorption. Thus, in the sporogenes–antisporogenes system (Tables 4–6) homologous absorption removed all the spore antibodies in the serum whereas heterologous absorption removed a substantial proportion of spore fluorescent

https://doi.org/10.1017/S0022172400025778 Published online by Cambridge University Press

antibodies. In the botulinum–antibotulinum system (Tables 7, 8), homologous and heterologous absorptions removed all the spore antibodies in the serum thus confirming that the proteolytic strains of *Cl. botulinum* types A and B have identical spore antigens. In the botulinum–antisporogenes (and vice versa) cross-absorption systems, spores of *Cl. botulinum* types A and B removed the low titre cross-reacting antibodies in *Cl. sporogenes* antisera, thus rendering the antisera specific for *Cl. sporogenes* types; the reverse held good when *Cl. botulinum* antisera were absorbed with spores of *Cl. sporogenes*.

DISCUSSION

Cl. botulinum is divided into six types (A–F) on the basis of antigenically distinct toxins that they produce but their biochemical activity separates them into proteolytic and non-proteolytic groups. Because of serological differences in their vegetative antigens, the two biochemical groups have been considered as two separate species. Thus, the proteolytic group, consisting of *Cl. botulinum* types A, B and F have been considered as representing or belonging to *Cl. parabotulinum* whilst the non-proteolytic types B, C, D, E and F are members of *Cl. botulinum* (Bengtson, 1924). In this investigation we have not followed this nomenclature rigidly, but have used both names when appropriate. However, since our other organism of study is *Cl. sporogenes*, which is a strongly proteolytic species, the comparative studies were made between *Cl. sporogenes* and *Cl. parabotulinum* although we have continued to use the term *Cl. botulinum* for these.

On morphological, cultural and biochemical grounds, non-toxigenic *Cl. parabotulinum* is indistinguishable from *Cl. sporogenes*. Somatic and flagellar cross-agglutination between the two species has been reported (Mandia, 1951, 1955; Solomon *et al.* 1971; Lynt, Solomon & Kautter, 1972). Meisel & Rymkiewicz (1959), however, did not find any cross-reactions by spore agglutination among strains of *Cl. sporogenes* and *Cl. botulinum* types A and B (proteolytic). This is not surprising as they used only a small number of strains (three) of each species. In this study, FAT was used to examine 31 strains of *Cl. parabotulinum* (types A, 18; B, 10; and F, 3). Table 2 shows that the spores of the strains fluoresced in *Cl. sporogenes* spore antisera but there was no regular pattern of reactions; antisera of the same type of *Cl. sporogenes* did not consistently react with the same strains of *Cl. botulinum*; the cross-reacting strains of *Cl. botulinum* did not correspond to the toxigenic types; the three strains of *Cl. botulinum* type F and some strains of types A and B showed no reaction with any of the *Cl. sporogenes* antisera. These latter results are reminiscent of the experience of Meisel & Rymkiewicz (1959).

A two-way cross-fluorescence test (see Table 3) shows that the two antisera to *Cl. botulinum* types A (Strain 188) and B (strain 172) fluoresced to full titre with all the strains of the homologous and heterologous types. This again shows that there is no relation between the spore antigens of *Cl. botulinum* and the potential toxin production by types of this organism. This result does not agree with the findings of Meisel & Rymkiewicz (1959), who did not show any cross-agglutination between spores of *Cl. botulinum* types A and B. This is surprising since sharing of vegetative antigens among the proteolytic *Cl. botulinum* (i.e. *Cl. parabotulinum*)

has been reported by Mandia (1951) and others. It is possible that Meisel & Rymkiewicz (1959) were dealing with examples of incomplete antibodies which failed to bring about agglutination of the spore suspensions; FAT might have given a positive reaction. The two-way cross-fluorescence tests show a great deal of cross-reaction among strains of *Cl. sporogenes* and *Cl. parabotulinum* types A and B, even though the cross-reacting titres are lower than those given by specific homologous antisera.

Cross-absorption of the antisera suggests the presence of more than one antigenic component (Tables 4–8). The different types of *Cl. sporogenes* share a component with *Cl. parabotulinum* types A and B; this component removes the cross-reacting antibody thereby rendering the botulinum antisera specific for this species. It is also responsible for the slight lowering of the titre in the sporogenes antisera when absorbed with botulinum spores. Another component is shared exclusively by types of *Cl. botulinum* including the three type-F strains (not shown in the Tables) and this is responsible for species specificity. We name this L and the group-specific component M (following the sequence of the spore antigenic components described by Princewill, 1979*a*, *b*), which is possessed by some strains of *Cl. sporogenes*. Some strains of *Cl. histolyticum* type II (Princewill, 1979*b*) also possessed component M which was absent from the strains of *Cl. bifermentans* and *Cl. butyricum* studied by Princewill (1979*b*). Table 9 shows the distribution of spore antigenic components among strains of the species of *Clostridium* studied in the series.

This work was supported in part by a scholarship grant awarded by the Nigerian Federal Ministry of Education. The author is also indebted to Mr J. Wolf for suggesting the problem and for his invaluable advice and help throughout this investigation.

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