A SEROTYPING SYSTEM FOR *CLOSTRIDIUM WELCHII* (*C. PERFRINGENS*) TYPE A, AND STUDIES ON THE TYPE-SPECIFIC ANTIGENS

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CLOSTRIDIUM WELCHII (*C. perfringens*) type A is now firmly established as an important cause of food poisoning in Britain and throughout the world. Between 1969 and 1974, it was responsible for 200 reported general outbreaks of food poisoning (20.4% of all general outbreaks) in England and Wales in which 8881 persons were affected (18.5% of all food poisoning; Vernon and Tillett, 1974; Vernon, personal communication).

Type-specific antisera were first prepared by Hobbs *et al.* (1953) against heat-resistant strains of *C. welchii* isolated from foods incriminated in food-poisoning outbreaks. The number of sera in use was increased markedly after it had been established that the more heat-sensitive classical strains were also responsible for *C. welchii* food poisoning (Hall *et al.*, 1963; Taylor and Coetzee, 1966; Sutton and Hobbs, 1968). By 1973, 48 antisera were available for determining possible relationships between food and faecal isolates of *C. welchii*, and the set comprised 57 antisera by the end of 1975.

This paper describes the application of the serotyping scheme to the investigation of 153 outbreaks of suspected *C. welchii* food poisoning over the 3-year period 1973–75. Results of related epidemiological studies and studies on the location of the strain-specific antigen are also presented.

MATERIALS AND METHODS

Preparation of antisera. Antisera were prepared by the method of Henderson (1940). The cells were grown in 1% Glucose Broth (Oxoid), washed twice, and re-suspended in 0.4 ml of formalin in 100 ml 0.85% saline to give a concentration of 2×10^{10} cells per ml as determined with Brown's opacity tubes. A 2-week course of intravenous injections in rabbits consisted of six doses (0.1, 0.2, 0.4, 0.8, 1.0 and 1.0 ml) administered at 3-day intervals; this usually gave a serum with a titre of 800–6400. Details of the sources of the 57 serotypes at present comprising the serotyping scheme are given in table I. All but one of the strains were isolated from food or faecal samples examined in connexion with outbreaks of food poisoning.

Serotyping of strains. Strains of *C. welchii* mostly in cooked-meat medium were submitted for serotyping by public health laboratories and hospital pathology departments throughout Great Britain. Full information about the outbreaks was obtained when possible by the use

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TABLE I

Details of strains used for the preparation of antisera to Clostridium welchii type A developed in the Food Hygiene Laboratory

Serotype no.	Culture reference no.	Source and history*	Serotype no.	Culture reference no.	Source and history*
1	NCTC8797	Boiled salt beef C	30	FH3794/67	Tongue NC
2	NCTC8238	Boiled salt beef C	31	FH3853/67	Tongue PC
3	NCTC8239	Boiled salt beef C	32	FH3911/73	Faeces C
4	NCTC8247	Meat paste C	33	FH4436/73	Tongue C
5	NCTC8678	Faeces C	34	FH5337/73	Minced beef C
6	NCTC8679	Faeces C	35	FH50/72	Blancmange NC
7	NCTC8449	Stewed lamb C	36	FH4552/72	Turkey NC
8	NCTC8235	Stew C	37	FH10209/66	Faeces C
9	NCTC8798	Rissole C	38	FH1073/67	Roast pork PC
10	NCTC8799	Roast meat C	39	FH9222/67	Brisket C
11	NCTC9851	Braised heart C	40	FH2932/67	Roast beef C
12	NCTC10239	Faeces C	41	FH3663/73	Pork C
13	NCTC10240	Chicken C	42	FH2925/74	Faeces NC
14	NCTC10611	Faeces C	43	FH1922/74	Semolina C
15	NCTC10613	Mincemeat C	44	FH2889/74	Beef C
16	NCTC10614	Faeces C	45	FH2890/74	Beef C
17	FH6849/67	Faeces C	46	FH1774/74	Gravy NC
18	FH10486/67	Faeces C	47	FH1185/74	Faeces NC
19	FH1149/67	Faeces C	48	FH3158A/74	Faeces C
20	FH3483/68	Faeces C	49	FH3158B/74	Faeces C
21	FH3746/68	Faeces C	50	FH3565/74	Faeces NC
22	FH3858/68	Minced kidney C	51	FH3579/74	Faeces NC
23	FH7869/66	Faeces NC	52	FH3799/74	Faeces NC
24	FH7870/66	Faeces C	53	FH4137/74	Lesion of C
25	FH4062/70†	Cream doughnut NC			necrotic enteritis C
26	FH1086/67	Roast pork PC	54	FH4314/74	Faeces C
27	FH7871/66	Faeces NC	55	FH4930/74	Chicken pie C
28	FH1543/67	Mincemeat C	56	FH5049/74	Faeces NC
29	FH1539/68	Faeces C	57	FH5069/74	Faeces NC

* C = causative of outbreak, i.e., isolated from food or the majority of faeces associated with the outbreak or both; NC = not causative of outbreak; PC = probably causative; evidence insufficient for definite inference.

† = This strain replaces the original strain used to prepare the serum.

of a questionnaire. On a number of occasions, counts of *C. welchii* in the food and faecal samples were done by the laboratories investigating the outbreaks and receiving the specimens; the methods used were those described by Sutton, Ghosh and Hobbs (1971). Isolates of *C. welchii* from these laboratories were plated on horse-blood agar that had been spread with three drops of a 1% neomycin solution and incubated anaerobically at 37°C overnight. The blood agar was made up of a base layer of peptone water agar and a top layer of nutrient agar (Oxoid Nutrient Broth no. 2 with 1% Davis New Zealand Agar) containing 5% defibrinated horse blood (Burroughs Wellcome). Haemolysis and colony form were noted, bacterial morphology was checked by Gram's stain, and colonies were picked to plates of egg-yolk agar formulated according to the method of McClung and Toabe (1947). The strains were serotyped from the blood agar or egg-yolk plates by slide-agglutination tests.

Determination of the strain-specific antigen. To demonstrate the presence of capsules, smears of the cells were suspended in Indian ink, dried in air, stained with 1% carbol fuchsin for 1 min. and re-dried for microscopic inspection.

The relationship of the strain-specific antigen to the capsule was studied by means of the

TABLE II
*Application of the Clostridium welchii type A serotyping system
 to the investigation of food-poisoning outbreaks*

Food-poisoning outbreaks			Number of isolates of <i>C. welchii</i>	
Year of occurrence	Number investigated	Number (and percentage) in which the causative serotype was determined	examined	typed
1973	39	21 (54%)	443	305 (69%)
1974	50	22 (44%)	739	443 (60%)
1975*	64	41 (64%)	1037	700 (67%)
1973-75	153	84 (55%)	2219	1448 (65%)

* = First ten months only.

following absorption tests. Cells from 18-h glucose broth cultures of *C. welchii* types 21, 26, 29 and 31 were collected by centrifugation, washed twice, and separately re-suspended in 20-ml volumes of 0.85% saline. Each suspension was divided into two equal portions A and B. Cells of portion A were heated with an equal volume of 10% trichloroacetic acid (TCA) in a boiling water bath until no capsules were visible in test samples. The suspension was neutralised with 0.1M sodium hydroxide and the cells were centrifuged and washed twice. Cells of portion B did not receive this TCA treatment. The pelleted cells from each portion were re-suspended in 2 ml of homologous serum, incubated at 50°C for 2 h and then removed by centrifugation. The resulting serum samples were tested for changes in their homologous titres and heterologous cross-reactions. Antisera were prepared against the acid-treated cells and the respective homologous and heterologous titres were measured.

Loss of strain-specific antigen in naturally rough C. welchii. Rough forms of *C. welchii* types 12, 26 and 31 were isolated by the selection of colonies with consistently rough characteristics on repeated subculture. Antisera were prepared against these rough strains and serum titres were determined by the indirect fluorescent antibody technique. Tube agglutination tests were also performed with suspensions prepared from the respective smooth cultures.

Approximately equal numbers of the rough and smooth cells of *C. welchii* types 12 and 26 were used separately to absorb the corresponding homologous antisera prepared with the smooth forms, and the titres of the absorbed sera were determined. Preparations of the smooth and rough forms were stained with Indian ink and 1% carbol fuchsin to determine the presence or absence of capsules.

Reversion of rough forms to smooth. Attempts were made to bring about reversion of a rough form of *C. welchii* (strain no. FH3701/67) to the typical smooth form. Media used included the sporulation medium of Duncan and Strong (1968), Columbia Base Agar (Oxoid) with 5% horse or sheep blood, tryptone-sulphite-neomycin agar (Marshall, Steenbergen and McClung, 1965) and veal cooked-meat prepared according to Cruickshank (1968) but with boneless veal in place of bullocks' hearts.

Animal-passages experiments were also performed: adult monkeys, mice and newly hatched chicks were fed and suckling mice were given intragastric inoculations of the viable rough cells. Cultures from faeces and intestinal contents of mice and chicks obtained at necropsy were searched for smooth colonial forms. Parenteral inoculations were made in mice; these animals were killed at 24 h and 48 h and the viscera cultured. Embryonated eggs

were given inoculations by the yolk sac route and cultures were made from the dead embryos. Isolates were re-passaged whenever there was an increase in smoothness of colonies.

RESULTS

Serotypes

Over the 3-year period 1973-75, 1591 of 2499 (64%) *C. welchii* type A isolates were successfully serotyped. They were placed in three categories according to source: (1) strains from outbreaks of food poisoning; (2) strains from human infections other than those associated with food poisoning; and (3) strains from the environment, faecal specimens from healthy persons, and foods not associated with outbreaks of food poisoning.

It was possible to serotype 1448 of 2219 (65%) isolates from 153 food-poisoning outbreaks (table II); in 84 (55%) of the outbreaks, the causative serotype was established by demonstration of a common *C. welchii* serotype in the majority of faecal samples and also in the suspected food when this was available. A predominance of certain serotypes was noted (table III). Non-typable isolates from earlier outbreaks were used to prepare new antisera for expansion of the series. In a number of the outbreaks, faecal sampling or the number of isolates submitted for typing was inadequate; the causative serotypes could not be established with certainty and epidemiological conclusions could not be drawn.

In 15 of the outbreaks, the numbers of *C. welchii* in faecal samples or the implicated food, or both, were determined. The median count of *C. welchii* in 68 faecal specimens from nine of the outbreaks was 2.5×10^5 per g (range 1×10^3 to 8.5×10^6 per g). The median *C. welchii* counts in foods incriminated in 10 of the outbreaks was 4.5×10^5 per g (range 2×10^3 to 2.5×10^8 per g).

Of 176 isolates from 32 patients in the second category, 104 (59%) could be typed; the potential value of the serotyping system for epidemiological investigations of clinical infections with *C. welchii* was demonstrated. In several of the incidents, however, inadequate numbers of isolates were submitted, so that little epidemiological significance could be attached to the typing results.

A heterogeneous collection of serotypes was found among 39 (37.5%) typable isolates out of 100 in category 3.

Determination of the strain-specific antigen

The results of absorption tests with capsule and decapsulated cells of *C. welchii* types 21, 26, 29 and 31 are shown in table IV. The samples of homologous sera absorbed with acid-treated cells showed no loss of titre, but there was a considerable loss when these sera were absorbed with untreated cells. Similar results were obtained with the cross-reacting antigens. There was variation in the natural degree of capsulation of each serotype, designated as +, ++ or +++ on the basis of capsule staining, but this variation did not affect the homologous and heterologous titres.

TABLE III

*Distribution of serotypes of Clostridium welchii type A among isolates from 153 incidents of food poisoning, 32 cases of clinical infection and 99 sources not associated with food-poisoning outbreaks or clinical infections**

Serotype	Number isolated from				Number not associated with food-poisoning outbreaks or clinical infections but isolated from		
	food-poisoning incidents		clinical infections		faeces	foods and food-working surfaces	hospital environment (floors and air)
	C	NC	C	NC			
NT	44	NR	14	NR	25	29	7
3/4†	20	8	0	2	0	0	0
4	9	7	2	0	0	0	1
1	9	0	0	0	0	0	0
41	9	3	0	0	0	0	0
17	0	7	0	0	8	1	0
29	6	5	1	0	0	1	0
7	5	3	0	0	0	0	0
31	5	9	0	0	0	0	0
10	3	5	0	0	8	2	0
25	3	13	3	0	0	0	0
28	3	3	1	2	0	1	2
38	3	7	2	1	0	0	0
55	3	0	0	0	0	0	0
3	2	2	0	0	0	0	0
5	2	9	2	0	0	2	0
11	2	11	0	1	0	0	0
16	2	0	0	0	0	0	0
18	0	1	2	1	0	0	0
23	1	5	0	1	0	0	2
27	2	6	0	1	0	1	0
32	2	1	0	0	0	2	0
33	2	6	0	2	0	0	0
34	2	9	2	0	0	0	0
39	2	4	2	1	0	0	0
9	1	2	0	1	0	1	0
12, 13	1	2	0	0	0	0	0
20	0	3	1	1	1	1	0
21	1	0	0	0	0	1	0
30, 35	0	6	0	0	0	1	0
37	0	1	0	1	0	0	1
40	0	2	0	0	0	0	1
41	0	3	0	0	0	0	0
43‡	1	1	0	0	0	0	0
44, 45, 46, } 48, 49, 51, 54 }	1	0	0	0	0	0	0
50	0	3	0	0	0	0	0
52, 56	0	1	0	0	0	0	0

* C = causative; NC = not causative; NR = not reported; NT = not typable.

† = Causative isolates were agglutinated by both antisera.

‡ = Serotypes nos. 43-56 were added to the scheme in 1975.

TABLE IV
*Titres of Clostridium welchii antisera before and after absorption
 with normal and decapsulated cells*

Homologous serotype no.	Degree of capsulation*	Treatment of serum†	Titre	Cross-reacting serotype no.	Degree of capsulation*	Treatment of cross-reacting serum†	Titre
21	++	{ A B C	512 512 4	18	++	{ A B C	4 4 2
26	+++	{ A B C	512 512 4	...	...	...	...
29	+++	{ A B C	128 128 8	8	++	{ A B C	16 16 8
31	+	{ A B C	256 256 2	FH3697/67‡	+++	{ A B C	128 128 2

* Degree of capsulation designated as +, ++ or +++ according to the result of capsule staining.

† A = untreated serum; B = serum absorbed with homologous acid-treated cells; C = serum absorbed with homologous normal cells.

‡ = Serotype not yet assigned a position in the serotyping system.

TABLE V
*Results of tests with antisera produced against rough and acid-treated
 Clostridium welchii cells of three serotypes*

Serotype no.	Antigen preparation*	Agglutinin titre determined by†	
		FA	TAS
26	Rough	20	<2
26	Acid-treated	>50	<2
29	Acid-treated	>50	<2
31	Rough	>50	<2
31	Acid-treated	>50	<2

* = No capsules were visible by capsule stain with any of these preparations.

† FA = serum titre determined by fluorescent-antibody technique; TAS = agglutinin titre determined in tube tests with parent-type smooth cells of corresponding serotype.

Results of experiments with the rough forms of *C. welchii* types 12, 26 and 31 are shown in tables V and VI. Little capsular material was demonstrable around rough cells as compared with their smooth counterparts. The rough cells agglutinated in normal saline; no means was found to overcome this effect and serum titres could not be determined by tube agglutination. By fluorescent antibody tests, antisera produced against rough cells of *C. welchii* types 26 and 31, and acid-treated cells of types 26, 29 and 31 were shown to

TABLE VI

Clostridium welchii antisera absorbed with smooth and rough cells

Cells used for absorption		Agglutinin titre of homologous serum‡	
Serotype no. (and S/R state)*	Degree of capsulation†	before absorption	after absorption
12 S	++	1024	64
12 R	—	1024	512
26 S	+++	256	8
26 R	—	256	256

* S = smooth; R = rough.

† = Degree of capsulation graded — to +++ according to results of capsule staining.

‡ = Agglutination titre determined in tube tests with suspensions of respective smooth cells.

contain homologous antibodies, but tube agglutination tests with the parent smooth forms revealed that the original type-specific and cross-reacting antibodies were not present (table V). Absorption of sera with rough cells of types 12 and 26 did not cause a significant loss of titre as compared with the loss of titre after absorption with the smooth forms (table VI).

Efforts to change a rough strain back to its smooth form were unsuccessful. A slight alteration in appearance of colonies towards the smooth form was obtained in the initial passages through suckling mice and chick embryos, but this did not progress with subsequent passages and the cells remained auto-agglutinating. α -Toxin (lecithinase) production, detected by the production of opalescence around colonies on egg-yolk agar, was also greatly reduced in some rough forms; this was found to be increased to some extent by passage of rough strains through suckling mice, chicks and chick embryos. Different media produced slight variations in colony form, but the degree of capsulation and auto-agglutinability in saline remained unaltered. During these studies, it was evident that the use of Oxoid Columbia Base horse-blood agar enhanced growth and gave larger colonies; the medium has now been adopted in this laboratory for the isolation and enumeration of *C. welchii* and for observation of the haemolytic effects of its colonies on blood agar.

DISCUSSION

The majority of strains of *C. welchii* type A forming the basis of the serotyping system used in these studies were originally isolated from cases of food poisoning; recently, strains from other sources have been included (table I). In practice it has become apparent that it is not justifiable to maintain the previous distinction between heat-resistant and heat-sensitive *C. welchii* isolates and new Arabic numerals have been assigned to heat-sensitive serotypes

TABLE VII
Revised serotype designations for heat-sensitive *Clostridium welchii* type A*

Former type no.	New type no.	Former type no.	New type no.
i, xii	23	x	29
ii	24	xi	39
iii, v	25	xiii	17
iv	E†	xiv	4
vi	26	xv	9
vii	38	xvi	40
viii	27	xvii	30
ix	28	xviii	31

* See Hobbs *et al.* (1973).

† E = eliminated; stock culture not viable.

originally designated by Roman numerals (table VII; Hobbs *et al.*, 1973); serotype numbers have been combined where strong cross-reactions have been found consistently over the period of study.

C. welchii can be confirmed as the causative agent of a food-poisoning outbreak by the demonstration of a common serotype in the majority of faecal samples and, if available, the suspect food. It is necessary to demonstrate this common serotype since typable strains are frequently isolated from the stools of healthy persons or those with diarrhoeal disease from other causes (Sutton, 1969; Akama and Otani, 1970; Sutton *et al.*, 1971; J. A. Hughes, unpublished data) and from the environment.

The results of applying the serotyping scheme to the investigation of 153 food-poisoning outbreaks and 32 cases of gas gangrene or other clinical conditions have clearly demonstrated the value of the scheme in the epidemiological study of food-borne disease and clinical infections caused by *C. welchii* type A.

Since *C. welchii* is usually present in faeces, serological typing in the investigation of food-poisoning outbreaks can be of value only if a sufficient number of isolates from each sample of faeces—and food, if available—is picked for typing. In a quarter of the outbreaks, inadequate numbers of isolates were submitted for typing and the investigations were inconclusive. Outbreaks due to more than one serotype may occasionally be encountered.

Guidelines for investigating food-poisoning outbreaks by means of the serotyping scheme are as follows: (1) details of the outbreak should be noted on a questionnaire form such as that suggested by Hobbs (1969); (2) at least three isolates from each faecal and food sample should be picked for serotyping; and (3) the numbers of *C. welchii* organisms in faecal and food samples should be determined when possible.

Sutton *et al.* (1971) showed that faeces from persons with *C. welchii* food poisoning contain significantly larger numbers of *C. welchii* than those from healthy persons or patients with diarrhoea due to other causes; it was proposed

that this constitutes a useful diagnostic criterion for *C. welchii* food poisoning. In the nine outbreaks in which faecal *C. welchii* counts were determined, the median count of 2.5×10^5 per g was significantly higher than the median and mean faecal counts in healthy persons reported by Sutton *et al.* (1971) and Hauschild, Hilsheimer and Griffith (1974) respectively.

C. welchii counts in the implicated foods were obtained in 10 of the outbreaks and, in all but two instances, were abnormally high. *C. welchii* is not infrequently found in foods, but numbers are usually small and enrichment is required for their isolation.

These results demonstrate the value of determining faecal and food counts of *C. welchii* in conjunction with serotyping for epidemiological investigations of food poisoning. Although standard plate-count techniques are the ideal, where shortages of time and manpower exist, useful approximate *C. welchii* counts can be obtained by plating 10-fold dilutions of suspensions of faecal or food samples in physiological saline with disposable calibrated loops (Nunc, Roskilde, Denmark).

In clinical investigations, as in food-poisoning investigations, it is important to submit several isolates from each sampling site or specimen being investigated.

Decapsulated *C. welchii* cells used in absorption experiments did not lower the titres of antisera to the respective parent smooth (capsulate) forms, and type-specific antibodies were not produced in rabbits inoculated with decapsulated cells; these results indicate that the type-specific and cross-reacting antigens reside in the capsular layer, thus supporting the results of Baine and Cherniak (1971), Lee and Cherniak (1974) and Paine and Cherniak (1975). Evidence that development of roughness represents a natural loss of the capsule, and hence loss of the type-specific antigen, is also given. Development of roughness is most frequently associated with passage on artificial media or with prolonged storage of the organism but, on rare occasions, rough forms are encountered in the field; these cannot be typed or used for preparation of type-specific antisera.

Attempts to transform a rough strain to the smooth form were unsuccessful but the indications were that occasional passage of stock strains through suckling mice, chicks or chick embryos might help to prevent loss of capsule and development of roughness.

Serotyping schemes for *C. welchii* type A have been developed independently in America and Japan as well as in Britain. Co-operative work is in progress to combine the three schemes in order to develop internationally acceptable serotyping facilities for *C. welchii* type A and a separate report is expected in the near future.

SUMMARY

A serotyping scheme for *Clostridium welchii* (*C. perfringens*) type A employing 57 antisera has been used to investigate the epidemiology of 153 food-poisoning outbreaks and 32 cases of gas gangrene and other clinical infections. Respectively 65% and 59% of the isolates were typable, and in 55% of the

food-poisoning outbreaks the causative serotypes were established. Isolation and reporting methods that would render the typing scheme of even greater epidemiological value are described.

The type-specific antigen was shown to reside in the capsule and to be lost from strains that had become rough. Development of roughness and its prevention are described.

A great range of antisera and an internationally acceptable serotyping scheme is expected after integration of this set with those developed independently in America and Japan.

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