CHARACTERISTICS OF *CLOSTRIDIUM PERFRINGENS* STRAINS ASSOCIATED WITH FOOD AND FOOD-BORNE DISEASE[1]

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ABSTRACT

HALL, HERBERT E. (Robert A. Taft Sanitary Engineering Center, Cincinnati, Ohio), ROBERT ANGELOTTI, KEITH H. LEWIS, AND MILTON J. FOTER. Characteristics of *Clostridium perfringens* strains associated with food and food-borne disease. J. Bacteriol. **85**:1094–1103. 1963.—A total of 83 strains of *Clostridium perfringens*—30 from England, Europe, and Asia, associated with food-poisoning outbreaks; 28 from the United States, associated with outbreaks or contaminated foods; and 25 from natural or pathological sources—have been studied to determine their serological relationships, sporulation and heat-resistance of spores, and their hemolytic activity on mammalian bloods. A comparison of the results obtained with these three groups of strains reveals that the Eurasian group is characterized by serological typability, poor sporulation with the production of heat-resistant spores, and a hemolytic activity limited to the production of partial hemolysis on horse, ox, and sheep bloods, whereas the strains from natural and pathological sources in this country are not serologically typable, sporulate well but the spores are not heat-resistant, and are hemolytically active, producing both partial and complete hemolysis on horse, ox, and sheep bloods. The American food-poisoning strains have a wide variety of characteristics. Some strains resemble the Eurasian in their serological typability and the production of heat-resistant spores, but sporulation and hemolytic activity are more like the strains from classical sources. On the basis of these data, it appears unlikely that *C. perfringens* food-poisoning outbreaks in the United States are restricted to strains meeting the criteria of classification described by British workers and that the isolation of large numbers of any strain of this organism from an incriminated food must be considered as having a possible bearing on the etiology of the outbreak.

[1] Mention of commercial products throughout has been made only to identify items which cannot be completely described, and is not to be construed as endorsement by the Public Health Service.

The experience of British, European, and Japanese investigators (Østerling, 1952; Hobbs et al., 1953; McNicol and McKillop, 1958; McKillop, 1959; Hayoshi, 1961) reveals that consumption of meats in which certain strains of *Clostridium perfringens* have grown extensively may result in illness characterized by acute abdominal pain and diarrhea, accompanied by little or no nausea and vomiting. The incubation period is usually from 8 to 22 hr, and the illness is of short duration (1 day or less). Fever, chills, headache, or other signs of infection are rarely observed. According to the English workers (Hobbs et al., 1953), the organisms responsible for this type of food poisoning are atypical strains of *C. perfringens* type A, differing from other members of the type A group by the production of low levels of alpha toxin (lecithinase), little or no theta toxin production (hemolysin), formation of heat-resistant spores (100 C for 1 hr or more), and by antigenic constituents which allow them to be grouped into 13 provisional serotypes.

Recent experiences in our own laboratory with cultures of *C. perfringens* from foods implicated in outbreaks or from patients' stools indicate that, in some instances at least, strains may be recovered which do not conform with the above criteria for food-poisoning types. This observation has prompted us to undertake a detailed study of the characteristics of *C. perfringens* from several sources to determine whether the organisms responsible for food poisoning in the United States are distinguishable from classical type A strains, as is the case in England (Hobbs et al., 1953).

This report describes the characteristics of several strains of *C. perfringens* type A obtained from food-poisoning outbreaks, natural sources, or as isolates from pathological specimens. Their

ability to produce heat-resistant spores, their activities on several mammalian bloods, and their serological relationships to Hobbs' 13 "heat-resistant" types are presented.

Materials and Methods

Grouping of the strains of C. perfringens. The 83 strains of *C. perfringens* studied were divided into three groups on the basis of strain origin. Group 1 was composed of 30 strains which were isolated from foods or feces in England, Europe, or Asia and were all associated with outbreaks of *C. perfringens* food poisoning (Table 1). Group 2 was composed of 28 strains of American origin which were isolated from foods or feces associated with food-poisoning outbreaks or from edible foods not so associated (Table 2). Group 3 was composed of 25 strains of American origin which were isolated from normal feces, soil, or pathological specimens (Table 3).

Biochemical and cultural characteristics. All of the 83 strains were classified as *C. perfringens*, according to the schema provided in *Bergey's Manual of Determinative Bacteriology* (Breed, Murray, and Smith, 1957). Their ability to produce lecithinase was determined on McClung-Toabe egg medium (McClung and Toabe, 1947), and hydrogen sulfide production was observed in sulfadiazine-polymyxin-sulfite agar (Angelotti et al., 1962).

Toxicological typing. All strains were typed on the basis of the lethal toxins produced by means of a mouse protection test. Toxic filtrates were prepared by growing the organisms in Noyes veal broth at 37 C in a water bath (Angelotti et al., 1962) with two transfers to fresh medium at 24-hr intervals. At 4 hr after the last transfer, the toxic material was recovered by centrifuging the culture at 2,000 × *g* and filtering the supernatant through Seitz or fritted-glass filters. Six mice, weighing 12 to 15 g, were used for each test. Each of three control mice was injected intraperitoneally with 0.4 ml of sterile 0.85% sodium chloride solution, and each of three test mice was similarly injected with 0.4 ml of a 1:2 dilution of Wellcome type A antiserum (Burroughs Wellcome & Co., Inc., Tuckahoe, N.Y.). Approximately 1 hr after the above injections, each mouse was injected intraperitoneally with 1 ml of undiluted toxic filtrate. Mice were observed for approximate time of death up to 72 hr; death rarely occurred after this interval. Preliminary studies had shown that the Noyes veal broth was in itself nonlethal and in effect caused no overt symptoms of illness.

Sporulation. All of the strains were tested for their ability to produce spores. Because of the difficulty associated with obtaining spores of *C. perfringens*, five sporulation media were employed. The formation of spores by a strain in one or more of these media, regardless of the extent of sporulation, was accepted as evidence of the ability of the culture to sporulate. The sporulation media employed were those of Ellner (1956) and of Zoha and Sadoff (1958); Sames' modification of Ellner broth, substituting 0.01% $MnSO_4$ for 0.01% $MgSO_4$ in Ellner's formula (*personal communication*); Sames' medium (containing yeast extract, 0.5%; Casamino Acids, technical, 0.5%; starch, 0.2%; NaCl, 0.5%; and $Na_2HPO_4 \cdot 7H_2O$, 0.9%; *personal communication*); and the media of Angelotti et al. (1962). The organisms to be tested were grown in Noyes veal broth (Angelotti et al., 1962) for 18 to 24 hr in a water bath at 37 C. A 1-ml portion of the supernatant culture suspension was transferred to a deep tube of Fluid Thioglycollate Medium (Difco) and incubated in a water bath at 37 C for 4 hr. After the 4-hr incubation, 0.3 ml of the Thioglycollate culture was transferred to screw-capped tubes (150 × 15 mm) containing 15 ml of each of the sporulation media, which were incubated at 37 C for 24 hr. After incubation, smears of the sediment from each of the tubes of sporulation media were stained with Bartholomew and Mittwar cold stain (Manual of Microbiological Methods, 1957) and examined microscopically. Samples of the sporulation media were also heated at 80 C for 10 min, subcultured to Fluid Thioglycollate Medium, incubated at 37 C for 72 hr, and examined for anaerobic growth of gram-positive bacilli.

Production of heat-resistant spores. Though several of the sporulation media described in the preceding section yielded spores of the test strains, only the medium of Angelotti et al. (1962; henceforth referred to as SEC broth) consistently yielded spore crops capable of resisting exposure to 100 C for prolonged intervals. Consequently, all 83 strains were tested for production of heat-resistant spores as follows. The cultures were passed through the several preliminary cultural stages, as described above, for obtaining spores, and 0.3 ml of the 4-hr

Fluid Thioglycollate culture was used to inoculate screw-capped tubes (150 × 15 mm) containing 15 ml of SEC broth. Prior to inoculation, the SEC broth tubes were heated in a boiling-water bath to dispel dissolved oxygen and then cooled rapidly in tap water. The inoculated tubes of broth were incubated in a water bath at 37 C for 18 to 24 hr, after which they were inverted several times to distribute the sediment, and 3-ml samples of each were transferred to sterile screw-capped tubes (150 × 15 mm). The tubes containing the samples to be tested for heat-resistant spores were placed in racks and submerged in a 100-C, thermostatically controlled bath (American Instrument Co., Silver Springs, Md.) containing a mixture of water and ethylene glycol. Approximately 2 in. of the tubes extended above the liquid level in the bath. The lid was replaced on the bath and remained in place throughout the exposure interval to insure temperature equilibrium between the liquid in the bath and the vapor phase above. It was assumed that those portions of the tubes above the liquid level of the bath and the air they contained reached 100 C, as evidenced by the constant escape of flowing steam from the small exhaust vent in the top of the bath. Exposure times of 30, 60, and 90 min were employed. After exposure, the heated tubes were cooled rapidly in an ice bath, and 2-ml samples were removed from each. The samples were subcultured to duplicate tubes of Fluid Thioglycollate Medium and incubated in a water bath at 37 C for 72 hr. After incubation, the tubes were observed for the presence of growth (turbidity) in the lower, anaerobic portion of the tubes and absence of growth in the upper, aerobic portion of the tubes. Gram stains were made and observed for the presence of gram-positive rods with cellular morphology resembling that of *C. perfringens*. The presence of gram-positive rods typical of *C. perfringens* and anaerobic growth was accepted as evidence of the survival of spores in the heated samples of SEC broth.

Hemolytic activity. Blood agar plates were prepared by adding 5% of the desired blood to Brain Heart Infusion Agar (Difco). All strains were tested on ox, horse, and sheep blood; 46 were also tested on human, guinea pig, rabbit, and swine blood. The ox, horse, sheep, and swine bloods were obtained commercially (Colorado Serum Co., Denver, Col.) as defibrinated bloods; and the human, guinea pig, and rabbit bloods were drawn in the laboratory and added directly to the medium without the use of an anticoagulant. To test for hemolytic activity, the strains were grown in tubes of Noyes veal broth (Angelotti et al., 1962) for 18 to 24 hr in a 37-C water bath. After incubation, the tubes were shaken vigorously and allowed to settle, and a 1-mm loop was used to spot-inoculate the blood agar plates (10 cultures per plate) with the turbid supernatant fluid.

The inoculated blood agar plates were incubated anaerobically in a Case Anaero Jar (Case Laboratories, Inc., Chicago, Ill.) for 18 to 24 hr at 37 C and then removed to a table top and held for 4 hr at room temperature to allow full development of the hemolytic activity of the alpha toxin, a hot-cold lysin. Hemolysis was observed macroscopically by transmitted light and recorded as: P (partial), a zone of discoloration surrounding the colony; C (complete), a zone of complete clearing of the blood agar surrounding or under the colony; and PC (partial and complete), a clear zone under or immediately surrounding the colony contiguous with a broader peripheral zone of partial hemolysis.

Serological relationships. The serological relationships of the strains to Hobbs' "heat-resistant" types 1 through 13 were determined by tube agglutination tests. The antigens were prepared by growing the 83 strains in Fluid Thioglycollate Broth, containing 0.5% dextrose but without agar, for 18 to 24 hr in a water bath at 37 C. The tubes were centrifuged at 2,000 × *g* in an International refrigerated centrifuge equipped with head #269 at 15 to 18 C for 20 min. The supernatant fluid was discarded, and the sediment was suspended in 10 ml of distilled water. Centrifugation and washing in distilled water were repeated and followed by two similar washings in 10 ml of formalinized saline (0.85% NaCl with 0.4% by volume of formalin). After the final washing, the sediment was suspended in a volume of formalinized saline to yield an optical density of 0.23 to 0.25 at 640 mμ in a Coleman Junior spectrophotometer, using optically matched screw-capped test tubes (15 × 150 mm). The antigens were stored at 4 C in screw-capped bottles. Stored in this manner, the antigens maintained their reactivity and specificity for at least 6 months.

The antisera to the Hobbs' "heat-resistant" types 1 through 13 were produced by the intravenous injection of rabbits with six successive, increasing doses of the above-described antigens.

The initial dose was usually 1 ml, and increases of 1 ml were made at 2- to 3-day intervals until a 5-ml dose was reached. The animal was then rested for 1 week and bled by cardiac puncture. A booster dose of 5 ml of antigen was then given, and second and third bleedings made 1 and 2 weeks later. Titers of 1:640 to 1:2,560 were obtained with this schedule.

The agglutination tests were performed in Pyrex tubes (10 × 75 mm) by adding 0.5 ml of the above-described antigen to 0.5 ml of appropriate serum dilutions. The tubes were incubated in a water bath at 37 C for 2 hr and stored at 4 C overnight. The tubes were read for agglutination after centrifuging at 250 × *g* for 10 min. For screening tests, the sera were used at a 1:50 dilution; for titrations, twofold dilutions from 1:10 through 1:2,560 were used. Titers were recorded as the reciprocal of the highest serum dilution showing clumps that did not break up when the base of the tube was flicked sharply.

RESULTS

Toxicological typing. The filtrates of 53 of the 83 strains tested for the production of lethal toxins killed unprotected mice, but failed to kill those protected with anti A serum. Of these 53 strains, 13 were in group 1, 20 in group 2, and 20 in group 3. Filtrates of the remaining 30 strains failed to kill the unprotected mice. Such findings may be considered to indicate a low level of alpha toxin production by a *C. perfringens* type A (Smith, 1955). These weakly toxigenic strains included 17 (57%) of the group 1 strains (Table 1), 8 (28%) of the group 2 strains (Table 2), and 5 (20%) of the group 3 strains (Table 3). This distribution would seem to add weight to the contention that food-poisoning strains are frequently poor alpha toxin-producing strains.

Sporulation. With the exception of F4733/59 (Table 1), every strain of *C. perfringens* tested, regardless of source, produced spores in SEC broth. It was possible to observe spores by direct microscopic examination with 77 of the strains. The five remaining strains, four of which were members of the Hobbs' 13 serotypes, produced too few spores to find by microscopic examination; however, positive results for spore production were obtained for these five cultures by means of the heating and subculturing method described earlier.

The degree of sporulation of the various strains in SEC broth was evaluated in terms of the ease

TABLE 1. *Characteristics of group 1 Clostridium perfringens strains isolated from food-poisoning outbreaks in England, Europe, or Japan*

Strain designation and source	Serological relationship to Hobbs' heat-resistant types	Relative spore production	Resistance to 100 C	Hemolytic activity[a] on		
				Horse	Ox	Sheep
			min			
NCTC-8797[b]	1	+[c]	60	P	P	P
NCTC-8238	2	++[d]	90	—	P	P
NCTC-8239	3	++	90	—	P	P
NCTC-8247	4	+	90	—	P	P
NCTC-8678	5	±[e]	90	—	P	P
NCTC-8679	6	±	90	—	P	P
NCTC-8449	7	+	90	—	P	P
NCTC-8235	8	+	90	—	P	P
NCTC-8798	9	±	90	P	PC	PC
NCTC-8799	10	+	90	P	P	P
NCTC-9851	11	++	90	—	P	P
NCTC-10,239	12	++	90	P	P	P
NCTC-10,240	13	+	90	P	P	P
F143/60[f]	—	±	—	P	P	P
F4733/59	—	−	—	—	—	P
F4824/59	—	++	90	—	—	P
IU-1157[g]	—	++	90	—	P	P
IU-1158	—	++	90	—	P	P
IU-1159	3	++	90	—	P	P
IU-1205	1	+	90	—	—	P
IU-1332	1	+	—	—	P	P
IU-1333	2	+	90	—	P	P
IU-2110	2	+	30	—	P	P
IU-2114	6	++	90	—	P	P
IU-2117	9	+	60	—	—	P
IU-1500	3	++	—	—	P	P
IU-1505	—	++	—	PC	C	PC
IU-2131	2	++	—	—	—	P
IU-2159	2	++	—	—	P	P
IU-2307	11	+	90	—	P	P

[a] Hemolysis recorded as P, partial; C, complete; and PC, partial and complete.

[b] Obtained from National Collection of Type Cultures, London, England.

[c] Many fields examined to detect a spore.

[d] Many spores in each field examined.

[e] Demonstrated by heating at 80 C for 10 min and subculturing.

[f] All F strains obtained from B. C. Hobbs, Central Public Health Laboratory, London, England.

[g] All IU strains obtained from L. S. McClung, Indiana University, Bloomington.

TABLE 2. *Characteristics of group 2 Clostridium perfringens strains isolated from food-poisoning outbreaks or edible foods in the United States*

Strain designation and source	Serological relationship to Hobbs' heat-resistant types	Relative spore production	Resistance to 100 C	Hemolytic activity[a] on blood		
				Horse	Ox	Sheep
			min			
Food poisoning						
B-1 (beef)[b]	9	+[c]	—	PC	PC	PC
B-10 (feces)[b]	9	++[d]	—	C	PC	PC
B-11 (feces)[b]	—	++	—	PC	PC	PC
S-34 (turkey)[e]	—	++	—	C	PC	PC
1362 (salami)[f]	3	++	—	C	C	PC
A38 (chili beans)[b]	9	+	90	C	PC	PC
S-40 (turkey)	—	++	—	PC	PC	PC
IU-686 (chicken)[g]	—	+	—	C	PC	PC
IU-690 (chicken)	—	++	—	—	P	P
IU-2129 (turkey)	—	++	—	C	PC	PC
IU-2825 (shrimp)	7	++	—	C	PC	PC
A77 (turkey)[h]	—	++	—	PC	PC	PC
S-79 (beef)	—	++	—	—	PC	—
S-80 (chicken)	4	+	90	—	—	—
6866 (chili beans)[b]	—	++	—	PC	—	C
6867 (chili beans)[b]	6	++	90	PC	PC	PC
S-88 (cold cuts)	10	++	—	—	P	P
3388 (feces)[b]	10	+	—	PC	—	P
2428 (chili beans)[b]	—	++	—	—	C	C
IU-1168 (feces)	12	++	90	—	P	P
Edible food						
S-45 (dried beef)	—	++	90	C	PC	PC
S-46 (dried beef)	—	++	—	C	PC	PC
S-47 (tuna pie)	7	++	—	C	PC	PC
IU-687 (chicken)	—	++	90	—	P	P
IU-689 (chicken)	—	++	—	C	PC	PC
IU-1306 (herring)	8	++	—	C	C	PC
IU-1340 (herring)	—	++	—	—	P	P
IU-2826 (shrimp)	—	+	90	—	P	P

[a] Hemolysis recorded as P, partial; C, complete; and PC, partial and complete.

[b] Obtained from Alcor Browne, California Department of Health, Berkeley.

[c] Many fields examined to detect a spore.

[d] Many spores in each field examined.

[e] All S— strains isolated at Robert A. Taft Sanitary Engineering Center, Cincinnati, Ohio.

[f] Obtained from Cleveland Department of Health, Cleveland, Ohio.

[g] All IU strains obtained from L. S. McClung, Indiana University, Bloomington.

[h] Obtained from J. E. McCroan, Georgia Department of Health, Atlanta.

with which spores were observed in stained preparation. This technique was employed to determine the ability of the various strains to sporulate; the results are shown in Table 4.

The examination of the stained smears of the sediments from the various sporulation media revealed marked differences in spore morphology. Spores produced in SEC broth were small and did not swell the cell; whereas, in the other media, numerous strains produced large atypical spores that caused a distinct swelling of the cell. These morphologically atypical spores occurred more frequently in Ellner and modified Ellner broths than in the other media.

Heat resistance of spores. Although, in most instances, Ellner broth was superior to SEC broth in terms of the number of spores produced, the former was unsatisfactory for heat-resistance studies. It seems probable that the atypical spores noted so frequently in this medium did not possess

the heat-resistant characteristics of those produced in foods by the parent strain. Consequently, all heat-resistance studies were carried out with spores produced in SEC broth. Of the 83 strains studied, 30 (or 36%) produced spores that resisted heating at 100 C for 30 min, or more. Of these 30 strains, 27 resisted an exposure interval of 90 min, whereas 2 strains resisted 60 min, and the remaining strain 30 min only. Of these same 30 strains, 23 were in group 1 (Table 1) and seven were in group 2 (Table 2). Of the 23 in group 1, 13 were the prototype strains from the NCTC in England, 5 were similar prototype cultures from L. S. McClung's collection, and the remaining five cultures were isolates from outbreaks in Sweden or Japan. Of the seven strains in group 2 which produced heat-resistant spores, three were isolated from incriminated foods, three from edible foods, and one from the feces of a patient recovering from food poisoning. It is noteworthy that in no instance were heat-resistant spores produced by group 3 strains (Table 3).

TABLE 3. *Characteristics of group 3 Clostridium perfringens strains isolated from normal feces, soil, or pathological specimens*[a]

Strain designation and source	Relative spore production	Hemolytic activity[b] on blood		
		Horse	Ox	Sheep
S89 (feces)[c]	++[d]	PC	PC	PC
S90 (feces)	++	PC	PC	PC
S98 (feces)	++	PC	PC	PC
S99 (feces)	++	PC	PC	PC
S100 (feces)	++	PC	PC	PC
S101 (feces)	++	PC	PC	PC
S102 (feces)	++	PC	PC	PC
S74 (feces)	++	PC	PC	PC
S75 (feces)	++	P	C	P
S76 (soil)	++	P	PC	PC
A91 (Pathology)[e]	++	PC	PC	PC
4336 (Pathology)[e]	++	PC	PC	PC
3861 (Pathology)[e]	++	PC	PC	PC
1526 (Pathology)[f]	++	PC	PC	PC
6442 (Pathology)[f]	±[g]	PC	PC	PC
8843 (Pathology)[f]	++	PC	PC	PC
8721 (Pathology)[f]	++	PC	PC	PC
5382 (Pathology)[e]	++	PC	PC	PC
7716 (Pathology)[f]	++	PC	PC	PC
3338 (Pathology)[f]	++	PC	PC	PC
664 (Pathology)[f]	++	PC	PC	PC
2553 (Pathology)[f]	++	PC	PC	PC
5755 (Pathology)[f]	++	PC	PC	PC
ATTC-3624 (Pathology)[h]	++	C	PC	PC
47-E-2 (Pathology)[h]	++	C	PC	PC

[a] None of the group 3 strains was serogically related to Hobbs' heat-resistant types or produced heat-resistant spores.

[b] Hemolysis recorded as P, partial; C, complete; PC, partial and complete.

[c] All S— strains isolated at Robert A. Taft Sanitary Engineering Center, Cincinnati, Ohio.

[d] Many spores in each field examined.

[e] Obtained from Ruth Holtz, Cincinnati General Hospital, Cincinnati, Ohio.

[f] Obtained from Alcor Browne, California Department of Health, Berkeley.

[g] Demonstrated by heating at 80 C for 10 min and subculturing.

[h] Obtained from Howard Noyes, Walter Reed Army Institute of Research, Washington, D.C.

TABLE 4. *Sporulation of Clostridium perfringens strains from various sources*

Source	Group no.	No. of strains in group	Per cent of strains in group producing spores	No. of strains in group displaying various abilities to sporulate*			
				++	+	±	−
Food poisoning in England, Sweden, and Japan	1	30	96.7	13	12	4	1
Food poisoning or unincriminated food in the U.S.	2	28	100	22	6	0	0
Pathology, feces, and soil in the U.S.	3	25	100	24	0	1	0

* Symbols: ++, many spores observed in each microscopic field; +, many microscopic fields examined to observe a single spore; ±, spores not observed in stained preparations but detectable by means of heating (80 C for 10 min) and subculture; −, spores not demonstrable by test methods employed.

Hemolytic activity. C. perfringens is described by most texts as being hemolytic, but the types of blood used and types of hemolysis produced are not always indicated. Hobbs (Hobbs et al., 1953) described the food-poisoning type characteristic of most English outbreaks as being nonhemolytic. Of the 12 recognized toxins of *C. perfringens*, only three, alpha, delta, and theta toxins, are hemolytic. In addition to these, there is the non-

alpha-delta-theta type of hemolysis described by Brooks, Sterne, and Warrack (1957), which is not related to the production of a recognized toxin. Since these authors found this latter type to occur only rarely with type A strains and since type A strains do not produce the delta toxin, the hemolytic activity of type A strains is limited to the actions of the alpha and theta toxins. The hemolytic activities of these two toxins are easily recognized, since the alpha toxin produces only partial hemolysis, whereas the theta toxin produces a complete clearing of the blood, similar to the beta-type hemolysis of the staphylococci and streptococci.

In general, our findings agreed with those of previous workers (Smith, 1955) and, in particular, with the reactions on ox and horse bloods as described by Brooks et al. (1957). The "hot-cold" lysis by the alpha toxin followed the pattern of activity described by MacFarlane (1950) in that its activity was most obvious on sheep blood.

The reactions of the 30 strains of group 1 on ox, horse, and sheep bloods are shown in Table 1. Only two of these strains (6.6%) produced zones of complete hemolysis on any blood, thus emphasizing their general lack of theta toxin-producing ability. Most of the strains (70%) produced partial hemolysis on the ox and sheep bloods, but only seven strains (23%) similarly affected horse blood. This is confirmatory evidence for their characteristically poor alpha toxin-producing ability, since it requires a higher concentration of alpha toxin to affect horse erythrocytes than those of the ox or sheep. These findings correlated well with the similar indications obtained from the typing procedure in which it was shown that many of these strains failed to kill mice.

Of the 28 strains in group 2 (Table 2), only 6 (21%) produced the same hemolytic pattern with ox, horse, and sheep bloods as that characteristic of the majority of the strains in group 1. Three of these six produced heat-resistant spores, and two of the six were serologically related to the "heat-resistant" types. Of the remaining 22 strains, 19 (68%) showed appreciable theta toxin production, as indicated by the complete hemolysis produced, in most instances, on all three bloods. The remaining three strains (11%) showed limited and irregular hemolytic activities.

A rather characteristic finding of the organisms in group 2 was the production of complete hemolysis on horse blood or one of the other bloods, or on both, without concomitant partial hemolysis. This probably reflects the relatively greater effect of theta toxin and its more rapid diffusion, as compared with the alpha toxin, rather than a lack of alpha toxin production.

The hemolytic pattern produced by the 25 strains of group 3 was relatively consistent (Table 3). Of these, 23 strains (92%) produced either complete or a combination of partial and complete hemolysis on all three bloods. These results indicate a relatively high level of alpha toxin production and a consistent theta toxin-producing capacity. Since this pattern appears to be characteristic of the classical type *C. perfringens* type A associated with infectious processes, it is interesting to note that, though many of the strains in group 2 produced patterns resembling the classical more closely than that of the British strains, only three (12%) of the strains in group 2 had this identical pattern.

The hemolytic activity of 46 strains (26 from group 1 and 20 from group 2) on all seven mammalian bloods was examined to determine whether characteristic patterns of activity could be detected. No one pattern of reaction was exhibited by a majority of the strains. It was observed, however, that the group 1 strains tended to produce more partial reactions without concomitant complete reactions than did the group 2 strains. The action of 22 of the 26 group 1 strains on rabbit and swine bloods fell into two catagories as follows: 11 strains effected complete lysis of rabbit blood, but did not affect swine blood; whereas the reverse was true in the remaining 11 strains. The remaining four strains in group 1 yielded either no reaction on both bloods or complete hemolysis on both. Furthermore, the group 1 strains were relatively inactive on horse blood, whereas the group 2 strains usually produced complete hemolysis on this blood. Since the complete hemolysis produced by all strains on guinea pig blood did not correspond to the known alpha and theta toxin-producing abilities of some of the strains, it was postulated that the lysis was not due to these toxins. The fact that hemolysis was still produced in guinea pig blood agar plates containing antitoxins to the alpha, theta, and delta toxins strengthens this hypothesis. However, the hemolysin must differ from the non-alpha-delta-theta type described by Brooks et al. (1957), since it was produced by all of these type A strains.

In general, the reactions on human, rabbit, or

TABLE 5. *Source, group, and serotype of Clostridium perfringens strains related to Hobbs' heat-resistant types*

Source	Group no.	Hobbs' serotype												
		1	2	3	4	5	6	7	8	9	10	11	12	Total
Food poisoning in England, Sweden, and Japan	1	2	4	2			1			1		1		11
Food poisoning in the U.S.	2			1	1		1	1	1	3	2		1	11
Unincriminated food in the U.S.	2							1						1
Pathology, feces, and soil in the U.S.	3	—	—	—	—	—	—	—	—	—	—	—	—	0

swine bloods were relatively difficult to interpret in that partial lysis was not a clearcut reaction as separated from a weakly complete reaction. The use of guinea pig blood did not allow any separation of the hemolytic toxins; whereas the use of horse blood minimized the effect of the alpha toxin in the presence of theta toxin. Though Brooks et al. (1957) originally reported good differentiation between strains on the basis of their reactions on horse and ox bloods, the data presented above indicate that the use of sheep blood in addition to horse and ox blood yields excellent estimates of the toxin-producing abilities of the strains under study. This is more clearly evident in view of our finding that of 83 strains studied only 1 failed to give a reaction on sheep blood and that with 5 strains alpha toxin activity was detected on sheep blood only.

Serological relationships. Of the 83 strains studied, 36 were found to be serologically related to Hobbs' 13 "heat-resistant" serotypes. Of these, 13 were the prototype cultures with which the typing sera were prepared. The remaining 23 strains are listed in Table 5, which indicates their source, serotype, and group (1, 2, or 3).

All 23 of these strains were serologically related to the prototype cultures, but it appears doubtful that they are all serologically identical. Of the 23 strains, 11 agglutinated to titer or to one dilution less than titer; whereas 12 had titers two or more dilutions less than the homologous strains. However, all of the latter 12 strains had titers of 1:160 or more, which was well above the dilution (1:10) that sometimes yielded cross reactions between the prototype strains.

Comparison of the characteristics of the three groups of C. perfringens strains. A comparison of the characteristics of the 30 strains in group 1 is shown in Table 1. Of these strains, 24 (80%) agglutinated in sera produced against the prototype cultures. As a whole, the group displayed consistent but poor spore production. Although spores were produced by every strain except F4733/59, seven strains (23%) did not produce spores capable of resisting 100 C for 30 min or more. The hemolytic pattern of this group was characterized generally by a negative reaction on horse blood and partial hemolysis on ox and sheep bloods.

Group 2, composed of 20 strains from food-poisoning outbreaks and 8 from edible food, is treated as a unit, since the characteristics of both subgroups are so similar (Table 2). The group is characterized by an extreme degree of variability of reactions. Although 12 strains (43%) were serologically related to Hobbs' "heat-resistant" types, only 6 strains (21%) sporulated poorly, and only 7 strains (25%) produced heat-resistant spores. The hemolytic pattern was also extremely variable. Six strains resembled the strains of group 1. One strain (S79) produced no hemolysis on horse, ox, or sheep bloods. The majority of the strains in this group produced complete or partial and complete hemolysis on all three bloods.

The group 3 strains are characterized by a high degree of uniformity of reaction (Table 3). None was serologically related to Hobbs' heat-resistant types, all but one (6442) sporulated readily, and none produced heat-resistant spores. The hemolytic pattern was very uniform in that 23 strains (92%) produced either complete or a combination of partial and complete hemolysis on horse, ox, and sheep bloods.

DISCUSSION

When we examine the dominating characteristics of these three groups, we find that 20 strains (67%) in group 1 had the characteristics described by Hobbs (Hobbs et al., 1953) for heat-resistant strains of *C. perfringens* type A in that they agglutinated with typing sera, produced heat-resistant spores, and displayed a hemolytic pic-

ture of only partial hemolysis on horse, ox, and sheep bloods. Although this includes a majority of the strains, it is not as high a percentage as might be expected from the English reports. It should be noted, however, that three strains (F143/60, F4733/59, and F4824/59) were sent to us as being atypical of the group and that four additional strains (IU 1332, 1500, 2131, and 2159) had been maintained in stock culture for some time and may have lost their heat-resistant characteristics. On the other hand, the findings of McNicol and McKillop (1958) and McKillop (1959) indicate that food poisoning may be caused by strains of *C. perfringens* not fitting the usually accepted criteria.

The characteristics of the strains in group 3 formed a more uniform pattern than did those of either of the other two groups. None agglutinated in typing sera or produced heat-resistant spores. Most produced a combination of partial and complete hemolysis on horse, ox, and sheep bloods. These classical *C. perfringens* type A strains are commonly associated with wound infections and are referred to by the English workers as the hemolytic, or "beta" hemolytic strains. In Great Britain, these are not usually considered food-poisoning strains, because they are assumed to be destroyed by cooking, owing to their inability to produce heat-resistant spores.

The strains of group 2 failed to correspond exactly to either of the above groups. Only one (IU-1168) was identical to the majority pattern of group 1 and only seven to the pattern of group 3. The remaining 20 strains displayed certain characteristics of both groups 1 and 3 in an apparently random fashion which defied interpretation. Since most of the strains belonging to group 2 were isolated from foods implicated in food-poisoning outbreaks in the United States, these data tend to foster the conclusion that many of these *C. perfringens* food-poisoning outbreaks were caused by strains which, in the British view, are not accepted as food-poisoning types.

It should also be kept in mind, however, that the English methods of isolation tend to select the type of organism they believe is of primary importance. On the other hand, American methods have tended to isolate all types of *C. perfringens* present, and it is suspected that in many instances little attention has been paid to accurate enumeration. In spite of this, until a much larger number of outbreaks have been carefully studied, it seems advisable that the presence of large numbers of *C. perfringens* in implicated food, regardless of their sporulation and hemolytic and serological characteristics, should be considered of etiological significance. If the organisms were present before cooking and possessed heat-resistant spores, then the development of numbers of vegetative cells large enough to cause food poisoning would indicate inadequate refrigeration after cooking. On the other hand, if the strain of *C. perfringens* involved did not possess heat-resistant spores, the food must have been contaminated through improper handling after cooking and the contamination permitted to develop to a significant level.

In view of the findings presented in this report, it appears inadvisable to emphasize any one criterion in attempts to define the nature of the *C. perfringens* strains associated with foodborne disease in this country. Since the evidence from outbreaks here and in England (Hobbs et al., 1953) and from experimentally produced disease (Hobbs et al., 1953; Dische and Elek, 1957) indicates that high levels of contamination are necessary for disease production, it would seem more appropriate to emphasize a determination of the numbers of *C. perfringens* present in implicated foods, rather than the serological or heat-resistant types present. However, valuable epidemiological evidence may be gathered by additional studies of these characteristics of the strains. For example, determinations of the serological types present may yield information concerning food and patient relationships or implicated food and food-handler relationships. A determination of the ability of the strains involved to produce heat-resistant spores may indicate whether sanitary precautions were neglected prior to or after cooking.

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