Characterization of *Clostridium perfringens* (*welchii*) Isolated from Market Poultry[1]

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ABSTRACT

YAMAMOTO, RICHARD (University of California, Davis), WALTER W. SADLER, HENRY E. ADLER, AND GEORGE F. STEWART. Characterization of *Clostridium perfringens* (*welchii*) isolated from market poultry. Appl. Microbiol. **9**:337–342. 1961.—Strains of *Clostridium perfringens* capable of producing heat-resistant spores, characteristic of the food-poisoning types, were not recovered in a random survey of feces and livers of market poultry. Favorable growth response with a known food-poisoning strain indicated that the media and methods employed were adequate. Spores produced in vitro from this strain survived at 100 C for several hours. Animal feeding experiments with this strain showed that heat-resistant spores (surviving for 1 hr at 100 C) could be readily demonstrated 24 hr after oral instillation of vegetative cells in mouse feces, but not in chicken feces. One experiment suggests that this strain might adapt to the environment of the intestinal tract of chickens, but not all of the spores recovered were as heat resistant as those of the parent culture.

Clostridium perfringens (*welchii*) was conclusively established as an etiological agent of food poisoning by Hobbs et al. (1953); McClung (1945) had earlier assigned it this role in two such outbreaks in the United States. Work by Dische and Elek (1957) with human volunteers confirmed Hobbs' findings that ingestion of cultures of this organism would produce food poisoning.

Recent reports (McNicol and McKillop, 1958; National Office of Vital Statistics (NOVS), 1959) have implicated poultry products in three outbreaks as the source of a food-poisoning strain of *C. perfringens*, as had McClung s much earlier report on two such outbreaks. Since routine laboratory procedures in investigating food poisonings do not detect this anaerobe and since the NOVS (1959) listed poultry products as the source of infection in 27% of the episodes of gastroenteritis of unknown etiology in 1958 (Dauer and Davids, 1959), these reports take on added significance. Shapiro and Sarles (1949) examined the intestinal flora of normal fowl and reported the principal obligate anaerobe to be *C. perfringens*. Their finding that anaerobic agar plate counts paralleled the aerobic suggest that large numbers are present in the intestines.

The study reported herein was conducted to characterize the strains of *C. perfringens* found in market poultry and to compare them with known food-poisoning strains.

MATERIALS AND METHODS

Source, cultural, and serological characteristics. In a random sampling of market poultry for *Salmonella* (Sadler et al., 1961), 804 fecal and 1,819 liver samples were cultured for anaerobes. A loopful of feces was inoculated into Ellner's sporulation medium (Ellner, 1956), incubated 24 hr at 37 C, and heated 10 to 12 min at 80 C. One milliliter was then subcultured to freshly prepared thioglycolate medium with resazurin (BBL)[3] and incubated 24 hr at 37 C. Cultures showing gas formation and large gram-positive rods were streaked to blood agar (bovine or horse) and incubated under hydrogen at 37 C for 24 to 72 hr. Colonies suspected as *C. perfringens*-type were picked for Gram-stain examination and catalase reaction. To facilitate isolation, the plates were held overnight at room temperature. Aerobes that continue to grow under these conditions are recognized readily in subsequent examination (Hobbs et al., 1953). The surface of the liver was seared with a hot spatula, and deep stab cultures were taken and placed in thioglycolate medium. Cultures showing gas formation and large gram-positive rods after 24 to 48 hr of incubation were streaked to blood agar for anaerobic incubation. Subsequent isolation was performed in a manner similar to that used with the fecal cultures.

Strains of *C. perfringens* were isolated from the feces of 9.6% of the fryers, 16.4% of the adult fowl, and 15.5% of the turkeys, and from the livers of four fryers and one fowl. On bovine blood agar all isolates produced the characteristic double zone of hemolysis; on horse blood agar a single zone of clear hemolysis was usual.

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The colonies were medium-sized, opaque, smooth, and catalase negative. Smears of pure cultures stained by the Gram method showed large gram-positive rods with rounded ends. Shorter forms, almost oval, were predominant upon continued subculture on artificial media. No spores were seen in preparations from thioglycolate or meat piece media. All of the isolates produced acid in sucrose, glucose, maltose, and lactose. Salicin was fermented by a few isolates. Nitrate was reduced, indole was not produced, gelatin was liquefied, and stormy fermentation occurred in iron milk. Toxin neutralization tests in mice with 11 isolates from 11 separate samplings indicated that they were type A strains. Several isolates inoculated intravenously in mice produced hemoglobinurea and death, indicating the presence of α-toxin.

Hobbs et al. (1953) listed several characteristics as useful criteria in classifying strains of *C. perfringens* as food-poisoning strains. They reported that food-poisoning strains are type A, produce heat-resistant spores, have recognizable somatic antigens, produce feeble toxins, and have nonhemolytic colonies. Reported of greatest significance was the characteristic of producing spores that will withstand 100 C for 60 min. Eight distinct serological types were reported among the food-poisoning strains at that time.

A slide agglutination test was performed by the method of Hobbs et al. (1953). The somatic antigens prepared from the poultry isolates were tested against antisera prepared from 10 food-poisoning strains of *C. perfringens*. The type sera, provisional types 1 through 10, were received through the courtesy of Dr. Hobbs. Of the cultures tested, 14.5% were agglutinated by one of the 10 sera. Serological typing is of value only from an epidemiological standpoint, as pointed out in earlier reports (Hobbs et al., 1953; Dische and Elek, 1957; and McNicol and McKillop, 1958).

Heat resistance of spores. Sixty-three isolates were initially tested for the heat stability of their spores. One-milliliter amounts of actively growing cultures were subcultured to Ellner's medium in triplicate. After 24-hr incubation at 37 C, spores were readily observed in all except eight isolates of fecal origin. One series of spore cultures was heated 10 min at 80 C, the second for 15 min at 100 C, and the third for 60 min at 100 C. The cultures were then rapidly cooled and subcultured to thioglycolate medium, which was held 10 days for detection of growth. As a further check on viability, a representative number of cultures were plated to blood agar and incubated anaerobically. All cultures, including those that appeared negative by spore stain, survived heating for 10 min at 80 C, but none survived the more severe heat treatments.

A study was conducted with three of the isolates to determine: (i) the extent of sporulation in Ellner's medium, (ii) the effect on spore heat stability of prolonged incubation in Ellner's medium, and (iii) the protection during heating provided by different media. The oval tube method of Mossel et al. (1956) was used to enumerate vegetative inoculum and subsequent spore harvest. Ellner's medium, inoculated with a 6-hr culture of each strain, was harvested 42, 90, and 208 hr after incubation at 37 C. Ringers diluent (quarter strength) was used to suspend the spore harvest (Gibbs and Hirsch, 1956). Total counts were on unheated suspensions, and spore counts were on suspensions heated 20 min at 75 C (Gibbs and Hirsch, 1956). Spore yield, 10% from one strain and 40% from the other two, did not increase from the 42- to the 208-hr periods. One milliliter of spore suspension (adjusted to contain about 10^7 spores per ml) from each interval was inoculated in duplicate in iron milk, meat piece medium and thioglycolate medium. The sets were heated at 100 C, one for 15 min and the other for 60 min. Spores so treated failed to survive, as determined by subsequent incubation at 37 C.

The next consideration was the possibility that another type of sporulation medium might yield spores of greater heat stability. An alkaline egg broth (Robertson, 1915) was chosen since Willis (1957) obtained favorable spore yields with this medium in his study of *C. perfringens* from a water supply. Furthermore, he found that spores produced in this medium survived 100 C for at least 20 min.

Spores produced in this medium from 19 isolates were tested for heat stability by the methods and media described. For comparison, spores produced in Ellner's medium were also tested. The spore inocula from each source of harvest contained 10^5 to 10^7 spores. Heat-resistant spores again appeared absent; spores from either medium failed to survive even 15 min at 100 C.

RESULTS

The negative results of the first portion of the survey of market poultry for strains of *C. perfringens* that produced heat-resistant spores (failure to survive treatment of 100 C for 1 hr) were felt to be of equivocal significance. Earlier reports had stated (Hobbs et al., 1953) that some strains produced heat-resistant spores in mammalian feces but lost this characteristic in artificial media.

Therefore a series of 160 paired fecal samples was taken from 12 lots of turkeys, fowls, and fryers. One of each pair was cultured as described above, the other was inoculated into either meat piece or nutrient broth media (Hobbs et al., 1953) and immediately heated for 1 hr at 100 C. These cultures were then rapidly cooled, incubated 18 to 24 hr at 37 C (aerobically for the meat piece cultures and anaerobically for the nutrient broth cultures), and plated to blood agar for pure culture study as outlined above. The results are

presented in Table 1. Although 41 of the samples yielded vegetative cells or heat-labile spores, none yielded spores that would withstand 100 C for 1 hr (heat-resistant spores).

Six of the isolates possessing somatic antigens common with Hobbs' food-poisoning strains were fed to 6-week-old Webster strain mice, and attempts were made to recover heat-resistant spores from their feces after various intervals. Heat-resistant spores from these strains had not been demonstrated in the feces of birds or in vitro, but the possibility was considered that another living host might be conducive to the development of such spores. The mouse was chosen since it has been shown that heat-resistant spores can develop in the intestinal tract of this animal (Hobbs et al., 1953). Although 3 of the 6 serotypes were recovered, heat-resistant spores could not be demonstrated in the feces of mice, either 2 or 6 days after feeding.

Susceptibility to sodium azide. A report by Mossel et al. (1956) indicated that sodium azide added to the medium in a concentration of 0.02% selectively inhibited the growth of strains of *C. perfringens* of the food-poisoning type. A quantitative comparison of growth in Mossel's medium, with and without sodium azide, was made with inocula containing both vegetative cells and spores of 19 strains. The growth of two were inhibited by this compound, but the inhibition was not complete: a narrow band of growth was observed near the surface of the tube.

Comparison with C. perfringens strain Hobbs 8797. Failure to demonstrate heat-resistant *C. perfringens* from poultry feces or from subcultures of poultry isolates left unanswered the question of the adequacy of culture media and methods employed. To help answer this question a strain of *C. perfringens* (8797) isolated from a food-poisoning outbreak was obtained from Dr. Hobbs for comparative studies. Dische and Elek (1957) had demonstrated that this strain was active in producing symptoms of food poisoning in human volunteers. A 24-hr culture on blood agar developed a very faint zone of hemolysis unlike those seen with the non-heat-resistant poultry isolates.

TABLE 1. *Results of Clostridium perfringens isolation from feces by two methods of culture*

Class	No. lots examined	No. samples	Culture method*		
			100 C heating for 1 hr; no. positive	Ellner's medium	
				No. positive	No. positive 1–10†
Turkeys	7	110	0	28	10
Fowl	3	30	0	10	5
Fryers	2	20	0	3	1
Total	12	160	0	41	16

* Refer to text for culture methods.

† Number of those positive in Ellner's medium that were serologically typed as being in Hobbs sera groups 1 to 10.

Heat resistance studies were conducted using spores from strain 8797. These studies indicated that when strain 8797 spores were produced in akaline egg broth or Ellner's medium: (i) as few as 5 of these spores suspended in meat piece medium survived heating for at least 1 hr at 100 C and (ii) some portion of an inoculum of 20,000 spores suspended in meat piece medium was viable after being heated for 5 hr at 100 C, the longest period tested. Quantitative studies indicated that the growth of this strain was completely inhibited in media with sodium azide (0.02%).

Several feeding trials were conducted to determine the ability of a known food-poisoning strain (8797) to establish itself in the intestinal tract of poultry and produce heat-resistant spores in that environment. In several of the trials a poultry isolate, strain UU7, was fed to the control group. This strain produced no heat-resistant spores and produced distinct double zones of hemolysis on bovine blood agar, although sodium azide markedly inhibited its growth and the organism was agglutinated by antiserum to Hobbs' serotype 5. No strain was isolated that was antigenically similar to 8797 (Hobbs' serotype 1), which facilitated accurate identification of the isolates from experimental inoculates.

Cultures of the two strains were grown for 18 hr in meat piece medium with glucose. For strain 8797, an aliquot of 1 ml containing 5×10^8 cells was administered per os into each of two adult male White Leghorn chickens, and 0.2 to 0.5 ml (1×10^8 to 2.5×10^8) of the same culture was given by the same route to each of four Webster mice. Strain UU7 was inoculated similarly—in doses of 7×10^8 organisms into each of two chickens and 1.4×10^8 to 3.5×10^8 organisms into each of four mice. Spore counts indicated that fewer than 10 spores were present per ml of inoculum for each strain. Both the chickens and mice had previously been demonstrated to be free of *C. perfringens*. Heat-resistant spores (cultures viable after heating 1 hr at 100 C) were isolated from mouse feces taken at 24 and 48 hr but not at 144 hr after feeding strain 8797, whereas cultures of chicken feces were negative at each sampling. Neither mouse nor chicken feces from those fed strain UU7 yielded heat-resistant spores.

In a second trial 14 chickens and 10 mice were inoculated with strain 8797. The inoculum consisted of a greater ratio of spores to vegetative cells in one group of chickens, the culture was placed in feed in another, and rectal administration of the culture was made in a third. Strain 8797 spores were not recovered from feces of birds from all groups taken 8, 24, or 48 hr after inoculation. The culture technique was also varied in attempts to recover the organism. Pooled feces taken

at various intervals and stored for 8 days in a refrigerator were cultured with negative results. Two birds from the group that received a high ratio of vegetative cells to spores, and two that received a low ratio, were killed at 24 hr, and various parts of the intestinal tract were cultured to determine whether localization of the organism had occurred. Material from the crop, proventriculus, anterior and posterior portions of the duodenum, cecum, and large intestine were negative for both the vegetative and spore form of this organism. Cloacal swabs taken from the remaining birds at 48 hr failed to yield either form of strain 8797. Mouse feces yielded heat-resistant spores of 8797 at 8 and 24 hr, but not at 48 hr.

Tests were made of the ability of strain 8797 to sporulate in chicken feces (10%) suspended in distilled water. Since the pH of fresh mouse feces was 7.8 and that of chicken feces was 7.0, the suspension was divided in two aliquots, one adjusted to pH 7.0 and one to pH 7.8. The larger particles were allowed to settle out, and the fluid was poured off, distributed into tubes, and autoclaved. The sterile medium was inoculated with a meat piece culture of strain 8797 in duplicates. The two series were incubated anaerobically for 72 hr, one at 37 C and the other at 42 C. Suspensions from each culture prepared by centrifugation were counted for spores, and aliquots were inoculated in duplicates into meat piece medium for heat studies. The results (Table 2) indicate that the spores that developed in chicken feces at pH 7.0 and 7.8 were heat resistant, regardless of temperature of incubation.

It is apparent that the intestinal tract of the chicken did not actively support the development of strain 8797. One would have expected to recover the original inoculum from the voided feces, however, and, further, the experiments in vitro showed that chicken feces did not adversely affect sporulation.

The samples were, perhaps, not taken early enough in these trials since the average time of passage of food through the intestinal tract of chickens is about 3 to 4 hr (Hillerman et al., 1953). Furthermore, since vegetative cells were originally inoculated, time for sporulation might have been insufficient. Experiments in vitro showed that heat-resistant spores from strain 8797 in Ellner's medium develop in about 18 hr. Consequently, in a further study, feces voided at short intervals after inoculation were sampled. An oral dose of 3.5×10^9 vegetative cells was given to each of 5 chickens. The birds were divided into two groups, respectively, 3 and 2 birds, and at each sampling period, feces were pooled from each group of birds. As a control, a similar dose of strain UU7 was fed to a similar number of birds.

TABLE 2. *Heat resistance of spores developed from Clostridium perfringens strain 8797 in natural enrichment cultures*

Media	Incubation temp	Vegetative cells (inoculum)	Spore count at 72 hr	Heat resistance*	
				No. spores in the inoculum	Survival, 60 min at 100 C
	C				
Chicken feces, pH 7.0	37	18×10^7	7.6×10^2	1.9×10^2	+
	42	18×10^7	2.4×10^3	6.0×10^2	+
Chicken feces, pH 7.8	37	36×10^7	4.0×10^4	1.0×10^4	+
	42	36×10^7	7.2×10^4	1.8×10^4	+

* Spores harvested at 72 hr were inoculated in numbers as indicated in meat piece medium for heat survival test.

TABLE 3. *Recovery of food poisoning Clostridium perfringens strain 8797 by various culture techniques from feces of chickens after feeding*

Method of culture†	Preinoculation		Interval between feeding and fecal collection (in hours) in groups* 1 and 2											
			(0–3)		(3–5)		(5–8)		(8–24)		(24–48)		(48–72)	
	1	2	1	2	1	2	1	2	1	2	1	2	1	2
Routine	c‡	c	—	c	c	c	c	c	c	c	c	c	H	—
S-15	—	—	—	—	—	—	—	—	H	—	—	—	H	—
S-60	—	—	—	—	—	—	—	—	—	—	—	—	—	—
S-15r	n	n	H	—	—	—	—	H	H	H	—	—	—	—
S-60r	n	n	—	—	—	—	—	H	—	H	—	—	—	—

* Feces was pooled from 3 chickens in group 1 and from 2 chickens in group 2 at each interval.

† Method of culture: (refer to text for details).

Routine: use of Ellner's medium for primary enrichment.

S-15: selective spore heating method (15 min at 100 C), feces cultured immediately.

S-60: selective spore heating method (60 min at 100 C), feces cultured immediately.

S-15r: selective spore heating method (15 min at 100 C), feces held at room temp. for 2 to 3 days prior to culture.

S-60r: as in S-15r except that the cultures were heated for 60 min.

‡ Cultures identified by agglutination test and cultural and heat-resistance characteristics; c = positive chicken strain but not strain 8797; H = positive for strain 8797; — = negative for *C. perfringens*; n = not done.

Cultures were made immediately after sampling at each interval and again after the samples were held at room temperature for 2 to 3 days, presuming that this holding period would permit sporulation. Similar to the earlier trials, heat-resistant organisms were not recovered from birds inoculated with strain UU7 at any interval. However, isolations were made from the group receiving strain 8797 as shown in Table 3. In general, it appears that holding the samples at room temperature 2 to 3 days before culturing increased the effectiveness of the isolation procedures. Contrary to the earlier trials, however, the feces from one of the two groups yielded the organism at the 8- to 24-hr and again at the 48- to 72-hr intervals when cultured immediately after sampling. It should be noted that at both intervals the organism was recovered when the feces was heated in culture medium for 15 min at 100 C but not when heated for 60 min at 100 C. Of interest is the fact that the feces voided at the 48- to 72-hr interval in one group yielded the organism by the routine method of culture, the only instance of recovery of this organism by this method in all of the feeding trials conducted. As mentioned earlier the success of this method depends on the presence of vegetative cells in the inoculum.

The more frequent isolation of strain 8797 from samples held at room temperature but not from fresh feces indicates that the organism was present in the fresh feces primarily in the vegetative form. The question then arises, why were not these vegetative cells detectable when fresh feces were cultured by the routine method? As indicated earlier, the method employed depends on sporulation of the vegetative cells upon inoculation into Ellner's medium, the primary enrichment medium used. In this medium, several of the isolates from chickens have been shown to sporulate quite readily, giving a high ratio of spores to vegetative cells. On the other hand it has been shown that only a fraction of the cells of strain 8797 sporulate in this medium. It is thus conceivable that, in the presence of a highly sporulative strain, strain 8797 may be masked when this method of culture is employed.

Discussion

The primary objective was to determine whether *C. perfringens* of the food-poisoning type was present in the intestinal tract of poultry. The main criterion for differentiation of this type of organism from the classical type A strains appears to be the marked heat resistance of the spores of the former. We were not able to recover such organisms from the livers or feces of the market poultry surveyed.

One might conclude that poultry, per se, are not an important reservoir of food-poisoning strains of *C. perfringens*. It would be too optimistic and without basis, however, to state that such organisms do not exist in this environment. Although studies with a known food-poisoning strain of *C. perfringens* (8797) indicate that the intestinal tract of poultry was not an ideal environment for its development, it was nevertheless able in some instances to support the development of spores of some degree of heat resistance. Studies in vitro also indicated that heat-resistant spores from strain 8797 could develop in the presence of chicken feces and at the chicken's normal body temperature, 42 C.

Aside from the fact that poultry meat may become contaminated with heat-resistant spores from other sources during the process of food handling (e.g., man is a very good reservoir), there is the possibility that heat-resistant spores may develop during storage of edible tissue contaminated with vegetative cells from the intestines. In Hobbs' survey (Hobbs et al., 1953) only 1.7% of the bovine fecal samples were positive although 24.1% of samples of raw beef were found to harbor this organism.

Possibly, the basic problem is not whether such organisms exist in poultry but rather the conditions under which heat-resistant spores are developed and the relationship of classical strains to their heat-resistant counterparts. The isolation in four instances of strain 8797 spores that would withstand 100 C for 15 min but not 60 min suggests that heat resistance of spores is not necessarily a stable characteristic of a given strain.

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Degeneration of *Streptomyces niveus* with Repeated Transfers

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ABSTRACT

REUSSER, F. (The Upjohn Company, Kalamazoo, Mich.), H. J. KOEPSELL, AND G. M. SAVAGE. Degeneration of *Streptomyces niveus* with repeated transfers. Appl. Microbiol. **9**: 342-345. 1961.—Novobiocin production by *Streptomyces niveus* decreased drastically as the culture was transferred at regular intervals under both sporulating and nonsporulating conditions. Addition of degenerated live mycelium as second inoculum to shake flask fermentations already inoculated with a high-producing strain resulted in sharply depressed novobiocin formation. Fractionated medium of low-producing strain containing either no cells or dead cells had no adverse effect on the antibiotic yield of the high-producing mycelium. It appears that the low-producing mycelium was outgrowing the high-producing mycelium. A study of the growth rates of the two types of mycelium in a clear broth medium indicated no differences in generation time. However, the low-producing strain proved to have a higher efficiency of carbohydrate utilization, thus overgrowing the high-producing strain. It was speculated that culture instability of *S. niveus* is due to heterocaryosis.

Maintenance of culture stability, particularly with respect to the development of continuous fermentations, is of prime importance in industrial antibiotic fermentations. Williams and McCoy (1953) and Perlman, Greenfield, and O'Brien (1954) have described changes in morphology and loss of antibiotic production when cultures of *Streptomyces griseus* were transferred at regular intervals.

We have found that *Streptomyces niveus* strains, selected for their ability to produce high novobiocin[1] titers, lose this ability markedly upon serial transfer of spores or vegetative mycelium.

[1] The trade-mark of The Upjohn Company for novobiocin is Albamycin.

The present study deals with the investigation of possible physiological bases responsible for the degeneration phenomenon observed with *S. niveus*.

MATERIALS AND METHODS

Cultural methods. Stock cultures of high-producing *S. niveus* strain BC-333 were stored on agar slants having the following composition: maltose, 10 g; Bacto-Tryptone, 5 g; K_2HPO_4, 0.5 g; NaCl, 0.5 g; $FeSO_4 \cdot 7\ H_2O$, 0.1 g; Bacto agar, 20 g; tap water to 1 liter; pH adjusted to 7.0 before sterilization. This strain originated from a single-spore isolate selected for its ability to produce novobiocin titers of 1,000 to 1,200 μg/ml (courtesy of Dr. B. W. Churchill).

The low-producing variant originated from the high-producing strain BC-333. It was transferred continuously at 3-day intervals in shake flasks in the vegetative state to insure a steady supply of low-producing cells during the experiments.

All fermentation studies were carried out in 500-ml wide mouth Erlenmeyer flasks containing 100 ml of medium. The flasks were incubated at 28 C on a rotary shaker at 250 rev/min with 2-in. eccentricity. The sulfite oxidation number as determined by the method of Cooper, Fernstrom, and Miller (1944) was 0.3 mM O_2/liter-min.

The fermentation medium used, unless indicated otherwise, was glucose monohydrate, 40 g; distiller's solubles,[2] 40 g; tap water to 1 liter; pH adjusted to 8.0 with NaOH before sterilization.

Analytical methods. Mycelial weight was determined by drying the centrifuged and washed cells in a vacuum oven at 65 C for 48 hr. Total carbohydrate was determined by the anthrone method of Neish (1952). Novobiocin was determined spectrophotometrically as described by Smith et al. (1958) and the values were uncorrected for isonovobiocin.

[2] Brown Forman Company, Louisville, Kentucky.