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Heat resistance of spores of *Clostridium welchii**

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INTRODUCTION

Extensive investigations have been undertaken by British scientists in order to determine the epidemiology of *Clostridium welchii* food poisoning (Hobbs *et al.* 1953; Hobbs, 1965). According to these workers the organisms responsible for this type of food poisoning are atypical strains of *Cl. welchii* 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 (haemolysin), formation of heat-resistant spores (100° C. for 1 hr. or more), and by antigenic constituents which allow them to be grouped into thirteen provisional serotypes.

Hall, Angelotti, Lewis & Foter (1963) working at the Robert A. Taft Sanitary Engineering Center, in the United States, examined 83 strains of *Cl. welchii* from a wide variety of sources for their serological relationships, sporulation, heat-resistance of spores, and their haemolytic activity on mammalian bloods. They found that the American food-poisoning strains have a wide variety of characteristics and concluded that *Cl. welchii* food-poisoning outbreaks in the United States were not restricted to strains meeting the criteria of classification described by the 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.

On the other hand, Sutton (1966*b*) investigated an outbreak of *Cl. welchii* food poisoning and isolated heat-resistant *Cl. welchii* from ten of the twelve persons attending a small gathering and from all seven who developed symptoms of food poisoning. In another study, Sutton (1966*a*) studied the incidence of heat-resistant *Cl. welchii* in selected classes of a rural population and found that the carrier rate for the general population was low (1·5–6·0 %), but persons associated with communal feeding and poor hygienic conditions had a much higher carrier rate (15·1–25 %). Sutton (1966*a*) suggested that heat-resistant spores of *Cl. welchii* withstood the cooking process, particularly if the portion of meat cooked is large and bulky, germinated, multiplied, and caused food poisoning upon ingestion by a susceptible person. It had been previously shown by Collee, Knowlden & Hobbs (1961) that heat-resistant *Cl. welchii* grows rapidly within a temperature range of 23–50° C.

The purpose of the present study was to examine the heat-resistance of the

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spores of *Cl. welchii* isolated from soil, faeces, and food poisoning outbreaks in order to determine if patterns of heat-resistance are related to the source of isolation

MATERIALS AND METHODS

Strains of Clostridium welchii

Eight strains of *Cl. welchii* type A were employed in this study. They were strain BP6K (Boyd, Logan & Tytell, 1948), source unknown; UM 115, isolated from soil at the University of Montana; F 106, isolated from a human faecal specimen; FH 153, isolated by Dr H. E. Hall, Robert A. Taft Sanitary Engineering Center, Cincinnati, from a faecal specimen from a food handler; A 91, isolated from a pathological specimen at the Cincinnati General Hospital and obtained from Dr H. E. Hall; A 48, recovered from chicken broth that caused human food poisoning by Dr L. S. McClung, Indiana University, Bloomington (McClung, 1945); Hobbs's serotype 2, National Collection of Type Cultures number 8238, isolated from boiled beef suspected to have caused food poisoning; Hobbs's serotype 3, National Collection of Type Cultures number 8239, isolated from boiled beef suspected to have caused food poisoning. The cultures were maintained in Bacto-Cooked Meat Medium (Difco). The stock cultures were routinely checked for purity using morphological, biochemical, and haemolytic criteria.

Preparation of spore suspensions

The method recently developed by Schneider, Grecz & Anellis, (1963) for the sporulation of *Clostridium botulinum* and *Cl. perfringens* (*welchii*) in dialysis sacs was used in the present study for the production of spores. The apparatus consisted essentially of a telescoped cellulose bag immersed into the sporulation medium in a large Pyrex culture tube (see schematic illustrations in the publication by Schneider *et al.*). This method was used successfully in producing spores of *Cl. welchii* in four laboratory media (Groom & Strong, 1966).

Cultures of *Cl. welchii* were transferred at 12 hr. intervals in 20 ml. of Bacto-Fluid Thioglycollate Medium (Difco) in order to obtain vigorous growth of the vegetative cells. Then 150 ml. of broth were inoculated and incubated for 20 hr. at 37° C. The cells were centrifuged, resuspended in physiological saline and recentrifuged three times, and then the packed cells were resuspended in 10 ml. of sterile physiological saline. The entire contents were placed in the sterile sporulation dialysis bag which was suspended in a modified Wagenaar and Dack medium (Schneider *et al.* 1963). This medium consisted of 5·0 % Trypticase, 1·0 % peptone proteose (Difco) and 0·5 % sodium thioglycollate (pH 7·2). The sporulation apparatus was incubated at 37° C. for 9 days. The spores were harvested according to the procedures described by Schneider *et al.* (1963) and washed three times with 50 ml. sterile Sorensen's phosphate buffer (M/15, pH 7·0). The method of Long & Williams (1958) was used to separate vegetative cells from the spores. The final suspension of spores, in 10 ml. Sorensen's buffer, was stored at 4° C. Immediately after the preparation of spores the spore suspensions were stained with malachite green and examined microscopically to determine the degree of sporulation achieved.

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Heat resistance studies

Thermal death time determinations were made using the stock spore suspensions which were diluted (1/100) with sterile Sorensen's phosphate buffer. Twenty-nine ml. of phosphate buffer, pH 7·0, was added to each reaction tube. The reaction tube consisted of a long Pyrex screw-capped culture tube containing a small ($\frac{5}{16}$ in. diameter) teflon-coated bar magnet. The reaction tubes were placed in a 6×10 in. basket and the basket was completely immersed in water contained in a constant temperature water bath at 90° C ($\pm$ 0·5° C). A stirring apparatus was used to rotate a small propeller-type blade to which was attached a teflon-coated bar magnet. As the blade with the magnet rotated in the hot water it agitated the small magnets nearby in the reaction tubes, inducing convection and cavitation, thereby exposing the spores to a more uniform water temperature. One of the reaction tubes contained a thermometer to record internal temperature. The tubes were allowed to reach equilibrium temperature and 1 ml. of the spore suspension was added to the tubes. Exposure to heat was timed 10 min. after the spores were introduced to the reaction tubes. The initial 10 min. period was considered a pasteurization period as well as a period for heat activation of the spores.

One-tenth ml. of the heated spore suspension was removed by a sterile 1 ml. pipette at designated intervals of time and immediately mixed in a culture tube containing 9·9 ml. of 1 % peptone-water which was previously cooled in an ice-water bath. The cooled contents were plated upon recovery medium consisting of 37 g. Bacto-Beef Heart for Infusions (Difco), 29·8 g. Bacto-Fluid Thioglycollate Medium (Difco), 15 g. agar, and 1000 ml. distilled water. Triplicate plates were inoculated. In addition culture tubes containing 10 ml. of fluid thioglycollate medium were inoculated. The plates and tubes were incubated in an anaerobic jar (Case Anaero-Jar) evacuated and filled with nitrogen gas at 37° C. for 48 hr. Plate counts were made with the aid of a Quebec darkfield colony counter and tubes were examined for the presence of growth.

Treatment of data

A logarithmic order of death was assumed and the data were treated according to the methods of Stumbo, Murphy & Cochran (1950), using the formula $D = U/(\log a - \log b)$. Using this formula D-values were computed from the straight-line portions of the thermal death time curves, where U = time of heating (minutes), a = initial spore population, and b = number of spores surviving at the end of heating period U.

Survival curves of the heat-treated spores were prepared by averaging arithmetically the three individual plate counts for each sample removed and determining the percentage survival and plotting these values as a function of time (exposure to heat). The trend in the data was analysed using the least-squares method. Only the data from the linear portion of the survival curves were treated by this method.

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RESULTS

The sporulation method employed resulted in the complete production of spores. No vegetative cells were observed when the spore suspensions were stained with malachite green and examined microscopically. The spores were stored at 4° C. for several months without any apparent change in the numbers of spores. This procedure for sporulation was consistently reproducible.

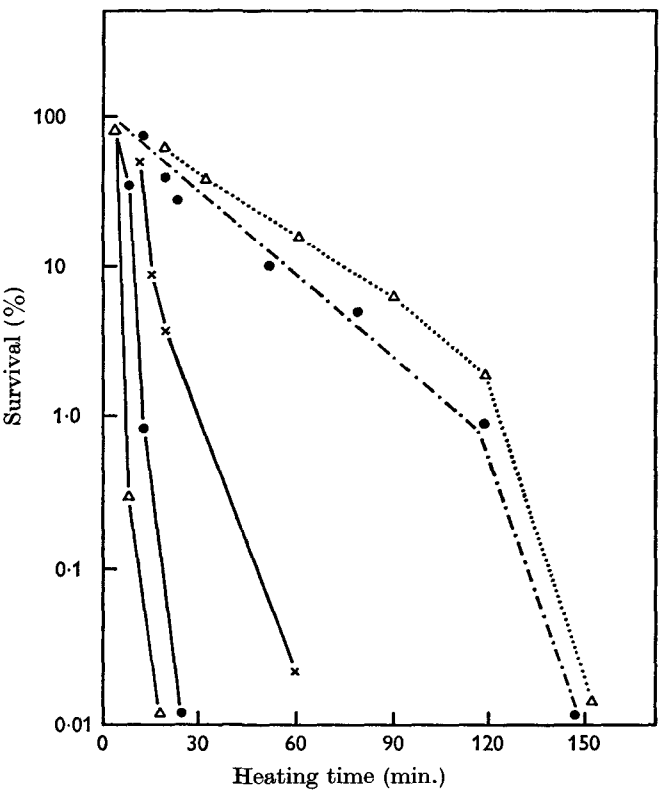

Fig. 1. Thermal survival curves for six strains of *Clostridium welchii*. △——△, UM 115; ●——●, BP6K, F 106; ×——×, FH 153; ●—·—●, Hobbs 3; △ - - - - △, Hobbs 2.

The effect of heat (90° C) on spores produced from different strains of *Cl. welchii* as a function of time is shown in Fig. 1. All strains exhibited a thermal death rate that was essentially exponential. Thermal destruction for strains UM 115, BP6K, F 106, and FH 153 was immediate and complete. On the other hand, strains Hobbs 2 and Hobbs 3 displayed considerable resistance to heat destruction. The heat-resistant spores could not be recovered after approximately 150 min. on the recovery plates but growth did occur in fluid thioglycollate medium. In some instances survivors grew in fluid thioglycollate medium after heating for 180 min. Weiss & Strong (1967) also reported similar findings.

D-values are presented in Table 1 for eight strains of *Cl. welchii*. The Hobbs strains displayed a significantly higher *D*-value. Weiss & Strong (1967) reported a

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similar relationship, although their *D*-values were somewhat greater. A strain, UM 115, recently isolated from soil had the lowest *D*-value. This value was two-and-a-half orders below that of the food-poisoning strains.

The *D*-values were computed with the least-squares method and the trend of the data is shown in Table 2. When the values were plotted on graph paper a straight line trend was obtained.

Table 1. *D-values of* Clostridium welchii *spores heated at* 90° C

Strain of *Cl. welchii*	*D*-values
UM 115	0·015
BP 6K	0·63
F 106	1·45
FH 153	2·28
A 48	7·07
A 91	4·93
Hobbs 3	6·05
Hobbs 2	8·71

Table 2. *Computed trend values of the D-values using the least-squares method*

Length of time heated at 90° C in minutes	Strain of *Cl. welchii*					
	A 48	A 91	F 106	FH 153	Hobbs 2	Hobbs 3
10	14·49	10·17	2·71	4·72	12·81	9·11
20	12·29	8·50	0·20	3·04	11·82	7·89
30	10·10	6·83	0	1·36	10·84	6·67
60	7·90	5·16	—	0	9·86	5·45
90	5·71	3·50	—	—	8·86	4·23
120	3·52	1·83	—	—	7·20	2·96
150	1·32	0·16	—	—	6·92	0

DISCUSSION

The heat-resistance of spores of *Cl. welchii* has been studied by numerous workers (Hobbs *et al.* 1935; Sutton, 1966*a*; Weiss & Strong, 1967; Hall *et al.* 1963). Since *Cl. welchii* is ubiquitous its heat sensitivity has been studied in many different parts of the world. It is apparent that heat-resistant and non-heat-resistant spores occur in nature (Collee *et al.* 1961). Hall & Angelotti (1962) reported that the spores of only 5 of 19 food-poisoning strains possessed heat resistance. The spores of *Cl. welchii* isolated by Dam-Mikkelsen, Petersen & Skovgaard (1962) from food-poisoning outbreaks in Denmark did not survive heating at 100° C. for 2 min. On the other hand Tong *et al.* (1962) investigated a food poisoning traced to turkey meat and isolated an aerobic sporulating bacillus which withstood boiling temperatures for 8 hr. Therefore, it appears that there is considerable variation in the reported findings with regard to the heat-resistance of spores of *Cl. welchii* isolated from food-poisoning incidents.

In the present study, although the number of strains studied was admittedly small, the organisms isolated from food-poisoning sources possessed a greater degree of heat-resistance than the strains isolated from other sources, particularly

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soil. It has been suggested that some strains loose their heat resistance on repeated laboratory culture (McKillop, 1959). One strain of *Cl. welchii* which had its origin in a food-poisoning outbreak was readily killed at 80° C. for 10 min. (Canada, Strong & Scott, 1964). However, this strain had been carried on laboratory media for approximately 10 years.

It is conceivable that *Cl. welchii* isolated from food-poisoning outbreaks tend to exhibit heat-resistant spores (Brooks, Sterne & Warrack, 1957) primarily because the heat-sensitive spores were destroyed during the process of cooking the food. This could select the heat-resistant spores, although initially the spore population may have comprised both heat-resistant and heat-sensitive spores. It is well established that different strains of the same species may possess spores of unequal resistance to heat (Williams, 1929; Williams & Zimmerman, 1951; Mehl & Wynne, 1951; Briggs, 1966).

Nevertheless, it is reasonable to assume that the heat-resistant spores are primarily involved in *Cl. welchii* food poisoning because these are the spores that are more likely to resist destruction during the cooking or re-heating processes.

Currently, we are engaged in a study of the heat-resistance of spores isolated from various sources, namely, faeces, soil, food, and food-poisoning cases, and hope to correlate this property with lecithinase activity of these strains.

SUMMARY

Eight strains of *Cl. welchii* were studied for the heat-resistance of their spores. Spores of *Cl. welchii* isolated from food-poisoning cases had greater heat-resistance than strains isolated from soil or faeces. *D*-values and trend values were calculated from the thermal death curves.

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