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Enumeration of *Clostridium welchii* in the faeces of varying sections of the human population

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INTRODUCTION

The work of Hobbs *et al.* (1953) showing that heat-resistant, non-haemolytic *Clostridium welchii* was a cause of food poisoning began a series of investigations to determine the incidence of this organism in varying sections of the population (Leeming, Pryce & Meynell, 1961; Collee, Knowlden & Hobbs, 1961).

Most of such investigations centred on the qualitative distribution of the organism, and little quantitative work has been carried out on the viable cell counts of *Cl. welchii* in faeces. Smith & Crabb (1961), while investigating the development of faecal flora of animals, carried out counts on a small group (ten persons) of human faeces. They obtained a median count of 1585 *Cl. welchii* per gram. Collee *et al.* (1961) and Goudie & Duncan (1956) both used semi-quantitative techniques and recorded large numbers of *Cl. welchii* present on many occasions. No actual values were given. In the light of these results it was decided that a series of quantitative counts of *Cl. welchii*, both haemolytic and non-haemolytic, would be of some value.

The investigation was intended to compare the counts obtained in groups of healthy individuals, in symptomless excretors of heat-resistant *Cl. welchii* and in a small group of persons associated with an outbreak of food poisoning due to *Cl. welchii*. It was also hoped to determine the value of viable cell counts as a diagnostic tool in investigating outbreaks of food poisoning attributed to *Cl. welchii*.

MATERIALS AND METHODS

Media used

The egg yolk medium was that of Willis & Hobbs (1958) with the medium base adjusted to pH 7·0. Other media used were blood agar (7·5% defibrinated horse blood added to oxoid blood agar base) and Robertson's cooked meat medium.

Neomycin sulphate (Upjohn) was added to the above media, excepting the cooked meat medium, at a concentration of 100 μg./ml. At this concentration the growth of most other organisms found in faeces was suppressed, while the growth of *Cl. welchii* was unaffected. Increasing the concentration to 250 μg./ml. reduced the count obtained with some strains of *Cl. welchii* by 50%.

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Sources of faecal specimens

Two hundred and fifty faecal specimens were collected in sterile glass containers from three distinct classes of the population. These were: class A, primary school children; class B, members of the general population attending their own general practitioner for miscellaneous minor illnesses; class C, aboriginal persons. In addition faecal specimens were collected from a small group of persons associated with an outbreak of food poisoning due to *Cl. welchii*.

Method of examination

All faecal specimens were examined for *Cl. welchii* by the following three techniques.

1. *Total viable cell counts* of *Cl. welchii* in faeces were carried out by a modified Miles and Misra technique of Smith & Crabb (1961). One gram of faeces was inoculated into 9 ml. of Ringer's solution and four to six glass beads (2 mm. diameter) were added. The mixture was shaken on a mechanical shaker at low speeds for 5 min. This was found to be sufficient to emulsify the faeces. It was then filtered through sterile muslin, and used as a 1/10 dilution of faeces. From this, 1/100 and 1/1000 dilutions were prepared and counts were made using the technique of Miles & Misra (1938). The plates were incubated anaerobically for 24 hr. at 37° C.

2. *Heat-resistant spore counts* were carried out on the filtered 1/10 faecal suspension used above. After doing the total cell counts the 1/10 suspension was held at 100° C. for 30 min., then allowed to cool. The counts were carried out by inoculating 5 drops from a 50 drop per ml. pipette on one plate, and in addition by flooding a second plate with 1 ml. of the boiled suspension. On this latter plate spores could be detected in numbers as low as 10/g. of faeces. The experimental error associated with such low counts would of course be high.

3. *Enrichment technique for the detection of heat resistant spores of* Cl. welchii. This was a modified method of Hobbs *et al.* (1953) One to two grams of faeces were inoculated into tubes of cooked meat medium which were then held at 100° C. for 60 min. and incubated overnight at 37° C. In this way any heat-resistant spores present were concentrated. The tubes were then subcultured on to the egg yolk and blood agar media and incubated anaerobically for 24 hr. Results were recorded as presence or absence of heat resistant spores of *Cl. welchii*.

In order to test the reliability of the Miles & Misra method with faecal suspensions, a known inoculum of *Cl. welchii* was added to a series of tubes containing 9 ml. of Ringer's solution with and without the addition of 1 g. of sterilized faeces, and counts were carried out. The counts in the presence of faeces were slightly lower than those without faeces, but they were quite reproducible.

RESULTS

It was found impossible to make accurate separate counts of haemolytic and non-haemolytic colonies, as faecal matter, particularly in the 1/10 dilution,

obscured observation of haemolysis. Values given in the quantitative section (Table 2) therefore represent total counts.

Of the 250 specimens examined *Cl. welchii* was identified in 175. In the majority of cases only haemolytic *Cl. welchii* was detected; non-haemolytic *Cl. welchii* were isolated from only thirty-nine of the specimens. Twenty-two of the specimens showing non-haemolytic colonies also yielded heat-resistant spores by the enrichment technique. The additional seventeen non-haemolytic strains, isolated from specimens that did not produce spores by the enrichment technique, were further examined. The spore cultures were obtained by the methods described by Hobbs *et al.* (1953). These spores failed to survive 100° C. for 30 min. in cooked meat medium.

Table 1. *Distribution of haemolytic and non-haemolytic* Clostridium welchii *in selected classes of the population*

Class	Number examined	Number showing haemolytic	Number showing non-haemolytic	Heat resistant spores
A	100	69	8	1
B	50	35	5	3
C	100	71	26	18
Total	250	175	39	22

Table 2. *Primary data: viable counts of* Clostridium welchii *in faeces of selected classes of the population; counts are expressed as colonies per drop* (0·02 *ml.*) *of a* 1/100 *faecal suspension*

Range Colonies/drop (0·02 ml.)	Number of specimens within range			
	Class A	Class B	Class C	Overall
Less than 1	33	16	30	79
1– 10	21	10	24	55
11– 20	16	8	14	38
21– 40	12	5	11	28
41– 60	7	4	9	20
61–100	7	4	6	17
101–200	1	1	3	5
201–300	2	2	2	6
350	1	—	1	2

The results for the individual classes are given in Table 1.

By quantitative methods it was possible to record *Cl. welchii* in as small a number as 100/g. of faeces, but because of sampling error such low counts would of course be unreliable. Table 2 gives the primary data for the counts obtained with the varying classes. It can be seen that over 50 % of the specimens in all classes were within the range $0–5{\cdot}0 \times 10^4$ organisms per gram. (i.e. less than ten colonies per drop as shown in Table 2), while 95 % of the specimens contained 5×10^5 or less organisms per gram.

From the primary data given in Table 2, the mean, standard deviation and median count were calculated. These values are given in Table 3.

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Inspection of these results immediately shows that the counts are scattered widely about the mean, with a very large standard deviation. They obviously do not represent a normal frequency distribution curve. In such a series of results one or two extreme readings will unduly influence the mean count, so it was considered that the median was the more informative way of expressing these results.

The variation in the daily excretio n of *Cl. welchii* was investigated by examining six specimens—2 per week for 3 weeks—from the same person using the same techniques. The results ranged from $1{\cdot}5 \times 10^3$ to $2{\cdot}05 \times 10^5$ organisms per gram. This is a very significant variation, and might help to explain the large standard deviation and wide range of results given in Tables 2 and 3.

Table 3. *Viable counts of* Clostridium welchii *present in faeces of* (*a*) *selected classes of the population, and* (*b*) *persons associated with an outbreak of* Cl. welchii *food poisoning. Results are expressed in organisms per gram and were calculated from the primary data given in Tables* 2 *and* 4

Class	Median	Mean	Standard deviation
(*a*) A	$3{\cdot}5 \times 10^4$	$1{\cdot}30 \times 10^5$	$2{\cdot}65 \times 10^5$
B	$4{\cdot}5 \times 10^4$	$1{\cdot}49 \times 10^5$	$2{\cdot}72 \times 10^5$
C	$4{\cdot}0 \times 10^4$	$1{\cdot}44 \times 10^5$	$2{\cdot}78 \times 10^5$
Overall	$3{\cdot}7 \times 10^4$	$1{\cdot}40 \times 10^5$	$2{\cdot}71 \times 10^5$
(*b*) Persons associated with food poisoning	$1{\cdot}025 \times 10^7$	$1{\cdot}01 \times 10^7$	$2{\cdot}11 \times 10^6$

Viable cell counts were also carried out after heating the 1/10 faecal suspension to 100° C. for 30 min. (method 2). Although heat-resistant spores could be isolated by enrichment techniques from twenty-two of the specimens examined, only two of these contained heat-resistant spores of *Cl. welchii* in numbers sufficiently high to enable them to be detected by direct methods. The spore counts in these cases were 200 and 500 viable spores per gram.

An actual outbreak of Clostridium welchii *food poisoning*

During the course of these studies, only one small outbreak of food poisoning attributed to *Cl. welchii* was investigated.

Of twelve persons attending a small gathering, seven developed diarrhoea within 12 hr. This was mild, with no vomiting, slight nausea and persisted for only 24 hr. Heat-resistant *Cl. welchii* were present in ten of the twelve persons concerned, and in all seven who developed symptoms of food poisoning.

Total *Cl. welchii* counts were carried out on all ten specimens that contained heat-resistant *Cl. welchii*. The results are given in Tables 3 and 4. These figures differ from those of the normal population in two striking ways.

1. The values are all much higher than those of the normal population.

2. The results are distributed evenly about the mean value; the median and mean being very nearly the same. This therefore represents a special group of persons, and the deviation from the normal viable cell count is significant

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(t = 12·5, D.F. = 259, P = less than 0·01). The viable count has increased at least 200-fold (comparing median and not mean counts).

Almost all the organisms present in the above cultures were of the non-haemolytic type—the haemolytic *Cl. welchii* appeared to be present in numbers similar to those found in the general population. It is therefore likely that almost all the *Cl. welchii* present were the heat-resistant type, introduced with the infected meat dish.

Table 4. *Spore and vegetative cell counts of* Clostridium welchii *isolated from faeces during an outbreak of* Cl. welchii *food poisoning*

Viable count per gram of faeces		
Vegetative cells	Spores	Ratio
$5{\cdot}0 \times 10^6$	$1{\cdot}5 \times 10^3$	1/3,333
$6{\cdot}0 \times 10^6$	$3{\cdot}0 \times 10^3$	1/2,000
$7{\cdot}5 \times 10^6$	—	> 1/60,000
$8{\cdot}0 \times 10^6$	$2{\cdot}0 \times 10^3$	1/4,000
$1{\cdot}0 \times 10^7$	$5{\cdot}0 \times 10^2$	1/20,000
$1{\cdot}05 \times 10^7$	$1{\cdot}0 \times 10^3$	1/10,500
$1{\cdot}05 \times 10^7$	$2{\cdot}5 \times 10^3$	1/4,200
$1{\cdot}25 \times 10^7$	$5{\cdot}0 \times 10^2$	1/25,000
$1{\cdot}30 \times 10^7$	$3{\cdot}0 \times 10^3$	1/4,300
$1{\cdot}8 \times 10^7$	$5{\cdot}0 \times 10^3$	1/3,600

Counts were also carried out on the two specimens that did not contain heat-resistant spores. The results were $2{\cdot}1 \times 10^6$ and $3{\cdot}6 \times 10^4$ *Cl. welchii* per gram.

Direct counts, performed on the boiled 1/10 faecal suspension, were able to detect heat-resistant spores in nine of the ten specimens. The spore vegetative cell ratios in these cases were 1:2000 to greater than 1:60000. In the carriers discussed earlier, however, spores were only able to be directly demonstrated in two of the twenty-two specimens examined.

These results are in agreement with the assumption, stated above, that most of the organisms present during active infection are of the non-haemolytic, heat-resistant type. If this had not been the case, there is little likelihood that nine of ten specimens would contain heat-resistant spores in numbers large enough to permit isolation by direct culture methods.

An antiserum prepared from one organism agglutinated all ten organisms isolated from the patients. It also agglutinated a strain of *Cl. welchii* isolated from the incriminated meat dish—a beef caserole—which was eaten by all persons concerned. Serologically this organism did not belong to any of the Hobbs types 1–13 at present considered capable of causing food poisoning.

DISCUSSION

The enumeration of *Cl. welchii* from faeces appears to be possible by the method described. Although there is some reduction in count, the counts are reproducible. Smith & Crabb (1961) used this method to examine the faecal flora of man and animals. They performed numerous daily counts and concluded that as a whole the results were reliable and consistent. Smith (1959) demonstrated by experiments

similar to those described here that the counts on faeces were reproducible. He used a swab and what he terms 'A standardized technique' and one would not expect the results to be as reliable as those using a calibrated dropping pipette.

It was shown that the viable cell count on the same patient varied from week to week. It seems reasonable to expect that the quality and quantity of food and drink, environmental conditions, general health and other factors would have a profound effect on the growth of *Cl. welchii* in the gut, and that under favourable conditions a rapid increase in numbers could occur in a short time, corresponding to the logarithmic phase of growth of organisms in culture. Smith & Crabb (1961) while working with animals noticed that a change in diet produced a change in the bacterial flora of the gut, and that in infants the number of *Cl. welchii* excreted occasionally undergoes a sudden increase in numbers. If the average be taken over a long period of time, however, they point out that the results are consistent. Goudie & Duncan (1956) claim that there is little difference in the day-to-day excretion of *Cl. welchii* provided the consistency of the stool does not change.

When carrying out viable counts from faeces, many variables exist that cannot satisfactorily be eliminated. It is, however, important to know that they do exist. No single figure obtained for a viable count on faeces can be expected to indicate the exact number of organisms being excreted. The method described does, however, offer a reproducible method whereby the bacterial counts of series of specimens can be compared.

Analysis of the results of the quantitative studies shows that the variations between all three classes examined do not represent significant differences. At the beginning of this work it was expected that the aboriginals, having a much higher incidence of heat-resistant *Cl. welchii* (Sutton, 1966) would contain a large group with a much higher viable count. This was not demonstrated, the distribution for all classes being somewhat similar (Table 2). From this, two assumptions can be drawn.

(*a*) The heat-resistant, non-haemolytic *Cl. welchii* are present in much smaller numbers than the haemolytic strains.

(*b*) The growth of the non-haemolytic strain almost completely suppresses the growth of the haemolytic strain, and so is present as a replacement for, and not in addition to the haemolytic strain. This is unlikely as all plates showed many more haemolytic than non-haemolytic colonies. Smith & Crabb (1961) concluded that other organisms generally have little antagonistic effect on the growth of flora normally found in the intestine, and so it would be unlikely that one strain of *Cl. welchii* would completely suppress the other. It is, therefore, more likely that (*a*) above is the correct interpretation of the results. The failure to demonstrate heat-resistant spores readily in the faeces of carriers by direct methods, adds weight to this assumption.

Counts carried out on persons associated with an outbreak of *Cl. welchii* food poisoning show that there is a significant increase in the number of *Cl. welchii* in the faeces of such persons. This could be due to the actual infection leading to multiplication within the host or simply to the diarrhoea associated with the food poisoning leading to an increased output of organisms, even though there is no

actual increase within the intestine. Goudie & Duncan (1956) were able to demonstrate (semi-quantitatively) that *Cl. welchii* could be isolated more often, and in larger numbers, from persons with diarrhoea or after purging. In the results of this work, however, not only was there an increased viable count but heat-resistant spores could be readily isolated by direct methods. It would therefore appear that the increased counts were due to the presence of large numbers of heat-resistant *Cl. welchii*. As this organism was associated with the food poisoning, and is not present in large numbers in the normal population, it is probable that the increased count was due to an actual increase in the organisms present in the intestine, and not simply due to diarrhoea increasing the output of organisms. This predominance of heat resistant *Cl. welchii* in outbreaks must be due to the survival of spores in cooked fresh meats in 'niches', providing favourable conditions for anaerobic growth during long slow cooling

Although the viable counts as a whole have given some useful information, it has been shown that individual counts are subject to too many variables to be of use in laboratory diagnosis. The finding of heat-resistant spores during infection, however, offers what could be a useful tool in investigating an outbreak of food poisoning. Heat-resistant spores could be isolated directly from only two of twenty-two carriers, but from nine of ten persons associated with the outbreak of food poisoning. This is a significant difference ($t = 7{\cdot}16$). Finding spores by direct culture after 30 min. at 100° C. would therefore appear to be evidence for infection due to heat-resistant *Cl. welchii*. This was the case in the single outbreak described here, and as the heat-resistance of spores varies so greatly and different outbreaks have varied so greatly with regard to the number of persons at risk who were actually infected, more figures are needed before this can be adopted as a useful aid in investigating outbreaks of food poisoning due to heat-resistant *Cl. welchii*.

SUMMARY

A method of doing viable counts of *Cl. welchii* has been described that gives reproducible results from faeces. By this method counts were carried out on the faeces of persons in the general population, and those associated with an outbreak of food poisoning due to *Cl. welchii*. There was a significant increase in viable count in those with symptoms of food poisoning. Owing to many variables, single viable counts do not appear to be useful in the laboratory diagnosis of food poisoning, but the detection of heat-resistant spores, by direct culture after boiling, may be of some use. More work must be carried out to substantiate this point.

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