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Laboratory techniques

Evaluation of ELISA, RPLA, and Vero cell assays for detecting *Clostridium perfringens* enterotoxin in faecal specimens

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SUMMARY Three hundred and ninety two faecal specimens from 70 separate outbreaks of suspected *Clostridium perfringens* food poisoning were examined by enzyme linked immunosorbent assay (ELISA), reversed passive latex agglutination (RPLA), and Vero cell assays for the presence of enterotoxin. Although the most time consuming method, ELISA was the most specific and reproducible. RPLA was slightly more sensitive than ELISA, but it showed some non-specific reactions. The Vero cell assay was the least sensitive and least reproducible method, being affected by some non-specific cytotoxic and cytotonic reactions. Normal rabbit serum should be included in the Vero cell assay as a control for the neutralisation of cytotoxic effects.

Clostridium perfringens was responsible for 68 outbreaks (1716 cases) of food poisoning in England and Wales in 1984.[1] Although the illness is typically mild (abdominal pain and diarrhoea lasting 12–24 hours), fatalities may occasionally occur in debilitated patients, especially the elderly.

The illness results from ingestion of large numbers of viable vegetative cells of *C perfringens*. The organisms multiply in the small intestine, and when they sporulate there is an accompanying release of enterotoxin. It is this toxin which is responsible for the symptoms.[2,3] The primary action of the enterotoxin produces changes in membrane permeability, leading to leakage of intracellular contents.[4,5] Horiguchi *et al* showed that enterotoxin activity includes a temperature independent binding step, a temperature dependent ion-transport step, and a calcium-ion dependent step change leading to release of intracellular macromolecules.[6]

Laboratory confirmation of an outbreak of *C perfringens* food poisoning is usually based on four criteria[7]: large numbers ($> 10^5$/g) of *C perfringens* in the food; large numbers ($> 10^6$/g) of the organism in the faeces of ill patients; the presence of the same serotype in most of the ill patients; and the presence of the same serotype in the food and faeces. Although these criteria have been extremely valuable when investigating suspected *C perfringens* food poisoning outbreaks, they have sometimes proved inconclusive—for example, when no food was available, when the strains were non-typable or were autoagglutinable, or when the incident concerned geriatric inpatients who may carry large numbers of the same serotype without signs and symptoms of food poisoning.[8,9]

An additional method of laboratory confirmation of the cause of the outbreak is the detection of *C perfringens* enterotoxin in the faecal specimens. Several methods for the detection of this enterotoxin have been described[10] with enzyme linked immunosorbent assay (ELISA), reversed passive latex agglutination (RPLA), and Vero cell assays the most commonly used. In this paper we compare the three methods.

Material and methods

Faecal specimens (392) from 70 separate suspected outbreaks of *C perfringens* food poisoning in the United Kingdom between January 1986 and January 1987 were obtained from public health and hospital laboratories.

Faecal specimens (2 g) were mixed in 2 ml 0·01 M phosphate buffer (pH 7·2) containing 0·15 M sodium chloride (PBS) in a vortex mixer. These suspensions were centrifuged at 12000 *g* for 20 minutes at 4°C and the supernatant fluids removed and passed through 0·22 μm membrane filters (Gelman Sciences Ltd, Northampton, England). The resulting filtrates were tested by the three assay procedures.

The sandwich ELISA procedure was carried out as described by Bartholomew and coworkers.[11] The RPLA test was carried out as described previously,[12] using the kit commercially available from Oxoid Ltd, Basingstoke, England.

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The Vero cell assay was based on the method of Giugliano *et al.*[13] Doubling dilutions (25 μl volumes) of faecal extract in saline were prepared across 12 wells of a 96 well tissue culture microtitre plate (Nunc, Gibco Ltd, Uxbridge, England). A sample of the extract, incubated at 37°C for 30 minutes with either normal rabbit serum or rabbit anti-enterotoxin diluted 1 in 5 with saline, was tested in parallel. Doubling dilutions of the anti-enterotoxin treated extract were prepared across nine wells of a second row, with the normal rabbit serum treated sample double diluted across the final three wells of the same row. Freshly trypsinised Vero cells in E199 medium (Flow Laboratories, Rickmansworth, England) (100 μl volumes, 2 × 10^5 cells/ml) were then added to each well. One row of the plate was used for titration of a standard enterotoxin preparation, and several wells for Vero cell control. The plate was sealed and incubated at 37°C overnight. Results were scored visually, and classified as 4, 3, 2, 1 or 0 (that is, 100%, 75%, 50%, 25% or 0% cytotoxicity). A specimen was considered to contain *C perfringens* enterotoxin when the cytotoxicity titre of the treated sample was reduced by two wells or more compared with the untreated sample, provided that the normal rabbit serum did not also reduce the cytotoxicity. The "minimum" cytoxic effect regarded as positive was a score of 3 in the first well and 2 in the second.

The serological types of isolates of *C perfringens* associated with the food poisoning outbreaks were determined using the 143 antisera available in the Food Hygiene Laboratory.[7]

Results

Over the 13 months of the study, 392 faecal specimens from 70 suspected outbreaks were examined. The number of specimens in which *C perfringens* enterotoxin was detected by the three methods is shown in table 1, together with the number of incidents thereby regarded as true *C perfringens* outbreaks by each method. Vero cell assay was the least sensitive method, detecting toxin in only 30% of specimens compared with about 40% for ELISA and RPLA. All specimens found to contain enterotoxin by Vero cell assay were also positive by ELISA and RPLA. When considering the number of outbreaks, the Vero cell assay appears almost as sensitive as the ELISA, failing to detect enterotoxin in only two outbreaks. The reason for this was that, using the ELISA, toxin could be detected in four or five specimens in each outbreak, while the Vero assay, although less sensitive, detected at least one positive specimen in each outbreak.

Table 1 *Detection (No/%) of C perfringens enterotoxin in 392 faecal specimens from 70 food poisoning incidents*

	ELISA	*RPLA*	*Vero cell assay*
No of specimens containing enterotoxin	157 (40)	165 (42)	116 (30)
No of incidents considered to be a *C perfringens* outbreak	35 (50)	42 (60)	33 (47)

Table 2 *Analysis of nine incidents giving equivocal results*

No of outbreaks in which enterotoxin detected		
ELISA	*RPLA*	*Vero cell assay*
2* (2)†	2* (2)†	0
0	7 (2)†	0

*Same outbreaks; †number of outbreaks also confirmed by serotyping.

Table 3 *Enterotoxin concentrations in faecal specimens detected by ELISA*

Enterotoxin (μg/g faeces)	*No of specimens*	
0·001– 0·01	18	
0·01 – 0·1	27	68
0·1 – 1·0	23	
1·0 – 10·0	52	
10·0 – 100·00	28	89
>100·0	9	

The seven incidents shown to be positive by the RPLA but not by the ELISA may appear to represent only eight specimens (table 1). In fact, there were 14 specimens from the seven incidents—the apparent difference being due to the fact that there were a number of specimens from other outbreaks which were shown to contain enterotoxin by the ELISA and not by the RPLA. In these cases the discrepancy did not affect the overall numbers, as other specimens in the same outbreaks contained enterotoxin. This slight variation was found also in earlier studies.[12 14]

Thirty three outbreaks were positive and 28 outbreaks negative by all three methods, giving an agreement value of 87%. Nine incidents gave a positive result by only one or two of the methods (table 2). In four of these nine outbreaks, serological typing of faecal isolates confirmed a *C perfringens* aetiology. In two (positive by RPLA alone) the symptoms were typical of *C perfringens*, in three the symptoms and incubation period were atypical.

The range of levels of enterotoxin found in the faeces from confirmed outbreaks, detected by ELISA is shown in table 3. ELISA was used to determine the toxin ranges encountered in preference to RPLA or Vero cell assay because ELISA is a quantitative method, whereas the other two are only semiquantitative.

Discussion

As the United Kingdom Reference Laboratory for *C perfringens* serotyping, we have a unique opportunity to investigate outbreaks of *C perfringens* food poisoning. The laboratory also offers facilities for the detection of enterotoxin. Although many laboratories may have a method for toxin detection such as RPLA or Vero cell assay, most are unlikely to experience more than one outbreak each year, and so will have little opportunity to study the methods available.

The ELISA has been used in this laboratory since 1983 for the detection of *C perfringens* enterotoxin in faeces and has been found to be specific, sensitive, and reproducible.[11] Specificity is achieved by the incorporation of a sample neutralised by antiserum of each specimen in the test. A sensitivity of about 2–4 ng/g of faeces ensures that even toxin present in specimens collected several days after the onset of illness can be detected. Other workers have found that similar sensitivities can be achieved using ELISA.[15–17]

The results obtained with the RPLA confirm those of earlier studies,[12 14] in which the method was found to be both reproducible and sensitive. In fact, this test detected seven outbreaks missed by ELISA, consisting of a total of 44 specimens of which 14 were positive. Those specimens had RPLA titres of 4 or 8—that is, almost at the limit of detection of the test. Of these seven outbreaks, two were also confirmed by serotyping; in two the incubation period and symptoms were typical of *C perfringens* food poisoning, while in the remaining three outbreaks the symptoms and incubation period were atypical. It is therefore possible that these latter three outbreaks, representing six positive specimens, were not *C perfringens* food poisoning, and the reactions were non-specific. This is supported by detection of Norwalk virus in one outbreak, in which it was considered to be the aetiological agent. Neutralisation of the low positive RPLA reactions was carried out in an attempt to clarify the result, but the outcome was inconclusive.

The Vero cell assay was the least sensitive and reproducible method. Repeat testing of samples showed that there could be a four-fold change in cytotoxicity titres, so some weak positives could become negative and vice versa. This variability, combined with the fact that a cytotoxicity score of 2 or 3 was often encountered in the first well alone (due to the 'non-specific' toxicity of faeces on tissue culture cells), meant that the "minimum" cytotoxicity score that could be judged positive was 3, 2, 1/0 across the first three wells. *C perfringens* enterotoxin was considered to be responsible for the outbreak if the cytotoxic effect was inhibited by the anti-enterotoxin serum. We found that it was necessary to include a "control" serum—normal rabbit serum—in the test to ensure that any inhibition of response was due to specific anti-enterotoxin antibodies. Thirty one specimens seemed to contain enterotoxin, but their cytotoxicity was reduced or totally inhibited by normal rabbit serum. Whether the Vero cells were detecting some other cytotoxic agent that was specifically inhibited by antibodies in the normal rabbit serum is unknown. Welch *et al* incorporated normal horse serum in their tissue culture assay for the detection of cytotoxin in faeces, but did not give details of problems encountered.[18] Normal serum has also been shown to inhibit other biological activities.[19 20]

The three methods gave good general agreement overall when comparing outbreaks. The results in table 2 confirm those of earlier workers[11] that most faecal specimens from food poisoning incidents have enterotoxin concentrations exceeding 1 μg/g faeces. Therefore, even the less sensitive assay systems, such as Vero cells, would be adequate for laboratory confirmation of most outbreaks of *C perfringens* food poisoning. Tissue culture is probably of less value when examining small outbreaks with few specimens, or when specimens are collected later than two or three days following onset of symptoms, when enterotoxin concentrations may be low. The lower sensitivity of Vero cells may be related partly to the fact that the biological activity of *C perfringens* enterotoxin is less stable than the immunological activity.[21] A sensitivity of 1 ng/ml for enterotoxin detection by Vero cells has been quoted by some workers,[21] but they were using pure toxin in saline and a plating efficiency assay which is not as easy to perform and is less adaptable to routine use.

A comparison of the three methods is given in table 4. We envisage using ELISA for investigation of large outbreaks (where we receive more than four or five specimens) and RPLA when investigating small outbreaks, or where we receive only a few specimens. Considering the possibility that non-specific reactions may sometimes occur with RPLA it would be prudent

Table 4 *General comparison of ELISA, RPLA, and Vero cell assays*

	ELISA	*RPLA*	*Vero cell assay*
Approximate cost (pence) per specimen (materials)	20	250	15
Time (hours) required for completion of test (per batch, 16 samples)	<8	24	24
Time (hours) spent on test (per batch)	2½	½	¾
Sensitivity	2–4 ng/g	2–4 ng/g	40 ng/g
Specificity	Excellent	Good	Good
Subjectivity	No	Yes	Yes
Specialised equipment	Yes	No	Yes

to check any low titre positive samples with ELISA, particularly if attempts at neutralisation of the agglutination reaction prove inconclusive.

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