The assessment and application of a bacteriocin typing scheme for *Clostridium perfringens*

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SUMMARY

A collection of 50 bacteriocins was assembled and used to type 802 isolates of *Clostridium perfringens* from food poisoning outbreaks and a variety of other sources. It was found that strains of the same serotype within an outbreak showed similar patterns of susceptibility to bacteriocins, and the use of a 'one difference' rule is proposed for interpretation of the typing patterns of epidemiologically related strains. Isolates of different serotype or of the same serotype isolated from different sources produced many variations in bacteriocin susceptibility patterns.

Two computer programs were developed to assist in the interpretation of bacteriocin typing patterns. Their use showed that related and unrelated strains formed different clusters and enabled a range of the 20 most discriminatory bacteriocins to be selected.

Isolates of *C. perfringens* from a wide range of sources were screened for their ability to produce bacteriocins. A much greater proportion of the strains from food poisoning outbreaks was bacteriocinogenic than were isolates from human and animal infections, various foods and the environment. The relevance of these findings to the occurrence of *C. perfringens* food poisoning is discussed.

INTRODUCTION

Clostridium perfringens may be isolated with relative ease from the faeces of healthy persons, and to demonstrate that it is the cause of an outbreak of food poisoning it is necessary to show a relation between isolates from the faeces of most of the patients and from the incriminated food.

There are several available techniques for the subdivision of *C. perfringens*. For some years a serological typing method has been applied in the Food Hygiene Laboratory (Stringer, Turnbull & Gilbert, 1980) and this has recently been complemented by an ELISA method for the detection of *C. perfringens* enterotoxin in faecal specimens from suspected food poisoning outbreaks (Bartholomew & Stringer, 1983). While serological typing has successfully identified the causative strains of almost 70 % of the outbreaks referred to the Food Hygiene Laboratory in recent years, there remain those incidents in which the majority of the isolates are serologically non-typable (Stringer, Watson & Gilbert, 1982).

The initial observation of bacteriocin-like activity in *C. perfringens* was made by Smith (1959) in his study of the bacteriophages of the organism. Subsequent

reports gave details of the occurrence and some properties of the bacteriocins, and the feasibility of both active and passive typing methods (Mahony & Li, 1978; Satija & Narayan, 1980a, b; Scott & Mahony, 1982).

Active bacteriocin typing methods, such as the colicin typing scheme for shigella (Abbott & Shannon, 1958), involve the comparison of activity ranges of bacteriocins produced by test strains against a standard set of indicator organisms. This approach was adopted by Uchiyama (1966), who was able to divide 74 strains of C. perfringens into four groups on the basis of bacteriocin production, and by Satija & Narayan (1980a), who noted a relation between bacteriocin production and the countries of origin of 90 strains examined.

A number of reports have described the use of passive bacteriocin typing, by which the sensitivity of test strains to a standard range of bacteriocins is assessed. Mahony (1974) proposed a passive typing scheme using ten bacteriocins which was later applied to the examination of faecal isolates of C. perfringens from 51 individuals (Mahony & Swantee, 1978). One bacteriocin type, designated C3, accounted for over half of the isolates tested, but an attempt to subdivide this group by the addition to the typing set of a further five bacteriocins was not successful. This range of bacteriocins was used in the investigation of a food poisoning incident involving a spiced vegetable dish. Isolates of C. perfringens from the remaining food, spices and stool specimens belonged to bacteriocin type C3 (Moors et al. 1980).

Antohi (1977) used seven bacteriocins (selected from a range of 27) to divide 318 strains of C. perfringens into 18 groups. Although 97·2% of the isolates were susceptible to one or more of the bacteriocins, the largest group contained over half of the strains tested. When this scheme was used in the investigation of food-borne illness, food and faecal isolates of C. perfringens were recovered which could not be distinguished by either active or passive bacteriocin typing techniques (Bittner et al. 1980).

The potential of passive bacteriocin typing in the investigation of C. perfringens food poisoning was demonstrated by Watson et al. (1982). Forty-nine bacteriocins prepared in the Food Hygiene Laboratory and a further 20 from Dalhousie University, Canada, were used to type isolates from a small number of outbreaks. This paper records the selection of 20 bacteriocins of C. perfringens and their use for the first time in the examination of large numbers of epidemiologically related isolates, allowing the evaluation of bacteriocin typing in the laboratory investigation of C. perfringens food poisoning incidents.

MATERIALS AND METHODS

Bacteriocin preparation

Seven hundred and fifty-four isolates of C. perfringens from a wide range of sources were screened for the ability to produce bacteriocins. Of these, 213 had been identified as the causative serotypes in unrelated food poisoning outbreaks, 64 were isolates from cases of clinical infection, 240 were 'normal' faecal isolates, 188 from the environment and 49 from foods not implicated in outbreaks.

A strip of 0·22 μm membrane filter (Millipore Ltd, London) was laid across the surface of a Columbia base blood agar (CBA) plate, inoculated with a suspension of a test strain using the method of Riley & Mee (1981), and incubated anaerobically

at 37 °C for 36 h. The filters were discarded and the plates streaked with five reference indicator strains (strain nos. M88, T6, T17, F1726/76 and F3600/79) at right angles to the line of original growth. After a further 18 h the plates were examined for inhibition of growth of the indicator strains.

Fifty bacteriocinogenic strains were selected for further study and bacteriocins prepared by the method of Watson (1983). An actively growing Robertson's cooked meat culture (RCM) of a bacteriocin-producing strain was subcultured to 100 ml of Brain Heart Infusion Broth (Difco) with a 1 % glucose supplement (1 % GBHI) using a 1 ml inoculum and incubated at 37 °C for 3 h. A 1 ml inoculum of this primary culture was transferred to a second 100 ml volume of 1 % GBHI broth and incubated at 30 °C for up to 6 h. The secondary culture was centrifuged at 6000 g for 15 min, the supernatant sterilized by 0·22 μm membrane filtration and the bacteriocin titre determined using the critical dilution method described by Mayr-Harting, Hedges & Berkeley (1972).

Bacteriocin typing

Isolates of *C. perfringens* were typed by a modification of the procedure described by Mahony (1974). A tenfold dilution of an overnight culture of each strain in Robertson's cooked meat medium was spread using a swab over the surface of CBA plates. The plates were each inoculated with 20 μl drops of ten different bacteriocins, and after anaerobic incubation at 37 °C for 18 h the zones of inhibition were recorded.

Clearly defined zones greater than 6 mm in diameter were scored as positive. Incomplete or partial inhibition of the lawn, or the growth of resistant colonies within a defined zone of the above dimensions was scored as a weak reaction, while other effects were scored negative.

Reproducibility studies

In order to assess the discriminatory abilities of the bacteriocins and the reproducibility and stability of the typing patterns obtained, the range of 50 bacteriocins was used to type 202 unrelated strains of *C. perfringens* on two occasions eight months apart. This collection of strains was made up of 40 % food poisoning isolates and the remainder made up equally of isolates from cases of clinical infection, from foods not implicated in outbreaks, from the environment and faecal specimens from healthy persons. During the interval between examinations the strains were stored in RCM at ambient temperature.

Computer analysis of bacteriocin typing results

Two FORTRAN IV programs, CLST31 and RANGER, were developed to assist in the interpretation of results.

Program RANGER. A Similarity Coefficient Sj (Sneath & Sokal, 1962) was calculated for each pair of bacteriocins from the typing patterns of the 202 unrelated strains. Using these values, bacteriocins of similar activity ranges were clustered together by the Unweighted Pair-Group Method using Arithmetic Averages (UPGMA) described by Sneath & Sokal (1973). A listing of the similarity levels at which groups of bacteriocins became indistinguishable was used to construct a dendrogram (Fig. 1).

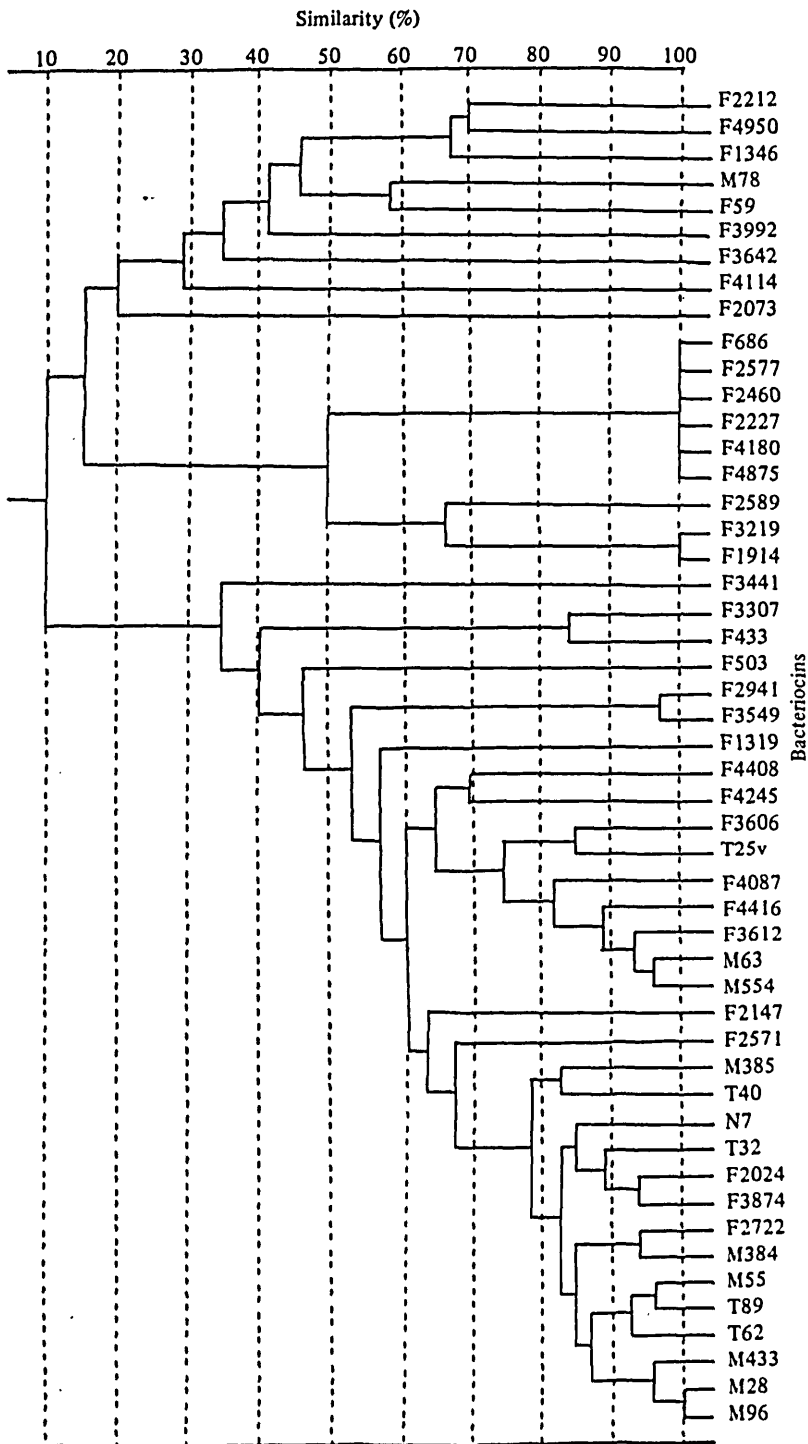The range of 20 bacteriocins with the greatest discriminatory capability was

Fig. 1. A dendrogram to illustrate the similarity of 50 bacteriocins.

Table 1. *Changes in the typing patterns of* 202 *unrelated isolates of* C. *perfringens over an eight-month period*

Number of reaction differences	Number of strains showing stated number of reaction differences with weak reactions counted as		
	Negative	Positive	Not scored
0	92	107	137
1	50	53	47
2	24	12	7
3	12	9	6
4+	24	21	5

Table 2. *Alteration of individual bacteriocin typing reactions*

Total number of reaction differences	Alteration in response between		
	Weak–negative	Negative–positive	Weak–positive
726	164	110	452

chosen by selecting dissimilar preparations giving as close as possible to 50 % of positive responses (i.e. those most able to distinguish between unrelated strains). Those bacteriocins giving predominantly positive or predominantly negative reactions were omitted, as were those giving more than 10 % of weak reactions.

Program CLST31. This program grouped together strains showing similar patterns of bacteriocin sensitivity using UPGMA. Clusters formed as progressively larger numbers of reaction differences were tolerated in the bacteriocin typing patterns of test strains. The cluster analysis was performed three times, with weak reactions counted initially as negative, then positive, and finally not scored.

Epidemiological application

The 20 selected bacteriocins were used to type 600 strains of *C. perfringens* received by the Food Hygiene Laboratory from 38 outbreaks of food poisoning in the United Kingdom during the period October 1978 to February 1984.

RESULTS

Stability of bacteriocin typing patterns

Only a small minority of the 202 unrelated strains showed more than three changes in bacteriocin susceptibility pattern in the interval between examinations (Table 1). A marked improvement in reproducibility was noted when weak reactions were scored positive rather than negative, suggesting a greater degree of overlap between weak and positive than between weak and negative reactions (Hall, 1971). This is supported by the analysis of changing individual typing reactions shown in Table 2, which demonstrates that weak reactions are involved in the majority of changes in bacteriocin typing pattern. When weak reactions were

not scored in the analysis, 91 % of the strains showed fewer than two strong reaction differences when typed on two occasions.

Selection of bacteriocins

The similarity of the bacteriocins is illustrated in Fig. 1. Although few identical pairs or groups of bacteriocins were observed, several clusters formed once a similarity of 90 % was reached. In the selection process, only one member of each such cluster was included in the reduced set of bacteriocins. Nine of the 50 bacteriocins tested (F3549, F2147, T32, F3874, T25v, F4408, F433, F3606, F503) were eliminated from the set as each produced more than 10 % of weak reactions, and a further nine (F686, F2577, F2460, F2227, F4180, F4578, F2589, F3219, F1914) were discarded due to their predominantly negative response.

Of the 32 remaining bacteriocins, the following 20 were selected as the most useful range: M384, M433, M385, N7, T40, F2571, F1319, F4245, F2024, F2941, T89, T62, M78, F3307, F3441, M554, F4416, F4087, F2212 and F4114. These bacteriocins were able to divide the collection of 202 unrelated strains into 86 groups, and although the largest cluster contained 11·9 % of the strains, many of the typing patterns observed were represented by a single strain.

Epidemiological application

Example 1 (outbreak 18). Sixteen of 44 residents and staff of an old people's home suffered diarrhoea about 10 h after a meal which included beef stew. *C. perfringens* serotype 21 was recovered from four, and serotype PS68/PS80 from all of the nine patients investigated. A sample of the incriminated food yielded both serotypes.

Bacteriocin typing results (Table 3) showed the serotype 21 isolates to be divided into two clusters, while a third cluster contained the 17 isolates of serotype PS68/PS80. Once one reaction difference was accepted the isolates fell into two clusters according to serotype.

Example 2 (outbreak 22). A meal including roast beef and chicken at a large nursing home was responsible for diarrhoea in 64 of 200 elderly residents. Both of the foods and faecal specimens from 10 of 25 victims yielded a strain of *C. perfringens* which could not be typed serologically and which produced only a weak lecithinase reaction. A further five patients yielded *C. perfringens* serotype TW5, and various typable and non-typable isolates were also present.

Twelve distinct bacteriocin typing patterns were observed among the 32 isolates tested. If one reaction difference was allowed a large cluster of strains developed which contained all the weakly lecithinase-producing non-typable strains and one other isolate (serotype 36).

An analysis of the results of serotyping and bacteriocin typing 600 isolates from 38 outbreaks of food poisoning is summarized in Table 4.

Examination of unrelated isolates

The bacteriocin typing results of 47 unrelated isolates of *C. perfringens* serotype 3/4, 19 of serotype 41 and 16 of serotype 11/13 (three of the serotype groups most frequently implicated in food poisoning), and 202 strains from a wide range of sources were analysed using CLST31 in the same way as for outbreaks. The clustering patterns are included for comparison in Table 4.

Table 3. *Outbreak 18, an example of bacteriocin sensitivity patterns*

Strain number	Source	Serotype	F4245	F3207	F2914	F2671	F4087	F4416	F4114	M384	T40	M78	F2212	F3441	M554	N7	M433	F2024	M385	T62	T89	F1319
												Sensitivity recorded to bacteriocin no.:									
F4069	Patient 1	PS68/PS80	−	−	+	−	+	+	−	+	+	−	−	+	+	−	−	−	−	−	−	−
F4070	Patient 1	PS68/PS80	−	−	+	−	+	+	−	+	+	+	−	+	+	−	−	−	−	+	−	−
F4071	Patient 2	PS68/PS80	−	−	+	−	+	+	−	+	+	+	−	+	+	−	−	−	−	+	−	−
F4072	Patient 2	21	+	+	−	+	−	+	−	+	+	−	−	+	+	+	+	+	+	+	+	−
F4074	Patient 3	PS68/PS80	−	−	+	−	+	+	−	+	+	+	−	+	+	−	−	−	−	+	−	−
F4075	Patient 4	21	+	+	−	+	−	+	−	+	+	+	−	+	+	+	+	+	+	+	+	−
F4076	Patient 4	PS68/PS80	−	−	+	−	+	+	−	+	+	−	−	+	+	−	−	−	−	−	−	−
F4077	Patient 5	PS68/PS80	−	−	+	−	+	+	−	+	+	−	−	+	+	−	−	−	−	−	−	−
F4078	Patient 5	PS68/PS80	−	−	+	−	+	+	−	+	+	+	−	+	+	−	−	−	−	+	−	−
F4079	Patient 6	PS68/PS80	−	−	+	−	+	+	−	+	+	−	−	+	+	−	−	−	−	−	−	−
F4080	Patient 6	21	+	+	−	+	+	+	−	+	+	−	−	+	+	+	+	+	+	+	+	−
F4081	Patient 7	PS68/PS80	−	−	+	−	+	+	−	+	+	−	−	+	+	−	−	−	−	−	−	−
F4082A	Patient 7	PS68/PS80	−	−	+	−	−	+	−	+	+	+	−	+	+	−	−	−	−	+	−	−
F4082B	Patient 7	21	+	+	−	+	+	+	−	+	+	−	−	+	+	+	+	+	+	+	+	−
F4083	Patient 8	PS68/PS80	−	−	+	−	+	+	−	+	+	−	−	+	+	−	−	−	−	−	−	−
F4084	Patient 8	PS68/PS80	−	−	+	−	+	+	−	+	+	−	−	+	+	−	−	−	−	−	−	−
F4085	Patient 9	PS68/PS80	−	−	+	−	+	+	−	+	+	+	−	+	+	−	−	−	−	+	−	−
F4086	Patient 9	PS68/PS80	−	−	+	−	−	+	−	+	+	−	−	+	+	−	−	−	−	−	−	−
F4087	Beef stew	21	+	+	−	+	+	+	−	+	+	−	−	+	+	+	+	+	+	+	+	−
F4088	Beef stew	21	+	+	−	+	−	+	−	+	+	−	−	+	+	+	+	+	+	+	+	−
F4089	Beef stew	PS68/PS80	−	−	+	−	+	+	−	+	+	−	−	+	+	−	−	−	−	−	−	−
F4090	Beef stew	PS68/PS80	−	−	+	−	+	+	−	+	+	−	−	+	+	−	−	−	−	−	−	−
F4091	Beef stew	PS68/PS80	−	−	+	−	+	+	−	+	+	−	−	+	+	−	−	−	−	−	−	−

Table 4. *Formation of clusters within outbreaks and groups of unrelated strains as a result of bacteriocin typing*

Outbreak number	Causative serotype	Number of strains of causative type	Number of strains in outbreak	Percentage of strains in largest cluster with reaction differences			
				0	1	2	3
1	27	3	3	100·0	—	—	—
2	11/13	5	7	100·0	—	—	—
3	6	8	10	100·0	—	—	—
4	3/4	9	9	100·0	—	—	—
5	3/4	7	18	100·0	—	—	—
6	11/13	8	12	100·0	—	—	—
7	19	13	18	100·0	—	—	—
8	TW24	9	16	100·0	—	—	—
9	PS73	18	20	100·0	—	—	—
10	3/4	12	12	100·0	—	—	—
11	11/13	3	3	100·0	—	—	—
12	10	11	18	90·9	100·0	—	—
13	11/13	8	9	75·0	87·0	100·0	—
14	1	3	3	100·0	—	—	—
15	TW23	18	18	100·0	—	—	—
16	3/4	9	9	88·9	100·0	—	—
17	32	8	8	62·5	100·0	—	—
18	PS68/PS80	17	23	82·6	100·0	—	—
19	PS68/PS80	21	28	71·5	100·0	—	—
*A	TW22	13	13	69·2	100·0	—	—
B	1	11	13	90·9	100·0	—	—
C	41	20	22	25·0	95·0	100·0	—
D	NT†	6	6	100·0	—	—	—
E	NT†	10	28	30·0	70·0	70·0	100·0
F	NT†	65	75	96·9	96·9	98·5	98·5
G	NT†	25	32	88·0	100·0	—	—
20	NT	?7	8	100·0	—	—	—
21	NT	?7	7	28·6	42·9	71·4	71·4
22	NT	?14	32	42·9	100·0	—	—
23	NT	?5	5	60·0	80·0	80·0	80·0
24	NT	?3	3	100·0	—	—	—
25	NT	?5	5	100·0	—	—	—
26	NT	?6	12	66·7	83·3	83·3	100·0
27	NT	?28	42	82·1	85·7	89·3	100·0
28	NT	?13	15	76·9	100·0	—	—
29	1	9	10	90·0	100·0	—	—
30	3/4	18	20	90·0	100·0	—	—
31	23	6	8	100·0	—	—	—
Unrelated strains	3/4	47		4·2	6·4	12·7	19·1
	41	19		10·5	15·8	15·8	21·1
	11/13	16		—	12·5	12·5	18·8
	Various	202		11·9	13·4	13·4	17·8

* Outbreaks labelled alphabetically were described by Watson *et al.* (1982).

† Denotes the preparation of an antiserum against a representative strain.

? The following number indicates the number of non-typable isolates associated with the outbreak.

Incidence of bacteriocin production by C. perfringens

Of 541 unrelated isolates from the faeces of healthy persons, human and animal infections, various foods and the environment, only 82 (15·2 %) produced bacteriocins. In contrast, bacteriocin production was demonstrated in 167 (78·4 %) of 213 strains implicated in food poisoning outbreaks.

DISCUSSION

Several studies have noted the advantages of active over passive typing schemes due to the greater stability of typing reactions (Edmondson & Cooke, 1979; Hardy, 1982; Simoons-Smit *et al.* 1983). Since only a minority of strains of *C. perfringens* is bacteriocinogenic, a passive typing method is of more value as long as the variability of typing patterns can be assessed.

It has been shown in a number of studies that minor variations occur in the typing patterns of epidemiologically related strains. By multiple typing of strains of klebsiella, Edmondson & Cooke (1979) showed that only 67 % reproducibility could be expected if patterns differing by one reaction were regarded as distinct. Anderhub *et al.* (1977), studying clinically related strains of *Serratia marcescens*, found that 52 % and 73 % of 89 cultures gave identical typing results on two occasions by cross-streaking and mitomycin-induction methods respectively.

Discrepancies which arise solely because of the occurrence of weak reactions would make it inadvisable to accept that any difference in typing patterns, however small, constitutes evidence that two strains are unrelated. It is apparent that a 'difference rule' must be applied in the interpretation of typing patterns even though this may adversely affect the discriminatory power of the typing scheme.

As illustrated by Tables 3 and 4, isolates of the same serotype within an outbreak generally show similar patterns of sensitivity to bacteriocins and readily cluster when small numbers of reaction differences are allowed. This is in marked contrast to the results of typing strains of different serotype, or of the same serotype isolated from different sources. Such strains show little tendency to form significant clusters until relatively large numbers of reaction differences are tolerated. In 30 of the 38 outbreaks shown in Table 4, strains of the causative serotype are indistinguishable if a 'one difference' rule is applied when using 20 bacteriocins. In the examination of groups of unrelated isolates, the largest clusters contain less than 16 % of the strains under the same conditions.

Two explanations may be proposed for the much higher incidence of bacteriocin production observed among strains of *C. perfringens* from food poisoning outbreaks. The production of bacteriocin in a foodstuff could be a factor in the selection of strains which may then proliferate to levels capable of causing illness, indicating the possibility of a relationship between bacteriocin production and the ability to produce enterotoxin *in vivo*. Alternatively, the production of either or both of these substances may be stimulated in the chain of events which culminates in an outbreak of food poisoning.

The serological typing scheme was developed by the Food Hygiene Laboratory primarily for use in the investigation of food-borne illness. Serotyping of

C. perfringens type A gives reproducible results, and in addition to a typability of 82 % of isolates associated with outbreaks, some 60 % of isolates of toxigenic types B, C, D and E show agglutinations with the set of antisera. The scheme requires, however, the use of 142 different antisera which are not commercially available, and the preparation of new antisera from serologically non-typable strains implicated in outbreaks is a time-consuming process.

In contrast, bacteriocin typing is a simple technique to perform, and with a relatively small set of bacteriocins may achieve a typability of 100 % of isolates of *C. perfringens* type A. There would appear to be little correlation between the bacteriocin type and serotype of unrelated strains, since serotyping succeeded in subdividing the common C3 group of Mahony & Swantee (1978), and in similar fashion common serotypes may be divided into groups of different sensitivity to bacteriocins.

This paper is the first record of the use of passive bacteriocin typing in the examination of large numbers of epidemiologically related strains of *C. perfringens*, and in each of the outbreaks investigated the causative strain showed a distinct bacteriocin typing pattern. In conclusion, passive bacteriocin typing has been shown to be a valuable technique in the investigation of *C. perfringens* food poisoning, especially when the majority of isolates associated with an outbreak are serologically non-typable.

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