

## The use of SDS–polyacrylamide gel electrophoresis in epidemiological studies of *Corynebacterium diphtheriae*

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(Accepted 3 September 1987)

### SUMMARY

Polyacrylamide gel electrophoresis of cell proteins was investigated as a possible typing method for *Corynebacterium diphtheriae*. A method was developed using stock strains which were representatives of the five gravis serotypes described by Robinson & Peeney (1936). This technique was then applied to recent isolates sent to our laboratory for identification.

### INTRODUCTION

*Corynebacterium diphtheriae* was initially classified into three varieties, gravis, mitis and intermedius. The biotypes were later subdivided using either serotyping, bacteriocin or phage typing or in some studies, a combination of all three (Gibson & Colman, 1973). There are however some disadvantages in using these methods. For example the phage-typing scheme was established for gravis strains (Saragea, Maximescu & Meitert, 1979) and the typability rate for mitis strains which are more common now, is low. It would be a time-consuming task to prepare serotyping antisera as most of the strains causing disease have originated abroad. A large number of sera would probably be required to type such a heterogeneous collection of strains.

The purpose of this study was to investigate a different method of typing which would be more suitable or adaptable to the small numbers of strains currently seen in the UK. Polyacrylamide gel electrophoresis of cell proteins in the presence of sodium dodecyl sulphate (SDS–PAGE) was investigated because Larsen *et al.* (1971) found that this method gave distinct patterns for different serotypes of *C. diphtheriae*. Also this technique has been used by Jackman (1982, 1986) to classify other coryneform organisms.

### MATERIALS AND METHODS

#### *Source of strains*

Strains of *C. diphtheriae* were selected from cultures which had been sent to the Division of Hospital Infection, Central Public Health Laboratory, Colindale for identification. Strains isolated following a case of diphtheria in Manchester were

kindly provided by Dr T. Riordan. Robinson & Peeney serotypes I–V (Robinson & Peeney, 1936) were obtained from the National Collection of Type Cultures, Central Public Health Laboratory.

### *Biochemical tests*

Carbohydrate fermentations were performed by standard methods. The reduction of nitrates was tested using a commercial 'dip-stick' (Keen & Mitchell, 1986). *C. diphtheriae* strains were classified as *gravis* or *non-gravis* in accordance with WHO guidelines.

### *Toxigenicity*

The toxigenicity of strains was examined in Elek plates using the method of Davies (1974). Sterile filter paper strips (Mast Laboratories, Brooks pers. comm.) were soaked in antitoxin, dried and stored at 4 °C. The dried antitoxin strip was laid across the inoculated Elek plate at right angles to the inoculum streaks. The plates were examined after incubation at 37 °C for 24 and 48 h.

### *Preparation of samples for electrophoresis*

Columbia horse blood agar plates were inoculated to produce confluent growth after overnight incubation at 37 °C. The growth was removed from the plate using a disposable loop. The organisms were resuspended in a 200 µg/ml solution of lysozyme in distilled water and incubated at 37 °C for 3 h. An equal volume of double strength sample buffer (Laemmli, 1970), was then added and the mixture was heated for 15 min at 100 °C.

After centrifugation for 5 min at 11 600 g the supernate was removed and stored at –20 °C prior to electrophoresis. Samples were separated by SDS–PAGE in 12.5% acrylamide gels using the method and buffers described by Laemmli (1970) with the stacking gel buffer of Palva & Mäkelä (1980) substituted for that given in the earlier publication.

## RESULTS

### *Protein patterns of stock strains*

Growth on agar plates was utilized because the organisms could be harvested more easily than from liquid cultures. The strains grew well on Columbia blood agar (Oxoid, CM331) and nutrient agar plates (Oxoid nutrient broth no. 2 CM67 + Japanese agar 1.5% w/v) after overnight incubation in air at 37 °C. Protein patterns of the five strains grown on both media were compared using the same sample volume. More bands were obtained with the cultures grown on nutrient agar and it was sometimes more difficult to detect differences in patterns. The cultures grown on Columbia blood agar resulted in more simple patterns; serotypes I, II and IV were identical (Fig. 1) whereas types III and V had distinct patterns. Columbia agar was therefore used in further work.

### *Protein patterns of recent isolates*

Eleven strains were examined, four of which were single, unrelated isolates. One of the cultures was from Switzerland the remainder were isolated from patients in

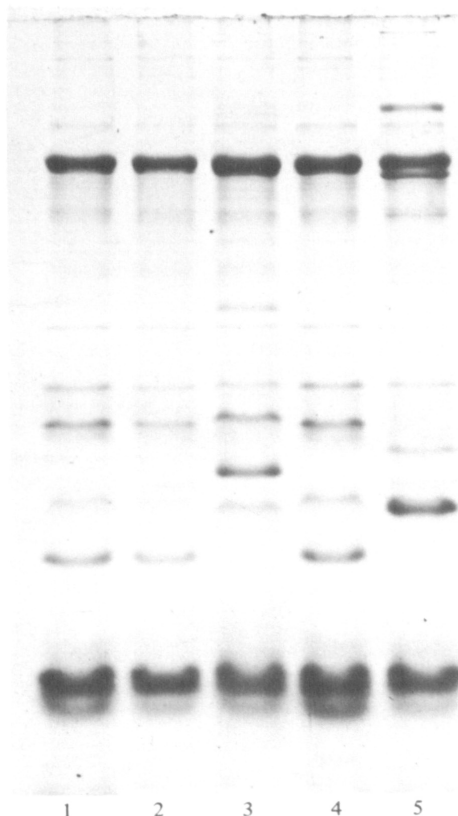


Fig. 1. Protein patterns of Robinson & Peeney serotypes grown on Columbia blood agar. Lane 1, Serotype I; 2, Serotype II; 3, Serotype III; 4, Serotype IV; 5, Serotype V.

the UK. During the period of this study, there were two cases of diphtheria, one in Manchester and the other in Hertfordshire, in which isolates were available from contacts of the patients. Cultures from the skin and throat of the Hertfordshire case were compared with two isolates from a sibling. Similarly, the strain from the index case of the Manchester incident was compared with strains isolated from two children at the same school. The electrophoretic protein patterns are shown in Fig. 2. Differences in protein profiles were easily identified visually. The strains associated with the case of diphtheria in Hertfordshire had identical patterns which were different from the set of cultures from Manchester. In contrast each of the four unrelated isolates produced an individual pattern that could be distinguished from all the others. These results clearly showed that a number of different patterns could be obtained from strains of *C. diphtheriae* and that this method was thus potentially useful for testing the relationship or identity of isolates.

#### *Protein patterns of the Manchester strains*

A total of 20 strains were examined from this incident (Table 1). The isolate from the index case was identified as a toxigenic *gravis* biotype. All strains

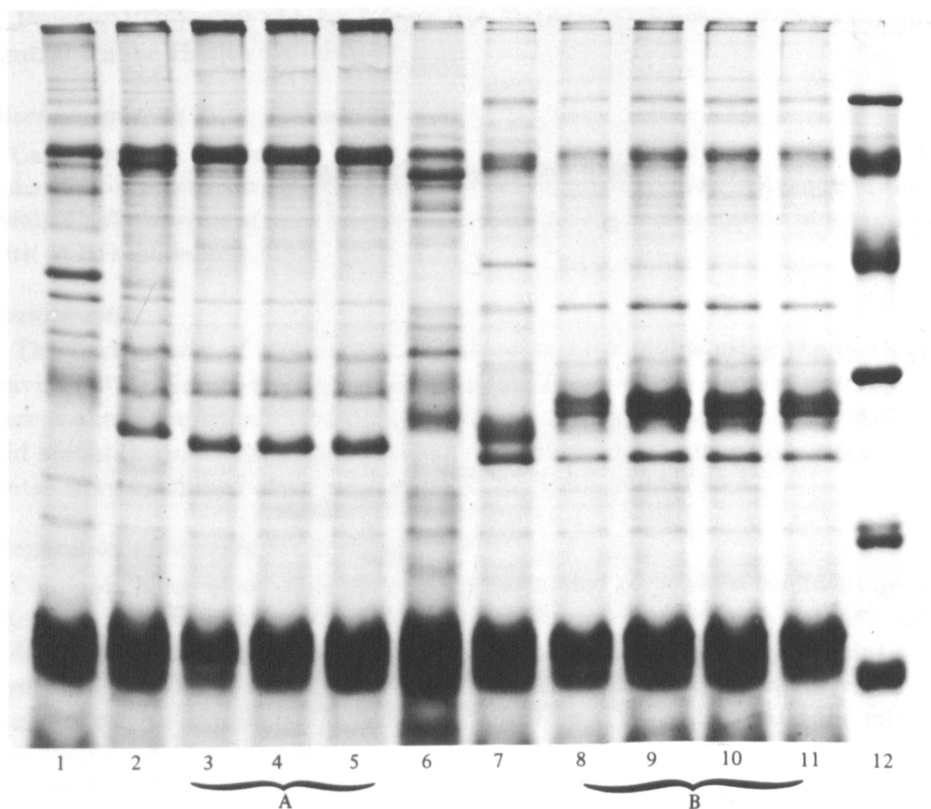


Fig. 2. Protein patterns of recent isolates of *C. diphtheriae*. Unrelated strains: lane 1, non-gravis, tox<sup>+</sup>; 2, gravis, tox<sup>-</sup>; 6, non-gravis, tox<sup>-</sup> (sucrose fermenter); 7, non gravis, tox<sup>+</sup>. (A) Manchester, all strains gravis, tox<sup>+</sup>: lane 3, index case; 4, carrier; 5, carrier. (B) Hertfordshire, all strains non-gravis, tox<sup>+</sup>: lane 8, index case (Skin/S); 9, index case (T/S); 10, sibling (T/S); 11, sibling (Skin/S). Molecular-weight markers: lane 12, phosphorylase b (94 000) BSA (67 000) ovalbumin (43 000) carbonic anhydrase (30 000) trypsin inhibitor (20 000) and lactalbumin (14 000).

obtained from contacts were indistinguishable from this isolate by biochemical and toxigenicity tests. The school contacts of the case included one child with a positive throat culture and skin lesion who had recently returned from Bangladesh. Extracts of all of these strains were tested on two gels electrophoresed simultaneously in the same tank. All but one of the isolates and identical protein patterns to that of the culture from the index case. One major band was absent from the exceptional strain (Fig. 3). This organism was isolated from an adult contact of the Asian child.

#### *A probable laboratory-acquired infection*

A strain which we identified as a non-toxicogenic *C. belfanti* was isolated from a patient in South London. A member of staff of the referring laboratory who had handled this and other *C. diphtheriae* cultures subsequently developed clinical diphtheria. These two isolates and six laboratory strains from the referring hospital laboratory which included control strains for the Elek test were examined

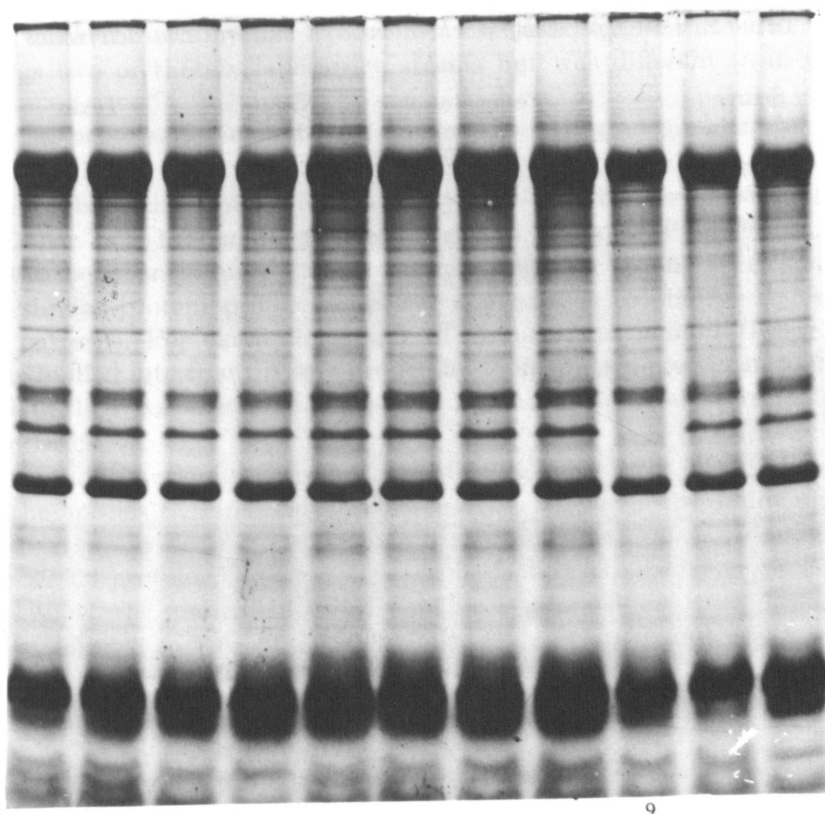


Fig. 3. Protein patterns of additional isolates from Manchester. All strains produced identical patterns with the exception of strain in lane 9.

Table 1. *Strains examined from an incident of diphtheria in Manchester*  
No. of individuals with *C. diphtheriae* isolated from

	Throat	Throat and nose	Throat and skin lesion
Index case	1	—	—
School contacts of index case	7	2	1
Contacts of two pupils who were carriers	3	—	—
Additional contacts of pupil with skin lesion	3	—	—

by gel electrophoresis. Toxigenicity studies and biochemical tests were also performed.

The patient's strain could be distinguished from the isolate from the laboratory worker by biochemical and toxigenicity tests (Table 2). Four of the six cultures handled in the laboratory were indistinguishable biochemically from the culture isolated from the member of staff. An examination of the protein patterns of all the strains helped to clarify the relationship between them (Fig. 4). Three cultures of the patient's strain were examined, one sent by the hospital attended by the patient and two from the referring laboratory. All three samples produced

Table 2. *Distinguishing biochemical and cultural characteristics*

Source of strain	Fermentation of starch	Nitrate reduction	Toxin production
Patient	-	-	-
Laboratory worker	-	+	+
Laboratory stock strains			
1	-	+	+
2	-	+	+
3	-	+	+
4	-	+	+
5	+	+	+
6	-	+	-

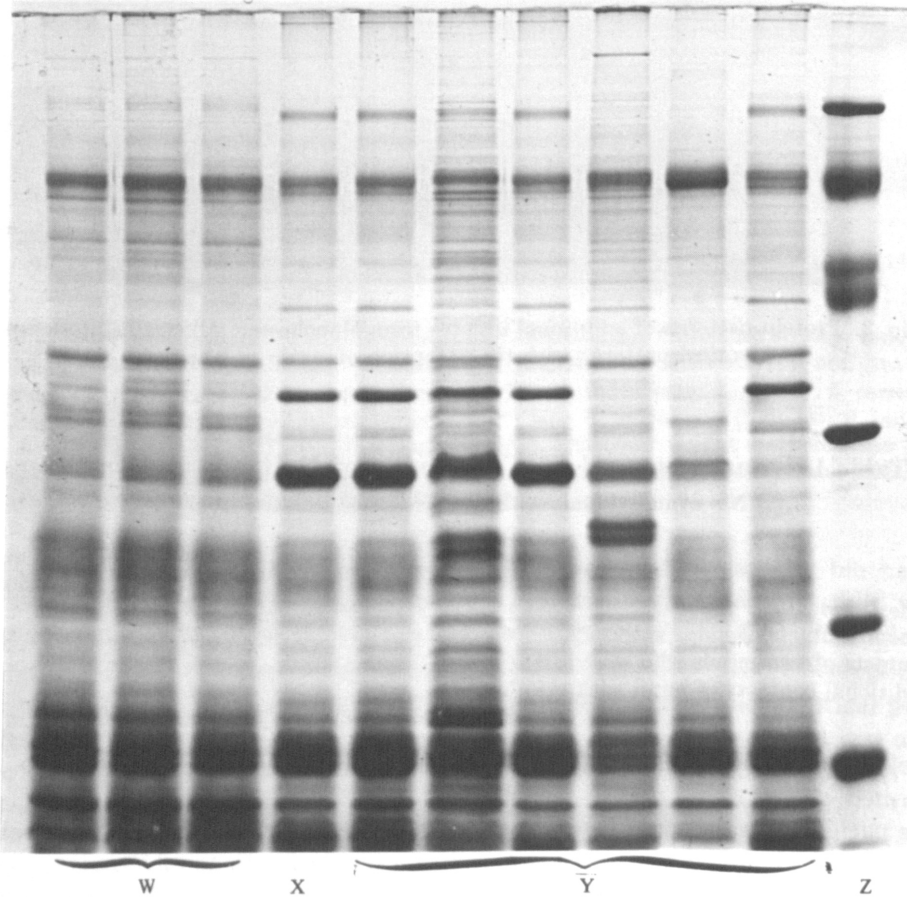


Fig. 4. Protein patterns of patient's strains and laboratory strains. W (lanes 1-3) three cultures of patient's strain; X (lane 4), strain from member of staff; Y (lanes 5-10) six laboratory stock strains; Z (lane 11) molecular-weight markers.

identical protein patterns which were readily distinguished from the pattern obtained with the strain that caused the infection. This isolate had the same pattern as two of the six laboratory strains but was different from the other four.

#### DISCUSSION

Profiles of bacterial proteins separated by SDS-PAGE are studied in one of two ways. Either as a taxonomic tool when cluster analysis is performed to show similarities between strains. Strains brought together in one species will often share a similar protein profile. A second approach is to observe differences in protein patterns. For example, strains of *Streptococcus mutans* (Russell, 1976) could be divided into four distinct groups by their protein profiles in SDS-PAGE and each of these corresponded to the groups obtained by analysis of their genetic characteristics.

In this study, marked differences in protein patterns were obtained for strains within the species *C. diphtheriae*. This enabled biochemically indistinguishable strains to be subdivided further. The method provided information on the mol. wt. of some 30–60 proteins. Because it is a reflection of the many genes coding for these proteins, the method provides a stable typing system in comparison with phage typing for example, where differences in phage patterns may be obtained amongst strains that are clearly related epidemiologically.

The compositions of media and conditions of growth of the organism were important in establishing a reproducible method. For this reason, stock items of laboratory media were employed. Growth harvested from solid media had two advantages. It was easier to collect the growth from an agar surface than from broth and secondly the amount of growth used to prepare the samples for electrophoresis could be standardized simply. Using the technique described for the preparation of the cell extract the differences in electrophoretic patterns between strains remained distinct and reproducible although the resolution of the individual protein bands may show some minor gel to gel variation. An advantage of this technique for typing diphtheria strains is that the number of cultures to be studied is unlikely to be large. The strains therefore can be compared directly as all the samples can be electrophoresed under the same conditions. To maintain a reproducible system in taxonomic studies in which large numbers of strains are examined, extracts of reference strains are usually included in each gel for comparison.

It is not possible to compare directly the results presented in this paper with previous reports because the methods vary. In Jackman's investigation (1982) sonicates of *Corynebacterium* species were separated on gradient gels. The gels were scanned with a densitometer and a similarity matrix constructed. Representatives of Robinson & Peeney's serotypes formed a related group whereas other isolates of *C. diphtheriae* from skin infections were scattered in the dendrogram.

The study of Larsen and colleagues was made over 15 years ago and individual samples were applied to gel rods. Present day electrophoresis equipment allows multiple samples to be compared more easily on slab gels. Larsen *et al.* found that 17 gravis strains examined formed a homogeneous group as judged by their protein profiles. More protein patterns were obtained with mitis strains (non-

starch fermenters) and this was consistent with the large number of serotypes found by earlier workers with these organisms. In our study of recent isolates, gravis strains were isolated from the Manchester incident and from one other unrelated case. These two sets of cultures had distinct gel patterns. However, most of the strains currently isolated in the UK do not ferment starch and this may be the reason for the variety of protein profiles obtained in this study. This study has shown that the separation of cell proteins of *C. diphtheriae* by SDS-PAGE provided a rapid alternative to other typing methods and should be helpful in epidemiological studies of diphtheria.

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