Polyacrylamide Gel Electrophoresis of
Corynebacterium diphtheriae: a Possible Epidemiological Aid

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In this preliminary study, the use of polyacrylamide gel electrophoresis as an aid in the characterization of Corynebacterium diphtheriae was evaluated and a standardized method was developed. The electrophoretic patterns of 17 gravis, 14 mitis, and 2 intermedius types of C. diphtheriae were compared with the electrophoretic patterns of 5 Robinson and Peeney stock serotype strains. Each of the 5 stock serotype strains had different electrophoretic patterns, although some common bands were present. The 17 gravis strains isolated in the United States showed patterns identical to those of the stock gravis serotype II strain. The 14 mitis strains examined produced 6 different electrophoretic patterns, irrespective of geographical location. One mitis pattern corresponded with the pattern of gravis serological type II. The two intermedius strains examined had identical electrophoretic patterns that resembled the pattern of gravis serotype IV. Polyacrylamide gel electrophoresis of C. diphtheriae strains may prove to be a useful epidemiologic tool in establishing the distribution and occurrence of various C. diphtheriae types.

Recent outbreaks of Corynebacterium diphtheriae in the United States (22) have reemphasized the epidemiological need for a standardized method for typing these organisms to determine epidemiological associations during outbreaks and the dynamics of circulation of bacterial types. At present, epidemiologic analysis of an outbreak is based on historical information obtained from persons with cases and their contacts and the laboratory confirmation of the Anderson (1) C. diphtheriae type.

C. diphtheriae has been traditionally classified into three groups or types (mitis, gravis, and intermedius) on the basis of colonial form, morphological structure, starch fermentations, hemolytic activity, clinical severity of the infection, and serological behavior (9). More recently, differentiation of C. diphtheriae has been made on the basis of phage type (7).

Many investigators have established their own serological typing system (4, 9, 11, 15, 19, 21, 23, 24). Hewitt (9) described 13 gravis types, 40 mitis types, and 2 distinct intermedius types. At the present time, according to Gundersen (8), the only commonly recognized serological types are those of Robinson and Peeney (15) which comprise gravis types I to V of Hewitt (9) and Tarnowski (21). Because of conflicting serotyping results and the difficulties of preparing standardized antisera, still newer methods for detecting strain-specific differences are being established.

Phage typing as used by Saraegea and Maximescu (18) has proved successful for differentiating 13 gravis, 3 mitis, and 3 intermedius strains. Although promising, this technique does not differentiate many strains within mitis and intermedius types, and its complexity is not well suited for the routine diagnostic laboratory.

Recently, polyacrylamide gel electrophoresis has been successfully employed for strain identification by Razin and Rottem (14) and Sacks et al. (17) who use whole cells of organisms such as Mycoplasma and the Enterobacteriaceae. Our study is a preliminary evaluation and development of a relatively simple procedure that uses disc electrophoresis to distinguish strain-specific differences within C. diphtheriae Anderson types.

MATERIALS AND METHODS

Strains. The C. diphtheriae strains used were obtained by nasal, throat, or skin culture of diphtheria cases or carriers across the United States (Table 1). In addition, stock gravis serotype strains, Robinson and Peeney types I to V, were obtained from H. Lautrop at the Serum Institute, Copenhagen, Denmark. All cultures were confirmed by methylene blue
TABLE 1. Number of C. diphtheriae strains by geographical origin, type, and toxigenicity

| Geographical origin (areas of U.S.) | Mitis | Gravis | Intermedius |
|-----------------------------------|-------|--------|-------------|
| East North Central                 | 1     | 1      | 1           |
| West North Central                | 91    | 1      | 1           |
| South Atlantic                    | 1     | 1      | 8           |
| East South Central                | 1     | 1      | 5           |
| West South Central                | 1     | 1      | 8           |
| Mountain                          | 1     | 1      | 3           |
| Pacific                           | 1     | 1      | 2           |
| Total                             | 11    | 3      | 14          |

No. of type

|       | AT | T  | AT | T  | AT |
|-------|----|----|----|----|----|
| T     | 1  | 1  | 1  | 1  | 1  |
| AT    | 1  | 1  | 1  | 1  | 1  |
| T     | 1  | 1  | 1  | 1  | 1  |
| AT    | 1  | 1  | 1  | 1  | 1  |

* T and AT represent strains which were toxigenic and atoxigenic, respectively.

smears, colonial morphology, biochemical reactions, and toxigenicity tests (6).

Media. Three media were evaluated for growth of C. diphtheriae for electrophoresis. We inoculated a washing of 18-hr-old growth from a Pae slant into 250 ml of Heart Infusion Broth (HIB) plus 0.1% NaHPO₄, supplemented with 5 ml each of 10% solutions of NaHCO₃ and glucose. Elek's agar plates were prepared according to Elek's formula (3), except that the serum was omitted and 0.3% Tween 80 was added. HIB agar plates were prepared by using the supplemented HIB described above plus 1.5% agar. All plates were prepared in petri dishes (15 by 150 mm) with 75 ml of medium. Plates were inoculated by swabbing them with growth from an 18-hr-old HIB culture. All inoculated media were incubated at 37°C for 24 hr.

Preparation of strains for electrophoresis. Strains grown in liquid media were killed by the addition of formalin [40% (w/v) formaldehyde] to a final concentration of 1% (v/v). Strains grown on duplicate plates of solid media were harvested in 30 ml of 0.5% formal physiological saline. In both instances, the cells were held in the formal solutions for 18 hr at room temperature before processing. Cells were washed three times in physiological saline and stored overnight at 4°C. The protein content of the strains was determined by the method of Lowry et al. (12) immediately before the cells were solubilized. Cells were solubilized in phenol-acetic acid-water (2:1:0.5; reference 14) for 1 hr on a shaker and were then centrifuged at 24,000 X g for 15 min to remove insoluble material. Cells held at 4°C for several weeks could still be used if a Lowry protein determination was made before solubilization.

Electrophoresis. Samples of the phenol-acetic acid-water extracts adjusted to contain 250 μg of protein were electrophoresed by the method of Rottem and Razzin (16) as modified by Larsen et al. (10) with the difference that electrophoresis was carried out for 1 hr at room temperature at 5 ma/tube; gels were stained with 1% naphthol blue black for 30 min and then destained by several washings in 7% acetic acid. Samples were electrophoresed in duplicate simultaneously and on different days to establish reproducibility.

RESULTS

Several media were evaluated for their ability to support sufficient growth of C. diphtheriae strains, especially the intermedius strains, without introducing components into the organisms which would interfere with analysis for strain-specific electrophoretic patterns. Elek's plates containing maltose yielded large amounts of growth of all three Anderson types. Unfortunately, when the strains grown on Elek's plates were electrophoresed, all patterns for the mitis, gravis, and intermedius strains were identical. Growth of the intermedius strains was poor on the supplemented HIB-agar plates, but the mitis and gravis grew well on these plates and produced individual electrophoretic patterns distinct from the patterns of the same strains in HIB. In the supplemented HIB, all strains grew well although the growth of the intermedius strains was still somewhat lighter than it was for the gravis and mitis strains. Therefore, since the most distinct electrophoretic patterns were from strains grown in the supplemented HIB, it was selected as the medium of choice.

The electrophoretic patterns of the gravis stock strains, Robinson and Peenie serotypes I to V, are shown in Fig. 1 and 2. Although all patterns were similar, they could be distinguished from each other by differences in the locations or in the absence of one or more bands.

![Fig. 1. Electrophoretic patterns of stock C. diphtheriae gravis strains Robinson and Peenie serotypes I–V, in supplemented HIB. (A) Serotype I, (B) serotype II, (C) serotype III, (D) serotype IV, (E) serotype V.](image-url)
(Fig. 5 and 6). The pattern of one mitis strain from Georgia was similar to the electrophoretic pattern of the gravis serological type II (Fig. 5 and 6). The other mitis patterns did not resemble any of the stock gravis patterns. In this preliminary study, no single mitis pattern was characteristic for any one geographical area.

Because of some difficulty encountered with light growth of the intermedius strains, only two strains have been electrophoresed to date. Both strains produced electrophoretic patterns similar to the gravis serological type IV pattern.

The reproducibility of the technique was excellent, as previously confirmed (10), as long as the amount of protein applied, length of electrophoretic run, and growth conditions remained constant.

DISCUSSION

The failure of gravis, mitis, and intermedius strains to produce characteristic electrophoretic patterns on Elek's medium is difficult to explain without a review of the biochemistry of carbohydrate utilization by *C. diphtheriae*. Some differences in the fermentation of maltose and glucose have been noted (2). Whether this has any relationship to electrophoretic patterns is unknown. Nikolaeva and Safonov (13) in their study on the complex of soluble proteins in the cells of *Clostridium perfringens* found that alterations in the medium composition did cause varia-

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**Fig. 2.** Densitometric tracings of the same poly-arylamide gels shown in Fig. 1.

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**Fig. 3.** Electrophoretic patterns of gravis strains grown in supplemented HIB. (A) Gravis, toxigenic, Texas; (B) gravis, atoxigenic, Florida; (C) gravis, toxigenic, Arizona; (D) gravis, atoxigenic, Texas.
when all diagnostic gravis strains examined had the same electrophoretic pattern, considering the findings of other investigators. Suslova (20) in his serological study of C. diptheriae in Russia found 90.8% of the 1,019 cultures serologically typed to be of type II. Ferris (5) in Australia found 88% of the 499 gravis strains serologically typed to be type II also, although the prevalence of this strain in Australia may have recently changed (7). Robinson and Peeney (15) reported that, although type II has almost worldwide distribution, types I and III were predominant in Great Britain and type IV in Egypt. They also stated that even though type II strains were predominant in the United States type V strains have been isolated only in the United States. When additional gravis strains are subjected to electrophoresis, perhaps we will also find type V.

Ferris (5) as well as Suslova (20) and Hewitt (9) noted a specific relationship between gravis serological type II strains and mitis serological type II strains. Hewitt states that the gravis and mitis type II strains are identical in serological behavior and differ only in starch fermentability and hemolytic activity. He further suggests that mitis type II may be a gravis type II which has lost its ability to ferment starch and hemolyze blood. Therefore, finding a mitis strain that has an electrophoretic pattern similar to the gravis serological type II strain may not be unusual. In phage typing, the similarity of mitis to gravis has also been noted. Gibson et al. (7) have reported a serologically untypable gravis strain.

![Fig. 4. Denstiometric tracings of the same polyacrylamide gels shown in Fig. 3.](image)

![Fig. 5. Electrophoretic patterns of mitis strains grown in supplemented HIB. (A) Mitis, toxigenic, Georgia; (B) mitis, toxigenic, Georgia; (C) mitis, toxigenic, Illinois; (D) mitis, atoxigenic, Louisiana; (E) mitis, atoxigenic, Washington; (F) mitis, toxigenic, Georgia (electrophoretic pattern similar to that of gravis serotype II).](image)
and a mitis strain of the same phage type and have suggested that these strains were from the same source.

The wide variety of electrophoretic patterns produced by the mitis strains correlates well with Hewitt's (9) finding of some 40 mitis serological types. Hewitt suggests that perhaps some mitis strains readily change their serological type. Only with the electrophoretic examination of numerous mitis strains can a definite epidemiological pattern for these types be established.

The serological relationship of an intermedius strain to a gravis strain was noted by Wright and Christison (24). Hewitt (9) also suggested that there may be a serological relationship between these two Anderson types. Before any definite relationship can be drawn between the electrophoretic patterns of stock serotype IV and the intermedius strains that we examined, many more strains must be tested.

It is unfortunate that the electrophoretic analysis of *C. diphtheriae* strains did not aid in the separation of atoxigenic strains from toxigenic strains.

Judging from these preliminary tests, we think the use of acrylamide gel electrophoresis as a means of characterizing *C. diphtheriae* is promising. The simplicity of this electrophoretic method, the reproducibility of the results, the small quantities of cells required, and the elimination of the need for expensive and difficult to produce antisera should encourage further investigation into the use of this method as a means of classifying *C. diphtheriae* strains.

**LITERATURE CITED**

1. Anderson, J. S., F. C. Happold, J. W. McLeod, and J. G. Thomson. 1931. On the existence of two forms of diphtheria bacilli—*B. diphtheriae* gravis and *B. diphtheriae* mitis—and a new medium for their differentiation and for bacteriological diagnosis of diphtheria. J. Pathol. Bacteriol. 34:667–681.

2. Barkadale, L. 1970. *Corynebacterium diphtheriae* and its relatives. Bacteriol. Rev. 34:378–422.

3. Elek, S. D. 1949. The plate virulence test for diphtheria. J. Clin. Pathol. 2:250–258.

4. Ewing, J. O. 1933. The serological grouping of the starch fermenting strains of *C. diphtheriae*. J. Pathol. Bacteriol. 37:345–351.

5. Ferris, A. A. 1950. Type-specific agglutinins in *Corynebacterium diphtheriae* infections. J. Pathol. Bacteriol. 62:157–174.

6. Frobisher, M. 1963. Diphtheria, p. 245. In A. H. Harris and M. B. Coleman (ed.), Diagnostic procedures and reagents, 4th ed. American Public Health Association, Inc., New York.

7. Gibson, L. F., G. N. Cooper, A. Saragea, and P. Maximeanu. 1970. A bacteriological study of strains of *Corynebacterium diphtheriae* isolated in Victoria and South Wales. Med. J. Aust. 1:412–417.

8. Gundersen, W. B. 1959. Investigation on the serological relationships of *Corynebacterium diphtheriae* type mitis and *Corynebacterium belfanti*. Acta Pathol. Microbiol. Scand. 47:65–74.
9. Hewitt, L. F. 1947. Serological typing of *C. diphtheriae*. Brit. J. Exp. Pathol. 28:338–346.

10. Larsen, S. A., C. D. Webb, and M. D. Moody. 1969. Acrylamide gel electrophoresis of group A streptococcal cell walls. Appl. Microbiol. 17:31–33.

11. Lautrop, H. 1950. Studies on antigenic structure of *Corynebacterium diphtheriae*. Acta Pathol. Microbiol. Scand. 27:443–447.

12. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265–275.

13. Nikolaeva, S. A., and V. I. Safonov. 1970. A study on the complex of soluble proteins in the cells of *Clostridium perfringens* by electrophoresis in polyacrylamide gel. Mikrobiologiya 39:87–90.

14. Razin, S., and S. Rottem. 1967. Identification of *Mycoplasma* and other microorganisms by polyacrylamide gel electrophoresis of cell protein. J. Bacteriol. 94:1807–1810.

15. Robinson, D. T., and A. L. P. Peeney. 1936. Serological types amongst gravis strains of *C. diphtheriae* and their distribution. J. Pathol. Bacteriol. 43:403–418.

16. Rottem, S., and S. Razin. 1967. Electrophoretic patterns of membrane proteins of *Mycoplasma*. J. Bacteriol. 94:359–364.

17. Sacks, T. G., H. Haas, and S. Razin. 1969. Polyacrylamide gel electrophoresis of cell protein of enterobacteriaceae. Israel J. Med. Sci. 5:49–55.

18. Sareaga, A., and P. Maximescu. 1966. Phage typing of *Corynebacterium diphtheriae*. Bull, W. H. O. 35:681–689.

19. Sia, R. H. P., and C. H. Huang. 1939. Serological classification of *C. diphtheriae*. Proc. Soc. Exp. Biol. Med. 41:348–349.

20. Suslova, V. S. 1964. On the problem of the serological properties of diphtheria bacteria. J. Hyg. Epidemiol. Microbiol. Immunol. 8:207–215.

21. Tarnowski, C. 1942. The type classification of Park Williams strain no. 8. Acta Pathol. Microbiol. Scand. 19:300.

22. United States Public Health Service, Center for Disease Control. 1970. Morbidity and Mortality rep. 19:487.

23. Wong, S. C., and T. T'ung. 1940. Further studies on type-specific protein of *Corynebacterium diphtheriae*. Proc. Soc. Exp. Biol. Med. 43:749–754.

24. Wright, H. A., and M. H. Christison. 1935. Further observations on the types of *Corynebacterium diphtheriae*. J. Pathol. Bacteriol. 41:447–467.