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Epidemiology of Diphtheria: Polypeptide and Restriction Enzyme Analysis in Comparison with Conventional Phage Typing

T. Krech[1]*, J. de Chastonay[2], E. Falsen[3]

Several methods for epidemiological typing of *Corynebacterium diphtheriae* were compared with the well accepted phage typing analysis. For this purpose, isolates from outbreaks of diphtheria in specific areas of the FRG and Sweden were analyzed by phage typing, their bacterial polypeptide profiles were examined and their phage-DNA restriction enzyme patterns were compared. All techniques were able to identify whether certain outbreaks were epidemiologically linked or not. Phage typing and phage-DNA restriction enzyme fragment analysis were limited in their application to lysogenic strains, whereas bacterial polypeptide analysis was universally applicable. Analysis of bacterial polypeptides was superior to all methods, especially in terms of speed and simplicity.

During the last 30–50 years the incidence of diphtheria has declined in Europe and the disease has become uncommon. In the Federal Republic of Germany, however, an increase of cases was recorded between 1975 and 1984: 109 epidemic incidents with 22 deaths occurred during this period (1). Also in Sweden in 1984 the disease resurged (2).

The causative agent of diphtheria, *Corynebacterium diphtheriae*, is spread by aerosol from man to man. The diphtheria toxin is the main virulence factor. It is produced only by strains possessing a temperate phage (prophage) which carries the *tox* gene. For epidemiological purposes, therefore, the bacteria, the phages, or the prophages can be investigated. In the past, epidemiological typing of *Corynebacterium diphtheriae* has been done by classifying strains as one of three variants: gravis, intermedius and mitis. These biotypes were then subdivided using either serotyping, bacteriocin or phage typing (3). Although phage typing has become a standard method, it gave a rather low typability rate in our isolates. Therefore more recent epidemiological markers were used, such as polypeptide analysis of bacterial proteins, which has been used successfully for typing other bacteria (4, 5). This method was adapted to *Corynebacterium diphtheriae*. Furthermore, the novel restriction enzyme analysis of phage-DNA was performed. We compared the efficacy of these techniques for identifying *Corynebacterium diphtheriae* strains during epidemics.

[1] Institute for Medical Microbiology and Virology of the University, Moorenstr. 5, 4000 Düsseldorf, FRG.
[2] Institute for Hygiene and Medical Microbiology of the University, Friedbühlstr. 51, 3010 Berne, Switzerland.
[3] Culture Collection University of Göteborg, Guldhedsgatan 10, 41346 Gothenburg, Sweden.

Materials and Methods

Microorganisms. The *Corynebacterium diphtheriae* isolates used for this study are listed in Table 1. Nontoxigenic strains, being of minor epidemiological importance, were not included. All isolates were of the mitis biotype. For some experiments *Corynebacterium diphtheriae* NCTC 10356 was used as a standard strain. For phage typing, 35 phages from the original and additional sets (Institutul Cantacuzino, Bucharest) were used. For reverse phage typing, a total of 88 different indicator *Corynebacterium diphtheriae* strains were applied.

Phage Typing. Phage typing was performed according to the methods described by Saragea and co-workers (3). In principle, each isolate was characterized by using three standard variations of phage typing. First, the lysotype was determined. Then reverse phage typing was performed. The isolate's prophage was converted into a free phage by UV-irradiation, and then its lytic activity was tested on a number of indicator *Corynebacterium diphtheriae* strains. Finally, we attempted to adapt a phage with high "plasticity" (951 kg) to each of the isolates.

Polypeptide Analysis. Polypeptide profiles were produced by SDS-polyacrylamide gel-electrophoresis (PAGE) according to the method of Lämmli (6). Briefly, bacteria were grown overnight on blood agar to produce confluent growth, which was then removed from the plate by floating it in destilled water. One milliliter of the suspension was transferred to glass vials and the amount of bacteria was equilibrated in all tubes. After centrifugation (1200 rpm, 10 min), the sediment was resuspended in 200 µl sample buffer consisting of 1.6 % SDS, 3.2 % 2-mercaptoethanol, 0.2 % bromophenol blue, 8.3 % sucrose in 0.01 M Tris at pH 7.8. Then the suspension was boiled for 15 min, and 35 µl of the sample were electrophoresed on discontinuous gel slabs consisting of a 5 % stacking gel and 12 % separating gel by using a vertical LKB slab gel apparatus. Electrophoresis was run with a constant current of 30 mA until the tracking dye entered the separating gel. Thereafter, the current was raised to 60 mA. Proteins were made visible with Coomassie blue stain.

Restriction Enzyme Digestion. For analysis of phage-DNA, the bacterial isolates were UV irradiated to liberate the pro-

Table 1: Phage typing characteristics of *Corynebacterium diphtheriae* isolates.

Isolate	Source and year of isolation	Phage type
7/83	endemic cases,	resist./8974[a]
1/84	Wuppertal/FRG,	resist./8974[a]
20/84	1983–1984	resist./8974[a]
31/82	imported from India, 1982	not typable
2/82	epidemic, Dortmund/FRG, 1982	I/406, 6003, 15235
3/82		I/406, 6003, 15235
4/82		I/406, 6003, 15235
GS 1		I/406, 6003, 15235
GS 2		I/406, 6003, 15235
GS 3		I/406, 6003, 15235
CCUG 15935	Gothenburg 1984	08,09/n.lg.[b]
CCUG 16574		08,09/n.lg.[b]
CCUG 17903	Gothenburg 1985	not typable
CCUG 17269	Stockholm 1984	not typable
CCUG 17995	Copenhagen 1985	not typable
CCUG 18673	Stockholm 1986	not typable
CCUG 18957	Stockholm 1986	not typable

[a] Result of lysotyping/reverse phage typing.
[b] n.lg.: not lysogen.

phage. The phage was then propagated on an indicator strain to a concentration of about 100 routine test dilutions (RTD). This was done in the same way as the multiplication of test phages for phage typing (3). After treatment with chloroform, the phage containing phase was centrifuged in a SW 27 rotor at 24,000 rpm for 3 h. The sediment was resuspended in 50 mM Tris containing 1 % SDS, treated with proteinase K and then the DNA was phenol extracted and ethanol precipitated. This and the following restriction enzyme digestion of the now purified DNA was performed according to standard procedures (7) using the enzymes mentioned under individual experiments. The fragments were then separated on a 1 % agarose gel containing ethidium bromide and made visible with UV light (366 nm).

Results

Reproducibility, Practicability and Discriminating Potential of the Different Methods

As is well known, reproducibility of phage typing was not absolute. Certain bacterial strains were inconsistently lysed by some phages. Also there was not always full agreement between our results and those obtained in the reference laboratory in Bucharest, although the same isolates were identified as such in both institutes (results not shown).

Polypeptide analysis by SDS-PAGE of reference strain NCTC 10356 grown on different days or in parallel and run on the same gel revealed identical profiles in all lanes (Figure 1). If run on different gels, some doubt arose as to whether the isolates were identical or not. Therefore, in subsequent experiments, all isolates with similar patterns on different gels were retested in parallel on one gel. The discrimination rate of 15 well-characterized *Corynebacterium diphtheriae* strains otherwise used as indicator strains for the phages of the typing set (3) was 100 % (Figure 2). Even the two strains 9408 and 430+, which represented an identical lysotype but were isolated in different parts of the world, were

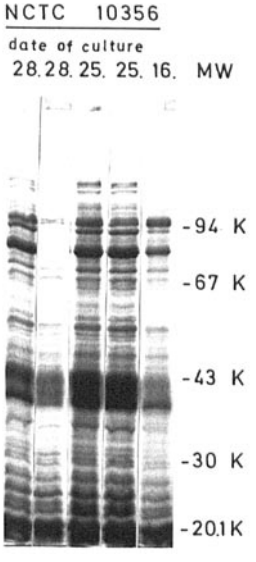


Figure 1: SDS-PAGE of the *Corynebacterium diphtheriae* NCTC 10356 reference strain grown on different days or the same day but in separate culture and run in parallel on the same gel. Identical profiles were obtained in all lanes.

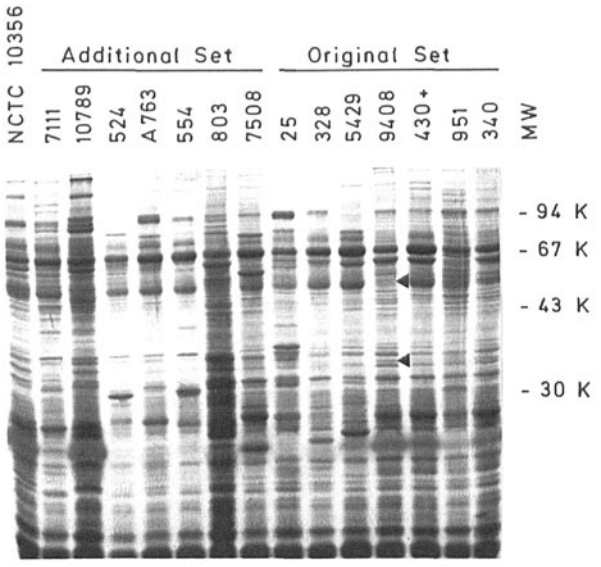
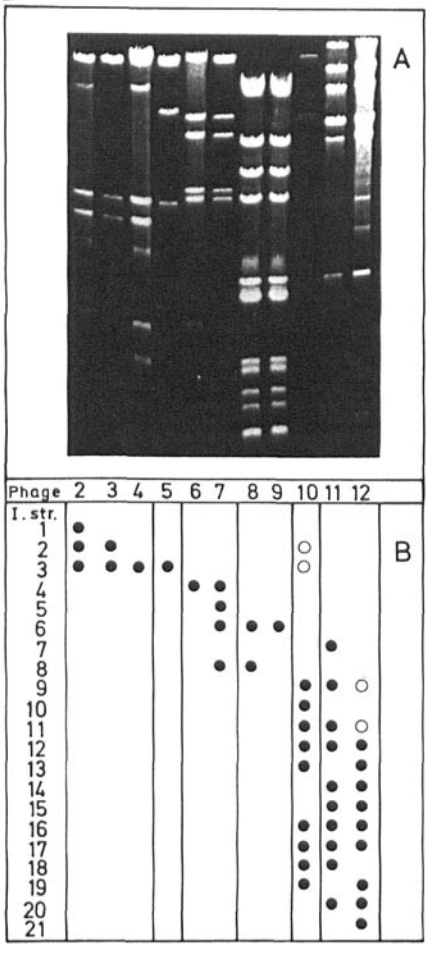


Figure 2: Discriminating potential of SDS-PAGE demonstrated with 15 well-characterized *Corynebacterium diphtheriae* strains of the original and additional set (see text) and the reference strain NCTC 10356. Strains 9408 and 430+ are identical by phage typing but not in SDS-PAGE (◀).



distinguished by SDS-PAGE. Furthermore, SDS-PAGE was less time consuming than phage typing, results being obtained after 2 days. Phage-DNA analysis by restriction enzymes proved to be a highly reproducible method leading to clear patterns. However, as demonstrated in Figure 3A, phages that were similar but not identical in lytic patterns could not always be distinguished with this method if only one restriction enzyme was used. Figure 4 shows symbolically how these apparently identical strains can be distinguished by using a panel of restriction enzymes. However, the wild-type isolates used in our studies were so different that one enzyme readily discriminated among them.

Comparative Analysis of the German Isolates

To test the efficiency of the different methods for epidemiological typing, isolates from two geographic

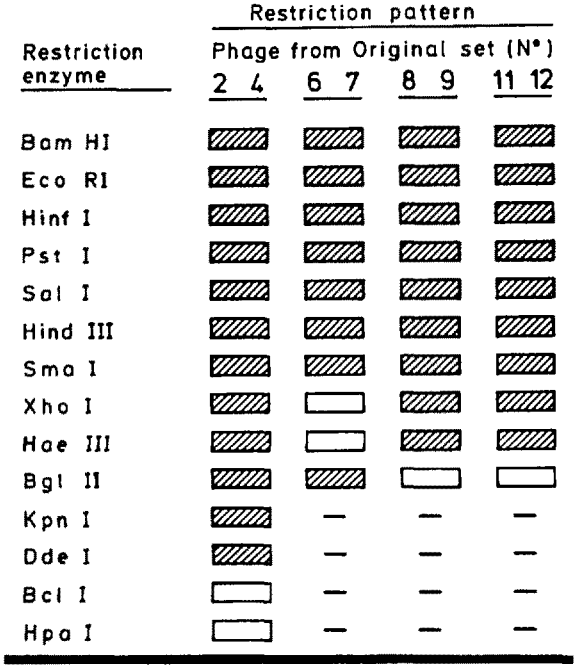


Figure 4: Differentiation of similar phages (from Figure 3) using a panel of enzymes. Symbols indicate that the similar strains tested by pairs give an identical (▨) or distinct (□) restriction enzyme pattern. ___, not tested.

Figure 3: Capability of phage-DNA analysis to discriminate between similar phages using one restriction enzyme. Phages were shown to be similar by comparing the lytic patterns on indicator *Corynebacterium diphtheriae* strains. A. DNA profile obtained with Bam HI. B. Lytic patterns on indicator strains. Phage numbers refer to the original set (3). I. str. indicator strain, (●) constant lysis, (○) inconstant lysis.

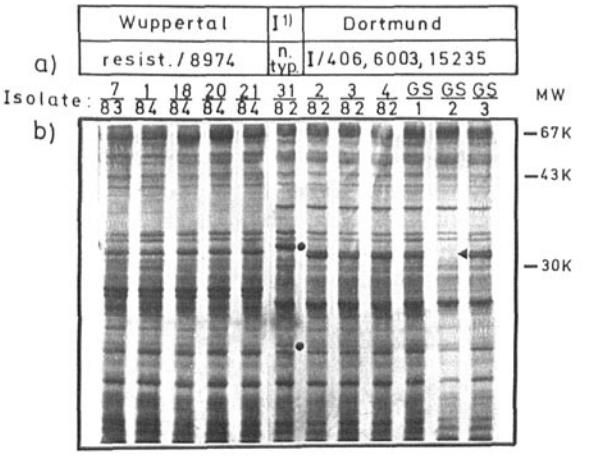


Figure 5: Isolates collected from endemic cases in Wuppertal and during the epidemic in Dortmund. a) Results of phage typing (lysotype/reverse type); imported from India; n. typ., not typable; b) SDS-PAGE profiles (•) prominent unique feature of strain 31/82; ◄, lacking band in GS/2.

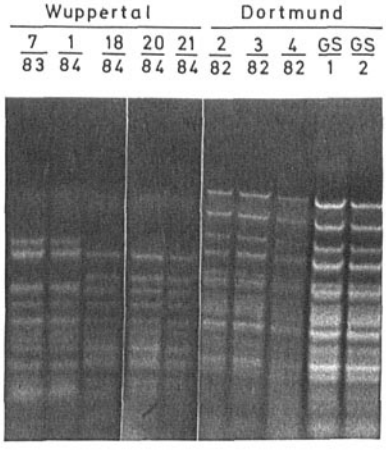


Figure 6: Phage-DNA profiles (Hae I) of the Wuppertal and Dortmund isolates. A genetic alteration can be recognized in isolates 18/84, 20/84 and 21/84, where the uppermost band is lacking. Instead of this band an additional shorter DNA fragment can be recognized somewhat lower on the gel.

locations about 50 km apart (Wuppertal and Dortmund) were analysed. On the basis of the results obtained by phage typing (Table 1, Figure 5) all Wuppertal isolates were concluded to be of the same origin. All of the Dortmund isolates except one (31/82) could be phage typed and were shown to be identical. (The isolate 31/82 was imported from India and discovered by chance.) Polypeptide analysis revealed that the Wuppertal and Dortmund isolates were not identical, but that, with the exception of isolate 31/82, one strain was responsible for all cases in the respective geographic location. Polypeptide analysis clearly identified isolate 31/82 as being distinct. A missing polypeptide band in isolate GS 2 of the Dortmund strain points to a mutation that occurred in the course of the epidemic. This alteration, however, was minor and did not jeopardize the epidemiological classification of GS 2. By phage-DNA analysis of the Wuppertal isolates the occurrence and spread of a genetic alteration were traced (Figure 6). The DNA-fragment profile obtained with restriction enzyme Hae I showed a slight alteration in the bacterium 18/84 isolated in 1984 and was also observed in two further isolates obtained later (20/84, 21/84). This confirms that one single strain successively caused the five cases during the 20-month endemic. Again, all Dortmund isolates except 31/82 had identical DNA fragment profiles. The imported isolate (31/82) could not be analyzed with this method since it was non-lysogenic.

Characterization of the Swedish Isolates

Many of the Swedish isolates could not be phage typed (Table 1) and therefore, no definite epidemiological information was obtained by this method. Polypeptide analysis, however, showed that all isolates had identical patterns (Figure 7). The strain isolated in Stockholm during the first outbreak in 1984 (CCUG 17269) and later in 1986 (CCUG 18673 and CCUG 18957) was shown to have the same polypeptide profile as the isolate from Copenhagen (CCUG 17995) and the three isolates from Gothenburg (CCUG 15935, CCUG 16574 and CCUG 17903), where the epidemic started, strongly suggesting a common source. It is not clear why two of those isolates (CCUG 15935 and CCUG 16574) were susceptible for phages nos. 8 and 9 of the typing set, whereas the others could not be phage typed.

Discussion

The purpose of this study was to find a rapid and reliable technique for epidemiological typing of *Corynebacterium diphtheriae* and to analyze the epidemiology of diphtheria in the FRG and in Sweden. Polypeptide analysis of whole bacteria using SDS-PAGE fulfilled these criteria best. SDS-PAGE correlates well with the "gold standard" phage typing and also has several additional advantages. It has a typing rate of 100 %, is rapid and easy to perform, and does not depend on a stock of phages or antisera. Therefore, the technique can be applied by virtually every laboratory for every kind of bacterial species. Polypeptide analysis has been successfully used for typing *Haemophilus influenzae* (4) and *Clostridium difficile* (5). SDS-PAGE profiles also have a few minor disadvantages. Results cannot be reported

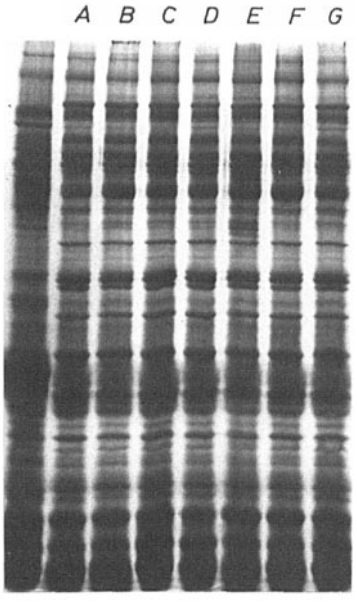


Figure 7: Selected *Corynebacterium diphtheriae* isolates from Sweden analysed by SDS-PAGE to show a possible epidemiological relationship (see Table 1). Lanes A to C contain isolates from Gothenburg (CCUG 15935, CCUG 16574, CCUG 17903), lane D contains a strain from Stockholm isolated in 1984 (CCUG 17269) and lane E an isolate from Copenhagen (CCUG 17995). Lanes F and G contain isolates originating from Stockholm in 1986 (CCUG 18673 and CCUG 18957). The unlabelled lane to the left contains reference strain NCTC 10356.

numerically and reproducibility from gel to gel is not complete. Therefore, exact comparison of isolates with similar profiles can only be accomplished by running them on the same gel slab.

In contrast, results obtained with restriction enzyme analysis from phage-DNA are very clear cut and reproducible. However, if the isolate's phage-DNA is cut with only one enzyme, some strains shown to be similar but not identical by phage typing cannot be differentiated. Surprisingly, the epidemiological evaluation of all wild-type strains tested in this study was readily accomplished by using only one enzyme. In fact, it has been demonstrated before that *tox* gene bearing corynebacteriophages share great sequence homologies (8). Apparently, however, their restriction enzyme profiles vary enough to allow for their use in epidemiological investigations.

In phage-DNA analysis, virulent phages have to be propagated on a sensitive host strain of *Corynebacterium diphtheriae*. This limits the typability rate, since phages cannot be propagated from all strains. Possible reasons for this are that either the prophage does not convert into a vegetative phage under the selected conditions, that only defective phages are produced, or that a sensitive host strain for the phage cannot be found. This problem could be overcome by prophage analysis, by means of which the phage-DNA containing bacterial DNA is isolated, cleaved with restriction enzymes, electrophoresed, and the fragments transferred to a suitable membrane to allow for hybridization with DNA isolated from easily propagated phages. Rappuoli and co-workers (9), using a cloned fragment of phage-DNA present in most prophages, applied this technique to epidemiologically characterize some of the Swedish isolates used in our study. Their results confirmed ours obtained with polypeptide analysis, since they also showed that isolates CCUG 15935, CCUG 16754, CCUG 17903 and CCUG 17269 were identical.

Despite the fact that restriction enzymes provide important epidemiological information, the methods consist of many working steps and are too time consuming for routine use. Nevertheless, they are well suited to answer more precise questions. For instance, the appearance of "subtypes," characterized by a slightly modified restriction fragment profile, and their spreading in the course of an epidemic can be followed. This was achieved in the case of the Wuppertal strains by using Hae I. Also, with a DNA probe, Pappenheimer and Murphy (10) proved that both toxigenic and non-toxigenic isolates recovered in the course of an outbreak derived from the same original strain.

With the methods described we were able to elucidate the epidemiological background of some recent diphtheria outbreaks in the FRG and in Sweden. Corresponding epidemiological information was obtained with all methods used. It became obvious that the outbreak in Dortmund was caused by one single strain. A different strain was isolated from only one carrier who had previously been in India. The five cases reported in Wuppertal over a 20 month period were also caused by one strain, illustrating how long a pathogenic strain can persist in a population, occasionally causing disease. The spreading potential of a pathogenic strain is illustrated by the data obtained from the Swedish isolates. The first diphtheria outbreak occurred in Gothenburg in 1984. The strain isolated from patients and healthy carriers during this outbreak was shortly thereafter cultured from diphtheria cases in Stockholm (1984) and was also found in Copenhagen. The second outbreak in Stockholm in 1986 again was very likely caused by the same strain.

In conclusion, our results suggest that SDS-PAGE polypeptide analysis of *Corynebacterium diphtheriae* is superior to all other methods tested. This method should also become invaluable for epidemiological typing of other bacterial species. In certain cases, however, it can be worthwhile to look for additional epidemiological markers using restriction enzymes and other methods.

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