

Molecular Epidemiology of *Corynebacterium diphtheriae* from Northwestern Russia and Surrounding Countries Studied by Using Ribotyping and Pulsed-Field Gel Electrophoresis

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A selection of 100 *Corynebacterium diphtheriae* isolates from asymptomatic carriers and clinical cases from five regions in northwestern Russia were examined. Six additional isolates from patients in Finland and Estonia with epidemiological links to Russia were also examined. All isolates were characterized by biotyping, toxigenicity testing, ribotyping, and pulsed-field gel electrophoresis (PFGE). Hybridization of genomic DNA digested with *Bst*EII revealed five ribotype patterns among the biotype *gravis* isolates (G1 through G5) and two patterns among the biotype *mitis* isolates (M1 and M2). PFGE using *Sfi*I was not able to distinguish between ribotypes G1, G2, and G4. The predominant ribotype pattern, G1, found in cases of disease in all the areas studied, appears to be disseminating, in view of the isolates received from imported cases in Finland and Estonia. Among the 106 isolates examined, 68 produced pattern G1 and 24 produced pattern M1. Most of the M1 isolates were from the Leningrad Oblast region. Distinct ribotypes such as G2, G3, G4, G5, and M2 could represent endemic disease.

Diphtheria is now the most widespread infectious fatal disease in Russia and the Ukraine. In Russia, there were over 15,000 cases in 1993 (10.1 per 100,000 people), compared with nearly 4,000 cases in 1992 (2.6 per 100,000). About 3,000 people were diagnosed with diphtheria in the Ukraine in 1993 (5.7 per 100,000), compared with just over 1,500 cases in 1992 (3.0 per 100,000). Imported cases of diphtheria contracted in Russia and the Ukraine by overseas travellers have been diagnosed recently in Finland, Estonia, Norway, Poland, Latvia, Lithuania, and Germany (16).

During the past few years, DNA fingerprinting of *Corynebacterium diphtheriae* has become a reliable method in epidemiology. In 1983, Pappenheimer and Murphy (10) clearly demonstrated the resolving power of molecular typing when they analyzed *C. diphtheriae* isolates in a surveillance study after a diphtheria case in Manchester, United Kingdom. Using restriction fragment patterns and patterns of hybridization with three DNA probes, they found good evidence for *in vivo* lysogenic conversion to toxin production. Rappuoli et al. (13) in 1988 used a recently discovered insertion element of *C. diphtheriae* as a DNA probe to characterize 34 Swedish strains from a diphtheria outbreak in an alcoholic and drug-abusing population. In 1989, Coyle et al. (2) studied the molecular epidemiology of the three biotypes of *C. diphtheriae* in the Seattle outbreak (1972 to 1982) by analyzing DNA restriction fragment patterns with three DNA probes. They found that during the outbreak there was little variability within the *intermedius* and *gravis* biotypes but considerable variability in the *mitis* biotype. In 1992, Gruner et al. (6) used a pBR322-derived plasmid carrying rRNA as a DNA probe to characterize a cluster of nontoxicogenic *C. diphtheriae* isolates among Swiss intravenous drug abusers. We studied the molecular epidemi-

ology of a selection of 100 *C. diphtheriae* isolates from clinical cases and asymptomatic carriers from five areas in northwestern Russia. Also, six additional isolates from Finland and Estonia linked epidemiologically to Russia were examined. All isolates were characterized by biotyping, toxigenicity testing, and restriction fragment length polymorphisms of rRNA genes (ribotyping) using the restriction endonuclease *Bst*EII. Pulsed-field gel electrophoresis (PFGE) using *Sfi*I enzyme digestion of the *C. diphtheriae* genome was used in an attempt to discriminate the ribotype patterns further.

MATERIALS AND METHODS

Bacterial strains. A total of 100 *C. diphtheriae* isolates collected randomly during 1993 from asymptomatic carriers and clinical cases of diphtheria in St. Petersburg, Republic of Carelia, and the Leningrad, Murmansk, and Kaliningrad Oblast regions in Russia were first identified by standard microbiology techniques at the local microbiology laboratory and then sent to the Diphtheria Laboratory at the Pasteur Institute in St. Petersburg for confirmation of species and toxigenicity. For comparative studies, two Finnish and four Estonian *C. diphtheriae* isolates from 1993 were also studied. These strains were identified as *C. diphtheriae* in the local microbiology laboratory. All isolates were then sent to the Diphtheria Laboratory at the National Public Health Institute, Helsinki, Finland, where they were biotyped, and then they were transported to the Respiratory and Systemic Infection Laboratory at the Central Public Health Laboratory, London, United Kingdom, for additional typing.

Biotyping and toxigenicity testing. The isolates were characterized by biotyping, by using a commercial kit (API Coryne; API-bioMérieux, Basingstoke, United Kingdom) according to the manufacturer's instructions. Toxigenicity was determined by the Elek immunoprecipitation test (5).

Ribotyping. Chromosomal DNA was prepared by a modified version of the method described by Pitcher et al. (12). The purified DNA was cleaved with *Bst*EII (Boehringer) according to the manufacturer's instructions. The restriction fragments were subjected to agarose (1%) gel electrophoresis and blotted onto Hybond-N (Amersham International, Amersham, Buckinghamshire, United Kingdom) as described previously by Saunders et al. (14). Membranes were hybridized with a biotin-labelled cDNA probe derived from the total rRNA of the *C. diphtheriae* type strain, NCTC 11397. The rRNA was isolated and labelled by the method described by Pitcher et al. (11). A ribotyping pattern was interpreted on the basis of a single band difference. *Bst*EII profiles were compared with the computer program GelManager (BioSystemica), using simple matching coefficients and the unweighted pair group method using averages.

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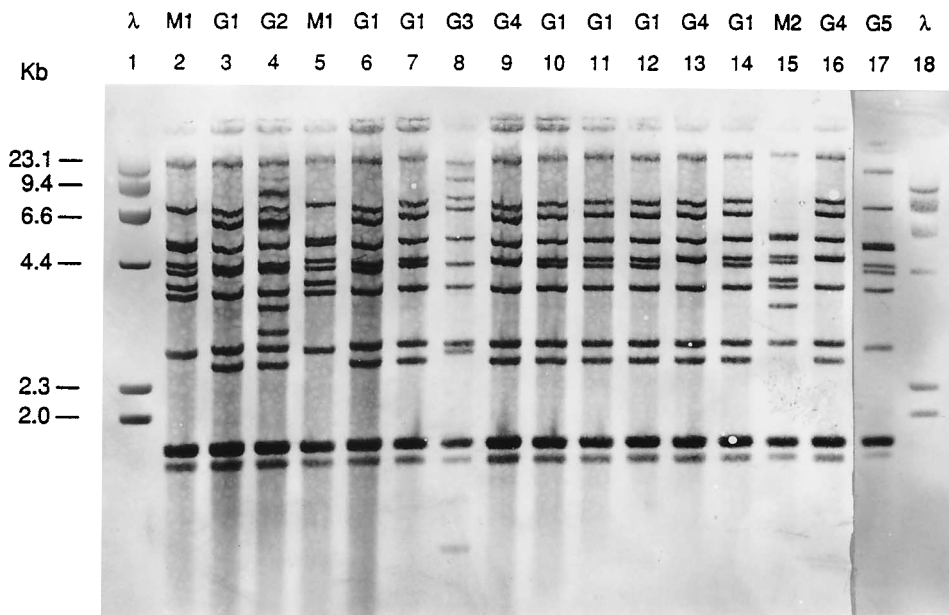


FIG. 1. *BstEII* rRNA gene profiles of *C. diphtheriae* biotype gravis and mitis isolates from northwestern Russia and surrounding countries. Lanes 1 and 18, lambda *HindIII* digests as a size standard (sizes are indicated on the left); lanes 2 to 17, ribotypes for 16 isolates from St. Petersburg (lanes 2, 3, 4, and 17), Leningrad Oblast (lanes 5 and 6), Murmansk Oblast (lanes 7 to 9), Finland and Estonia (lanes 10 and 11, respectively), Carelia (lanes 12 and 13), and Kaliningrad Oblast (lanes 14 to 16). Ribotypes are indicated above the lanes.

PFGE. Genomic DNA was prepared by a modified version of the method described by Murray et al. (9). The agarose blocks were incubated overnight at 37°C in lysis buffer, and this incubation was followed by deproteination in proteolysis buffer for 48 h. The DNA was cleaved with *Sfi*I (Boehringer) according to the manufacturer's instructions. PFGE was carried out in 0.5× Tris-borate-EDTA-1.5% agarose gels at 14°C by using a CHEF DRII system (Bio-Rad, Hertfordshire, United Kingdom). The pulse times were 5 to 20 s over 20 h and 1 to 5 s over 18 h. A lambda DNA concatemer (Bio-Rad) was used as a molecular

size marker. Differences of three or more bands were used to distinguish PFGE types.

RESULTS

A total of 106 *C. diphtheriae* isolates were examined (100 isolates from five regions in Russia, 2 from Finland, and 4 from

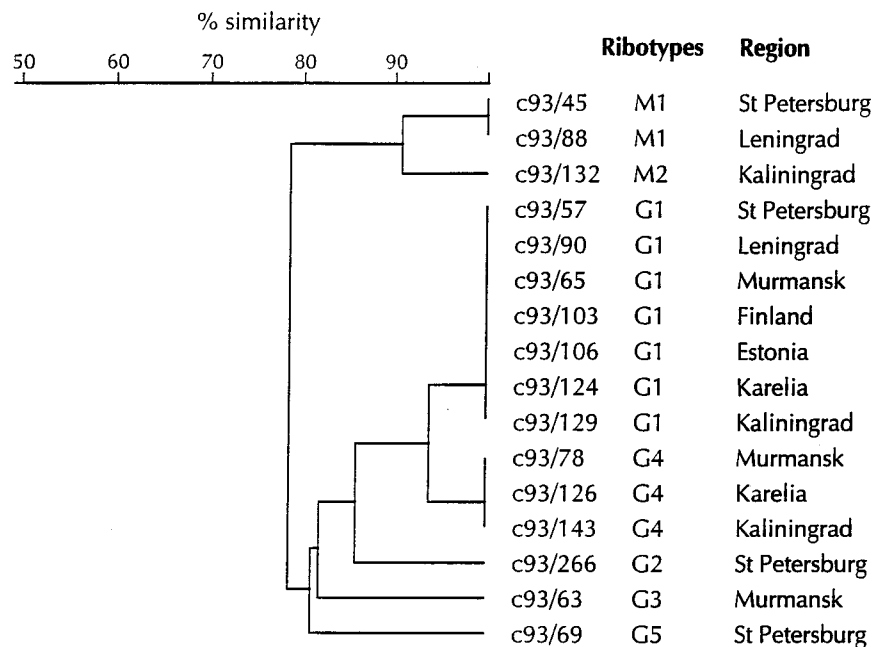


FIG. 2. Cluster analysis of rRNA gene profiles of *C. diphtheriae* isolates from northwestern Russia and surrounding countries.

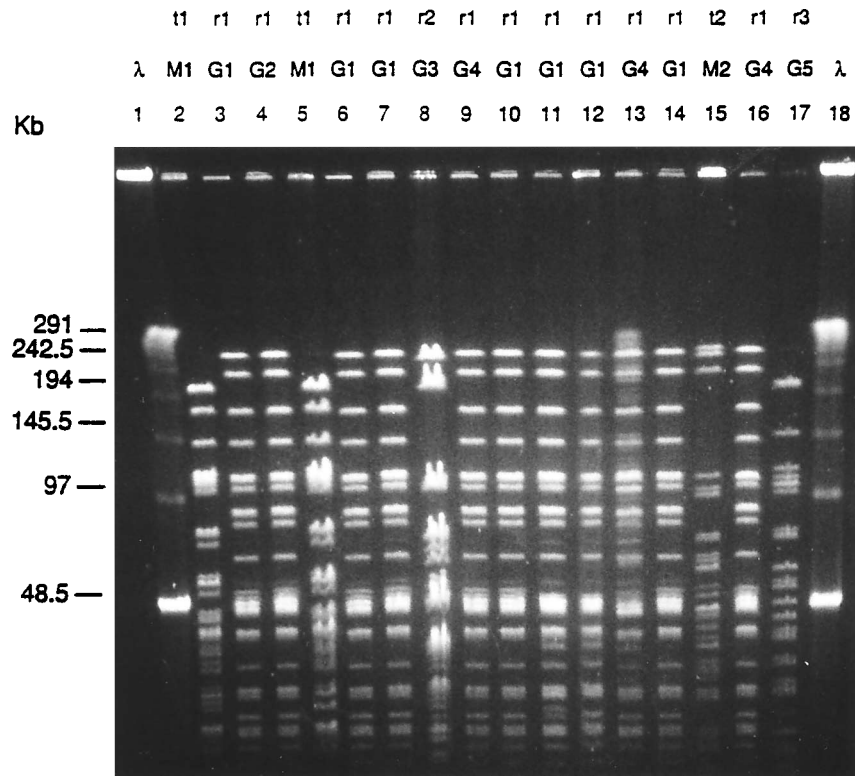


FIG. 3. *Sfi*I PFGE profiles of *C. diphtheriae* biotype gravis and mitis isolates from northwestern Russia and surrounding countries. Lanes 1 and 18, lambda concatemer as a size standard (sizes are indicated on the left); lanes 2 to 17, PFGE types obtained for the 16 isolates which were ribotyped. Ribotype designations (G1 to G5 and M1 and M2) and PFGE types (r1 to r3 and t1 and t2) are indicated above the lanes.

Estonia). Among the 100 isolates from northwestern Russia, 75 belonged to biotype gravis and 25 were of the mitis biotype. The two Finnish and the four Estonian isolates belonged to biotype gravis, and all the strains examined were toxigenic.

Examination of the restriction fragment length polymorphisms of ribosomal genes of the 106 strains revealed five distinct patterns of restriction fragments hybridizing with the probe (G1 through G5) among the biotype gravis isolates and two distinct patterns (M1 and M2) among the biotype mitis isolates (Fig. 1). The dendrogram (Fig. 2) represents the relationships between the *Bst*EII restriction digest patterns, determined by using GelManager.

Ribotype distribution of *C. diphtheriae* isolates in various regions in northwest Russia. Of the 42 isolates received from St. Petersburg, 33 belonged to biotype gravis and 9 belonged to biotype mitis. Twenty-four isolates produced the ribotype pattern G1, eight produced pattern G2, and one produced pattern G5, which was not noted in any of the other geographical areas. The nine biotype mitis isolates produced pattern M1.

Sixteen isolates were received from the Leningrad Oblast region; 14 belonged to the mitis biotype, and only 2 were of the gravis biotype. The 14 biotype mitis isolates produced pattern M1, and the two biotype gravis isolates produced pattern G1.

All 17 isolates received from the Murmansk Oblast region belonged to biotype gravis. Fifteen of them produced ribotype pattern G1, one produced pattern G3, and one produced pattern G4.

Twenty isolates were received from the Kaliningrad Oblast region. Eighteen were of the gravis biotype; 17 of the isolates produced pattern G1, and 1 isolate produced pattern G4. Two isolates belonged to the mitis biotype; one of them produced

pattern M1, and the other produced pattern M2, which was not seen in any of the other regions.

All five isolates received from the Republic of Carelia belonged to biotype gravis. Four isolates produced pattern G1, and the fifth isolate produced pattern G4. The six isolates received from Finland and Estonia were of the gravis biotype, and they all produced pattern G1.

PFGE of macrorestriction fragments. *Sfi*I was used in order to further discriminate the ribotypes. Examination of the PFGE profiles revealed three distinct patterns among the biotype gravis isolates, and they were designated r1 (ribotypes G1, G2, and G4), r2 (ribotype G3), and r3 (ribotype G5). Two patterns were revealed among the biotype mitis isolates, and they were designated t1 (ribotype M1) and t2 (ribotype M2) (Fig. 3). The restriction endonuclease *Sfi*I produced PFGE profiles consisting of 18 to 25 DNA fragments ranging in size from 24 to 290 kb, as determined by comparison of the mobilities of the fragments with those of lambda concatemers. PFGE using *Sfi*I was not able to discriminate ribotype pattern G1 further. PFGE was able to distinguish ribotype patterns G3, G5, M1, and M2, but it was not able to distinguish ribotypes G1, G2, and G4 (Table 1).

DISCUSSION

For identifying the sources and monitoring the spread of an epidemic, epidemiological markers such as biotype and toxigenicity have been useful, but phenotypic systems have limitations in their typing ability and stability and have a low discriminatory ability. We have used restriction fragments derived from a small portion of the *C. diphtheriae* genome as markers

TABLE 1. Association of ribotypes and PFGE profiles of *C. diphtheriae* isolates from northwestern Russia and surrounding areas

PFGE profile	n	No. of isolates with ribotype:						
		G1	G2	G3	G4	G5	M1	M2
r1	79	68 ^a	8 ^a		3 ^a			
r2	1			1				
r3	1					1		
t1	24						24	
t2								1
Total	106	68	8	1	3	1	24	1

^a Isolates differentiated by ribotyping but not by PFGE.

to distinguish between strains. The method is based on the detection of minor differences in rRNA sequences. The restriction endonucleases *EcoRI*, *PvuII*, and *BstEII* were tested on the *C. diphtheriae* genome before *BstEII* alone was chosen for ribotyping. *BstEII* was more discriminatory than *EcoRI* and *PvuII*, and it also generated sufficient fragments complementary to the probe to provide useful results (3).

Hybridization of genomic DNA digested with *BstEII* revealed characteristic rRNA gene restriction patterns among the 100 isolates received from northwestern Russia. Five ribotype patterns, G1 through G5, were identified among the biotype gravis isolates. Of the biotype gravis isolates, 84% (68 of 81) produced pattern G1. One predominant pattern, M1, was apparent among the biotype mitis isolates (24 of 25), with the identification of another distinct pattern, M2, which was recognized only in the Kaliningrad Oblast region.

The predominant pattern G1 was present in St. Petersburg, the Republic of Carelia, and the Murmansk, Kaliningrad, and Leningrad Oblast regions, and G1 appears to be disseminating in view of the imported cases in Finland and Estonia (4). The majority of biotype mitis isolates were from the Leningrad Oblast region, but they were also received from St. Petersburg and the Kaliningrad Oblast region. The recognition of distinct patterns such as G2 through G5 and M2 could represent possible endemic disease in St. Petersburg, the Republic of Carelia, and the Murmansk and Kaliningrad Oblast regions, as opposed to the predominant epidemic clone G1, which is causing disease in all the areas studied.

The ribotype patterns obtained within the gravis biotype from the Russian epidemic have not so far been identified among small numbers of *C. diphtheriae* biotype gravis strains from other parts of the world, including the United Kingdom, which were examined at the Central Public Health Laboratory.

Recent data suggest that PFGE is the most discriminating of the currently available genotypic methods (1, 8, 15). A variety of restriction enzymes (*NotI*, *DraI*, *SalI*, *SmaI*, *XhoI*, *SacI*, *RsaI*, *HincII*, *HinfI*, *AluI*, and *SfiI*) were tested on the *C. diphtheriae* genome before *SfiI* was chosen for use. The restriction enzyme *SfiI* generated reasonable-size fragments which could be separated by PFGE. The enzymes *NotI*, *DraI*, *SmaI*, and *SalI* produced very small fragments, and it was therefore difficult to obtain clear, definitive restriction fragment profiles by PFGE. We encountered problems in applying some of the other enzymes in that digestions inconsistently went to completion and some enzymes did not digest *C. diphtheriae* DNA at all (3). Unlike the case for ribotyping, in which we used a one-band difference to distinguish the patterns, for PFGE we used a difference of three or more bands to distinguish PFGE types (7). The PFGE profiles produced by isolates which produced ribotype patterns G1, G2, and G4 are identical or very

similar. The minor variations in PFGE patterns between the G1, G2, and G4 ribotypes which are barely visible on Fig. 3 were not reproducible from run to run. Therefore, on the basis of the similarity of the PFGE profiles and the fact that ribotype patterns G1 and G2 differ from each other by three bands and pattern G4 differs from G1 by only a single band, whereas G2 differs from G4 by four bands. We can assume that ribotype patterns G1, G2, and G4 could have arisen from a single clonal group.

Interpretation of chromosomal patterns can be difficult when isolates differ by only a few bands. Such differences could arise within a single individual from inversions, deletions, or other rearrangements of the chromosome or from the acquisition or loss of a prophage, transposon, insertion sequence, or plasmid (9). Thus, ribotypes G1, G2, and G4 may represent possible variants within the same clonal group.

Further studies of the discriminatory power of ribotyping and PFGE may include the use of different or additional enzymes.

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