Genotypic and Phenotypic Characteristics of *Corynebacterium diphtheriae* Strains Isolated from Patients in Belarus during an Epidemic Period

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One hundred two *Corynebacterium diphtheriae* strains (93 of the gravis biotype and nine of the mitis biotype) isolated from clinical cases during the Belarus diphtheria epidemic were characterized by biotyping, toxigenicity testing by the Elek test and an indirect hemagglutination assay, phage typing, and ribotyping. The gravis biotype strains were characterized as high and medium toxin producers, and strains of biotype mitis were characterized as low and medium toxin producers. Most strains (82 of 102) were distributed among five phage types. Seventy-two strains (64 of the gravis biotype and 8 of the mitis biotype) belonged to phage type VI ls5,34add. Hybridization of genomic DNA digested with *Bst*EII and *Pvu*II revealed five ribotype patterns, namely, D1, D4, D6, D7, and D13. The majority of gravis biotype strains belonged to ribotypes D1 (49 of 93) and D4 (33 of 93) and included one clonal group of *C. diphtheriae*. This clone predominated in all regions in Belarus. There was a statistical association between ribotypes and phage types but not between ribotypes and levels of toxin production.

The diphtheria epidemic that started in the early 1990s in Eastern Europe was exceptional. It was the first major epidemic during the vaccination era and occurred in countries where diphtheria toxoid vaccine had been used successfully for decades. The epidemic spread to many countries of the Newly Independent States of the former Soviet Union, including Belarus, very rapidly. In the mid-1960s, diphtheria cases in Belarus were almost eliminated due to the 97.4% vaccination coverage rate of the population. In 1981, there were no diphtheria cases. However, since 1982 the disease has reemerged in Belarus. Between 1982 and 1989, a total of 138 diphtheria cases and seven deaths were reported, with a peak incidence in 1987 (35 cases). Between 1990 and 1999, there were 1,137 cases and 30 deaths, with a peak incidence in 1995 (319 cases; incidence rate, 3.10 per 100,000 population).

The epidemic in Belarus is currently under control. Since 1998, Belarus has been one of the countries that have achieved and maintained the World Health Organization (WHO) target of <1 diphtheria case per 100,000 population. Success would not have been possible without mass immunizations. At that time it was impossible to determine the origin of the causative organism, *Corynebacterium diphtheriae*, and its transmission without microbiological and molecular characterization.

For many decades, the epidemiological subdivision of *C. diphtheriae* was based primarily upon the biotype, serotype, and phage type (7). The limitation of *C. diphtheriae* identifica-

tion by microbiological methods stimulated development of new molecular and biological typing systems. Previously, molecular epidemiological studies were based on restriction fragment length polymorphisms of the *C. diphtheriae* genome (20) and hybridization of genomic DNA to a variety of molecular probes targeting the insertion element (24, 25), diphtheria toxin gene, and corynephage β and its attachment sites (1, 20, 23). However, these methods did not provide information on the true genetic structure of *C. diphtheriae* (8). Intraspecies genetic diversity is one of the most important factors determining the mechanism of transmission and survival of epidemic agents. Multilocus enzyme electrophoresis (MEE) is considered the gold standard for determination of the genetic structure of bacterial species (27). Grimont and Grimont (11) showed the usefulness of rRNA gene restriction patterns in the taxonomy and identification of gram-negative and gram-positive bacterial species.

The analysis of *C. diphtheriae* isolates from the epidemic in Russia using not only MEE but also ribotyping and a randomly amplified polymorphic DNA assay showed heterogeneity among *C. diphtheriae* isolates and the clonality of the biotype gravis (3, 5, 14, 22). *C. diphtheriae* strains that caused epidemic diphtheria in Russia formed a separate clonal complex, which included strains of the biotype gravis, among 14 related electrophoretic types and were designated electrophoretic type 8 complex (ET 8 complex). The main characteristics of the epidemic strains are that they belonged to the ET 8 complex and ribotypes D1 (Saint Petersburg, Russia) and D4 (Russia) (21). Prior to 1990, strains of these ribotypes were rarely isolated in Russia. These strains began to be isolated more frequently, and in 1994, they were more than 80% of all identified ribotypes (22). The same

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FIG. 1. Normalized graph showing migration patterns of rRNA gene restriction fragments of DNA from *C. diphtheriae* ribotypes found in Belarus after digestion with *BstEII* and *PvuII* and hybridization with the digoxigenin-labeled Oligo 5 Mix probe.

clonal *C. diphtheriae* group was the causative organism of the diphtheria epidemic in Georgia (29).

It has been shown that strains of this clonal complex were occasionally isolated from the residents of the countries of the European Union and the United States. Imported diphtheria cases, caused by *C. diphtheriae* biotype gravis ribotype D1 (Saint Petersburg), were reported from Finland, Estonia, and Germany (5). However, the geographical origin of epidemic *C. diphtheriae* strains and their association with native and epidemic strains circulating worldwide are not clear.

The purpose of this investigation was to identify the ribotypes of *C. diphtheriae* epidemic strains isolated from patients in Belarus and to correlate clonal type with phage type and toxin production.

Bacterial strains. A total of 102 strains from diphtheria cases isolated during the diphtheria epidemic in Belarus in 1996 to 1997 were selected. Bacteriological identification was performed according to the *WHO Manual for the Laboratory Diagnosis of Diphtheria* (6).

Toxicogenicity testing. Toxicogenicity was determined by the Elek immunoprecipitation method (Elek test). The toxin production level of isolates was determined by a passive hemagglutination assay. This test was performed with sheep red blood cells that had been treated with formalin and tannin and then sensitized with diphtheria antitoxin. The sensitivity of the diagnostic test using erythrocyte diphtheria antibody, which was prepared at a concentration of 0.0015 Lf/ml (where Lf is the limit of flocculation) compared to the reference diphtheria toxoid was included in the investigation at a dilution of 0.1 Lf/ml. One full loop of each isolate grown on Columbia agar blood was suspended in 3 ml of Elek broth medium supplemented with 10% (vol/vol) newborn bovine serum and incubated at 37°C in air for 18 h. Serial 25- μ l dilutions of the culture supernatant were aseptically dispensed into microtiter plates prior to the addition of 25 μ l of the erythrocyte diphtheria antibody solution. The plates were sealed and incubated for 1.5 to 2 h at 20°C. Toxin concentrations of culture supernatant (in Lf per milliliter) are calculated by taking the last dilution of culture supernatant at which there was hemagglutination and multiplying the dilution factor by the sensitivity of the diagnostic erythrocyte diphtheria antibody test.

Depending upon the toxin production level, *C. diphtheriae* strains were classified as strains of high toxicogenicity (toxin

production level from 0.05 to 0.38 Lf/ml), medium toxicogenicity (toxin production level from 0.01 to 0.02 Lf/ml), or low toxicogenicity (toxin production level from 0.003 to 0.006 Lf/ml).

Ribotyping. Ribotyping included DNA restriction with *BstEII* and *PvuII* and hybridization with a digoxigenin-labeled Oligo 5 Mix probe (prepared in our laboratory) for typing 102 toxigenic isolates of the gravis and mitis biotypes. The patterns were interpreted using the Taxotron program by cluster analysis using the unweighted pair group method. Admissible error was 5% (12).

Phage typing. A total of 102 isolates were typed using two typing schemes for *C. diphtheriae*, the original scheme described by Saragea and Maximescu in 1964 (28) and another scheme described by Maximescu et al. in 1972 (16). Lysoreistant (LR) or lysosensitive (ls) *C. diphtheriae* strains were classified as nontypeable.

Statistical methods. The statistical correlation between ribotype, phage type, and level of toxin production was estimated by the Crosstabs procedure using the program SPSS for Windows, release 8.0.0 (SPSS, Inc., Chicago, Ill.). The significance level for analysis was $P \leq 0.05$.

Biotyping and toxicogenicity testing. Most (93 of 102) *C. diphtheriae* strains isolated from diphtheria patients belonged to biotype gravis; nine strains belonged to biotype mitis. All were toxigenic by the Elek test. The gravis biotype strains were characterized as producing high (≥ 0.05 Lf/ml) or medium (from 0.006 up to 0.05 Lf/ml) levels of toxin, which corresponded to 33.3 and 48.4% of the total. Only 18.3% of isolates produced low levels of toxin (≤ 0.006 Lf/ml). Strains of biotype mitis produced low (two of nine strains) to medium (seven of nine strains) levels of toxin.

Ribotyping. Three distinct ribotype patterns were revealed among the 93 biotype gravis strains examined, while two distinct patterns were seen for the mitis biotype. The majority of gravis biotype strains belonged to ribotypes D1 (Saint Petersburg) (49 of 93 strains) and D4 (Russia) (33 of 93 strains). Only three gravis isolates belonged to ribotype D6 (Lyon, France). Five gravis isolates could not be identified by the reference database and formed a potential new group.

A small number of biotype mitis isolates were distributed between two ribotypes: D7 (Otchakov, Belarus) (six isolates) and D13 (Schwarzenberg, Belarus) (two isolates). The ribotype of one isolate was also novel.

TABLE 1. Relationship between ribotype, phage type, and toxin production level of *C. diphtheriae* isolates from Belarus

Ribotype	No. of isolates in each ribotype of phage type:									Total no. of strains	No. of strains with toxin production level ^a :		
	VI ls5,34add	VI ls34add	VI	VI ls16o	XIV	ls34Add	ls5,34add	ls19,20o	lsLR		Low	Medium	High
D1	40	2		1	1	1	1		3	49	12	19	18
D4	18		2		1		11		1	33	5	22	6
D7	8							1		9	1	7	1
D6	1				2					3		1	2
D13	1								1	2	1	1	
New	4				1		1			6		2	4
Total	72	2	2	1	5	1	13	1	5	102	19	52	31

^a The toxin production levels were as follows: low (0.003 to 0.006 Lf/ml), medium (0.01 to 0.02 Lf/ml), and high (0.05 to 0.38 Lf/ml).

As shown in Fig. 1, the restriction patterns of ribotypes D1 and D4 are closely related and D1 and D4 would be considered one clonal complex.

Thus, the diphtheria cases were caused primarily by one clone, a toxigenic strain of the gravis biotype, which predominated in Belarus and accounted for 80.4% of all epidemic strains included in this study.

Phage typing. Most strains (82 of 102) were distributed among five phage types (VI ls5,34add, VI ls34add, VI, VI ls16o, and XIV). Of 102 strains, 15 were atypical (ls34add, ls5,34add, and ls19,20o) (Table 1), and 5 were nontypeable (phage resistant or only phage sensitive). More than half of the gravis strains (64 of 93 [68.8%]) belonged to phage type VI ls5,34 add. The other four phage types comprised one to five isolates. All nine strains of the mitis biotype, except for one strain that was phage resistant, belonged to the predominant phage type (VI ls5,34 add).

Correlation of ribotyping, associated phage type, and level of toxin production. As shown in Table 1, the predominant ribotypes D1 and D4 (82 of 102 strains) comprised most of the isolates examined. The genetically related isolates of biotype gravis (58 of 82 [70.7%]) belonged to one phage type (VI ls5,34 add). The remainder of these two ribotypes correlated with four other phage types or were atypical or nontypeable. Strains of other ribotypes included isolates of phage type VI ls5,34add. However, strains of this phage type also predominated among ribotype D7 and in a new group (eight of nine strains and four of six strains), respectively. The correlation between ribotypes and phage types appeared to be significant ($\chi^2 = 75.5$; degrees of freedom [df] = 40; $P < 0.01$).

A statistical correlation between ribotype and level of toxin production was not demonstrated ($\chi^2 = 20.0$; df = 30; $P > 0.05$). Ribotype D1 strains were those strains producing medium to high levels of toxin, whereas more than half of the strains of ribotype D4 (66%) produced medium levels of toxin. Strains of other ribotypes correlated with various levels of toxin production (Table 1).

Discussion. Epidemic diphtheria has reemerged in Belarus after almost three decades of successful control. The epidemiological data have shown that the main reasons for the epidemic in Belarus are the same as in other countries of the Newly Independent States of the former Soviet Union: decreased immunization among children, gaps in adult immunity, and increased migration throughout the territory (4, 9, 10, 13, 30). There have been hypotheses concerning the changes in the

genome, which probably contributed to changes within biological properties and hence, an increase in toxigenicity. The investigation of the structural gene of diphtheria toxin (*tox*) and its regulatory element (*dtxR*) by Nakao et al. (18, 19) revealed the insignificant changes in the *tox* gene and some mutations in the *dtxR* region. The point mutations in the *tox* gene did not lead to amino acid substitutions, which confirmed a highly conserved *tox* gene that preserves diphtheria toxin properties. Mutation in the *dtxR* region probably led to high levels of toxin production among epidemic strains.

Bacteriological and molecular epidemiological monitoring of diphtheria is an important part of epidemiological and microbiological surveillance (4, 15). The National Reference Laboratory for Diphtheria was established at the Belarussian Research Institute for Epidemiology and Microbiology in 1996 to improve control. During this period (1996 to 1998) a collection of isolates from all regions of Belarus was obtained. All isolates were identified and characterized by the regional bacteriology laboratories using classical bacteriological methods and were sent to the National Reference Laboratory for Diphtheria for confirmation and molecular typing. This important microbiological and epidemiological problem had been solved successfully, thanks to the collaboration within the European Laboratory Working Group on Diphtheria network and also the participation within the European Commission INCO Copernicus international research project "Microbiological surveillance of diphtheria in Eastern Europe."

A number of molecular epidemiological typing methods, including ribotyping, pulsed-field gel electrophoresis, and MEE, have been applied to the study of *C. diphtheriae* strains during the diphtheria epidemic. The clone of toxigenic *C. diphtheriae* biotype gravis that caused the epidemic in Russia was revealed. The study of *C. diphtheriae* strains associated with the diphtheria epidemic in Belarus using phenotypic and genotypic methods (ribotyping) showed that toxigenic strains of the biotype gravis ribotypes D1 and D4 predominated in the clinical cases (48.0 and 38.4%, respectively). The correlation of these ribotypes to the epidemic clone that caused the diphtheria epidemic in Russia confirmed that a similar or indistinguishable *C. diphtheriae* clone also caused the epidemic in Belarus.

Phage typing revealed a predominant phage type among strains that caused disease—toxigenic gravis biotype VI ls5,34 add, which accounted for 72 of 102 (70.6%) isolates examined. These results correlated well with the data of McClosley et al. (17) and Riegel et al. (26) concerning the effectiveness of

phage typing in investigations of diphtheria outbreaks. Similarly, 20% of strains examined were atypical or nontypeable.

Toxigenic strains of biotype gravis produced medium to high levels of toxin (48.4 and 33.3%). However, such consistency was not identified among each ribotype. There was no definitive association between ribotypes and toxin genotypes in typing *Vibrio cholerae* O1 strains as shown by Damian et al. (2). The combination of the two methods could make it possible to create a more robust and discriminatory typing system to allow the study of the dissemination of gram-negative and gram-positive bacterial species.

Currently, ribotyping is one of the most basic and effective discriminatory typing methods for *C. diphtheriae* and is the main tool that has been used to explore the epidemiology of current outbreaks not only within Belarus but also in countries of the European Union. The global collection of *C. diphtheriae* ribotypes along with database analysis should allow the rapid recognition and determination of the geographic origin of epidemic strains globally.

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