GENETIC RELATIONSHIPS of Corynebacterium diphtheriae STRAINS ISOLATED FROM A DIPHTHERIA CASE AND CARRIERS BY RESTRICTION FRAGMENT LENGTH POLYMORPHISM of rRNA GENES

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SUMMARY

In the present study we report the results of an analysis, based on ribotyping of Corynebacterium diphtheriae intermedia strains isolated from a 9 years old child with clinical diphtheria and his 5 contacts. Quantitative analysis of RFLPs of rRNA was used to determine relatedness of these 7 C. diphtheriae strains providing support data in the diphtheria epidemiology. We have also tested those strains for toxigenicity in vitro by using the Elek’s gel diffusion method and in vivo by using cell culture method on cultured monkey kidney cell (VERO cells). The hybridization results revealed that the 5 C. diphtheriae strains isolated from contacts and one isolated from the clinical case (nose case strain) had identical RFLP patterns with all 4 restriction endonucleases used, ribotype B. The genetic distance from this ribotype and ribotype A (throat case strain), that we initially assumed to be responsible for the illness of the patient, was 0.450 showing poor genetic correlation among these two ribotypes. We found no significant differences concerned to the toxin production by using the cell culture method. In conclusion, the use of RFLPs of rRNA gene was successful in detecting minor differences in closely related toxigenic C. diphtheriae intermedia strains and providing information about genetic relationships among them.

KEYWORDS: Corynebacterium diphtheriae; Ribotyping; Epidemiology.

INTRODUCTION

Diphtheria is an acute infectious and communicable disease involving primarily the tonsils, pharynx, larynx, or nose, and occasionally other mucous membranes or skin.2,3,10,15,33 Corynebacterium diphtheriae, the causative agent, is an obligate aerobe that grows rapidly on the surface of many types of artificial media. The organisms are club-shaped gram-positive nonspore-bearing rods, and are without flagella or capsules. Its capacity to produce toxin and the immunity state of the individual decide whether diphtheria will evolve. Toxigenic strains of C. diphtheriae spread directly from person-to-person by droplet infection. It has been demonstrated recently that a given toxigenic strain may directly colonize the nasopharyngeal cavity. Alternatively, the tox gene may spread indirectly by the release of toxigenic corynebacteriophage and lysogenic conversion of autotrophic non toxigenic C. diphtheriae in situ.14,27

Since effective immunization has been available, the occurrence of epidemic disease has been limited almost entirely to countries with localized populations with inadequate or prior immunization. Because individuals with clinical disease may transmit organisms to others with comparative ease, carrier rates of 5 or 10% are frequently seen among close contacts; carriage of

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Diphtheria is typically a disease of communities, schools and households with children of susceptible ages. Closeness and duration of contact play a major part in determining the spread of the disease. Contact during sleeping hours in common dormitories is far more dangerous than casual contact during waking hours and a search for contacts should be made promptly prior to the final determination of the toxigenicity of the original isolate.

Following initial identification, C. diphtheriae may be identified as gravis, intermedius or mitis biotype on the basis of glycogen fermentation, hemolysis on sheep blood agar plates, colonial form on tellurite mediums, and different features of growth in broth media. These characteristics were very important when first observed, for there appeared to be an association between the genotypic biotype and the degree of severity in the clinical manifestations of diphtheria. The toxins can be produced by the three biotypes and they can be equally neutralizable by the ordinary antitoxic sera. By now it is more or less generally agreed that the differentiation of these biotypes is not significantly related to clinical severity but has been useful from an epidemiological point of view in some cases.

The bacterin and bacteriophage typing methods have also been used for epidemiological studies and control purposes for several decades. In addition to the determination of biotype, bacterin type and lysotype of C. diphtheriae isolates, it is now possible to use molecular biological techniques in the study of diphtheria outbreaks. Restriction endonuclease digestion patterns of chromosomal DNA as well as the use of a cloned corynebacterial insertion sequence as a genetic probe, have been used effectively in the study of C. diphtheriae isolates. More recently, the typing method based on restriction fragment length polymorphism (RFLPs) of rRNA genes (ribotyping) has been successfully applied to study the molecular epidemiology and taxonomy of microorganisms belonging to different genera and species; Salmonella spp., Campylobacter spp., H. pylori, N. meningitidis, and others.

In the present study we report the results of the analysis, based on ribotyping of C. diphtheriae strains isolated from a 9 years old child with clinical diphtheria and his 5 contacts. Quantitative analysis of RFLPs of rRNA was used to determine relatedness of these C. diphtheriae strains providing support data in the diphtheria epidemiology. We have also tested these strains for toxigenicity in vitro by using the Elek's gel diffusion method and in vivo by using cell culture method on cultured monkey kidney cell (VERO cells). The goal of this study was to verify the possible genetic linkage between strains isolated from the clinical case and the contact strains by using ribotyping.

MATERIALS AND METHODS

Case description: On 15 September 1994, a nine years old child suffering from diphtheria was admitted to the Emílio Ribas Hospital for Infectious Diseases in Greater São Paulo, Brazil. After his admission to hospital, a toxigenic strain of C. diphtheriae was isolated from his throat and nose. Nasopharyngeal swabs from 10 family members of the same house and 30 children from the same classroom of the school were taken in order to trace diphtheria carriers. All 30 children had received basic immunization against C. diphtheriae in childhood and later in school while only one member of the family had received it (a 2 years old cousin). Five carriers of toxigenic strains of C. diphtheriae were found among the family and anyone among the students (Table 1).

### Table 1

<table>
<thead>
<tr>
<th>Isolation site</th>
<th>Strain from</th>
<th>Age</th>
<th>Biotype</th>
<th>Toxin</th>
<th>Banding pattern no. obtained</th>
<th>Ribotype</th>
<th>VERO Log_2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Site</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>EcoRI</td>
<td>CleI</td>
<td>AccI</td>
</tr>
<tr>
<td>70T</td>
<td>Case</td>
<td>9 years</td>
<td>intermedius</td>
<td>+</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>70N</td>
<td>Case</td>
<td></td>
<td>intermedius</td>
<td>+</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>71T</td>
<td>Sister</td>
<td>19 years</td>
<td>intermedius</td>
<td>+</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>72T</td>
<td>Mother</td>
<td>39 years</td>
<td>intermedius</td>
<td>+</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>74N</td>
<td>Brother</td>
<td>5 months</td>
<td>intermedius</td>
<td>+</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>75T</td>
<td>Cousin</td>
<td>2 years</td>
<td>intermedius</td>
<td>+</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>76T</td>
<td>Brother</td>
<td>5 years</td>
<td>intermedius</td>
<td>+</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

* a: Isolation site: Throat (T) or Nose (N)
* b: The biotypes were determined as described in Material and Methods.
* c: Toxin production detected by Elek's gel Method
* d: Derived as explained in Material and Methods
* e: Log_2 of the titer of culture cell test with VERO cells
Bacterial strains, biotypes and toxin testing. We analyzed 8 strains of C. diphtheriae. Two of these strains were recovered from the clinical case, one from throat (T) and one from nose (N), these strains are referred from now on throat case strain, and nose case strain. Five other strains were isolated from his household contacts (Table 1). One C. diphtheriae strain (2T) was used as control for ribotyping analysis. It was selected arbitrarily being isolated 3 months before the case described here at the same city from a 6 years old child with diphtheria. The strains were isolated and identified in the Bacteriology Division of Adolfo Lutz Institute as previously described by Sacıchi et al. 

The biotypes of C. diphtheriae were characterized as described by Saragea et al. 

The strains were tested for toxigenicity in vitro by using the Elek’s gel diffusion method and in vivo by using cell culture method as described by Solltenek et al. with some minor modification. The strains were grown in flasks with 20ml of Elek’s Broth for 24 h in a gyratory shaker at 36°C. The cells were removed by centrifugation and the supernatant was sterilized by filtration. Aliquots of the supernatants were immediately stored at -20°C. The possible differences in toxin production were assayed by a micro-cell-culture test. Serial dilutions of the supernatant were inoculated on cultured monkey kidney cell (VERO cells), and incubated at 37°C during 48 hours. The toxigenicity was evaluated by daily microscopic examination, according to the cytopathic effect of diphtheria toxin on the tissue culture. The toxin titer was determined by the Log of the reciprocal supernatant dilution yielding a 50% reduction of viable cells.

Bacterial DNA extraction. Chromosomal DNA was extracted as previously described by Schiller et al. and Groman et al. with minor modifications. Cells grown overnight on Mueller-Hinton agar (Difco Laboratories, Detroit, Mich.) at 37°C were inoculated in 100 ml of tryptic soy broth (Difco Laboratories, Detroit, Mich.) and grown for 3 h with aeration. Penicillin G was added to a final concentration of 1 mg/ml and reincubated for 2 h with aeration. Cells were harvested by centrifugation and washed in 20 ml of 10 mM Tris (pH 8.0). The pellet was resuspended in 4 ml of 10 mM Tris - 0.5 M sucrose - 5 mg of hen egg white lysozyme (Sigma Chemical Co., St. Louis, Mo.) per ml. The cell suspension was incubated for 2 h at 37°C. The cells were harvested by centrifugation, and the pellet was resuspended in 9 ml of 20 mM Tris - 10 mM EDTA (pH 8.0). Sodium dodecyl sulfate was added to a final concentration of 1% and mixed well. The cell suspension was heated to 65°C for 20 min to achieve complete lysis and high-molecular weight DNA was purified as described by Brenner et al. An aliquot of each DNA sample was examined spectrophotometrically to determine concentration and purity.

Ribotyping. Preliminary experiments were conducted with 6 enzymes (Clal, EcoRI, EcoRV, Acel, HindIII, and XhoI) with the throat case strain (70T) and one of his contacts (74N) to determine the most appropriate enzyme for restriction digestion. Approximately 10 μg of DNA was digested with each restriction enzyme in a final volume of 20 μl during 18 hours at the conditions recommended by the supplier (New England BioLabs, Beverly, MA). The pBR322 plasmid DNA was used as a probe. The hybridization of the plasmid vector and the C. diphtheriae chromosomal was evaluated by using the pBR322 plasmid as a probe and the 70T and 74N DNA strain digested with EcoRI endonuclease. The two probes were labeled with digoxigenin-11-dUTP by the random-primed method of the Genius 1 DNA labeling and detection kit (catalog no. 103637; Boehringer GmbH, Mannheim, Federal Republic of Germany). Hybridization of blots with digoxigenin-labeled probes and colorimetric detection were done with the same kit as described by the manufacturers. Haemophilus aegyptius 3031 EcoRI DNA digest was included in each gel, and used as reference. Strains with identical patterns were given the same banding pattern number. When banding pattern numbers for the four restriction endonucleases were combined, each unique group was considered as a separate ribotype and designated with a letter of the alphabet.

Genetic Distance. The genetic distance (GD) was calculated by determining the total number of unique bands produced by each restriction endonuclease and then assembling these as a set of bands of decreasing size. The banding patterns of each strain were compared with this total set of bands (master set), and for each band in a strain profile that matched a band in the master set, the number 1 was placed in that position. When a band was missing, the position was marked with a 0. The patterns of sequences of 1's and 0's for each strain were compared with the patterns for the other strains, and a relatedness index was calculated as the proportion of mismatches of bands.

RESULTS

Biotyping and toxic testing. All 8 strains of C. diphtheriae were identified as biotype intermedius and toxic producers by Elek’s gel diffusion method after 5 to 7 days incubation at 37°C. In the micro-cell-culture test the first cytopathic changes show up in the arrangement of the cells. Groups of more refractory cells delimited by different sized lacunae appear. Hence holes in the cell monolayer, and cell detachment are observed. The tissue culture layer disappears almost completely within 48 h. The titers obtained by cell culture method are shown in Table 1.

Restriction endonuclease digestion of DNA. The Clal, EcoRI, EcoRV, and Acel were the only enzymes tested that gave proper digestion of DNA for all strains tested in Table 1. Digestion did not occur when HindIII was used, and a few bands were demonstrated with XhoI. Suitable discrimination following hybridization with RNA probe was seen when Acel, EcoRV, Clal, and EcoRI were used. (Fig.1).
The lack of hybridization of the pBR322 plasmid vector with the 70T and 74N isolates was confirmed. No homology between this plasmid and the genome of *C. diphtheriae* was present, meaning that the hybridization patterns obtained were due to specific reaction with the rRNA genes.

**DISCUSSION**

To highlight RFLP, investigators have probed bacterial chromosomal DNA with genes encoding toxin production in *C. diphtheriae*. This technique produces one to 11 hybridization bands for analysis; however, the utility of these probes is restricted to the species containing nucleotide sequences that hybridize with the specific probe. A major disadvantage of this approach is that poor or no evolutionary correlation can be made among the isolates. The interference of phylogenetic relationships from molecular data is contributing greatly to our understanding of the bacterial evolution. The merits of rRNA for phylogenetic inference include universality, functional constancy, ease of identification and apparent lack of lateral gene transfer. The 16S + 23S rRNA genes do provide great amount of phylogenetic information for resolving closely spaced evolutionary branching of phylogenetic trees.

Here, hybridization results revealed that the 5 *C. diphtheriae* strains isolated from contacts and one isolated from the clinical case (nose case strain) had identical RFLP patterns (RbB) with all 4 restriction endonucleases used. The GD from this ribotype and ribotype A (throat case strain), that we initially assumed to be responsible for the illness of the patient, was 0.450 showing poor genetic correlation among these two ribotypes. The *C. diphtheriae* control strain RbC, had 0.717 and 0.500 of GD with the ribotype A and B respectively.

No definite knowledge as to how the 6 persons were infected with *C. diphtheriae* has been obtained. It probably did not come from the school since anyone from those 30 school contacts were culture positive for *C. diphtheriae*. Special selective culture techniques for detection of *C. diphtheriae* are seldom used nowadays unless there is clinical suspicion of the disease. Consequently small numbers of diphtheria bacilli may be unrecognized during cultural examination, particularly in the presence of streptococci or some other microbial cause for the lesion under investigation, and in such an instance infection will not be diagnosed. Nevertheless it seems likely that the number of persons harboring *C. diphtheriae*, with or without lesions or symptoms, is decreasing.

Another important observation which has been made in this investigation is that all children of the same classroom of the clinical case had received basic immunization against diphtheria in childhood and later in school. Only one of the 10 family members had received the basic and booster immunization against diphtheria and also carried toxigenic strain of *C. diphtheriae*.
(75T strain). The complete absence of pharyngeal diphtheria in these carriers can be consequence of antitoxic immunity and does not indicate eradication of the causative organism.

RAPPUIOLI et al.22.23 suggest that virulence might be correlated with toxin dosage and/or the presence of genes other than tox genes for diphtheria toxin14. Studies on the citotoxic activity of diphtheria toxin demonstrated the possibility of detection of very small amount of toxin production by tissue culture test. We found no significant differences concerned to the toxin production among all strains by using the cell culture method. Four of the 5 family contacts that carry the toxigenic strains have not received the basic booster immunization against diphtheria. We believe that some other factors concerned with the virulence and host defenses could be responsible for the illness.

It is possible that both strains (70T, Rba and 70N, Rbb) have contributed for the illness of the case, however, the presence of Rbb strains was not enough to produce disease even in a non immunized 5 months old child (74N). Besides, the fact that these family members have received antibiotic treatment immediately after the clinical diagnosis of diphtheria must be considered since it could have no time for these carriers to become sick. Nevertheless, the data do not permit to answer questions on the occurrence of disease in the group studied, and for the moment these must remain speculative. Undoubtedly many variables determine pathogenesis in addition to toxin, and a simple correlation seems unlikely, except in animal studies of isogenic tox+ and tox- strains. As concluded from a recent Swedish study that measured antitoxic antibody levels15, it seems likely that severity of disease in the Seattle outbreak was related to the patients' immune status rather than to strain variation.

In conclusion, the use of RFLPs of rRNA gene was successful in detecting minor differences in closely related toxigenic C. diphtheriae intermedia strains and in providing information about genetic relationships between them.

RESUMO

Relação genética de cepas de Corynebacterium diphtheriae isoladas de caso e seus contatos por RFLP de rRNA Gene

No presente estudo, nós reportamos os resultados de uma análise, baseada na ribotipagem de cepas de C. diphtheriae intermedia isoladas de uma criança de 9 anos com difteria e seus 5 contatos. Análise quantitativa por RFLP de rRNA foi usada para determinar a relação destas 7 cepas de C. diphtheriae fornecendo dados de interesse epidemiológico. Nos tambem testamos estas cepas para toxicidade in vitro usando método de difusão de Elek e in vivo usando método de cultura celular com células VERO. Os resultados de hibridização revelaram que as 5 cepas de C. diphtheriae isoladas dos contatos e uma isolada do caso (cepa isolada do nariz) tiveram padrões idênticos com as 4 enzimas de restrição usadas, ribotipo B. A distância genética deste ribotipo e o ribotipo A (cepa isolada da garganta do caso) que nos inicialmente assumimos ser responsável pela doença do paciente, foi de 0,450 mostrando pouca relação genética entre estes dois ribotipos. Nós não encontramos diferenças relativas à produção de toxina usando método de cultura celular entre as cepas. Em conclusão, o uso de RFLP de rRNA gene foi satisfatório em detectar pequenas diferenças em cepas de C. diphtheriae toxigênicas epidemiologicamente relacionadas e em fornecer informação sobre a relação genética entre elas.

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