Rev. Inst. Med. trop. São Paulo
37(4): 291-296, julho/agosto, 1995.

GENETIC RELATIONSHIPS of *Corynebacterium diphtheriae* STRAINS ISOLATED FROM A DIPHTHERIA CASE AND CARRIERS BY RESTRICTION FRAGMENT LENGTH POLYMORPHISM OF *r*RNA GENES

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SUMMARY

In the present study we report the results of an analysis, based on ribotyping *of Corynebacterium diphtheriae intermedius* strains isolated from a 9 years old child with clinical diphtheria and his 5 contacts. Quantitative analysis of RFLPs of *r*RNA 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 of 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 *r*RNA gene was successful in detecting minor differences in closely related toxigenic *C.diphtheriae intermedius* 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[6,30,33] *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 autochthonous nontoxigenic *C.diphtheriae* in situ[16,27].

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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virulent diphtheria bacilli is uncommon, although have been found[3, 19, 30, 33].

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 proceed promptly prior to the final determination of the toxigenicity of the original isolate[3, 19, 30, 33].

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[27]. These characteristics were very interest when first observed, for there appeared to be an association between the phenotipical biotype and the degree of severity in the clinical manifestations of disease. The toxins can be produced by the three biotypes and they can be equally neutralizable by the ordinary antitoxic serums. 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[7].

The bactericin and bacteriophage typing methods[27,32] have also been used for epidemiological studies and control purposes for several decades. In addition to the determination of biotype, bactericin type and lysotype of *C. diphtheriae* isolates, it is now possible to use molecular epidemiological techniques in the study of diphtheria outbreaks. Restriction endonuclease digestion patterns of chromosonal DNA[8,15,24] as well as the use of a cloned corynebacterial insertion sequence as a genetic probe, have been used effectively to study outbreaks of clinical diphtheria[8,15]. More recently, the typing method based on restriction fragment length polymorphism (RFLPs) of *r*RNA genes (ribotyping)[10,20] has been successfully applied to study the molecular epidemiology and taxonomy of microorganisms belonging to different genera and species: *Salmonella* spp.[2], *Campylobacter* spp.[14], *H. aegyptius*[12], *N.meningitidis*[13,31,34], *C. diphtheriae*[9,29], and others[35].

In the present study we report the results of an 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 *r*RNA 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 difffusion 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 Emilio 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

Characteristics of the 8 *Corynebacterium diphtheriae* strains by using 4 different restriction enzymes and by micro-cell-culture test

Isolation site[a]	Strain from	Age	Biotype[b]	Toxin[c]	Banding pattern no. obtained with[d]				Ribotype	VERO[e] Log_2
					*Eco*RI	*Cla*I	*Acc*I	*Eco*RV		
70T	Case	9 years	*intermedius*	+	1	1	1	1	A	19
70N	Case		*intermedius*	+	2	2	2	2	B	19
71T	Sister	19 years	*intermedius*	+	2	2	2	2	B	19
72T	Mother	39 years	*intermedius*	+	2	2	2	2	B	19
74N	Brother	5 months	*intermedius*	+	2	2	2	2	B	20
75T	Cousin	2 years	*intermedius*	+	2	2	2	2	B	20
76T	Brother	5 years	*intermedius*	+	2	2	2	2	B	16
2T	Control	6 years	*intermedius*	+	3	3	3	3	C	16

[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 at Adolfo Lutz Institute as previously described by SACCHI et al.[25,26]. The biotypes of *C.diphtheriae* were characterized as described by SARAGEA et al.[27]. The strains were tested for toxigenicity *in vitro* by using the Elek's gel diffusion method[27], and *in vivo* by using cell culture method as described by SOTTNEK et al.[30] with some minor modification. The strains were grown in flasks with 20ml of Elek's Broth[30] for 24 h in a gyrator 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[17,18]. 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 citotoxic effect of diphtheria toxin on the tissue culture. The toxin titer was determined by the Log_2 of the reciprocal supernatant dilution yielding a $\geq 50\%$ reduction of viable cells.

Bacterial DNA extraction. Chromosomal DNA was extracted as previously described by SCHILLER et al.[28] and GROMAN et al.[11], with minor modifications. Cells grown overnight on Mueller-Hinton agar (Difco Laboratories, Detroit, Mich.) at 37°C were inoculated in 100ml 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.[5]. An aliquot of each DNA sample was examined spectrophotometrically to determine concentration and purity.

Ribotyping. Preliminary experiments were conducted with 6 enzymes (*Cla*I, *Eco*RI, *Eco*RV, *Acc*I, *Hind*III, and *Xho*I) with the throat case strain (70T) and one of his contacts (74N) to determine the most appropriate enzyme for restriction digestion. Approximately 10µg of DNA was digested with each restriction enzyme in a final volume of 20µl during 18 hours at the conditions recommended by the supplier (New England BioLabs, Beverly, MA). The pKK3535 plasmid DNA was used as a probe[1]. 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 *Eco*RI 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. 1093657; Boehringer GmbH, Mannheim, Federal Republic of Germany)[4]. Hybridization of blots with digoxigenin-labeled probes and colorimetric detection were done with the same kit as described by the manufacturers. *Haemophilus aegyptius* 3031 *Eco*RI DNA digest was included in each gel, and used as reference[12]. 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[9].

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 cytotoxic 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 48h. The titers obtained by cell culture method are shown in Table 1.

Restriction endonuclease digestion of DNA. The *Cla*I, *Eco*RI, *Eco*RV, and *Acc*I were the only enzymes tested that gave proper digestion of DNA for all strains tested in Table 1. Digestion did not occur when *Hind*III was used, and a few bands were demonstrated with *Xho*I. Suitable discrimination following hybridization with *r*RNA probe was seen when *Acc*I, *Eco*RV, *Cla*I, and *Eco*RI were used, (Fig.1).

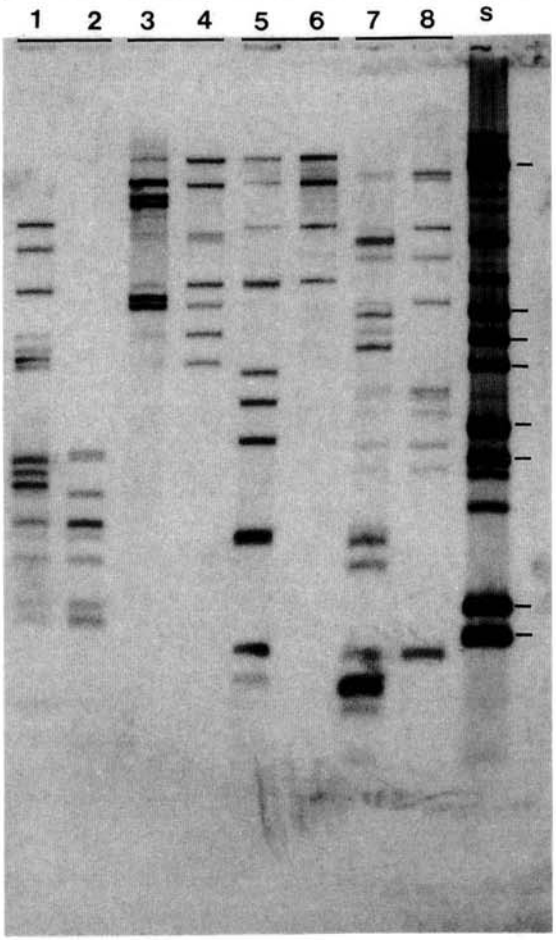

Fig. 1 - Restriction fragments of two *C. diphtheriae* strains hybridized with pKK3535 plasmid DNA labeled with digoxigenin-11dUTP.
Lanes 1, 3, 5 and 7, *C. diphtheriae* strain 70T digested with *Acc*I, *Eco*RV, *Cla*I, and *Eco*RI respectively. Lanes 2, 4, 6 and 8, *C. diphtheriae* strain 70 N digested with the same restriction enzymes respectively. Lane S, molecular weight standard DNA from *H. aegyptius* 3031 digested with *Eco*RI (17.6, 6.3, 5.6, 4.9, 3.8, 3.2, 1.7, 1.5 kb from the top).

Ribotyping and Genetic Distance. When the 8 *C.diphtheriae* strains were analyzed by ribotyping, 3 different ribosomal DNA hybridization profiles were obtained with each of the enzymes used (Table 1). When the profiles for all 4 restriction endonucleases were considered together, 3 different ribotypes were obtained (Table 1). While all the contact strains had the same ribotype, designated as RbB, the throat case and control strains were classified as RbA and RbC respectively. Quantitative analysis of patterns indicated that the RbA (throat case strain) have at least 0.450 of GD with the strain isolated from carriers (RbB) and 0.717 of GD with the control strain used (RbC). The nose case strain was of the same ribotype (RbB) as the strains isolated from carriers.

The lack of hybridization of the pBR322 plasmid vector with the 70T and 74N isolated 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 *r*RNA genes.

DISCUSSION

To highlight RFLP, investigators have probed bacterial chromosomal DNA with genes encoding toxin production in *C.diphtheriae*[11,21]. 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 *r*RNA for phylogenetic interference include universality, functional constancy, ease of identification and apparent lack of lateral gene transfer[20]. The 16S + 23S *r*RNA genes does provide great amount of phylogenetic information for resolving closely spared 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 of 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 steptococci 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 erradication of the causative organism.

RAPPUOLI 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 toxin[24]. Studies on the citotoxic activity of diphtherial 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 carrier the toxigenic strains have not received the basic and 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 diagnose 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 levels[3], 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 *r*RNA gene was sucessful in detecting minor differences in closely related toxigenic *C. diphtheriae intermedius* 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 RLFP de *r*RNA Gene

No presente estudo, nós reportamos os resultados de uma análise, baseada na ribotipagem de cepas de *C. diphtheriae intermedius* isoladas de uma criança de 9 anos com difteria e seus 5 contatos. Análise quantitativa por RFLP de *r*RNA foi usada para determinar a relação destas 7 cepas de *C. diphtheriae* fornecendo dados de interesse epidemiológico. Nós também 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 nós 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 RLFP de *r*RNA 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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Recebido pa publicação em 30/01/1995
Aceito para publicação em 28/06 1995