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## Evidence of increased carriage of *Corynebacterium* spp. in healthy individuals with low antibody titres against diphtheria toxoid

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### SUMMARY

This study evaluated whether a correlation exists between carriage of corynebacteria and the lack of immunity to diphtheria toxoid. Samples of both nasal and pharyngeal secretions were taken from 500 apparently healthy subjects of both sexes and of all ages and inoculated onto Tinsdale's medium. A serum sample was also taken for ELISA test to determine the titre of diphtheria toxin antibodies. None of the subjects carried *Corynebacterium diphtheriae*. Ninety-three strains of *Corynebacterium* spp. were isolated from 93 subjects and 86 of these were classified to species or group level by biochemical tests. *C. xerosis* was the most common (25·8%) followed by *C. pseudodiphthericum* (16·1%), *C. jeikeium* and *C. striatum* (both 10·8%), and *C. urealyticum* (9·7%). Three other species accounted for approximately 20% of strains and seven were unclassified as biochemically atypical corynebacteria. Non-protective antibodies to diphtheria toxin were found in 80 of the 93 subjects and a strong statistical association was demonstrated between carriage of corynebacteria and non-protective levels of anti-toxin antibodies. The remaining 13 subjects had protective levels of antitoxin antibodies. In contrast, only 45 of the 407 non-colonized subjects had non-protective antitoxin titres. The prevalence of carriage increased with age among males as did the percentage of non-protected subjects. The prevalence of female carriers of corynebacteria was significantly lower. Serum samples from 12 subjects with different antibody titres to diphtheria toxoid reacted to varying degrees with whole-cell lysates of a number of species of corynebacteria. The results suggest that a causal relationship may exist between nasopharyngeal carriage of corynebacteria and a low anti-diphtheria toxin immune response.

### INTRODUCTION

Over the last 50 years, routine immunization of infants with diphtheria toxoid has resulted in a dramatic decline in the incidence of diphtheria in many industrialized countries [1]. So much so that at the beginning of the 1980s it was envisaged by the year

2000 indigenous respiratory diphtheria would be eliminated from Europe [2]. Nevertheless, in recent years, the World Health Organization (WHO) has reported epidemics of diphtheria in several countries of eastern Europe [3] and the major causal factors identified were the decline in immunization coverage in children and waning of immunity to diphtheria in adults [4].

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Recent studies [5–7] have shown that in Europe the percentage of the population with protective levels of diphtheria antibody is below the threshold limit (75–80%) that is required to prevent the onset of a diphtheria epidemic [8]. Moreover, it appears that the progressive decline in immunity to diphtheria is inversely proportional to age, indicating that a large segment of the adult population is not protected against the disease [8]. Despite the current low incidence of toxigenic strains of *Corynebacterium diphtheriae*, the rare cases that do occur in unimmunized subjects suggest that reservoirs of the organism have not been eradicated [9] and once toxigenic strains are harboured by a sufficient number of carriers, the spread of infection in a susceptible population is facilitated.

The recent diphtheria epidemics in eastern Europe underline the need for stricter epidemiological surveillance. We therefore undertook an investigation to determine the incidence of nasopharyngeal carriers of *C. diphtheriae* and other corynebacteria in a sample of the Italian population. We also examined the relationship between carriage of corynebacteria and levels of antibodies to diphtheria toxin in these individuals.

## MATERIALS AND METHODS

### Subjects and samples

In the period from November 1996 to March 1997 a total of 500 subjects (226 males, 274 females) were recruited at random from outpatients attending the S. Anna Hospital, Ferrara, for blood chemistry tests as part of a periodic check-up or screening for employment purposes. This sample represented approximately 0.3% of the local population (170 453 inhabitants) covered by the Health Service Local Unit of the Emilia-Romagna Region. All subjects were residents of Ferrara or its suburbs and were divided by sex and by age to reflect the distribution in the general population according to the 1991 national census. The dimension of the sample was calculated according to the significant threshold indicated by the Probably Proportional to Size (PPS) method. Subjects were divided into age groups of 0–20 years, 21–40 years, 41–60 years, and > 60 years old.

A serum sample was taken from each subject and nose and pharynx samples were collected with flexible calcium alginate swabs. Swabs were preserved in silica gel tubes at room temperature for a maximum of

24–48 h before use [10]. Serum samples were frozen and stored at  $-20^{\circ}\text{C}$ .

### Isolation and identification of corynebacteria

Swabs were cultured on Tinsdale's medium with supplement (Oxoid, UK) and plates were incubated in air at  $37^{\circ}\text{C}$  for 48 h. Suspected corynebacteria were tested for production of cystinase [11], urease and gelatinase, hydrolysis of aesculin, hippurate, tyrosine and casein [12], and reduction of nitrate [13]. Carbohydrate fermentation tests (glucose, maltose, saccharose, mannose, xylose, fructose, arabinose, lactose) were also performed by the disk technique [12]. Species identity was confirmed by the API Coryne system (BioMérieux, France). Toxin production was evaluated by Elek's immunoprecipitation test [14], with control strains of *C. diphtheriae* CN 2000 (C7, CB) which were obtained from the Istituto Ricerche Immunologiche Siena, Siena, Italy.

### ELISA test

Plastic microplates with high-binding capacity were coated with  $0.3\ \mu\text{g}$ /well of purified diphtheria toxoid (specific activity: 2063 Lf/mg protein nitrogen). Sera were diluted 1 in 100, in duplicate, in 0.1 M phosphate buffer (pH 7.4 with 0.05% Tween 20 and 1% bovine albumin; PBST) and 100  $\mu\text{l}$  volumes were dispensed to each well of the microtitration plate. After incubation at  $37^{\circ}\text{C}$  for 30 min, the plates were washed 5 times with PBST and 100  $\mu\text{l}$  of conjugate (anti-IgG monoclonal antibody conjugated with alkaline phosphatase; Sclavo Diagnostics, Italy) was then added and reincubated for 30 min. The substrate was paranitrophenylphosphate diluted in 0.1 M ethanalamine buffer (pH 9.6) and colour was developed for 30 min at  $37^{\circ}\text{C}$ . The reaction was stopped by the addition of 50  $\mu\text{l}$  of 3 N sodium hydroxide and the optical density was determined at 405 nm. Antitoxin titres were expressed as IU/ml by reference to a standard calibration curve of a positive control serum pool of 100 human sera with an antitoxin concentration of 0.1 IU/ml determined against the WHO diphtheria antitoxin equine serum (1st International Standard, Statens Seruminstitut, Copenhagen, Denmark) by an *in vivo* neutralization test [15]. Negative and positive serum controls were included in each run. The serum immunity of subjects was classified according to internationally accepted criteria [16]; anti-toxin level < 0.01 IU/ml (non-protective); 0.01–

Table 1. *Nasopharyngeal carriage of corynebacteria by 93 subjects according to age and sex*

Age group (years)	Males			Females		
	Nose	Pharynx	Nose + Pharynx	Nose	Pharynx	Nose + Pharynx
0-20 (n = 64)	6.0*	3.0	3.0	3.2	0	0
21-40 (n = 138)	5.9	8.9	2.9	11.2	5.6	4.2
41-60 (n = 143)	13.6	10.6	6.0	11.68	3.8	1.2
> 60 (n = 155)	10.0	8.3	5.0	8.42	3.1	3.1
Total (n = 500)	9.2	8.4	4.4	9.4	3.6	2.5

\* All values are percentages.

0.099 IU/ml (basic immune protection);  $\geq 0.1$  IU/ml (full protection).

#### Cell wall antigens of corynebacteria

Isolates of different corynebacterial species were grown to mid to late log phase in 250 ml of trypticase soy broth in conical flasks with shaking at 37 °C according to the method of Cheung [17]. The bacteria were centrifuged at 2000 g for 10 min, washed twice with 0.05 M Tris hydrochloride (pH 7.8) and re-suspended in the same buffer containing 0.002 M MgCl<sub>2</sub>. The cell suspension was vortexed and sonicated on ice with short pulses at 80-100 W and then heated immediately to 75 °C for 10 min to inhibit autolysis. Cell debris was removed by centrifugation at 2000 g for 10 min and the crude cell walls were deposited at 15000 g for 30 min. Contaminating cytoplasmic membrane was removed by incubation twice in Triton-X100 for 30 min and cell walls were recovered at 15000 g for 30 min. Lysostaphin was added to a final concentration of 75 µg/ml in Tris-HCl buffer with stirring for 2 h at 37 °C in 0.145 M NaCl buffer. Insoluble material was removed by centrifugation and the cell walls were dialysed against distilled water and standardized to a protein concentration of 10 mg/ml. Lysates were diluted in sodium carbonate buffer (pH 9.5) and coated to microtitration plates at 37 °C overnight.

#### Statistical methods

The results of serum immunity levels and carriage of corynebacteria were analysed by the  $\chi^2$  test and the

Yates modification with  $P < 0.01$  indicating significance. To examine whether antibodies against diphtheria toxoid cross-reacted with antigens of non toxigenic corynebacteria, Spearman's non-parametric correlation test was used. A  $r_s$  value of = 0.746 (chance-limit = 1%) was considered to be significant as a non-causal finding.

## RESULTS

#### Carriage of corynebacteria

Culture of 1000 nasopharyngeal samples yielded 93 strains of corynebacteria in an equal number of subjects. *C. diphtheriae* was not recovered from any of the samples. Eighty-six of the 93 strains were classified according to species or group; the remaining 7 gave atypical reactions and could not be assigned to a species. *C. xerosis* was the most frequently isolated (no. 24, 25.8%), followed by *C. pseudodiphthericum* (no. 15, 16.1%), *C. jeikeium* (no. 10, 10.8%), *C. striatum* (no. 10, 10.8%), *C. urealyticum* (no. 9, 9.7%), *C. accolens* (no. 8, 8.6%), *C. ulcerans* (no. 6, 6.4%) and *C. matruchotii* (no. 4, 4.3%). All *C. ulcerans* strains were toxin negative [18].

Table 1 shows the site of carriage of corynebacteria according to sex and age. The nose was the most commonly colonized site in both sexes and markedly more males harboured coryneforms in the pharynx than females. Only 5 subjects under 20 years were colonized compared with 60 in the 21-60 age groups; rates increased with age in both groups but peaked for males at 41-60 years and 21-40 years for females.

Eighty of the 93 carriers of corynebacteria gave a serum titre to diphtheria toxin of  $< 0.01$  IU/ml and

Table 2. Nasopharyngeal carriage of corynebacteria by male and female subjects according to age and diphtheria immune status

Age classes (years)	Males (n = 226)						Females (n = 274)					
	Susceptibles		Basic protection		Full protection		Susceptibles		Basic protection		Full protection	
	Carriers	Non carriers	Carriers	Non carriers	Carriers	Non carriers	Carriers	Non carriers	Carriers	Non carriers	Carriers	Non carriers
0-20 (n = 64)	12.1	6.1	0	57.5	0	24.2	3.2	12.9	0	64.5	0	19.3
21-40 (n = 38)	13.4	10.4	4.4	55.2	0	16.4	16.9	12.6	4.2	54.9	0	11.2
41-60 (n = 143)	28.7	13.6	1.5	50.0	0	6.0	11.6	10.3	5.1	57.1	0	15.5
> 60 (n = 155)	18.3	5.0	3.3	68.3	1.6	3.3	13.6	3.1	1.0	70.5	0	11.5
Total (n = 500)	19.0	9.2	2.6	57.5	0.4	11.0	12.7	8.7	2.9	8.7	0	13.5

so had no immune protection to diphtheria. The remaining 13 subjects had protective levels of antitoxin antibodies. In contrast, 45 of the 407 non-colonized subjects had a non-protective antitoxin titre. The difference between the non-protective antibody group of subjects and antibody protected non-carriers was statistically significant ( $\chi^2 = 208.493$ ,  $P < 1 \times 10^{-6}$ ; Yates modified  $\chi^2 = 228.327$ ). This difference held for all age groups and both sexes ( $\chi^2$  males = 105.234,  $P < 1 \times 10^{-6}$ ,  $\chi^2$  females = 108.175;  $P < 1 \times 10^{-6}$ ).

### Corynebacteria carriage and diphtheria immunity

The association of nasopharyngeal carriage of corynebacteria with diphtheria antibody titre according to sex and age is given in Table 2. Among the male population, the prevalence of carriers increases with age as does the percentage of non-protected subjects. The latter feature increased from 12.1% in 0-20 years group to 28.7% in subjects above 40 years of age. Among non-carriers, the prevalence of fully protected subjects ( $\geq 0.1$  IU/ml) showed an age related decrease, falling from 24.2% in the 0-20 years old to 3.3% in the over-60s age group while the prevalence of individuals with basic immune protection (0.01-0.099 IU/ml) remained almost constant. Overall, more females than males had protective titres to diphtheria.

### Cross reactivity with corynebacterial antigens

A panel of 3 groups of 4 sera from 12 subjects positive in diphtheria toxin ELISA was selected to represent subjects with full, basic and no antibody protection. These sera were tested against cell wall antigens from seven species of corynebacteria. Table 3 shows that each of the 12 sera reacted with lysates prepared from *C. xerosis*, *C. pseudodiphthericum*, *C. jeikeium*, and *C. ulcerans*. Sera with titres  $\geq 0.01$  IU/ml reacted with lysates from *C. striatum* and only sera with the highest titres  $\geq 0.1$  IU/ml reacted with antigens of *C. urealyticum*, and *C. accolens*. According to Spearman's test, there was good correlation between antitoxin titres and antibodies reactive with cell wall antigens of corynebacterial species. The results showed a chance-limit  $< 1\%$  ( $r_s = 0.997$ ) suggesting that this finding may be due to specific cross-reactions against diphtheria toxin and other corynebacterial antigens.

Table 3. Reactivity in ELISA of a panel of sera from subjects, with varying degrees of immunity to diphtheria toxin, with cell antigens of different species of corynebacteria

Coated antigens	Serum reactions according to antitoxin titre											
	= 0.1 IU/ml				0.01–0.099 IU/ml				< 0.01 IU/ml			
	1	2	3	4	5	6	7	8	9	10	11	12
Diphtheria toxoid	0.105	0.152	0.162	0.164	0.010	0.041	0.098	0.024	0.009	0.005	0.008	0.003
	11.1	14.9	15.7	15.9	3.37	5.90	10.5	4.51	3.29	2.96	3.21	2.80
<i>C. xerosis</i>	0.035	0.052	0.056	0.058	0.003	0.014	0.031	0.007	0.003	0.001	0.002	0.001
	5.41	6.80	7.13	7.29	2.80	3.70	5.09	3.12	2.80	2.63	2.72	2.63
<i>C. pseudodiph.</i>	0.042	0.061	0.064	0.069	0.004	0.016	0.036	0.008	0.003	0.002	0.003	0.001
	5.98	7.54	7.78	8.19	2.88	3.86	5.94	3.21	2.80	2.72	2.80	2.63
<i>C. jeikeium</i>	0.025	0.031	0.032	0.033	0.002	0.008	0.021	0.004	0.001	†	0.001	†
	4.60	5.09	5.17	5.25	2.72	3.21	4.27	2.88	2.63	‡	2.63	‡
<i>C. striatum</i>	0.036	0.038	0.065	0.071	0.004	0.021	0.031	0.007	0.003	†	†	†
	5.49	5.74	7.86	8.35	2.88	4.27	5.09	3.12	2.80	‡	‡	‡
<i>C. urealyticum</i>	0.004	0.006	0.008	0.007	†	†	0.004	†	†	†	†	†
	2.88	3.04	3.21	3.12	‡	‡	2.88	‡	‡	‡	‡	‡
<i>C. accolens</i>	0.005	0.008	0.009	0.010	†	0.002	0.005	†	†	†	†	†
	2.96	3.21	3.29	3.37	‡	2.72	2.96	‡	‡	‡	‡	‡
<i>C. ulcerans</i>	0.036	0.041	0.052	0.057	0.003	0.010	0.031	0.006	0.002	†	0.002	†
	5.49	5.90	6.80	7.21	2.80	3.37	5.09	3.04	2.72	‡	2.72	‡

Serum titres are expressed according to IU/ml and Ratio [OD of test serum sample/OD of negative control serum]. Cut-off = 1.5.

† Titre <  $1 \times 10^{-3}$  IU/ml.

‡ OD = negative control serum (OD = 113 at 405 nm).

## DISCUSSION

We set out to determine whether there was a link between colonization of the nasopharynx of individuals with corynebacteria and their immunity to diphtheria toxin. We chose 500 apparently healthy subjects and screened their nose and pharynx for corynebacteria by culture on Tinsdale's medium. Serum samples were tested in a standard ELISA for antibody levels to toxin. No patients were known to be taking immunosuppressive therapy, intravenous drugs, or recent antimicrobial therapy. Whenever possible, a vaccination history was taken. Only 68 recorded immunization with diphtheria toxoid in childhood.

Blood agar and tellurite containing media are generally accepted to be optimal for the primary isolation of corynebacteria, in particular *C. diphtheriae*, while Tinsdale's medium is recommended for subsequent confirmation of suspicious colonies [14]. The latter was preferred for this survey as it appeared to give greater selectivity than the Loeffler or Pergola media [12]. *C. xerosis* was the most common species isolated in the survey. This species is reported to be rare in clinical samples [20–22], and is difficult to differentiate from *C. amycolatum* [21]. However, we are confident of the identification of *C. xerosis* by its weak production of propionic acid from glucose fermentation and confirmation by the API Coryne database 2.0.

Despite the fact that diphtheria vaccination for all newborns became compulsory in Italy in 1939, routine immunization was sometimes incomplete in many areas until the 1950s and after. Nevertheless, as 345 individuals (69%) of the population sampled here were less than 60 years (mean 40) of age and were born and now live in the Emilia-Romagna Region where immunization against diphtheria began in the late 1940s, we can assume that the majority of the subjects received primary vaccination. Diphtheria toxoid vaccination protects only against the phage-mediated toxin and not against infection organisms. Past experience suggests that mass active immunization may also confer a degree of herd immunity by eliminating the selective advantage for toxigenic *C. diphtheriae* transmission *in vivo* [23]. Antibodies to the toxin also appear to be effective in suppressing the survival of virulent *C. diphtheriae* which leads to the proliferation of carriage of nontoxigenic strains [24]. We did not isolate any strains of *C. diphtheriae* from the study population which is consistent with the fact

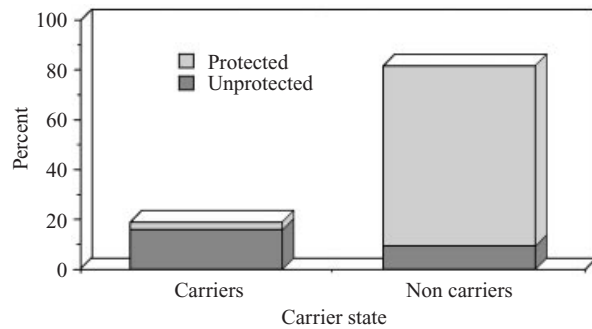


Fig. 1. Nasopharyngeal carriage of corynebacteria according to immune status against diphtheria.

that no cases of diphtheria have been found in the territory since 1968 [25] which suggests that *C. diphtheriae* is very rare or even absent from the general population. In the absence of repeated exposure to toxigenic strains of *C. diphtheriae* or periodic administration of booster doses of toxoid, immunity to diphtheria wanes with time and adults may become susceptible to the disease [26].

At the first International Meeting of the European Laboratory Working Group on Diphtheria, it was agreed that the toxin neutralization test in animals or cell cultures is the most reliable test for titration of diphtheria antitoxin levels. However, this assay is laborious, expensive and requires 5–7 days to obtain a result. The need for a simple, rapid, and cheap assay for measuring antibody levels in large numbers of serum samples directed the choice towards a commercially available ELISA method (ELISA Diphtheria IgG Test, Sclavo Diagnostics, Italy). For the quantitative determination, the ELISA test was previously compared with the standard neutralization test and regression analysis did not show any tendency for ELISA to overestimate antibody concentration [6].

The principal finding of the study was that of the 93 subjects who had nasal and/or pharyngeal reservoirs of corynebacteria, 80 (86.0%) had a non-protective antibody titre ( $< 0.01$  IU/ml) to diphtheria toxin. There was a strong association between an anti-diphtheria serum titre below the relative protection threshold ( $= 0.01$  IU/ml) and carriage of corynebacteria in the nasopharynx (Fig. 1). This finding held for both sexes and all age groups with the greatest incidence in those over 21 years. Calculation of relative risk ( $RR = 52.023\%$ ) implied an association which was not due to chance or to sampling distortion. Indeed, this association reinforces the hypothesis of causal effect between a low anti-diphtheria toxin

immune titre and nasopharyngeal colonization with corynebacteria [27].

In other words, vaccination with diphtheria toxoid may induce the production of different antibodies directed against diphtheria toxin and antigenic determinants commonly located in *C. diphtheriae* and coryneform bacteria. This might be explained by impurities in the toxoid, such as bacterial somatic and cellular constituents arising through formalization of broth cultures [28], which may stimulate an antibody response that protects against colonization of the nasopharynx by corynebacteria. Indeed, Relyveld and colleagues [29] reported that Arthus-type allergic reactions were due to impurities in diphtheria toxoid which could not be removed after interaction with formaldehyde. Further attempts to obtain toxin free of other contaminating substances proved to be laborious and the yields of toxin comparatively low [30], so that today the diphtheria toxoid universally used for immunization is not much different from that originally proposed by Ramon [31]. This suggests that the lack of immunity may increase susceptibility to colonization by corynebacteria in the nasopharynx.

We tested the hypothesis with sera from subjects with varying degrees of protective immunity to diphtheria and screened corynebacterial strains representative of the seven species recovered. Our findings suggested that those with highest titres of antibody to toxin reacted most strongly with soluble cell wall antigens of several species. This was supported by statistical analysis which indicated that these results were neither due to chance nor to technical limitations of the ELISA method.

In the literature, ELISA tests that use the diphtheria toxoid as an antigen are considered to be adequate to monitor the protective status for anti-toxin titres  $\geq 0.1$  IU/ml. The test used here compared with the *in vivo* neutralization test, did not show a tendency to overestimate the immune status of individuals [6]. This might be due to the conditions of use of the test which optimise exposure of the neutralizing epitopes of the toxin on the solid phase, the reduced incubation period and relatively high dilution of the test serum, which together encourage high affinity bonding with neutralizing antibodies. Our findings show a possible correlation between nasopharyngeal colonization with coryneform bacteria and immune status to diphtheria toxin. The mechanisms causing antibodies to diphtheria toxoid to protect against colonization by non-toxigenic strains of corynebacteria are not known. Should others confirm this correlation both exper-

imentally and in different populations, it may be valuable to provide follow-up vaccination of those adults with the most marked decline in anti-diphtheria immunity and thus benefit a large potentially susceptible segment of the population.

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