

DATA REGARDING THE INCIDENCE OF *CORYNEBACTERIUM SPP.* IN THE ROMANIAN HEALTHY POPULATION

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ABSTRACT

Although controlled by vaccination long ago, diphtheria may become a re-emergent disease as a result of at least two factors: the circulation of toxigenic strains in endemic areas (countries of former CSI, some of them neighbouring countries of Romania) and the lack of interest of the local health institutions for surveillance of this very low incidence disease.

The aim of this study was to evaluate the carriage of potentially toxigenic *Corynebacterium* strains in healthy people in Romania.

In this study, 1120 biological samples were analysed consisting of swabs, nasal exudates and conjunctival secretions collected from 730 healthy individuals from eight different geographic regions of the country, in order to establish carriage of potentially toxigenic strains belonging to the *Corynebacterium* genus.

129 strains of the *Corynebacterium* species were isolated and identified by phenotypic and molecular methods: *Corynebacterium pseudodiphtheriticum* (n = 76), *C. propinquum* (n = 34), *C. striatum* (n = 9), *C. macginleyi* (n = 2), *C. glucuronolyticum/seminale* (n = 2), *C. group F1* (n = 6). These species are saprophytic species for the upper respiratory tract, some of them being an opportunistic pathogen especially in immunocompromised hosts.

The present data require the attention of clinicians, epidemiologists and microbiologists since diphtheria is an infectious disease that could re-emerge.

Keywords: *Corynebacterium spp.*, diphtheria, corynebacteria healthy carriers

INTRODUCTION

The *Corynebacterium* genus is a heterogeneous taxonomic group, consisting of over 120 species

and subspecies isolated from human, animal and environmental, many of them being medically relevant.

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The genus includes three potentially toxigenic species: *C. diphtheriae* with 4 biotypes (gravis, mitis, intermedius and belfanti), *C. ulcerans* and *C. pseudotuberculosis*. *C. diphtheriae* and *C. ulcerans* are the only two species that cause diphtheria, a notifiable infectious disease, characterized by pseudomembranous angina accompanied by toxemia.

Diphtheria is a serious infectious disease with high epidemic potential. The disease is vaccine preventable if vaccination coverage at age one is at minimum of 95% and 90% in adults.

This is a re-emerging disease as it was demonstrated by the diphtheria epidemics which occurred during the 1990's in all CIS (former USSR) countries. Toxigenic strains and diphtheria cases continue to be reported in some of these countries and all over the world.

In Europe, in 2000-2010 there were 895 clinically and microbiologically confirmed cases of the diphtheria disease. This data was obtained from 25 European countries participating in the DIPNET Project (Diphtheria Surveillance Network) (1).

In the time frame 1998-2003, the USA reported seven cases of respiratory diphtheria to the CDC. The last confirmed case of diphtheria-like illness in the USA in 2010 was determined by a strain of *Corynebacterium ulcerans* in a person under the age of 80 years with unknown vaccination history (2).

The *C. diphtheriae* toxigenic strains circulating in developing countries, where diphtheria is still

endemic, can be imported in the developed and diphtheria-free countries and the illness may sporadically occur there.

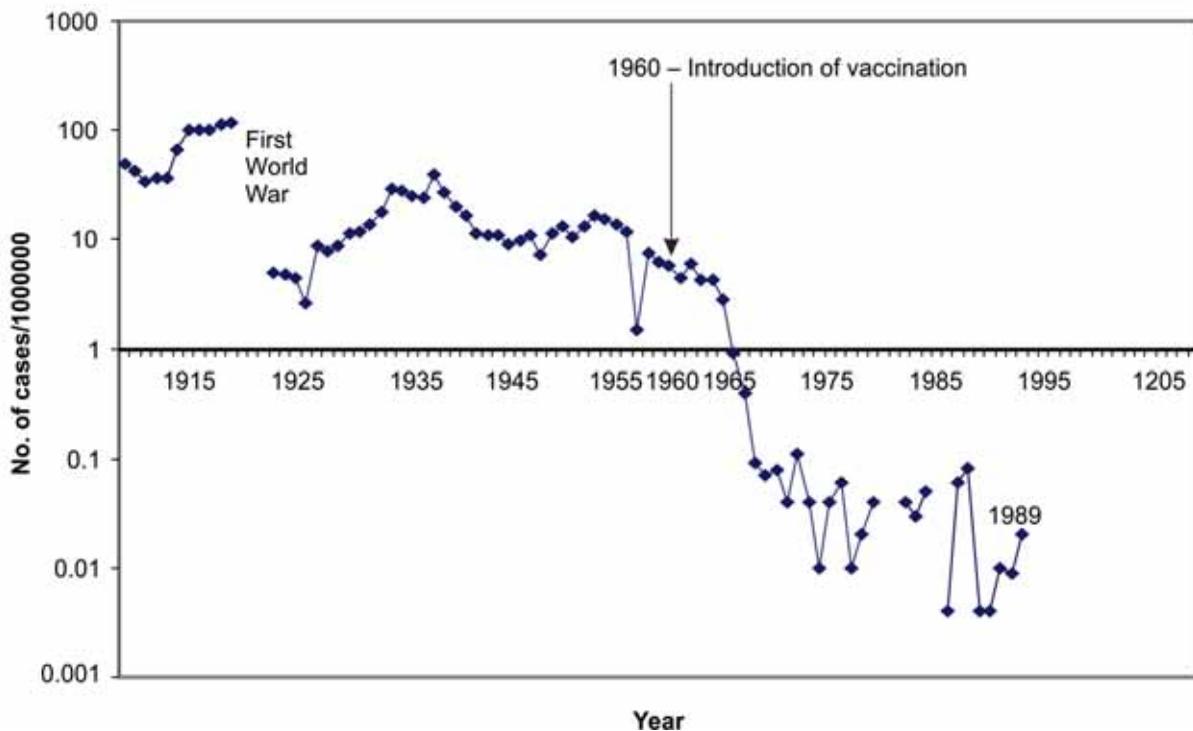
Many of the species belonging to the *Corynebacterium* genus are part of the normal microbiota of the human skin and upper respiratory tract. Saprophytic strains belonging to these species have become known as important agents involved in infections in individuals with normal or depressed immune status. In recent years numerous cases of invasive infections caused by species belonging to this genus, such as *C. jeikeium*, *C. striatum*, *C. urealyticum* and *C. pseudodiphtheriticum* are described. These species are isolated more frequently than others and act as the first opportunistic aetiology in immunocompromised or debilitated patients. Many isolates from invasive infections have been described as multi-drug resistant phenotypes.

In Romania, the epidemiology of diphtheria is controlled through the National Program for Diphtheria Surveillance based on case definition (www.insp.gov.ro).

The differential diagnosis with viral pharyngitis, Vincent angina, white angina, mononucleosis, oral syphilis and candida is performed.

Vaccination against diphtheria was introduced in 1960 and a spectacular waning of disease was recorded in the next years (Fig. 1).

Although in Romania there were no reported cases of diphtheria since 1989, in 1995 a National



Source: NCCDSC (National Centre for Communicable Diseases Surveillance and Control)

FIGURE 1. The annual incidence of diphtheria cases in Romania during 1905-2005

Diphtheria Control Programme was introduced under the circumstances of diphtheria imported risks (due to the epidemic evolution of disease in former USSR countries and intensified international travel).

During the last 19 years few *C. diphtheriae* strains were isolated at country level: 1995 (n = 356) 2004 (n = 7), 2005 (n = 3) and 2007 (n = 40). Since then no strain was referred to the National Reference Laboratory. We can't conclude that we are passing through a period of considerable reduction of *C. diphtheriae* circulation or we are assisting to a decrease in the concern of the territorial laboratories regarding this issue. Is this the epidemiologic reality or are we facing an underreporting situation?

Also, the carriage of toxigenic *C. ulcerans* strains, the second species able to cause diphtheria, is not known.

Regarding the role of other species of *Corynebacterium* in infectious pathology, there are no epidemiological studies in Romania. The only existing data in the literature refers to three strains of *Corynebacterium urealyticum* isolated from urine in children in 1997 (3), a strain of *Corynebacterium striatum* isolated from a lung infection (4) and a few *Corynebacterium striatum/amycolatum* strains isolated in ventilator-associated pneumonia in a Cardiovascular Surgery Clinic (5).

The present study aims a. to perform a screening for non-toxigenic and/or potential toxigenic species belonging to the genus *Corynebacterium* in healthy people in Romania and b. to evaluate the carriage of corynebacteria by military personnel coming back from Afghanistan.

MATERIAL AND METHODS

A number of 1,120 samples were analysed in the current study. They were collected from the phar-

ynx, nose and conjunctiva of a total of 730 subjects living in very different regions of the country (Fig. 2):

- 462 people from eight different geographical areas of the country, namely the counties Brasov, Dolj, Maramures, Neamt, Suceava, Timis, Tulcea and Bucharest (Table 1 and Fig. 2). The individuals selected for study were children, students and nursing staff from open and closed communities, homes of healthy and disabled children in weekly boarding homes.

- 102 people were present for pharyngeal exudates sampling type and/or nasal discharge for various diseases for laboratory analysis at the "Cantacuzino" Institute, Bucharest.

- 166 subjects were Romanian soldiers returning from peacekeeping missions in Afghanistan.

The analyses of samples were performed according to WHO Laboratory Diagnosis of Diphtheria protocols. (6)

Samplings were collected by sterile cotton-tipped plastic rod and Amies transport medium was used. Time from sampling to laboratory processing did not exceed 24 hours.

Samples spread on Columbia agar with the addition of 7% sheep blood (Columbia Blood Agar Oxoid) and Tinsdale medium (Oxoid Agar Base Tinsdale + Tinsdale supplement Oxoid) were incubated at 37°C for 24-48 hours. The first examination of the plates was performed at 18-24 hours of incubation, using a stereomicroscope. Suspected colonies were analysed by classical microbiological methods: Gram stain, catalase and oxidase tests and subcultivated on Columbia agar containing 7% sheep blood.

Identification was performed using API Coryne kit, VITEK2 type ID cards ANC (anaerobic and corynebacteria identification) and by RNA 16S sequence analysis.

TABLE 1. Clinical specimens and geographical areas

County	Source of isolation			Total samples	Mean age (years)
	Pharynx	Nose	Conjunctiva		
Brasov	81	7		88	13.06
Bucharest	Group 1	79	28	107	15.79
	Group 2	93	39	2 conjunctiva secretion	134
Dolj	67	64		131	-
Maramures	50			50	16.08
Neamt	50	50		100	13.44
Suceava	50	50		100	15.04
Timis	27			27	2.33
Tulcea	50			50	6.16
Military personnel	166	166	1 conjunctiva secretion	333	33.84
TOTAL	713	404	3	1120	



FIGURE 2. Map illustrating the counties where the investigation was performed

RESULTS

A total of 131 strains, with characteristic morphology for *Corynebacterium*, were isolated. Among them 49 were isolated from pharyngeal samples, 81 from nose and one from conjunctival secretion. (Fig. 3)

An acceptable, good or very good identification profile was obtained for a total of 127 strains by API Coryne kit identification.

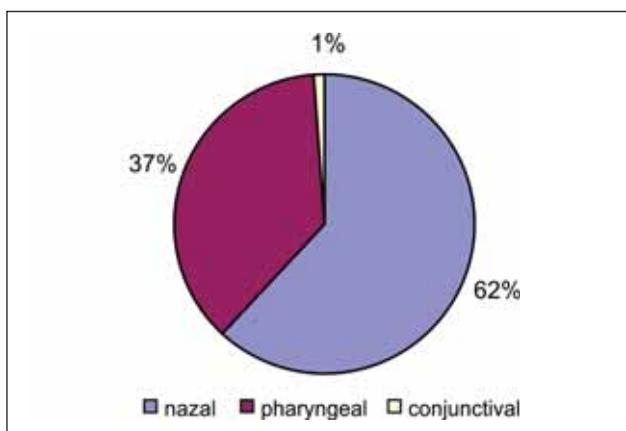


FIGURE 3. The origin of the strains: nasal, pharyngeal and conjunctival samples

In the case of four strains we obtained a low discrimination unacceptable identification profile - using the API Coryne kit. These strains were subsequently identified using the Vitek II system ID-ANC identification cards and 16S RNA gene sequencing (Fig. 4). Six samples were identified as *Corynebacterium striatum/amycolatum* (good or very good identification) using the Api Coryne kit and for species identification RNA 16S sequence analysis was also performed.

Using all these three methods (API Coryne, Vitek 2 and 16S RNA sequence analysis) to analyse the 131 strains of *Corynebacterium* spp. we obtained the results reported in Fig. 3.

Nasal and pharyngeal carriage of corynebacteria by 730 subjects according to age and sex is presented in Table 2.

DISCUSSION

In this study, analysing a total of 1120 throat swabs, nasal and three conjunctival secretions, no potentially toxigenic strain was isolated but 131 strains of *Corynebacterium* spp. were identified. In

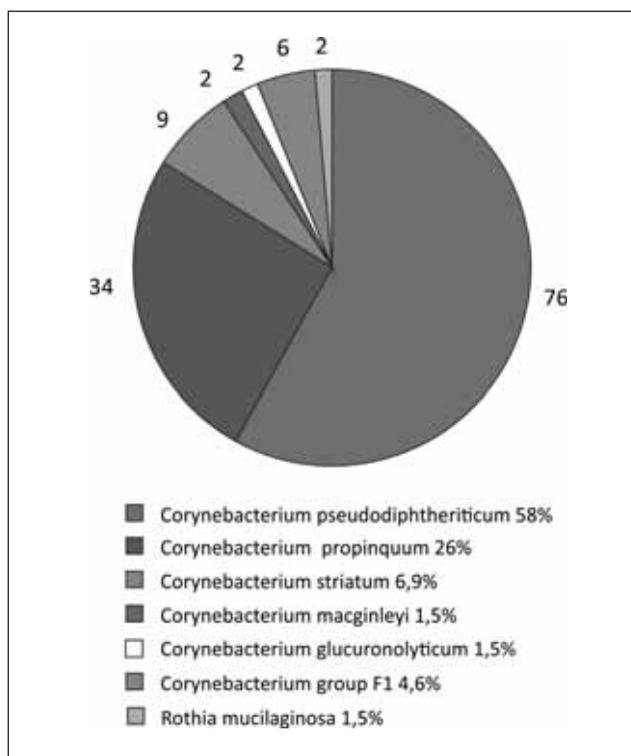


FIGURE 4. Species of *Corynebacterium* identified in this study

a similar study conducted in 2007-2008 in ten European countries, DIPNET network members, between 968 and 8551 swabs taken from people with upper respiratory tract infection for each country were analyzed (7). The study revealed that there are countries where toxigenic strains of *Corynebacterium diphtheriae* are still circulating (Latvia, Lithuania), countries where non toxigenic *Corynebacterium diphtheriae* strains are reporting (Estonia, Latvia, Lithuania, Turkey, UK) and others where no *C. diphtheriae* and/or *C. ulcerans* strains were identified (Bulgaria, Greece) (7).

In a study performed in Italy, from a total of 253 apparently healthy vaccinated subjects (135 males and 118 females), 6-14 years old, only nine isolates of *Corynebacterium spp.* were identified (8).

Other Italian authors studied the correlation between carriage of corynebacteria strains and the level of protection against diphtheria. A strong statistical association between carriage of corynebacteria and non-protective levels of anti-toxin antibodies was demonstrated. The nasal and pharyngeal secretions were taken from 500 apparently healthy subjects of both sexes and of all ages. A number of ninety three subjects were positive for *Corynebacterium spp.* Non-protective levels of antibodies to diphtheria toxin were found in 80 of these 93 subjects, while among the 407 non-colonized subjects only 45 had non-protective antitoxin titres. None of the subjects carried *Corynebacterium diphtheriae* (9).

In this study, most strains of *Corynebacterium spp.* were isolated from subjects enrolled in the age group 10-14 years. The two age groups that include more carriers of corynebacteria are 5-9 respectively 15-19, both age groups being located close to mandatory vaccination ages, 4-5 and 14 years, according to the National Immunization Program. This can be explained by the fact that these age groups were the best represented in the study (122 subjects in the age group 5-9 years, 152 subjects in the 10-14 years group and 92 subjects belonging to the 15-19 years group), given that the literature supports the cantonment carriers of *C. diphtheriae* in closed communities children. We could make a correlation with anti-diphtheria immunity for we have not performed any serologic testing of subjects, but in the future it would be interesting to conduct a study of seroprevalence extended to different age groups.

TABLE 2. Nasal and pharyngeal *Corynebacterium spp* carriers selected from 730 subjects according to age and sex

Age (years)	Women		Men		Total
	Pharyngeal secretion	Nasal secretion	Pharyngeal secretion	Nasal secretion	
< 1 year	0	0	0	0	0
1-4 years	1	2	3	2	8
5-9 years	5	9	1	6	21
10-14 years	6	16	14	14	50
15-19 years	1	3	12	10	26
20-24 years	2	1	1	2	6
25-29 years	1	0	0	3	4
30-34 years	0	1	1	4	6
35-39 years	0	1	1	3	5
> 40 years	0	2	0	3	5
Total	16	35	33	47	131

The reports published in the past 3 years in Romania, by National Institute of Public Health – National Centre for Communicable Diseases Surveillance and Control (www.insp.gov.ro) reveal that the coverage after 4 doses of DTP vaccine was systematically below the target of 95% in children aged 18 months. It is extremely important to analyse the causes of such decreased vaccination coverage.

From the data presented in these 3 reports, we note the preservation of a relatively constant level of the percentage corresponding to medical contraindications, the insignificant variations in terms of the number of children born abroad and those not declared; the number of cases of unvaccinated children decreased due to the lack of vaccine and what seems worrying is the alarming and dramatic increase of the percentage of people who refused vaccination, from 13.2% in 2011 to 23.4% in 2012 and 24.3% in 2013.

As regards the immunization in the adult population in Romania, there is no coherent management of the dT vaccine after age 14. As a consequence, diphtheria could become a real and serious public health problem in Romania, taking into consideration that both toxigenic strains are circulating in geographically close countries and the free movement of the population in regions where the endemic diphtheria is still evolving.

We should not forget that in Romania there is an uncontrolled consumption of antibiotics, which can decrease carriage of *Corynebacterium diphtheriae*, although in recent years there have been cases of isolated strains that showed patterns multi-resistant to common antibiotics (10,11).

In recent years, numerous studies have shown the involvement of several species belonging to the *Corynebacterium* genus, different from potential toxigenic strains, such as the *Corynebacterium stri-*

atum and *C. pseudodiphtheriticum* (species isolated in our study) in non-invasive and invasive infections (12-19). In Romania there are no such relevant studies and the current study aims to evaluate the circulation of these bacteria in healthy people.

Phenotypic identification of strains belonging to the *Corynebacterium* genus is difficult. For accurate identification, molecular techniques were needed, and RNA sequence analysis of the 16 S gene was applied.

Diphtheria should remain in the specialist's attention. This statement was demonstrated by the last two reported cases of cutaneous diphtheria in people who had returned from African countries in Europe, in Norway and Austria. (20,21)

Our study represents the initiation after many years of a laboratory surveillance program for a potential re-emerging disease. The surveillance program should be maintained and continued in order to be prepared and be able to offer a prompt, correct and efficient response in terms of curative and public health 21st Century medicine.

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The authors declare that they have no conflict of interest.

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