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An increase in non-toxigenic *Corynebacterium diphtheriae* infections in Poland — molecular epidemiology and antimicrobial susceptibility of strains isolated from past outbreaks and those currently circulating in Poland

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ABSTRACT

Objectives: An increase in non-toxigenic *Corynebacterium diphtheriae* infections – mainly invasive infections – has been observed in countries with high vaccination coverage. However, reasons for this situation are unknown. In this study we characterized and compared human clinical isolates of non-toxigenic *C. diphtheriae* strains isolated from infections that have occurred over recent years and *C. diphtheriae* strains isolated from diphtheria cases from past outbreaks in Poland.

Methods: We determined biotypes, genotypes, the occurrence of plasmids, and antimicrobial susceptibilities of 19 clinical *C. diphtheriae* strains. Genotypes were determined using pulsed-field gel electrophoresis (PFGE) and enterobacterial repetitive intergenic consensus polymerase chain reaction (ERIC-PCR) techniques.

Results: The non-toxigenic *C. diphtheriae* strains isolated over the last few years were found to belong to biotype *gravis* and were genetically indistinguishable using PFGE and ERIC-PCR techniques. No plasmids were detected in the strains. All tested strains were susceptible to penicillin and erythromycin, as well as to imipenem, vancomycin, daptomycin, gentamicin, tetracycline, clindamycin, trimethoprim/sulfamethoxazole, rifampin, quinupristin/dalfopristin, and linezolid. Of the strains tested, 47% were intermediate for cefotaxime.

Conclusions: The genetic similarity of non-toxigenic *C. diphtheriae* strains causing infection suggests that the strains represent a single clone. They may possess additional virulence genes in a chromosome, related with higher pathogenicity and invasiveness. The genetic changes have not been followed by resistance to antibiotics.

Introduction

Corynebacterium diphtheriae is the etiologic agent of toxin-induced classic diphtheria – an acute infectious disease of humans affecting the upper respiratory tract and occasionally the skin. The toxin produced by *C. diphtheriae* also affects other parts of the body including the heart and nervous system, causing paralysis and cardiac failure. Diphtheria is rare in countries with high vaccination coverage. However, an increase in non-toxigenic *C. diphtheriae* infections has been observed in many countries, for example in France,[1] Italy,[2] Switzerland,[3] Germany,[4] and Canada.[5,6] Probably the most spectacular increase in the number of non-toxigenic isolates has been observed in England and Wales – from one isolate in 1986 to 294 in the year 2000.[7,8] A lot of the recorded cases are of invasive disease. A similar situation has been observed in Poland since 2004, when the first case of invasive infection due to non-toxigenic *C. diphtheriae* was recorded.[9] Since then, non-toxigenic *C. diphtheriae* strains have been isolated from infections – mainly invasive infections – almost every year.

Observations made during recent years point towards non-toxigenic *C. diphtheriae* being an important and dangerous pathogen. The fatality rate in invasive *C. diphtheriae* infections is very high. Patey et al.[1] stated that in France between 1987 and 1993 the fatality rate of *C. diphtheriae* infections was 36%, despite specific antibiotic treatment. These observations, together with increased international travel, show the necessity of monitoring the spread of *C. diphtheriae* strains, not only those that are toxigenic but also non-toxigenic strains.

The aim of this study was to characterize human clinical isolates of *C. diphtheriae* isolated from diphtheria cases of past outbreaks and non-toxigenic isolates from infections that have

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Table 1
Characteristic of tested *Corynebacterium diphtheriae* strains

Strain	Year of isolation	Region of origin	Site of isolation	Biotype	Toxigenicity (Elek test)	*tox* gene PCR	PFGE types	ERIC-PCR types
1/A	1960s	ND	Nasopharynx	mitis	+	+	C	X
2/A	1960s	ND	Nasopharynx	mitis	+	+	C1	X
3/A	1960s	ND	Nasopharynx	mitis	+	+	C	X
4/A	1960s	ND	Nasopharynx	intermedius	+	+	H	XI
5/A	1960s	ND	Nasopharynx	gravis	+	+	G	IV
6/B	1990s	Otmuchów	Nasopharynx	gravis	–	–	F	VII
7/B	1990s	ND	Nasopharynx	mitis	+	+	B1	I
8/B	1990s	ND	Nasopharynx	intermedius	+	+	B	III
9/B	1990s	ND	Nasopharynx	mitis	+	+	B	II
10/C	2000	Suwałki	Nasopharynx	belfanti	–	–	E	VIII
11/D	2001	Suwałki	Nasopharynx	belfanti	–	–	D	IX
12/E	2004	Warszawa	Blood	gravis	–	–	A2	V
13/E	2006	Bydgoszcz	Blood	gravis	–	–	A	V
14/E	2007	Gdynia	Blood	gravis	–	–	A	V
15/E	2007	Bydgoszcz	Wound	gravis	–	–	A	V
16/E	2007	Warszawa	Fistula	gravis	–	–	A	V
17/E	2007	Bydgoszcz	Wound	gravis	–	–	A1	VI
18/E	2007	Gdynia	Blood	gravis	–	–	A	V
19/E	2008	Rzeszów	Blood	gravis	–	–	A	V

PCR, polymerase chain reaction; PFGE, pulsed-field gel electrophoresis; ERIC, enterobacterial repetitive intergenic consensus; ND, not determined.

occurred in recent years in Poland, and to compare variability in these two groups of strains.

Materials and methods

Bacterial strains

Nineteen *C. diphtheriae* isolates were investigated. Ten isolates were from past outbreaks (five isolated in the 1960 s and the remaining during 1990–2000); all these isolates were from the nasopharynx. Eight isolates were from blood and wound swabs isolated during 2004–2008. One isolate was from a nose swab isolated in 2001. Details of the tested *C. diphtheriae* strains are presented in Table 1. The strains were isolated from patients in different parts of Poland. No risk factors were identified for the infections. All the strains had been stored at −70 °C. Investigated strains were identified and biotyped according to the World Health Organization (WHO) guidelines.[10] Toxigenicity was tested using standard tests[10] and a modified Elek test.[11] PCR for the *tox* gene was performed according to Efstratiou and Maple.[10]

Pulsed-field gel electrophoresis (PFGE)

Bacteria from overnight BHI broth (containing 10% horse serum and 0.5% glycine) culture were centrifuged and resuspended in 100 μl of Tris–EDTA (TE) buffer. An equal volume of 1.2% SeaKem Gold agarose (BMA, USA) was added, and plugs were cast with a standard casting tray. After the plugs solidified, they were incubated in 1 ml of buffer I (1 M NaCl, 10 mM Tris–HCl pH 7.6, 0.5% sodium deoxycholate, 0.25 M EDTA pH 7.5, 0.5% sarcosyl, 0.5% Brij 58, 1 mg/ml lysozyme). After overnight incubation at 37 °C the plugs were washed in TE buffer and incubated in buffer II (0.25 M EDTA pH 9–9.5, 1% sarcosyl, 50 μg/ml proteinase K) at 50 °C overnight. The plugs were then washed several times in TE buffer.

DNA in agarose plugs was digested with *Sfi*I (Fermentas, Lithuania), and fragments were separated in 1% pulsed-field certified agarose (Bio-Rad Laboratories, USA) in 0.5 Tris–borate–EDTA buffer (pH 8.0) in a CHEF-DR II system (Bio-Rad Laboratories, USA). The electrophoresis conditions were as described by De Zoysa et al.[12] DNA fragments were photographed over a UV light after staining with ethidium bromide.

Enterobacterial repetitive intergenic consensus polymerase chain reaction (ERIC-PCR) typing

Total DNA was extracted with the DNeasy Tissue Kit (Qiagen, Germany) according to the manufacturer's instructions. ERIC-PCR was carried out as described by Steinbruechner et al.[13]

Ribotyping

C. diphtheriae strains isolated in 2004–2008 were ribotyped according to von Hunolstein et al.[2]

Plasmid isolation

Two methods were used for plasmid isolation: alkaline lysis according to Sambrook and Russel[14] and a commercial kit – Plazmid Mini (A&A Biotechnology, Poland). As a control we used *Escherichia coli* V517 strain, which possesses eight cryptic plasmids of different sizes.

Antimicrobial susceptibility testing

The minimum inhibitory concentrations (MICs) to 14 antibiotics and chemotherapeutics were determined by the E-test method (AB Biodisk, Sweden) on Mueller–Hinton sheep blood agar plates in accordance with the manufacturer's instructions and Clinical and Laboratory Standards Institute (CLSI) guidelines.[15] A direct colony suspension equivalent to a 0.5 McFarland standard was inoculated onto the plates for all antibiotics and chemotherapeutics used in the test. For intermediate strains the test was repeated with an inoculum of density 1 McFarland. Results were interpreted according to CLSI standards.[15]

Dendrograms

Data analysis was performed with GelCompar II 5.0 for Windows (AppliedMaths). Dendrograms were constructed by the unweighted pair group method with arithmetic averages (UPGMA), using Dice correlation (with an optimization of 1.5% and a position tolerance of 1.5%). The computer-assisted analysis was performed according to the manufacturer's instructions.