POLYMORPHISM OF THE *DTXR* GENE IN THE CURRENTLY EXISTING STRAINS OF *CORYNEBACTERIUM DIPHTHERIAE*

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The pathogenic mechanism used by *Corynebacterium diphtheriae* is attributed to the ability of the diphtheria toxin to disrupt protein synthesis in human cells. Diphtheria toxin production is regulated by the DtxR protein. The latter is involved in the iron-mediated repression of the toxin gene and coordinates activities of other genes essential for the survival of *C. diphtheriae*. The DtxR-encoding gene occurs in both toxigenic and non-toxigenic strains; therefore it can be used to analyze the population structure of the species. In our work we have studied 45 strains of *C. diphtheriae* isolated in the Russian Federation in 2010–2015. These strains were analyzed to reveal that gene *dtxR* is a highly conservative region of *C. diphtheriae* genome that can be found in all members of the studied species. The majority of the discovered polymorphisms were synonymous (16 of 18 single nucleotide polymorphisms identified). In spite of the low phylogenetic signal, the allelic variant of *dtxR* was associated with the strain's phenotype (biovar, toxigenicity). The obtained data indicate the presence of aggressive negative selection aimed to maintain the existing protein sequence in the population. Based on the results, we recommend *dtxR* polymerase chain reaction as an additional technique for pathogen identification, which is especially relevant considering the increasing prevalence of the disease associated with non-toxigenic *C. diphtheriae* strains.

Keywords: diphtheria, *Corynebacterium diphtheriae*, *dtxR*, multilocus sequence typing, metalloregulatory proteins

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ПОЛИМОРФИЗМ ГЕНА *DTXR* У СОВРЕМЕННЫХ ШТАММОВ *CORYNEBACTERIUM DIPHTHERIAE*

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Считается, что патогенез *Corynebacterium diphtheriae* основан на воздействии дифтерийного токсина на синтез белка в клетках человека. Регуляция синтеза токсина находится под контролем белка DtxR. Данный белок осуществляет железоопосредованную репрессию гена дифтерийного токсина, а также координирует работу множества других генов, необходимых для нормальной жизнедеятельности *C. diphtheriae*. Ген, кодирующий DtxR, можно использовать для анализа популяционной структуры вида, так как он присутствует в геноме как токсигенных, так и нетоксигенных штаммов. В работе было изучено 45 штаммов *C. diphtheriae*, выделенных на территории Российской Федерации в 2010–2015 гг. Анализ этих штаммов показал, что ген *dtxR* обнаруживается у всех представителей вида и является высококонсервативным участком генома *C. diphtheriae*. Большинство выявленных полиморфизмов были синонимичны (16 из 18 однонуклеотидных замен). Несмотря на низкий уровень филогенетического сигнала, аллельный вариант *dtxR* был ассоциирован с биологическими признаками штамма (биовар, токсигенность). Полученные данные свидетельствуют о высокой активности отрицательного отбора, направленного на поддержание в популяции существующей последовательности белка, и позволяют рекомендовать наработку фрагментов гена *dtxR* методом полимеразной цепной реакции в качестве дополнительного метода идентификации возбудителя, что особенно актуально в условиях растущего числа заболеваний, ассоциированных с нетоксигенными штаммами *C. diphtheriae.*

Ключевые слова: дифтерия, *Corynebacterium diphtheriae*, *dtxR*, мультилокусное сиквенс-типирование, металлорегуляторные белки

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In spite of successful vaccination strategies, sporadic cases of diphtheria still occur, and the infection remains a serious health issue. Virulence of *Corynebacterium diphtheriae* is associated with its ability to produce a diphtheria toxin encoded by the *tox* gene [1]. Its pathogenic mechanism is based on ADP ribosylation of the elongation factor 2 that disrupts protein synthesis in human cells [2]. It should be noted that the presence of the *tox* gene in the *C. diphtheriae* genome does not necessarily confer toxigenicity. There are nontoxigenic tox-bearing strains (NTTB strains) that have lost their ability to synthesize a fully functional toxin following a series of mutations [3, 4].

Although *tox* is a part of the phage genome, iron-mediated regulation of toxin expression is exerted by the iron-sensing regulator DtxR, the product of the chromosomal gene *dtxR*. Thus, *tox* transcription directly depends on iron homeostasis, as low iron levels trigger *tox* expression followed by synthesis of the diphtheria toxin [5].

The *dtxR* gene is present in both toxigenic and nontoxigenic strains [6] meaning that it has functions other than regulation of diphtheria toxin synthesis. The DtxR regulon is reported to contain 20 more loci, including genes responsible for iron metabolism. To date, the DtxR protein of *C. diphtheriae* is known to regulate siderophore synthesis, a high-affinity transport system (ciuABCDEFG) and transcription of 3 loci involved in heme-monooxygenase (*hmuO*) activity [1, 7]. DtxR may also have a role in regulating bacterial virulence [7].

Microorganisms need large amounts of iron which is not so easy to acquire. However, iron excess stimulates production of toxic reactive oxygen species. Mammals have developed a mechanism of nonspecific defense against infections that relies on reducing the levels of unbound iron by specific iron-binding proteins [8, 9].

Therefore, survival and dissemination of the pathogen in the host depends on its ability to acquire different metal ions from protein complexes. For that, the pathogen employs various uptake mechanisms. Gene expression is controlled by metalloregulatory proteins - highly conserved transcriptional regulators [10, 11]. Once they bind to a specific metal ion, these regulators change their conformation and trigger or repress binding of the active site to the gene operator [12].

DtxR is a typical example of a metalloregulatory protein. Crystallography demonstrates that in its inactive state DtxR is a monomer that consists of two domains. A large conserved N-terminal domain contains two binding sites for iron ions and a helix-turn-helix motif that can bind to DNA; a smaller, less conserved C-terminal domain resembles the SH3 domain of eukaryotes. Ferrous ions bind to the binding sites rendering the repressor active. Once it is activated, dimerization occurs [13].

The two DtxR domains are linked by a proline-rich peptide segment. When the repressor is inactive, this segment binds to the SH3-resembling domain resulting in the formation of a prolylpeptide-SH3 complex (Pr-SH3) and stabilizing the repressor in its inactive state. After ferrous ions bind to the N-terminal domain triggering DtxR activation, the Pr-SH3 complex dissociates and the proline segment stabilizes helical segments of the N-terminal domain, which leads to dimerization of two protein subunits [14].

Considering the role of DtxR in *C. diphtheriae* survival, the *dtxR* gene must be studied to evaluate the pathogenic potential of *C. diphtheriae*, elucidate dynamics of circulating strains and assess feasibility of *dtxR* as a target for the PCR-based diagnosis of diphtheria or other infections associated with nontoxigenic *C. diphtheria* strains. The aim of this work was to identify genetic polymorphisms of DtxR and to analyze the population structure of *C. diphtheriae* strains circulating in Russia.

METHODS

We studied genotypic characteristics of 45 strains of *C. diphtheriae* (bv. gravis and bv. mitis) isolated in 2010–2015. The study was conducted at the Reference center for Measles, Parotitis, Rubella, Pertussis, and Diphtheria of Gabrichevsky Moscow Research Institute of Epidemiology and Microbiology. *C. diphtheriae* strains were obtained from bacterial laboratories of the institutions for disease prevention and centers for hygiene and epidemiology located in 14 different regions of Russia, where the strains had been isolated for diagnostic or preventive screening or for the purpose of epidemiological research. The following collection strains were used: *C. diphtheriae* (State Research Center for Applied Microbiology & Biotechnology, Obolensk, Russia) and *C. diphtheriae* PW 8 (Therapeutic Products Regulatory Research Center, Moscow, Russia). Besides, the following strains were used as PCR negative control, 1 strain per species: *C. ulcerans*, *C. pseudotuberculosis*, *C. amycolatum*, *C. glucuronolyticum*, *C. xerosis*, *C. afermentans subsp. afermentans*, *C. afermentans subsp. lipophiium*, *C. coyleae*, *C. pseudodiphtheriticum*, *C. macifaciens*, *C. simulans*, and *C. durum* from the collection of Gabrichevsky Research Institute.

The strains were isolated following the guidelines of the *Laboratory Diagnosis of Diphtheria* manual (Guidelines 4.2.698–98 and 4.2.3065–13). The isolates were seeded onto the solid tellurite blood agar base containing 2 % agar (Microgen, Russia), 10 % bovine blood (LeiTran, Russia), and 0.02 % potassium tellurite (State Research Center for Applied Microbiology & Biotechnology, Russia). Then the cultures were thermostated for 24–48 h at 37 °C. Morphological, toxigenic and biochemical profiles of the grown cultures were prepared according to Guidelines 4.2.698-98 and 4.2.3065–13 mentioned above using the biochemical test system DS-DIPH-CORYNE (Diagnostic Systems, Russia).

Chromosomal DNA was extracted by boiling from a freshly grown 24-hour old *C. diphtheriae* culture. A culture sample was picked up with a sterile loop and suspended in 100 µl of deionized water, incubated for 20 min at 95 °C and centrifuged. The supernatant was used for the PCR assay.

PCR amplification of the *dtxR* gene of *C. diphtheriae* was performed using a pair of primers to cover the entire region of the studied gene: a previously proposed F1 5'-GGGACTACAACGCAACAAGAA-3' [15] and R1 5'-TCATCTAATTTCGCCGCCTTTA-3' designed by the PerlPrimer application, v1.1.21 [16]. Specificity of primers was checked using BLASTn by comparing their sequences to similar sequences of other *Corynebacterium* species obtained from the NCBI Nucleotide database.

The reaction mix contained a PCR buffer with 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 µM of each primer, 200 µM of each dNTP, 1 µL of the DNA solution, and 1 unit/50 µL Taq DNA polymerase (Fermentas, Lithuania). Amplification was performed in the Tertsik amplifier (DNA Technology, Russia) operated in the automatic mode The amplified fragments were analyzed by 1.5 % agarose gel electrophoresis. Sequencing of the obtained fragments was performed by Evrogen, Russia.

C. diphtheriae strains were genotyped by multilocus sequence typing (MLST) according to the international protocol [17] using fragments of sequences of 7 housekeeping genes, namely *atpA* (encodes the α-subunit of ATP synthase),

dnaE (encodes the α-subunit of the DNA polymerase III holoenzyme), *dnaK* (encodes the Hsp70 chaperone), *fusA* (encodes elongation factor G), *leuA* (encodes 2-isopropylmalate synthase), *odhA* (encodes components E1 and E2 of the 2-oxoglutarate dehydrogenase complex), and *rpoB* (encodes the β-subunit of RNA-polymerase). Allelic profiles were identified for each strain.

The obtained sequences were compared to the nucleotide sequences published in GenBank. The sequence of the *dtxR* gene of *C. diphtheriae* PW8 was used as a reference (Genbank NC_016789.1).

Nucleotide sequences were aligned using the MUSCLE algorithm [18]. Polymorphisms were mapped to the protein structure based on the PDB data. Queries to the NCBI Nucleotide database were run using BLASTn. To identify alleles of the housekeeping genes, the PubMLST software was used. To estimate selection pressure on the *dtxR* gene, we applied the Nei-Gojobori method and calculated the K_a/K_s ratio, where K_a is the number of nonsynonymous substitutions per site and K_s is the number of synonymous substitutions per site [19]. Fisher's exact test was performed on contingency tables using the AS159 algorithm [20] and R 3.3.2. Phylogenetic trees were reconstructed by neighbor-joining based on the comparison of *dtxR* nucleotide sequences and included sequences of *C. diphtheriae* PW8, *C. diphtheriae* NCTC 13129 and *C. diphtheriae* 178-01 (Genbank NC_016789.1, BX248353.1 and NZ_JZUJ01000001.1). Evolutionary distances were computed using the Maximum Composite Likelihood method [21] and scaled as units of substitutions per site. Evolutionary analysis was performed by MEGA7 v.7.0.21 [22]

RESULTS

PCR amplification revealed the presence of the *dtxR* gene in all studied toxigenic and nontoxigenic strains of *C. diphtheriae*. Besides, PCR results came out negative for all allied species.

The samples of 45 *C. diphtheriae* strains were sequenced and the obtained sequences were compared to the *dtxR* sequences retrieved from GenBank revealing polymorphisms at 18 positions: 66, 126, 225, 273, 358, 402, 440, 474, 504,507, 516, 558, 564, 579, 639, 640, 654 and 685 (Table 1).

The majority of substitutions relative to the reference sequence were synonymous. Specifically, the most frequent single nucleotide substitution at position 273 of the *dtxR* gene did not affect the protein sequence. This substitution was observed in 14 strains.

Polymorphisms at positions 440 and 640 that resulted in A147V and L2141substitutions, respectively, seemed to have no significant effect on the DtxR function. According to the 3D protein structure published in PDB (ID 2QQ9), amino acid at position 147 is found in the unstructured proline-rich (Pr) region. Since this segment participates in protein dimerization, we cannot rule out a possible effect of the amino acid substitution on DtxR activation. It appears that substitution of leucine for isoleucine at position 214 of the C-terminal domain does not have any effect on protein folding because these amino acids have similar properties. Thus, nonsynonymous substitutions are very likely to produce no effect on the DtxR function.

Many of the identified sequence variants are well known and were described previously by other researchers. However, we were able to identify a new single nucleotide polymorphism at position 358 that also has no effect on the amino acid sequence. A nucleotide query to the NCBI Nucleotide database performed by BLASTn returned no results.

We observed various combinations of polymorphisms in the *dtxR* gene relative to the reference sequence. Based on the discovered combinations of nucleotide sequences, *C. diphtheriae* isolates were distributed into several groups (Table 2).

More than a half (55 %) of identified nucleotide sequences differed from the reference sequence. It should be noted that the new substitution at position 358 was observed in one strain only (group 5) that had the least number of substitutions compared to the reference sequence, including nonsynonymous polymorphisms.

Based on the alignment of *dtxR* sequences, we constructed a phylogenetic tree (see the Figure).

Table 1. Frequency of nucleotide substitutions in the *dtxR* gene of the studied strains of *C. diphtheriae* relative to the strain PW8

Position of nucleotide in the *dtxR* gene	Nucleotide substitution	Encoded amino acid	Amino acid substitution	Number of strains
66	A–T	22	–	5
126	C–T	42	–	5
225	T–C	75	–	6
273	C–T	91	–	14
358	T–C	120	–	1
402	T–A	134	–	1
440	C–T	147	Alanine (A) – Valine (V)	6
474	C–T	158	–	6
504	T–A	168	–	6
507	C–T	169	–	6
516	T–C	172	–	6
558	C–T	186	–	2
564	T–A	188	–	6
579	C–T	193	–	2
639	C–T	213	–	4
640	C–A	214	Leucine (L) – Isoleucine (I)	2
654	T–C	218	–	2
685	C–T	229	–	1

Table 2. Combinations of polymorphisms of the dtxR gene in *C. diphtheriae* strains

Group of strains	Number of strains (n = 45)	Position of nucleotide in *dtxR* nucleotide sequence																	
		66	126	225	273	358	402	440	474	504	507	516	558	564	579	639	640	654	685
1	20 (44 %)	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
2	5 (11 %)	*	T	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
3	9 (20 %)	*	*	*	T	*	*	*	*	*	*	*	*	*	*	*	*	*	*
4	5 (11 %)	*	*	C	T	*	*	*	*	*	*	*	*	*	*	T	*	*	*
5	1 (2 %)	*	*	C	*	C	A	**T**	T	A	T	C	T	A	T	T	**A**	C	T
6	1 (2 %)	T	*	*	*	*	*	**T**	T	A	T	C	T	A	T	T	**A**	C	*
7	4 (9 %)	T	*	*	*	*	*	**T**	T	A	T	C	*	A	*	*	*	*	*
Reference strain PW8	–	A	C	T	C	T	T	C	C	T	C	T	C	T	C	C	C	T	C

Note. * — a match with the reference; **T**, **A** — polymorphism with amino acid substitution.

The branching order is an approximation to some extent, due to the similarity of the analyzed sequences and hence a weak phylogenetic signal. The tree in the dendrogram is not rooted due to the lack of possibility to select an appropriate outgroup.

We also analyzed correlations between group composition and toxigenicity, biovars and sequence types (ST) determined by MLST (Table 3). Profiles of NTTB strains were previously described in [23].

Based on the distribution of toxigenic and nontoxigenic strains of different biovars with regard to the allelic variants of *dtxR*, Fisher's exact test was performed. The test was performed on 2 × 7 contingency tables to examine the association between a biovar type and the allelic variant of *dtxR* ($p = 0.00078$) and on 3 × 7 contingency tables to examine the association between toxigenicity and the allelic variant of *dtxR* ($p = 2.8 \cdot 10^{-9}$). The obtained results prompt us to conclude that the associations between a biovar type/toxigenicity and the allelic variant of *dtxR* are not accidental and implicate a phylogenetic signal — a sum of associations between the allelic variant of the gene and biological characteristics of the strains. Distribution of sequence types was nonuniform, identical sequence types were rarely found in one group.

To assess selection pressure on the *dtxR* gene, we calculated the K_a/K_s ratio (0.0526). The obtained value ($K_a/K_s < 1$) indicates a strong negative selection, i.e., selection pressure is aimed at maintaining the current protein sequence [24].

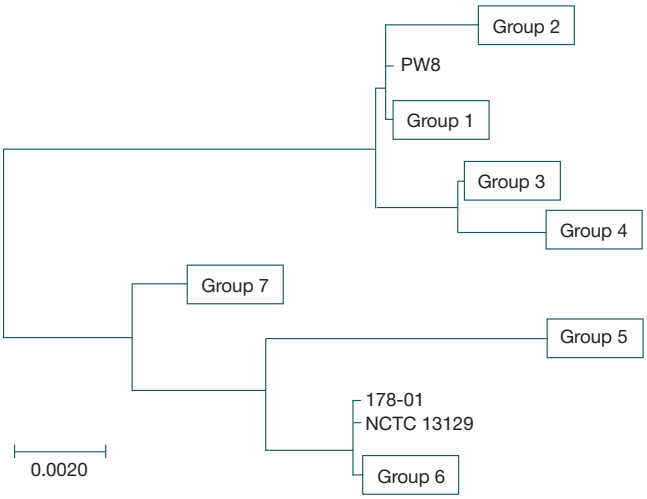
DISCUSSION

Our study confirmed the essential role of DtxR in the viability of toxigenic and nontoxigenic strains of *C. diphtheriae*. The observed polymorphisms provide new information of the variability of its strains in Russia. *dtxR*–related nucleotide and amino-acid substitutions were studied previously under various conditions with regard to diphtheria dissemination [25–27]. This work was conducted against the background of sporadic incidence. We performed a comprehensive analysis of nucleotide sequences and assessed their correlation with strain toxigenicity, biovars and sequence types. This approach allowed us to better understand the structure of the population of currently circulating *C. diphtheriae* strains.

The hypothesis about the significant effect of horizontal gene transfer on the structure of *C. diphtheriae* population was discussed earlier [17]. There are works describing transfer mechanisms for genes conferring antibiotic resistance [28] and virulence [29]. Components of the DtxR regulon may vary in different strains of *C. diphtheriae* due to the loss, acquisition, or partial deletion of genes responsible for iron provision and hence *tox* expression [1, 30]. The discovered correlation between a sequence type and the allelic variant of *dtxR* proves the idea that homologous recombination in *C. diphtheriae* does not completely block the phylogenetic signal [17]. At the same time, the analysis of the population structure did not reveal any direct correlation between a biovar and the *dtxR* allele meaning that there is no phylogenetic basis for such classification, which is mainly determined by the frequency of horizontal gene transfer [31].

Less than a half (45 %) of the studied sequences were found to be identical to the reference sequence. Of 13 polymorphisms, only 1 was identified as new (position 358), but it did not affect the amino acid sequence. The most frequent was the synonymous polymorphism at position 273 observed in 14 strains. Our work demonstrates that Russian strains carry a smaller range of variants of the primary DtxR structure [26, 27].

It should be noted that although *dtxR* affects bacterial resistance to oxidative stress, under normal conditions it is not a critical gene for *C. diphtheria* [32], as was proved in the experiment with the mutant DtxR-defective strain [33]. However, we did not observe any significant changes in the studied sequences that could result in the synthesis of



The phylogenetic tree of *C. diphtheriae* strains based on the *dtxR* gene sequences

The tree is scaled to 0.1 substitutions per 200 b. p.; branch lengths correspond to the evolutionary distances used to construct the tree. Sequences within groups are identical.

Table 3. Group composition according to polymorphism combinations in the *dtxR* gene of *C. diphtheriae* strains

Group	Number of substitutions in *dtxR* relative to strain PW8	Number of strains	Biovar (number of strains)	Toxigenicity (number of strains)	Sequence type
1	–	20	mitis (18), gravis (2)	notoxigenic (15)	*
				NTTB strains (3)	76
				toxigenic (2)	5, 46
2	1	5	mitis	NTTB strains	40
3	1	9	gravis (1), mitis (8)	toxigenic (8)	25
				notoxigenic (1)	123
4	3	5	mitis	toxigenic	28, 67
5	15	1	mitis	notoxigenic	–
6	12	1	gravis	toxigenic	8
7	7	4	gravis	toxigenic	8

Note. * — MLST non performed, – — ST not found in the database.

a functionally inactive protein. There were two nucleotide polymorphisms that did result in amino acid substitutions, but the analysis of PDB data showed that those substitutions did not affect protein folding. Perhaps, functionally important polymorphisms of the *dtxR* gene may impair strain adaptation to the mammalian host limiting distribution of alleles that lead to the synthesis of the defective protein among the *C. diphtheriae* population. The value of the K_a/K_s ratio proved that DtxR is controlled by the stabilizing selection.

The pathogenic potential of *C. diphtheriae* does not always depend on strain's ability to produce the diphtheria toxin. For example, nontoxigenic strains of *C. diphtheriae* are becoming an increasing source of severe infection causing endocarditis [34], arthritis [35] and osteomyelitis [36]. Nontoxigenic strains are especially dangerous for patients with compromised immunity [37, 38]. This necessitates a more comprehensive approach to *C. diphtheriae* identification as currently existing methods are aimed at detecting toxigenic strains only.

Our findings confirmed the presence of the *dtxR* gene in both toxigenic and nontoxigenic strains of *C. diphtheriae* proving the feasibility of PCR-based identification proposed earlier [39]. Sequencing demonstrated that polymorphisms occurred mainly in the C-terminal domain of DtxR [15]. However, nucleotide substitutions were also observed in other gene regions (positions 66, 126, 225, 273, and 358). One of such substitutions (position 126) was found in 5 strains (group 2) in the region corresponding to the primer that had been proposed earlier for PCR- *dtxR* [15, 39]. The pair of primers used in this study proved their high specificity confirmed by zero false-positive results. This, PCR-based identification of *C. diphtheriae* is a promising technique for the diagnosis of diphtheria and infections associated with nontoxigenic strains.

CONCLUSIONS

We analyzed the structure of *C. diphtheriae* population based on the allelic variants of the *dtxR* gene. The analysis revealed that 55 % of strains had sequences different from the reference sequence. The majority of the discovered polymorphisms were synonymous. The absence of wild strains with defective DtxR and a high similarity of the analyzed nucleotide sequences indicate a strong negative selection aimed to maintain the currently existing repressor sequence.

Homologous recombination attenuates the phylogenetic signal but does not block it completely. We discovered associations between the allelic variants of *dtxR* and toxigenicity/biovar type. The obtained results allow us to conclude that *dtxR* represents a conserved sequence. We recommend PCR-*dtxR* as an accurate method for identification of toxigenic and nontoxigenic strains of *C. diphtheriae*.

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