GENOTYPIC CHARACTERISTICS OF BORDETELLA PERTUSSIS, CANDIDATE STRAINS FOR PRODUCTION OF PERTUSSIS COMPONENT OF VACCINES (STATEMENT I)

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Vaccination is an effective means of preventing pertussis infection. The purpose of this work was to improve vaccines available in the Russian Federation in general and actualize vaccine strains used for the production thereof in particular. We studied *B. pertussis* strains isolated in Moscow, Voronezh, Novosibirsk, Ulyanovsk and Chelyabinsk regions, and eight production strains part of the adsorbed diphtheria-pertussis-tetanus (DPT) vaccine. Multilocus antigenic sequence typing (MAST) and whole genome multilocus sequence typing (wgMLST) were used for genotyping. We studied cultural morphological, enzymatic, serological, and genotypic properties of the candidate *B. pertussis* strains, and compared their genotypic properties to those of B. pertussis vaccine strains from the current composition of the DPT vaccine. Candidate strains belong to four genotypes: *ptxA1/ptxB2/ptxC2/ptxP3/fim2-2/fim3-2/prn2*, *ptxA1/ptxB2/ptxC2/ptxP3/fim2-2/fim3-2/prn9, ptxA1/ptxB2/ptxC2/ptxP3/fim2-1/fim3-1/prn1* and *ptxA1/ptxB2/ptxC2/ptxP3/fim2-2/fim3-1/prn2*. Current vaccine strains were from other six genotypes: *ptxA2/ptxB1/ptxC1/ptxP1/fim2-1/fim3-1/prn1, ptxA2/ptxB2/ptxC1/ptxP2/fim2-1/fim3-1/prn1, ptxA4/ptxB1/ptxC1/ptxP2/fim2-1/fim3-1/prn1, ptxA2/ptxB2/ptxC1/ptxP1/fim2-1/fim3-1/prn1, ptxA4/ptxB2/ptxC1/ptxP2/fim2-1/fim3-1/prn1* and *ptxA1/ptxB2/ptxC1/ptxP1/fim2-1/fim3-1/prn1*. With the help of wgMLST, we established affiliation of all candidate strains of *B. pertussis* to ST2.

Keywords: *Bordetella pertussis*, vaccines, genotyping, multilocus antigenic sequence typing

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ГЕНОТИПИЧЕСКАЯ ХАРАКТЕРИСТИКА ШТАММОВ *BORDETELLA PERTUSSIS* — КАНДИДАТОВ ДЛЯ ПОЛУЧЕНИЯ КОКЛЮШНОГО КОМПОНЕНТА ВАКЦИННЫХ ПРЕПАРАТОВ (СООБЩЕНИЕ I)

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Вакцинация является эффективным средством предупреждения заболевания коклюшной инфекцией. Целью работы было усовершенствование вакцинных препаратов в РФ, в том числе актуализация производственных вакцинных штаммов. Изучали штаммы *B. pertussis*, выделенные в г. Москве, Воронежской, Новосибирской, Ульяновской и Челябинской областях, и восемь производственных штаммов для адсорбированной коклюшно-дифтерийно-столбнячной (АКДС) вакцины. Для генотипирования использовали мультилокусное антигенное сиквенс-типирование (MAST) и полногеномное мультилокусное сиквенс-типирование (wgMLST). Изучены культурально-морфологические, ферментантивные, серологические и генотипические свойства штаммов *B. pertussis* — кандидатов в производственные вакцинные штаммы и проведен сравнительный анализ генотипических свойств этих штаммов и производственных вакцинных штаммов *B. pertussis* для вакцины АКДС. Кандидатные штаммы являются представителями четырех генотипов — *ptxA1/ptxB2/ptxC2/ptxP3/fim2-2/fim3-2/prn2, ptxA1/ptxB2/ptxC2/ptxP3/fim2-2/fim3-2/prn9*, *ptxA1/ptxB2/ptxC2/ptxP3/fim2-1/fim3-1/prn1* и *ptxA1/ptxB2/ptxC2/ptxP3/fim2-2/fim3-1/prn2*. Производственные вакцинные штаммы принадлежали к другим шести генотипам: *ptxA2/ptxB1/ptxC1/ptxP1/fim2-1/fim3-1/prn1, ptxA2/ptxB2/ptxC1/ptxP2/fim2-1/fim3-1/prn1, ptxA4/ptxB1/ptxC1/ptxP2/fim2-1/fim3-1/prn1, ptxA2/ptxB2/ptxC1/ptxP1/fim2-1/fim3-1/prn1, ptxA4/ptxB2/ptxC1/ptxP2/fim2-1/fim3-1/prn1* и *ptxA1/ptxB2/ptxC1/ptxP1/fim2-1/fim3-1/prn1*. С помощью wgMLST установлена принадлежность всех кандидатных штаммов *B. pertussis* к ST2.

Ключевые слова: *Bordetella pertussis*, вакцинные препараты, генотипирование, мультилокусное антигенное сиквенс-типирование

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Pertussis is a dangerous disease that may end in a fatality, especially among newborns and infants [1-3]. Growing prevalence of pertussis, spread of its severe forms among babies below 1 year of age, and lethal outcomes underpin relevance of the study. In 2023, there were 52,727 pertussis cases registered in the Russian Federation, yielding the incidence rate of 36.2 per 100,000 population), which is 16.4 times more than in 2022 (2.2 per 100,000 population) and 7.5 times higher than the average long-term incidence rate (4.8 per 100,000 population). Over 80% of the patients were children under 14 years of age, according to the Pertussis and Diphtheria Monitoring Reference Center of G.N Gabrichevsky

Research Institute for Epidemiology and Microbiology (based on the analysis of form No. 2 "Information on infectious and parasitic diseases") [4–6].

Routine vaccination against pertussis in the first year of life triggers development of immunity and resistance to infection [1, 3]. By the age of 6–7, this post-vaccination immunity weakens. Starting school, children enter new groups, some members of which may be infected. In such groups, unvaccinated and those who have lost their post-vaccination immunity contract the disease most often, with the latter having it in a light form with atypical cough [7–9]. Previously, 7 to 17% of prolonged cough in adolescents have been shown to be associated with *B. pertussis*. Various authors estimate that in 75% of cases, infants under 1 year of age catch this infection from school-age children [9, 10]. Therefore, vaccination is an effective means of preventing pertussis in youngest populations.

With the incidence of pertussis on the rise, medical professionals and researchers have expressed their concerns about efficacy of the currently applied prevention strategy [11, 12]. The reasons that, most likely, underpin the said rise, are as follows: large number of parents refusing vaccination of their infants under the age of 1, which delays the entire vaccination schedule [2, 6]; growing number of non-immune individuals among older children; deterioration of specific immunity in adults [10, 13]; genotypic variability of the pathogen under selective action of the vaccines [14–23]; spread of *B. pertussis* by asymptomatic carriers [24]. In addition, wide adoption of PCR tests has significantly increased the number of detected cases of pertussis, which now includes mild, subclinical courses of the disease, as well as cases registered when investigating group infections (unpublished data from the Pertussis and Diphtheria Monitoring Reference Center).

For more than 60 years, DPT vaccine includes a whole-cell pertussis component. Though highly effective, such component is reactogenic, which necessitated development of cell-free vaccines that have been widely used throughout the world since the second half of the 1990s. These vaccines contain from 1 to 5 purified pertussis antigens (pertussis toxin (PT), filamentous hemaglutinin (FHA), pertactin *(PRN*), type 2 and type 3 fimbriae (*Fim2* and *Fim3*)). Nevertheless, vaccination of the population cannot fully prevent the spread of pertussis, the incidence of which is nowadays growing in many countries of the world. Since the 2000s, against the background of widespread use of cell-free pertussis vaccine in Europe, Japan, the USA, and Australia, the prevalence of this disease has been growing, in some cases — to the point of epidemic outbreaks [https://www.who.int/health-topics/pertussis#tab=tab_1]. According to several researchers, there is a correlation between preference for such vaccines and the increasing incidence of pertussis, which raises a number of questions about the their efficacy and ability to control the disease [11, 12, 22, 23].

Numerous monitoring studies investigating emergence of the new strains of *B. pertussis* have shown that some of them acquire genetic mutations affecting the structure of protective antigens, thus granting ability to evade the immune response [14–23]. Multilocus antigenic sequence typing (MAST) focuses on changes in the population of *B. pertussis*. In particular, genotyping studies gene sequences encoding protective antigens, including those part of the currently common cell-free vaccines: *ptxA* (encodes the S1 subunit of pertussis toxin), *ptxB* (encodes the S2 subunit of pertussis toxin), *ptxC* (encodes the S3 subunit of pertussis toxin), *prn* (encodes pertactin, the adhesive protein), *fim2* and *fim3* (encode the fimbrial proteins *Fim2* and *Fim3*, respectively). Pertussis toxin's promoter, *ptxP*, is also part of typing isolates, since it has been established that the *ptxP3* allele enhances toxin production [25].

In the Russian Federation, both whole-cell and cell-free pertussis vaccines (as a component of combined preparations) are common. Thus, the purpose of this work was to study the biological and genotypic properties of the vaccine candidate *B. pertussis* strains.

METHODS

Under regulations of the Russian Federation, Pertussis and Diphtheria Monitoring Reference Center of G.N Gabrichevsky Research Institute for Epidemiology and Microbiology receives bacterial strains of *Bordetella* and clinical samples obtained from pertussis patients for the purposes of *B. pertussis* verification and genotyping. We selected eight verified strains of *B. pertussis* (smooth form phase of development) with good growth potential, and, as prescribed by the applicable regulations, studied their morphological, cultural, enzymatic, serological, and genotypic properties in order to select candidates to design a pertussis vaccine on. The origin of the strains was one of the inclusion criteria: they were supposed to be from various regions of the Russian Federation.

The selected *B. pertussis* strains were isolated from samples donated by patients of different ages in 2016 through 2020 in Moscow, Voronezh, Novosibirsk, Ulyanovsk, and Chelyabinsk regions. For comparison, we used eight vaccine strains from the collection of Scientific Centre for Expert Evaluation of Medicinal Products that are in production of the pertussis component of DPT vaccine (Table 1).

The strains were cultured on Bordetelagar (State Research Center for Applied Biotechnology and Microbiology, Obolensk, Russia), a dense nutrient medium, for 72 hours at +36–37 °C. The identification of microorganisms relied on their cultural morphological, tinctorial and biochemical properties. The cultural and morphological properties of the resulting colonies were uncovered using a SteREO Discovery V12 stereoscopic microscope (Carl Zeiss; Germany) with a PlanApo S 1.0 × FWD 60 mm lens objective and a PI 10 × 23 Br foc eyepiece. Gram staining (ECOlab; Russia) allowed gauging the tinctorial properties. The stained smears were examined through an Axio Scope A1 light microscope with EC Plan-NEOFLUAR 100 × 1.3 lens and PI 10 × 23 Br foc eyepiece (Carl Zeiss; Germany).

The identification of *B. pertussis* strains by biochemical properties was carried out in accordance with the applicable regulations [26].

To learn the antigenic structure (serotypes) of the strains, we staged an extended agglutination reaction using pertussis sera to agglutinogens 1,2,3 dry adsorbed (NPO Microgen; Russia).

We carried out a whole genome sequencing of the eight strains of *B. pertussis* currently used for the pertussis component of DPT vaccine and eight strains of *B. pertussis* selected as candidates to design such component on based on their microbiological and growth characteristics. For this task, we employed MAST, which enabled analysis of the sequence of genes encoding protective antigens of the pathogen (*ptxA*, *ptxB*, *ptxC*, *ptxP*, *prn*, *fim2* and *fim3*), as recommended in [27] and relying on the data from GenBank and BIGSdb databases, and whole genome multilocus sequence typing (wgMLST).

Genomic DNA was isolated from bacterial culture using the ExtractDNA Blood&Cells kit (Eurogen, Russia). For whole genome sequencing, we used the GenoLab platform (GeneMind Biosciences; China) and SG GM kits (Raissol; Russia), as recommended by the manufacturer. The data were assembled in SPAdes-3.15.4, the quality of the array was verified with

Table 1. Genotypic characteristics, vaccine production *B. pertussis* strains

№	№ Piece	Isolation year, location	Year introduced to production	Serovar	*ptxA*	*ptxB*	*ptxC*	*ptxP*	*prn*	*fim2*	*fim3*
1	305	Russia, 1957	1958	1.2.0	2	1	1	1	1	1	1
2	312	Russia, 1959	1962	1.2.3	2	2	1	2	1	1	1
3	475	Russia, 1966	1967	1.2.3	4	1	1	2	1	1	1
4	267	Russia, 1967	1968	1.0.3	2	1	1	1	1	1	1
5	38	Russia, 1966	1967	1.2.0	2	2	1	1	1	1	1
6	39	Russia, 1970	1980	1.2.3	2	2	1	1	1	1	1
7	345	Russia, 1959	1962	1.2.3	4	2	1	2	1	1	1
8	703	Russia, 1970	1976	1.0.3	1	2	1	1	1	1	1

the help of QUAST 5.2.0 (https://github.com/ablab/quast; Russia). We employed the BIGSdb server to identify alleles of the genes of interest. For the purpose of comparison of the nucleotide sequences yielded by MAST, we used the following reference gene numbers: ptxA gene (*ptxA1* (AJ245366), *ptxA2* (AJ245367), *ptxA4* (AJ245368)); *ptxA4* gene (HM185483.1) (*ptxA41*, *ptxA42*); *ptxC* gene (AJ420987) (*ptxC1* (M13223), *ptxC2* (AJ420987)); *ptxP*, pertussis toxin promoter (*ptxP1* (FN252323.1), *ptxP2* (FN252322.1), *ptxP3* (FN252324.1)); *fim2* gene (*fim2-1* (KT194049), *fim2-2* (AJ420988)); *fim3* gene (*fim3-1* (X51543.1) and *fim3-2* (AY464180.1)); *prn* gene (*prn1* (AJ011091.1), *prn2* (AJ011092.1), *prn9* (AJ315611.1)). To find the alleles needed to build the tree based on wgMLST, we used pyMLST 2.1.65 (https://github.com/bvalot/pyMLST /; France). All complete ST2 genomes available in the NCBI Refseq public database, as well as the ST1 Tohama as an external representative, were used for the purpose of comparison. Based on the sequences of 2974 genes, we compiled the allele profile of each strain, then calculated the distance matrix reflecting the number of mismatched alleles between the strains. Using the distance matrix and the rapidNJ 2.3.2 software (https://github.com/somme89/rapidNJ; Denmark), we then built the employing the Neighbor-Joining algorithm.

Two clades were taken from the said tree, one of which consisted entirely of the candidate strains, while the other included strain 3–20 and five comparison strains. For these strains (as well as for Tohama I), we took sequences of all 2974 studied genes, concatenated and used them to build a tree applying the Neighbor-Joining algorithm and the Kimura distance model. This approach was applied to evaluate bootstrap support levels in the above-described clades of the previous tree.

RESULTS

Cultured on Bordetelagar for 72 hours, all eight studied strains of *B. pertussis* developed convex round shiny smooth surface colonies of grayish-white color, up to 1.5 mm in size, with oily consistency, which could be easily removed by a loop. Observing the colonies through a SteREO Discovery V12 stereoscopic microscope with a PlanApo S 1.0 × FWD 60 mm lens and a PI 10 × 23 Br foc eyepiece (Carl Zeiss; Germany), we noted a narrow beam of light ("tail") radiating from their centers. Microscopy also revealed small randomly arranged gram-negative rods. All studied strains of *B. pertussis* exhibited catalase and oxidase activity, did not grow on blood and meat-peptone agar, did not produce enzymes tyrosinase and urease, did not grow on Simmons' citrate agar, did not reduce nitrates to nitrites, and were immobile. Considering the antigenic structure, all studied strains of *B. pertussis* had agglutinogen 1 (species feature), and agglutinated with adsorbed type-specific sera to agglutinogens 1,2,3 not lower than 1 : 280. The production vaccine strains belonged to three different serotypes: 1.2.0, 1.0.3 and 1.2.3; candidate strains to two serotypes: 1.0.3 and 1.2.0 (Tables 1, 2).

By the sequence of the *ptxA* gene, which encodes S1 subunit of pertussis toxin, most of the production strains of *B. pertussis* correspond to the two alleles of *ptxA*, *ptxA4* and *ptxA2*, and only one production strain has the *ptxA1* allele, like all current candidate strains. The *ptxA1* allele differs from other alleles by significant mutational changes with amino acid substitution in the T-epitope of pertussis toxin's S1 subunit (nucleotide positions 204, 586, 668 and 96), which has a continuous immunodominant structure recognized by monoclonal protective m-antibodies (mAT). The *ptxA1* allele differs from the *ptxA4* allele at positions D68E, I228M and I232V, and differs from the *ptxA2* allele at position I228M [12, 27].

By the sequence of the *ptxB* gene, which encodes pertussis toxin's S2 subunit, three production strains of *B. pertussis* correspond to the *ptxB1* allele of the gene, and most production strains (five) and all current candidate strains have the *ptxB2* allele. The *ptxB1* allele differs from the *ptxB2* allele by the changed G18S in the S2 subunit of pertussis toxin.

Studying the *ptxC* gene, which encodes S3 subunit of the pertussis toxin B complex, we found strains of two nucleotide sequence variants, *ptxC1* and *ptxC2*. All production vaccine

Table 2. Genotypic characteristics, candidate *B. pertussis* strains

№	№ Piece	Year of isolation	Serotype	*ptxA*	*ptxB*	*ptxC*	*ptxP*	*prn*	*fim2*	*fim3*
1	16–16	2016	1.0.3	1	2	2	3	2	2	2
2	31–2–17	2017	1.0.3	1	2	2	3	9	2	2
3	25–16	2016	1.0.3	1	2	2	3	2	2	2
4	37–18	2018	1.2.0	1	2	2	3	2	2	1
5	30–18	2018	1.0.3	1	2	2	3	2	2	2
6	1–20	2020	1.0.3	1	2	2	3	2	2	2
7	2–20	2020	1.0.3	1	2	2	3	2	2	2
8	3–20	2020	1.2.0	1	2	2	3	2	2	1

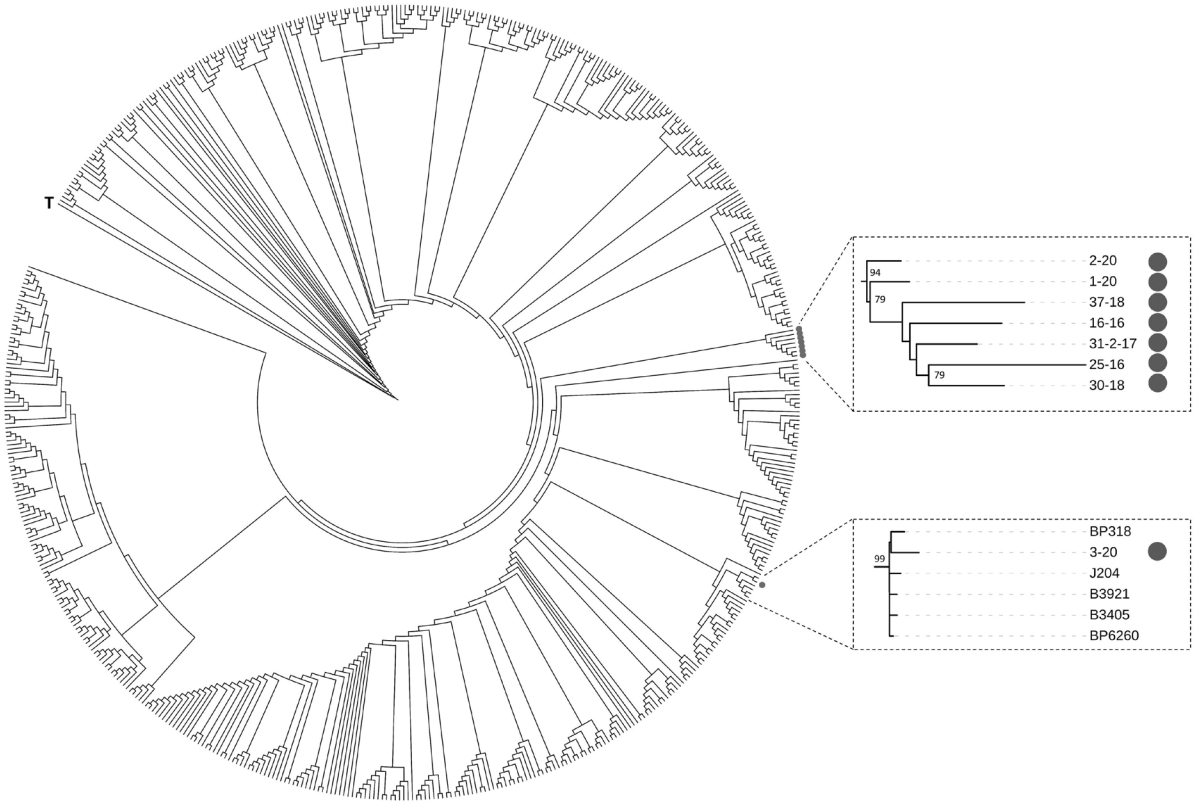

Fig. Phylogenetic tree of bacteria of ST2 type based on wgMLST. Vaccine candidate strains are *gray dots*. Sections of the tree containing these strains are presented magnified on the right, with bootstrap support levels marked in the nodes (only values above 70). The ring diagram does not show lengths of branches because some strains have abnormally long branches. Letter T denotes the Tohama I strain used as an external representative

strains of *B. pertussis* carry the *ptxC1* allele, while all current candidate strains have the *ptxC2* allele, which differs from the *ptxC1* allele by a replaced nucleotide at position C681T, this replacement entailing no changes at the amino acid level.

Although *ptxP,* pertussis toxin's promoter, is not part of the cell-free vaccine, it is usually used in strain typing as a marker of the emerging genetic lineage that has spread throughout the world [14–23, 27]. Analysis of the *ptxP* region's sequence has shown that the studied strains have three *ptxP* alleles: *ptxP1*, *ptxP2*, and *ptxP3*. Five *B. pertussis* vaccine production strains carried the *ptxP1* allele, three — *ptxP2* allele, and all candidate strains carried the *ptxP3* allele. This allele differs from *ptxP1* and *ptxP2* by a mutation at position –65, which reinforces binding to the BvgA dimer and thus increases production of the pertussis toxin [25].

Sequencing of the *fim3* gene, which encodes fimbrial protein Fim3, revealed two variants of the nucleotide sequence, *fim3-1* and *fim3-2*. All vaccine production strains were found to carry a similar *fim3-1* allele, six candidate strain — *fim3-2*, and two remaining candidate strains — *fim3-1*. The nucleotide sequence of *fim3-2* differs from that of *fim3-1* allele by a significant mutation, which entails changes at A87E in the Fim3 protein molecule.

Sequencing of the *fim2* gene, which encodes fimbrial protein *Fim2*, revealed two variants of the nucleotide sequence, *fim2-1* and *fim2-2*. All production strains had a similar allele, *fim2-1*, and all candidate strains of *B. pertussis* — *fim2-2*. The identified allelic variants of *fim2-1* and *fim2-2* differed in R174K of Fim2 protein molecule.

The *prn* gene contains about 2800 bases that encode a large adhesive precursor protein: the 5' gene end encodes a part of the pertactin precursor that is exported from the cell, while the 3' end encodes the integral outer membrane protein that enables the export. Sequencing of the pertactin (*prn*) gene in the studied *B. pertussis* strains revealed three variants of its alleles, *prn1*, *prn2* and *prn9*, with *prn1* identified in the vaccine production strains, and *prn2* and *prn9* — in 7 and 1 candidate strains, respectively. The nucleotide sequences of the *prn2* and *prn9* alleles differ from that of the *prn1* allele: the former contain significant mutational changes in six positions (828, 831, 832, 833, 834 and 836), which entail V279G and A278F substitutions on the amino acid level. The *prn2* allele's nucleotide sequence has an insert in the 15 bp, GGCGGCCTCGGTCC in the gene's 1st region (positions 841-855), and the gene's prn9 allele has an extra fragment in the 30 bp, GGCGGCCTCGGTCCTCGGTCC (1st region, positions 841–871). All changes in the *prn2* and *prn9* alleles are in the *Prn* protein's 1st and 2nd regions, which are immunogenic and participate in the development of the B-cell immune response.

This study has shown that all vaccine production strains belong to six genotypes: *ptxA2/ptxB1/ptxC1/ptxP1/fim2-1/fim3-1/prn1, ptxA2/ptxB2/ptxC1/ptxP2/fim2-1/fim3-1/prn1, ptxA4/ptxB1/ptxC1/ptxP2/fim2-1/fim3-1/prn1, ptxA2/ptxB2/ptxC1/ptxP1/fim2-1/fim3-1/prn1, ptxA4/ptxB2/ptxC1/ptxP2/fim2-1/fim3-1/prn1 and ptxA1/ptxB2/ptxC1/ptxP1/fim2-1/fim3-1/prn1* (Table 1). Candidate strains belong to four genotypes: *ptxA1/ptxB2/ptxC2/ptxP3/fim2-2/fim3-2/prn2, ptxA1/ptxB2/ptxC2/ptxP3/fim2-2/fim3-2/prn9, ptxA1/ptxB2/ptxC2/ptxP3/fim2-1/fim3-1/prn1* and *ptxA1/ptxB2/ptxC2/ptxP3/fim2-2/fim3-1/prn2* (Table 2).

Based on the candidate strains' complete genome sequences, we have built a phylogenetic tree that shows the evolutionary position of these strains among all the representatives of the ST2 sequence type (Figure). It was established that all of them, except one, form a single cluster, apparently inherent in the Russian Federation.

DISCUSSION

All the *B. pertussis* genome sequences learned in the context of this study were added to the National Catalog kept by the State Research Center for Applied Biotechnology and Microbiology as part of the Federal Project "Sanitary Shield of the Country — Health Safety (Prevention, Detection, Response)."

Long-term monitoring of the genotypic properties of B. pertussis has shown that more than 60 years of routine immunization of children triggered spread of pertussis pathogens with new genotypes (allelic profiles) [14–16], which is consistent with the changes in the genetic structure of *B. pertussis* protective antigens registered worldwide [18–23]. Studies conducted in different countries of the world have also revealed genotypic differences between vaccine production strains and circulating pertussis pathogens [11, 18–23, 28, 29].

This study describes and suggests as candidates eight strains of *B. pertussis* that belong to four different genotypes different from the currently used DPT vaccine production strains. Graduation of these candidates to vaccine production strains requires animal studies designed to investigate their immunobiological, hemagglutinating, hemolytic and leukocytosis-stimulating properties, virulence and toxicity, as well as deposition in the National Collection of Pathogenic Microorganisms (NCPM-Obolensk).

Circulating strains of *B. pertussis* change continuously, which necessitates ceaseless monitoring of the strains' genetic properties that would allow assessing the effect of the said changes on the efficacy of the vaccine. Reasoned assessment of the adequacy of the currently common whole-cell and cell-free pertussis vaccines as means of preventing the respective infection requires further research of the circulating *B. pertussis* strains' genotype and specifics of development of post-infection and post-vaccination immunity. Evaluation of protective properties of the produced vaccines also calls for animal model experiments that involve strains of *B. pertussis* with modern genotypes.

CONCLUSIONS

This study yielded *B. pertussis* strains suggested as candidates for pertussis vaccines, and presents assessment of their growth, cultural, morphological, and genotypic properties.

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