Molecular and genetic characteristics of group A rotaviruses detected in Moscow in 2015–2020

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Abstract
The aim of the study was to analyze genetic characteristics of strains belonging to group A rotaviruses (RVA) circulating in Moscow in 2015–2020, including rare strains non-typeable by polymerase chain reaction (PCR).

Materials and methods. A total of 289 stool samples were tested; the samples were collected from children aged 1 month to 17 years, hospitalized with acute gastroenteritis. Immunochromatography and real-time reverse transcription-polymerase chain reaction (real-time RT-PCR) assays were used for detection of rotaviruses in the samples. The rotavirus genome sequencing was performed using the Sanger technique and nanopore sequencing.

Results and discussion. RVA RNA was detected in 131 clinical samples, and the G/[P] genotype was identified in 125 samples. The general profile showed prevalence of RVA strains with the G9P[8]I1 genotype (37%) followed by G3P[8]I2, G4P[8]I1, G2P[4]I2, G1P[8]I1, and G3P[8]I1 variants (18, 15, 11, 5, and 2%, respectively). Seven (5%) isolates were identified as GxP[8]. In 2015–2020, the region reported a decline in G4P[8]I1 genotype prevalence (from 39% to 9%) and an increase in the proportion of the G9P[8]I1 genotype (from 6% to 37%) as compared to 2009–2014. In 2018–2020, a large number of cases with the previously unknown DS-1-like reassortant strain with the G3P[8]I2 genotype were reported; the above strain has become widely common worldwide in the recent years. Nanopore sequencing was performed to analyze the genome of the G3P[8]I2 strain and the rare G4P[6]I1 strain. It was found that the G4P[6]I1 strain was phylogenetically related to porcine rotaviruses.

Conclusion. In the recent years, the genetic diversity of RVA circulating in the Moscow Region has changed significantly. The obtained results prove the importance of continuous monitoring of rotavirus infection and selective sequencing of RVA genes to fine-tune data of the type-specific real-time RT-PCR. The ever-changing genetic composition of the circulating RVA strains calls for regular optimization of RVA genotyping systems based on real-time RT-PCR.

Keywords: group A rotaviruses, acute gastroenteritis, G/[P]-genotyping, nanopore sequencing

Ethics approval. The study was conducted with the informed consent of the patients. The research protocol was approved by the Ethics Committee of the I. Mechnikov Research Institute of Vaccines and Sera (Protocol No. 6, September 24, 2021).

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Молекулярно-генетические особенности ротавирусов группы А, выявленных в Москве в 2015–2020 гг.

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Аннотация

Цель работы — анализ генетических характеристик штаммов ротавирусов группы А (РВА), циркулировавших в Москве в 2015–2020 гг., включая редкие штаммы, нетипируемые методом полимеразной цепной реакции (ПЦР).

Материалы и методы. Исследовали 289 фекальных образцов от детей в возрасте от 1 мес до 17 лет, госпитализированных с острым гастроэнтеритом. Выявление ротавирусов в образцах проводили методами иммунохроматографии и обратной транскрипции (ОТ) с ПЦР в реальном времени (ОТ-ПЦР-РВ). Секвенирование ротавирусного генома проводили по Сэнгеру и методом нанопорового секвенирования.


Заключение. За последние годы в генетической структуре РВА, циркулировавших на территории московского региона, произошли существенные изменения. Полученные результаты свидетельствуют о необходимости постоянного мониторинга ротавирусной инфекции и выборочного секвенирования генов РВА для уточнения данных типоспецифической ОТ-ПЦР-РВ. Из-за постоянных изменений генетического состава циркулирующих штаммов РВА требуется периодическая оптимизация систем генотипирования РВА на основе ОТ-ПЦР-РВ.

Ключевые слова: ротавирусы группы А, острый гастроэнтерит, G/P-генотипирование, нанопоровое секвенирование

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Introduction

Group A rotaviruses (RVA) are a major cause of acute gastroenteritis hospitalizations of children aged under 5 years in countries characterized by a low rotavirus vaccination coverage. In 2016, rotavirus infection (RVI) was responsible for 258 million episodes of diarrhea and 128,500 deaths among children younger than 5 years worldwide [1].

In 2020, vaccination against RVA was included in the National Immunization Programs in 107 countries1. In Russia, immunization against RVI is included in the National Vaccination Schedule as required for epidemic reasons. Currently, four rotavirus vaccines have been

1 URL: https://preventrotavirus.org/vaccine-introduction/global-introduction-status
recommended by WHO and approved for application: pentavalent vaccines — RotaTeq (Merck & Co., Inc.), ROTASIIL (Serum institute of India Pvt. Ltd.); and monovalent vaccines — Rotarix (GlaxoSmithKline), ROTAVAC (Bharat Biotech). Mass vaccination against RVA decreases incidence and acute gastroenteritis hospitalization rates in all age groups, especially among infants and people over 65 years [2].

RVAs belong to species Rotavirus A, genus Rotavirus, family Reoviridae. The rotavirus genome consists of 11 segments of double-stranded RNA, which encode 12 proteins [3]. The present-day system of rotavirus classification offers genotyping for all 11 genes (Gx–P[x]–I[x]–R[x]–C[x]–M[x]–A[x]–N[x]–T[x]–E[x]–H[x]), most of the circulating human RVAs belong to 3 evolutionary lines different in genome constellations: Wu-like strains (G1–P[8]–I1–R1–C1–M1–A1–N1–T1–E1–H1), DS-I-like (G2–P[4]–I2–R2–C2–M2–A2–N2–T2–E2–H2), and AU-I-like strains (G3–P[3]–I3–R3–C3–M3–A3–N3–T3–E3–H3) [3]. The previously used binary system of typing addressed only VP7 (G) and VP4 (P) proteins. G/[P]-genotyping of RVA strains is performed by an reverse transcription followed by multiplex polymerase chain reaction (RT-PCR) [4] or sequencing of the respective genes.

At present, G1P[8], G2P[4], G3P[8], G4P[8], G12P[8], and G9P[8] genotypes, homotypic or partially heterotypic towards the licensed vaccines (RotaTeq and Rotarix), prevail among circulating RVA strains worldwide [5]. There have been described cases of interspecies transmission of RVA from animals to humans as well as cases when humans were infected with strains resulting from reassortment of animal and human rotavirus strains [6–8].

The evolutionary changes of RVA strains over time [9], territorial differences in distribution of circulating strains, their increased diversity after implementation of immunization in some regions, changes in the genotypic structure both in countries with and without scheduled vaccination require continuous epidemiological monitoring of RVI [10].

The aim of the study is the analysis of genetic characteristics of the RVA strains circulating in Moscow in 2015–2020, including rare strains, which are non-typeable by PCR tests.

### Materials and methods

#### Clinical samples

During 5 years (2015–2020), a total of 289 stool samples were collected from children aged 1 month to 17 years, displaying symptoms of acute gastroenteritis and hospitalized to the Vladimisky Moscow Regional Research Clinical Institute. Children vaccinated against RVI were not included in the study. The informed consent was received from the parents or legal guardians of all tested children.

A total of 45 samples were identified as positive for the RVA antigen using immunochromatography (the RIDA Quick Rotavirus reagent kit, R-Biopharm AG). Stool samples were collected from patients with diarrhea (not later than 72 hours after the onset of symptoms) into sterile containers; before they were shipped to the laboratory, samples were stored at −20°C, then were shipped to the Mechnikov Research Institute for Vaccines and Sera for genetic analysis of rotaviruses.

### RNA extraction

To isolate RNA, we used 10% fecal extracts diluted in sterile saline and cleared through centrifugation (5,000 rpm, 5 min). To isolate RNA from the extracts, we used an AmpliSens® MAGNO-sorb kit (InterLab-Service) in accordance with the manual. The RNA samples were stored at 80°C.

### Detection of rotavirus RNA

To detect RVA in clinical samples by real-time RT-PCR, we used single-tube reaction with primers and probes described earlier [11]. Genotyping RVA using two-stage multiplex real-time PCR for VP7 proteins (G1, G2, G3, G4, G9), VP4 (P[4], P[6], P[8]), and VP6 (I1, I2) was performed in accordance with the procedure requirements [12]. The analysis of RVA genotyping based on multiplex RT-PCR as well as the subsequent analysis of PCR products using agarose gel electrophoresis were used as reference methods and performed in accordance with the WHO recommendations [4].

### Amplification and sequencing of VP7 and VP4 genes

For sequencing of VP7 and VP4 genes of G3P[8] I2 strains, we performed amplification using the previously described VP7F/VP7R [13] and VP4F/VP4R [14] primers generating amplicons 881 and 663 base pairs long, respectively. Aliquots of the extracted RNA (10 μl) were mixed with 3 pmol VP7F or VP4F primers, incubated at 95°C for 1 min and cooled down for 2–3 min to room temperature. The RT test was performed in the 25 μl reaction mixture containing an RT primer, 25 units of MMLV reverse transcriptase (Syntol, Russia). The RT stage included incubation for cDNA synthesis at 45°C for 30 min and inactivation of the MMLV reverse transcriptase at 95°C for 5 min. The temperature and time parameters for the real-time PCR were as follows: 95°C — 120 sec; 95°C — 60 sec, 52°C — 40 sec; 72°C — 40 sec (45 cycles); 72°C — 40 sec. PCR-amplicons of each gene were purified with a Cleanup Standard kit (Eurogen); the sequencing of both DNA strands was performed using a NANO-PHORE®05 genetic analyzer (Syntol) and a reagent kit from the Syntol Company.
Nanopore sequencing of the rotavirus genome and bioinformatic analysis

The full-genome cDNA of rotavirus gene segments was produced using a mix of unRAf1, unRAf2, unRAf3, unRAr1, unRAr2, and unRAr3 primers (Table 1) for the RT test. For concurrent amplification of all segments of the RVA gene, we used universal primer Up [15] (Table 1). The amplification was performed in the following reaction mixture: TaKaRa LA Taq polymerase (2.5 units), LA PCR Buffer II (Mg²⁺ plus), 25 mM MgCl₂, (final concentration Mg²⁺ 5 mM) (TaKaRa), dNTP 25 mM (Syntol), 40 pmol Up primer. The amplification parameters: 95°C — 2 min, then 40 cycles at 95°C, each for 30 sec, 65°C — 30 sec, 68°C — 3.5 min. PCR amplicons were purified using phenol/chloroform extraction. The concentration and purity of amplicons were measured by spectrophotometry (A260/230, 260/280).

The DNA library for nanopore sequencing (NPS) was constructed using a Rapid Sequencing reagent kit with a portable MinION sequencer and standard flowcell R9 (Oxford Nanopore Technologies). We developed a pipeline for accurate classification of NPS data. The Python programming language-based pipeline identified the received reads, actuating the BLAST tool-based analysis for the database of reference sequences of rotavirus genome segments².

Then, the reads were mapped to the reference sequence using the Minimap2 program [16]; the consensus sequence was created using the script³ written in the Python language. In the consensus sequence, we selected a nucleotide with the highest frequency at the alignment position. In rare cases, when two nucleotides were found at the same position in the equal quantity, the second nucleotide was ignored. The RVA genotype was identified by the nucleotide sequence of gene segments using the Rotavirus A Genotype Determination online service⁴ based on the software from Dan Katznel [17].

The method of nanopore sequencing of the rotavirus genome is described in detail in the article by Faizuloev et al. [18].

GenBank accession numbers (NCBI)

The nucleotide sequences corresponding to 10 segments of the genome of Moscow-40/2020 (G3P[8]I2) and Moscow-1P/2015 (G4P[6]I1) isolates have been deposited to GenBank under numbers MW558493–MW558502 and MT876633–MT876642, respectively. The partial sequences of three other strains with the G3P[8]I2 genotype have been deposited under numbers MT648671, MT648671, MT648671 for VP7 and MT814324, MT814326 for VP4.

### Phylogenetic analysis

Phylogenetic trees were built using the MEGA-X program [19] and the maximum likelihood method (the Kimura two-parameter model [20]). The bootstrap test included 1,000 replications. The RVA strains recommended by the Rotavirus Classification Working Group [3] and the strains having, based on the BLAST data, at least 99.5% similarity to the studied strains were used as reference strains.

### Results

A total of 289 stool samples were tested for presence of the RNA or RVA antigen; all of them were collected from children hospitalized with symptoms of acute gastroenteritis. RNA was detected in 131 (45%) samples and was further used for G/[P]-genotyping by real-time RT-PCR and/or sequencing. The RVA G/[P]-genotype was detected in 125 samples (95.4%), and in 7 (5.3%) samples. RVA was defined only by the P gene, while no genotype was identified for 6 (4.6%) samples.

The genetic composition of the studied RVA strains is presented in Fig. 1. In 2015–2020, RVA strains with the G9P[8]I1 genotype prevailed in the overall profile (37%), the second place was taken by G3P[8]I2 (a new DS-1-like strain) - 18%, which was followed by G4P[8]I1 (15%), G2P[4]I2 (11%), G1P[8]I1 (5%), and G3P[8]I1 (2%). Seven (5%) strains defined only by the P gene belonged to the P gene variant [8]. We have also found a single case of co-infection with two genotypes (G9P[8]I1 and G2P[4]I2) and a rare strain with the G4P[6]I1 genotype.

Fig. 2 shows the year-to-year distribution of the RVA genotypes identified in the Moscow Region. In the last years, the proportion of the G9P[8]I1 genotype has increased significantly, ranging from 36% to 41% during 2015-2020. At the same time, the prevalence of G4P[8]I1 genotypes decreased from 38% to 9%, while the prevalence of the G2P[4]I2 genotype increased to 14%.

### Table 1. Primers for amplification of full length rotavirus gene segments

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer sequence 5′–3′</th>
</tr>
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<tbody>
<tr>
<td>unRAf1</td>
<td>GCCGGAGCTCGCAGAAATTCGCTGWWAA</td>
</tr>
<tr>
<td>unRAf2</td>
<td>GCCGGAGCTCGCAGAAATTCGCGGTTTTTTTTT</td>
</tr>
<tr>
<td>unRAf3</td>
<td>GCCGGAGCTCGCAGAAATTCGCGGTTTTTTTTTAAT</td>
</tr>
<tr>
<td>unRAr1</td>
<td>GCCGGAGCTCGCAGAAATTCGCGGCTGACATC</td>
</tr>
<tr>
<td>unRAr2</td>
<td>GCCGGAGCTCGCAGAAATTCGCGGCTGACAWA</td>
</tr>
<tr>
<td>unRAr3</td>
<td>GCCGGAGCTCGCAGAAATTCGCGGCTGACATC</td>
</tr>
<tr>
<td>Up</td>
<td>GCCGGAGCTCGCAGAAATTCGCGGCTGACATC</td>
</tr>
</tbody>
</table>

² URL: https://github.com/lioj/bioinformatics/blob/master/py/classificationStat.py
³ URL: https://github.com/lioj/bioinformatics/blob/master/py/bam2consensus.py
⁴ URL: https://www.viprbrc.org/brc/rvaGenotyper.spg?method=S howCleanInputPage&decorator=reo
Note that the partial sequencing of VP7 (G) and VP4 (P) genes was required for G/P-genotyping of some strains with the GxP[8]I2 genotype, as the real-time RT-PCR-based laboratory assay was not able to identify their G/P-genotype [12]. The sequencing and phylogenetic analysis of RVA G and P genes (the GenBank numbers: MT648671, MT648671, MT648671 for the G gene and MT814324, MT814326 for the P gene) in the sample with detected GxP[8]I2 showed that they belong to the G3P[8]I2 genotype (Fig. 3). It was found that the absence of a signal during real-time RT-PCR genotyping was caused by the mismatch of nucleotides between the VP7 gene with the G3P[8]I2 genotype and the respective probe. The non-typeable samples with the GxP[8]I2 genotype were tested with mono-specific PCR with primers to G3 and electrophoretic detection, which identified amplicons with the expected mobility in all samples (the data are not provided), thus confirming that they belonged to the G3P[8]I2 genotype.

The BLAST search and the phylogenetic analysis demonstrated the similarity of the sequenced VP7 and VP4 genes to the same RVA genes of the new G3-DS-1-like constellation detected worldwide. The similar G3-DS-1-like strains were detected in Australia (2013) [21], Spain (2014–2015) [22], Hungary (2016) [23], Brazil (2016) [24], Indonesia (2016) [25], Russia (2019) [28], and other countries (Fig. 3).

In addition, the nanopore sequencing of the Moscow-40/2020 isolate with the G3P[8]I2 genotype was performed (Table 2). It helped identify gene variants of 10 genome segments (the GenBank numbers MW558493–MW558502) and demonstrated a high degree (91.0–99.8%) of similarity between the consensus sequence and the reference strain RVA/Human-wt/THA/SKT-281/2013/G3P[8] (the GenBank numbers LC086714–LC086724). The rare Moscow-1P/2015 strain with the G4P[6]I1 genotype was detected in the clinical sample collected from an 8-year-old patient in 2015. Real-time PCR was able to identify only the P[6] gene variant. Sanger sequencing and NPS helped identify the genotype of this strain by 10 genes: G4-P6-I1-R1-C1-M1-A1-N1-T1-Ex-H1 (the GenBank numbers...
Fig. 3. Phylogenetic trees based on the sequenced genes of the VP4 and VP7 proteins of the new G3P[8]I2 strain.

▲ — segments sequenced using the Sanger technique; ♦ — the gene sequenced using the NPS technique; ∆ — the equine RVA VP7 gene.

The respective GenBank accession number, name of strains and G/P-genotype were used for designation of strains.
The obtained results are indicative of significant changes, which took place in the "genetic landscape" of RVA strains circulating in the Moscow Region. Based on the data from the previous studies [12, 26], before 2015, most of the genotyped RVA belonged to genotype G4P[8]. Our data indicate a gradual decrease in the prevalence of this genotype, from 38% to 9% in 2017–2018. At the same time, the proportion of the G9P[8] genotype increased to 36–41% in the Moscow Region. These data are consistent with the data obtained during the independent studies that were performed in Moscow [27, 28], Nizhny Novgorod [29, 30], and Orenburg [31].

Our study was focused on clinical samples collected from children hospitalized with acute gastroenteritis. The actual "genetic landscape" and the distribution of RVA genotypes circulating in Moscow can differ from the obtained data, since we did not include patients with mild or moderate gastroenteritis, not requiring hospitalization, in our study. Previously, in the study by E.R. Meskina, it was pointed out that severe rotavirus gastroenteritis could be associated with specific RVA genotypes [32].

The strain with genotype G3P[8]I2, which was discovered by us in 2018, is of special interest. It accounted for 81.7% of the genome (Table 2).

The phylogenetic analysis of genes VP7, VP6, and VP4 strain Moscow-1P/2015 (Fig. 4) demonstrates a high degree of similarity of the analyzed sample to genes of porcine RVA (the GenBank numbers: VP4 — KX363402, MK227950, KX363435, MK227948; VP6 — MK227391, MK227402, KX363414, MG066585, KJ126830; VP7 — JX498957, JX498956, MK227392, MN133419, MN133444) or RVA strains (strain RVA/Human-wt/CHN/R1954/2013/G4P[6] having the GenBank numbers: KF726066–KF726076, KF726056) and isolated from human feces, though having the confirmed origin from porcine RVA [7]. The BLAST analysis of the other 7 genes also demonstrates a high degree of similarity (92–98%) of the nucleotide sequence to porcine RVA strains. Thus, the phylogenetic analysis indicates that the G4P[6] strain is of porcine RVA origin.

### Table 2. Results of nanopore sequencing of the genome of RVA strains with G3P[8]I2 and G4P[6]I1 genotypes

<table>
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<tbody>
<tr>
<td></td>
<td>number of reads</td>
<td>segment coverage, %</td>
<td>genotype</td>
</tr>
<tr>
<td>1</td>
<td>VP1</td>
<td>1,372</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>VP2</td>
<td>3,795</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>VP3</td>
<td>1,995</td>
<td>100</td>
</tr>
<tr>
<td>4</td>
<td>VP4</td>
<td>633</td>
<td>100</td>
</tr>
<tr>
<td>5</td>
<td>NSP1</td>
<td>546</td>
<td>100</td>
</tr>
<tr>
<td>6</td>
<td>VP6</td>
<td>15,187</td>
<td>100</td>
</tr>
<tr>
<td>7</td>
<td>NSP3</td>
<td>14,566</td>
<td>100</td>
</tr>
<tr>
<td>8</td>
<td>NSP2</td>
<td>175</td>
<td>100</td>
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<tr>
<td>9</td>
<td>VP7</td>
<td>1,465</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>NSP4</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>11</td>
<td>NSP5/6</td>
<td>77</td>
<td>100</td>
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</table>

MT876633–MT876642, MG271938), accounting for 81.7% of the genome (Table 2).

The obtained results are indicative of significant changes, which took place in the "genetic landscape" of RVAAs circulating in the Moscow Region. Based on the data from the previous studies [12, 26], before 2015, most of the genotyped RVA belonged to genotype G4P[8]. Our data indicate a gradual decrease in the prevalence of this genotype, from 38% to 9% in 2017–2018. At the same time, the proportion of the G9P[8] genotype increased to 36–41% in the Moscow Region. These data are consistent with the data obtained during the independent studies that were performed in Moscow [27, 28], Nizhny Novgorod [29, 30], and Orenburg [31].

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Another atypical strain — Moscow-1P/2015 with the G4P[6]I1 genotype, which was detected only in one case, may have also resulted from the reassortment of the human and animal RVA or may have the animal origin. In this particular case, it is not clear whether the infection with this strain has a human-to-human transmission route, or the patient was infected with the virus from animals. Based on the literature data, the proportion of RVA with the G4P[6]I1 genotype is not high in the total diversity of RVA strains detected in humans [??????]. It may be caused by some factor limiting the G4P[6]I1 spread in the human population, for example, a species barrier, if we assume that this strain is of animal origin. On the other hand, it should be remembered that most of the related studies tend to focus on children hospitalized with rotavirus enteritis, while mild cases are generally left out of studies. Therefore, the available data do not give any reliable information about the actual prevalence of any of the RVA strains.

Porcine RVA strains are phylogenetically related to strains of human rotaviruses [34]; therefore, it is difficult to find out whether the studied strain resulted from...
a direct transmission or reassortment [37–39]. Cases of reassortment or direct interspecies transmission of RVA with the G4P[6]I2 genotype have been reported and described both in Asia [6, 25, 40] and in Europe [36].

**Conclusion**

In 2015–2020, the genetic profile of RVA circulating in the Moscow Region changed significantly: The detection frequency of the G4P[8]I1 genotype, which was the most prevalent in the previous years, decreased; at the same time, the number of hospitalizations with RVA caused by the G9P[8]I1 genotype have been reported and described both in Asia [6, 25, 40] and in Europe [36].

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