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Original Article

Virological and genetic characteristics of human respiratory syncytial viruses isolated in Russia, 2017-2018

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Abstract

Isolation of human respiratory syncytial virus (HRSV) from clinical samples and storage of isolates for long period remains a considerable problem. We describe in detail the optimized conditions of HRSV isolation and cultivation in three cell cultures HeLa, HEp-2, and Vero. HRSV was detected in 35.2% (166/471) specimens by real-time PCR from symptomatic infants and children up to 15 years from October 2017 to March 2018 in Russia. HRSV-positive samples were used for virus isolation in HeLa, HEp-2, and Vero cells in different manners (in monolayer or suspension). To optimize the conditions of HRSV cultivation, these cell cultures were treated or not with receptor-destroying enzyme (RDE). Ten isolates were successfully obtained by the way of infection of the suspension of cells with subsequent RDE treatment. Among them, several isolates induced the cytopathogenic effect (CPE) by the syncytium formation in both Hela and HEp-2 cell cultures. The genetic analysis revealed that the manners of isolation by using monolayer or suspension and subsequent RDE treatment did not influence the nucleotide and amino acid structures of obtained HRSVs. The CPE characteristics of obtained viruses were the same in HeLa, HEp-2, and Vero cell cultures, and were described as large syncytium up to 150 microns or more in size with the nuclei peripheral location and an optically bright zone in the center of the formation. We showed that infection of cell suspension with the subsequent RDE treatment increased the chance of HRSVs isolation from clinical samples.

Key words: Human respiratory syncytial virus infection; cultivation; isolation; suspension; monolayer; sequences.

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Introduction

Human respiratory syncytial virus (HRSV) is one of the main etiological agents that cause acute infections of the human respiratory system. Acute respiratory infection (ARI) is dangerous for infants and young children and is the most frequent reason for death among children under one-year-old [1]. It is believed that more than 95% of children are infected with HRSV by the age of 2 years [2]. Human RSV can cause severe lesions of the lower respiratory tract with bronchial obstruction, bronchiolitis, and increases the risk of developing asthma [3,4].

Human RSV has two main proteins embedded in the bilipid membrane of the virion - fusion protein (F) and attachment protein (G) [5]. Protein F may mediate the fusion of viral particles with the host cell's surface, as well as the apoptosis and hemifusion of infected cells with each other that causes the appearance of syncytia. An indicator of syncytium formation is the cytopathic effect (CPE) [6,7].

To date, the treatment of HRSV disease is mostly symptomatic, although HRSV has been studied for more than 40 years. Vaccine against HRSV is being development, but at the moment is not widely used [8] and therefore requires further studies of the biological properties of HRSVs and their pathogenic potential. One of the most effective directions is the creation of vaccines based on attenuated wild-type HRSVs. However, one of the main problems of HRSVs isolation is the complexity of cultivation in different cell cultures. Also, is known that HRSVs may degrade rapidly in laboratory conditions and are unstable during storage [1,9-11]. Many actual virological articles emphasize the difficulty of HRSVs cultivation [12-14],

but over the past 5-10 years there have been no works devoted to a detailed description of the isolation and cultivation methods of currently circulated HRSVs. In many morphological works, authors used stock of Long strain HRSV (ATCC VR-26; RSV/A Long) and A2 (ATCC CCL-23), which were isolated in 1956 and 1960s, respectively [15-17]. Using such historical viruses confirms our hypothesis about the difficulty in isolation and cultivating current circulated HRSVs.

The purpose of this study was the necessity of optimization conditions of HRSV isolation and cultivation that will help to isolate genetically relevant HRSVs.

Methodology

Ethics issues

All aspects of the study were approved by the Ethics Committee of the Federal State Budgetary Institution "Research Center of Clinical and Experimental Medicine" protocol No. 2013-23 and by the Ethics Committee of the "Federal Research Center of Fundamental and Translational Medicine" protocol No. 2021-10. Written informed consent was obtained from all parents/legal guardians prior to sample taking.

Sample Collection

Nasal and throat swabs were collected from 471 infants and children up to 15 years suffering from ARIs and were hospitalized in Novosibirsk Children's Municipal Clinical Hospital between October 2017 and March 2018 with symptoms of ARIs, such as fever, sore throat, cough, sputum, shortness of breath, lung auscultation abnormalities, tachypnea, and chest pain. Nasal and throat swabs were placed in tubes with transport medium consisting of Dulbecco's modified Eagle's medium (Capricorn Scientific, Germany) containing 0.5% bovine serum albumin, 100 μg/mL of gentamicin sulfate (BioloT, Russia), and 50 units/mL of amphotericin B (BioloT, Russia). Specimens were stored at 4 °C not more than 48 hours before sending to the laboratory and then were frozen at -70 °C and stored until virus isolation that were occurred usually within 14 days.

Virus detection

All samples were tested for HRSV and other respiratory viruses such as influenza A and B viruses, parainfluenza virus types 1–4, metapneumovirus, four coronaviruses (NL63, 229E, OC43, and HKU1), rhinovirus, adenovirus, and bocavirus by real-time polymerase chain reaction (PCR) using «AmpliSens ARVI-screen-FL» and «AmpliSens Influenza virus A/B-FL» RT-PCR Kits (Interlabservice, Russia) according to the manufacturer's instructions [18].

Cells

HeLa, HEp-2, Vero (cell cultures of collection of State Research Center of Virology and Biotechnology «VECTOR», Russia) were maintained in Dulbecco's modified Eagle's medium (Capricorn Scientific, Germany), 10% fetal bovine serum (FBS) (Capricorn Scientific, Germany), and 50 μg/mL of gentamicin sulfate (BioloT, Russia). All cells were incubated at 37 °C, 5% CO_2.

Virus isolation and cultivation

The samples positive for HRSV in real-time PCR were used for virus isolation on HeLa, HEp-2, and Vero cells. 96-well plates with daily HeLa, HEp-2, and Vero cells were inoculated with 30 μL aliquots of the clinical samples. Intact HeLa, HEp-2, and Vero cells were used as controls in each plate. Human RSV infection of the cell culture plates was evaluated and confirmed under the microscope (Micromed I, Russia) by the presence of a CPE by the syncytium formation on day 4-5 post-infection with further confirmation by real-time PCR using the AmpliSens ARVI-screen kit (Interlabservice, Russia).

Infection of a cell monolayer

The 96-well plates were seeded with 3 × 104 cells per well in a growth medium. In 24 hours of the incubation cell monolayer was pre-washed with a Hanks solution; then the 30 μL aliquots of clinical samples were inoculated into cells, and plates were incubated at 37°C and 5% CO_2 for 1 hour for virus adsorption. Upon removing the supernatant, a maintenance medium consisting of DMEM (Capricorn Scientific, Germany) with 2% of FBS (Capricorn Scientific, Germany) and 100 μg/mL of gentamicin sulfate (BioloT, Russia) was added into the wells.

Infection of cell suspension

Sample aliquots and a cell suspension in a growth medium (DMEM, with 10% FBS (Capricorn Scientific, Germany) and 50 μg/mL of gentamicin sulfate (BioloT, Russia) were added to a 96-well plate. The plates were incubated at 37 °C and 5% CO_2 for 24 hours, then the growth medium was removed and a supportive medium (DMEM Capricorn Scientific (Germany), with 2% FBS (Capricorn Scientific, Germany) and 100 μg/mL of gentamicin sulfate (BioloT, Russia) was added.

RDE treatment

Receptor-destroying enzyme (RDE) was used for cell treatment if needed (Receptor Destroying Enzyme Kit Cosmos Biomedical Ltd, United Kingdom; which is the enzyme neuraminidase of vibrio cholerae). Firstly, the culture medium was removed from the plates, then RDE solution was added, and incubated at 37 ℃ and 5% CO_2 for 1 hour, after which the RDE solution was replaced by maintenance medium as described above. The concentration of RDE solution was 1:4 of the initial concentration, and it was non-toxic for cells, and did not cause visible changes in the monolayer.

Titration of HRSV isolates

For virus titration, 10-fold dilutions of the isolates were made in Hanks' solution. One hundred microliters of each dilution were added to 96-well plates (8 wells per dilution) and 3×10^4 cells/well of HeLa and HEp-2 suspension in growth medium and were repeated two times. Plates were incubated at 5% CO_2 37 °C for 24 hours. Then one plate of the duplicate was treated with RDE solution as described above.

Supernatant was replaced by 200 µL/well of maintenance medium (as described above) in both plates and cells were incubated at 5% CO_2 and 37 °C and observed daily for CPE for 7 days.

Virus titers were calculated by the Kerber method with Ashmarin–Vorobyov modification [19], as follows: $\log_{10}TCID_{50}/mL = lgDn - \delta(\Sigma Li - 0.5) + 1$. Calculations were made in Excel for Mac.

Light Microscopy and Immunocytochemical analysis

Daily, HeLa, HEp-2, and Vero cells were checked for syncytium formation (on 3rd - 5th day post-infection) and then were fixed with a 4% formalin solution for 10 minutes, then cells were stained with azur and eosin for 30 seconds (Minimed, Russia). The evaluation of CPE was estimated under an inverted AxioVert 40 microscope (Carl Zeiss, Germany) with AxioCam ICc 3 camera (Carl Zeiss, Germany), Bright Field mode. Intact HeLa, HEp-2, and Vero cells were used as controls.

The localization of HRSV in the infected HeLa, HEp-2, and Vero cells were revealed by immunocytochemical method [20]: cells on the coverslips were fixed with a 4% formalin solution for 10 minutes and were treated by diagnostic kit with fluorescent antibodies to HRSV (LLC "Enterprise for the production of diagnostic drugs", St. Petersburg, Russia). The presence of HRSV was identified by green immunofluorescent signal in the cells. Light microscopy and photography were carried out using an AxioImager Z1 microscope (Carl Zeiss, Germany) under direct light, or differential interference contrast (DIC).

An immunofluorescence (IF) assay of the HRSV localization in the infected HeLa, HEp-2, and Vero cells were done additionally. Upon pre-staining cells with fluorescent antibodies to HRSV (as mentioned above) the cell nuclei were contrasted with 5µM 4,6-diamino-2phenyl indole (DAPI), briefly washed with deionized water, and mounted in the Prolong Antifade/Slow Fade mounting medium. The presence of HRSV was identified by green immunofluorescent signal in the cells. Cells were imaged using an LSM710/NLO confocal microscope (Carl Zeiss, Germany), and images were processed with Adobe Photoshop CS6 software.

Sequencing

RNA was isolated by using a GeneJET viral DNA/RNA purification kit (Thermo Fisher Scientific, Waltham, MA, USA) and treated with TURBO DNase (Thermo Fisher Scientific, Waltham, MA, USA). Up to 200 ng of RNA was used for the DNA libraries, which were prepared using the NEBNext Ultra RNA Library Prep kit (New England Biolabs, Ipswich, MA, USA). Sequencing of the DNA libraries was conducted with a Reagent kit, Version 3 (600-cycle), on a MiSeq genome sequencer (Illumina, San Diego, CA, USA) in SB RAS Genomics Core Facility (ICBFM SB RAS, Novosibirsk, Russia). Full-length genomes were assembled de novo with CLC Genomics Workbench version 9.5.3 (Qiagen, Hilden, Germany).

The genome sequences of HRSV were deposited in GISAID EpiRSV. Accession ID: HRSV/B/Russia/Novosibirsk-50Hl/2018 (EPI_ISL_2575499); HRSV/B/Russia/Novosibirsk-50Hp/2018 (EPI_ISL_2575500); HRSV/B/Russia/Novosibirsk-66Hl/2018 (EPI_ISL_2575546); HRSV/B/Russia/Novosibirsk-66Hp/2018 (EPI_ISL_2575547).

Sequence analysis

Human RSV sequences being investigated were combined with sequences retrieved from the GenBank database. For multiple alignments, a MUSCLE program was used. Comparative pairwise sequence alignment of 2 investigated viruses was performed via BioEdit. Phylogenetic trees were built via MEGA 5 using Maximum Likelihood, utilizing the general time reversible (GTR) nucleotide substitution model. Bootstrap support values were generated using 500 rapid bootstrap replicates.

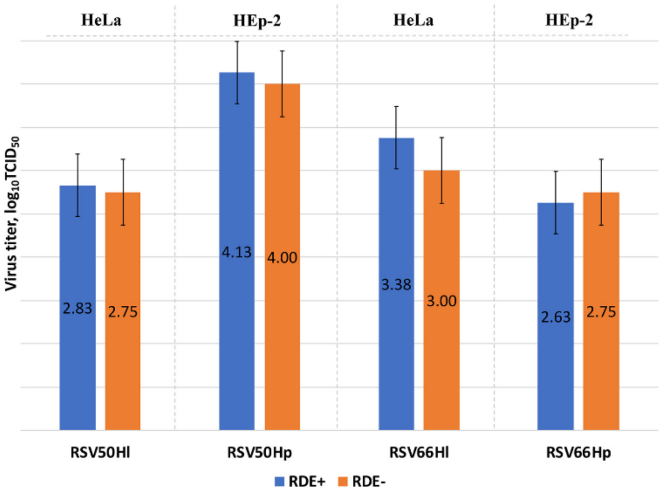
Results

Isolation and cultivation of HRSV

As part of the annual monitoring of ARIs we analyzed 471 clinical samples from hospitalized patients for the presence of genetic material of the respiratory viruses by using real-time PCR. Positive for HRSV was 35.2% (166/471) samples. All HRSV-positive samples were used for virus isolation on the three cell lines: HeLa, Hep 2, and Vero. At the same time, we tried to isolate the viruses on each cell culture in four different manners: on the cells monolayer or in the suspension of cells, and with or without RDE treatment (Table 1). Because it was previously shown that neuraminidase treatment increased syncytium formation in cell cultures infected with HRSV [21], we applied the RDE 24 hours after infection. Then, we performed the assessment of CPE under a microscope. In the case of CPE presence, the PCR analysis for confirmation of HRSV infection was done. Samples positive for HRSV that were confirmed by both methods with the PCR and the CPE on one or more cell cultures (Table 1) were taken for further passaging in cell cultures to optimize the conditions for HRSV isolation.

Totally, 4 samples were successfully cultured in the Hela cell culture and 6 samples in the HEp-2 cell culture. Some isolates were selected in both Hela and HEp-2 cultures, some – only in one. It is noteworthy, that two samples - 'N55 and 'N60' - were isolated in both mentioned cultures, but only in case of suspension infection with subsequent RDE treatment. Because these two samples were equally cultivated on both HeLa and HEp-2 cell cultures they were selected for further study and were named as HRSV/B/Novosibirsk/50Hl/2018 (RSV50Hl), HRSV/B/Novosibirsk/50Hp/2018 (RSV50Hp), HRSV/B/Novosibirsk/66Hl/2018 (RSV66Hl), and HRSV/B/Novosibirsk/66Hp/2018 (RSV66Hp). Viral titers of these viruses did not differ significantly from each other during cultivation on the HeLa and HEp-2 cell cultures (Figure 1). In addition, we noted that RDE treatment also did not influence to increasing of viral propagation in the cell culture of previously isolated HRSVs.

Figure 1. Virus titers of HRSV-isolates in HeLa and HEp-2 cells, log10TCID50/mL.



It is interesting to note, that we did not observe the CPE in Vero cells after inoculation of clinical samples under the same conditions of infection and it was not possible to isolate any virus from this cell culture. However, infection of Vero cells by HRSVs isolated in HeLa and HEp-2 cells led to syncytium formation in Vero cells.

Four viruses (RSV50Hl, RSV50Hp, RSV66Hp, and RSV66Hl) further were cultivated in the three cell cultures with subsequent treatment by RDE, or without it. The 10-fold dilutions of viruses were made in Hanks' solution for titration. Suspension of HeLa, HEp-2, and Vero cells were infected with 100 $TCID_{50}$ of the RSV50Hl, RSV50Hp, RSV66Hl, RSV66Hp and incubated at 5% CO_2 37 °C.

Whole genome analysis of isolates

For the phylogenetic analysis of Novosibirsk viruses (RSV50Hl, RSV50Hp, RSV66Hp, and RSV66Hl) the dendrograms were constructed based on

Table 1. Total number of HRSVs isolated in cell cultures.

Cell culture	**HeLa**				**HEp-2**				**Vero**			
Condition	suspension		monolayer		suspension		monolayer		suspension		monolayer	
Treated /untreated	RDE[1]		RDE[1]		RDE[1]		RDE[1]		RDE[1]		RDE[1]	
	+	-	+	-	+	-	+	-	+	-	+	-
Samples successfully cultivated	N17 N50 N66 N79	0	0	0	N32 N45 N50 N66 N74 N80	0	0	0	0	0	0	0

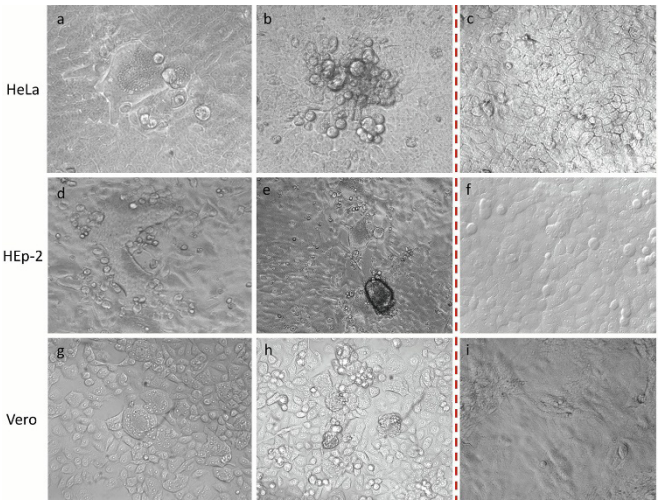
[1]: laboratory strain number; +: treated with receptor destroying enzyme (RDE); -: untreated with RDE.

genome-wide sequences of their F and G genes compared to closely related viruses based on the results of BLAST analysis. Multiple alignments and phylogenetic analysis of 503 genome-wide sequences of HRSV deposited worldwide were carried out to determine the phylogenetic relationships of the Novosibirsk strains.

The gene encoding the G-protein of the HRSV is the main marker of belonging to a certain genetic group. According to the analysis of amino acid sequences, G proteins of all studied HRSVs contain duplications of fragments 239-258 and 259-279 [22]. According to the phylogenetic analysis of the G-gene that is available in the database, Novosibirsk's viruses significantly differ from St. Petersburg`s viruses. The St. Petersburg and Novosibirsk viruses form two separate phylogenetic clusters of HRSVs corresponding to subtypes A and B, accordingly (Figure 2). At the same time, the viruses from Novosibirsk are phylogenetically related to the European and South American variants of the HRSV by the nucleotide sequence of the G-gene.

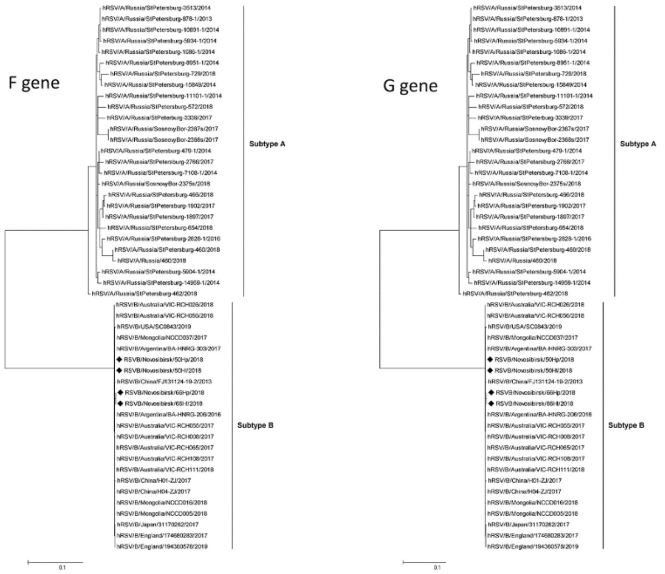
The F-protein is a surface glycoprotein of HRSV. Genetic differences between the F-genes of HRSVs cause features in their biological properties [23]. Phylogenetic analysis of the F-gene confirmed the belonging of Novosibirsk viruses to subtype B and St. Petersburg viruses to subtype A (Figure 2). Also, as in the case of the G-gene, the F-gene of the Novosibirsk

Figure 3. Transformations of infected with HRSV HELA, HEp-2 and Vero cell cultures.



a,d,g: on day 4-5 post infection with HRSV were seen syncytium formations in HeLa, HEp-2, and Vero, respectively; **b,e,h**: on day 6-7 post infection with HRSV were seen sequential fragmentation of syncytia with the formation of apoptotic cells in HeLa, HEp-2, and Vero, respectively; **c,f,i**: control cells of HeLa, HEp-2, and Vero cultures, respectively. Unstained. Bar 50 μm.

Figure 2. Phylogenetic dendrogram based on the nucleotide sequences of the F- and G-genes of the Russian and closely related strains worldwide respiratory syncytial viruses.



HRSVs is genetically similar to the F-genes of viruses from Europe and South America.

According to the multiple alignments and phylogenetic analysis, the Novosibirsk viruses (RSV50Hl, RSV50Hp, RSV66Hp, and RSV66Hl) belong to the genetic group BA9 subtype B and are related to viruses detected worldwide: in Australia, China, Argentina, UK, and in Japan (Supplementary Figure 1).

Sequences of genome-wide nucleotide and single genes of RSV50Hl, RSV50Hp, RSV66Hp, and RSV66Hl viruses revealed that these variants remain identical in relation to their own primary isolates. Thus, isolation on the different cell cultures did not affect changes in the nucleotide and amino acid sequences of HRSVs.

Morphological study of HeLa, HEp-2, and Vero cultures cell infected with HRSV

Three cell cultures HeLa, HEp-2, and Vero were infected with the viruses RSV50Hl, RSV50Hp, RSV66Hl, and RSV66Hp, and next were daily evaluated for the presence of CPE. On the 4th or 5th day of CPE detection, the cells were fixed and stained for further analysis under light or confocal microscopes. Because the observed cytopathic effects of these viruses were the same, we selected one virus RSV66Hl to demonstrate the morphological changes in infected HELA, HEp-2, and Vero cell cultures.

The HRSV was identified by the syncytium formation. The first signs of the morphological

transformations in infected cells were registered on days 4-5 post-infection. The cytopathic changes were identical in all studied cell cultures: formation of the large syncytium up to 150 microns or more with a nuclei peripheral location and an optically bright zone in the center of syncytium (Figure 3,4). In the following 1-2 days, sequential fragmentation of syncytia with the formation of apoptotic cells and destruction of multinucleation in HeLa, HEp-2, and Vero cell cultures were observed (Figure 3 B, E, H).

Identification of CPE correlated well with IF results. All studied cell cultures were IF positive (Figure 5, Supplementary Figure 2), which in turn confirms the obtained virological and molecular biological data on the cultivation of HRSVs in these cell cultures. In infected HeLa, HEp-2, and Vero cell cultures with RSV66Hl virus were observed specific diffuse or granular fluorescence of the syncytium central zone, which indicates the localization of HRSVs in this part of the formation, and the nuclei of infected cells were located along the periphery.

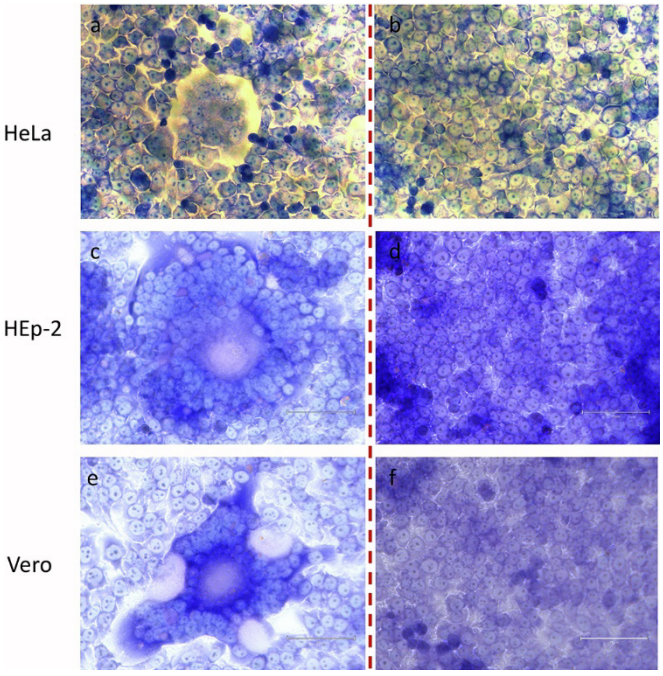
Discussion

Human RSV is an epidemiologically relevant pathogen that cause hospitalization of children worldwide, and may to lead a death of infants less than 1 year of age [1]. In our previous research were shown that HRSVs were the most frequently detected agents among other acute respiratory viruses (influenza A and B viruses, parainfluenza virus types 1–4, metapneumovirus, four coronaviruses (NL63, 229E, OC43, and HKU1), rhinovirus, adenovirus, and bocavirus) that affected children in the Novosibirsk, Russia, during 2013 – 2017 period [24]. In the current work, we detected 35.2% (166/471) HRSV-positive samples during the 2017 – 2018 period that showed the continuing predominance of HRSV among children virus-infection incidence.

It is known that various cell cultures are used for the cultivation of HRSV: HeLa, HEp-2, Vero, RhMK, MRC, WI-38, and A549 [13,14,25] among which may widely differ in the sensitivity in detection. Some authors used a combination of two or more cell cultures to enhance the isolation of HRSV [13]. Difficulties in isolating and cultivating HRSVs also arise due to high viral thermolability. The ability to infect may be lost from 50% up to 100% as a result of slow freezing at –20 ºC and then thawing, or only one freeze-thaw cycle. Storage for a long time (more than 12 months) is also can cause a loss of virulence [14].

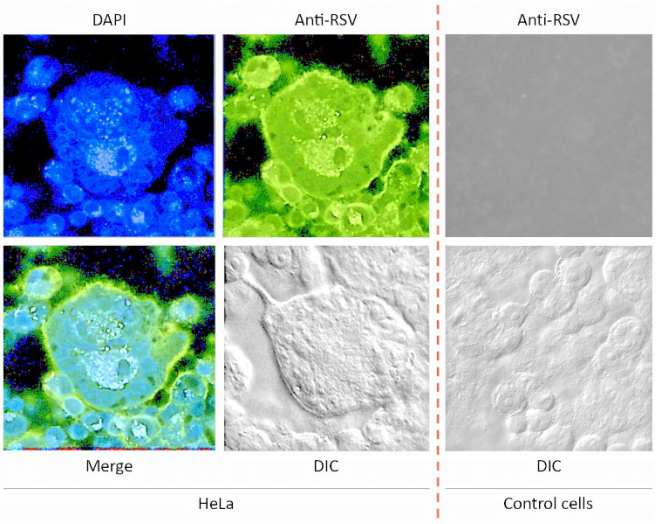
Isolation of HRSVs in cell cultures is a very difficult process. All known morphological works were done by use of historical HRSVs such as Long strain or

Figure 4. Syncytium formations by HRSV on HeLa, HEp-2, and Vero cell cultures.



a,c,e: infected with HRSV HeLa, HEp-2, and Vero cell cultures, respectively; **b,d,f**: control cells of HeLa, HEp-2, and Vero cultures, respectively. Azur and eosin staining. Bar 75 µm.

Figure 5. Immunofluorescent analysis of Hela cell cultures infected with HRSV.



Hela cells were treated by diagnostic kit with fluorescent antibodies to HRSV and the cell nuclei were contrasted with 5uM 4,6-diamino-2phenyl indole (DAPI); photography was carried out using a LSM710/NLO confocal microscope and AxioImager Z1 microscope (Carl Zeiss, Germany) under differential interference contrast (DIC). Bar 20 µm.

A2 [15,26,17]. The purpose of this study was to search for optimized conditions for HRSVs isolation and cultivation that may help to isolate new genetically relevant variants. In this work, we used tree cell cultures (HeLa, HEp-2, and Vero) that were infected in four different manners: on the cell monolayer or in the suspension of cell with or without subsequent RDE treatment. Only 10 isolates from 166 clinical samples were successfully obtained in case of infection of the HeLa and HEp-2 suspension of cells with subsequent RDE treatment. Thus, our study shows the same level of replication of HRSVs in both HEP-2 and HeLa cell cultures. This data allows us to recommend both cultures for virus cultivation not only HEP-2 which is consistent with the research of Rajan *et al.* [27], but also HeLa.

Among ten isolates 4 samples were produced in HeLa cell culture and 6 samples were produced in Hep-2 cell culture. At the same time, we got no results of virus production in the Vero cell culture. It is known that RDE treatment removes non-specific inhibitors from cells, and also enhances the subsequent binding stage, such as fusion [21]. We assumed that RDE treatment can contribute to the successful cultivation of HRSVs. Our result showed that it is true in the case of viral isolation in the suspension of HeLa and HEp-2 cell cultures, but at the same time, RDE treatment didn't significantly increase the virus production of being isolated HRSVs in the same cell cultures. Interestingly, RDE did not help to isolate any viruses in the Vero cell culture even in case of infection of the cell suspension. Thus, the treatment of RDE does not always determine the success of cultivation.

It is noteworthy, that two clinical samples the 'N55' and 'N60' were equally cultivated in the suspension of Hela and HEp-2 cell cultures with RDE treatment, and those were selected for further study. The genetic analysis of obtained viruses (RSV50Hl, RSV50Hp, RSV66Hp, and RSV66Hl) was carried out based on sequences of genome-wide nucleotide and single genes. It was shown that variants of the same viruses when isolated in two different cell cultures remain identical – the nucleotide and amino acid sequences did not change in depending on the isolation system. Four obtained viruses - RSV50Hl, RSV50Hp, RSV66Hl, and RSV66Hp - were effectively cultivated not only in the cell culture on which they were firstly obtained, but also in the Vero, and showed the same viral load in both Hela and HEp-2 cell cultures.

As a result of the analysis of HRSV sequences presented in the GenBank database, it was found that previously genome-wide sequences of HRSVs isolated in Russia were not deposited in this database and all Russian viruses are represented by sequences of two genes: F and G. Amino acid substitutions in antigenic sites often lead to the virus escaping from the immune response of the host organism. F and G proteins of HRSVs are the main targets of neutralizing antibodies [26] and contain the main antigenic sites.

According to the genetic analysis, the viruses RSV50Hl, RSV50Hp, RSV66Hp, and RSV66Hl belong to the group BA9 subtype B and are related to viruses detected worldwide: in Australia, China, England, Japan, Argentina, etc. In addition, were revealed the similarity of our HRSVs from Novosibirsk, Russia, with viruses isolated in different geographical regions of the world during several epidemic seasons, which, in turn, indicates the existence of conservative genetic clusters of similar HRSV variants that persist for several years and spread over considerable distances in the human population.

Morphological analysis showed an equal cytopathic effect in the cell culture of all studied viruses. This fact is also caused by the absence of fundamental genetic differences. For example, in a virus RSV66Hl we described the syncytium formation in HeLa, HEp-2, and Vero cell cultures. Cytopathic changes on monolayers appeared at the same time - on days 4-5 post-inoculation. The typical parameter of syncytium reached 150 microns or more in size with a peripheral arrangement of nuclei and an optically bright zone in the center of the syncytium. On days 5-6 post-inoculation sequential fragmentation of syncytia with the formation of apoptotic cells and destruction of multinucleation were observed. Identification of CPE correlated well with IF results. Infected HeLa, HEp-2, and Vero cell cultures had specific diffuse or granular fluorescence of the syncytium in the central zone with periphery-located nuclei.

Our previous research showed that the RSV66Hl virus is suitable for use in diagnostic purposes by preparing an antigen-containing drug [28]. In addition, it can be used as a reference control sample for evaluating the specificity of PCR-based test systems and investigating the effectiveness of antiviral drugs in vitro.

There is a difficulty in the isolation and cultivating of HRSVs. We have tested four cultivation manners, of which the most effective for the isolation of relevant HRSVs was the infection of a suspension of cells (HeLa or HEp-2) with subsequent RDE treatment. Human RSVs that were isolated during the 2017-2018 period in Russia belong to group B and the nucleotide sequences of complete genomes have been determined for them.

Isolation in the different cell cultures did not affect the nucleotide and amino acid sequences of HRSVs.

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Authors' Contributions

Elena A. Prokopyeva: conceptualization, data curation, formal analysis, project administration, resources, supervision, validation, visualization, writing—original draft, writing—review and editing. Olga G. Kurskaya: conceptualization, data curation, formal analysis, methodology, project administration, resources, validation, visualization, writing—original draft, writing—review and editing. Mariya V. Solomatina: data curation, methodology, visualization Ivan A. Sobolev: formal analysis, methodology, software, validation, visualization, writing—review and editing. Tereza A. Saroyan: data curation, methodology. Nikita A. Dubovitsky, Anastasiya A. Derko, Peter M. Kozhin, Tatyana Yu. Alikina, and Marsel R. Kabilov: helped with data curation. Alexander M. Shestopalov: project administration. Kirill A. Sharshov: conceptualization, data curation, formal analysis, project administration, validation, writing - original draft, writing - review and editing. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement

The genome sequences of HRSV were deposited in GISAID EpiRSV. Accession ID: HRSV/B/Russia/Novosibirsk-50Hl/2018 (EPI_ISL_2575499); HRSV/B/Russia/Novosibirsk-50Hp/2018 (EPI_ISL_2575500); HRSV/B/Russia/Novosibirsk-66Hl/2018 (EPI_ISL_2575546); HRSV/B/Russia/Novosibirsk-66Hp/2018 (EPI_ISL_2575547).

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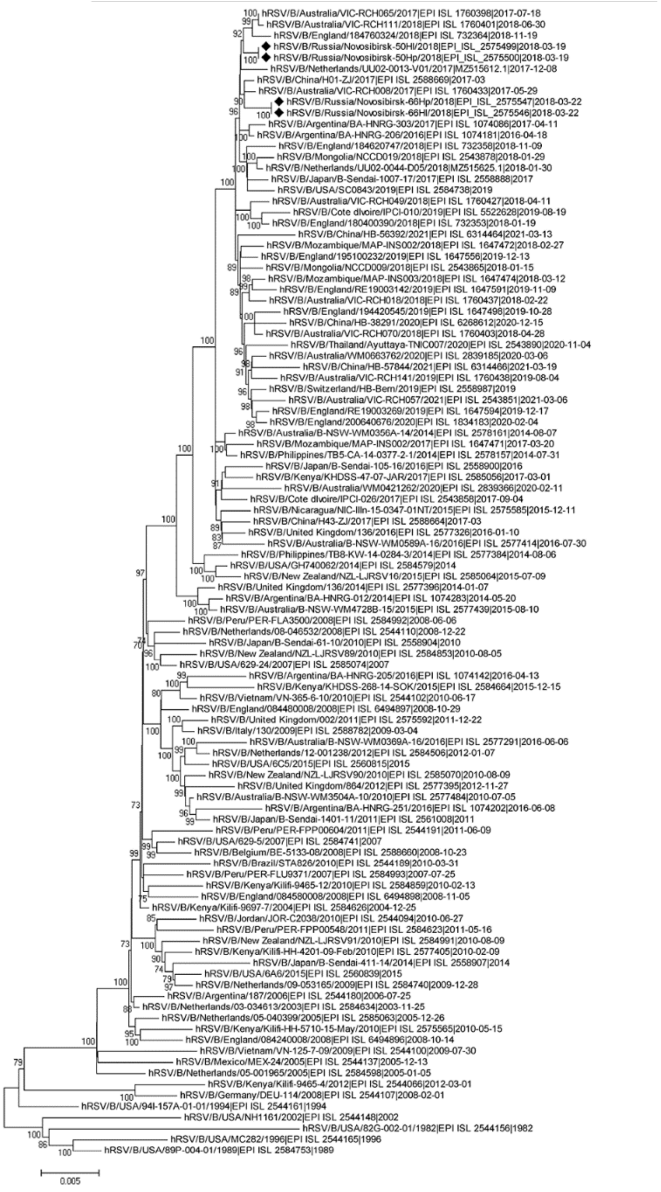
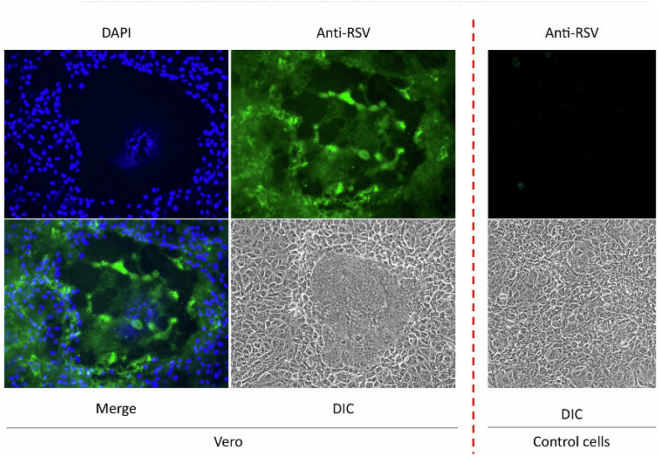
Annex – Supplementary Items

Supplementary Figure 1. Phylogenetic dendrogram based on the genome-wide nucleotide sequences of 503 respiratory syncytial viruses.



Supplementary Figure 2. Immunofluorescent analysis of Vero cell cultures infected with HRSV.



Vero cells were treated by diagnostic kit with fluorescent antibodies to HRSV and the cell nuclei were contrasted with 5uM 4,6-diamino-2phenyl indole (DAPI); photography was carried out using a LSM710/NLO confocal microscope and AxioImager Z1 microscope (Carl Zeiss, Jena, Germany) under differential interference contrast (DIC). Bar 100 µm.