

1 **Replication of SARS-CoV-2 in human respiratory** 2 **epithelium**

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29 www: <http://virogenetics.info/>.

30 **Abstract**

31 SARS-CoV-2 emerged by the end of 2019 to rapidly spread in 2020. At present, it is of utmost
32 importance to understand the virus biology and to rapidly assess the potential of existing drugs
33 and develop new active compounds. While some animal models for such studies are under
34 development, most of the research is carried out in the Vero E6 cells. Here, we propose fully
35 differentiated human airway epithelium cultures as a model for studies on the SARS-CoV-2.
36 Further, we also provide basic characteristics of the system.

37

38 Keywords: HAE, human airway epithelium, ALI, COVID-19, NCoV-2019, SARS-CoV-2,
39 coronavirus, Coronaviridae, model, culture, FISH

40 **Introduction**

41 Coronaviruses constitute a large family of RNA viruses that infect mainly mammals and
42 birds. In humans, there are four species associated with mild-to-moderate respiratory infections.
43 While these viruses are present in the human population for a long time, they are believed to
44 enter the human population in a zoonotic event, and one may speculate that they may have
45 caused epidemics similar to the one observed for the SARS-CoV-2. Time to the most recent
46 ancestor analysis suggests that human coronavirus HCoV-NL63 is the oldest species in humans,
47 followed by its cousin HCoV-229E and two betacoronaviruses, which emerged in humans in a
48 relatively near past^{1,2,3,4}. In the 21st century, we already faced the emergence of the three novel
49 coronaviruses in humans, of which SARS-CoV disappeared after one season never to come
50 back, and MERS-CoV never fully crossed the species border, as its transmission between
51 humans is highly ineffective^{5,6,7}. The 2019 zoonotic transmission, however, resulted in the
52 emergence of a novel human coronavirus, which seems to carry an optimal set of features
53 allowing for its rapid spread with considerable mortality. Whether the virus will become
54 endemic in humans is an open question^{8,9,10}.

55 At present, the studies on the virus are carried out using a surrogate system based on the
56 immortalized simian Vero E6 cell line¹¹. While this model is convenient for diagnostics and
57 testing of some antiviral drugs, it has serious limitations and does not allow for the
58 understanding of virus biology and evolution. To make an example, the entry route of human
59 coronaviruses varies between the cell lines and differentiated tissue, not mentioning the immune
60 responses or virus-host interactions^{12,13,14}.

61 Here we used the fully differentiated epithelium cultures to study the infection with the
62 novel human coronavirus SARS-CoV-2. We observed an efficient replication of the virus in
63 the tissue, with the maximal replication at 2 days post-infection. At the time of the study no

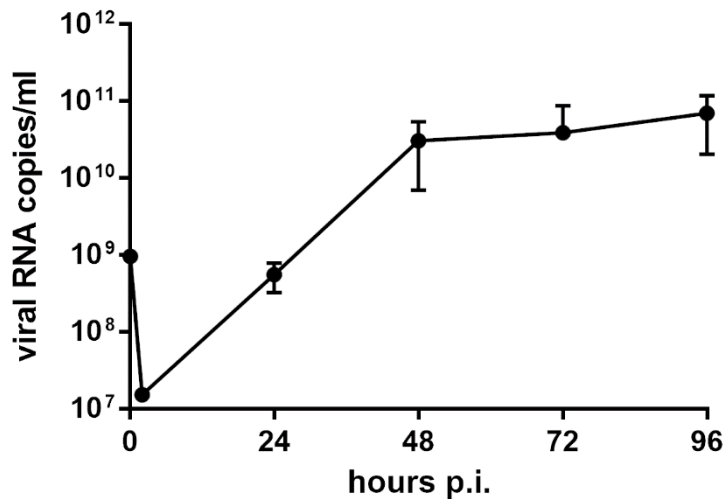
64 antibodies were available. Therefore we developed immuno-FISH to show that the virus infects
65 primarily ciliated cells of the respiratory epithelium.

66

67 **Results and discussion**

68 The HAE cultures reconstitute the tissue lining the conductive airways of humans. Fully
69 differentiated, are among the best tools for studying the viral infection in a natural
70 microenvironment¹⁵. These air-liquid interphase cultures contain a number of cell types (e.g.,
71 basal, ciliated, and goblet). At the same time, they also functionally reflect the natural tissue
72 with extensive crosstalk and production of protective mucus and surfactant proteins^{16,17,18}. The
73 cultures were previously shown by us and others to be superior to the standard cell lines in
74 terms of ability to support coronaviral replication of the HCoV-HKU1, but also as a model to
75 study the biology of the infection¹⁹. To make an example, human coronaviruses were shown
76 some time ago to use a very different entry pathway in immortalized cell lines and in the natural
77 human epithelium. While in the first one they enter via pH-dependent endocytic pathway, in
78 the latter one they utilize surface serine proteases as TMPRSS2 or kallikreins for activation and
79 the fusion occurs on the cell surface. This may have grave consequences not only for the basic
80 science, but also the antiviral drug development^{12,13,14,20}.

81 Here we verified whether HAE cultures may be used to study the SARS-CoV-2 infection
82 and identified the cellular targets in the tissue. First, the HAE cultures were inoculated with the
83 SARS-CoV-2 stock and cultured for 5 days. Every day (days 0-4) the apical and basolateral
84 release of the virus was evaluated with the RT-qPCR and the results for the apical release of
85 the virus are presented in **Figure 1**.



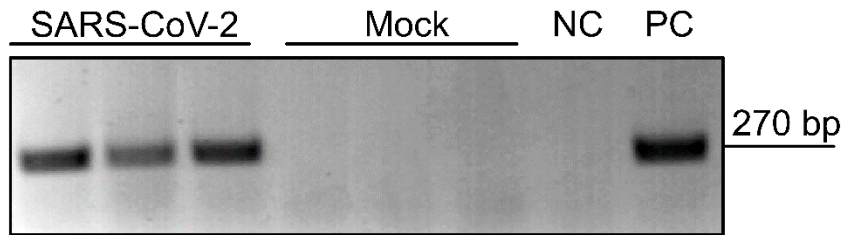
86

87 **Figure 1. SARS-CoV-2 replicates in HAE cultures.** Replication of SARS-CoV-2 was evaluated using
88 an RT-qPCR, and the data are presented as RNA copy number per ml. The experiment was
89 carried out in triplicate, and average values with standard deviation are presented.
90

91 Clearly, the increase in virus titer on the apical side is visible already 24 h post-inoculation,
92 to reach the plateau at 48 h post-inoculation. We did not observe any release of the virus from
93 the basolateral side of the HAE culture and therefore we do not show the relevant data on the
94 graph. The results we observe are consistent with the previously reported polarity of the HAE
95 cultures and apical infection / apical release reported previously for other human coronaviruses.
96 Similarly as for other human coronaviruses, the apical-apical polarity of SARS-CoV-2
97 infection-release restricts the virus to the airway lumen¹⁶.

98 Further, we sampled the tissue at 96 h post-infection, to verify whether the subgenomic
99 mRNAs are present. The analysis was carried out with RT-PCR and the results are presented in

100 **Figure 2.**



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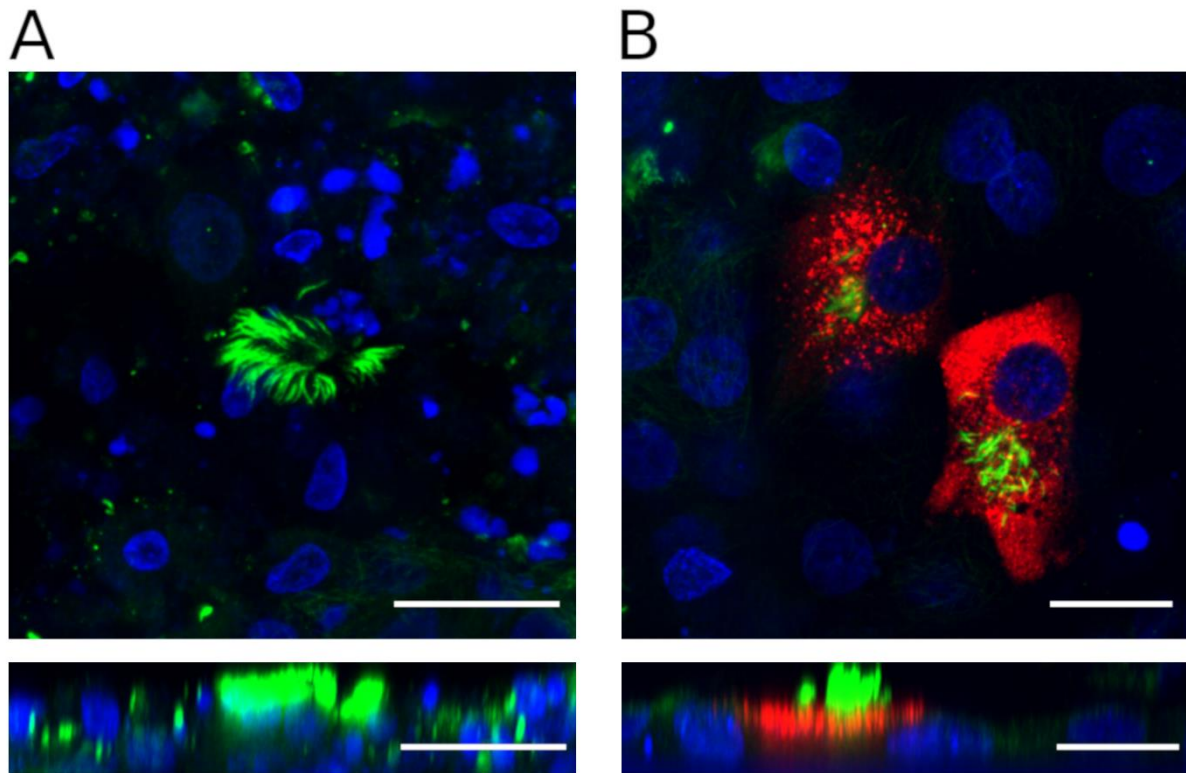
102 **Figure 2. sg mRNAs of the SARS-CoV-2 in HAE cultures.** The presence of the N sg mRNAs 4 days
103 p.i. in the HAE cultures infected with the SARS-CoV-2 was evaluated using an RT-PCR. NC =
104 negative control, PC = positive control.

105

106 The analysis clearly showed that the sg mRNA are abundant in the infected HAE cultures.

107 As this is generally considered to be the hallmark of an active replication, we believe that it
108 provides sufficient proof that the virus is indeed actively replicating in the cultures.

109 Next, we made an effort to visualize the infection in the tissue. As at the time of the study
110 no antibody for the confocal microscopy was available, we developed an immuno-FISH assay,
111 where the viral RNA was visualized in the context of the cell using 20 sequence-specific probes
112 and signal amplification. At the same time, the β -tubulin was visualized using specific
113 antibodies to visualize the ciliated cells. Obtained results are shown in **Figure 3**.



114
115 **Figure 3. SARS-CoV-2 infects ciliated cells of the human airway epithelium.** Three-dimensional
116 Immuno-RNA FISH demonstrating localization of SARS-CoV-2 subgenomic RNA in ciliated HAE
117 cultures. Three-dimensionally reconstructed confocal image stacks of cells infected with SARS-
118 CoV-2 (A) and mock control cells (B). The bottom lanes of panels A and B show the xz plane in
119 orthogonal views. SARS-CoV-2 RNA is visualized by FISH using a set of probes against viral
120 nucleocapsid RNA and is shown in red. Cilia are visualized by an anti- β 5 tubulin antibody and
121 are shown in green. Nuclei are stained with DAPI and are shown in blue. Bar = 20 μ M.

122
123 Summarizing, we show that the SARS-CoV-2 effectively replicates in the HAE cultures
124 and that this *ex vivo* model constitutes a convenient tool to study the viral infection. We also
125 show that the virus infects ciliated cells. The infection is polarized - the infection and release
126 occurs at the apical side of the epithelium. It is worth to note that in the absence of the
127 immunodetection tools the new generation of immune-FISH tools offers an interesting
128 alternative.

129

130 **Materials and Methods**

131 **Cell culture**

132 Vero E6 (*Cercopithecus aethiops*; kidney epithelial; ATCC: CRL-1586) cells were
133 maintained in DMEM (Thermo Fisher Scientific, Poland) supplemented with 3% FBS (heat-
134 inactivated fetal bovine serum; Thermo Fisher Scientific, Poland) and streptomycin
135 (100 µg/ml), penicillin (100 U/ml), and ciprofloxacin (5 µg/ml). Cells were cultured at 37°C in
136 atmosphere containing 5% CO₂.

137

138 **Human airway epithelium (HAE) cultures**

139 Human epithelial cells were isolated from conductive airways resected from transplant
140 patients. The study was approved by the Bioethical Committee of the Medical University of
141 Silesia in Katowice, Poland (approval no: KNW/0022/KB1/17/10 dated 16.02.2010). Written
142 consent was obtained from all patients. Cells were mechanically detached from the tissue after
143 protease treatment and cultured on plastic in BEGM media. Subsequently, cells were transferred
144 onto permeable Transwell insert supports ($\phi = 6.5$ mm) and cultured in BEGM media. After the
145 cells reached full confluency, the apical medium was removed, and the basolateral medium was
146 changed to ALI. Cells were cultured for 4-6 weeks to form differentiated, pseudostratified
147 mucociliary epithelium. All experiments were performed in accordance with relevant
148 guidelines and regulations.

149

150 **Virus**

151 SARS-CoV-2 (isolate 026V-03883; kindly granted by Christian Drosten, Charité –
152 Universitätsmedizin Berlin, Germany and provided by the European Virus Archive - Global
153 (EVAg); <https://www.european-virus-archive.com/>). Virus stock was prepared by infecting
154 fully confluent Vero E6 cells at a TCID₅₀ of 400 per ml. Three days after inoculation,

155 supernatant from the cultures was aliquoted and stored at -80°C . Control Vero E6 cell
156 supernatant from mock-infected cells was prepared in the same manner. Virus yield was
157 assessed by titration on fully confluent Vero E6 cells in 96-well plates, according to the method
158 of Reed and Muench. Plates were incubated at 37°C for 2 days, and the cytopathic effect (CPE)
159 was scored by observation under an inverted microscope.

160

161 **Virus infection**

162 Fully differentiated human airway epithelium (HAE) cultures were inoculated with the
163 SARS-CoV-2 at a TCID₅₀ of 1000 per ml (as determined on Vero E6 cells). Following 2 h
164 incubation at 37°C , unbound virions were removed by washing with 200 μl of $1 \times \text{PBS}$, and
165 HAE cultures were maintained at an air-liquid interphase for the rest of the experiment. To
166 analyze virus replication kinetics, each day p.i., 100 μl of $1 \times \text{PBS}$ was applied at the apical
167 surface of HAE and collected following the 10 min incubation at 32°C . All samples were stored
168 at -80°C and analyzed using RT-qPCR.

169 Additionally, 48 h post-infection, selected HAE cultures were collected, and the presence
170 of sg mRNA was determined as hallmarks of an active infection.

171

172 **Isolation of nucleic acids and reverse transcription (RT)**

173 Viral DNA/RNA Kit (A&A Biotechnology, Poland) was used for nucleic acid isolation
174 from cell culture supernatants and Fenzol (A&A biotechnology, Poland) was used for total
175 RNA isolation from cells. RNA was isolated according to the manufacturer's instructions.
176 cDNA samples were prepared with a High Capacity cDNA Reverse Transcription Kit (Thermo
177 Fisher Scientific, Poland), according to the manufacturer's instructions.

178

179 **Quantitative PCR (qPCR)**

180 Viral RNA was quantified using qPCR (CFX96 Touch Real-Time PCR Detection System,
181 Bio-Rad, Poland). cDNA was amplified using 1 × qPCR Master Mix (A&A Biotechnology,
182 Poland), in the presence of probe (100 nM, FAM / BHQ1, ACT TCC TCA AGG AAC AAC
183 ATT GCC A) and primers (450 nM each, CAC ATT GGC ACC CGC AAT C and GAG GAA
184 CGA GAA GAG GCT TG). The heating scheme was as follows: 2 min at 50°C and 10 min at
185 92°C, followed by 30 cycles of 15 s at 92°C and 1 min at 60°C. In order to assess the copy
186 number for N gene, standards were prepared and serially diluted.

187

188 **Detection of SARS-CoV-2 N sg mRNA**

189 Total nucleic acids were isolated from virus or mock-infected cells at 4 days p.i. using
190 Fenzol reagent (A&A Biotechnology, Poland), according to the manufacturer's instructions.
191 Reverse transcription was performed using a high-capacity cDNA reverse transcription kit (Life
192 Technologies, Poland), according to the manufacturer's instructions. Viral cDNA was
193 amplified in a 20 µl reaction mixture containing 1 × Dream *Taq* Green PCR master mix
194 (Thermo Fisher Scientific, Poland), and primers (500 nM each). The following primers were
195 used to amplify SARS-CoV-2 subgenomic mRNA (sg mRNA): common sense primer (leader
196 sequence), 5' – TAT ACC TTC CCA GGT AAC AAA CCA -3'; nucleocapsid antisense, 5' –
197 GTA GCT CTT CGG TAG TAG CCA AT – 3'. The conditions were as follows: 3 min at 95°C,
198 35 cycles of 30 s at 95°C, 30 s at 52°C, and 20 s at 72°C, followed by 5 min at 72°C and 10
199 min at 4°C. The PCR products were run on 1% agarose gels (1 Tris-acetate EDTA [TAE] buffer)
200 and analyzed using molecular imaging software (Thermo Fisher Scientific, Poland).

201

202 **RNA Fluorescent *in situ* Hybridization (RNA-FISH) and Immunofluorescence**

203 HAE cultures were infected with SARS-CoV-2 [TCID₅₀=1000, as assessed for the Vero
204 E6 cells] and fixed at 5 days post-infection with 3.7% paraformaldehyde (PFA) overnight. The
205 next day, cells were subjected to RNA-FISH protocol using hybridization chain reaction (HCR)
206 technology from Molecular Instruments, Inc. Briefly, cells were permeabilized with 100%
207 methanol overnight and then subjected to graded rehydration with methanol/PBS, Tween 0.1%.
208 The set of DNA HCR v3.0 probes complementary to SARS-CoV-2 nucleocapsid RNA was
209 incubated for 12 h at 37°C, extensively washed, and hybridized with HCR amplifiers for 12 h
210 at room temperature in the dark. Next, cells were subjected to immunostaining with antibodies
211 against mouse β 5-tubulin from Santa Cruz Biotechnology (sc-134234, 1:100), rinsed three
212 times with PBS, 0.1% Tween-20 and followed by 1 h incubation with Alexa fluorophore 488
213 secondary antibodies (Invitrogen, 1:400). The cells were finally washed three times with PBS,
214 0.1% Tween-20, cell nuclei were stained with DAPI (4',6'-diamidino-2-phenylindole)
215 (Thermo Fisher Scientific, D1306) and mounted on slides with Prolong diamond antifade
216 mounting medium (Invitrogen, P36970). Fluorescent images were acquired using a Zeiss LSM
217 710 confocal microscope (Carl Zeiss Microscopy GmbH) with ZEN 2012 SP1 Black Edition
218 and processed in ImageJ Fiji (National Institutes of Health, Bethesda, MD, USA).

219

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224 the design of the study; in the collection, analyses, or interpretation of data; in the writing of
225 the manuscript, or in the decision to publish the results.

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