# Replication of SARS-CoV-2 in human respiratory epithelium

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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

**Introduction**

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

At present, the studies on the virus are carried out using a surrogate system based on the immortalized simian Vero E6 cell line[11]. While this model is convenient for diagnostics and testing of some antiviral drugs, it has serious limitations and does not allow for the understanding of virus biology and evolution. To make an example, the entry route of human coronaviruses varies between the cell lines and differentiated tissue, not mentioning the immune responses or virus-host interactions[12,13,14].

Here we used the fully differentiated epithelium cultures to study the infection with the novel human coronavirus SARS-CoV-2. We observed an efficient replication of the virus in 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

**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　　microenvironment[15]. 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 infection[19]. 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
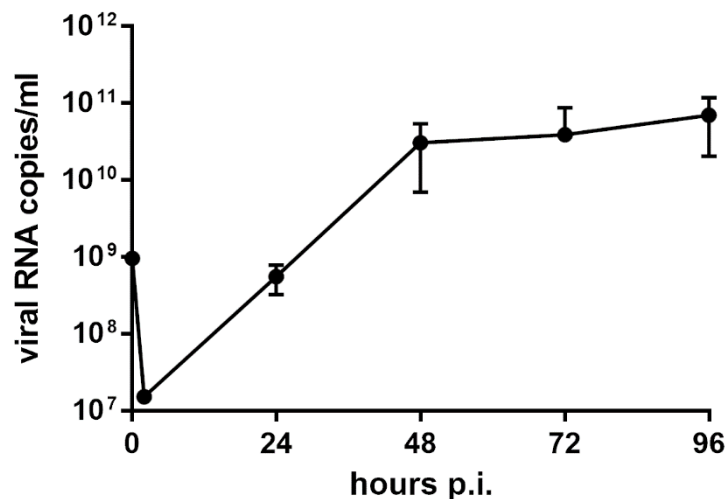
Figure 1. SARS-CoV-2 replicates in HAE cultures. Replication of SARS-CoV-2 was evaluated using an RT-qPCR, and the data are presented as RNA copy number per ml. The experiment was carried out in triplicate, and average values with standard deviation are presented.

Clearly, the increase in virus titer on the apical side is visible already 24 h post-inoculation, to reach the plateau at 48 h post-inoculation. We did not observe any release of the virus from the basolateral side of the HAE culture and therefore we do not show the relevant data on the graph. The results we observe are consistent with the previously reported polarity of the HAE cultures and apical infection / apical release reported previously for other human coronaviruses. Similarly as for other human coronaviruses, the apical-apical polarity of SARS-CoV-2 infection-release restricts the virus to the airway lumen[16].

Further, we sampled the tissue at 96 h post-infection, to verify whether the subgenomic mRNAs are present. The analysis was carried out with RT-PCR and the results are presented in **Figure 2**.

101
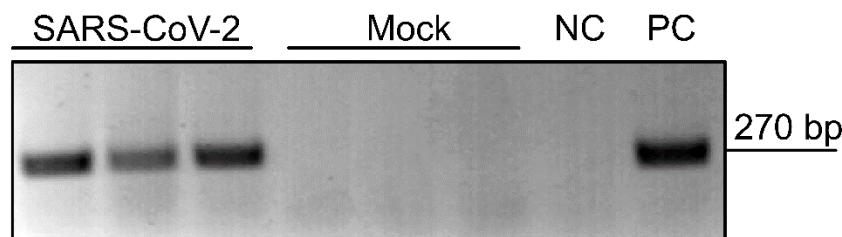
102 **Figure 2. sg mRNAs of the SARS-CoV-2 in HAE cultures.** The presence of the N sg mRNAs 4 days
103 p.i. in the HAE cultures infected with the SARS-CoV-2 was evaluated using an RT-PCR. NC =
104 negative control, PC = positive control.
105

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**.
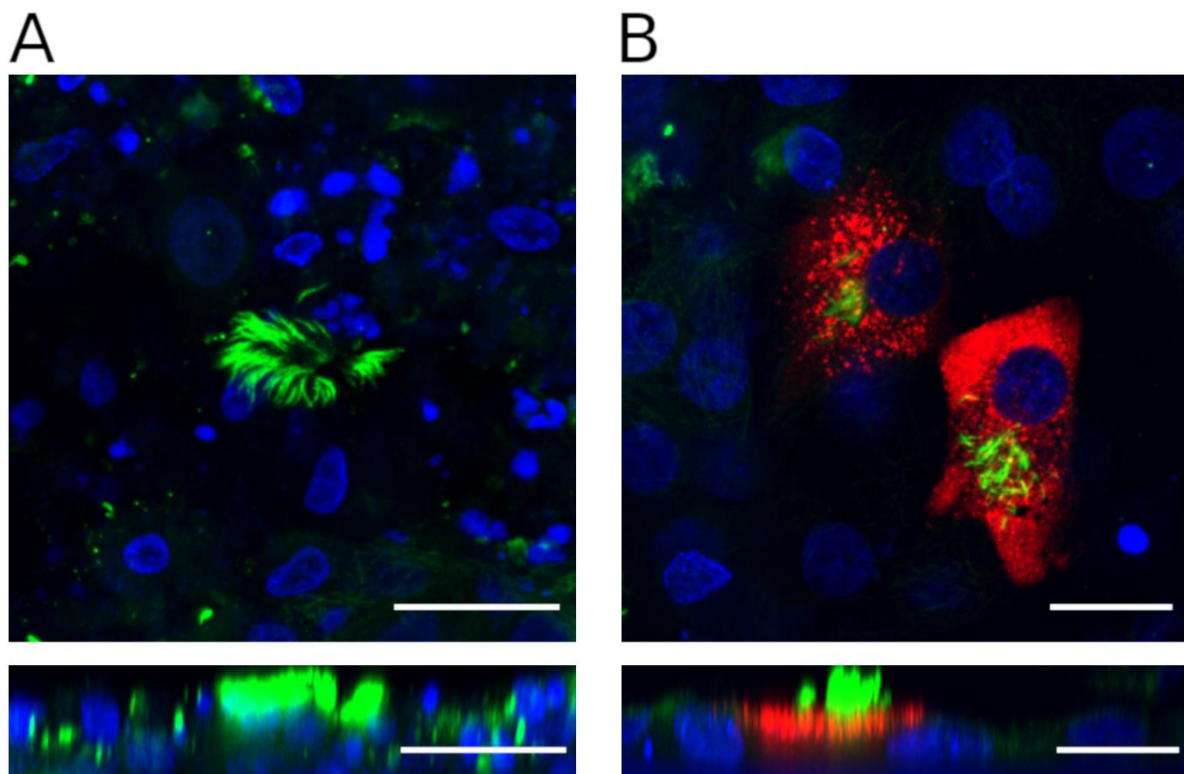
114
115 **Figure 3. SARS-CoV-2 infects ciliated cells of the human airway epithelium.** Three-dimensional
116 Immuno-RNA FISH demonstrating localization of SARS-CoV-2 subgenomic RNA in ciliated HAE
117 cultures. Three-dimensionally reconstructed confocal image stacks of cells infected with SARS-
118 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-β5 tubulin antibody and
121 are shown in green. Nuclei are stained with DAPI and are shown in blue. Bar = 20μM.

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% $CO_2$.

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 (ϕ = 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 $TCID_{50}$ 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 $TCID_{50}$ 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 $1 \times$ 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 $1 \times$ 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 Fenozol (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

**Quantitative PCR (qPCR)**

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

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

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

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

HAE cultures were infected with SARS-CoV-2 [$TCID_{50}$=1000, as assessed for the Vero E6 cells] and fixed at 5 days post-infection with 3.7% paraformaldehyde (PFA) overnight. The next day, cells were subjected to RNA-FISH protocol using hybridization chain reaction (HCR) technology from Molecular Instruments, Inc. Briefly, cells were permeabilized with 100% methanol overnight and then subjected to grated rehydration with methanol/PBS, Tween 0.1%. The set of DNA HCR v3.0 probes complementary to SARS-CoV-2 nucleocapsid RNA was incubated for 12 h at 37°C, extensively washed, and hybridized with HCR amplifiers for 12 h at room temperature in the dark. Next, cells were subjected to immunostaining with antibodies against mouse β5-tubulin from Santa Cruz Biotechnology (sc-134234, 1:100), rinsed three times with PBS, 0.1% Tween-20 and followed by 1 h incubation with Alexa fluorophore 488 secondary antibodies (Invitrogen, 1:400). The cells were finally washed three times with PBS, 0.1% Tween-20, cell nuclei were stained with DAPI (4 =,6 =-diamidino-2-phenylindole) (Thermo Fisher Scientific, D1306) and mounted on slides with Prolong diamond antifade mounting medium (Invitrogen, P36970). Fluorescent images were acquired using a Zeiss LSM 710 confocal microscope (Carl Zeiss Microscopy GmbH) with ZEN 2012 SP1 Black Edition and processed in ImageJ Fiji (National Institutes of Health, Bethesda, MD, USA).

**Conflicts of Interest:** The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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