Health Sciences Department

PhD XXXV Cycle

PhD course in Health Sciences

Curriculum in Epidemiology and Vaccine Prevention

“Unusual aspects of the SARS-CoV-2 pandemic. From the development of a new protocol for reviving virions from faeces to the test of a new drug”

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I. Abstract

Severe Acute Respiratory Syndrome-Coronavirus-2 (SARS-CoV-2) belongs to the *Coronaviridae* family, genus *Betacoronavirus.* Infection by SARS-CoV-2 can cause Coronavirus Disease (COVID-19). This is an infectious disease that can have a wide range of symptoms. Most people infected show mild to moderate respiratory illness and recover without special treatment. However, some patients can develop a serious illness and require medical attention. The virus can spread mainly from an infected person's mouth or nose in small liquid particles when they cough, sneeze, speak or breathe. These particles range from larger respiratory droplets to smaller aerosols and can stay suspended in the air in an indoor space for minutes or even hours. It has also been widely demonstrated that the virus can be found in the environment such as in waste-waters. It remains unclear and controversial if the faecal transmission has a crucial role in the human-to-human transmission of the virus.

The experiments reported in this PhD thesis were conducted with the Virology Research Group and The Clinical Virology Research Group at the University of Oslo/Oslo University Hospital, Norway. They had two aims: first, to investigate the possibility of reviving SARS-CoV-2 virions from faeces of COVID-19-positive patients; and second, to study a new solution called Drug X as a new treatment for defeating SARS-CoV-2.

Our preliminary results showed that for the 13,5% of faeces samples, it was possible to revive SARS-CoV-2 viable virions using the Vero-E6 cell line. This ideally indicated the possibility for positive patients to transmit the virus to naïve people. At the same time, it was possible to establish the highest concentration of Drug X can be used, so the highest concentration where the cells survive. It was compared with other acid solutions concluding that although our results showed that Drug X does not affect SARS-CoV-2 infection, this research lays the foundations to explore other similar solutions as a valuable treatment for COVID-19.

A detailed exploration of the cellular and molecular functions of SARS-CoV-2 infection is still necessary to better understand virus-host cell interactions to provide novel ways to treat COVID-19 and limit virus circulation.

II. Abbreviations

Abbr.	Explanation	Abbr.	Explanation
ACE2	Angiotensin-Converting Enzyme 2	**M protein**	Membrane protein
Ag-RDTs	Antigen-detection Rapid Diagnostic Tests	**MERS-CoV**	Middle East Respiratory Syndrome Coronavirus
CDC	Centers for Disease Control and Prevention	**ms**	milliseconds
COVID-19	Coronavirus Disease 2019	**N protein**	Nucleocapsid protein
CPE	Cytopathic Effect	**NAATs**	Nucleic Acid Amplification Tests
DMEM	Dulbecco´s Modified Eagle´s Medium	**ng**	nanogram
DMSO	Dimethyl sulfoxide	**NGS**	Next-Generation Sequencing
E protein	Envelope protein	**PBS**	Phosphate Buffered Saline
ECDC	European Centre for Disease Prevention and Control	**PhA**	Phosphoric Acid
ELISA	Enzyme-Linked Immuno Sorbent Assay	**RBD**	Receptor binding domain
ER	Endoplasmic Reticulum	**RdRp**	RNA-dependent RNA polymerase
FCS	Fetal calve serum	**RT-qPCR**	Reverse Transcriptase quantitative Polymerase Chain Reaction
FDA	Food and Drug Administration	**S protein**	Spike protein
FIA	Antigen Fluorescence Immunoassay	**SARI**	Severe Acute Respiratory Infection
g	Unit for relative centrifugal force	**SARS-CoV-2**	Severe Acute Respiratory Syndrome Coronavirus 2
HA	Haemagglutination Assays	**ssRNA**	single stranded RNA
HCl	Hydrochloric acid	**SuA**	Sulphuric Acid
HCoV	human coronaviruses	**TMPRSS2**	Transmembrane Protease Serine 2
hpi	hours post infection	**TPN**	Total Parenteral Nutrition
hpt	hours post treatment	**VBM**	Variants Being Monitored
IC50	Half maximal inhibitory concentration	**VOC**	Variant Of Concern
IIF	Indirect Immuno Fluorescence	**WHO**	World´s Health Organization
ILI	Influenza Like Illness	**µg**	microgram
ISS	National Institute of Health (Italy)	**µl**	microliter
LAMP	Loop-Mediated Isothermal Amplification	**µM**	micromolar

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1) Introduction

1.1 SARS-CoV-2

1.1.1 Morphology, molecular characteristics, and replication

Severe Acute Respiratory Syndrome-Coronavirus-2 (SARS-CoV-2) belongs to the *Coronaviridae* family, genus *Betacoronavirus* [1]. The virion is spherical, approximately 80 to 160mm in diameter. Based on the genetic and antigenic characteristics, coronaviruses can be divided into four genera: α, β, γ, and δ. Among them, coronaviruses α and β infect mammals only, while γ and δ mainly infect birds, although some may also infect mammals [2]. Except for SARS-CoV and Middle East respiratory syndrome coronavirus (MERS-CoV), most coronaviruses do not cause severe disease in humans. The genome is a positive-sense single-strand RNA of 26 to 32Kb, representing the longest known genome among RNA viruses, with a single linear RNA segment expressing four structural proteins known as protein S (spike), E (envelope), M (membrane), and N (nucleocapsid). The N protein contains the viral genome, while the S, E, and M proteins create the viral capsid.

Thanks to the S protein, the virus binds to the target cell receptor called Angiotensin Converting Enzyme 2 (ACE2) on the surface of the epithelial cells of the respiratory tract. The virus fusion with the host cell membrane starts with the cleavage of protein S into the S1 and S2 subunits by host proteases. The virus can release its internal components and replicate in the cytoplasm of the cell and release newly formed virions in the airways [3]. Please see a more detailed description in Figure 1.

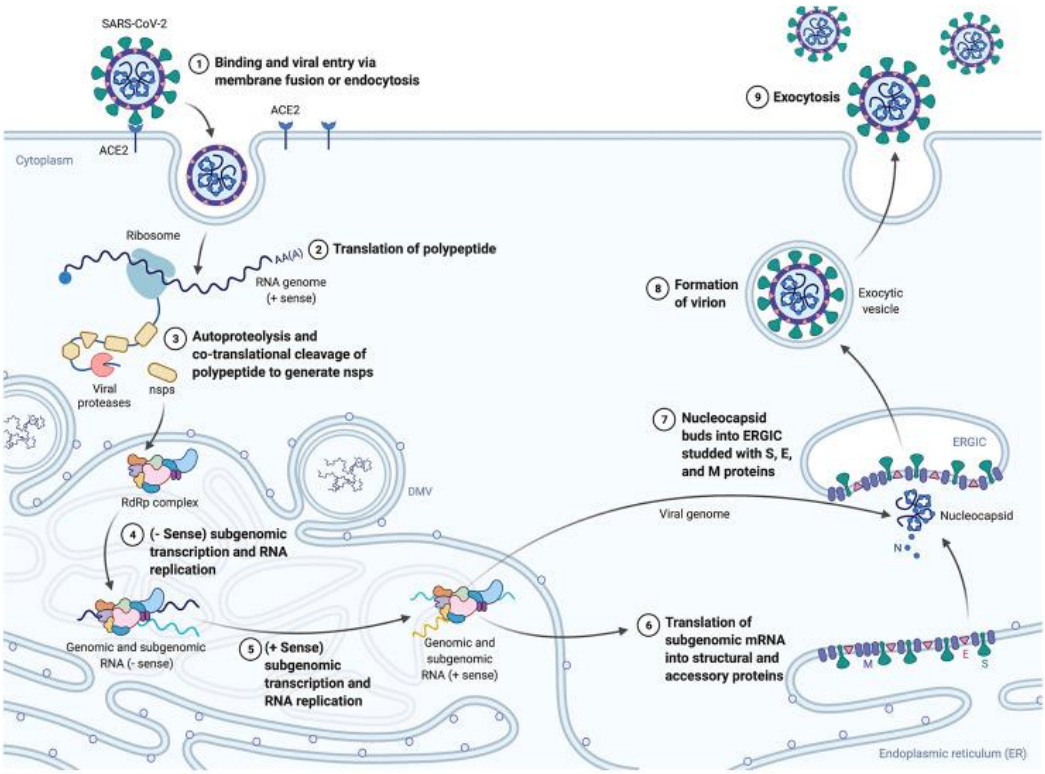


Figure 1. Schematic representation of the SARS-CoV-2 biogenesis. SARS-CoV-2 enters host cells by interacting with the angiotensin-converting enzyme 2 (ACE2) receptor by the surface spike (S) protein. Upon entry of the virus into the host cell, viral genomic RNA is released into the cytoplasm, where it is translated into viral polymerase proteins. Here, sub-genomic (–) RNAs are synthesized and used as templates for sub-genomic (+) messenger RNAs (mRNAs). The nucleocapsid (N) structural protein and viral RNA are replicated, transcribed, and synthesized in the cytoplasm. In contrast, other viral structural proteins, including the S protein, membrane (M) protein, and envelope (E) protein, are transcribed and then translated into the endoplasmic reticulum (ER). The structural proteins traverse the ER-Golgi intermediate compartment for virion assembly, followed by the release of the nascent virion from the host cell via exocytosis [3].

1.1.2 Clinical features

Infection by SARS-CoV-2 can cause Coronavirus Disease (COVID-19). This is an infectious disease that can have a wide range of symptoms. Most people infected show mild to moderate respiratory illness and recover without special treatment. However, some patients can develop a serious illness and require medical attention[4]. Older people and those with underlying medical conditions like

cardiovascular disease, diabetes, chronic respiratory disease, or cancer are more likely to develop severe illness [4]. Still, anyone at any age can have severe symptoms and complications [5].

Symptoms that may appear 2-14 days after exposure to the virus include [6,7]:

- Fever or chills
- Cough
- Shortness of breath or difficult breathing
- Fatigue
- Muscle or body aches
- Headache
- New loss of taste or smell
- Sore throat
- Congestion or runny nose
- Nausea or vomiting
- Diarrhea

1.1.2.1 ILI and SARI definitions

European Centre for Disease Prevention and Control (ECDC) defines the Influenza-Like Illness or ILI case as a person with sudden onset of symptoms of an acute respiratory infection with:

- at least one respiratory sign or symptom (cough, sore throat, shortness of breath)
- and at least one systemic sign or symptom (fever or feverishness, headache, myalgia, generalized malaise)

with onset within the last 10 days [8].

Similarly, a Severe Acute Respiratory Infection or SARI case is every person with sudden onset of symptoms of a severe acute respiratory infection with:

- at least one respiratory sign or symptom (cough, sore throat, shortness of breath)
- and at least one systemic sign or symptom (fever or feverishness, headache, myalgia, generalized malaise)

with onset within the last 10 days.

- and requires hospitalization [9].

1.1.3 Epidemiology, the pandemic emergency, and transmission

SARS-CoV-2 was first identified in late 2019 in Wuhan, China, and quickly spread worldwide, representing the most significant public health problem second only to the "Spanish" influenza pandemic in the early 20th century [10]. The virus can spread mainly from an infected person's mouth or nose in small liquid particles when they cough, sneeze, speak or breathe. These particles range from larger respiratory droplets to smaller aerosols and can stay suspended in the air in an indoor space for minutes or even hours. It is also known that human coronaviruses such as SARS, MERS or endemic human coronaviruses (HCoV) can persist on inanimate surfaces like metal, glass or plastic for up to 9 days, but can be efficiently inactivated by surface disinfection procedures with 62–71% ethanol, 0.5% hydrogen peroxide or 0.1% sodium hypochlorite, within one minute [11].

Infectious exposure to respiratory fluids carrying SARS-CoV-2 occurs in three principal ways [12,13]. First, by inhalation of very small fine droplets and aerosol particles that contain the infectious virus. The risk of transmission is greatest within one to two meters from the infectious source where the concentration of droplets and particles is greatest. Second, by the deposition of droplets and particles onto exposed mucous membranes. Third, by touching mucous membranes with hands soiled by exhaled respiratory fluids containing the virus or from touching inanimate surfaces contaminated with the virus [12,13].

It has also been widely demonstrated that the virus can be found in the environment such as in waste-waters but it remains unclear and controversial if the faecal transmission has a crucial role in the human-to-human transmission of the virus (see section 1.4.1).

During the pandemic, governments worldwide needed to estimate the virus transmission and take prompt containment and public health measures such as social distancing, wearing face masks and using hand sanitisers. For this reason, it was essential to consider the basic reproduction number (R0) value. The WHO defines it as "the average number of people that one person with SARS-CoV-2 is

likely to infect in a population without any immunity (from the previous infection) or any interventions", equally saying a naïve population [14]. R_0 is usually estimated retrospectively, and it varies across populations and time. It depends on the duration of infectivity after the patient gets infected; the likelihood of infection transmission per contact between a susceptible person and an infectious individual; as well as the contact rate. A separate but related parameter is the effective or time-varying reproduction number (Re or Rt), which estimates the average transmission in a population with mitigation measures and immunity [15], assessed that the R_0 of SARS-CoV-2 is like, or higher than, the R_0 of SARS-CoV and the pandemic influenza of 2009 (Table 1) [15,16]. In Italy, during the first months of the pandemic in 2020, the R_0 of SARS-CoV-2 ranged between two and three, but, as of 8th June 2022, R_0 was less than one, clearly indicating a decline of the virus circulation [17,18].

Table 1. Characteristics of SARS-CoV-2, SARS-CoV, and pandemic influenza (2009) [15].

			influenza 1918	influenza 2009	
Transmissibility, R_0	2·5	2·4	2·0	1·7	SARS-CoV-2 has the highest average R_0
Incubation period, days	4–12	2–7	Unknown	2	Longer incubation period; SARS-CoV epidemics form slower
Interval between symptom onset and maximum infectivity, days	0	5–7	2	2	SARS-CoV-2 is harder to contain than SARS-CoV
Proportion with mild illness	High	Low	High	High	Facilitates undetected transmission
Proportion of patients requiring hospitalisation	Few (20%)	Most (>70%)	Few	Few	Concern about capacity in the health sector
Proportion of patients requiring intensive care	1/16 000	Most (40%)	Unknown	1/104 000	Concern about capacity in the health sector
Proportion of deaths in people younger than 65 years out of all deaths	0·6–2·8%	Unknown	95%	80%	SARS-CoV-2 might cause as many deaths as the 1918 influenza pandemic, but fewer years of life lost and disability-adjusted life-years, as deaths are in the older population with underlying health conditions
Risk factors for severe illness	Age, comorbidity	Age, comorbidity	Age (<60 years)	Age (<60 years)	..

Data from the following references.[2,35-36] MERS-CoV=Middle East respiratory syndrome coronavirus. SARS-CoV=severe acute respiratory syndrome coronavirus. SARS-CoV-2=severe acute respiratory syndrome coronavirus 2.

Since the beginning of the COVID-19 pandemic in 2020, over 526 million confirmed cases and over six million deaths related to the SARS-CoV-2 infection have been reported worldwide (Figure 2). These trends should be cautioned as several countries have been progressively changing COVID-19

testing strategies, resulting in lower overall numbers of tests performed and, consequently, lower cases detected. In Italy, the National Institute of Health (ISS) reports the data collected by the integrated surveillance system for COVID-19 weekly. The number of diagnosed COVID-19 cases as of 21st November 2022 was 23,823,192, of which 179,985 deaths [17,19].

From the beginning of the pandemic, many thousands of variants have circulated, but when this PhD thesis was written, there were four main variants being monitored (VBM) [20]:

- Alpha: UK, Sep 2020.
- Beta: South Africa, May 2020.
- Gamma: Brazil, Nov 2020.
- Delta: India, Oct 2020.

And one worldwide prevalent variant of concern (VOC) [20]:

- Omicron: South Africa, 2021 B.1.1.529, BA.1, BA.1.1, BA.2, BA.3, BA.4 and BA.5 lineages.

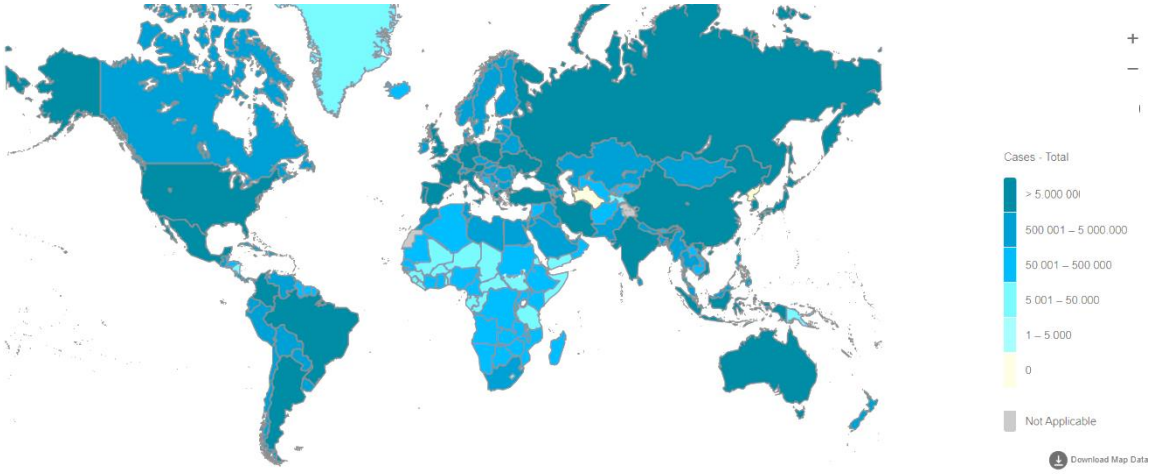


Figure 2. COVID-19 cases per 100 000 population reported by WHO countries, territories, and areas as of 21st November 2022 [21].

1.2 Laboratory diagnostics in the COVID-19 pandemic era

A quick and accurate laboratory diagnostic technology to early detect a pathogen and take appropriate and effective public health decisions is crucial for controlling epidemics. In past times, the techniques

were limited to cell cultures and a restricted panel of serological reactions. The advent of highly sensitive molecular technologies (PCR and derivatives) and reagents obtained by biotechnology (e.g., probes and monoclonal antibodies –mAbs–) allowed to perform analyses much faster, more reliably, with maximum safety and changed the role of laboratory diagnostics in the clinical management of patients. In general, the modern virus diagnostic laboratory is characterized by high test throughputs, rapid turnaround times, and a close liaison with clinical staff. Many of the older and slower diagnostic approaches, such as animal virus inoculation, virus isolation in cell culture, and serological antibody titration, are now less important [22].

Viral diagnosis in the laboratory can be performed in two ways (Figure 3):

– Directly: identification of the virus (or components) in the organic material (Microscopy; Electron Microscopy; Cell Cultures –CPE detection–; PCR, etc.); or

– Indirectly: determination of the presence of specific antibodies versus a specific etiological agent (Enzyme-Linked Immuno Sorbent Assay –ELISA–; Indirect Immuno Fluorescence –IIF–; Neutralization assays; Haemagglutination Assays –HA–; Western blot; etc.)

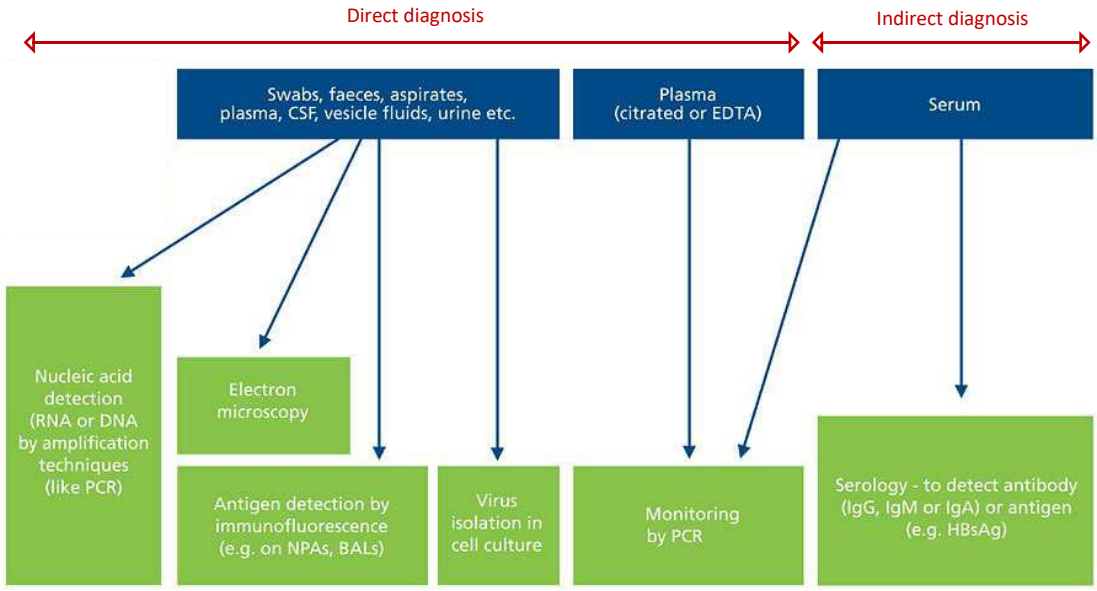


Figure 3. Types of samples commonly used in the laboratory diagnostic routines (blue) and the techniques widely performed(green). Serum samples analyzed with the serology techniques are the only ones representing the indirect way of laboratory diagnosis.

In 2020, with the rise of the worldwide SARS-CoV-2 pandemic and its health emergency, the molecular analysis demand increased, raising several critical issues such as the requirement of special equipment, laboratory reagents and skilled staff. Therefore, the laboratory routine had to adapt quickly and find a wide variety of laboratory methods for a rapid and accurate diagnosis of COVID-19. Based on clinical criteria, COVID-19 cannot be reliably distinguished from infections by other respiratory viruses and bacteria such as influenza, seasonal CoV, adenovirus, *Bordetella pertussis*, *Mycoplasma pneumoniae* etc. In this context, laboratory-based diagnosis assumes a role in the clinical management of patients and the implementation of disease control measures and looking for alternative diagnostic solutions to implement a molecular screening strategy has become a priority for the healthcare systems[23].

When a patient is a suspected SARS-CoV-2 case, two tests commonly performed to prove the patient is infected are:1) the molecular Nucleic Acid Amplification Tests, or NAATs (such as the Reverse Transcriptase-Polymerase Chain Reaction –RT-PCR–; and the isothermal amplification tests like the Loop-mediated isothermal amplification –LAMP–); 2) or the antigen-detection rapid diagnostic tests (Ag-RDTs) (Figure 4). Both these assays can detect the virus, but additional testing is required to differentiate the variants and subvariants.

The SARS-CoV-2 Ag-RDTs are a quick and cheap solution also suggested by the WHO because they are self-tests, and the result can be easily interpreted [24]. In the last years, several easy-to-perform rapid antigen detection tests were developed and used in some countries as first-line laboratory strategies for COVID-19 diagnostic [25,26]. For example, our research group demonstrated that the COVID-19 antigen fluorescence immunoassay (FIA) FREND™ test showed high sensitivity (93.3 %, 95 % CI: 83.8-98.2) and specificity (100% (95% CI: 92.9-100) in nasopharyngeal swabs compared to RT-PCR results [27,28].

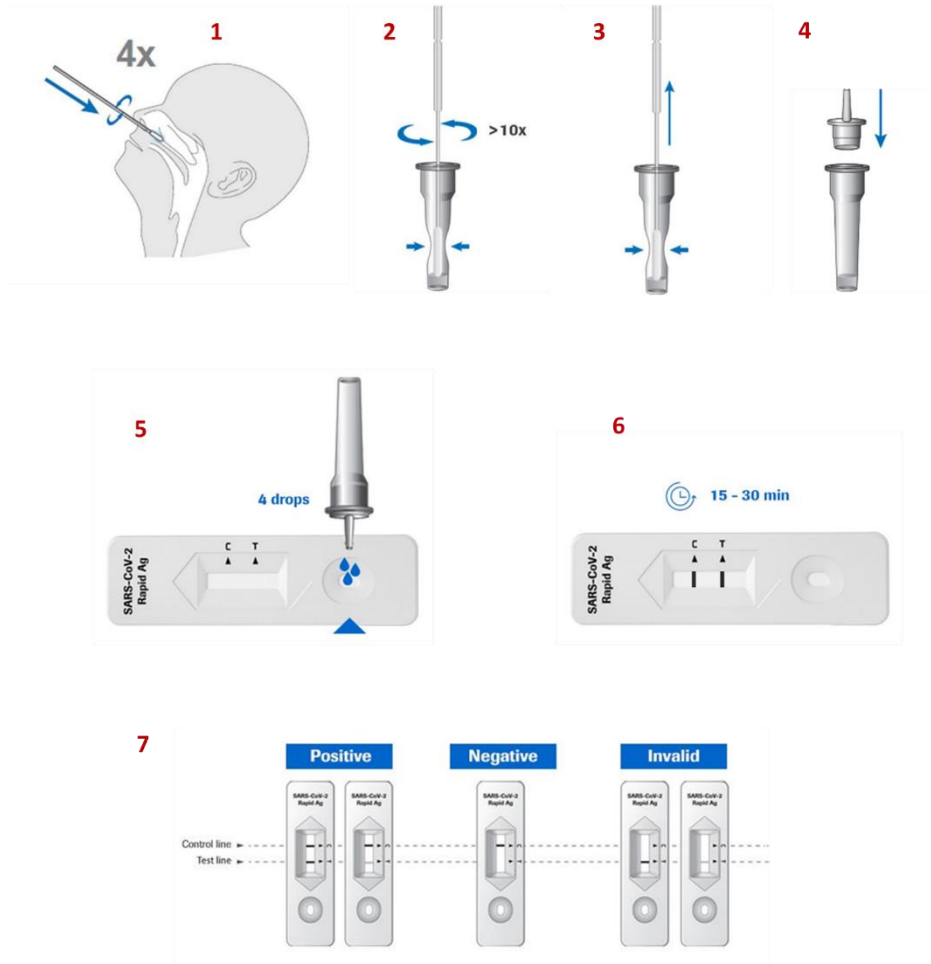


Figure 4. Example of how to perform an antigen-detection rapid diagnostic test (Ag-RDT). 1) The sterile swab is inserted into the nostril and/or mouth by rotating the swab towards the throat until it meets resistance and is removed from the nostril. NB Samples must be taken from both nostrils using the same swab. 2) The swab is inserted into the tube containing the extraction buffer and simultaneously the tube is squeezed and rotated for more than 10 times. 3) The swab is removed by squeezing the sides of the tube to extract the liquid. 4) A cap is pressed firmly onto the tube. 5) The test strip is placed on a flat surface and four sample drops are applied at a 90º angle. 6) The test result can be read within 15-30 min based on manufacturer instructions. 7) A coloured line appears in the upper section of the strip indicating that the test is working correctly. This is the control line (C). Even if the control line is fuzzy, the test should be considered to have been performed correctly. If no control line is visible, the test is invalid. In case of a positive result, a coloured line appears in the lower section of the strip. This is the test line (T).

However, Ag-RDTs are less accurate than NAATs in asymptomatic individuals as they work best for patients with a high viral load during the early phases of illness. Thus, if a molecular test is needed, the first step is to collect a naso/oro-pharyngeal sample. RT-PCR is the gold-standard test for

laboratory diagnosis of SARS-CoV-2, and the RT-PCR kits usually target the E, RdRp and N genes. Once the case is confirmed, the next-generation sequencing (NGS) technique can be used to define the variant and subvariant(S) of the virus (or to discover a new one). Sequencing is now the most frequently used approach for identifying, classifying, and tracking SARS-CoV-2 variations but this is expensive, time-consuming and needs specialist mechanisms and interpretation.

Before introducing vaccination on a vast scale, in the laboratory diagnosis of SARS-CoV-2, immunological assays were also used and the human antibody response to viral infection could be considered an independent prognostic indicator. Indeed, when a virus enters the body and releases viral antigens into circulation, the human immune system produces IgA, IgM and IgG antibodies that are more effective and long-lasting than the antigenic proteins. Serum and other body fluids can be tested for antibodies using serological tests.

1.3 New molecules as a strategy against SARS-CoV-2

Since the SARS-CoV-2 pandemic in 2020, many new molecules and drugs have been developed and studied to treat the infection. Unfortunately, misinformation was spread. Thus, WHO dedicated a section of the official website to common misinformation about general beliefs on SARS-COV-2 and COVID-19 [29]. The most famous example is when, in March 2020, ex-US President Donald Trump promoted the use of chloroquine and hydroxychloroquine, two related anti-malarial drugs, for treating COVID-19. The Food and Drug Administration (FDA) later clarified that they have not approved these drugs to treat COVID-19 but their administration caused severe side effects, illness, and death in many people who used them.

Another example is given by the suggestion that Ivermectin, a medication used to treat parasitic infections, was a possible COVID-19 treatment. Importantly, the concentration of the drug that was required to achieve the antiviral effects was several times higher than what can be achieved in the bloodstream of patients. The promotion of Ivermectin as a COVID-19 treatment has led to increases in Ivermectin-related poisoning reports in the United States, as well as national shortages of the drug

in Australia [30,31]. The highest quality evidence published so far suggests that Ivermectin is ineffective at treating COVID-19 [32]. Nevertheless, in this scenario, were also developed many useful medical devices trying to reduce the viral load in the upper airways and so quicken the patient's recovery. An example is given by the Acid-Oxidizing solution (AOS2020) containing pure and stable HClO in a liquid carrier solution (Tehclo Technology™ APR Applied Pharma Research, Switzerland) [33]. The Hygiene research group at Hospital San Martino, Genoa, tested it in a post-market, interventional, randomized clinical trial to evaluate the safety of AOS2020 and whether it could be a potential solution for upper respiratory hygiene when the SARS-CoV-2 infection occurs [34].

1.4 The Norwegian experience (10/2021-08/2022)

During my PhD, I spent ten months with the Virology Research Group and The Clinical Virology Research Group at the University of Oslo/Oslo University Hospital to gain more experience in basic scientific research. It is known that a significant challenge with the SARS-CoV-2 pandemic is the occurrence of SARS-CoV-2 variants resistant to vaccines and antiviral treatments. Much effort is put into understanding the transmission and pathogenesis of the virus. Still, many questions remain elusive, including which mechanisms SARS-CoV-2 utilizes to interact with the host cell to facilitate replication and pathogenesis. With these premises, I was involved in three main projects where we wanted to provide a new crucial understanding of SARS-CoV-2 and COVID-19 and the virus transmission routes in humans.

1.4.1 Reviving SARS-CoV-2 virions from faeces of COVID-19 positive patients: studying other transmission routes of the virus

Experience from the SARS pandemic outbreak in Hong Kong 21 years ago [35], has taught us that the faecal transmission route is important for understanding the epidemiology of SARS-like viruses, especially if facilities and structures for fragile patients such as the elderly, children, and immunocompromised patients are considered. Several studies have shown that the RNA of SARS-CoV-2 can be found in the faeces of positive patients even up to 47 days from the symptom onset [36].

Similarly, SARS-CoV-2 can survive for up to 25 days in water sources [37,38], and, in Italy, wastewater has been crucial for investigating the early circulation of the virus in the population [39,40]. Although SARS-CoV-2 can be found in faeces and urine, there are no consistent data about culturing stool samples of COVID-19 patients. All these premises considered it is clear how necessary it is to understand 1) the pathogen characteristics associated with virulence; 2) the replication dynamics and in-host evolution of the pathogen; 3) the dynamics of the host response; 4) the pharmacology of antimicrobial or host-directed therapies; 5) the transmission dynamics; 6) and factors underlying individual susceptibility. More specifically, the Virology Research Group (VRG) and the Clinical Virology Research Group (ClinVir) (University of Oslo (UiO)/Oslo University Hospital (OUS), had the possibility of collecting a significant number of faeces samples from the early stages of the pandemic in 2020 until the beginning of 2022 in different hospitals all over Norway, as a part of the Norwegian SARS-CoV-2 study, to carry out an observational study [41].

1.4.2 Drug X as a new solution for defeating SARS-CoV-2: drug testing experiments

From the early stages of the pandemic, the focus worldwide was to find efficient preventive measures to avoid the circulation of SARS-CoV-2 and proper treatments for curing the patients. New therapies and medical devices were adopted, but the vaccine remains the best solution to try to stop the spreading of the virus. However, as some people do not respond to vaccines (or do not want to be vaccinated), developing efficient drugs to treat COVID-19 remains important. With the preclinical "Drug X project", we tested a new drug for treating COVID-19 patients. The study was conducted in cell cultures to test the toxicity and the efficacy of the drug to inhibit SARS-CoV-2 replication compared to vehicle treatment. Components of Drug X are confidential, but it is dissolved in Phosphoric Acid (PhA) and that is why this acid is used as a vehicle and comparison in all the experiments. The PhA, indeed, is an ingredient commonly used for electrolyte replenishment and total parenteral nutrition (TPN) therapy, and for the relief of upset stomach associated with nausea but also in dentistry and orthodontics as an etching solution, to clean and roughen the surfaces of teeth where dental appliances or fillings are placed [42].

2) Aims of study

2.1 Reviving SARS-CoV-2 virions from faeces of COVID-19 positive patients: studying other transmission routes of the virus

Primary objectives

This study is part of the bigger "Norwegian SARS-CoV-2 study" carried out by the VRG and ClinVir (UiO/OUS). This study focuses on:

- Describing the clinical features of the illness or syndrome caused by SARS-CoV-2;
- Describing, when possible, the response to treatment, including supportive care and novel therapeutics;
- Observing the pathogen replication, excretion and evolution, within the host, and identifying determinants of severity and transmission using high throughput sequencing of pathogen genomes obtained from the respiratory tract, blood, urine, stool, CSF and other samples;
- Characterizing, the host responses to infection and therapy over time, including innate and acquired immune responses, circulating levels of immune signalling molecules and gene expression profiling in peripheral blood;
- Understanding transmissibility and the probabilities of different clinical outcomes following exposure and infection;
- Facilitating effective triage and clinical management of patients with relevant infections; determining infectivity and appropriate infection control measures of the various pathogens; developing clinical guidance documents and offering clinical recommendations to policymakers based on evidence obtained;
- Understanding the broader epidemiology of COVID-19 infection through studying potential contacts and asymptomatic individuals in collaboration with the Norwegian Institute of Public Health.

Secondary objectives

One of the secondary objectives of the study is to develop a new protocol for reviving SARS-CoV-2 virions from faeces from COVID-19-positive patients. From our knowledge, this is the first study of this sort and may give new information about the study of other SARS-CoV-2 transmission routes.

2.2 Drug X as a new solution for defeating SARS-CoV-2: drug testing experiments

In this "in vitro" pre-clinical research part of the study we wanted to:

a) Establish the highest concentration Drug X can be used at, the highest concentration where the cells survive and specifically:
   - The chronic exposure to measure toxicity/viability at two-time points incubating the cells with Drug X, for 24 and 72 hours (h) post-treatment (hpt)
   - The acute exposure to measure toxicity/viability at two-time points incubating with Drug X for 1, 2 and 5 h, washing with PBS after each time point and replacing with 2% FCS, P/S, DMEM

b) Establish if Drug X inhibits the virus directly

c) Establish a better knowledge about the origin of the antiviral effects of Drug X on VeroE6 cells.

3) Materials and methods

3.1 Norway exchange period, 10/2021 – 08/2022

3.1.1 The biosafety level 3 laboratory

All the lab work concerning SARS-CoV-2 was performed in a biosafety level three laboratory (BSL-3) at Rikshospitalet, Oslo Universitetssykehus (OUS). BSL-3, in general, is used to work with infectious agents, which may cause serious or potentially lethal diseases because of exposure to a specific pathogen. It is designed with a specific airflow pressure (negative) that prevents infectious

particles from being transmitted through the air from inside the lab to other areas. Safety routines regarding donning and doffing PPE were followed according to international safety instructions [43,44]. The lab has a self-closing double-door access system. In the for-entry room, shoe covers, contagion coat, hair cover, double hand gloves, conical respirator FFP3 mask with valve and a visor mask were put on, in the respective order. Virkon™ disinfectant tablets (LanXess, Germany) were added to water according to the manufacturer's instructions and used for disinfecting virus waste and equipment before taking them carefully in and out of the biosafety cabinet (BSC) inside the lab. After finishing each session in the BSC, the hood was adequately cleaned with 70% EtOH and ultraviolet (UV) light was lit up inside the cabinet for 30 minutes to inactivate any remnants of the virus.

3.1.2 Cell culturing

For all the projects, the VeroE6 (C1008, CRL-1586™) cell line was used. According to ATCC (American Type Culture Collection)[44], the VERO cell line is derived from the kidney of a normal adult African green monkey (*Cercopithecus aethiops*) isolated in 1962. VeroE6 line is a clone cell line that grows adherently. Cells exhibit contact inhibition after forming a monolayer and are therefore valuable for growing slow-replicating viruses.

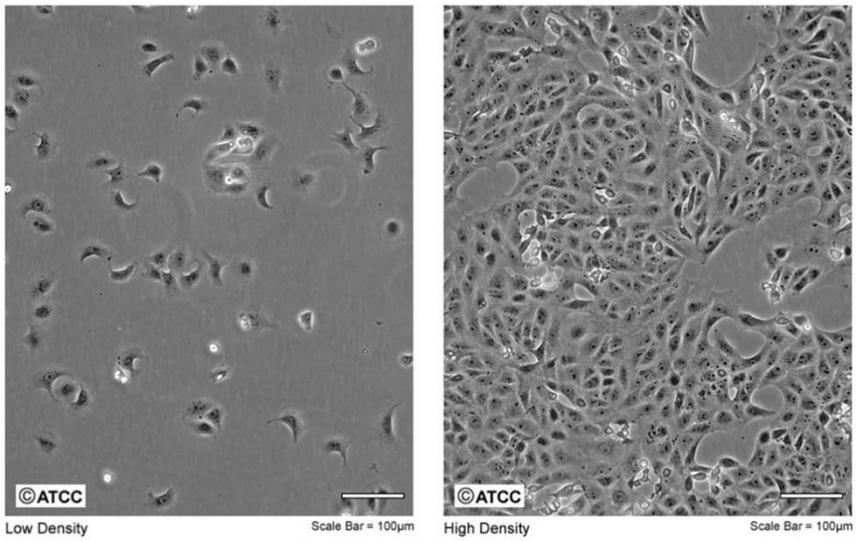


Figure 7. VeroE6 cells displayed in a low density (confluence) and high density (confluence) from ATCC (American Type Culture Collection)[45].

The cells were grown in Gibco™ Dulbecco's Modified Eagle Medium (DMEM) 1X + GlutaMAX™ - I (Thermo Fisher Scientific Inc., US), with 10% Fetal Calf serum (FCS) (Sigma-Aldrich® Solutions, Merck KGaA, Germany), and antibiotics (Penicillin/Streptomycin, Thermo Fisher Scientific Inc., US) to prevent bacterial contamination at a temperature of 37°C; an atmosphere of 95% air and 5% carbon dioxide (CO_2).

3.1.2.1 Revival of cells from liquid-N2

The cells were quickly thawed in a 37°C water bath (cells must still be cold). Then, they were transferred to a 15 mL tube. 5mL growth medium (37°C) (GM) was slowly added in droplets, and then more growth medium (37°C) was added up to 10mL. After gently mixing cells and growth medium by inverting, the cells were pelleted by spinning down the tubes in a centrifuge at 300xg for 5 minutes, and the supernatant (SN) was discarded from the pellet. The cell pellet was resuspended in 2mL growth medium (37°C) and transferred to a cell T75/T175 culture flasks (Thermo Fisher Scientific Inc., US) with an appropriate amount of GM.

3.1.2.2 Subculturing and maintenance

For subculturing, GM was removed and discarded, and the cells were washed with Gibco™ Phosphate Buffered Saline (PBS) (Thermo Fisher Scientific Inc., US) (37°C) twice and then trypsinized with 0.05% Trypsin-EDTA (1X) (Gibco™, Thermo Fisher Scientific Inc., US). Enough trypsin was added to cover the cells and trypsin in excess was removed using a pipette. Then, the flasks were left for 2-5 minutes at 37°C to help the cells detach, then complete GM was added to inhibit the trypsin activity and cells were resuspended by gently pipetting. Lastly, an appropriate amount of the cell suspension was transferred to the new culture flask(s) and an appropriate amount of complete GM was added.
A subcultivation ratio of 1:4 (generally from 1:3 to 1:6) is recommended for Vero E6 cells and medium renewal of two or three times per week.

3.1.2.3 Cryopreservation

Freezing medium (FM) was made by using 40% Complete GM, 50% FCS and 10% Dimethyl sulfoxide (DMSO) (Sigma-Aldrich® Solutions, Merck KGaA, Germany). DMSO is added to avoid cell death because of the formation of ice crystals in the cells. Cells should be actively growing to almost full confluence. The cryopreservation/freezing process was performed quickly to avoid the toxic DMSO destroying the cells while not frozen. After trypsinizing the cells as usual, they were spun down at 300xg for 10 minutes at 4°C. The cells were resuspended in FM and 1mL of cryopreservation medium/cells was added to the cryovials tubes (Thermo Fisher Scientific Inc., US). They were put in a Mr Frosty™ freezing Container (Thermo Fisher Scientific Inc., US) containing 100% isopropanol to the optimal cooling rate of -1°C/minute, at -80°C for one day. The next day, vials were transferred to the liquid nitrogen tank.

3.1.2.4 Viability testing and cell counting

Viability tests and cell concentration measurements were performed using the automated cell counter Countess 3 (Invitrogen, Thermo Fisher Scientific Inc., US) and Trypan Blue (Thermo Fisher Scientific Inc., US). 10µl of Trypan Blue was added to 10µl cell suspension just before the cell count. Due to its negative charge, Trypan Blue only interferes with cells with a destroyed membrane. Therefore, dark cells represent dead cells, while bright cells represent alive cells.

3.1.1.5 Mycoplasma testing with MycoAlert® Mycoplasma Detection Kit (Lonza, Switzerland)

During the experiments, a mycoplasma test was performed weekly to avoid contaminations. All the reagents were brought to RT and 1ml of cell culture SN was to eppy. One well was dedicated to medium only (negative ctr). Then the eppys were spun at 400xg for 5' to pellet the cells and then 30µl of cell-free SN were transferred in triplicates to a ½ area well white plate. 30µl of MycoAlert Reagent (R) was added to each well with SN/medium only and incubated for 5'. The plate was read on the luminometer (1st reading) (Reading A) and afterwards, 30µl of MycoAlert Substrate (S) was added to each well with SN/medium only. After incubation for 10', the plate was read again (2nd reading) (Reading B). The results were calculated as follows: Reading B/Reading A. Cells with a mycoplasma

ratio B/A< 1 were considered as negative for mycoplasma infection; ratio=1-1.2, borderline; ratio>1.2, positive.

3.1.1.6 Viability Assays of drug treated and/or infected cells

Viability assays were performed to measure the cytopathic effect (CPE) in drug-treated and/or SARS-CoV-2-infected VeroE6 cells both chemically (Adenosine 5′-triphosphate (ATP) measurement) and manually by microscopy. Specifically, for the Drug X experiments, the cells were infected with the original Wuhan strain (2019-nCoV/Italy-INMI 1) obtained from the European Virus Collection (EvaG) and first sequenced in China on the 7th of January 2020 [46]. For the SARS-CoV-2 infections, passages 3 and 4 (INMN1, p3 and p4) were used.

For the chemical viability assay, the Viral ToxGLO™ Assay (Promega Corporation, USA) was used. According to the manufacturer's instructions[47] it is a quantifiable method for determining viral-CPE caused by lytic virions in host cells. The assay measures cellular ATP level as an indication of host cell viability. ATP depletion can be assessed and associated with viral burden when CPE results from viral infection. The quantity of ATP observed can be used as an easy way to measure virally generated CPE because it immediately correlates with the number of viable host cells in the culture. The amount of ATP present generates a luminous signal that is similar to a "glow". If there is ATP present in the cells, luciferin will submit light observed by the plate reader, allowing the plate reader to quantify the vitality of the cells. We used a VICTOR® Nivo Multimode Microplate Reader (PerkinElmer Inc., US) to measure the signal (Figure 11). Half a volume (50µl) of ATP Detection Buffer was transferred to each well, and a white sticker was placed on the bottom of the 96-well plate (PerkinElmer ViewPlate-96 or 1/2 Area OptiPlate-96 microplates) (also wells without cells). The program was set up to orbital shake the cells for two minutes to induce cell lysis after adding the cell viability reagent, then to incubate for ten minutes to stabilize the luminescent signal (LUM). The plate reader measured the luminescence signal, and the values were calculated in relation to Mock (not treated).

Then, the "Reed & Muench" approach transformed into an Excel sheet, was used to determine the TCID50. This makes it perfect for high-throughput automated screenings (HTS) [48].

The CPE was also manually checked by an optical microscope for a double-check viability assay (Figure 8).

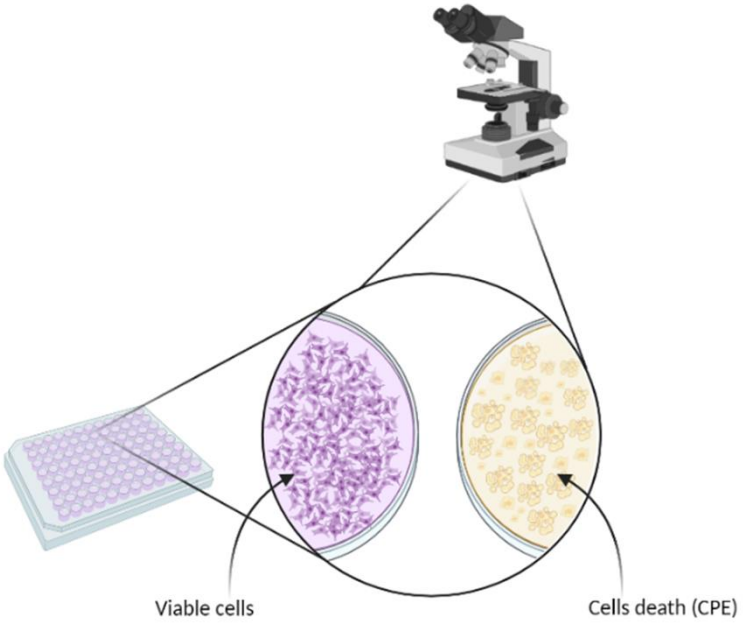


Figure 8. Manually check viability and cytopathic effect (CPE) of VeroE6 cells. A microscope was used to visualize the CPE of the cells in 96-well plates. Figure made with BioRender.com.

3.1.3 Reviving SARS-CoV-2 virions from faeces of COVID-19 positive patients: studying other transmission routes of the virus

3.1.3.1 Sample preparation

Samples were collected retrospectively from the ClinVir Research group at OUS, from hospitalized patients with a SARS-CoV-2 positive respiratory sample, since the pandemic's beginning (March 2020). The samples that were not taken at the first access of the patient in the hospital but during follow-ups were excluded from the experiment.

Before starting testing clinical samples at the official beginning of the experiment, we tested which was the best modality of preparation of the samples and if there was a difference between solid and liquid (Universal Transport Medium™, Copan Diagnostics) samples.

3.1.3.2 SARS-CoV-2 virion precipitation from the supernatant

After the preliminary testing, it was decided to use the Lenti-X Concentrator Precipitation Reagent (TaKaRa, Japan) because it provides a fast and simple method for concentrating SARS-CoV-2 virions in the samples (supernatant) by mixing it with the concentration reagent. This step was followed by incubation (one to 24 hours) and centrifugation in a standard centrifuge (Figure 9). After these first steps, the samples were mechanically filtrated. The procedure was applied regardless of the type of sample used.

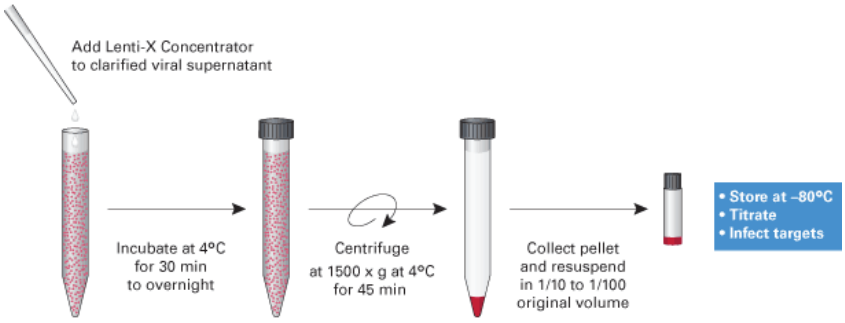


Figure 9. Workflow using the Lenti-X Concentrator Precipitation Reagent (TaKaRa, Japan)

In detail, the samples were thawed at room temperature, filtered using 0.22µm syringe PET filters and 5/10ml syringes, and then the clarified supernatant was transferred to a sterile container. One volume of Lenti-X Concentrator was combined with three volumes of clarified supernatant. The mixture was incubated at 4°C overnight. Then, the samples were centrifuged at 1,500xg for 45 minutes at 4°C, and afterwards, an off-white pellet was visible, containing virions. The supernatant was removed carefully, and the pellet was resuspended in 2 mL Hanks' Balanced Salt Solution, HBSS (Thermo Fisher Scientific Inc., US).

Secondly, the samples were filtered. The filter was pre-wet with HBSS and filtered using 0.22µm syringe PET filters and 5/10mL syringes to make 2 mL aliquots in 1.5mL Eppendorf tubes.

3.1.3.2.1 Infecting Vero-E6 cells for Passage 1 (P1)

Vero-E6 cells from T175 flasks were counted and plated out the day prior to infection with the faeces samples. Cells were plated out in 96-well plates at a concentration of 2-4 x 10^5 cells/mL (or 2-4 x 10^4 cells/ 96 well) obtained using 10% DMEM, P/S, enriched with 2% Antibiotic-Antimycotic (100X) (Thermo Fisher Scientific Inc., US) and 2% Amphotericin B (Thermo Fisher Scientific Inc., US). The cells were infected with 75µL of sample mixed with 125µL of medium per well. Three replicates were performed for every sample.

3.1.3.2.2 Infecting Vero-E6 cells for Passage 2 (P2) and Passage 3 (P3)

When CPE was present (usually between 72 and 120 hours post-infection), 100µL of scraped cells and supernatant, possibly containing secreted virions, were transferred to a new 96-well plate with fresh Vero-E6 cells. The more passages in fresh cells are done, the more the virions will be concentrated in the supernatant.

3.1.3.2.3 Manual extraction of nucleic acids

Total RNA was extracted from secreted supernatants using the NAxtra™ nucleic acid extraction kit (Lybe Scientific, Norway) protocol (see Appendix 1). 100µl of each sample was lysed with NAxtra lysis buffer. The beads containing RNA were resuspended in 20µl nuclease-free water and measured with RT-qPCR using KiCqStart One-Step Probe RT-qPCR ReadyMix, as described in section 3.1.3.2.4.

3.1.3.2.4 Detection of SARS-CoV-2 RNA by RT-qPCR

For quantifying SARS-CoV-2 in the supernatant from infected cells, the KiCqStart One-Step Probe RT-qPCR ReadyMix (manufacturer) was used [49].

For making the master mix, 2.5µl of KiCqStart One-Step Probe RT-qPCR (2X) and 0.083µl of SARS-CoV-2 target primer/probe assay (60X) were mixed for each reaction and added to each respective well in a 96-well PCR plate. Furthermore, 2.8µl of RNA template was added to the respective wells containing 2.2µl master mix, with a final volume of 5µl in each well. The 96-well plate was spun in a

PCR plate spinner (VWR) and analyzed in the qPCR instrument (Thermo Fischer). The RT-qPCR conditions are shown in Table 2.

The high temperature of 50°C for the primer annealing is to give a high specificity and to avoid the secondary structure of the RNA interfering with the synthesis of complementary DNA (cDNA). This step includes a reverse transcriptase step with an RNA-dependent DNA polymerase that generates a strand complementary to the RNA (cDNA). The 2-minute incubation at 95°C is to inactivate the reverse transcriptase and activate the polymerase before continuing with the PCR cycles.

Step	Description	Temperature (°C)	Duration	Cycles
1	Reverse transcription	50	15 min	Hold
2	Enzyme activation	95	2 min	Hold
3	Denaturation	95	15 sec	45
4	Annealing/extension/ signal detection	60	1 min	

Table 2. Parameters for one-step RT-qPCR using KiCqStart One-Step Probe RT-qPCR kit. The temperature of 50°C is to achieve an optimal synthesis of complementary DNA (cDNA). For inactivating the reverse transcriptase (RT) and for activating the DNA polymerase, the temperature is set to 95°C.

Every sample was tested in triplicates and negative control (water), and a standard curve (SC) were included (INMN1, P4 10-fold dilutions). To be able to detect and quantify the product in real-time, a thermocycler registers the fluorescent molecules added to the sample and quantifies the excited molecules that emit the fluorescent light during the PCR [50]. The number of cycles before the fluorescent signal rises above the background levels is defined as cycle threshold (CT) values. The CT values are explained by the concentration of the template. At the start, the concentrations of the template are too low for detection. As the amplification continues, a growth phase arises followed by a final plateau phase.

3.1.4 Drug X as a new solution for defeating SARS-CoV-2: drug testing experiments

In this “in vitro” pre-clinical research part of the study we wanted to:

a) Establish the highest concentration Drug X can be used at, the highest concentration where the cells survive

Phosphoric acid (PhA), as negative vehicle control, Drug X and culture medium (Mock), as representative of the 100% viability, were compared for the experiment.

For every solution, seven different concentrations in three-fold dilutions were made: from 10% (triplicates) concentration to 5 - 0.156 % (quadruplicates) concentration.

Eight 96-well plates with VeroE6 cells ($2x10^5$ cells/mL/$2x10^4$ cells/well) were plated out (Figure 11) to test:

- The chronic exposure to measure toxicity/viability at two-time points, incubating the cells with Drug X, 24 and 72 hours (h) post-treatment (hpt)
- The acute exposure to measure toxicity/viability at two-time points, incubating with Drug X for 1, 2 and 5 h, washing with PBS after each time point and replacing with 2% FCS, P/S, DMEM.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		10% PhA	PhA 5%	→	→	→	PhA 0.625%	→	→	→	0	No cells, only medium
C			PhA 2. 5%	→	→	→	PhA 0.3125%	→	→	→		
D			PhA 1.25%	→	→	→	PhA 0.156%	→	→	→		
E		10% Drug X	Drug X 5%	→	→	→	Drug X 0.625%	→	→	→		
F			Drug X 2.5%	→	→	→	Drug X 0.3125%	→	→	→		
G			Drug X 1.25%	→	→	→	Drug X 0.156%	→	→	→		
H												

Figure 11. Plate set-up example. Cells in yellow were treated with 100µl PhA, per well, whereas the cells in red were treated with 100 µl Drug X, per well. Every plate had a column with not treated VeroE6 cells and medium (green) and a column with no cells, only medium.

After incubation, the Viral ToxGLO™ Assay (Promega Corporation, USA) was performed. Then, the plate was put in the VICTOR® Nivo Multimode Microplate Reader (PerkinElmer Inc., US) (Figure 12), and the "shake&incubate" program was run on the dedicated software (12') to increase the

reproducibility of results in replicate wells before measuring luminescence. The luminescence was measured, and the viability values were calculated by plotting net RLU (relative luminescence units) values after subtracting the average of blank wells (see the results section).

b) Establish if Drug X inhibits the virus directly

Once the maximum dose had been identified whether Drug X inhibited the virus directly was tested. The PhA was used as the negative control at the equivalent pH of the diluted Drug X solution.

Protocol

The pH of PhA and Drug X was measured before incubating the Vero E6 cells with different corresponding concentrations (2.5% - 0.078%) for 5 hours. Then, cells were infected with 50µl of virus dilution (added 2µl of INMN1, P4 in 5 ml medium 2% FCS DMEM). After 72 hours, the toxicity/viability was tested with the Viral ToxGLO^{TM} Assay as described above.

Results:

c) Establish a better knowledge about the origin of the antiviral effects of Drug X on VeroE6 cells

The effects of the Drug X 1.25% and 0.156% concentrations were compared, at constant pH, against the same concentrations of:

- Phosphoric acid (H3PO4), PhA
- Hydrochloric acid (HCl), HCl
- Sulphuric acid (H2SO4), SuA

Drug X and phosphoric acid acted as positive controls. At constant pH, the proton concentration effect is constant. This allows determining the impact of the acids counter ions, phosphate versus chloride and sulphate. Eventually, it tells if the antiviral effect is due to the acidity, the counter ion, or a combination of the two. Drug X is dissolved in PhA and we wanted to test the potential inhibitory effect on SARS-CoV-2 infection. In this series of experiments, we focused on a range of Drug X concentrations that avoided a very low pH and so antiviral effect.

Protocol

Vero E6 cells were incubated with drug 1.25% (pH 1.5 or same as 0.156 % Drug X) or 0.156% (pH 6.17 or same as 0.156 % Drug X) concentration in 2% FCS in DMEM (PerkinElmer 1/2 Area OptiPlate-96 were used) for 5 hours (Figure 13). Then, drugs were removed, and the cells were infected with the standard virus dilution of 0.2 or 0.3 moi (added 2µl of INMN1, P4 in 5 ml 2%FCS DMEM) and also a reduced amount (add 0.5µl of INMN1, P4 in 15 ml 2% FCS in DMEM) of the virus was used as a comparison. The toxicity/viability 72 hours post-infection was measured with the Viral ToxGLO™ Assay (Promega Corporation, USA).

– SARS-CoV-2 **+ SARS-CoV-2**

	1	2	3	4	5	6	7	8	9	10	11	12
A		**0**	**PhA**	**HCl**	**SuA**	**Drug X**	**0**	**PhA**	**HCl**	**SuA**	**Drug X**	
B												
C												
D												
E												
F												
G												
H												

Figure 13. Plate set-up example. All cells (except the "0" yellow column) were treated with an acid/Drug X. The cells in red were also infected with SARS-CoV-2 after the 5hs incubation.

4) Results

4.1 Reviving SARS-CoV-2 virions from faeces of COVID-19 positive patients: studying other transmission routes of the virus. Preliminary results

For this experiment, 42 faeces samples (two solid, 40 swabs with transport medium) were analyzed. Five samples were excluded because they were not first-access samples but follow-ups. For every sample, three passages of the virions were performed in Vero-E6 cells. This way, the virus was amplified, as well as the other microorganisms from the faecal sample were gradually removed using antibiotics and antimycotics. Figure 14 shows the results from the viability assay. In total, viable

virions were revived from five samples. All the samples from #6 to #42 showed reduced viability compared to Mock in at least one passage, indicating that there was viral-CPE caused ideally by lytic virions in host cells. About 81% (30/37) of the samples showed reduced viability in all the cell passages and 11% (4/37) showed reduced viability only in passage 3. In general, for passage 1 and 2, the viability was lower compared to passage 3 and Mock. At the moment of the writing of this PhD thesis, 68% (25/37) of the samples were analyzed with RT-qPCR (the rest will be processed at a later time point).

As many of the RT-qPCR values had good triplicates results but were outside the lower linear range, it was not possible to obtain a linear correlation and so to calculate them using the standard curve method as planned. However, it indicates that there was virus present (especially for samples with valuable values for two passages or more). As a matter of fact, even if from the diagnostics point of view having just 2/3 results is not enough to assess the positivity of a patient, it is interesting to consider these samples because it could suggest that virus may be present at a low concentration. Also, in the samples where the triplicates were too far from each other or only one was positive, there might be virus as well. Further analysis should be performed.

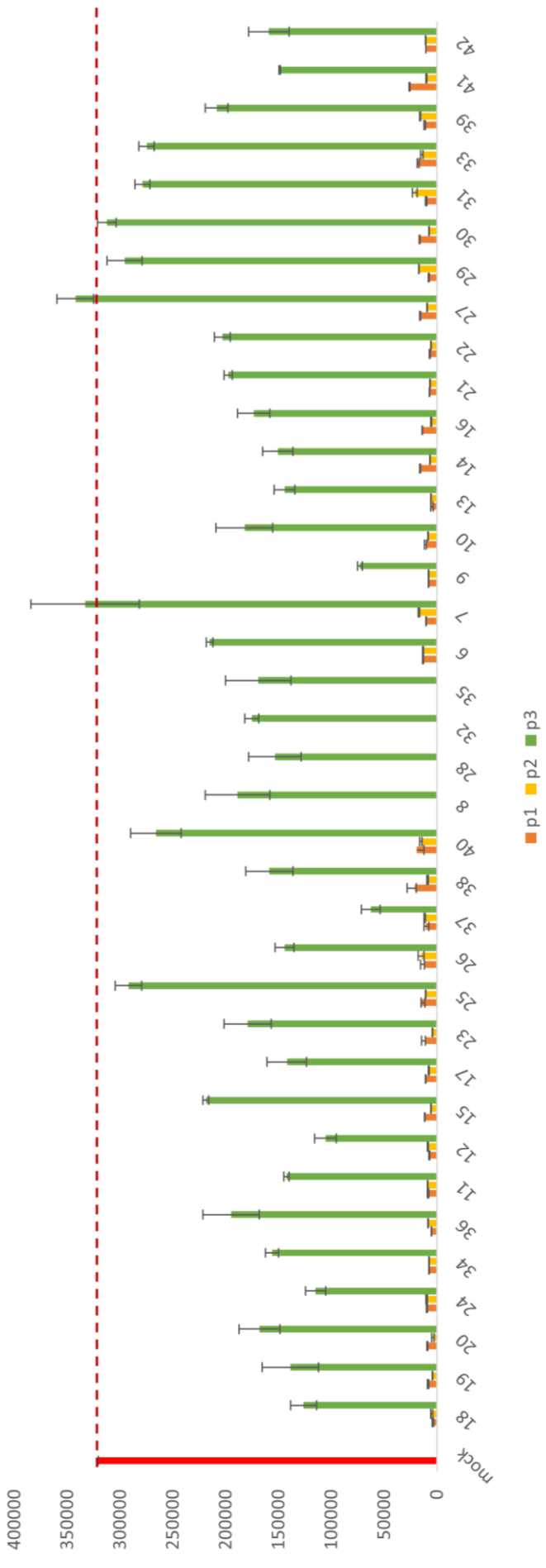


Figure 14. Results for the experiment concerning the revival of SARS-CoV-2 virions from faeces of COVID-19 positive patients. For every sample Passage 1 (P1) in orange, 2 (P2) in yellow, and 3 (P3) in green, in Vero-E6 cell culture are reported. The control (MOCK) is shown in red. All the samples from #6 to #42 showed reduced viability compared to Mock in at least one passage, indicating that there was viral-CPE caused ideally by lytic virions in host cells

4.2 Drug X as a new solution for defeating SARS-CoV-2: drug testing experiments

a) Establish the highest concentration Drug X can be used at, the highest concentration where the cells survive

To perform the toxicity/viability test at two time points (24h and 72h), the cells were incubated with Drug X or PhA for 24 or 72 h, simulating a chronic treatment. At 24 hours post-infection, PhA showed a good inhibitory effect for several dilutions (5%-1.25%) (Figure 15 *a)*) whereas Drux X was effective just for the 5% dilution (Figure 15 *b)*). After 72 hours, for both PhA and Drug X, it was clear that, regardless the dilution of the solution, the toxicity was too high (full CPE) (Figure 15 *c), d)*). So, it was not possible to determine the highest concentration of Drug X tolerated by the cells.

Then, the acute exposure was simulated. The toxicity/viability was measured at two-time points, incubating the cells with Drug X or PhA for 1, 2 and 5 h. Based on the results from the acute treatment (Figure 16), the 10% and 5% dilution should have been excluded from the experiment because the CPE was very high probably due to high toxicity.

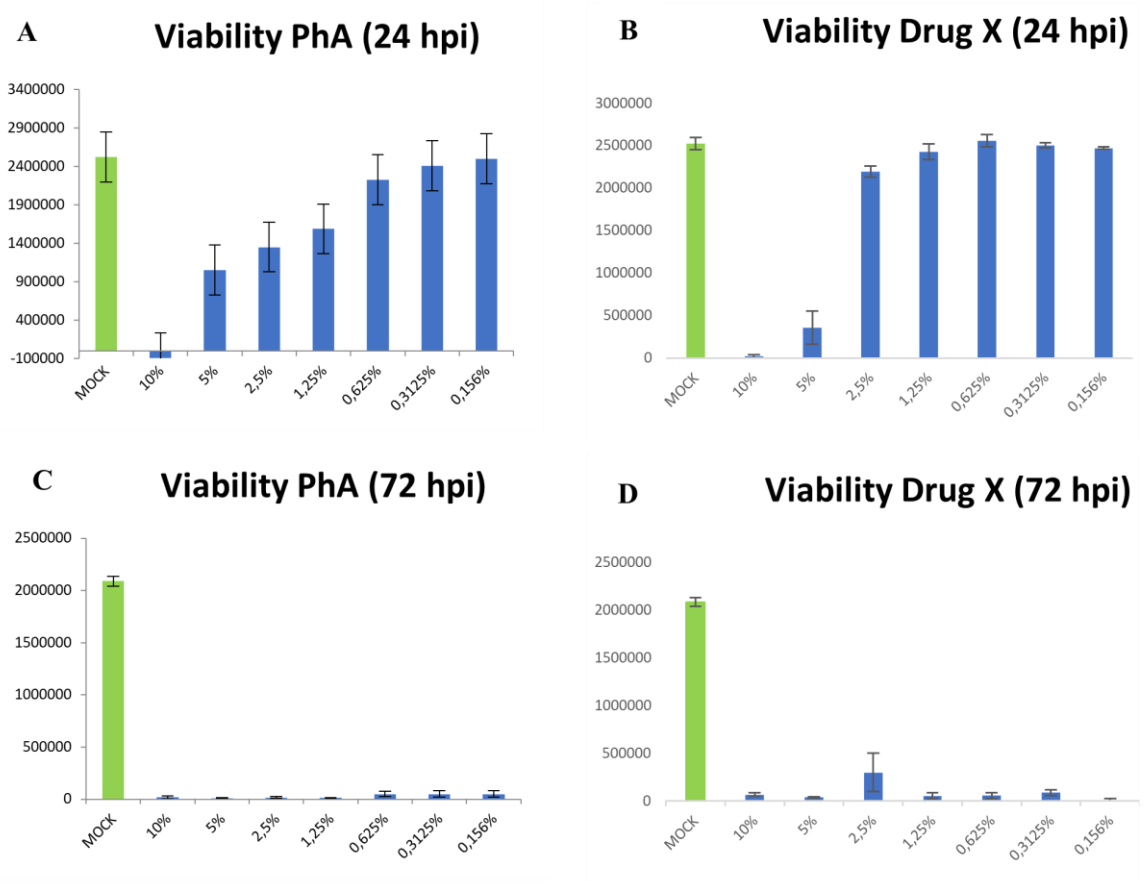


Figure 15. Results of the Viral ToxGLO™ Assay (Promega Corporation, USA) to measure the cell viability and toxicity. Data were acquired at two time points: 24h post-treatment and 72hours post-treatment. The Mock samples (green column) were used as a comparison. At 24 hours post-infection, a) PhA showed a good inhibitory effect for several dilutions (5%-1.25%), whereas b) Drux X was effective just for the 5% dilution. After 72 hours, c) and d) for both PhA and Drug X, it was clear that regardless of the dilution of the solution, the toxicity was too high (full CPE).

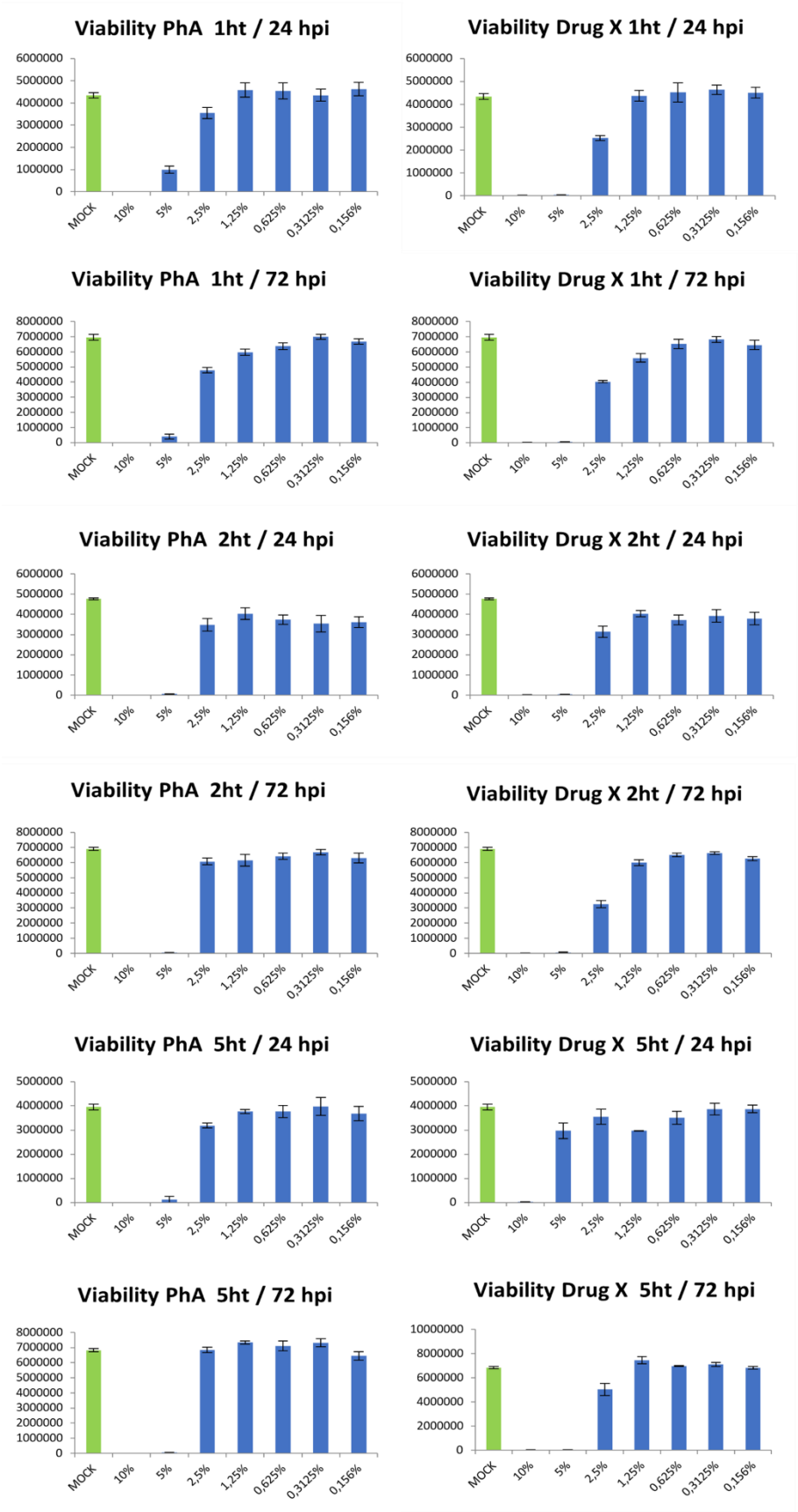
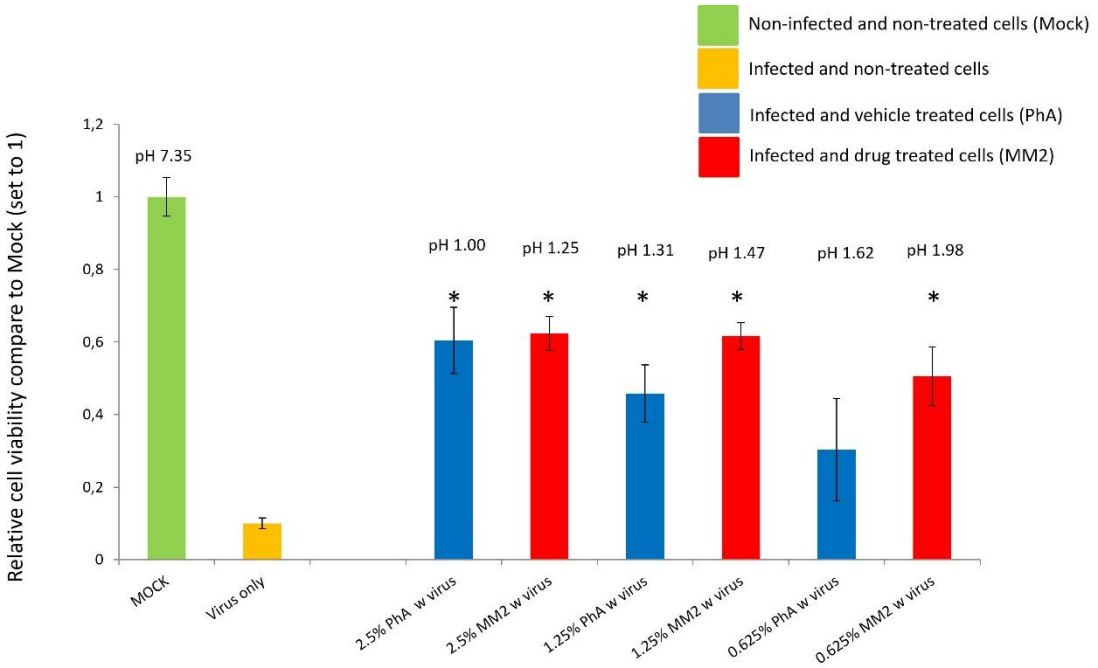


Figure 16. Cells were treated for 1h with PhA or Drug X and then the Viral ToxGLO™ Assay (Promega Corporation, USA) was performed 24 hours post-treatment (hpt) to measure the cell viability and toxicity. The Mock column (green) was used as a comparison.

b) Establish if Drug X inhibits the virus directly

Once the maximum dose experiment was performed, we tested whether Drug X inhibits the virus directly. The PhA was used as the negative vehicle control at the equivalent pH of the diluted Drug X solution.

Adherent Vero E6 cells were treated with different corresponding concentrations (2.5 %- 0.078%) of PhA and Drug X for 5 hours. Then, treated cells were infected with virus INMN1, P4 for 72 hours, and the inhibition of virus infection was measured by cell viability assay (Figure 17 and 18).



* p<0.05, increased viability for infected and treated cells (vehicle or drug) compared to only infected cells. Paired TTEST
No significance between vehicle or drug at the same concentrations. Paired TTEST

Figure 17. Treatment with higher dilutions (2.5% - 0.625%) of PhA and Drug X. The cells were treated for 5h with PhA or Drug X and then infected with the virus. The Viral ToxGLO™ Assay (Promega Corporation, USA) was performed 72 hours post infection (hpi) to measure the cell viability and toxicity. The Mock column (green) was used as a comparison. In general, no difference in inhibitory effect of SARS-CoV-2 infection was observed between vehicle (PhA) and drug (Drug X) at higher concentrations (2.5 %- 0.625%).

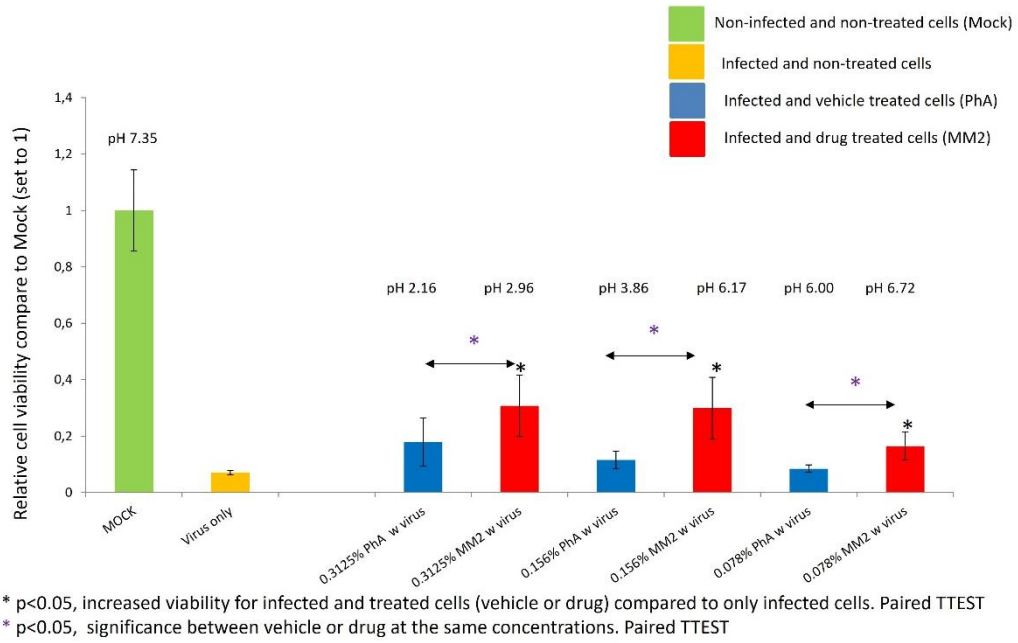


Figure 18. Treatment with lower dilutions (0.3125%-0.078%) of PhA and Drug X. The cells were treated for 5h with PhA or Drug X and then infected with the virus. The Viral ToxGLO™ Assay (Promega Corporation, USA) was performed 72 hours post infection (hpi) to measure the cell viability and toxicity. The Mock column (green) was used as a comparison. An inhibitory effect by Drug X compared to vehicle was observed. However, the inhibitory effect compared to non-infected cells was about 30%.

In general, no difference in inhibitory effect of SARS-CoV-2 infection was observed between vehicle (PhA) and drug (Drug X) at higher concentrations (2.5 %- 0.625%) (Figure 17). However, there was a good inhibitory effect by PhA only, and the inhibitory effect compared to non-infected cells was more than 60% for 2.5% PhA. At lower concentrations (0.3125%-0.078%) (Figure 18) an inhibitory effect by Drug X compared to vehicle was observed. However, the inhibitory effect compared to non-infected cells was about 30%. In general, efficient inhibition of CPE (most likely inhibition of virus replication) was seen when SARS-CoV-2 infected Vero E6 cells were treated with 1.25% and 0.156% Drug X, pH 1.47 and 6.17 respectively.

c) Establish better knowledge about the origin of the antiviral effects of Drug X on VeroE6 cells

The effects of the Drug X 1.25% and 0.156% concentrations were compared, at constant pH, against the same concentrations of PhA, HCl and SuA.

In general, no difference in inhibitory effect of SARS-CoV-2 infection was observed between vehicles (PhA, HCl, SuA) and Drug X both at higher and lower concentrations (1.25% - 0.156%). However, at a concentration 1.25% of vehicle and low concentration virus (0.5µl), there was a slight vehicle effect in the cells that were not infected; however, Drug X does not seem to be effective at all in the infected cells. Moreover, there was a good CPE effect by SARS-CoV-2 at higher concentration (2µl) compared to the lower concentration (0.5µl).

Thus, both 1.25% and 0.156% concentrations of Drug X do not seem to be effective compared to other vehicles and with MOCK (regardless of the concentration of the virus used for the infection) (see Figure 19).

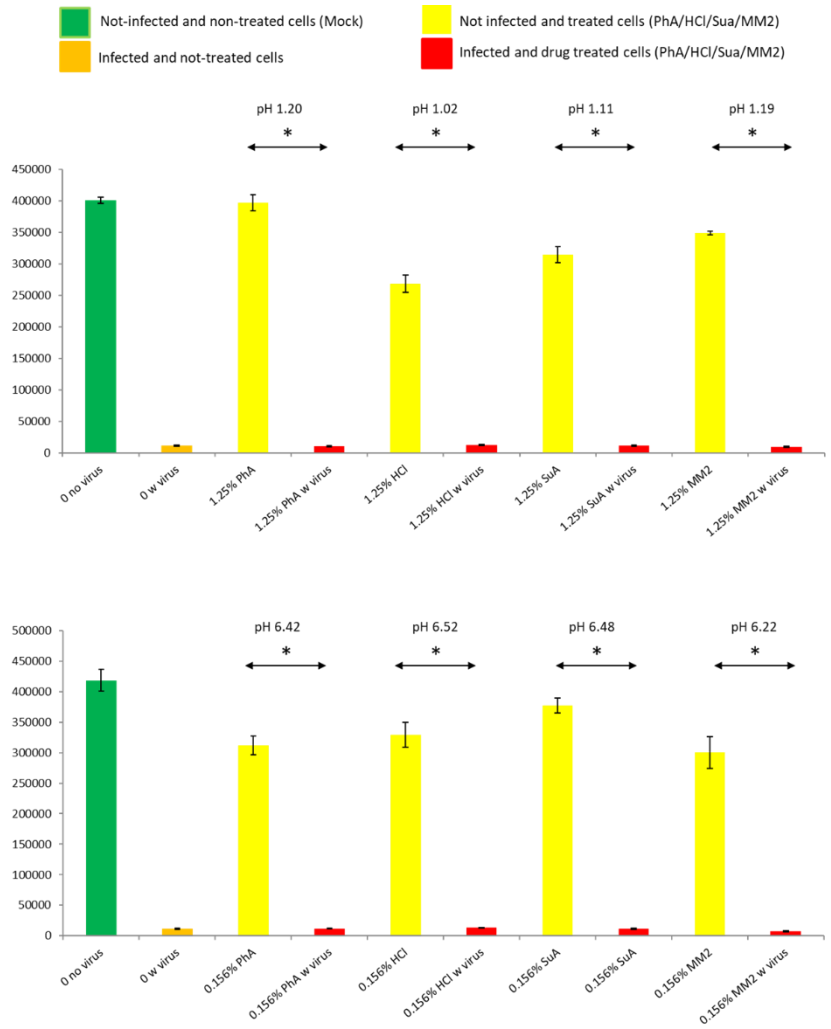


Figure 19. The effects of Drug X 1.25% and 0.156% concentrations were compared, at constant pH, against the same concentrations of PhA, HCl and SuA. a) 1.25% drug vs vehicles. b) 0.156% drug vs vehicles

5) Discussion

The first part of the aims of this PhD thesis was to investigate the possibility of reviving living virions from faeces of COVID-19 positive patients using the Vero-E6 cells. In addition, a new laboratory protocol was developed and evaluated on-field.

It is widely demonstrated in the literature that the main way of human-to-human transmission of SARS-CoV-2 is through contaminated respiratory secretions (droplets and aerosols). But some evidence points to faecal-oral contamination as a potential secondary human transmission route for the virus. First, clinical evidence of multiple infected patients exhibiting signs of gastroenteritis [51,52], significant stool excretion of viral genomes [53], histological analyses showing the presence of virions and infiltration of pro-inflammatory cells in the duodenum and rectum [54,55], had demonstrated the virus intestinal replication. Furthermore, intestinal organoids and intestinal human cell lines as well as animal models [56,57] have all shown evidence of viral replication. It is also hypothesized that the long-COVID may be caused by an intestinal viral reservoir [58]. Such a possibility was supported by the experience of the SARS-CoV faecal-oral infection in 2003 [59]. Despite this data, there are just a few cases of SARS-CoV-2 faecal shedding reported that have been proven to cause infection [60–63]. SARS-CoV-2 must be able to remain contagious in faeces and eventually start replication in the host gut or respiratory tract to establish transmission from contact with infected faeces. SARS-CoV-2 has a relatively fragile lipid membrane in comparison to enteric viruses. As well as influenza viruses, SARS-CoV-2 may be protected against such inactivation by mucosal secretions or food ingestion [64] as Zang R et all (2020) demonstrated in their study [65]. Our preliminary results showed that for the 13,5% of faeces samples it was possible to revive SARS-CoV-2 viable virions using the Vero-E6 cell line. This indicated the possibility for positive patients to transmit the virus to naïve people. Our data are in contrast with Wurtzer S et al (2022) [57] who used hamsters as an animal model to examine faecal-nasal transmission following intranasal inoculation of faecal materials infected with SARS-CoV-2 RNA. They demonstrated that despite a significant genomic viral input, inhaling stool preparations from infected hamsters did not cause infection in new animals. These findings supported earlier studies using the same animal model showing that the replicative virus or the least infectious dosage for this model was not present in the faeces of infected animals [56]. For our experiment, the lower CPE in

passage 3 detected by the cell viability assays is probably due to an early harvest, so the virus could not amplify enough and even if with the manual CPE checks a clear CPE was visible, it was not so consistent when analyzed with the Viral ToxGLO™ Assay (Promega Corporation, USA). We suppose that more passages in cell culture are needed to amplify the virus further so it can be detectable by RT-qPCR and not just by the luminescence assay. Moreover, from the statistical point of view, it would be necessary to perform the same experiment on a larger pool of samples. Also, the variability in faeces composition could be related to differences in diet, genetic background or gut microbiota or an alteration of this latter composition, throughout infection [66,67]. Then, the gut microbiota diversity and physico-chemical parameters of stools could also explain the interindividual variability of SARS-CoV-2 inactivation in faeces, as previously reviewed [68]. Comparing the viral persistence in the stools of infected patients with divergent gastric symptoms may provide insight into the factors that may promote enteric replication of SARS-CoV-2.

The second part of the aims of this PhD thesis was focused on a different topic. Drug X was tested as a new solution for defeating SARS-CoV-2. It is an acid solution and there is not much literature regarding Drug X, but it is documented the effect of the strong acids that we used as a comparison. For example, inorganic and organic derivatives of SuA help to inhibit the growth of bacteria, yeast, moulds, or other microorganisms in food, but they are also widely used in the medical practice as a disinfectant or as part of different drugs [69]. Whereas, HCl is commonly used to adjust the pH of injections and ophthalmic solutions [70], and PhA is an ingredient used for electrolyte replenishment and total parenteral nutrition (TPN) therapy and the relief of upset stomachs associated with nausea [42].
Given all the overmentioned information, there are different opinions about what is helping SARS-CoV-2 to infect and replicate successfully in host cells. Environmental factors which affect viral fusion include external pH, temperature, humidity and osmolarity. These factors are discussed along with their implications on mucus thick layer, proteases, the abundance of sialic acid, vascular permeability and exudate/oedema.

SARS-CoV-2 shares many of the characteristics of coronavirus and the general mechanism for SARS-CoV-2 infection has been identified based on knowledge of the other SARS-CoV and MERS-CoV. Indeed, there are many studies about the influence of pH and virus replication in humans. In general,

it is common opinion that low or high pH levels are inhibiting SARS-CoV-2 infection in the human airways [71,72] and that a pH range between 7.5 and 9 is the best condition for both SARS-CoV and SARS-CoV-2 to maintain complex structures such as ACE2 and the S protein [73].

For example, Cicconetti et al. (2021) [74] have illustrated one method of achieving physicochemical microenvironment modulation of the airway tissue, the first route of virus infection, through the administration of nebulized warm sodium bicarbonate by inhalation. It is an alkaline solution of approximately 8.5 pH and is an alkalinizing agent widely used in the treatment of metabolic acidosis.

On the contrary, Giarratana et al. [33] tested an acid-oxidizing solution containing hypochlorous acid on human coronavirus SARS-Cov-2 in Vero 76 cells with good results. Then, Panatto et al. carried out a phase IV clinical trial demonstrating that the overmentioned HClO-based solution in a sprayable formulation was effective in reducing the SARS-CoV-2 viral load in patients with mild COVID-19 disease [34].

In this PhD thesis, our results showed that it was not possible to establish the highest Drug X concentration where the cells survive. After 72h from infection with SARS-CoV-2, the effect of the virus on the cell culture was so significant that the presence of the drugs (both PhA and Drug X) was not relevant. Apparently, in a chronic condition, Drug X is not effective. We obtained a comparable result also when the acute treatment was simulated. At 72hpi the culture showed full CPE and no sign of virus inhibition caused by the drug. Then, we established if Drug X was able to inhibit the virus directly. In general, no difference in inhibitory effect of SARS-CoV-2 infection was observed between vehicle (PhA) and drug (Drug X) at higher concentrations (2.5 %- 0.625%) (Figure 17). On the other hand, there was a good inhibitory effect by PhA only, and the inhibitory effect compared to non-infected cells was more than 60% for the 2.5% concentration of PhA. At lower concentrations (0.3125%-0.078%) (Figure 18) an inhibitory effect by Drug X compared to vehicle was observed. However, the inhibitory effect compared to non-infected cells was about 30%. In general, efficient inhibition of CPE (most likely inhibition of virus replication) was seen when SARS-CoV-2 infected Vero E6 cells were treated with 1.25% and 0.156% Drug X, pH 1.47 and 6.17 respectively. Eventually, the origin of the antiviral effects of Drug X on VeroE6 cells was explored. Thus, both 1.25% and

0.156% concentrations of Drug X do not seem to be effective compared to other vehicles and with MOCK (regardless of the concentration of the virus used for the infection).

In conclusion, nevertheless, our results showed that Drug X does not affect SARS-CoV-2 infection, this research lays the foundations to explore other similar solutions as a valuable treatment for COVID-19. Indeed, early and timely intervention with specific treatments controlling virus replication and inflammation might help to modify the course of the disease progression, and improve patients' recovery time and rate, ultimately avoiding the risk of hospital collapse sadly experienced by most countries worldwide.

Thus, infection modalities are more complicated than so far reported, and more appropriate knowledge of the infection process is necessary for new drug discoveries not only for pre-exposure prophylaxis but also in protecting against SARS-CoV-2 antigenic drift and future virus pandemics.

6) Conclusion and limitations

From the present PhD thesis preliminary results, we conclude that human faeces seem to be a favourable environment for the persistence of SARS-CoV-2 in an infectious form when Vero E6 cells are used. Although SARS-CoV-2 transmission after exposure to naturally contaminated faeces is unlikely, we observed an inter-individual variability, and we cannot rule out the possibility of persistent viral particles in human or animal faeces, especially shortly after their excretion. To estimate the risk of contamination after exposure to contaminated faeces, the infectious viral titer in stools and the minimal infectious dose remain to be determined. Our results must also be implemented considering the different circulating virus variants which have shown differences in pathogenicity, transmissibility, and changes in tropism. If faecal-nasal contamination was involved in the spread of the virus in wildlife, this would potentially open new pathways for the virus's evolution.

Despite existing data in the literature demonstrating valuable chemical components for preventing and treating COVID-19, the experiments herein presented did not show relevant results regarding the efficacy of Drug X against SARS-CoV-2 infection.

A detailed exploration of the cellular and molecular functions of SARS-CoV-2 infection is still necessary to understand virus-host cell interactions to provide novel ways to treat COVID-19.

7) Bibliography

(1) Hu, B.; Guo, H.; Zhou, P.; Shi, Z.-L. Characteristics of SARS-CoV-2 and COVID-19. *Nat Rev Microbiol 19* **2021**, 141–154. https://doi.org/10.1038/s41579-020-00459-7.

(2) Fuk-Woo Chan, J.; Kok, K.-H.; Zhu, Z.; Chu, H.; Kai-Wang To, K.; Yuan, S.; Yuen, K.-Y. Genomic Characterization of the 2019 Novel Human-Pathogenic Coronavirus Isolated from a Patient with Atypical Pneumonia after Visiting Wuhan. *Emerg Microbes Infect* **2020**, *9:1*, 540–540. https://doi.org/10.1080/22221751.2020.1737364.

(3) Izadpanah, A.; Rappaport, J.; Datta, P. K. Epitranscriptomics of SARS-CoV-2 Infection. *Front. Cell Dev. Biol. 10:849298.* https://doi.org/10.3389/fcell.2022.849298.

(4) Centres for Disease Control and Prevention (CDC). *People with Certain Medical Conditions | CDC*. https://www.cdc.gov/coronavirus/2019-ncov/need-extra-precautions/people-with-medical-conditions.html (accessed 2022-11-21).

(5) World Health Organization (WHO). *Coronavirus disease (COVID-19)*. https://www.who.int/health-topics/coronavirus#tab=tab_1 (accessed 2022-11-21).

(6) Centres for Disease Control and Prevention (CDC). *Symptoms of COVID-19*. https://www.cdc.gov/coronavirus/2019-ncov/symptoms-testing/symptoms.html (accessed 2022-11-21).

(7) Huang, C.; Wang, Y.; Li, X.; Ren, L.; Zhao, J.; Hu, Y.; Zhang, L.; Fan, G.; Xu, J.; Gu, X.; Cheng, Z.; Yu, T.; Xia, J.; Wei, Y.; Wu, W.; Xie, X.; Yin, W.; Li, H.; Liu, M.; Xiao, Y.; Gao, H.; Guo, L.; Xie, J.; Wang, G.; Jiang, R.; Gao, Z.; Jin, Q.; Wang, J.; Cao, B. Clinical Features of Patients Infected with 2019 Novel Coronavirus in Wuhan, China. *The Lancet* **2020**, *395* (10223), 497–506. https://doi.org/10.1016/S0140-6736(20)30183-5.

(8) European Centre for Disease Prevention and Control. *EU case definitions - Influenza including Influenza A(H1N1)*. https://www.ecdc.europa.eu/en/surveillance-and-disease-data/eu-case-definitions (accessed 2022-11-21).

(9) European Centre for Disease Prevention and Control. *Severe Acute Respiratory Infections (SARI) Reporting Protocol Version 3.6*.

(10) Wu, Q.; Li, Q.; Lu, J. A One Health Strategy for Emerging Infectious Diseases Based on the COVID-19 Outbreak. *J Biosaf Biosecur* **2022**, *4* (1), 5–11. https://doi.org/10.1016/J.JOBB.2021.09.003.

(11) Kampf, G.; Todt, D.; Pfaender, S.; Steinmann, E. Persistence of Coronaviruses on Inanimate Surfaces and Their Inactivation with Biocidal Agents. *Journal of Hospital Infection* **2020**, *104* (3), 246–251. https://doi.org/10.1016/J.JHIN.2020.01.022.

(12) World Health Organization (WHO). *Water, sanitation, hygiene, and waste management for SARS-CoV-2, the virus that causes COVID-19*. https://www.who.int/publications/i/item/WHO-2019-nCoV-IPC-WASH-2020.4 (accessed 2022-11-21).

(13) Samet, J. M.; Burke, T. A.; Lakdawala, S. S.; Lowe, J. J.; Marr, L. C.; Prather, K. A.; Shelton-Davenport, M.; Volckens, J. SARS-CoV-2 Indoor Air Transmission Is a Threat That Can Be Addressed with Science. *Proc Natl Acad Sci U S A. 2021 Nov 9;118(45):e2116155118.* . https://doi.org/10.1073/pnas.2116155118.

(14) Centres for Disease Control and Prevention (CDC). *COVID-19 Pandemic Planning Scenarios*. https://www.cdc.gov/coronavirus/2019-ncov/hcp/planning-scenarios.html (accessed 2022-11-21).

(15) Petersen, E.; Koopmans, M.; Go, U.; Hamer, D. H.; Petrosillo, N.; Castelli, F.; Storgaard, M.; al Khalili, S.; Simonsen, L. Comparing SARS-CoV-2 with SARS-CoV and Influenza Pandemics. *Lancet Infect Dis* **2020**, *20* (9), e238–e244. https://doi.org/10.1016/S1473-3099(20)30484-9.

(16) Wilder-Smith, A. COVID-19 in Comparison with Other Emerging Viral Diseases: Risk of Geographic Spread via Travel. *Trop Dis Travel Med Vaccines* **2021**, *7* (1), 1–11. https://doi.org/10.1186/S40794-020-00129-9/METRICS.

(17) EpiCentro - Istituto Superiore di Sanità. *Infografica web - Dati della Sorveglianza integrata COVID-19 in Italia*. https://www.epicentro.iss.it/coronavirus/sars-cov-2-dashboard (accessed 2022-11-21).

(18) Ministero della salute, I. *Covid-19 - Situazione in Italia*. https://www.salute.gov.it/portale/nuovocoronavirus/dettaglioContenutiNuovoCoronavirus.jsp?lingua=italiano&id=5351&area=nuovoCoronavirus&menu=vuoto (accessed 2022-11-21).

(19) Protezione Civile. *COVID-19 situazione Italia*. https://opendatadpc.maps.arcgis.com/apps/dashboards/b0c68bce2cce478eaac82fe38d4138b1 (accessed 2022-11-21).

(20) Centres for Disease Control and Prevention (CDC). *SARS-CoV-2 Variant Classifications and Definitions*. https://www.cdc.gov/coronavirus/2019-ncov/variants/variant-classifications.html (accessed 2022-11-21).

(21) World Health Organization (WHO). *Coronavirus Disease (COVID-19) Situation Reports*. https://www.who.int/emergencies/diseases/novel-coronavirus-2019/situation-reports (accessed 2022-11-21).

(22) Burrell, C. J.; Howard, C. R.; Murphy, F. A. Laboratory Diagnosis of Virus Diseases. *Fenner and White's Medical Virology* **2017**, *9*, 135. https://doi.org/10.1016/B978-0-12-375156-0.00010-2.

(23) Mak, G. C.; Cheng, P. K.; Lau, S. S.; Wong, K. K.; Lau, C. S.; Lam, E. T.; Chan, R. C.; Tsang, D. N. Evaluation of Rapid Antigen Test for Detection of SARS-CoV-2 Virus. *Journal of Clinical Virology* **2020**, *129*. https://doi.org/10.1016/J.JCV.2020.104500.

(24) World Health Organization (WHO). *Use of SARS-CoV-2 antigen-detection rapid diagnostic tests for COVID-19 self-testing*. https://www.who.int/publications/i/item/WHO-2019-nCoV-Ag-RDTs-Self_testing-2022.1 (accessed 2022-11-21).

(25) Scohy, A.; Anantharajah, A.; Bodéus, M.; Kabamba-Mukadi, B.; Verroken, A.; Rodriguez-Villalobos, H. Low Performance of Rapid Antigen Detection Test as Frontline Testing for COVID-19 Diagnosis. *Journal of Clinical Virology* **2020**, *129*. https://doi.org/10.1016/J.JCV.2020.104455.

(26) Porte, L.; Legarraga, P.; Vollrath, V.; Aguilera, X.; Munita, J. M.; Araos, R.; Pizarro, G.; Vial, P.; Iruretagoyena, M.; Dittrich, S.; Weitzel, T. Evaluation of a Novel Antigen-Based Rapid Detection Test for the Diagnosis of SARS-CoV-2 in Respiratory Samples. *International Journal of Infectious Diseases* **2020**, *99*, 328–333. https://doi.org/10.1016/J.IJID.2020.05.098.

(27) Orsi, A.; Pennati, B. M.; Bruzzone, B.; Ricucci, V.; Ferone, D.; Barbera, P.; Arboscello, E.; Dentone, C.; Icardi, G. On-Field Evaluation of a Ultra-Rapid Fluorescence Immunoassay as a Frontline Test for SARS-CoV-2 Diagnostic. *J Virol Methods* **2021**, *295*. https://doi.org/10.1016/J.JVIROMET.2021.114201.

(28) Bruzzone, B.; de Pace, V.; Caligiuri, P.; Ricucci, V.; Guarona, G.; Pennati, B. M.; Boccotti, S.; Orsi, A.; Domnich, A.; da Rin, G.; Icardi, G. Comparative Diagnostic Performance of Rapid Antigen Detection Tests for COVID-19 in a Hospital Setting. *International Journal of Infectious Diseases* **2021**, *107*, 215–218. https://doi.org/10.1016/J.IJID.2021.04.072.

(29) World Health Organization (WHO). Coronavirus Disease (COVID-19) Advice for the Public: Mythbusters. *https://www.who.int/emergencies/diseases/novel-coronavirus-2019/advice-for-public/myth-busters#methanol (accessed 2022-11-21).*

(30) Centres for Disease Control and Prevention (CDC). *Rapid Increase in Ivermectin Prescriptions and Reports of Severe Illness Associated with Use of Products Containing Ivermectin to Prevent or Treat COVID-19*. https://emergency.cdc.gov/han/2021/han00449.asp (accessed 2022-11-21).

(31) Lind, J. N.; Lovegrove, M. C.; Geller, A. I.; Uyeki, T. M.; Datta, S. D.; Budnitz, D. S. Increase in Outpatient Ivermectin Dispensing in the US During the COVID-19 Pandemic: A Cross-Sectional Analysis. *J Gen Intern Med* **2021**, *36* (9), 2909–2911. https://doi.org/10.1007/S11606-021-06948-6.

(32) Popp, M.; Reis, S.; Schießer, S.; Hausinger, R. I.; Stegemann, M.; Metzendorf, M.-I.; Kranke, P.; Meybohm, P.; Skoetz, N.; Weibel, S. Ivermectin for Preventing and Treating COVID-19. *Cochrane Database of Systematic Reviews* **2022**, *2022* (6). https://doi.org/10.1002/14651858.cd015017.pub3.

(33) Giarratana, N.; Rajan, B.; Kamala, K.; Mendenhall, M.; Reiner, G. A Sprayable Acid-Oxidizing Solution Containing Hypochlorous Acid (AOS2020) Efficiently and Safely Inactivates SARS-Cov-2: A New Potential Solution for Upper Respiratory Tract Hygiene. *European Archives of Oto-Rhino-Laryngology* **2021**, *278* (8), 3099–3103. https://doi.org/10.1007/s00405-021-06644-5.

(34) Panatto, D.; Orsi, A.; Bruzzone, B.; Ricucci, V.; Fedele, G.; Reiner, G.; Giarratana, N.; Domnich, A.; Icardi, G. Efficacy of the Sentinox Spray in Reducing Viral Load in Mild COVID-19 and Its Virucidal Activity against Other Respiratory Viruses: Results of a Randomized Controlled Trial and an In Vitro Study. *Viruses* **2022**, *14* (5). https://doi.org/10.3390/V14051033.

(35) Shiu, L.; Ffcm, H. M. *The SARS Epidemic in Hong Kong: What Lessons Have We Learned?*; 2003; Vol. 96.

(36) Dergham, J.; Delerce, J.; Bedotto, M.; Scola, B. la; Moal, V. Clinical Medicine Isolation of Viable SARS-CoV-2 Virus from Feces of an Immunocompromised Patient Suggesting a Possible Fecal Mode of Transmission. *J. Clin. Med* **2021**, 10. https://doi.org/10.3390/jcm10122696.

(37) Patel, M.; Chaubey, A. K.; Pittman, C. U.; Mlsna, T.; Mohan, D. Coronavirus (SARS-CoV-2) in the Environment: Occurrence, Persistence, Analysis in Aquatic Systems and Possible Management. *Science of the Total Environment* **2021**, *765*. https://doi.org/10.1016/J.SCITOTENV.2020.142698.

(38) Shutler, J. D.; Zaraska, K.; Holding, T.; Machnik, M.; Uppuluri, K.; Ashton, I. G. C.; Migdał, Ł.; Dahiya, R. S. Rapid Assessment of SARS-CoV-2 Transmission Risk for Fecally Contaminated River Water. **2021**, *1*, 949–957. https://doi.org/10.1021/acsestwater.0c00246.

(39) la Rosa, G.; Mancini, P.; Bonanno Ferraro, G.; Veneri, C.; Iaconelli, M.; Bonadonna, L.; Lucentini, L.; Suffredini, E. SARS-CoV-2 Has Been Circulating in Northern Italy since December 2019: Evidence from Environmental Monitoring. *Science of the Total Environment* **2021**, *750*. https://doi.org/10.1016/J.SCITOTENV.2020.141711.

(40) Panatto Id, D.; Orsi, A.; Pennati, B. M.; Lai, P. L.; Mosca, S.; Bruzzone, B.; Caligiuri, P.; Id, C. N.; Bertamino, E.; Orsi, G. B.; Manini, I.; Loconsole, D.; Id, F. C.; Pandolfi, E.; Ciofi, M. L.; Atti, D.; Concato, C.; Linardos, G.; Muda, A. O.; Raponi, M.; Piccioni, L.; Rizzo, C.; Chironna, M.; Icardi, G. No Evidence of SARS-CoV-2 in Hospitalized Patients with Severe Acute Respiratory Syndrome in Five Italian Hospitals from 1st November 2019 to 29th February 2020. **2019**. https://doi.org/10.1371/journal.pone.0260947.

(41) Oslo University Hospital. *Norwegian SARS-CoV-2 study*. https://www.ous-research.no/corona/Projects/20822 (accessed 2022-11-18).

(42) DrugBank Online. *Phosphoric acid*. https://go.drugbank.com/drugs/DB09394 (accessed 2022-11-21).

(43) World Health Organization (WHO). *Laboratory biosafety manual, 4th edition*. https://www.who.int/publications/i/item/9789240011311?sequence=1&isAllowed=y (accessed 2022-11-21).

(44) Centres for Disease Control and Prevention (CDC). *Biosafety in Microbiological and Biomedical Laboratories, 6th Edition*. https://www.cdc.gov/labs/BMBL.html (accessed 2022-11-21).

(45) ATCC (American Type Culture Collection). *Vero | ATCC*. https://www.atcc.org/products/ccl-81 (accessed 2022-11-22).

(46) Lorente-González, M.; Suarez-Ortiz, M.; Landete, P. Evolution and Clinical Trend of SARS-CoV-2 Variants. *Open Respiratory Archives* **2022**, *4* (2). https://doi.org/10.1016/J.OPRESP.2022.100169.

(47) Promega Corporation USA. *Viral ToxGlo™ Assay*. https://no.promega.com/products/cell-health-assays/cell-viability-and-cytotoxicity-assays/viral-toxglo-assay/?catNum=G8941 (accessed 2022-06-29).

(48) Paul D. Scotti. *README for Excel Spreadsheet for calculation of "dose 50% (2013)*. https://www.researchgate.net/publication/258201259_README_for_Excel_Spreadsheet_for_calculation_of_dose_50?channel=doi&linkId=0deec52731c6303d2f000000&showFulltext=true (accessed 2022-08-18).

(49) Merck KGaA. *KiCqStart™ One-Step Probe RT-qPCR ReadyMix™*. https://www.sigmaaldrich.com/NO/en/technical-documents/protocol/genomics/qpcr/kicqstart-probe-rt-qpcr-readymixes (accessed 2022-08-22).

(50) Singh, C.; Roy-Chowdhuri, S. Quantitative Real-Time PCR: Recent Advances. *Methods Mol Biol* **2016**, *1392*, 161–176. https://doi.org/10.1007/978-1-4939-3360-0_15.

(51) Cheung, K. S.; Hung, I. F. N.; Chan, P. P. Y.; Lung, K. C.; Tso, E.; Liu, R.; Ng, Y. Y.; Chu, M. Y.; Chung, T. W. H.; Tam, A. R.; Yip, C. C. Y.; Leung, K. H.; Fung, A. Y. F.; Zhang, R. R.; Lin, Y.; Cheng, H. M.; Zhang, A. J. X.; To, K. K. W.; Chan, K. H.; Yuen, K. Y.; Leung, W. K. Gastrointestinal Manifestations of SARS-CoV-2 Infection and Virus Load in Fecal Samples From a Hong Kong Cohort: Systematic Review and Meta-Analysis. *Gastroenterology* **2020**, *159* (1), 81–95. https://doi.org/10.1053/J.GASTRO.2020.03.065.

(52) Parasa, S.; Desai, M.; Thoguluva Chandrasekar, V.; Patel, H. K.; Kennedy, K. F.; Roesch, T.; Spadaccini, M.; Colombo, M.; Gabbiadini, R.; Artifon, E. L. A.; Repici, A.; Sharma, P. Prevalence of Gastrointestinal Symptoms and Fecal Viral Shedding in Patients With Coronavirus Disease 2019: A Systematic Review and Meta-Analysis. *JAMA Netw Open* **2020**, *3* (6). https://doi.org/10.1001/JAMANETWORKOPEN.2020.11335.

(53) Wölfel, R.; Corman, V. M.; Guggemos, W.; Seilmaier, M.; Zange, S.; Müller, M. A.; Niemeyer, D.; Jones, T. C.; Vollmar, P.; Rothe, C.; Hoelscher, M.; Bleicker, T.; Brünink, S.; Schneider, J.; Ehmann, R.; Zwirglmaier, K.; Drosten, C.; Wendtner, C. Virological Assessment of Hospitalized Patients with COVID-2019. *Nature* **2020**, *581* (7809), 465–469. https://doi.org/10.1038/S41586-020-2196-X.

(54) Xiao, F.; Tang, M.; Zheng, X.; Liu, Y.; Li, X.; Shan, H. Evidence for Gastrointestinal Infection of SARS-CoV-2. *Gastroenterology* **2020**, *158* (6), 1831-1833.e3. https://doi.org/10.1053/J.GASTRO.2020.02.055.

(55) Qian, Q.; Fan, L.; Liu, W.; Li, J.; Yue, J.; Wang, M.; Ke, X.; Yin, Y.; Chen, Q.; Jiang, C. Direct Evidence of Active SARS-CoV-2 Replication in the Intestine. *Clin Infect Dis* **2021**, *73* (3), 361–366. https://doi.org/10.1093/CID/CIAA925.

(56) Monchatre-Leroy, E.; Lesellier, S.; Wasniewski, M.; Picard-Meyer, E.; Richomme, C.; Boué, F.; Lacôte, S.; Murri, S.; Pulido, C.; Vulin, J.; Salguero, F. J.; Gouilh, M. A.; Servat, A.; Marianneau, P.

Hamster and Ferret Experimental Infection with Intranasal Low Dose of a Single Strain of SARS-CoV-2. *J Gen Virol* **2021**, *102* (3). https://doi.org/10.1099/JGV.0.001567.

(57) Sharma, K.; Wurtzer, S.; Lacote, S.; Murri, S.; Marianneau, P.; Monchatre-Leroy, E.; Boni, M.; Ferraris, O.; Maday, Y.; Kébé, O.; Dia, N.; Peyrefitte, C.; Sokol, H.; Consortium, O.; Moulin, L.; Maréchal, V. Reduction in SARS-CoV-2 Virus Infectivity in Human and Hamster Feces. **2022**. https://doi.org/10.3390/v14081777.

(58) Natarajan, A.; Zlitni, S.; Brooks, E. F.; Vance, S. E.; Dahlen, A.; Hedlin, H.; Park, R. M.; Han, A.; Schmidtke, D. T.; Verma, R.; Jacobson, K. B.; Parsonnet, J.; Bonilla, H. F.; Singh, U.; Pinsky, B. A.; Andrews, J. R.; Jagannathan, P.; Bhatt, A. S. Gastrointestinal Symptoms and Fecal Shedding of SARS-CoV-2 RNA Suggest Prolonged Gastrointestinal Infection. *Med (N Y)* **2022**, *3* (6), 371-387.e9. https://doi.org/10.1016/J.MEDJ.2022.04.001.

(59) Yu, I. T. S.; Li, Y.; Wong, T. W.; Tam, W.; Chan, A. T.; Lee, J. H. W.; Leung, D. Y. C.; Ho, T. Evidence of Airborne Transmission of the Severe Acute Respiratory Syndrome Virus. *N Engl J Med* **2004**, *350* (17), 1731–1739. https://doi.org/10.1056/NEJMOA032867.

(60) Kang, M.; Wei, J.; Yuan, J.; Guo, J.; Zhang, Y.; Hang, J.; Qu, Y.; Qian, H.; Zhuang, Y.; Chen, X.; Peng, X.; Shi, T.; Wang, J.; Wu, J.; He, J.; Li, Y.; Zhong, N. Probable Evidence of Fecal Aerosol Transmission of SARS-CoV-2 in a High-Rise Building. https://doi.org/10.7326/M20-0928.

(61) McDermott, C. v.; Alicic, R. Z.; Harden, N.; Cox, E. J.; Scanlan, J. M. Put a Lid on It: Are Faecal Bio-Aerosols a Route of Transmission for SARS-CoV-2? *J Hosp Infect* **2020**, *105* (3), 397–398. https://doi.org/10.1016/J.JHIN.2020.04.024.

(62) Yuan, J.; Chen, Z.; Gong, C.; Liu, H.; Li, B.; Li, K.; Chen, X.; Xu, C.; Jing, Q.; Liu, G.; Qin, P.; Liu, Y.; Zhong, Y.; Huang, L.; Zhu, B. P.; Yang, Z. Sewage as a Possible Transmission Vehicle During a Coronavirus Disease 2019 Outbreak in a Densely Populated Community: Guangzhou, China, April 2020. *Clin Infect Dis* **2021**, *73* (7), E1795–E1802. https://doi.org/10.1093/CID/CIAA1494.

(63) Liu, Y.; Ning, Z.; Chen, Y.; Guo, M.; Liu, Y.; Gali, N. K.; Sun, L.; Duan, Y.; Cai, J.; Westerdahl, D.; Liu, X.; Xu, K.; Ho, K. fai; Kan, H.; Fu, Q.; Lan, K. Aerodynamic Analysis of SARS-CoV-2 in Two Wuhan Hospitals. *Nature* **2020**, *582* (7813), 557–560. https://doi.org/10.1038/S41586-020-2271-3.

(64) Hirose, R.; Nakaya, T.; Naito, Y.; Daidoji, T.; Watanabe, Y.; Yasuda, H.; Konishi, H.; Itoh, Y. Mechanism of Human Influenza Virus RNA Persistence and Virion Survival in Feces: Mucus Protects Virions From Acid and Digestive Juices. *J Infect Dis* **2017**, *216* (1), 105–109. https://doi.org/10.1093/INFDIS/JIX224.

(65) Zang, R.; Castro, M. F. G.; McCune, B. T.; Zeng, Q.; Rothlauf, P. W.; Sonnek, N. M.; Liu, Z.; Brulois, K. F.; Wang, X.; Greenberg, H. B.; Diamond, M. S.; Ciorba, M. A.; Whelan, S. P. J.; Ding, S. TMPRSS2 and TMPRSS4 Promote SARS-CoV-2 Infection of Human Small Intestinal Enterocytes. *Sci Immunol* **2020**, *5* (47). https://doi.org/10.1126/SCIIMMUNOL.ABC3582.

(66) Kalantzi, L.; Goumas, K.; Kalioras, V.; Abrahamsson, B.; Dressman, J. B.; Reppas, C. Characterization of the Human Upper Gastrointestinal Contents under Conditions Simulating Bioavailability/Bioequivalence Studies. *Pharm Res* **2006**, *23* (1), 165–176. https://doi.org/10.1007/S11095-005-8476-1.

(67) Sencio, V.; Machelart, A.; Robil, C.; Benech, N.; Hoffmann, E.; Galbert, C.; Deryuter, L.; Heumel, S.; Hantute-Ghesquier, A.; Flourens, A.; Brodin, P.; Infanti, F.; Richard, V.; Dubuisson, J.; Grangette, C.; Sulpice, T.; Wolowczuk, I.; Pinet, F.; Prévot, V.; Belouzard, S.; Briand, F.; Duterque-Coquillaud, M.; Sokol, H.; Trottein, F. Alteration of the Gut Microbiota Following SARS-CoV-2 Infection Correlates with Disease Severity in Hamsters. *Gut Microbes* **2022**, *14* (1). https://doi.org/10.1080/19490976.2021.2018900.

(68) Guo, M.; Tao, W.; Flavell, R. A.; Zhu, S. Potential Intestinal Infection and Faecal-Oral Transmission of SARS-CoV-2. *Nat Rev Gastroenterol Hepatol* **2021**, *18* (4), 269–283. https://doi.org/10.1038/S41575-021-00416-6.

(69) DrugBank Online. *Sulfuric Acids*. https://go.drugbank.com/categories/DBCAT001037 (accessed 2022-11-22).

(70) DrugBank Online. *Hydrochloric acid: Uses, Interactions, Mechanism of Action*. https://go.drugbank.com/drugs/DB13366 (accessed 2022-11-22).

(71) Xiao, S.; Yuan, Z.; Huang, Y. Disinfectants against SARS-CoV-2: A Review. *Viruses 2022, Vol. 14, Page 1721* **2022**, *14* (8), 1721. https://doi.org/10.3390/V14081721.

(72) Sharma, N. K.; Sarode, S. C. Low PH and Temperature of Airway Surface Liquid Are Key Determinants That Potentiate SARS-CoV-2 Infectivity. *Curr Mol Med* **2022**, *22* (6), 471–477. https://doi.org/10.2174/1566524021666210816095557.

(73) Xie, Y.; Guo, W.; Lopez-Hernadez, A.; Teng, S.; Li, L. The PH Effects on SARS-CoV and SARS-CoV-2 Spike Proteins in the Process of Binding to HACE2. *Pathogens* **2022**, *11* (2). https://doi.org/10.3390/PATHOGENS11020238/S1.

(74) Cicconetti, F.; Sestili, P.; Madiai, V.; Albertini, M. C.; Campanella, L.; Coppari, S.; Fraternale, D.; Saunders, B.; Teodori, L. Extracellular PH, Osmolarity, Temperature and Humidity Could Discourage SARS-CoV-2 Cell Docking and Propagation via Intercellular Signaling Pathways. *PeerJ* **2021**, *9*. https://doi.org/10.7717/PEERJ.12227.

8) Appendix

NAxtra™ nucleic acid manual extraction kit

- Ensure that you read and understand the information provided in this guide before you begin the extraction procedure.
- Review your assay documentation to determine if an extraction control is recommended to verify the efficacy of the nucleic acid preparation. Follow the extraction control guidelines provided in the assay documentation.
- Determine the number of required reactions based on the number of patient samples to be processed, plus one Negative Control per plate.
- Sample input volume may be **100 µl** or **200 µl**. Sensitivity may be increased if using 200 µl sample input volume.
- Ensure that all NAxtra™ MAGNETIC BEADS are resuspended by shaking the bottle.
- Prepare ready to use **BEAD MIX**: **20 µl** of the concentrated NAxtra™ MAGNETIC BEADS to **380 µl** (100µl sample input) or **580 µl** (200 µl sample input) Isopropanol per reaction, plus 10% overage. Amount of concentrated beads is independent of sample input volume.
- Prepare fresh **80% Ethanol** using Ethanol, Absolute, Molecular Biology Grade and Nuclease-free Water (not DEPC-Treated) for the required number of reactions, plus 10% overage.

Preparation of BEAD MIX

Sample input	Concentrated beads	Isopropanol	BEAD MIX
100 µl	20 µl	380 µl	400 µl
200 µl	20µl	580µl	600 µl

Protocol guide for manual extraction

1. Pipette out 200 µl NAxtra™ LYSIS BUFFER (pr. sample tube or pr. well if using well plate). The volume of NAxtra™ LYSIS BUFFER should be 200 µl independently of sample volume input.
2. Add 100 µl (or 200 µl) patient sample, mix by pipetting several times (at least 5 times up and down) and leave at room temperature with shaking (900 rpm) for 5 min.
3. Resuspend the ready to use NAxtra™ MAGNETIC BEADS diluted in Isopropanol. Vortex thoroughly to resuspend all beads.

4. Add 400 µl (or 600 µl) BEAD MIX to each sample/lysis tube and mix (pipetting/vortexing).
5. Keep the beads in solution for 10 min by shaking (900 rpm).
6. Place on a magnetic stand and wait until the liquid is clear (2–5 min). Remove and discard supernatant without disturbing the bead pellet.
7. Resuspend and wash the beads in 400 µl 100% Isopropanol, shaking 2 min (900 rpm).
8. Place on a magnetic stand and wait until the liquid is clear (2–5 min). Remove and discard supernatant without disturbing the bead pellet.
9. Resuspend and wash the beads in 400 µl 80% EtOH, shaking 2 min (900 rpm).
10. Place on a magnetic stand and wait until the liquid is clear (2–5 minutes). Remove and discard supernatant without disturbing the bead pellet.
11. Resuspend and wash the beads in 400 µl 80% EtOH, shaking 2 min (900 rpm).
12. Place on a magnetic stand and wait until the liquid is clear (2–5 min). Remove and discard supernatant without disturbing the bead pellet.
13. Dry the beads for 10 min at room temperature, NB! Important that the beads are dried completely.
14. Resuspend the beads in 50 µl Elution buffer, shaking 5 min (900 rpm).
15. Place on a magnetic stand and wait until the liquid is clear (2–5 min). Transfer the clear supernatant to a new RNase/DNase-free storage tube.

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Love you all♥