**University of Veterinary Medicine Budapest**

**Department of Microbiology and Infectious Diseases**

# Virus Neutralisation Test with Human Sera for COVID-19 and Bovine Coronavirus

# Vírusneutralizációs Tesztek Humán Szérumokkal a SARS-COV-2 és a Szarvasmarha Coronavirus Kimutatására

Trishabye Essoo

Supervisor: Dr. Lőrincz Márta, DVM

Department of Microbiology and Infectious Diseases

2024

## Abstract

The cell surface proteins of SARS CoV-2, including the spike protein containing the neutralisation epitope, are constantly changing, which can cause changes in infectivity and the effectiveness of protective antibodies. The latter is thought not to have happened yet. A host change is also suspected for the Russian flu that spread in the 1800s, which is thought to have been caused by the bovine coronavirus. It is now a possible cause of upper respiratory tract infections in autumn and winter. We originally intended to neutralise sera from the first wave of COVID-19 with a bovine coronavirus, but despite repeated attempts to isolate the virus, we were unsuccessful. A new study was performed with a sample from a male patient with a history of the disease who had been vaccinated three times but still showed symptoms of COVID-19. The SARS CoV-2 virus (negative for other upper respiratory pathogens) isolated from the nasal swab was used to neutralise sera from the first wave. Cytopathogenic effects were observed compared to expected. It is assumed that there was no association between the virus and the sera. A PCR for nucleocapsid was positive, but testing of a region of the gene encoding the spike protein was negative. The virus is currently undergoing whole genome sequencing.

## Absztrakt

A SARS CoV-2 sejtfelszíni fehérjéi – beleértve a neutralizációs epitópot tartalmazó tüskefehérjét is – folyamatosan változnak, ami a fertőzőképesség és a védő ellenanyagok hatásosságának változását okozhatja. Ez utóbbi vélekedések szerint nem történt még meg. Gazdafajváltást feltételeznek az 1800-as években terjedő orosz influenzáról is, melyet feltételezhetően a szarvasmarha coronavírusa okozta. Jelenleg az őszi-téli időszak felső légúti fertőzéseinek egyik lehetséges okozója. Eredetileg vizsgálataink során a COVID-19 első hullámából származó vérsavókat egy szarvasmarha coronavírussal szándékoztunk neutralizálni, de a vírus izolálása többszöri próbálkozás ellenére is sikertelen maradt. Újabb vizsgálatot végeztünk, egy, a korábban már betegségen átesett és három alkalommal vakcinázott, ennek ellenére a COVID-19 tüneteit mutató férfi beteg mintájával. Az orrtamponból izolált SARS CoV-2 vírus (egyéb felsőlégúti megbetegedést okozó patogénekre negatív) mintával az első hullámából származó vérsavókat neutralizáltuk. A várthoz képest sejtlekerekedést tapasztaltunk. Feltételezhetően a vírus és a savók között kapcsolat nem alakult ki. A nukleokapszid kimutatására elvégzet PCR pozitív, még a tüskefehérjét kódoló gén egy szakaszának vizsgálata negatív eredményre vezetett. A vírus jelenleg a teljes genomszekvenáláson esik át.

## Table of Contents

# 1. List of Abbreviations

| Abbreviation | Definition |
|---|---|
| **ACE2** | Angiotensin-Converting Enzyme 2 |
| **BCoV** | Bovine CoronaVirus |
| **BPI-3** | Bovine Parainfluenzavirus-3 |
| **CPE** | Cytopathogenic Effect |
| **COVID-19** | Coronavirus Disease 2019 |
| **CoV** | CoronaVirus |
| **E** | Envelope |
| **EID** | Emerging Infectious Diseases |
| **FBS** | Foetal Bovine Sera |
| **HCoV** | Human CoronaVirus |
| **HE** | Haemagglutinin-Esterase |
| **HKU1-CoV** | Human CoronaVirus HKU1 |
| **M** | Membrane |
| **MDBK** | Madin-Darby canine kidney cells |
| **MERS-CoV** | Middle East Respiratory Syndrome |
| **MHV** | Mouse Hepatitis Virus |
| **N** | Nucleocapsid |
| **NÉBIH ÁDI** | National Food Chain Safety Office |
| **NIAID** | National Institute of Allergy and Infectious Diseases |
| **PBS** | Phosphate Buffered Saline |
| **PCR** | Polymerase Chain Reaction |
| **S** | Spike protein |
| **SARS-CoV-2** | Severe Acute Respiratory Syndrome Coronavirus 2 |
| **TCID50** | Tissue Culture Infective Dose 50 |
| **TGEV** | Transmissible GastroEnteritis Virus |
| **Vero** | African green monkey kidney epithelial cells |
| **WHO** | World Health Organization |

## 2. Introduction

On a global scale, it has been reported by the World Health Organization (WHO) that as of August 16, 2023, over 700 million people have been confirmed to be infected with Coronavirus Disease 2019 (COVID-19). The disease, caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has also resulted in the death of about 7 million people [1]. This pandemic is the third major outbreak caused by a newly emerged strain of coronavirus in the last two decades. Previous outbreaks were caused by SARS-CoV and Middle East respiratory syndrome coronavirus (MERS-CoV) [2].

More than 13 billion vaccine doses have been administered since and vaccination has been on the forefront for protection against SARS-CoV-2 to not only to contain the spread of the virus but also to decrease the severity of symptoms in those infected. However, due to the rapid emergence of variants (for example the Delta and Omicron variants) more advanced and sustainable strategies are needed [1,2].

The Coronavirus genera of viruses affect not only humans but a vast number of mammals and birds as well. Hence, it is of great significance not only in human health but also in veterinary medicine. In this investigation, COVID-19 as well as the traits and similarities of the Coronavirus genera are discussed. Specifically, the immunological background of less-pathogenic strains such as Human coronavirus (HCoV) OC43 – which is part of the common cold in humans and is thought to be a spill-over disease originating from bovine coronavirus (BCoV). Furthermore, using lab methods such as polymerase chain reaction (PCR), we had to show the cross-link between BCoV and HCoV-OC43. As humans have most likely been exposed to the latter through colds, as well as through human enteric coronaviruses, it can be assumed that antibodies against BCoVs are present in the body.

# 3. Literature review

## 3.1. General Characteristics of Coronaviruses

Coronaviruses (CoVs) are positive-sense RNA viruses that are enveloped. The name corona is derived from the Latin for 'crown' relating to the outward-projecting glycoproteins seen under the lens of the electron microscope [3,4].

CoVs are contained within the order *Nidovirales* under the *Coronaviridae* family and subfamily *Coronavirinae* [3,5,6]. Nido, the Latin word for 'nest' refers to the common genome pattern shared between other viruses within this order [4]. Their RNA genome is the largest known, reaching a length of up to 32 kb [6]. CoVs produce a group or 'nest' of subgenomic mRNA material at the 3' end, encompassing the specific enzymes that serve as part of the RNA replication process. The family is further divided into four genera, namely, the *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus* and *Deltacoronavirus.* These genera, which hold both veterinary and medical significance, were grouped into their categories primarily due to their serological relationships [3,6].

There are four structural proteins common in all CoVs: a large surface glycoprotein or spike (S) protein, a relatively smaller envelope (E) protein, the integral membrane (M) glycoprotein and a protein for the phosphorylated nucleocapsid (N) [3]. These structural proteins are a crucial part of the strategy these viruses have in successful infection spread, from viral genome production, replication, attachment of the virion to the receptor, and promoting the entry into host organisms [5]. The receptor-binding domain (namely RBD) and other domains linked to fusion are carried by the S glycoprotein, an essential in the entry strategy of CoV [6]. The S protein is largely comprised of an S1 protein stalk and an S2 coil. The S1 amino acids are relevant as they are variable. This variation between virus serotypes, such as avian coronavirus and infectious bronchitis virus (IBV), can cause a difference of up to 40%. As the S1 protein is the main inducer of protective immunity, a variation in the S1 protein helps a different strain of the virus elude a previous immune response [3]. Lineage A β-CoVs (including bovine-CoV, OC43-CoV, human coronavirus HKU1 (HKU1-CoV) and mouse hepatitis virus (MHV)) also contain an additional envelope protein, hemagglutinin-esterase (HE) which act as receptor-destroying enzymes. The exact mechanisms are poorly understood but the function of the HE and S are thought to be linked, as the balance between receptor destruction and binding to receptors affects virus binding and release [7].

## 3.2. Transmission, Replication Sites and Diseases

Different transmission pathways are possible between coronaviruses (figure 1). Viruses can also cause respiratory and/or gastrointestinal diseases. In humans, respiratory symptoms are predominant and droplet transmission (>5 μm) is the most important mode of transmission. Direct contact is the most important mode of transmission of SARS-CoV-2 from an infected person to another uninfected person, especially in households with close interaction between family members. The ability of SARS-CoV-2 to be transmitted by contagious objects (e.g. door handles) has been investigated in several studies, but this mode of transmission is probably insignificant compared to the previous ones. In the case of coronaviruses, both airborne and faecal transmission may occur. The intestinal route of transmission of SARS-CoV-2 is not fully understood, but the virus RNA is shed by this route as well [8, 9, 10].

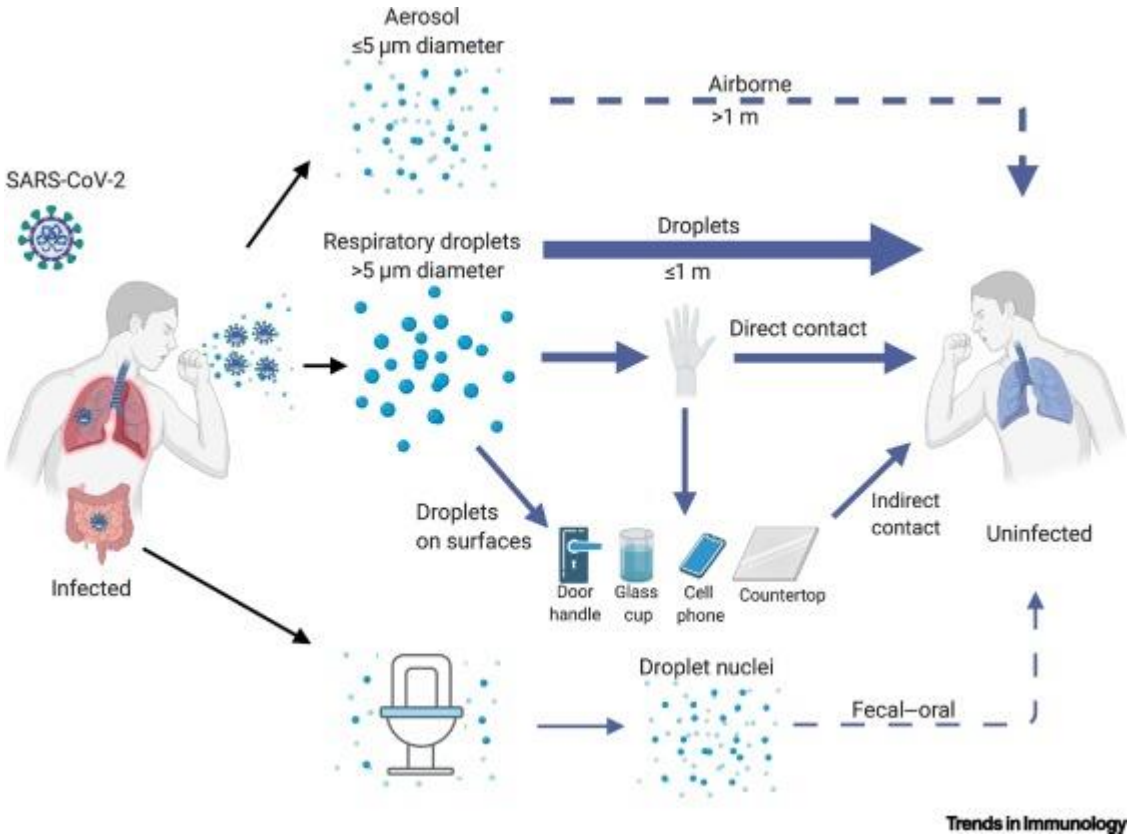


*Figure 1.: Possible routes of Coronaviruses' spread. Solid arrows show confirmed viral transfer from one infected person to another, with a declining gradient in arrow width denoting the relative contributions of each transmission route. Dashed lines show the possible propagation path. The SARS-CoV-2 symbol in the caption "infected patient" indicates where the RNA/infectious virus was detected. Figure is copied from [10].*

Coronaviruses enter the airway and intestinal epithelial cells via a receptor-mediated pathway. They multiply in the cells and then escape. They need cell surface proteins to do this. Some of the proteins and essential genes mentioned above are key factors in CoV

infection and replication**.** The spike (S) protein on the surface of the virus enables CoVs to bind to host cell receptors. Different coronaviruses use different receptors, influencing their host range and infectivity. Their genes can influence which species a virus can infect. Mutations in the S protein, for example, may allow a virus to jump species. Once inside a host cell, coronaviruses use their RNA polymerase gene to replicate. They also have accessory genes, which can vary between strains, that help evade the host immune response and improve viral persistence.

For coronaviruses that have adapted to humans, person-to-person transmission is facilitated through close contact, respiratory droplets, and potentially airborne particles (especially in crowded or enclosed spaces) [8,9].

When it comes to genes, as mentioned above, the following which encode the respective proteins, are of particular significance:

- **S gene** for the spike protein: binds to receptors on host cells and is crucial for host entry [8,9].
- **M gene** for the membrane protein: plays a key role in virus assembly and stability [8,9].
- **E gene** for the envelope protein: involved in viral assembly and release [8,9].
- **N gene** for the nucleocapsid protein: binds to viral RNA to package it within the virus particle [8,9].
- **ORF1ab:** a large replicase gene that encodes non-structural proteins essential for viral RNA synthesis [8,9].

Each coronavirus species has a unique configuration of these genes, which impacts its infectiousness and pathogenicity.

Coronaviruses enter cells via a receptor-mediated pathway (e.g. bound to angiotensin-converting enzyme 2 in SARS-CoV2) by the envelope protein S. The two subunits of the viral S protein (S1/S2) are cleaved by a transmembrane protease. Then, after fusion of the virus across the cell membrane, the RNA genome is transferred to the cytosol, where translation begins. The resulting polyproteins are cleaved into non-structural proteins by a viral protease. Replication begins in membrane vesicles derived from the endoplasmic reticulum. The full-length negative strand is then transcribed from the positive strand genome. This results in the production of structural proteins. The virion is assembled in the

endoplasmic reticulum-Golgi intermediate compartment and the positive-strand genome is inserted [3,8,9].

Although not always causing damage at those sites, it is thought that the replication of all CoVs take place in at least the epithelial cells of the respiratory and/or enteric tracts. Some CoVs like the avian CoV (Gammacoronaviruses); IBV not only cause respiratory disease but can also damage male and female reproductive organs and depending on the viral strain and even on the breed of the chicken, can cause severe kidney damage as well. This virus can practically use any epithelial site in the host to replicate. The focal location can be life-threatening as in the case of porcine CoV; transmissible gastroenteritis virus (TGEV). The replication site is in the alimentary tract leading to a mortality rate of more than 90% in newborn piglets. Human coronaviruses are more commonly manifest as respiratory and enteric diseases.
As in SARS-CoV, infected humans suffered from diarrhoea as well as severe respiratory illness. CoVs like MHV can reach the central nervous system resulting in acute or chronic demyelination [3].

## 3.3. Spill-over Diseases vs Zoonosis

Firstly, in relation to host spectrum, it is important to note the difference between the following terms: ‘spill-over’ and zoonosis. A "spillover" event, also known as an "evolutionary jump," occurs when a pathogen spreads from its natural animal host to a new or "novel" host, resulting in infection. Such an event is typically a chance encounter, potentially caused by initial or repeated exposures to the new host. Sometimes, genetic changes in the pathogen enable it to infect the new host. These jumps may either result in a limited, dead-end infection or continue spreading, fueling a cycle of transmission. The emphasis is on the chance nature of the phenomenon as opposed to it being a natural part of the pathogen’s infection cycle. The established jump from animals to humans is known as cross species spill-over [12,13].
On the other hand, zoonosis is “any infection that is naturally transmissible from vertebrate animals to humans” as defined by the WHO. It emphasizes the fact that the source of the

infection is maintained by an animal species, acting as a reservoir for the pathogens and will be passed on to humans from animals via direct contact or indirectly [14].

Another relevant classification, referred to as emerging infectious diseases (EID) are "diseases that have newly appeared in a population or have existed but are rapidly increasing in incidence or geographic range" as defined by the National Institute of Allergy and Infectious Diseases (NIAID)[15].

This terminology is slightly ambiguous with regards to etiology of the diseases however it helps to argue that COVID-19 should be more accurately classified as an "EID of probable animal origin". It helps to show that even though it has emerged as a human pathogen, it is not a true zoonosis and is instead a spill-over disease from an animal host.

Recognizing the difference between diseases that originate in animals but can spread independently among humans, and those that need an animal reservoir to maintain the pathogen, enables more focused research on infectious diseases. This distinction also supports the development of better prevention strategies and treatment methods [12].

## 3.4. Host spectrum of Coronaviruses

The hosts of CoVs extend to 45 different species not only concerning a risk to public health but also affecting livestock farming as the range goes from humans to poultry and other production animals [16,17,18,19]. Based on previous studies bats, rodents, poultry, and production animals, CoVs are initially thought to be host specific but it is not always the case [3,16]. For example, *Rhinolophus* bats are the source of severe acute respiratory syndrome–related coronavirus (SARSr-CoV) but human SARS-CoV virus is only found in the *Rhinolophus sinicus* bat species and for SARS-CoV-2 the relevant species is *Rhinolophus affinis* [16,20,21,22]. In contrast, the diversity of CoVs arise from their quick genetic variation as there are many uncorrected errors in the RNA genome replication as well as numerous instances of genome recombination [23]. To summarize; bats, rodents and birds serve as natural reservoirs and the CoVs are endemic in these species, among these, there is great genetic diversity [16]. As explained previously, it is difficult to classify CoVs as conventional zoonoses without accounting for the spill-over effect [3]. There is continuous

and accidental cross-species spread, that is through the fast evolution and overlap of habitats with animals [16,24].

It is evident that replication and the disease are not always limited to a single host species [3,16]. This can be seen in canine enteric CoV and feline CoV which are able to replicate and cause disease in swine. This occurs as the viruses have a similar amino acid identity to porcine TGEV. Furthermore, canine respiratory CoV also has a very high amino acid identity (more than 95%) to HCoV-OC43 and BCoV, including the S protein. This phenomenon also makes co-infection possible. There are also cases of pheasant CoV infecting chickens asymptomatically and the same with IBV infection in a specific type of duck species (teal). The profound example of the wide host range of CoVs is shown by SARS-CoV with their origin being thought to stem from bats, additionally being transmitted to many other species (for example the civet cat) via trade and finally causing the deadly disease in humans. The same spill-over background has been considered for the SARS-CoV-2 infection of humans, also thought to have bats at the source but the exact intermediate route is still unknown [3].

This is where spill-over may come into play but there are also other factors that may be involved causing and allowing to the change or variety in hosts which will be discussed. Research on SARS-CoV, SARS-CoV-2, and MERS-CoV shows that these viruses have a diverse range of possible hosts [24,25]. SARS-CoV-2 can infect and cause diseases in humans, dogs, cats, ferrets and other animals. It has also been reported that human contact with other animals caused by long-term viral epidemics can lead to spread of the virus among different species, and sharing habitats is crucial for breaking interspecies barriers and the subsequent interspecific transmission of viruses [16,27]. Acknowledging these overlaps in habitats and potential for transmission of the diseases to humans is crucial.

## 3.5. Triggers for Persistent Infections in New Hosts

For MHV, persistent infection is known however for other coronaviruses like IBV it is less common. The trigger for re-shedding of the virus after egg-laying in hens who were previously infected as young chickens is thought to be the stress of the egg-laying process [3].

As previously mentioned, the protein that is responsible for tissue tropism not only within a host but also determining host range is the S protein. A prime demonstration of this phenomenon has been through the genetic manipulation of the MHV genome which usually cannot bind to feline cells. When the MHV S protein gene is replaced with the S protein gene of CoV from the feline coronavirus, a recombinant virus is formed that can bind as well as replicate in the feline cells. Pathogenicity can also be affected by other proteins known as 'accessory proteins'. Through experiments with genetically modified CoVs and using targeted recombination or 'infectious clones', it is evident that modifying such accessory proteins usually found in the ORF1 and other small genes combined within the structural protein genes, the level of pathogenicity can be attenuated. The exact roles of these proteins are not clear but may be a potential route in the development of a new live vaccines. It is significant as often the control of these diseases is compromised due to the numerous alternatives of S1 protein. This protein also induces protective immunity [3].

## 3.6. Human Coronavirus-OC43 and Bovine Coronavirus

There are four endemic human coronaviruses, namely, HCoV-229E, -NL63, -OC43, and -HKU1 [28]. Even though they have zoonotic ancestry, they evolved into true human viruses distributed worldwide [29,30,31,32]. All 4 of are substantial contributors in several upper and lower respiratory tract infections in both adults and children. Clinically, they have similar manifestation to many other causes of the common cold. However, the relation between hosts as well as the history of the evolution natural can prove useful in understanding the past human pandemics [28].

There is strong evidence for two of the viruses that allude origins in production animal species even though the primordial background for all four viruses is connected to bats and rodents. As common for many other CoVs, HCoV-NL63 and -229E are believed to stem from bat reservoirs however, it is more likely that HCoV-OC43 and -HKU1 differentiated from their common ancestors. HCoV-HKU1 and -OC43 are related but individual viruses with different primordial zoonotic ancestors, moreover, reached the human population separately [29]. The emergence of HCoV-OC43 (a β-CoV) is more likely to be from domestic animals like cattle or swine. At the end of the 19$^{th}$ century, the emergence of HCoV-OC43 may have stemmed from the BCoV pandemic, indicating that it could have been a potential ancestor. In fact, early samples of HCoV-OC43 and BCoV are thought to share

97% of genome identity [29,32,33]. Furthermore, there is data to show that HCoV-229E may have been transmitted from Arabian camels like MERS. There are parallels of spill-over in both contexts [28].

Along with swine, canine, equine, and lagomorph species, HCoV-OC43 and BCoV are considered host range variants of the virus species *Betacoronavirus-1* [25,30]. HCoV-OC43 is believed to have emerged around 70 to 130 years ago from a single cross-species transmission occasion enabling the human-endemic virus to develop [29,32,33,34]. The other β1CoVs along with HCoV-OC43, are host specificity [29,32,36]. These observations demonstrate the potential for variability of host changes in β1CoVs, but since they are still independent, they cement the presence of host barriers, and how adaptive mutations can be selected when these barriers are overcome, consequently creating host specialization and eventually, virus differentiation. Thus, comparing BCoV and HCoV-OC43 would be useful to find the factors that encourage or limit the cross-species transmission of CoVs and help in understanding the conditions that allow the establishment of humans as hosts [29].

The strategy of using conventional antibodies is easily compromised by many factors including escape viral mutations, decreased stability, weak attachment, the large size of the antibodies, the amount of plasma needed as well as manufacturing costs. Especially with new emerging variants of SARS-CoV-2 in the human population and frequent coronavirus spillovers, neutralising antibodies that are not affected by these factors and further antigenic drift are required to potentially hinder new zoonotic infections in the future [28].

In this sense, bovine-derived antibodies can be significantly more effective and protective than conventional human antibodies. Inherently, they theoretically possess the necessary characteristics for cross-protection and large quantities can be produced. Furthermore, it has been reported that they can be effective against a wide spectrum of determinants, which reduces the possibilities the viral pathogens have for mutational escape [28].

## 3.7. The Variability of SARS-CoV-2

It is important to explore the inherent traits of SARS-CoV-2 that led to its rapid evolution and persistence within the human population.

Phenotypic diversification of SARS-CoV-2 into variants is evident in changes not limited to but including the transmissibility of the disease, the severity, and the capacity to evade the immune system. Explaining the factors and processes which propel these changes are crucial towards establishing courses of action for crisis management, prevention, and treatment [37]. SARS-CoV-2 and coronaviruses in general, follow their counterparts as RNA viruses, in that they tend to undergo rapid evolution. These transformations can be measured and observed over time periods of months or years. Several aspects can be taken into consideration such as the epidemiological profile, that is, the trend in infectious individuals over time, immune status, and the movement of human hosts. These dynamics as well as the viral evolution are characteristic of RNA viruses [37,38].

One of the key driving factors in the evolutionary process is the rate of arising mutations and it's spread throughout populations. Mutations that prove to be beneficial to the viral transmission will be compounded, such as the D614G mutation [37,39].

There is emphasis on the D614G mutation in SARS-CoV-2 for several reasons. D614G is the viral spike protein – it is a large glycoprotein, with trimeric structure consisting of S1 and S2 subunits. This protein is responsible for facilitating viral entry into the cells and has been broadly researched in other coronaviruses as well, including SARS-CoV [40,41,42] and MERS [39,43,44].

To enter the cell, the spike protein of SARS-CoV-2 binds to angiotensin-converting enzyme 2 (ACE2). Therefore, gene mutations at this site have the possibility to modify the binding affinity and infectivity of the receptor, furthermore, influencing viral immune evasion and ability for the antigen to provoke an immune response in the body of the host, that is the immunogenicity [45].

The significance of the D614G has been acknowledged based on observations in three distinctive categories. Firstly, studies using lentiviruses which have been phenotyped show that *in vitro,* D614G increases infectivity [41,46,47,48].

The second basis analyses the structure, which surmises that the receptor binding conformation is altered by D614G in such a way that the tendency of ACE2 binding and fusion is increased [37,47].

The third category follows evaluation of the frequency of the 614D and 614G variants over time (relative to global sequence databases), trends show that in locations that where 614D viruses were reported early in the pandemic, later they were regularly found to be overtaken by 614G viruses [37,46,49].

More recent experiments, by means of examining infectious cDNA clones from SARS-CoV-2 strains in circulation, demonstrate the contrast of spike 614 variants in animal models and human cell cultures.

In animal models of SARS-CoV-2 infection has been displayed that in the upper respiratory tract, replication is enhanced [37,50] as well as transmission [37,51] of the 614G variant. Linked with epidemiological patterns regarding discrepancies with upper respiratory tract viral loads [35,52,53], these results indicate the differences in transmission-mediated efficacy between spike 614 variants [37].

In general, the evolution of viruses is a layered process driven by not only the ability for viral replication and evolution within individuals to occur, but also their successful transmission from person to person, leading to augmented alterations. Variation can be lost at numerous points during the complex processes, some are lost during the bottleneck state in transmission, while other mutations are regularly transferred simply by chance, proving no selective benefit [37,58]. Furthermore, beyond variety among the population, through the branching out of viral lineages, including strains which may be antigenically distinct, more advanced processes like lineage competition and even extinction arise [37].

Diversity can arise based on the mutation rate, that is the inherent rate where changes in the genetics of the virus can emerge in a replication cycle. This is a biochemical feature established by the replication reliability or fidelity of the polymerase enzyme of the virus. Mutation rate is crucial in determining the pace of virus evolution. Selection can occur following these genetic alterations. As mentioned above, most mutations cause losses, genes can be deleted thus leading to failure in virion replication [37,55,56,57].

The mutation rate of SARS-CoV-2 is estimated to be between $1 \times 10^{-6}$–$2 \times 10^{-6}$ mutations per nucleotide per replication cycle, as with previously determined rates in other β-CoVs [37,55,58,59].

In relation to the typical rates for other RNA viruses, these rates are lower, for example hepatitis C virus (HCV) and human immunodeficiency virus (HIV) have rates of $\sim 10^{-5} \times 10^{-6}$ and $\sim 10^{-4} \times 10^{-6}$ mutations per nucleotide per replication cycle, respectively. But they lack a 3′ exonuclease mechanism for proofreading which CoVs possess in their replication apparatus [37,58,60,61,62]. Diversity is also generated through the insertions and deletions due to errors in replication. For example, the deletion at position 69–70 of the spike gene which resulted in a drop of the S-gene, a key factor in the detection of the SARS-CoV-2 alpha variant, and moreover has been linked to an exacerbation of infectivity [37,64].

## 4. Objectives

Our primary objective was to verify neutralisation between human serums from the first wave of COVID-19 and BCoV.

Our next objective is to serologically test a newly isolated SARS CoV-2 and its first wave sera.

# 5. Materials and Methods

## 5.1. Materials

### 5.1.1. Human Blood serum

Human blood serum samples (1,211 in total) were collected from the CMC Déli Klinika (Budapest, Kuny Domokos u. 13-15) in 2020. The samples were made available to us by Dr. István Jankovics. This was a national COVID-19 control study. The samples are from the first wave of the pandemic. Antibody production against the SARS-CoV2 virus was tested in the serum samples using an ELISA test.

We have a short history of each sample, which includes age, gender, COVID-19 result (positive/negative), vaccination status (vaccinated/not vaccinated), clinical symptoms. We did not look at these data during our study so that they could not influence the results of the study. If this was relevant, this data can be used in the discussion.

We tried to obtain bovine coronaviruses from several places. We got isolates from the virology department of NÉBIH ÁDI (National Food Chain Safety Office), on the other hand we got samples from the Department of Biology. We tried to propagate them, but we failed. We tried several times, but after five blind passages we found no cytopathogenic effect or other signs of virus replication. So, we tried to isolate the virus from a clinical sample. The sample was obtained by Vetcontroll Ltd. from a herd of growing cattle with respiratory disease. The sample was clearly positive by PCR.

Testing continued with the sample from Dr. Béla Dénes. He had been ill for several weeks in the spring without any previous history. His main symptoms were fever, lethargy and dry cough. A rapid COVID test was positive, and the virus was isolated.

## 5.2. Methods

In this experiment we tried to identify the agent using virus isolation techniques, and also performed serological tests.

### 5.2.1. Virus isolation in tissue culture

For BCoV, isolation is usually attempted ante-mortem from faeces. The virus was isolated by Dr. Ádám Bálint and Dr. Kinga Fornyos (Vetcontroll Kft. in Hungary). The viruses

were stored in -80 °C. One of the sample was a fresh nasal swab from a grower heifer. The virus was soaked from the nasal swab using phosphate buffered saline (PBS).
The SARS CoV-2 was isolated from nasal swab sample. I did not participate in human virus isolation process.

### 5.2.2. Cell culture

Monolayer cell culture is required for virus isolation. MDBK cells (Madin-Darby canine kidney cells, ATCC, USA) were used for BCoV and Vero cells (African green monkey kidney epithelial cells, ATCC, USA) for SARS CoV. Cells were stored in freezing medium containing 10% dimethyl sulfoxide. After thawing, cells were washed twice in 10 ml PBS after centrifugation for 10 minutes and 1500 g. After washing, the cells were plated on 75 $cm^2$ tissue culture flasks (NUNC) with 15 ml DMEM medium (Merck, USA) containing 10% heat-inactivated fetal bovine serum (FBS, Merck, USA), 10 ml/l antibiotic-antimicotic solution (Merck, USA) and 5% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Merck, USA). When the cells reached 100% confluence, they were passaged. To do this, we washed the cells three times with PBS (about 20 ml of PBS was poured into the flask and then drained). The cells were then digested with trypsin-EDTA solution (Merck, USA) (incubate at 37 °C for about 10 minutes). Cells were resuspended in fresh DMEM medium (Containing 10% FBS, 10 ml/l antibiotic) and were placed on new 75 $cm^2$ tissue culture plate for two days. The medium was replaced with post-inoculation medium containing the antibiotic-antimicrobial mixture added to the DMEM medium (see above) and 10 ml/l heat-stable trypsin (Thermo Fischer Scientic, USA). The cells were incubated at 37 °C for 1 hour in the presence of 5% $CO_2$. The virus was then added (the virus was first filtered through a Millipore 0.22 μm filter after soaking from the nasal swab) [60,61].

The cells were incubated at 37 °C in the presence of 5% $CO_2$ and the cytopathic effect (CPE) was monitored daily by light microscopy.

### 5.2.3. Virus neutralisation

For virus neutralisation, we used MDBK (BCoV) and Vero cells (SARS CoV-2) grown as described above. A 96-well tissue culture plate (Greiner Bio-One, Austria) was used for the assay and 10,000 cells were counted per well. When at least 95% of the cells in the wells had grown, the medium was replaced with post-inoculation medium. In the meantime, undiluted sera were mixed with diluted virus. Seras were performed at 60 μl/well. The same

amount of previously determined Tissue Culture Infective Dose 50 (TCID50) SARS-CoV-2 and BCoV virus was measured. Both sera and virus were diluted in FBS and trypsin-free DMEM medium. The sera-virus mixture was also incubated (cells in the presence of 5% $CO_2$) for 1 hour at 37 °C. At the end of the incubation period, the mixture was added to the cells and kept at 37 °C for three days in the presence of 5% $CO_2$. The CPE was checked daily by light microscopy and on the third day the cells were stained with neutral red stain (Merck, USA) [60]. The culture medium was removed from the wells and 10% neutral red staining in DMEM medium was added to each well. After incubation period (1 hour at 37 °C), the neutral red medium was removed, and the plates were washed twice with PBS. Then, acid alcohol (1% acetic acid in 50% ethanol) solution was added to each well. After (30 minutes at room temperature) the absorbance of neutral red stained plates was read at 540 nm using ELISA reader (MultiScan SkyHigh Microplate Spectrophotometer, Thermo Fischer Scientic, USA).

### 5.2.4. Tissue Culture Infective Dose 50

Infectious titrations have also been performed for bovine coronavirus and SARS-CoV-2. (I was not involved in the human coronavirus work).

Cells were first planted on a 96-well plate (MDCK for BCoV, Vero cells for SARS CoV-2). The cell culture medium was DMEM as described above. When the cells reached 70-80% confluence, they were infected with the virus (in post-inoculation medium). The plate was infected with dilutions of the virus as follows (figure 2):

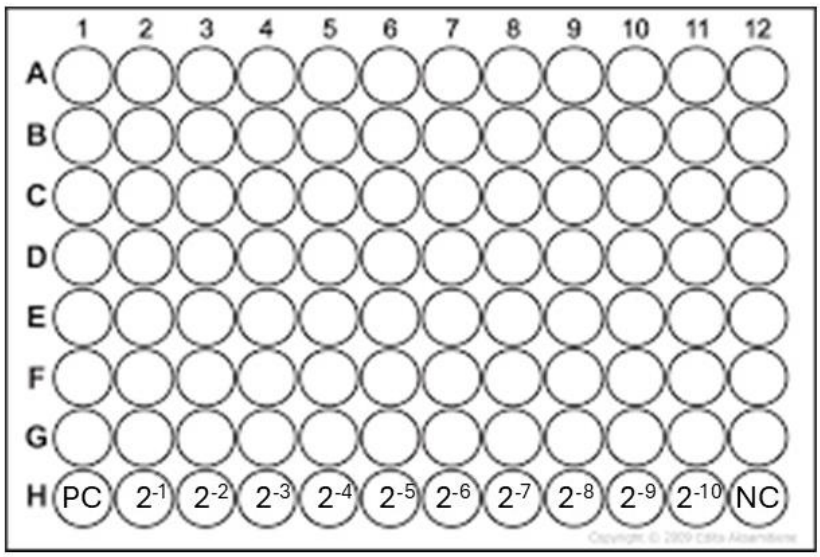


*Figure 2.: Form of TCID50 examination (PC: positive control, NC: negative control and the virus solutions)*

The bovine coronavirus test was read after three days and the result calculated (table 1) using the REED-MUENCH formula [62]:

*Table 1.: Result calculation of bovine coronavirus TCID50 test, using the REED-MUENCH formula.*

| | + | - | A | B | A/(A+B) |
|---|---|---|---|---|---|
| $2^{-5}$ | 8 | 0 | 26 | 0 | 26/26= 100% |
| $2^{-6}$ | 7 | 1 | 18 | 1 | 18/19= 94% |
| $2^{-7}$ | 5 | 3 | 11 | 4 | 11/15= **73%** |
| $2^{-8}$ | 4 | 4 | 6 | 8 | 6/14= **43%** |
| $2^{-9}$ | 2 | 6 | 2 | 14 | 2/14= 14% |
| $2^{-10}$ | 0 | 8 | 0 | 22 | 0/22= 0% |

This was based on a 192-fold dilution of the virus. The same was done for human coronavirus.

### 5.2.5. PCR

The nucleic acid of the viruses was also tested by PCR. In the case of SARS-CoV-2 virus, specific primer pairs designed for the nucleocapsid gene and the S gene coding for the spike protein were used by the staff of NÉBIH ÁDI.

In the case of bovine virus, nucleic acid was extracted using the Zymo Research (USA) Quick Viral DNA/RNA Kit according to the manufacturer's instructions.

The extracted RNA genomic material (10 µl) was reverse transcribed prior to PCR analysis. The Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Fisher Scientific, USA) was used for transcription according to the manufacturer's instructions. The protocol is available at the Thermo Fischer website [61].

For the PCR reaction, the DreamTaq Green PCR DNA Polymerase enzyme DreamTaq from Thermo Fischer Scientic (USA) and the dNTP set from the same company were used. Literature primers were ordered from Merck (USA). The final volume of the reaction was 50 µl and contained the following components:

- 5 µl cDNS

- 5 µl 10X Dream Taq Green puffer
- 1 µl 1 mM dNTP
- 1 µl primer-F
- 1 µl primer-R
- 1 unit Dream Taq DNS polymerase enzyme
- 50 µl was supplemented with twice distilled, sterile, pyrogen free water (Millipore, USA)

The sequences of bovine primers were as follows:

BCoV-F 5’ -GCCGATCAGTCCGACCAATC-3’ [62]

BCoV-R 5’ -AGAATGTCAGCCGGGGTAT 3’ [62]

BPI-3F 5’ -CCTGCCCTTTGGAGTTATGCGA-3’ [63]

BPI-3R 5’ -GCATCACGTGCCAC TGCTTG-3’ [63]

The predicted PCR product was 407 base pairs in case of coronavirus and 127 base pairs in case of bovine parainfluenzavirus. The reaction conditions were as follows: 5 minutes of preheating at 95 °C, 35 cycles of 30 seconds at 94 °C, 30 seconds at 58 °C, 1 minute at 72 °C, and a final 7 minutes incubation at 72 °C. Amplification was performed using a TGradient Thermocycler (Biometra, Germany). The DNA products were visualised on a 2% agarose gel (Lonza, Switzerland) stained with GR Safe DNA Stain I (Nzytech, Portugal) according to the manufacturer's instructions. Its size was determined using a molecular weight marker – DNA 1 kb ladder (Thermo Fischer Scientic, USA) –. Electrophoresis was performed at 110 V for approximately 20 minutes in TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM ethylenediaminetetraacetic acid).

# 6. Results

## 6.1. Virus isolation

Bovine coronavirus isolates showed no cytopathogenic effect on MDBK. No effect indicative of virus replication was observed in the blank passages.

Therefore, PCR was used to test for virus replication, but PCR gave negative results.

For the swab specimen, PCR gave a strong positive result in the diagnostic test. During virus isolation, the result of the second pass was positive. The cells were rounded and detached. Infectious titration was performed to determine TCID50. The bovine coronavirus test was read after three days using light microscope. Cytopathogenic effect was described for the evaluation of the test. The table 2 described the result of the light microscope. The result calculated using the REED-MUENCH formula [62]:

*Table 2.: Result of bovine coronavirus TCID50 test, using the REED-MUENCH formula*

| | CPE effect | No CPE effect |
|---|---|---|
| $2^{-5}$ | 8 | 0 |
| $2^{-6}$ | 7 | 1 |
| $2^{-7}$ | 5 | 3 |
| $2^{-8}$ | 4 | 4 |
| $2^{-9}$ | 2 | 6 |
| $2^{-10}$ | 0 | 8 |

On this basis, a 192-fold dilution of the virus was used for the virus neutralisation assay. The same was done for human coronavirus.

## 6.2. Neutralisation of bovine coronavirus

Blood sera were mixed with the diluted virus and applied to pre-treated MDBK cells. Samples were checked after three days. After three days, no positive results were seen when the blood sera were tested, with viruses showing cellular depletion throughout. The test was repeated, with the sera inactivated before the test, but the result was unchanged. Since humans have probably been exposed to the OC43 virus during colds, as well as to human

enteric coronaviruses, it can be assumed that antibodies against bovine coronaviruses are present in the body [60, 64]. Those are the reasons we had to check the virus with PCR.

Neutralisation of the human coronavirus has produced surprising results. The virus was tested by rapid test and PCR, but the neutralisation was still negative. In the positive/double positive cases, the acids were inactivated and filtered. The result was inconclusive. Figure 3 is a photograph of the plate where samples A1, A2 are the negative control. B1 and B2 are the samples diluted to TCID50. The evaluation with neutral red was straightforward using the method of Wang et al. [60]. The plate presented did not contain a standard. It can be clearly seen that there are marked differences between virus infected and uninfected samples. The threshold value was taken as 60% of the difference between the negative and positive control (50% difference is considered doubtful), i.e. in this table (table 3):

Negative control mean: 3.6628 and 3.5394= 3.6011

Positive control mean: 0.3635 and 0.3357= 0.3496

3.6011-0,3496)*0.6=**1.9509 positive,**

(3.6011-0.3496)*0.5=1.62575-1,9508 doubtful

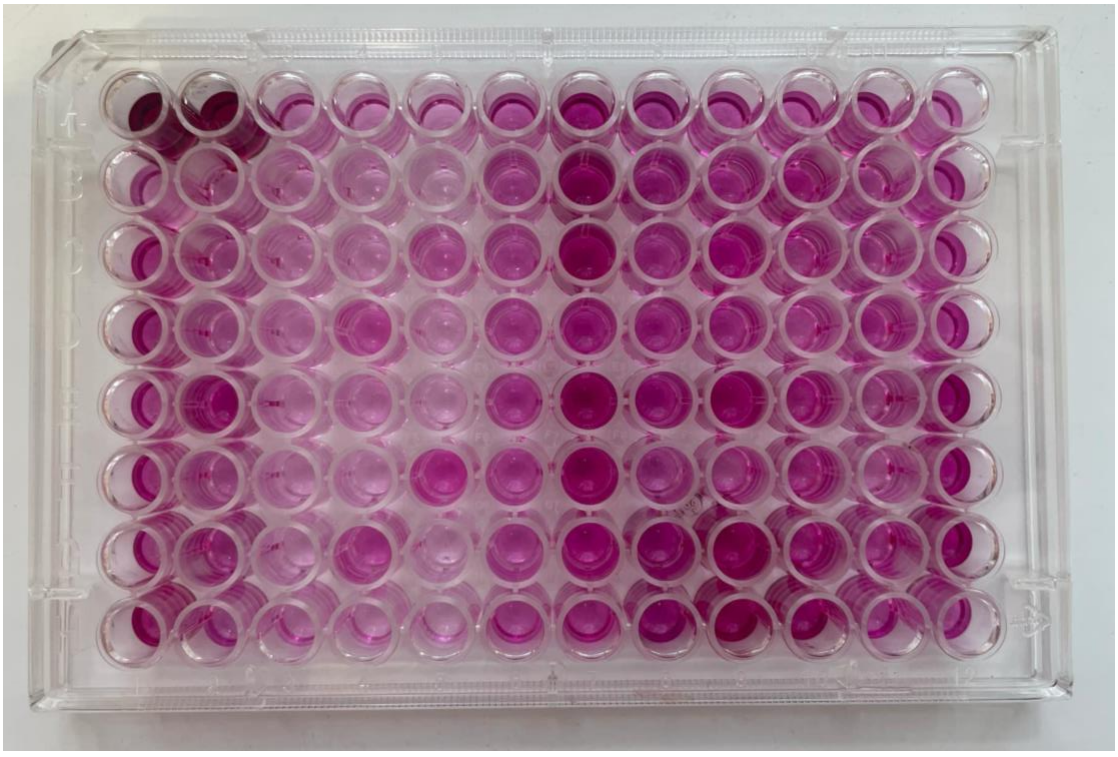

*Figure 3.: Virus neutralisation staining with neutral red. The first two wells are positive.*

*Table 3: Optical density of neutral red stained virus neutralisation*

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 3.6628 | 3.5394 | 0.3815 | 0.3343 | 0.2988 | 0.6042 | 1.1905 | 0.7970 | 0.6563 | 0.5951 | 0.6483 | 0.4413 |
| 0.3635 | 0.3357 | 0.2720 | 0.2259 | 0.1628 | 0.5534 | 1.8337 | 0.8974 | 0.7804 | 0.9300 | 0.6248 | 0.7672 |
| 0.7466 | 0.4876 | 0.2830 | 0.2464 | 0.4464 | 0.5220 | 1.9170 | 0.6997 | 1.1372 | 0.4076 | 0.6094 | 0.6993 |
| 0.7775 | 0.4158 | 0.2850 | 0.5705 | 0.1940 | 0.5614 | 0.8703 | 0.7900 | 0.6553 | 0.4176 | 0.8363 | 0.8249 |
| 0.5699 | 1.2266 | 0.2413 | 0.2814 | 0.1711 | 0.6395 | 2.0053 | 0.9548 | 1.6639 | 0.6838 | 0.4350 | 1.5183 |
| 0.6024 | 0.4898 | 0.2325 | 0.2039 | 0.7194 | 0.5595 | 1.6290 | 0.5925 | 0.3593 | 0.6336 | 0.3392 | 1.2271 |
| 0.3579 | 0.7042 | 0.2074 | 0.5962 | 0.1575 | 0.5482 | 0.9951 | 0.9571 | 1.8879 | 0.6501 | 0.6198 | 1.5900 |
| 0.7718 | 0.4621 | 0.2876 | 0.2164 | 0.1664 | 0.5115 | 0.6644 | 1.0118 | 1.8143 | 1.0122 | 0.5099 | 0.6544 |

In total, 52 cases were found where samples had to be filtered (B7, C7, E7, F7, E9, G9 and H9, samples of the plate shown in the figure 4). For these samples, the light microscopy results were negative, with a clear cytopathogenic effect in the cells (Figure 4).

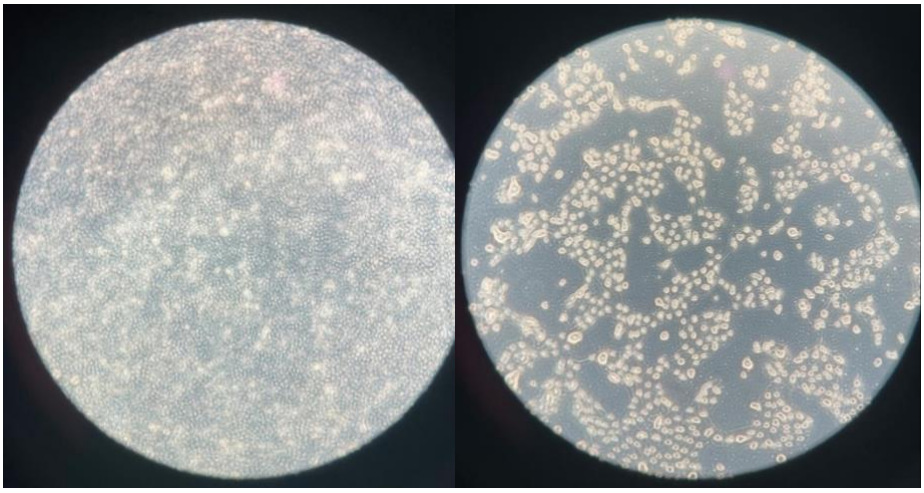

*Figure 4.: Negative and positive virus neutralisation test*

## 6.3. Cattle virus detection with PCR

Therefore, virus isolation was also tested by PCR. In this case, BCoV was not present in the isolate, but Bovine Parainfluenzavirus-3 (BPI-3) was. This was probably the cause of the problem [64], and the result was negative.

## 6.4. SARS CoV-2 detection with PCR

The human coronavirus sample was first tested by PCR. The PCR designed for the N gene, which encodes the coronavirus nucleocapsid protein, gave a positive result. However, the amplification reaction for the S gene did not work, although the control samples were perfect. Detection of this S gene has been attempted by others. Specific primers for this assay are available from the WHO [65]. Full sequence analysis of the sample is underway [65].

## 7. Discussion

In a previous study, also carried out by a student in the department, porcine coronavirus (transmissible gastroenteritis virus) was neutralised by 4.2% of the same human sera. We thought it would be interesting to see how these sera reacted to a β-CoV. We chose BCoV because this virus is easily accessible in winter.

During the autumn-winter season, 15-30% of human upper respiratory tract illnesses are caused by coronaviruses. These are usually caused by HCoV- 229E, HCoV-NL63, HCoV-OC43 and HCoV-HKU1. These viruses cause illness every 3-4 years. HCoV-OC43 is genetically related to BCoV. Sequencing studies by a group of Belgian researchers suggest that the two viruses are very similar at the nucleotide level [66]. An interesting correlation is that the virus was detected at the same time as the Russian influenza epidemic that caused pandemic fever in 1889-1890 [67]. The epidemic began in Turkestan and spread rapidly across Europe, reaching the United States via Siberia and Alaska. Unlike 'normal' influenza epidemics, it was not seasonal. Among the influenza viruses, H2N2 and equine H3N8 have been implicated as possible pathogens. At present, however, BCoV appears to be the most likely. At that time, the live cattle trade was expanding worldwide and Mycoplasma mycoides infection was also spreading. Herds were controlled by slaughtering at the abattoir, which exposed workers to bovine viruses. BCoV and HCoV-OC43 are associated with the same symptoms [67]. Zhang et al (1994) described BCoV as the underlying cause of diarrhoea in children. Later, HECV-4408 isolated from children could experimentally induce diarrhoea in calves [68]. The virus is a coronavirus that causes non-respiratory disease in humans. It has a higher similarity to the BCoV S gene than HCoV- OC43. And serological tests suggest that protection against either homologous or heterologous viruses is not significantly different. Therefore, it has been described that "HECV-4408 is probably a variant of bovine coronavirus" [69].

As described above, a high cross-reactivity between the salts and BCoV was expected. Therefore, it was surprising that we found a negligible (52/1211) suspect sample even with the neutral red test, and more than 95% of the sera gave a negative reaction (viral replication was detected on the plate). Therefore, we tested our isolate with BCoV detection PCR, which gave negative results. However, respiratory BPI-3 detection was successful.

Studies with monoclonal antigens for parainfluenza viruses have found that the cross-protection of antibodies to the haemagglutinin-neuraminidase protein between human and bovine viruses is low [70,71]. Thus, positive virus neutralization was not expected for parainfluenza. The parainfluenza virus grows faster than coronaviruses, both viruses are enveloped RNA viruses, and thus the conditions for replication in tissue culture are identical. Therefore, BPI-3 virus could not be eliminated from infected samples. In the case of coronaviruses, the group has previously experienced a failure to recover the virus from virus isolates that did not replicate well. Previously, in the case of porcine epidemic diarrhoea virus, freshly frozen isolates did not recover in all cases, but after a year, this failed altogether. In this case, the isolates were aged 3-5 years and stored at -80°C, not in liquid nitrogen.

The sera were derived from the first wave, samples from patients with clinical symptoms characteristic of COVID-19 or suspected infection. We therefore had a legitimate expectation that we would obtain results consistent with previous findings. We used Vero cells to propagate the virus in passage 0, as described in the literature [72,73]. We observed cell proliferation 72 h post- infection. Although such rapid replication is reported in the literature [72,73], so far in isolations, it has taken 7-10 days for CPE to appear in the second wave. Learning from the BCoV case, we also detected the virus by PCR. Due to secondary bacterial pathogens, screening of the sample was considered necessary. Complete genome sequencing of the virus has not been successful so far.

Mutations in the spike protein can essentially change the basic properties of the virus. The neutralising effect of antibodies may be reduced, which may even cause the loss of efficacy of marketed vaccines in extreme cases. In addition, the disease may worsen, possibly leading to new symptoms and an increase in the speed of virus spread. When a mutation occurs in a virus, new variants emerge, which change their behaviour and new strains are formed. Thus, we are currently talking about alpha, beta, gamma, epsilon, kappa, lambda, eta mu, iota, zeta, delta and omicron variants (Figure 5) [74]. The latest variant is Omicron XEC [75] which has increased its spreading speed. Although it is not known exactly which virus was detected, we do know that several patients in Hungary have reported cases with a longer and more severe course in previously infected individuals not at risk.

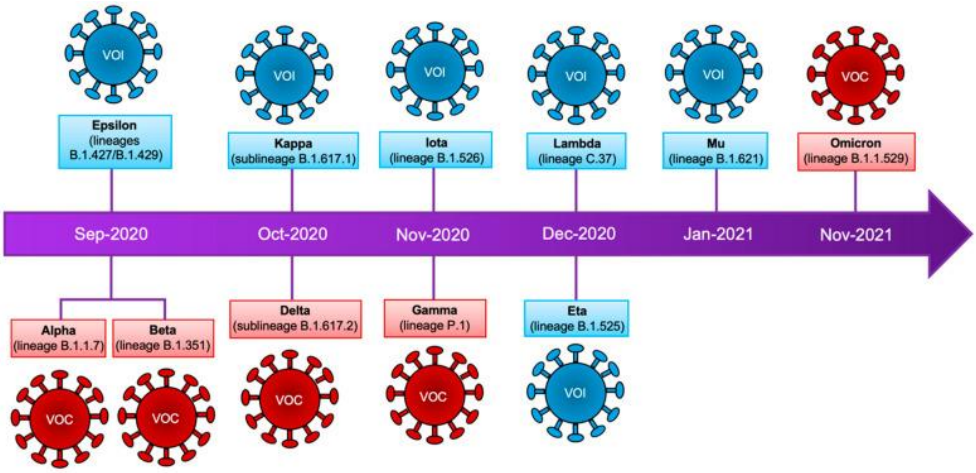


*Figure 5.: SARS-CoV-2 variants. Timeline that summarizes the emergence of SARS-CoV-2 variants. Based on Flores-Vega et al., 2022 [75]*

Based on recommendations, vaccines largely protect against infection, but there is an outbreak of Omicron strains. This is due to a change in the viral receptor binding site [77]. Therefore, new variants may require vaccine updates [78]. Although we are not 100% certain that our results indicate that the protection provided by the first wave is inadequate, our results are thought-provoking. If they are to be believed, then vaccination is recommended for updated protection, even if newer types of vaccines are not available. Our hypothesis is that even if we do not achieve complete protection, our immune system will be able to protect against new variants more quickly.

## 8. Summary

SARS-CoV-2 continues to evolve, with mutations in the spike protein that may enhance infectivity and potentially weaken immunity, though vaccine protection might still remain effective. Historical parallels can be drawn with the 19th-century "Russian flu," thought to be caused by a bovine coronavirus that crossed species to infect humans and spread globally. This virus persists as a cause of seasonal respiratory infections.

This study investigates the neutralization of bovine coronavirus and its implications in understanding cross-protection among coronaviruses and related viruses. Initially, sera from the first wave of COVID-19 were tested against BCoV grown in tissue culture; however, isolates largely failed to replicate, with BPI-3 dominating instead. A new study was launched, driven by the aggressive viral behavior seen in Human samples from 2024; tests with sera from earlier waves demonstrated minimal neutralization of BCoV despite high cross-reactivity expectations. Interestingly, PCR testing for BCoV was negative, while BPI-3 detection succeeded, revealing limited cross-protection between human and bovine haemagglutinin-neuraminidase proteins. Failure to recover BCoV isolates after long-term storage emphasized the challenges in viral stability. Whole-genome sequencing of the studied virus remains incomplete, raising questions about mutations in the spike protein that could alter viral behavior, reduce neutralization by antibodies, and potentially impair vaccine efficacy. The study underlines the dynamic evolution of viral variants, such as Omicron, which demand ongoing vaccine updates to enhance immune protection. Despite uncertainties, findings advocate for vaccination as a proactive measure against emerging variants to mitigate disease severity and enhance immune response adaptability.

## 9. References


[1] World Health Organization. (2023). *WHO Coronavirus (COVID-19)*. World Health Organization. https://covid19.who.int/ Downloaded: 2023, October 25

[2] Saied, A. A., Metwally, A. A., Alobo, M., Shah, J., Sharun, K., & Dhama, K. (2022). Bovine-derived antibodies and camelid-derived nanobodies as biotherapeutic weapons against SARS-CoV-2 and its variants: A review article. *International Journal of Surgery*, *98*, 106233. https://doi.org/10.1016/j.ijsu.2022.106233

[3] Britton, P. (2019). Coronaviruses: General Features (Coronaviridae). *Encyclopedia of Virology*, 193–197. Editors: Bamford, D. H., Zuckerman, M. https://doi.org/10.1016/B978-0-12-814515-9.00139-9

[4] Deng, X., & Baker, S. C. (2021). Coronaviruses: Molecular Biology (Coronaviridae). *Encyclopedia of Virology*, 198–207. Editors: Zuckerman, M. https://doi.org/10.1016/b978-0-12-814515-9.02550-9

[5] Satarker, S., & Nampoothiri, M. (2020). Structural Proteins in Severe Acute Respiratory Syndrome Coronavirus-2. *Archives of Medical Research*, *51*, 482-491. https://doi.org/10.1016/j.arcmed.2020.05.012

[6] Corman, V. M., Muth, D., Niemeyer, D., & Drosten, C. (2018a). Hosts and Sources of Endemic Human Coronaviruses. *Advances in Virus Research*, *100*, 163–188. https://doi.org/10.1016/bs.aivir.2018.01.001

[7] Lang, Y., Li, W., Li, Z., Koerhuis, D., Burg, A. C. S. van den, Rozemuller, E., Bosch, B.-J., Kuppeveld, F. J. M. van, Boons, G.-J., Huizinga, E. G., Schaar, H. M. van der, & Groot, R. J. de. (2020a). Coronavirus hemagglutinin-esterase and spike proteins coevolve for functional balance and optimal virion avidity. *Proceedings of the National Academy of Sciences*, *117*, 25759–25770. https://doi.org/10.1073/pnas.2006299117

[8] Su, S., Wong, G., Shi, W., Liu, J., Lai, A. C. K., Zhou, J., Liu, W., Bi, Y., & Gao, G. F. (2016). Epidemiology, genetic recombination, and pathogenesis of coronaviruses. *Trends in Microbiology*, *24*, 490-502. https://doi.org/10.1016/j.tim.2016.03.003

[9] Cui, J., Li, F., & Shi, Z. L. (2019). Origin and evolution of pathogenic coronaviruses. *Nature Reviews Microbiology*, *17*, 181-192. https://doi.org/10.1038/s41579-018-0118-9

[10] Harrison, A. G., Lin, T., & Wang, P. (2020). Mechanisms of SARS-CoV-2 Transmission and Pathogenesis. *Trends in immunology*, *41*, 1100–1115. https://doi.org/10.1016/j.it.2020.10.004

[11] World Health Organization. (2020). *Modes of transmission of virus causing COVID-19: implications for IPC precaution recommendations*. WHO. Retrieved from https://www.who.int Downloaded: 2023, October 26

[12] Haider, N., Rothman-Ostrow, P., Osman, A. Y., Arruda, L. B., Macfarlane-Berry, L., Elton, L., Thomason, M. J., Yeboah-Manu, D., Ansumana, R., Kapata, N., Mboera, L.,

Rushton, J., McHugh, T. D., Heymann, D. L., Zumla, A., & Kock, R. A. (2020). COVID-19—Zoonosis or Emerging Infectious Disease? *Frontiers in Public Health*, *8*, 596944. https://doi: 10.3389/fpubh.2020.596944. eCollection 2020.

[13] Plowright, R. K., Parrish, C. R., McCallum, H., Hudson, P. J., Ko, A. I., Graham, A. L., & Lloyd-Smith, J. O. (2017). Pathways to zoonotic spillover. *Nature Reviews Microbiology*, *15*, 502–510. https://doi.org/10.1038/nrmicro.2017.45

[14] *Zoonoses*. (2020, July 29). Who.int; World Health Organization. https://www.who.int/news-room/fact-sheets/detail/zoonoses#:~:text=A%20zoonosis%20is%20any%20disease Downloaded: 2023, October 26

[15] NIAID. (2018, July 26). *NIAID Emerging Infectious Diseases/ Pathogens | NIH: National Institute of Allergy and Infectious Diseases*. Nih.gov. https://www.niaid.nih.gov/research/emerging-infectious-diseases-pathogens Downloaded: 2023, October 26

[16] Zhou, Z., Qiu, Y., & Ge, X. (2021). The taxonomy, host range and pathogenicity of coronaviruses and other viruses in the Nidovirales order. *Animal Diseases*, *1*, 5. https://doi.org/10.1186/s44149-021-00005-9

[17] Jackwood, M. W., Hall, D., & Handel, A. (2012). Molecular evolution and emergence of avian gammacoronaviruses. *Infection, Genetics and Evolution*, *12*, 1305–1311. https://doi.org/10.1016/j.meegid.2012.05.003

[18] Graham, R. L., Donaldson, E. F., & Baric, R. S. (2013). A decade after SARS: strategies for controlling emerging coronaviruses. *Nature Reviews Microbiology*, *11*, 836–848. https://doi.org/10.1038/nrmicro3143

[19] Cui, J., Li, F., & Shi, Z.-L. (2018). Origin and evolution of pathogenic coronaviruses. *Nature Reviews Microbiology*, *17*, 181–192. https://doi.org/10.1038/s41579-018-0118-9

[20] Li, Q., Guan, X., Wu, P., Wang, X., Zhou, L., Tong, Y., Ren, R., Leung, K. S. M., Lau, E. H. Y., Wong, J. Y., Xing, X., Xiang, N., Wu, Y., Li, C., Chen, Q., Li, D., Liu, T., Zhao, J., Li, M., & Tu, W. (2020). Early Transmission Dynamics in Wuhan, China, of Novel Coronavirus-Infected Pneumonia. *The New England Journal of Medicine*, *382*, 1199–1207. https://doi.org/10.1056/NEJMoa2001316

[21] Hoffmann, M., Kleine-Weber, H., Schroeder, S., Krüger, N., Herrler, T., Erichsen, S., Schiergens, T. S., Herrler, G., Wu, N.-H., Nitsche, A., Müller, M. A., Drosten, C., & Pöhlmann, S. (2020). SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor. *Cell*, *181*, 271–280. https://doi.org/10.1016/j.cell.2020.02.052

[22] Boni, M. F., Lemey, P., Jiang, X., Lam, T. T.-Y., Perry, B. W., Castoe, T. A., Rambaut, A., & Robertson, D. L. (2020). Evolutionary origins of the SARS-CoV-2 sarbecovirus lineage responsible for the COVID-19 pandemic. *Nature Microbiology*, *5*, 1408-1417. https://doi.org/10.1038/s41564-020-0771-4

[23] Brian, D. A., & Baric, R. S. (2019). Coronavirus Genome Structure and Replication. *Current Topics in Microbiology and Immunology*, *287*, 1–30. https://doi.org/10.1007/3- 540-26765-4_1

[24] Hu, B., Ge, X., Wang, L.-F., & Shi, Z. (2015). Bat origin of human coronaviruses. *Virology Journal*, *12*, 221. https://doi.org/10.1186/s12985-015-0422-1

[25] Wang, Q., Qiu, Y., Li, J., Liao, C., Zhou, Z., & Ge, X. (2020). Receptor utilization of angiotensin converting enzyme 2 (ACE2) indicates a narrower host range of SARS-CoV-2 than that of SARS-CoV. *Transboundary and Emerging Diseases 68,* 1046-1053. https://doi.org/10.1111/tbed.13792

[26] Munnink, B. B. O., Sikkema, R. S., Nieuwenhuijse, D. F., Molenaar, R. J., Munger, E., Molenkamp, R., Spek, A. van der, Tolsma, P., Rietveld, A., Brouwer, M., Bouwmeester- Vincken, N., Harders, F., Honing, R. H. der, Wegdam-Blans, M. C. A., Bouwstra, R. J., GeurtsvanKessel, C., Eijk, A. A. van der, Velkers, F. C., Smit, L. A. M., & Stegeman, A. (2020). Transmission of SARS-CoV-2 on mink farms between humans and mink and back to humans. *Science, 371*172-177. https://doi.org/10.1126/science.abe5901

[27] Corman, V. M., Muth, D., Niemeyer, D., & Drosten, C. (2018b). Hosts and Sources of Endemic Human Coronaviruses. *Advances in Virus Research*, *100*, 163–188. https://doi.org/10.1016/bs.aivir.2018.01.001

[28] Lang, Y., Li, W., Li, Z., Koerhuis, D., Burg, A. C. S. van den, Rozemuller, E., Bosch, B.-J., Kuppeveld, F. J. M. van, Boons, G.-J., Huizinga, E. G., Schaar, H. M. van der, & Groot, R. J. de. (2020b). Coronavirus hemagglutinin-esterase and spike proteins coevolve for functional balance and optimal virion avidity. *Proceedings of the National Academy of Sciences*, *117*, 25759–25770. https://doi.org/10.1073/pnas.2006299117

[29] Woo, P., Lau, S., Yip, C., Huang, Y., & Yuen, K.-Y. (2009). More and More Coronaviruses: Human Coronavirus HKU1. *Viruses*, *1*, 57–71. https://doi.org/10.3390/v1010057

[30] Su, S., Wong, G., Shi, W., Liu, J., Lai, A. C. K., Zhou, J., Liu, W., Bi, Y., & Gao, G. F. (2016). Epidemiology, Genetic Recombination, and Pathogenesis of Coronaviruses. *Trends in Microbiology*, *24*, 490–502. https://doi.org/10.1016/j.tim.2016.03.003

[31] Vijgen, L., Keyaerts, E., Moes, E., Thoelen, I., Wollants, E., Lemey, P., Vandamme, A.-M. ., & Van Ranst, M. (2005). Complete Genomic Sequence of Human Coronavirus OC43: Molecular Clock Analysis Suggests a Relatively Recent Zoonotic Coronavirus Transmission Event. *Journal of Virology*, *79*, 1595–1604. https://doi.org/10.1128/jvi.79.3.1595-1604.2005

[32] de Groot, R.J., Baker, S.C., Baric, R., Enjuanes, L., Gorbalenya, A.E., Holmes, K.V., Perlman, S., Poon, L., Rottier, P.J.M., Talbot, P.J., Woo, P.C.Y. and Ziebuhr, J. (2012). Coronaviridae. *Virus Taxonomy*, 806–828. https://doi.org/10.1016/b978-0-12-384684-6.00068-9

[33] Lau, S. K. P., Lee, P., Tsang, A. K. L., Yip, C. C. Y., Tse, H., Lee, R. A., So, L.-Y., Lau, Y.-L., Chan, K.-H., Woo, P. C. Y., & Yuen, K.-Y. (2011). Molecular Epidemiology of Human Coronavirus OC43 Reveals Evolution of Different Genotypes over Time and Recent Emergence of a Novel Genotype due to Natural Recombination ▿ . *Journal of Virology*, *85*, 11325–11337. https://doi.org/10.1128/JVI.05512-11

[34] Mark J.G. Bakkers, Lang, Y., Feitsma, L. J., Ruben J.G. Hulswit, Stefanie, Vliet, van, Margine, I., D.F, J., Frank, Langereis, M. A., Huizinga, E. G., & Raoul. (2017). Betacoronavirus Adaptation to Humans Involved Progressive Loss of Hemagglutinin-Esterase Lectin Activity. *Cell Host & Microbe*, *21*, 356–366. https://doi.org/10.1016/j.chom.2017.02.008

[35] Markov, P. V., Ghafari, M., Beer, M., Lythgoe, K., Simmonds, P., Stilianakis, N. I., & Katzourakis, A. (2023). The evolution of SARS-CoV-2. *Nature Reviews Microbiology*, *21*, 1–19. https://doi.org/10.1038/s41579-023-00878-2

[36] Pybus, O. G., & Rambaut, A. (2009). Evolutionary analysis of the dynamics of viral infectious disease. *Nature Reviews Genetics*, *10*, 540–550. https://doi.org/10.1038/nrg2583

[37] Volz, E., Hill, V., McCrone, J. T., Price, A., Jorgensen, D., O'Toole, Á., Southgate, J., Johnson, R., Jackson, B., Nascimento, F. F., Rey, S. M., Nicholls, S. M., Colquhoun, R. M., da Silva Filipe, A., Shepherd, J., Pascall, D. J., Shah, R., Jesudason, N., Li, K., & Jarrett, R. (2021). Evaluating the Effects of SARS-CoV-2 Spike Mutation D614G on Transmissibility and Pathogenicity. *Cell*, *184*, 64-75.e11. https://doi.org/10.1016/j.cell.2020.11.020

[38] Belouzard, S., Chu, V. C., & Whittaker, G. R. (2009). Activation of the SARS coronavirus spike protein via sequential proteolytic cleavage at two distinct sites. *Proceedings of the National Academy of Sciences*, *106*, 5871–5876. https://doi.org/10.1073/pnas.0809524106

[39] Li, F. (2014). Receptor Recognition Mechanisms of Coronaviruses: a Decade of Structural Studies. *Journal of Virology*, *89*, 1954–1964. https://doi.org/10.1128/JVI.02615-14

[40] Li, F. (2005). Structure of SARS Coronavirus Spike Receptor-Binding Domain Complexed with Receptor. *Science*, *309*, 1864–1868. https://doi.org/10.1126/science.1116480

[41] Millet, J. K., & Whittaker, G. R. (2014). Host cell entry of Middle East respiratory syndrome coronavirus after two-step, furin-mediated activation of the spike protein. *Proceedings of the National Academy of Sciences*, *111*, 15214–15219. https://doi.org/10.1073/pnas.1407087111

[42] Yang, X., Chen, X., Bian, G., Tu, J., Xing, Y., Wang, Y., & Chen, Z. (2014). Proteolytic processing, deubiquitinase and interferon antagonist activities of Middle East respiratory syndrome coronavirus papain-like protease. *The Journal of general virology*, *95*, 614–626. https://doi.org/10.1099/vir.0.059014-0

[43] Watanabe, Y., Berndsen, Z. T., Raghwani, J., Seabright, G. E., Allen, J. D., Pybus, O. G., McLellan, J. S., Wilson, I. A., Bowden, T. A., Ward, A. B., & Crispin, M. (2020). Vulnerabilities in coronavirus glycan shields despite extensive glycosylation. *Nature Communications, 11,* 2688. https://doi.org/10.1038/s41467-020-16567-0

[44] Korber, B., Fischer, W. M., Gnanakaran, S., Yoon, H., Theiler, J., Abfalterer, W., Hengartner, N., Giorgi, E. E., Bhattacharya, T., Foley, B., Hastie, K. M., Parker, M. D., Partridge, D. G., Evans, C. M., Freeman, T. M., de Silva, T. I., McDanal, C., Perez, L. G., Tang, H., & Moon-Walker, A. (2020). Tracking changes in SARS-CoV-2 Spike: evidence that D614G increases infectivity of the COVID-19 virus. *Cell, 182*. 812-827. https://doi.org/10.1016/j.cell.2020.06.043

[45] Yurkovetskiy, L., Wang, X., Pascal, K. E., Tomkins-Tinch, C., Nyalile, T. P., Wang, Y., Baum, A., Diehl, W. E., Dauphin, A., Carbone, C., Veinotte, K., Egri, S. B., Schaffner, S. F., Lemieux, J. E., Munro, J. B., Rafique, A., Barve, A., Sabeti, P. C., Kyratsous, C. A., & Dudkina, N. V. (2020). Structural and Functional Analysis of the D614G SARS-CoV-2 Spike Protein Variant. *Cell, 183*, 739-751. https://doi.org/10.1016/j.cell.2020.09.032

[46] Zhang, L., Jackson, C. B., Mou, H., Ojha, A., Rangarajan, E. S., Izard, T., Farzan, M., & Choe, H. (2020). The D614G mutation in the SARS-CoV-2 spike protein reduces S1 shedding and increases infectivity. *bioRxiv : the preprint server for biology,* 2020.06.12.148726. https://doi.org/10.1101/2020.06.12.148726

[47] Furuyama, T. N., Antoneli, F., Carvalho, I. M. V. G., Briones, M. R. S., & Janini, L. M. R. (2020). Temporal data series of COVID-19 epidemics in the USA, Asia and Europe suggests a selective sweep of SARS-CoV-2 Spike D614G variant. *Molecular Biology: preprint,* 2020 06. 12. https://doi.org/10.48550/arXiv.2006.11609

[48] Plante, J. A., Liu, Y., Liu, J., Xia, H., Johnson, B. A., Lokugamage, K. G., Zhang, X., Muruato, A. E., Zou, J., Fontes-Garfias, C. R., Mirchandani, D., Scharton, D., Bilello, J. P., Ku, Z., An, Z., Kalveram, B., Freiberg, A. N., Menachery, V. D., Xie, X., & Plante, K. S. (2020). Spike mutation D614G alters SARS-CoV-2 fitness and neutralization susceptibility. *bioRxiv : the preprint server for biology*, 2020.09.01.278689. https://doi.org/10.1101/2020.09.01.278689

[49] Hou, Y. J., Chiba, S., Halfmann, P., Ehre, C., Kuroda, M., Dinnon, K. H., Leist, S. R., Schäfer, A., Nakajima, N., Takahashi, K., Lee, R. E., Mascenik, T. M., Graham, R., Edwards, C. E., Tse, L. V., Okuda, K., Markmann, A. J., Bartelt, L., de Silva, A., & Margolis, D. M. (2020). SARS-CoV-2 D614G variant exhibits efficient replication ex vivo and transmission in vivo. *Science, 370,* eabe8499. https://doi.org/10.1126/science.abe8499

[50] Lorenzo-Redondo, R., Nam, H. H., Roberts, S. C., Simons, L. M., Jennings, L. J., Qi, C., Achenbach, C. J., Hauser, A. R., Ison, M. G., Hultquist, J. F., & Ozer, E. A. (2020). A clade of SARS-CoV-2 viruses associated with lower viral loads in patient upper airways. *EBioMedicine, 62,* 103112. https://doi.org/10.1016/j.ebiom.2020.103112

[51] 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.

(2020). Virological assessment of hospitalized patients with COVID-2019. *Nature, 581*. 465-469. https://doi.org/10.1038/s41586-020-2196-x

[52] Clarke, D. K., Duarte, E. A., Moya, A., Elena, S. F., Domingo, E., & Holland, J. (1993). Genetic bottlenecks and population passages cause profound fitness differences in RNA viruses. *Journal of virology*, *67*, 222–228.https://doi.org/10.1128/jvi.67.1.222-228.1993

[53] Sanjuan, R., Nebot, M. R., Chirico, N., Mansky, L. M., & Belshaw, R. (2010). Viral Mutation Rates. *Journal of Virology*, *84*, 9733–9748. https://doi.org/10.1128/jvi.00694- 10

[54] Sanjuán, R., & Domingo-Calap, P. (2016). Mechanisms of viral mutation. *Cellular and Molecular Life Sciences*, *73*, 4433–4448. https://doi.org/10.1007/s00018-016- 2299-6

[55] Loewe, L., & Hill, W. G. (2010). The population genetics of mutations: good, bad and indifferent. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *365*, 1153–1167. https://doi.org/10.1098/rstb.2009.0317

[56] Fehr, A. R., & Perlman, S. (2015). Coronaviruses: An Overview of Their Replication and Pathogenesis. *Coronaviruses*, *1282*, 1–23. https://doi.org/10.1007/978-1-4939-2438- 7_1

[57] Amicone, M., Borges, V., Alves, M. J., Isidro, J., Zé-Zé, L., Duarte, S., Vieira, L., Guiomar, R., Gomes, J. P., & Gordo, I. (2022). Mutation rate of SARS-CoV-2 and emergence of mutators during experimental evolution. *Evolution, Medicine, and Public Health*, *10*, 142–155. https://doi.org/10.1093/emph/eoac010

[58] Ribeiro, R. M., Li, H., Wang, S., Stoddard, M. B., Learn, G. H., Korber, B. T., Bhattacharya, T., Jérémie Guedj, Parrish, E. H., Hahn, B. H., Shaw, G. M., & Perelson, A. S. (2012). Quantifying the Diversification of Hepatitis C Virus (HCV) during Primary Infection: Estimates of the In Vivo Mutation Rate. *PLOS Pathogens*, *8*, e1002881–e1002881. https://doi.org/10.1371/journal.ppat.1002881

[59] Rawson, J. M. O., Landman, S. R., Reilly, C. S., & Mansky, L. M. (2015). HIV-1 and HIV-2 exhibit similar mutation frequencies and spectra in the absence of G-to-A hypermutation. *Retrovirology*, *12*. 60, https://doi.org/10.1186/s12977-015-0180-6

[60] Wang, S., Sakhatskyy, P., Chou, T. H., & Lu, S. (2005). Assays for the assessment of neutralizing antibody activities against Severe Acute Respiratory Syndrome (SARS) associated coronavirus (SCV). *Journal of immunological methods*, *301*, 21–30. https://doi.org/10.1016/j.jim.2005.03.008

[61] Maxima First Strand cDNA Synthesis Kit for RT-qPCR. (2024). Thermofisher.com. https://www.thermofisher.com/order/catalog/product/K1642?SID=srch-srp-K1642. Downloaded: 2023, March 6

[62] Reed, L., J., & Muench, H. (1938) A simple method of estimating fifty-percent endpoints. *American Journal of Epidemiology, 27. 493–497,* https://doi.org/10.1093/oxfordjournals.aje.a118408

[63] Tsunemitsu, H., Smith, D. R., & Saif, L. J. (1999). Experimental inoculation of adult dairy cows with bovine coronavirus and detection of coronavirus in feces by RT-PCR. *Archives of virology*, *144*, 167–175. https://doi.org/10.1007/s007050050493

[64] Kamdi, B., Singh, R., Singh, V., Singh, S., Kumar, P., Singh, K. P., George, N., & Dhama, K. (2020). Immunofluorescence and molecular diagnosis of bovine respiratory syncytial virus and bovine parainfluenza virus in the naturally infected young cattle and buffaloes from India. *Microbial pathogenesis*, *145*, 104165. https://doi.org/10.1016/j.micpath.2020.104165

[65] Han, M. G., Cheon, D. S., Zhang, X., & Saif, L. J. (2006). Cross-protection against a human enteric coronavirus and a virulent bovine enteric coronavirus in gnotobiotic calves. *Journal of virology*, *80*, 12350–12356. https://doi.org/10.1128/JVI.00402-06

[66] *SARS-CoV specific RT-PCR primers*. (2024). https://www.who.int/publications/m/item/sars-cov-specific-rt pcr-primers. Downloaded: 2024, November 15

[67] Vijgen L., Keyaerts, E., Moës, E., Thoelen, I., Wollants, E., Lemey, P., Vandamme, A. M., & Van Ranst, M. (2005). Complete genomic sequence of human coronavirus OC43: molecular clock analysis suggests a relatively recent zoonotic coronavirus transmission event. *Journal of virology*, *79*, 1595–1604. https://doi.org/10.1128/JVI.79.3.1595-1604.2005.

[68] Berche P. (2022). The enigma of the 1889 Russian flu pandemic: A coronavirus?. *Presse medicale (Paris, France : 1983)*, *51*(3), 104111. https://doi.org/10.1016/j.lpm.2022.104111

[69] Zhang M., Herbst W., Kousoulas K.G., & Storz J. (1994) Biological and genetic characterization of a hemagglutinating coronavirus isolated from a diarrhoeic child. *Journal of medical virology*, *44*, 152–161. https://doi.org/10.1002/jmv.1890440207.

[70] Han, M. G., Cheon, D. S., Zhang, X., & Saif, L. J. (2006). Cross-protection against a human enteric coronavirus and a virulent bovine enteric coronavirus in gnotobiotic calves. *Journal of virology*, *80*, 12350–12356. https://doi.org/10.1128/JVI.00402-06

[71] Klippmark, E., Rydbeck, R., Shibuta, H., & Norrby, E. (1990). Antigenic variation of human and bovine parainfluenza virus type 3 strains. *The Journal of general virology*, *71*, 1577–1580. https://doi.org/10.1099/0022-1317-71-7-1577

[72] Rydbeck, R., Löve, A., Orvell, C., & Norrby, E. (1987). Antigenic analysis of human and bovine parainfluenza virus type 3 strains with monoclonal antibodies. *The Journal of general virology*, *68*, 2153–2160. https://doi.org/10.1099/0022-1317-68-8-2153

[73] Harcourt, J., Tamin, A., Lu, X., Kamili, S., Sakthivel, S., K., Murray, J., Queen, K., Tao,Y., Paden, C., R., Zhang, J., Li, Y., Uehara, A., Wang, H., Goldsmith, C., Bullock, H.,

A., Wang, L., Whitaker, B., Lynch, B., Gautam, R., Schindewolf, C., Lokugamage, K., G., Scharton, D., Plante, J., A., Mirchandani, D., Widen, S.,G., Narayanan, K., Makino, S., Ksiazek, T., G., Plante, K., S., Weaver, S., C., Lindstrom, S., Tong, S., Menachery, V., D., & Thornburg, N., J. (2020) Isolation and characterization of SARS-CoV-2 from the first US COVID-19 patient. *bioRxiv : the preprint server for biology*, 2020.03.02.972935.https://doi.org/10.1101/2020.03.02.972935.

[74] Banerjee, A., Nasir, J. A., Budylowski, P., Yip, L., Aftanas, P., Christie, N., Ghalami, A., Baid, K., Raphenya, A. R., Hirota, J. A., Miller, M. S., McGeer, A. J., Ostrowski, M., Kozak, R. A., McArthur, A. G., Mossman, K., & Mubareka, S. (2020). Isolation, Sequence, Infectivity, and Replication Kinetics of Severe Acute Respiratory Syndrome Coronavirus 2. *Emerging infectious diseases*, *26*, 2054–2063. https://doi.org/10.3201/eid2609.201495

[75] Flores-Vega, V.,R., Monroy-Molina, J., V., Jiménez-Hernández, L., E., Torres, A., G., Santos- Preciado, J., I., & Rosales-Reyes, R. (2022) SARS-CoV-2: Evolution and Emergence of New Viral Variants. Viruses. Mar 22;14(4):653. https://doi.org/10.3390/v14040653.

[76] *SARS-CoV-2 variants of concern as of 27 September 2024*. (2024). European Centre for Disease Prevention and Control. https://www.ecdc.europa.eu/en/covid-19/variants-concern Downloaded: 2024, October 5

[77] Kuhlmann C., Mayer, C. K., Claassen, M., Maponga, T., Burgers, W. A., Keeton, R., Riou, C., Sutherland, A. D., Suliman, T., Shaw, M. L., & Preiser, W. (2022). Breakthrough infections with SARS-CoV-2 omicron despite mRNA vaccine booster dose. *Lancet (London, England)*, *399*, 625–626. https://doi.org/10.1016/S0140-6736(22)00090-3

[78] Ma Q, Li M, Ma L, Zhang C, Zhang H, Zhong H, Wen J, Wang Y, Yan Z, Xiong W, Wu L, Guo J, Yang W, Yang Z, Zhang B. (2023) SARS-CoV-2 bivalent mRNA vaccine with broad protection against variants of concern. Front Immunol. 14,1195299. https://doi.org/10.3389/fimmu.2023.1195299.

# Thesis progress report for veterinary students

Name of student: TRISHABYE ESSOO

Neptun code of the student: HOH8CU

Name and title of the supervisor: DR. LŐRINCZ MÁRTA

Department: MICROBIOLOGY AND INFECTIOUS DISEASES

Thesis title: VIRUS NEUTRALISATION TEST WITH HUMAN SERA FOR COVID-19 AND BOVINE CORONAVIRUS

## Consultation – 1st semester

| | Timing | | | Topic / Remarks of the supervisor | Signature of the supervisor |
|---|---|---|---|---|---|
| | year | month | day | | |
| 1. | 2023. | 04 | 28 | Sample sorting | |
| 2. | 2023. | 05. | 02 | Cell culture preparing | |
| 3. | 2023. | 05. | 04 | Passage of cell culture | |
| 4. | 2023. | 05. | 05 | Virus neutralizing | |
| 5. | 2023. | 05. | 08. | Detection CPE | |

**Grade achieved at the end of the first semester:** ..... Tells (5) .....

## Consultation – 2nd semester

| | Timing | | | Topic / Remarks of the supervisor | Signature of the supervisor |
|---|---|---|---|---|---|
| | year | month | day | | |
| 1. | 2024 | 08 | 27 | Determination of infection titer | |
| 2. | 2024 | 09 | 03 | Checking the writing of a thesis (material & method) | |
| 3. | 2024 | 09 | 10 | Checking the writing of a thesis (result) | |
| 4. | 2024 | 10 | 01 | Checking the writing of a thesis | |
| 5. | 2024 | 11 | 18 | Correcting a thesis | |

**Grade achieved at the end of the second semester: ..... jeles (5) .....**

The thesis meets the requirements of the Study and Examination Rules of the University and the Guide to Thesis Writing.

I accept the thesis and found suitable to defence,

..... Zöldhegy .....
signature of the supervisor

Signature of the student: .....

Signature of the secretary of the department: .....

Date of handing the thesis in..... .....