COVID-19
as a Model for PCR Viral Respiratory Disease Diagnostics

Michal Pohludka
Lenka Piherová

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PREFACE

The COVID-19 pandemic has affected the whole world and influenced almost all areas of human life. Although each country has approached the situation differently, they had one activity in common – the effort to slow down the spread of the virus to prevent an overload of national health systems. The preferred testing method for medical diagnostics as well as for elimination of the spread within the population is the Real-Time Quantitative Reverse Transcription PCR (RT-qPCR).

In this book, we focus in detail on the methodology, good practice, and troubleshooting. The aim of the publication is to provide an advanced practical manual that can be utilized in any diagnostic laboratory using the PCR method.

Good practice in laboratory diagnostics is essential for medical as well as for epidemiological reasons. During an epidemic outbreak, laboratory diagnostics helps physicians to treat patients effectively and the laboratory big data provides information about the spread of the virus in different sociological groups. This is illustrated in the presented experiment studying the spread of the virus in child population at schools.

INTRODUCTION

The COVID-19 pandemic caused a global crisis affecting almost all aspects of our lives. Many countries implemented restrictions to prevent their health systems from overloading and to stop, or at least control, the spread of the infection. Governments applied widespread anti-epidemic measures that were kept for a long time. However, the infection and transmission of the SARS-CoV-2 virus has a fundamentally different course, consequences, and intensity than any other infection their expert teams have experienced. For less at-risk populations, these widespread measures had questionable, sometimes even negative effects. The most affected groups were children and students.

One of the key tools in the fight against the COVID-19 pandemic is laboratory diagnostics based on PCR methodology. Prior to the pandemic, there were just a few parameters measured by this technique. Currently, the PCR testing for SARS-CoV-2 is the most measured laboratory parameter and PCR has become one of the best-known methods even among the general public.

During the pandemic, we were experiencing a significant technological progress in SARS-CoV-2 PCR diagnostics, as there was a huge demand for the delivery of fast, reliable, and sensitive patient results. In a therapeutic context, it is essential to quickly deliver information of SARS-CoV-2 positivity to the physician to provide the patient with an effective treatment that works early in the course of the infection. From an epidemiological perspective, it is beneficial to look at the evolution of the virus and its spread in the population with a specific focus on different demographic and sociological groups.

The PCR method has become the diagnostic gold standard for determination of SARS-CoV-2 presence. It was used for monitoring of the spread within the population and consequently for the management of restrictive interventions to slow the spread of the virus and to keep the healthcare system running. It also makes it possible to provide government officials with the necessary data on the development of the epidemic with the possibility of predicting its future state.

The COVID-19 pandemic has opened up unsuspected possibilities in PCR diagnostics. This is one of the few positive effects of the pandemic. Nowadays, discussions are conducted on how to use all the equipment and laboratories to determine parameters other than SARS-CoV-2. This might result in a much faster and precise delivery of results to physicians for other infectious disease parameters such as HCV, HBV, or STDs.

The global pandemic of SARS-CoV-2 has brought and continues to bring dramatic changes in all social areas. There are significant ones in the healthcare sector, but perhaps the greater effects permeate into business, services, and the everyday activities of people. Even

though this is a global problem, different countries are approaching the complex issue of the pandemic and its impact differently. Some are using restrictive measures to slow the spread of the virus. Others are letting the epidemic run and instead focus on quality diagnosis and treatment, which is currently the most functional approach in the initial phases of any epidemic. In this context, the global strategy in the fight against the pandemic strongly influences laboratory diagnostics. In some countries, the priority is to test just people with symptoms and effectively use the testing capacities for patients, so that an efficient treatment can be applied already in the early stage of the infection. With such an approach, the results are delivered faster in comparison with countries that test the population more broadly for epidemiological reasons to prevent the transmission of the infection.

DEVELOPMENT OF PCR DIAGNOSTICS FOR DETECTION OF SARS-COV-2

At the beginning of the pandemic of COVID-19, knowledge regarding the virus and its impact was minimal. However, soon after, the viral genome has been sequenced and characterized and the function of individual genes of the virus has been determined. This was crucial for the establishment of the PCR methodology. In the early days of fighting the pandemic, the World Health Organization (WHO, https://www.who.int/) and Centers for Disease Control and Prevention (CDC, https://www.cdc.gov/) provided assistance to research teams worldwide in developing protocols and setting recommendations for PCR methodologies.

PCR diagnostics were set up very carefully in the early days. Two or even more genes were detected simultaneously in a multiplex assay, targeting conserved parts of the virus sequence, so the analysis would not be affected by possible mutations. This posed difficulties in terms of evaluation and there were situations where laboratories were not even able to evaluate the results easily. The lack of knowledge of the life cycle of the virus in the organism came into play. Samples were collected from people at different stages of infection, from the first contact with the virus all the way to the stage when the infection was no longer present in the organism, but residual mRNA remained on the mucous membranes of the nasopharynx. In all these cases, due to the sensitivity of the PCR methodology, positive results were reported. There were cases when only one gene came out positive, even after the analysis was repeated to rule out a faulty outcome. It was later found out that the mRNA stays on the mucosa longer and degrades gradually, with the *E gene*, which is one of the analyzed genes, being the last to degrade. Out of caution, these samples were considered positive, which caused a significantly negative social effect. People have been kept in quarantine for unreasonably long periods and in some cases had to undergo multiple exit tests.

This problem was solved by changing the PCR diagnostic setup. PCR kits that had two genes as targets in one channel during the analysis became tolerated. With this setup, there were no longer controversial results that would keep people in quarantines for unreasonably long periods. At the same time, this approach significantly increased throughput of laboratories that could automate the entire process. With this change, PCR diagnostics suddenly became one of the key tools not only for diagnosing people with symptoms, but also for tracing people potentially infected from a contact with a positive individual. This was the point at which laboratories began to automate the whole process and rely on automated evaluation of results, which led to a rapid increase in testing capacity.

The increase in laboratory testing capacity also brought some negative effects. The main one was and still is a lack of communication between physicians and laboratories. Patients and physicians are thus deprived of obtaining comprehensive information, even though far more conclusions can be made from PCR spectra than just positivity or negativity for SARS-CoV-2. Probably the most valuable information for the physician would be a determination of the stage of infection, which would help to decide the most efficient treatment or further sampling, which can be used to determine the dynamics of the progress of the infection and whether it is increasing or declining.

The reduction of the information to only SARS-CoV-2 positivity or negativity and the hindered communication between laboratories and physicians have a negative effect on the follow-up diagnosis and treatment. The preventive measures are important for populations at higher risk of a severe course of COVID-19. These include older individuals (60 and above), diabetic or obese people, or people with cardiovascular or renal problems. For these individuals, early diagnosis is essential, as therapeutic approaches are most effective in the early stages of infection.

Therefore, many hospitals have strongly focused on testing symptomatic individuals, mostly their own patients. This allows them to obtain results in a timelier manner and to set up a testing system. Patients are also diagnosed in more detail based on parameters other than the simple presence of SARS-CoV-2 infection. Blood parameters such as inflammatory markers are monitored to detect strong inflammation in the body that leads to an inappropriate immune response to infection and a severe course of the disease.

Currently, PCR methodology is set up to monitor the presence of SARS-CoV-2 in test subjects. Further information is generally not provided and personalization is impossible due to the high testing throughput in laboratories. This is negatively reflected in the failure of the medical approach in most cases. Thus, people are admitted to hospital only with severe symptoms, at which point no treatment is effective enough, and thus they are treated mainly with supportive methods to boost immunity and to prevent an inappropriate reaction of the body.

LABORATORY PCR DIAGNOSTICS AS A KEY PARAMETER FOR PLANNING A TREATMENT

Laboratory diagnostics can provide significantly more data to physicians and patients to ensure that the course of COVID-19 does not turn into a complicated and prolonged disease or even kill the patient. Nowadays, there are relatively effective treatments, both in the form of monoclonal antibodies and in the form of antivirals and supportive medications. The fundamental problem of COVID-19 is the inadequate immune response of the body, which is triggered by a cytokine storm. This cascade involves molecules such as interleukins and other acute-phase molecules. This results in many undesirable processes in the body affecting blood coagulation, blood oxygen saturation, and the efficiency of oxygen transfer in the lungs to blood in general. The body is also susceptible to other secondary infections, which can be both bacterial and viral. These in most cases cause pneumonia.

Another major promise to help in the fight against severe COVID-19 disease is to determine predictive markers. One of these has been developed in the Czech Republic (owned by GeneSpector Innovations and discovered by scientists at Charles University in Prague)

and has already been granted a patent. The marker is a level of expression of SAA1 (serum amyloid A1), which is found in the nasopharynx and its higher expression compared to the housekeeping gene means a likely severe disease course that occurs several days later. Providing this information gives physicians time to implement appropriate treatment and monitor the patient. The use of this marker is currently undergoing clinical trials.

BARRIERS TO INCREASING LABORATORY CAPACITY

Social and medical pressure to increase testing capacity has forced laboratories to consider maximizing automation. This relies on two basic areas – the analytical part and the IT part. Despite this, the throughput of laboratories was still insufficient, so laboratories everywhere began to focus on optimizing all the steps, and at the same time, laboratories tried to develop new approaches to achieve mass testing.

One possibility was the implementation of the pooling method. This allowed a dramatic increase in capacity, but only a few laboratories succeeded in achieving this, both technologically and in terms of personnel. It turned out that setting up such a system is not easy and has increased demands on the laboratory management and the evaluation of results. All this is the reason why only a very small number of laboratories have adopted the pooling method, those being the ones that were able to meet these increased demands for testing throughput.

EVOLUTION OF THE VIRUS AND RELATED CONCERNS

Monitoring the evolution of SARS-CoV-2 virus is one of the important activities in the fight against the pandemic. However, the most important thing is to monitor the clinical effects of each variant on the health of individuals. Unfortunately, this can only be determined from past illnesses and monitoring the impact of the infection on human health. This is the main cause for concern when new mutations and variants arrive, as relevant conclusions can only be reached in a matter of weeks, more likely months.

The SARS-CoV-2 virus, given its size, has a huge number of possible variants that can appear during its replication. However, the majority of variants are not compatible with the possibility of either surviving alone or entering the population in competition with other SARS-CoV-2 variants or viruses other than SARS-CoV-2. Therefore, we see an emergence of a new variant in the population at a time when it is gaining a competitive advantage across a spectrum of other variants, resulting in the uncontrolled spread throughout populations across the globe. This has been evident in all the new variants so far, and the essential precondition for its dominance appears to be a higher infectivity of the variant. This is directly linked to mutations in the S-protein, which is a key component in binding to receptors that subsequently carry the genetic information of the virus into human cells and drive its replication.

To be able to identify the representation of the different virus variants in the population, the above-mentioned discriminatory PCR tests were used. This technique identifies point mutations, so it is possible to monitor the representation of the various variants already known thanks to this diagnostic. However, the ability to track the evolution of mutations in other areas of the viral genetic information is lost.

To obtain information on changes in the genetic information of the virus, it is necessary to use a sequencing method. This method can be approached in two ways, the choice being influenced mainly by the difficulty of evaluation, cost, and volume of data. The more complex method is to characterize the whole genome sequence of the virus, which is complicated, but provides complete information. However, since it is essential to monitor the evolution of the S-protein region, which is used to deliver viral particles into cells and determines the affinity of the virus for receptors and its infectivity, sequencing is much more often performed only on this region. Another reason is that this region is the most susceptible to mutation, whereas other regions of the genome are more conserved and mutations in regions outside the S-gene do not directly affect the level of infectivity.

Each country approaches the sequencing program with a slightly different strategy. For example, the Czech Republic has designated centers where the analyses take place and where samples are sent from different parts of the country. At present, samples are sequenced somewhat randomly, with a few groups being analyzed preferentially – travelers from exotic or dangerous countries in terms of the epidemic and young people with a complicated medical course. So, it is not sequenced genetic material acquired from a representative sample of the population, which would help to monitor the evolution of the virus in a clearly defined group.

However, sequenced samples can potentially provide more information than is currently measured. Beside monitoring the evolution of the virus, the complete genetic information present in the collected sample can be sequenced, including the human one. This predetermines an area of investigating the immune response and, in general, the response of organisms to SARS-CoV-2 infection. When infection occurs in the cells of the nasopharyngeal mucosa, there is a significant immune response and the expression of many genes that are involved in the response in any way. Unfortunately, the stage of the organism's response to infection at the molecular level is currently not of direct interest. This topic is being addressed mostly by scientific groups who are looking to link ongoing infection to the organism's response, as there is a difference in expression profiles compared with the physiological situation that may lead to the discovery of new drugs and therapeutic approaches. It may also lead to an understanding of the mechanism of SARS-CoV-2 infection, which, in addition to its importance in drug development, may also answer many questions in the field of vaccination strategy and pharmacogenetics.

NEW EPIDEMIC WAVES AND A PREDICTION OF NEW VARIANTS

Epidemic management is from a medical and epidemiological perspective dependent on the ability to predict the future state in a horizon of at least weeks. Predicting the arrival of epidemic waves gives government officials the opportunity to take any steps necessary to slow down the spread of infection. It is also crucial to be able to predict the peak of an epidemic wave at a certain level of probability and to use the data to predict hospital occupancy and the impact on other areas of medical care – preventive care, scheduled interventions, and treatment of chronic and urgent medical problems.

This is aided by various mathematical models that consider many parameters – the number of people who have had a disease or infection, the proportion of people vaccinated, regional differences, etc. What is very difficult to predict, however, is the behavior of the virus itself,

the evolution of mutations and variants that affect the profile of the epidemic curve, and of course the effectiveness of the used restrictive measures.

In this book, we show a new model that can be used to predict the arrival of new epidemic waves with high accuracy and well in advance.

INTRODUCTION TO VIROLOGY

Virology is a biological science studying non-cellular organisms, especially viruses, but also viroids. Sometimes prions are also included in its scope. In many respects, it borders on the following other disciplines in particular: biochemistry, molecular biology, genetics, epidemiology, parasitology, and immunology (Schlesinger et al., 2004).

The father of virology is considered to be the Russian scientist Dmitri Ivanovsky, who in 1892, while filtering the cell sap of tobacco, realized that the objects causing tobacco mosaicism passed through this filter. Later, these objects were called viruses, and thus virology was founded. To this day, the tobacco mosaic virus is considered to be the first virus discovered. In the first half of the 20th century, relatively good microscopic images of viruses were already available. In the 1930s, Vinson and Petre precipitated the tobacco mosaic virus as if it was an ordinary protein. They even demonstrated its movement in an electric field, providing further evidence that viruses are proteins. However, Max Schlesinger showed in 1932 that the bacteriophages he studied contained phosphorus and deoxyribonucleic acid (DNA) in addition to proteins. Studies on other viruses had shown the presence of ribonucleic acid (RNA). The first successful crystallization of virus particles was a milestone (Stanley, 1935). In the 1950s, there were tremendous advances in the study of viruses – plant, animal, and bacterial as well (Fields et al., 2007). From the 1960s, scientists began to use viruses as model organisms to study general processes that could then be applied to all forms of life. From the 1970s onwards, a revolution in experimental biology began with the development of genetic engineering, and viruses were an integral part of this revolution. Currently, there are advances in knowledge about the role of viruses in cancer or, for example, the development of new vaccines against viral diseases. However, some viruses, such as HIV, still provide a major challenge for virologists.

The global pandemic of SARS-CoV-2 has extremely progressed the understanding of viruses. Scientific teams and commercial entities backed by large amounts of funding have begun to concentrate on different areas of virology. Virology and other related sciences have come to the forefront – not only of scientific interest, but also of social interest. In the last two years, there has been an unusual shift in the knowledge of virus behavior, its mechanism of entry into cells, and its life cycle. The field of molecular biology and genetics is playing an important role. With the SARS-CoV-2 pandemic, it is possible to study the mutagenesis of the virus as it spreads through the population. The immune response of humans to viral infection is no longer unknown and new mechanisms have been discovered. New technologies such as NGS (next generation sequencing) have been introduced to monitor not only the evolution of the virus, but also epidemiological parameters, such as spread of the virus geographically or

in different demographic groups. Methods and technologies of molecular biology have made it possible to respond rapidly to a pandemic and to develop new types of vaccines. Despite many disagreements in society, works in virology, immunology, and genetic engineering have made, as well as enabled, a significant progress.

Virology as a science is now seen as an integral part of the natural sciences and is becoming a center of interest not only for scientific groups, but also for pharmaceutical companies. Studying not only viruses themselves, but also their mechanisms and impact on human health, including possible effective treatments, is a new concept in virology.

NON-CELLULAR ORGANISMS

Non-cellular or subcellular organisms are a diverse group of structures at the interface between living and non-living systems. They include viruses, viroids, and possibly prions – if they can be considered organisms.

Viruses are small, non-cellular organisms that contain only one type of nucleic acid and replicate only in living cells using host proteosynthesis. They are organized only as particles; therefore, they are not organized as cells. Mature virions contain only one type of nucleic acid, and thus the viruses can be characterized as DNA or RNA viruses. Virion is the term used for a single viral particle, which can come in different shapes. They can be spherical, rod-shaped, filamentous. The inner part of the virion is called the nucleoid. It is composed of nucleic acid and is surrounded by a protein capsid. The capsid is composed of protein subunits – capsomers. Their shape allows them to fit together to form a larger aggregate. Regarding the type of symmetry, there are two main groups of capsids: cubic and helical. The complex of capsid and nucleic acid is called a nucleocapsid. The simplest virions are just single naked nucleocapsids (picornaviruses, papillomaviruses, adenoviruses). Enveloped viruses have, in addition to the capsid, an additional envelope of a double layer of proteins and lipids and virus-specific glycoproteins. These are embedded in the cell membranes of infected host cells and allow identification of the virus and virus-infected cells (Bednář et al., 1999).

A viroid is a small infectious circular autocatalytic ribonucleic acid (RNA) that does not encode any protein. The hosts of viroids are vascular plants, in which viroids cause diseases similar to viruses. Common symptoms of such diseases include stunting, epinasty, and yellow leaf spots (Lhotský, 2016).

Prions (derived from proteinaceous infectious particles) are infectious particles consisting of only a protein molecule. The prion theory was formulated in 1982 by Stanley Prusiner, who received the Nobel Prize in 1997. Prions are the causative agents of neurodegenerative diseases in humans and animals (Bednář et al., 1999).

STRUCTURE AND COMPOSITION OF VIRAL PARTICLES

Viral particles have a variety of shapes and sizes. They are measured in nanometers ranging between 20 and 750 nm. The basic structure of a virus is made up of a molecule containing genetic information and a protein layer that protects that information molecule. In general, there are four main morphological virus types: helical, icosahedral, enveloped and head-and-tail (Fig. 1).

A – Helical: The virus structure has a capsid with a central cavity or hollow tube that is made up of proteins arranged in a circular fashion, creating a disc-like shape. The disc shapes are attached helically. Usually, plant viruses belong to this group.

B – Icosahedral: An icosahedron is a geometric shape with 20 sides, each composed of an equilateral triangle. A typical example of viruses from this group are polioviruses or herpesviruses.

C – Enveloped: Viral envelopes consist of a lipid bilayer that closely surrounds a shell of virus-encoded, membrane-associated proteins. The exterior of the bilayer is studded with virus-encoded, glycosylated (trans-)membrane proteins. Animal viruses are frequently enveloped.

D – Head-and-tail: This is a variant of the icosahedral viral shape found in bacteriophages. Some viruses, regardless of their protein capsid shape, are enveloped with a lipid bilayer around their cap.
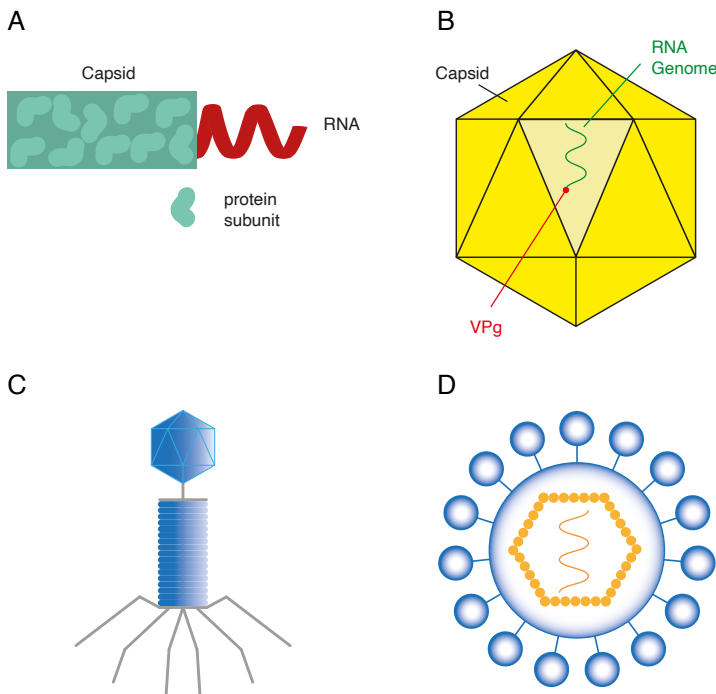

Fig. 1 Main morphological virus types: A – helical, B – icosahedral, C – head-and-tail, D – enveloped

There is also a classification of viruses according to the Baltimore classification groups, which is based on their mechanism of mRNA synthesis. Characteristics directly related to this include whether the genome is made out of DNA or RNA, which can be either single- or double-stranded and either positive or negative. There are seven groups (Tab. 1).

Tab. 1 Groups of viruses according to the Baltimore classification groups

Group	Name	Type of genetic information
Group I	double-stranded DNA viruses	dsDNA
Group II	single-stranded DNA viruses	ssDNA
Group III	double-stranded RNA viruses	dsRNA
Group IV	positive sense single-stranded RNA viruses	(+)ssRNA
Group V	negative sense single-stranded RNA viruses	(–)ssRNA
Group VI	single-stranded RNA viruses with a DNA intermediate in their life cycle	ssRNA-RT
Group VII	double-stranded DNA viruses with an RNA intermediate in their life cycle	dsRNA-RT

HUMAN CORONAVIRUSES

Human coronaviruses are among the viruses that cause respiratory diseases with varying severity. The severity scale ranges from common cold, through bronchiolitis, to death (Pene et al., 2003). In recent years, we have seen the human coronaviruses appear periodically in different places all around the world. The major issue with such outbreaks is high infectivity with fatal pneumonia in a significant number of cases (Wu et al., 2020). The first human coronavirus outbreak started in November 2002 in Foshan, China (Ge et al., 2015). This turned to a global issue with a significant lethal rate around 10% (Lee et al., 2003). Then, the globe faced another pandemic a decade later. The virus was called MERS-CoV and appeared in June 2012 in Saudi Arabia with a global fatality rate of over 30% (de Groot et al., 2013). The third pandemic started in December 2019 in Wuhan, China. The virus was called "severe acute respiratory syndrome coronavirus 2" (SARS-CoV-2) and it caused a disease known as COVID-19. Even though all of the mentioned pandemics share the same development (Zhu et al., 2020), the pandemic of SARS-CoV-2 had much stronger negative effects compared with the previous outbreaks, and it affected all the aspects of human life, sometimes with catastrophic consequences.

Human coronaviruses are enveloped viruses that contain non-segmented, single-stranded, positive-sense RNA genome (Masters, 2006). Their primary hosts are vertebrates. From the perspective of the classification, the coronaviruses are distributed into 39 species. What is important: they are genotypically and serologically separated into four major general AlphaCoV, BetaCoV, GammaCoV, and DeltaCoV, as established by the International Committee for Taxonomy of Viruses (Wu et al., 2020). The phylogenetic tree of human coronaviruses is shown in Fig. 2.
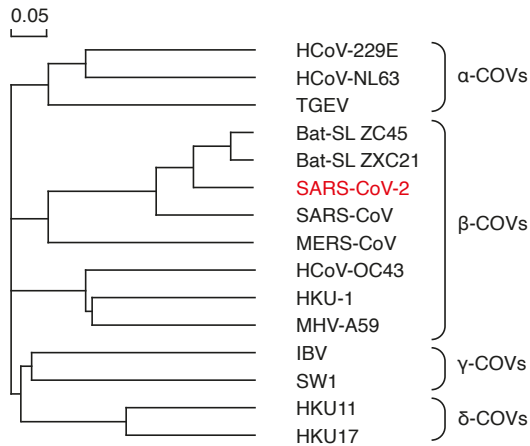
Fig. 2 Phylogenetic tree of human coronaviruses
(Biswas et al., 2020)

Coronavirus SARS-CoV-2

The coronavirus SARS-CoV-2 (Severe Acute Respiratory Syndrome Coronavirus 2) belongs to the genus Beta-CoV and is responsible for the disease called coronavirus disease (COVID-19). Primarily, the coronaviruses cause infections in birds and other mammals. However, they have the ability to cross species barriers even from birds to humans (Menachery et al., 2017). Coronaviruses have the largest genome out of all the RNA viruses and SARS-CoV-2 is not any exception and its viral transcript consists of a 5'-cap structure and a 3'poly-A tail (Lai & Stohlman, 1981).

The SARS-CoV-2 Genome Composition

The genome architecture of SARS-CoV-2 is depicted in Fig. 3. The length of the genome is around 30,000 bp.

In general, the genome of coronaviruses includes a variable number of open reading frames (ORF). The first 50 ORFs (ORF1a/b) correspond to about two thirds of the whole genome and is translated into pp1a (polyprotein 1a) and pp1ab proteins that are cleaved by proteases. This results in sixteen non-structural proteins called nsp1-16 (non-structural protein). The last third containing 30 ORFs consists of genes coding structural and accessory proteins (Khailany et al., 2020). There are four major genes encoding structural proteins: S protein, E protein, M protein, and N protein. The spike (S) protein is able to recognize the receptor of the host cell. The penetration of the virus into the host cell is mediated through angiotensin converting enzyme 2 (ACE2) (Wu et al., 2020). Next, the SARS-CoV-2 virus contains additional six proteins, encoded by the *ORF3a*, *ORF6*, *ORF7a*, *ORF8*, and *ORF10* genes. The functions of these proteins are still unclear. Most of the proteins encoded in the region of ORF1a and ORF1ab are crucial for virus replication and for the adaption to a new host (Yoshimoto, 2020). Some of the nsp proteins are important for creation of the replication
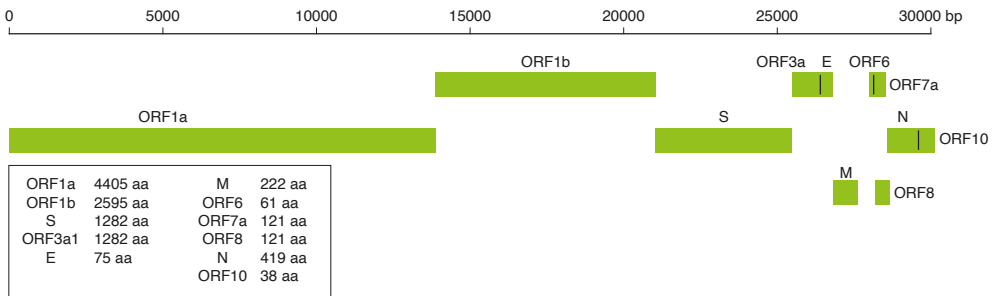
Fig. 3 Genome architecture of SARS-CoV-2

and transcription complex, e.g., nsp 12 is the RNA-dependent RNA polymerase. However, other proteins, such as nsp 7 and nsp 8, most probably even other nsp proteins, are also essential for its functionality.

The Genomic Changes of SARS-CoV-2

Coronaviruses are relatively stable due to their proofreading mechanism. Although SARS-CoV-2 is no exception and its mechanism is active in the process of replication, due to the enormous replication frequency, various mutations in the SARS-CoV-2 virus sequence occur. These come in all forms, from point deletions and insertions to more complex changes in the virus genome. However, only a very limited number of mutations have the possibility to gain an evolutionary advantage and start replicating to form a new variant. Therefore, despite the massive spread of the virus in the population, only a few virus variants are clinically relevant and have significantly different infectivity and evolutionary advantage over the previous forms. Nevertheless, the current character of the global world is an ideal environment for the most evolutionarily favored variant to prevail.

All the known sequences and variants of SARS-CoV-2 are reported into the GISAID database (https://www.gisaid.org/).

The SARS-CoV-2 Structure

The SARS-CoV-2 consists of phosphorylated nucleocapsid (N) protein with genomic RNA as a core enveloped by a bilayer of phospholipids. The particle has a spherical shape with a diameter between 80 and 120 nm. A characteristic property is the outer surface projecting the spike protein. The structure of the SARS-CoV-2 is shown in Fig. 4.

Generally, human coronaviruses, SARS-CoV-2 included, are composed of the following proteins: spike (S), membrane (M), envelope (E), nucleocapsid (N), and hemagglutinin (HA). The S, M, and E proteins are embedded in the viral envelop, while N protein protects viral RNA genome located in the core of the virus (Zhou et al., 2020). The S protein is heavily glycosylated and contains the receptor-binding domain that is critical for its binding with the ACE2 receptor. There are additional important parts in the protein sequence of S protein, such as polybasic cleavage sites (RRAR/S). They allow digestion by host's furin-like protease
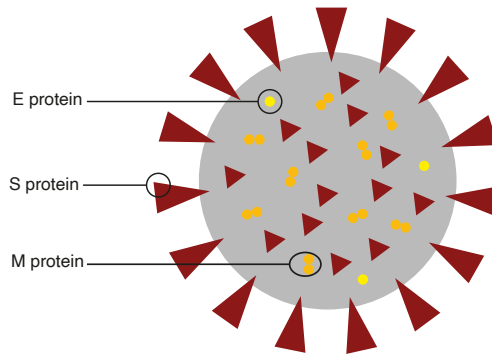
19

Fig. 4 Protein structure of SARS-CoV-2

during viral replication, which is most likely important for the infectivity (Nao et al., 2017). However, the whole functionality of the polybasic sites in still unknown.

The Life Cycle of SARS-CoV-2

All the SARS-coronaviruses, SARS-CoV, MERS-CoV, and SARS-CoV-2, exhibit a common strategy for replication and translation following infection in the host cells.

The initial step is the binding of SARS-CoV-2 virus to a cell receptor, and it determines the severity of infection and pathogenesis. The binding takes place through host cell surface by a densely glycosylated S protein. The protein is a trimeric fusion protein and consist of two major subunits: a receptor binding domain (S1; also known as RBD) and a second domain (S2) mediating viral fusion with host cell membrane. The fusion process of the SARS-CoV-2 membrane with the host cell membrane starts when the S1 domain binds to a host cell ACE2 receptor (Li et al., 2003; Wu et al., 2020). There are other receptors, such as CD209L (C-type lectin, also called L-SIGN) and dipeptidyl peptidase-4 (DPP4, also known as CD26), which can be used for the fusion of the virus with the host cells, but those have much lower affinity than ACE2 (Jeffers et al., 2004; Raj et al., 2013).

The ACE2 receptors are widely distributed on epithelial cells. The number of receptors is individual and in general, children have a much lower concentration of them compared with adults. In term of the epithelial cells, ACE2 receptors are present in the cells of trachea, bronchi, bronchial serous glands, and alveoli (Liu et al., 2011), as well as alveolar monocytes and macrophages (Kuba et al., 2005). ACE2 is also diffusely expressed on the endothelial cells of arteries and veins, cerebral neurons, immune cells, tubular epithelial cells of kidneys, mucosal cells of intestines, and epithelial cells of renal tubules (Gu & Korteweg, 2007; Guo et al., 2008). The SARS-CoV-2 attacks these cells with ACE2 receptors and virions are released to infect other new targets.

Once the virus is attached to host cells via ACE2 receptors, the virus entry follows. There are two known mechanisms based on the availability of the host cell protease to activate receptor-attached spike protein (Simmons et al., 2013). The first one is SARS-CoV-2 entering host cells as an endosome, which is mediated by clathrin-dependent and clathrin-independent endocytosis (Kuba et al., 2010). This leads to structural and conformational
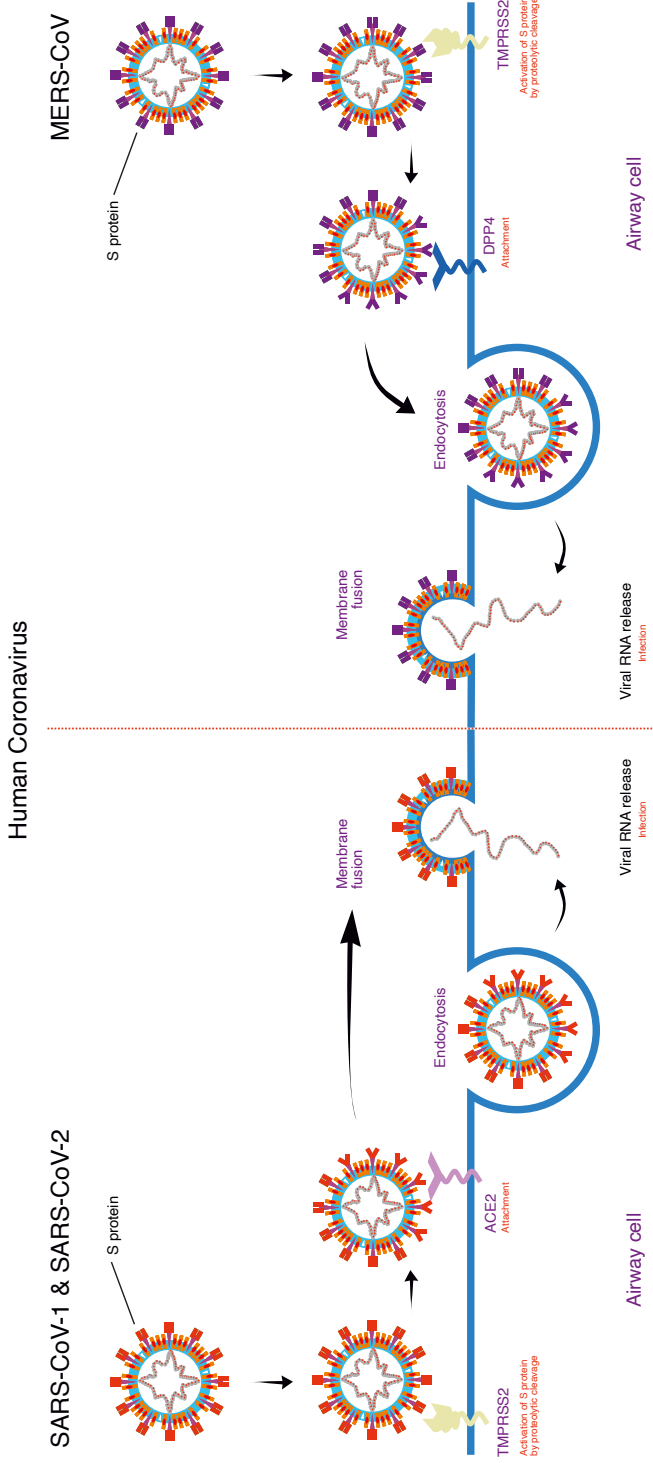
Fig. 5 The attachment of SARS-CoV-2/MERS-CoV and entry into airway cells

changes in the viral particle, specifically in the S protein, fusing the viral envelope with the endosomal wall (Simmons et al., 2013). The second possible way is a direct invasion of the virus into the host cell. This is possible thanks to the proteolytic cleavage of receptor-attached spike protein by the host's transmembrane serine protease 2 (TMPRSS2) or trans-membrane serine protease 11D (TMPRSS11D) on the cell surface (Heurich et al., 2014). Both paths are depicted in Fig. 5.

When the virus and the host cell membrane fuse, the virus releases the whole nucleocapsid with packed genomic RNA into the cytoplasm. The viral genome behaves as any other mRNA and the cell's ribosome translates two-thirds of this RNA. For the rest of the genetic information, there is a special mechanism. From the sequence responsible for ORF, two large overlapping polyproteins (pp) are formed: pp1a and pp1ab. More specifically, it comes to a translation of different proteins due to a frame shift triggered by slippery sequence (UUUAAAC) and downstream RNA pseudo knot at end of ORF1a (Masters, 2006). Protease
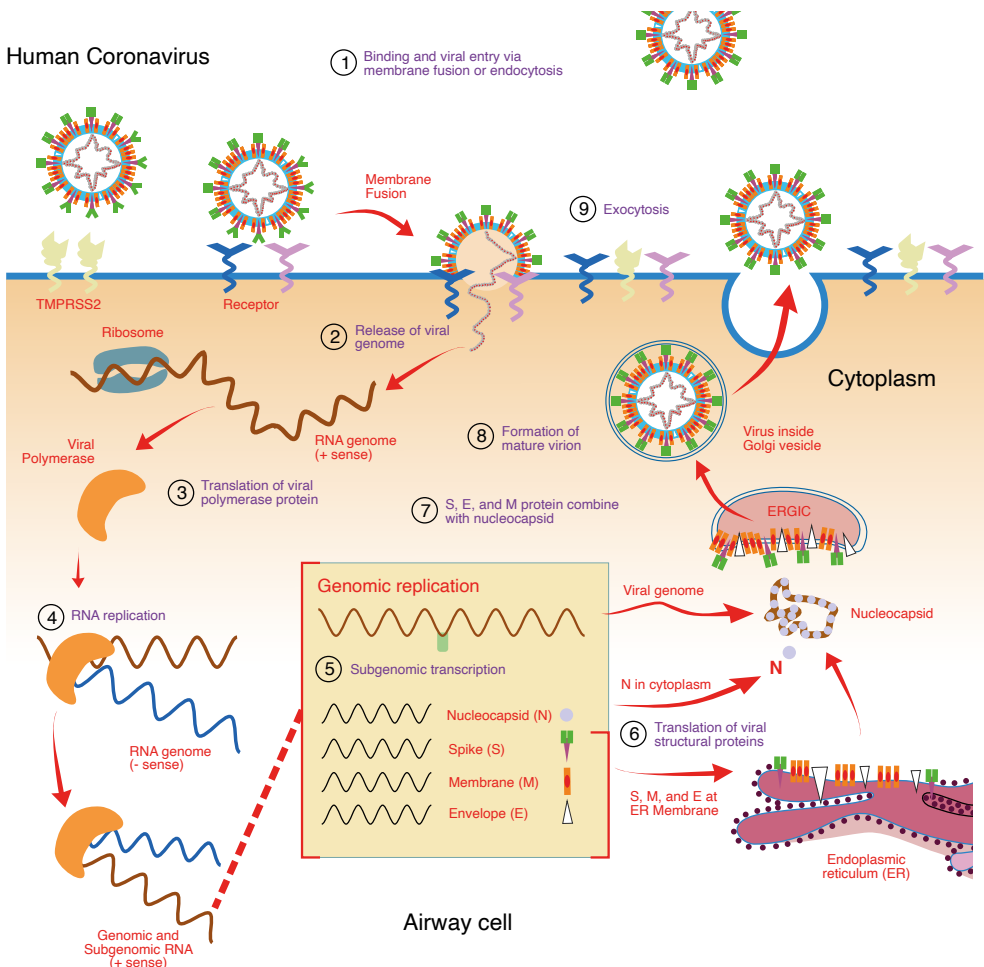

Fig. 6 An overview of the life cycle of HCoV in the host-cell (Song et al., 2019; Zumla et al., 2015)

activities follow and a whole spectrum of different proteins is created, such as nsp1-nsp16, RdRP, RNA helicase, and exoribonuclease (Fehr & Perlman, 2015).

The majority of the newly translated nsp proteins, together with structural proteins like N protein form a complex called RTC (multi-protein replicase-transcriptase complex), are responsible for the viral genome replication and transcription (Fehr & Perlman, 2015). The main protein in the complex is RdRP, which synthesizes negative-sense sub-genomic RNA strands of viral RNA from corresponding positive-sense mRNAs. Afterwards, it is used as a template for the production of positive-sense strands (mRNAs). These newly synthesized RNA strands represent genome for the generation of new viral progeny.

The final step of the SARS-CoV-2 life cycle is an assembly and release. The viral RNA translation process is driven inside the endoplasmic reticulum. The formation of structural proteins (S, E, M) is the leading step, and they are moving along the secretory pathway into the Golgi intermediate compartment. The mechanism is following: N protein packs the newly produced RNA genome and a nucleocapsid is constructed. Next, M protein is responsible for virion assembly through multiple protein–protein interactions, which assist in the incorporation of the nucleocapsid, envelope, and spike proteins into a virus particle (Fehr & Perlman, 2015). Finally, there is a process of secretion. The virion fuses with the plasma membrane and it is finally secreted from the host cell by exocytosis (Fehr & Perlman, 2015; Lim et al., 2016; see Fig. 6).

BIOLOGICAL MATERIALS SUITABLE FOR SARS-COV-2 DETECTION

Material commonly used worldwide for SARS-CoV-2 detection is a nasopharyngeal swab. The RT-qPCR detection of SARS-CoV-2 mRNA from the swab is the gold standard for diagnosing the COVID-19 infection both in symptomatic patients and asymptomatic individuals (Piras et al., 2020). This sample collection is based on a trivial procedure which, however, should be done by a trained operator to avoid false-negative results, as sufficient contact of the swab with the epithelial cells is essential for the proper sampling. The swab is then placed into a transport medium – we use the viRNAtrap collection tube (GeneSpector, Czech Republic) – and the sample is ready for transport to a laboratory for analysis. The viRNAtrap solution is a strong chaotropic and denaturing agent. It allows to destroy the viral capsid and due to it making the material non-infectious.

We have developed a method for SARS-CoV-2 detection from saliva that is currently used in several European states. The sample collection is based on Salivette (Sarstedt, Germany), a product originally used for cortisol testing (Costa et al., 2021). Major advantages over other solutions or a swab collection are its simplicity, low invasiveness, and a possibility of self-collection with no need for trained personnel and no leftover infectious waste. This method represents a very safe method of collecting biological material with virtually no risk of contamination of the environment with droplets or body fluids of the patient and lower aerosol formation compared with gargle methods. This type of collection is easy to perform. It is also painless, so it is suitable for more sensitive individuals or children over 3 years of age. In practice, due to the above-mentioned properties and the overall simple procedure, this sample collection method is commonly used for children's testing (Fig. 7).
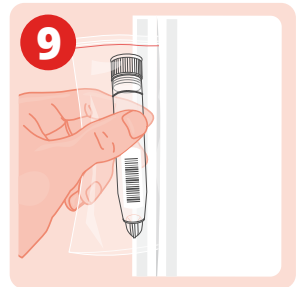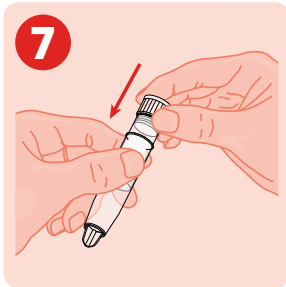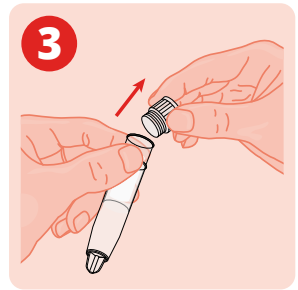
Fig. 7 User manual for the saliva sample collection using Salivette

EXTRACTION OF VIRAL RNA/DNA

The extraction of viral nucleic acids from nasopharyngeal swabs is based on a fast and robust extraction protocol using magnetic beads (GeneSpector, Czech Republic). The principle of this method is shown in Fig. 8.

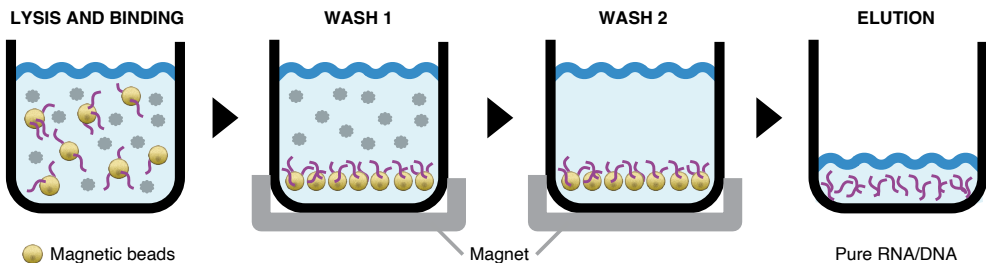The extraction kit utilizes the binding properties of silica-coated magnetic beads in a presence of high concentration of a chaotropic salt, which also acts as a strong denaturing agent.

LYSIS AND BINDING   **WASH 1**   **WASH 2**   **ELUTION**

Magnetic beads   Magnet   Pure RNA/DNA

Fig. 8 RNA/DNA isolation based on magnetic-beads extraction technology (GeneSpector, Czech Republic)

DETAILED EXPERIMENTAL PROTOCOL

100 µL of a sample from a nasopharyngeal swab in the viRNAtrap medium or saliva is taken into the extraction process. The sample is mixed with 300 µL of the viRNAtrap lysis buffer containing magnetic beads and 400 µL of pure isopropanol (Sigma, Germany). 10 µL of a PCR internal control (this step is related to the specific chosen detection kit) is added and mixed thoroughly by pipetting or vortex. The mixture is incubated for at least for 2 minutes at a room temperature and then placed on a magnetic stand for additional 2 minutes until the solution clears up. The supernatant is pipetted off and 250 µL of Wash Buffer 1 is added. After one minute of incubation, the supernatant is pipetted off and the beads are washed for a second time with 250 µL of Wash Buffer 2 for one minute. After this step, the critical point is to let the magnetic beads dry, which takes approximately 2 minutes. After the drying process, the tube with magnetic beads is removed from magnetic stand and the magnetic beads are resuspended using 50 µL of Elution Buffer. After 2 minutes of incubation with the elution buffer, the tube sample is placed back on the magnetic stand until the solution

clears up, which takes approximately 2 minutes. The supernatant containing nucleic acid is transferred into a new microtube or a well and the eluted RNA can be used immediately or be stored at –70 °C.

This extraction process can be adopted for any open automated instrument. We have successfully tested it on three different open platforms – KingFisher (Thermo, USA), Bravo (Agilent, USA), and Tecan (Tecan, Switzerland). This way, all the necessary steps are done automatically and on top of that, the temperature can be modified for specific steps to enhance the extraction process. In the laboratories, mainly the automated process is implemented, and the manual one is used only in small laboratories.

POLYMERASE CHAIN REACTION IN DIAGNOSTICS

Polymerase chain reaction (PCR) is a key method for research and development in molecular biology, genetics, and other related scientific fields. It has also found its application in routine clinical diagnostics, where, however, it used to be one of the underused methods. Before the arrival of the COVID-19 epidemic, it was mainly used for the diagnosis of infectious agents, but in many cases, they were determined by other, more widely used methodologies. PCR has also found other uses in the fields of clinical genetics, hematology, pharmacogenetics, oncology, and microbiology.

With the COVID-19 epidemic, the PCR method has become widely known not only in professional circles, but also to the general public. However, this method is only one part of the whole process of PCR testing for SARS-CoV-2.

If SARS-CoV-2 is present in the tested sample, it can be directly detected only if the virus concentration is very high. This direct detection is used, for example, by antigen tests where the main advantages are their speed and almost no requirements of expertise. Their huge disadvantage is a low sensitivity in relation to the incubation period of the infection and the infectivity. This method can detect positive individuals only when they are at the peak of infection. In contrast, the PCR method allows detection of the virus in samples at extremely low concentrations. Only hundreds or lower thousands of virus particles must be present in the tested medium to acquire a positive result.

In essence, the PCR method specifically amplifies pieces of genetic information to concentrations that can be visualized in some way. In other words, if the genetic information of SARS-CoV-2 is present in a sample, it is exponentially multiplied into a huge number of copies, while the multiplication is selective, and thus genetic information of other infections or human genes remains at low concentrations. In most cases, the PCR method is preceded by nucleic acid isolation, which disintegrates the viral envelope and releases its genetic information into solution and the sample is further purified, so that it contains only nucleic acids and is free of substances that could negatively affect the PCR reaction itself.

If we talk about PCR diagnostics of viruses, we can divide them based on the virus character into RNA and DNA approaches. This is very important in connection with the PCR method itself, which can amplify only strands of DNA. To detect RNA viruses, as in the diagnosis of COVID-19, reverse transcription has to be included, which involves the transcription of RNA into DNA. The whole process takes place in a single tube and is named RT-PCR (reverse transcription – polymerase chain reaction). The reaction protocol has two parts, the

first one involving reverse transcription of RNA to DNA and the second one involving a specific amplification of a segment of the DNA related to the studied gene (Tab. 2).

Tab. 2 Example of RT-qPCR protocol

	Temperature	**Time**	**Number of cycles**
Reverse transcription	42 °C	10 min	1
Initial denaturation	95 °C	3 min	1
Denaturation	95 °C	10 sec	45 cycles
Annealing and elongation + fluorescence acquisition	60 °C	30 sec	

The specifically amplified fragments can be visualized in several ways. Accordingly, PCR can be divided into end-point and real-time variants. In the end-point variant, visualization is achieved by a gel electrophoresis of the final product, and thus this method is qualitative, providing information of the present/absent type. This method is widely used when the fragment needs to be further processed by other molecular-biological methods.

Real-time PCR, on the other hand, allows quantification, hence the term RT-qPCR in the case of RNA analysis. As shown in Tab. 2, a cycle of 45 reactions takes place. In each one, in the first step, specifically designed primers bind to fragments of the diagnosed gene, while the DNA is in a single-strand form due to the set working temperature, and in the second step, the complementary DNA strand is amplified. Increase of the temperature in the next cycle causes the double-stranded DNA to be unlinked into two single-stranded DNA strands and the specific primers to reseed onto specific DNA sequences, and the reaction repeats approximately 45 times. After each cycle, a signal is read, which is caused by probes carrying fluorophores that light up at a specific wavelength. The C_T value represents the number of cycles where the fluorescence signal of the reaction crosses a set threshold (Fig. 9, red arrow).

Using more than one fluorophore makes it possible to observe the propagation of multiple genes (depending on the type of machine, but realistically up to five) in a single reaction, because there are specific primers for multiple genes and specific probes for individual genes that emit light at different wavelengths. Thus, it is possible to monitor the presence of different genes in a multiplex reaction and quantify them at the same time. At least two reactions or two channels are used in the PCR diagnosis of the COVID-19 disease, the first being for the detection of SARS-CoV-2 and the second being either for an internal control reaction added to the sample before nucleic acid isolation to ensure that the whole process starting with isolation has been carried out correctly or for a housekeeping gene.

The PCR reaction requires components listed in Tab. 3. Standard profile of a RT-qPCR reaction is shown in Fig. 9.

Tab. 3 List of components necessary for PCR reaction

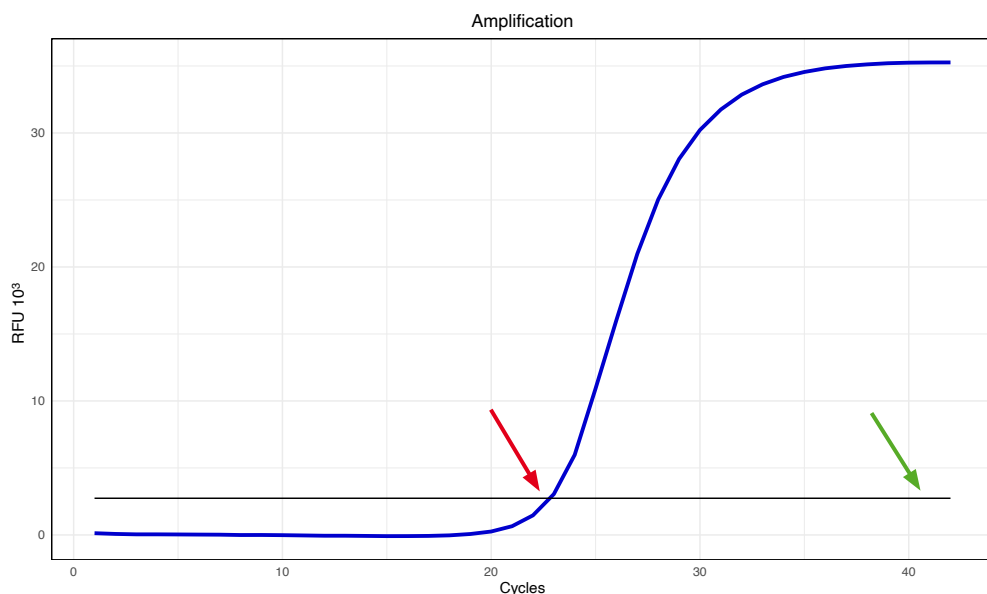| RT	qPCR
Primer complement to RNA	Primers
RNA	Probes with fluorophores
Mix of dNTPs (usually 10mM dATP, dCTP, dGTP and dTTP)	Mix of dNTPs (usually 10mM dATP, dCTP, dGTP and dTTP)
Enzyme buffer	Enzyme buffer
Dithiothreitol	DMSO
Enzyme	Enzyme

Fig. 9 Standard profile of a real-time PCR curve: red arrow – C_T value, number of cycles where the fluorescence signal of the reaction crosses the set threshold; green arrow – set threshold; x-axis – number of PCR cycles; y-axis – fluorescence intensity

The nature of the curve is in most cases very similar to Fig. 9, but sometimes there are changes to the curve that indicate an incident. This can signify a reaction problem, systematic error, change in the nature of the sample, etc. These anomalous curves are discussed in a later chapter.

DETECTION OF VIRAL PRESENCE BY RT-QPCR

The real-time reverse-transcription polymerase chain reaction (RT-qPCR) is a molecular assay used globally to detect RNA of SARS-CoV-2 in clinical samples of patients having signs and symptoms of COVID-19 (fever, fatigue, chills, dry cough, sneezing, dyspnoea, myalgia, lymphopenia, and radiographic findings of pneumonia) (Corman et al., 2020; Nagura-Ikeda et al., 2020) and in samples due to epidemiological reasons.

The procedure of RT-qPCR is similar for all the commercial kits. For the detection of presence of RNA of SARS-CoV-2, we used the "gb SARS-CoV-2 Combi" kit (Generi Biotech, Czech Republic), which is CE-IVD certified.

Detailed Experimental Protocol
The reaction containing 10 µL of Master Mix OneStep Multi and 5 µL of Assay CoV-2 E-RdRP Combi were mixed with 5 µL of extracted RNA (Fig. 10).
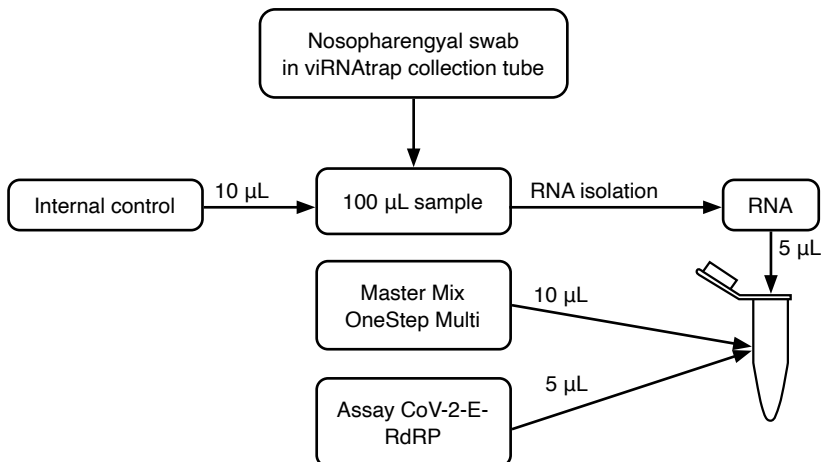
Fig. 10 Preparation of PCR reaction

The PCR reaction is centrifugated and placed into a real-time PCR instrument CFX-96 (Bio-Rad, USA). The program and temperature profile of the reaction is in Tab. 4.

Tab. 4 The program used for RT-qPCR

Reverse transcription	42 °C	10 min	
Initial denaturation	95 °C	3 min	
Denaturation	95 °C	10 sec	45 cycles
Annealing and elongation + fluorescence acquisition	60 °C	30 sec	

EVALUATION OF PCR DATA

The obtained data were evaluated using the CFX Manager 3.1 software (BioRad, USA). The detection kit "gb SARS-CoV-2 Combi" contains two fluorophores with excitation in different wavelengths (excitation/emission FAM 495/520 nm; HEX 537/553 nm). Probes labeled with FAM fluorophore are used for detection of presence of two genes located on the SARS-CoV-2 genome – *RdRP* and *E* gene. The presence of SARS-CoV-2 is determined by the value of the cycle threshold C_T in FAM channel and the internal control, present throughout the complete process of isolation, in HEX channel. C_T values inversely correlate with the viral load in the sample (i.e., the lower the C_T value, the higher the viral titer) and their interpretation is specific to each amplicon.

The described method is semiquantitative as there is no housekeeping gene used for a normalization process. A positive semiquantitative result, indicating the presence of SARS-CoV-2 RNA, is determined when the cycle threshold (C_T) value is less than 38 (Tab. 5). If the internal control has the cycle threshold (C_T) value of less than 35 and the FAM channel detecting the presence of SARS-CoV-2 has a negative signal, the result is negative with either no presence of the virus in the sample or in a concentration below the level of detection. If both channels have no signal, then the reaction and the result are invalid and must be repeated from the beginning.

Tab. 5 Interpretation of results from RT-qPCR assay

Valid results	FAM	HEX	Invalid results	FAM	HEX
Negative	-	+	Failed extraction; inhibition of RT-qPCR	–	–
Positive	$C_T < 35$	+/–			
Weak positive	$C_T < 38$	+			

The level of detection is 3,000 viral particles when using the combination of the "viR-NAtrap Extraction Kit" (GeneSpector, Czech Republic) and the "gb SARS-CoV-2 Combi" (Generi Biotech, Czech Republic) PCR detection kit. The theoretical LOD (limit of detection) of the PCR kit is 3.5 copies per reaction. The detection limits are shown in Fig. 11.

The data are automatically connected to a LIS (laboratory information system) and consequently reported to physicians, national authorities, or the tested individuals.

In general, there are two approaches to controlling the whole RT-qPCR process. The one described in this book is using the internal control system, which is very widely used, but does
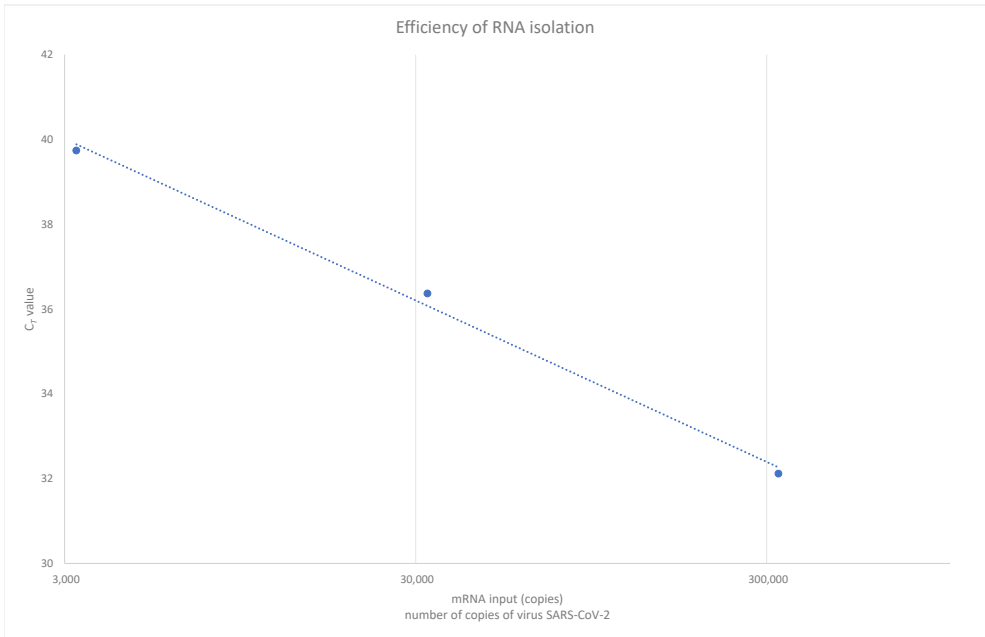
Fig. 11 Calibration curve and determination of the limit of detection using the combination of the viRNA-trap Extraction Kit and the gb SARS-CoV-2 Combi PCR detection kit

not detect an incorrect sampling collection. Therefore, some laboratories use a housekeeping gene as control. This is a parallel detection of a specific human gene that is always present in the mucous membranes of the nasopharynx. Its positive signal in the appropriate detection channel also indicates a good quality sample and a valid result for the detection of the presence of SARS-CoV-2. However, at the time of the peak of testing, laboratories began to move away from this approach, because the frequency of poor-quality collections was high and it was also very difficult to arrange a new patient collection, especially from people who were tested for epidemic purposes and were not symptomatic. Their willingness to be tested again was very low, which caused complications and slowed down the whole process in laboratories.

EVALUATION OF RESULTS FROM RT-QPCR DETECTION

Measuring one sample requires a different approach than analyzing thousands of samples in one day. The main difference is the level of automation that goes into the sample evaluation and subsequent reporting. However, both options share the same basic features and steps leading to the analytical outcomes.

Control Mechanisms

After the PCR run, the data is analyzed and all reaction controls are checked (Fig. 12).

33
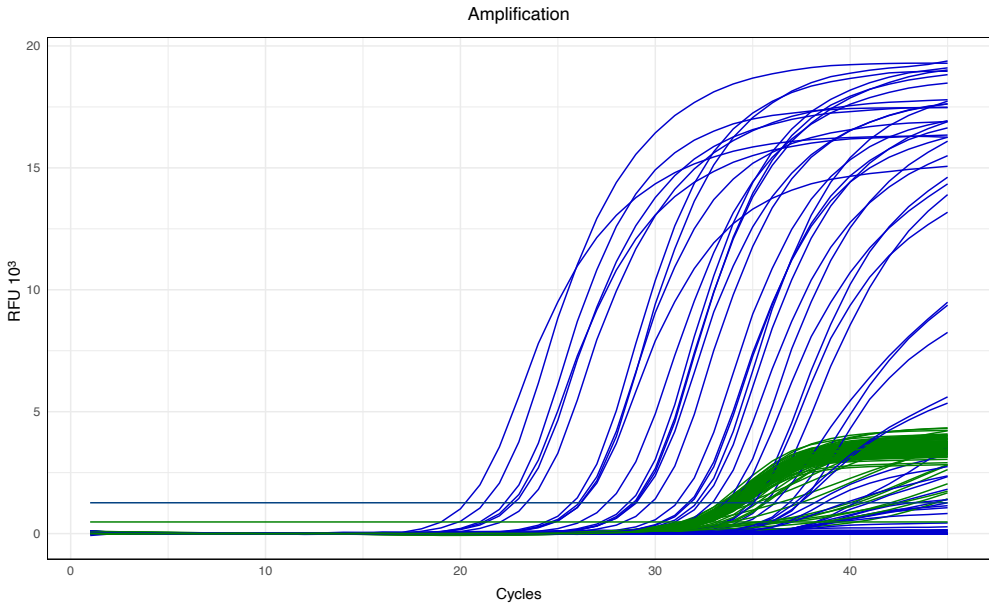
Fig. 12 Results of a PCR run: blue curves – positive samples detected in FAM channel; green curves – internal controls detected in HEX channel

In the first step, positive and negative controls are run simultaneously alongside each set of the analyzed samples. The expected runs are shown in Fig. 13.

The positive control run shows a standard curve containing a linear ground phase, an early exponential phase, a log-linear phase, and a plateau phase. Crucial is the C_T value of the positive control in the SARS-CoV-2 detection channel (FAM fluorophore), which should be between 20 and 30 for the combination of the RNA extraction and PCR kit we used (Fig. 13A). If the value is more than 30, then inhibition of the reaction can be expected. This may be mainly due to poor setup of the PCR instrument or inappropriate preparation of the PCR mix for the reaction. This control does not provide insight into the control of the extrac-
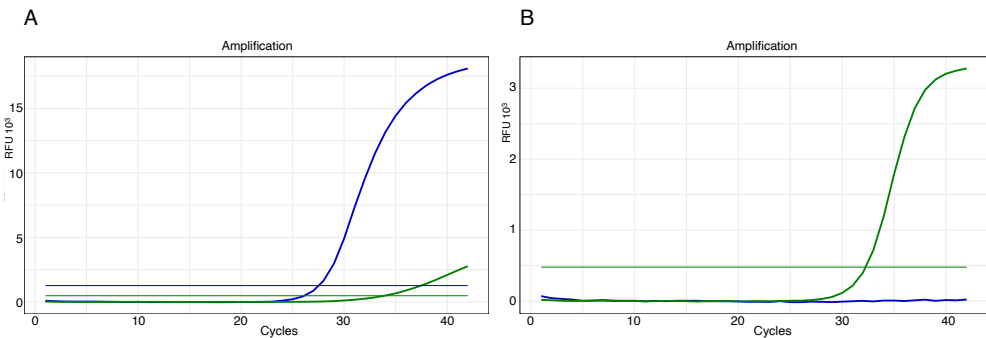
Fig. 13 The standard PCR profile of a positive (A) and negative (B) control in FAM and HEX channels

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tion process and the PCR reaction, as it is a combination of the PCR mix and the manufacturer's pre-prepared control that is not going through the extraction procedure.

The negative control is essential to monitor for adverse effects that may be caused by contamination or improper setup of the PCR protocol (Fig. 13B). The expected curve in the SARS-CoV-2 detection channel is a linear ground phase from the beginning to the end of the analysis. Any exceedance of the threshold indicates a problem in the reaction, or a poor machine setup and the problem must be addressed.

An internal control is added to each sample prior to the actual mRNA extraction to determine if the entire process, starting with the extraction followed by the PCR reaction, has gone well. This is detected in a different spectral channel than the detection of the SARS-CoV-2 sequence itself (HEX channel in the case of the kit we used).

The curve of the internal control can vary and, in most cases, differs from positive and negative samples for SARS-CoV-2 (Fig. 14).

For negative samples of SARS-CoV-2, a standard curve of the internal control with all the phases already mentioned can be expected, with a C_T value between 25 and 35. Usually, with the combination of the extraction and PCR kit we used, the C_T value is around 30 (shown in Fig. 15), and a significant deviation from this value to those borderline values marks the beginning of potential problems at some stage of the measurement process. If the internal control for negative samples for SARS-CoV-2 is within the expected range and both positive and negative controls ran as expected, then the negative result is valid and ready to be reported to the physician, patient, or other authority, such as a regional health center.
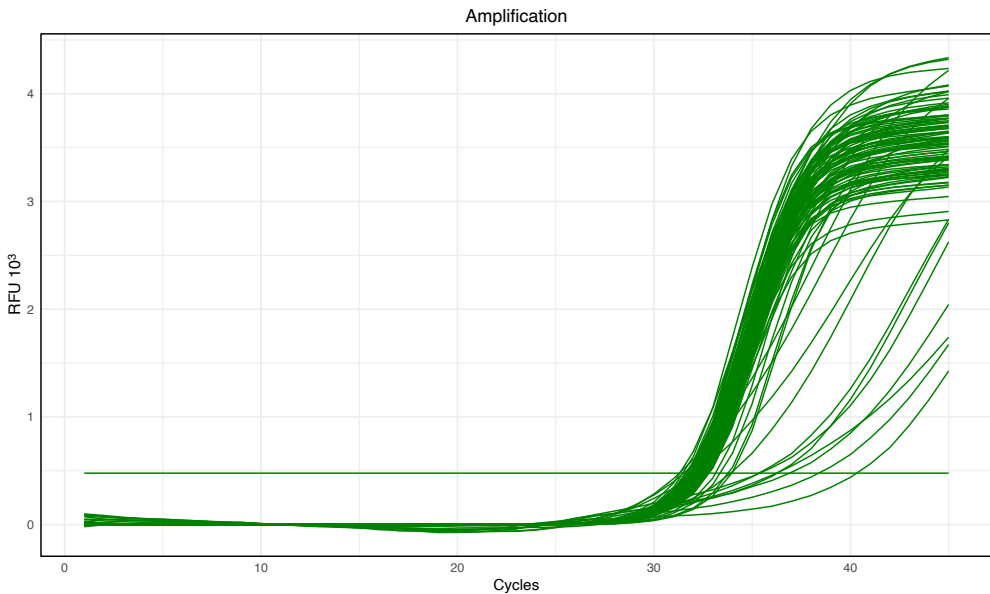
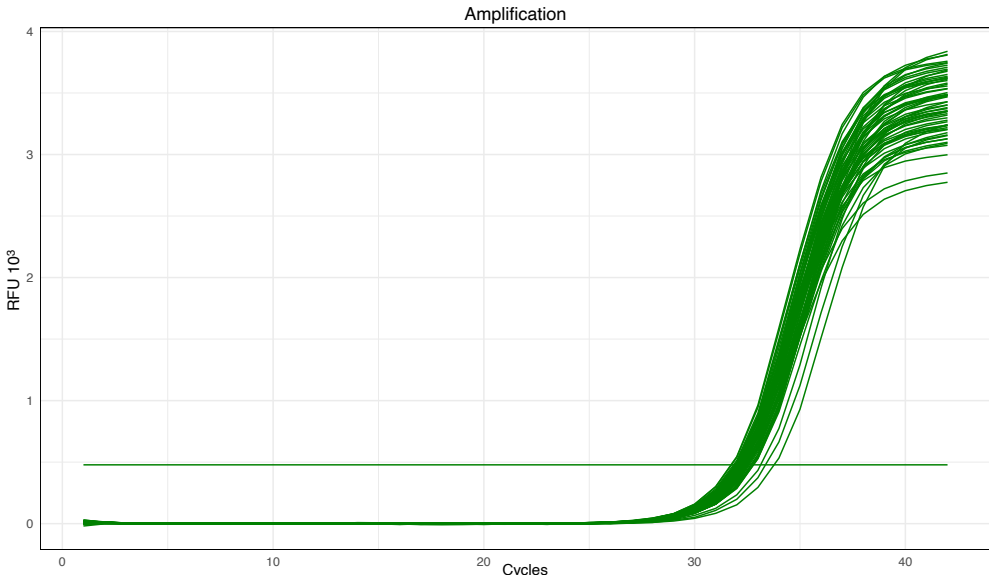**Fig. 14** Curve of the internal control

Fig. 15 Standard curve of the internal control in negative samples of SARS-CoV-2

In case of positive samples of SARS-CoV-2, the curve shows a different profile (Fig. 16A). This is because of a competition between two reactions – the production of a target for SARS-CoV-2 and the internal control. Since the same building molecules (deoxynucleotides) are used for their synthesis, the character of the internal control curve is dependent on the input SARS-CoV-2 mRNA concentration. At the same time, the reaction is designed (including the length of each product) to preferentially amplify SARS-CoV-2 fragments. In general, the higher the input mRNA concentration in the PCR reaction, the more is the synthesis of the internal control negatively affected or even does not occur at all. Realistically, it appears that in strongly positive samples of SARS-CoV-2, no synthesis of the internal control in the HEX channel occurs. However, this is not a given rule and during our analyses, strong samples of SARS-CoV-2 also appeared with synthesized internal
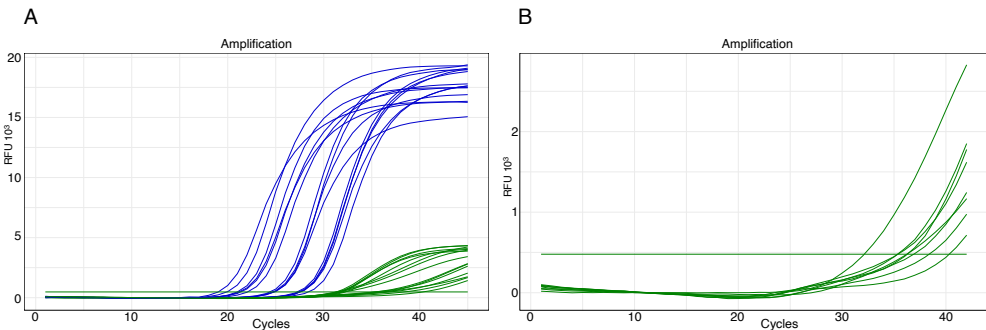
Fig. 16 Different positive samples of SARS-CoV-2 and their effect on the synthesis of the internal control (a strongly positive and moderately positive samples of SARS-CoV-2)

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control, albeit at a lower concentration, which is reflected by a significant shift of the C_T value towards higher values. The different positive samples of SARS-CoV-2 and their effect on the synthesis of the internal control are shown in Fig. 16.

Therefore, for positive samples of SARS-CoV-2, we have established a rule that in case of positivity, it is not necessary to monitor the value of the internal control due to the uncontrollable process of its synthesis. In the case that positive samples of SARS-CoV-2 also have the expected results of positive and negative controls in the same run, then the positive result for SARS-CoV-2 is valid and is ready to be reported to the physician, patient, or other authority, such as a regional health center.

Threshold Setting

Setting the threshold value is also key. Each lab has slightly different rules for setting the threshold value, but in general, the setting should not change the actual test results. What must be said, however, is that the threshold setting significantly affects the C_T value. This does not play a major role in qualitative or semi-quantitative testing of SARS-CoV-2. The value of C_T is important when the dynamics of ongoing infection are monitored, and in these studies, quantification is related to the C_T value of the housekeeping gene and is evaluated in relative terms, or an absolute quantification is performed using a calibration curve. However, neither approach is performed in routine diagnostic laboratories because of the large number of samples and the clinical irrelevance of such an approach.

Adjustment of the threshold value is generally done to ensure results independent of subjective influence, such as the person performing the evaluation of the results. Some laboratories chose the approach of applying percentage of the threshold value of the total signal. Usually, this percentage value is somewhere around 5–10%. Other labs use the absolute value of the threshold. This is possible if the lab uses the same RT-qPCR process including all kits and reagents every time. Even so, there are minor shifts between batches and individual measurements. However, in most cases, this shift is insignificant. For more accurate evaluation, various threshold correction functions are also used directly within the evaluation software. These allow better visualization and more accurate adjustment, but the main benefit is the elimination of poorly evaluated samples in the automatic evaluation, where some samples do not have a clean linear (straight) path in the initial cycles and could incorrectly cross the threshold value right at the beginning of the curve (Fig. 17). In these cases, a false positive sample would be reported.
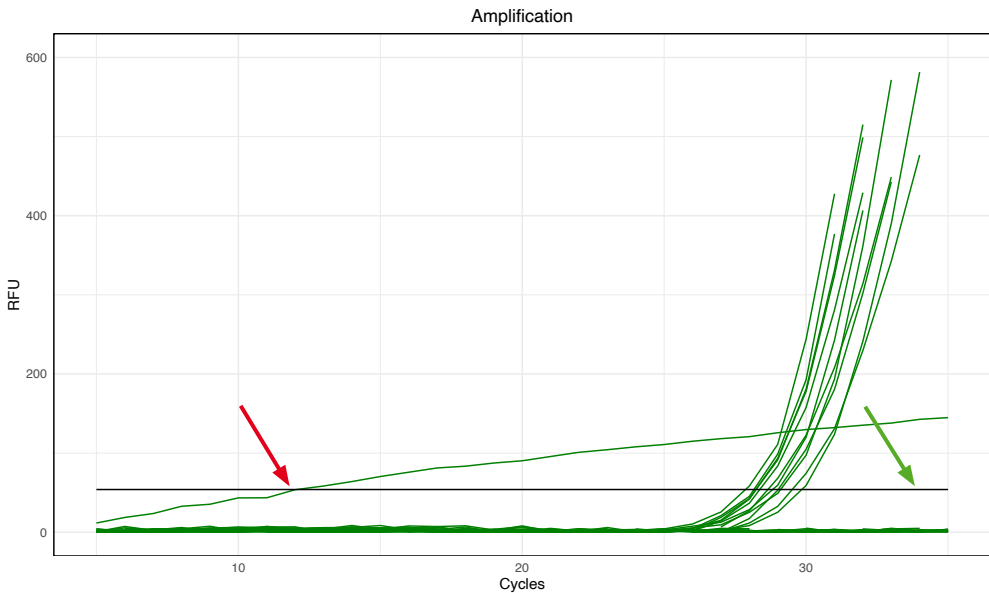
Fig. 17 Threshold settings: green arrow – the threshold line; red arrow – crossing point of a curve with false positive result

Evaluation of Results and Reporting

Using PCR, it is not possible to determine whether an individual is at the beginning, in the middle, or at the end of an infection. This could be the basis for further medical and epidemiological action. However, it is possible to estimate from the character of the curve whether the individual is in a late stage of the infection, or an early stage of the infection. Both are characterized by a late C_T value, which makes recognition very difficult. As this recognition is an advanced diagnosis, which is not supported by any study known to date and is based on the experience of people working in the laboratory, it is not possible to provide this information to doctors or patients. The late-phase infection curve shows a late threshold with a C_T of more than 35 with a maximum absorbance significantly lower than the positive samples or the positive control. Another characteristic of the late phase of the infection is the shape of the curve, which is more tilted and does not show the standard axis shape.

By contrast, the early-phase curve has a standard axis shape with a maximum absorbance close to that of the positive control. The difference can be seen in the two spectra in Fig. 18.

Such atypical samples with late C_T values are quite difficult to evaluate, especially if a larger number of samples is measured. We have optimized the automatic evaluation of results with subsequent transfer to the medical information system and government systems (IHIS). Only information whether an individual is positive or negative for SARS-CoV-2 is reported together with the C_T value, but without further comments and opinion from the laboratory personnel.

The frequency of these atypical results with late C_T values is influenced by the epidemiological situation in the population. As the strength of the epidemic increases and more people
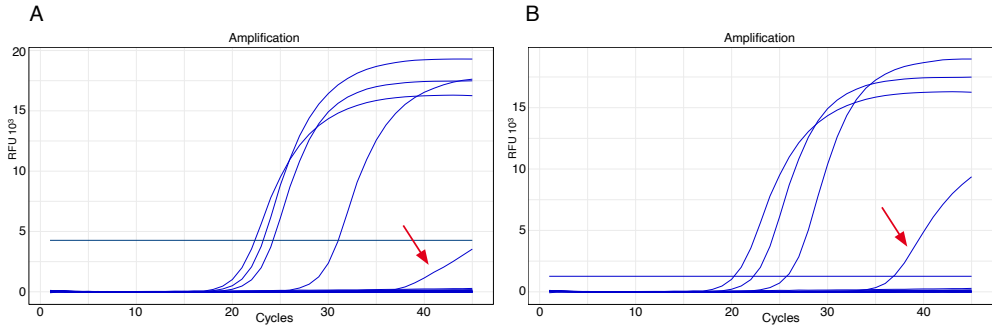
Fig. 18 Spectra of late-phase (A) and early-phase (B) of infection. The red arrows show typical curves for these conditions

are tested, the number of such samples also increases. There are several reasons for this. The main one is the higher probability of detecting people positive for SARS-CoV-2, as well as the dysfunctional tracing of people to prevent the spread of the epidemic. With epidemic peaks of high testing demand, people are contacted late, and even asymptomatic people are going for PCR testing and are often caught at a late stage of infection. This brings the negative social effect that these particular people are then meaninglessly left in quarantine even though they are already non-infectious.

However, most people tested as positive for SARS-CoV-2 show a standard curve, which allows for the aforementioned, and at least partial, automation in the evaluation of the results and fully automated transfers to the above-mentioned databases.

Problems Faced During Detection

Some of the spectra show atypical patterns, which may affect the results and subsequent treatment. In a very limited number of cases, this is due to a non-standard character of the sample itself. In most cases, the problem starts somewhere in the sample preparation and analysis process, i.e., in the isolation or PCR reaction. Such problems can be either one-time or systemic.

One-Time Problems

In the context of testing nasopharyngeal specimens for SARS-CoV-2, one-time problems include single contaminations, poorly sealed PCR plates, failure to add some components of the reaction, sample mix-ups, etc. These problems can be identified by simply repeating the entire sample preparation followed by PCR analysis. This step helps to eliminate the problems and report the results to the patient or physician. The spectra of one-time problems can be seen in Fig. 19.
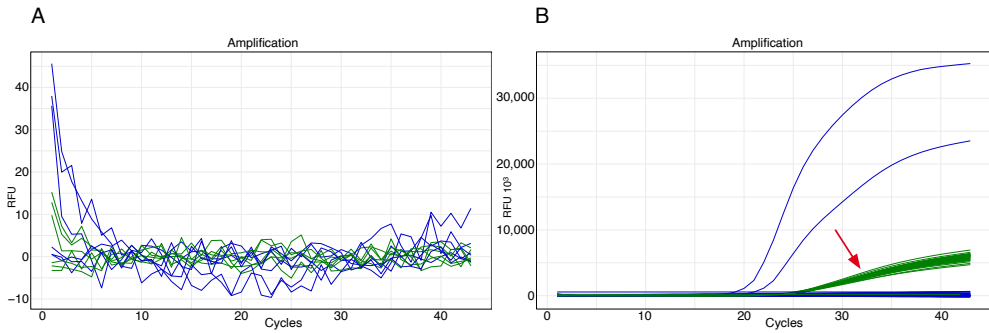
Fig. 19 One-time problems spectra: A – negative sample for both internal control and SARS-CoV-2 (either no internal control or other component of the reaction is omitted); B – poorly sealed PCR plate

Systemic Problems

The more difficult situation is the repeated reporting of non-standard spectra. The most common is systemic contamination, which is seen as positivity across the whole plate with a high C_T value. At this point, it is critical to detect the problem, because false positive results are being reported and the problem is gradually becoming more serious, as the C_T values across the PCR plate start to drop and the problem escalates. Another example of a systemic problem is when the internal control channel is oversaturated with the signal being extremely strong for physical or chemical reasons and the SARS-CoV-2 detection channel is silenced providing false-negative results. Spectra of systemic problems can be seen in Fig. 20.

In all cases, the expertise and experience of the people evaluating the results is essential to detect all forms of problems affecting the correct issue of patient results.
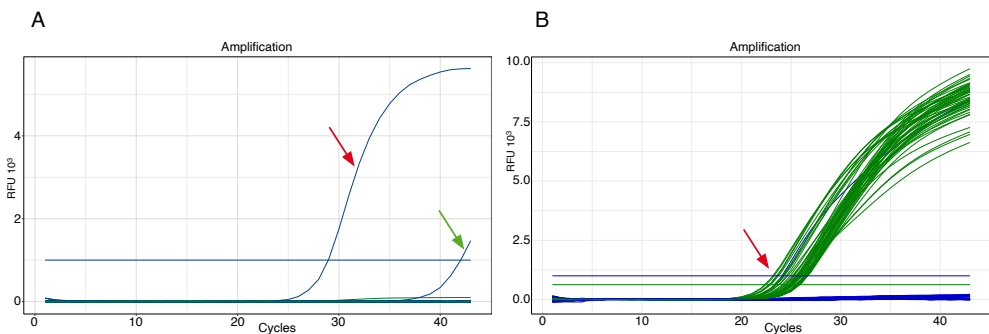
Fig. 20 Systemic problems: A – contamination, red arrow shows positive control, green arrow shows negative control; B – problems with internal control

EVALUATION PROCESS OF SALIVA TESTING

This chapter should be an inspiration for any other testing and evaluations in each laboratory.

The test tube Salivette (with cotton swab, cap: white) is used to detect the presence of the virus in saliva. The method utilizes a tampon made from a cotton-based absorbent material and a tube for its storage and transport. Saliva collection is performed by inserting the cotton tampon into the mouth of the subject for one to two minutes and then placing it in the transport tube (see Fig. 7).

During evaluation, individuals who underwent a reference nasopharyngeal swab also underwent saliva collection using a saliva collection tube (Salivette, Sarstedt, Germany). The fluid was separated from the cotton roll by centrifugation and RNA of SARS-CoV-2 was extracted using the viRNATrap extraction kit (total 200 μL) and detected by RT-qPCR analysis as described above.

In parallel, a validation in order to demonstrate the functionality of the sampling system Salivette at the end of its shelf life to detect SARS-CoV-2 virus particles in collected saliva was conducted. The sample integrity was proven over the whole product life cycle. For this validation, 18 pieces of Salivette tubes were used.

EVALUATION OF SARS-COV-2 DETECTION IN SALIVA

Samples were collected from patients using both ways (reference – nasopharyngeal swab, test – Salivette) and were isolated and analyzed by RT-qPCR immediately within the same day. The study group consisted of 249 women and 345 men. Age range of this group was from 4 years to 95 years.

The identified positivity or negativity of the sample obtained by both sample collection methods was compared.

A match between both collection methods was found in 587 of 594 samples tested. A discrepancy was found in 7 of 594 samples tested, with 1 sample taken by the test method being false positive and 6 samples taken by the test method being false negative. Conformity between the reference and test method was found in 98.8% of cases. The 6 samples that were positive in the nasopharyngeal swab and negative in the saliva samples had high C_T values, and therefore low viral loads. The one sample that came out positive in saliva and negative in a nasopharyngeal swab may be explained by the dynamics of viral RNA degradation, where in saliva, nucleic acid is more stable for a longer time.

Tab. 6 Results of validation of sample collection using Salivette

		Nasopharyngeal swab		
		Positive	**Negative**	**Total**
Salivette	**Positive**	90	1	91
	Negative	6	497	503
	Total	96	498	594

Based on the presented results, it can be concluded that collection of samples using a saliva collection tube, such as the Salivette, is functionally comparable to a nasopharyngeal swab.

VALIDATION OF FUNCTIONALITY OF SALIVETTE FOR SARS-COV-2 DETECTION IN SALIVA

To examine whether the sampling system is suitable for collecting samples over its entire expiration period, we designed an experiment to confirm or refute this hypothesis. At the end, the integrity of Salivette was proven over the whole product shelf life. For this validation, we used 18 pieces of collection tubes close to their expiration date.

Patients with previously positive SARS-CoV-2 results (10 men and 8 women) were contacted and, with informed consent, asked to do an additional test of saliva collection one day after the nasopharyngeal swab test. The time difference between the nasopharyngeal swab collection and the collection using the Salivette system did not exceed 36 hours. Samples were collected from patients at home due to quarantine regulations. All saliva samples were analyzed within 24 hours of collection. The results were following:

1. Of the total number of 18 patients enrolled, 14 were confirmed as SARS-CoV-2-positive.
2. Results of 2 patients were excluded for non-compliance with the collection conditions. Most likely, they ate just before the collection, as evidenced by the lack of endogenous control and very high pH value.
3. Results of 2 patients were affected by their dehydration causing a lack of saliva.

The selected patients were all significantly SARS-CoV-2-positive based on previous tests using swab collection from the nasopharynx. Saliva collection took place on the following day and there was no time to instruct patients about the drinking regimen, i.e., to drink 2 hours before saliva collection, due to which some of the patients were dehydrated and samples could not be included into this validation.

These results show that the Salivette sampling system is fully functional at the end of its shelf life, as all positive patients tested with nasopharyngeal swab also tested positive from saliva collected by the Salivette. No difference in functionality or sample integrity could be determined at the end of its shelf life.

SAMPLE POOLING FOR A CONSEQUENT DETECTION OF SARS-COV-2 BY RT-QPCR

The saliva pooling method has proven to be crucial in screening children at schools and employees in companies. In pooling, a defined number of biological samples are mixed. If the result of this mixture is positive, then individual samples are analyzed to identify the specific positive individual. This way, it is possible to test large groups of people where the percentage of positives is expected to be very low. Generally, the pooling method is used when the coincidence of positivity is below five percent. If the incidence rate is higher, the benefits of the method are lost and, in turn, the analyses become more expensive and slow down the whole process in the laboratory.

To determine the effect of sample dilution on the outcome of the RT-qPCR analysis, a study was designed involving saliva samples from SARS-CoV-2-positive patients. The C_T values of these samples were compared with the C_T values of pools generated by diluting the positive samples in different ratios. To make the conditions of the validation study as close to real conditions as possible, the positive samples were diluted with real saliva samples from SARS-CoV-2-negative patients.

For the pooling testing, we have collected saliva samples from SARS-CoV-2-positive patients and SARS-CoV-2-negative patients. These samples were diluted based on algorithm shown in Tab. 7 to form pools.

Tab. 7 The principle of dilution of samples for pooling

Dilution	Positive sample	Negative sample	Total
8×	1 sample 30 µL of saliva	7 samples 7 × 30 µL of saliva from different samples	240 µL
12×	1 sample 30 µL of saliva	11 samples 11 × 30 µL of saliva from different samples	360 µL
24×	1 sample 30 µL of saliva	23 samples 23 × 30 µL of saliva from different samples	720 µL

Subsequently, 200 µL was taken from each of the prepared pool and RNA isolation and detection was performed on each of these aliquots according to the previously described methodology.

POOLING AS A TOOL FOR HIGH-THROUGHPUT SARS-COV-2 PCR TESTING

Three pools of different dilutions, 8-fold, 12-fold, and 24-fold were formed from each positive sample. A total of 48 different pools were created for the study. The undiluted samples and diluted samples (pools) were then subjected to analysis by RT-qPCR. The C_T values were compared with each other and the effect of dilution of the positive sample on the result of the analysis was determined based on the comparison.

All 48 pools were assessed as SARS-CoV-2-positive by RT-PCR analysis. Results are shown in Tab. 8.

Tab. 8 Results of pooled samples

Dilution	8x	12x	24x
ΔC_T average	2.08	2.57	3.24
Std. deviation	0.72	0.94	0.81

The data show that the pooling method does not lead to false negative results by diluting positive samples. At the same time, the C_T values are not significantly affected by dilution. Pooling can therefore also be used for quantitative analysis of the so-called viral load.

In conclusion, pooling is suitable for screening large groups of people when a low positivity rate is expected. Using saliva as the testing material, pooling is a significantly more suitable method for screening than antigen testing. This is due to the significantly higher sensitivity, which makes it possible to catch individuals positive for SARS-CoV-2.

CHARACTERIZATION OF SARS-COV-2

There are three main strategies for the characterization of variants or sequence of SARS-CoV-2. Selected variants can be determined using specific multiplexing RT-qPCR as a reliable first-line tool to identify suspected variants in positive samples. Selected regions of the genome (e.g., S-protein sequences) (Fig. 21) are determined by Sanger sequencing or by one of the genome sequencing methods with amplicons prepared by polymerase chain reaction (PCR). The whole genome is determined by sequencing a set of PCR amplicons covering the whole genome region or by sequencing fragments of the virus genome obtained by their specific enrichment using synthetically prepared complementary oligonucleotides.

Next generation sequencing (NGS) technology is used not only to track the occurrence of individual variants, but also for tracing origins, understanding the evolution, and investigating the spread and transmission chains of outbreaks.
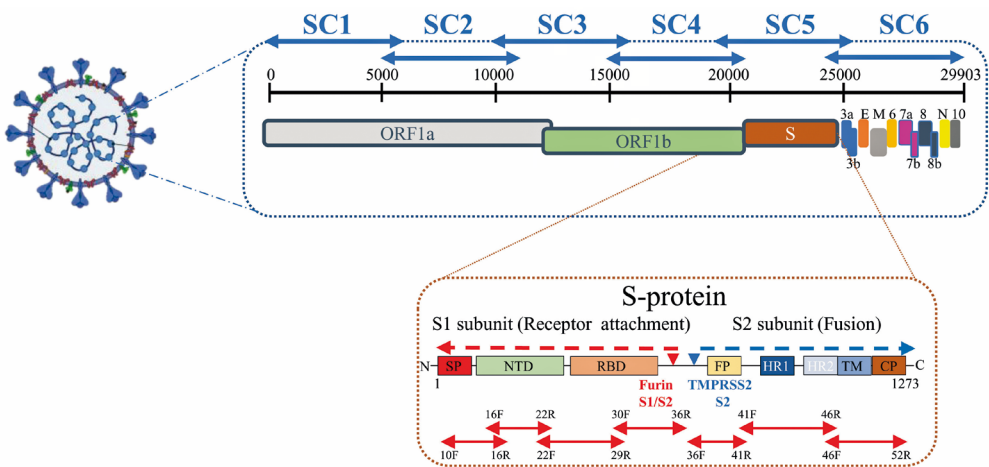
Fig. 21 Schema of different sequencing strategies for characterization of SARS-CoV-2 genome

SCREENING OF DIFFERENT SARS-COV-2 VARIANTS IN THE POPULATION BY RT-QPCR

Different commercial kits were used for screening of SARS-CoV-2 variants by RT-qPCR. To understand the profiles and evaluations, we show a few results with a kit from Generi Biotech – "gb SARS-CoV-2 Variant E484K, L452R". The detection of targets is done in parallel in four channels – SARS-CoV-2 (ROX), Internal control (Cy5), variant E484K (FAM), and variant L452R mutation B (HEX). The temperature profile is described in Tab. 9.

Tab. 9 The program used for RT-qPCR

Reverse transcription	42 °C	10 min	
Initial denaturation	95 °C	3 min	
Denaturation	95 °C	10 sec	45 cycles
Annealing and elongation + fluorescence acquisition	60 °C	30 sec	

A positive result, indicating the presence of SARS-CoV-2, is determined by cycle threshold (C_T) values being below 38 in ROX channel (Tab. 10). The presence of two variants is determined via signals in FAM and HEX channels. A positive result in the FAM or HEX channel is represented by an amplification curve that is shifted by a maximum of 7 cycles to the right of the curve in the ROX channel. A negative result either does not contain an amplification curve at all, or the curve is shifted to the right by more than 7 cycles from the ROX signal (Tab. 11). The assessment based on C_T values is possible when the threshold fluorescence in all channels is set to the same level.

Tab. 10 Validation of results from discriminated RT-qPCR assay

Valid results	ROX	Cy5	Invalid results	ROX	Cy5
Negative	–	+	Failed extraction; inhibition of RT-qPCR	–	–
Positive	+	+/–			

Tab. 11 Interpretation of results from discriminated RT-qPCR assay

	FAM	HEX	ROX
E484K positive	$C_{T\,FAM} < C_{T\,ROX} +7$	–	+
L452R positive	–	$C_{T\,HEX} < C_{T\,ROX} +7$	+
E484K and L452R negative	–	–	+

Positive samples were screened for selected variants based on the state health department's orders, which led to the identification of the SARS-CoV-2 variants (Fig. 22).

The discriminatory PCR tests were performed with different frequencies over the time of the epidemic. For example, during January 2022 in the Czech Republic, only 10% of positive samples where the C_T value was below 30 were analyzed. Then, between January and February, the discriminatory PCR was performed on all symptomatic samples and at the end, from February 2022, there was no need for the variant characterization, as only the Omicron variant was present in the Czech population.

In general, this method indirectly led to the identification of three variants – presence of E484K (Alpha), presence of L452R (Delta), absence of both (suspected Omicron) (Fig. 23).
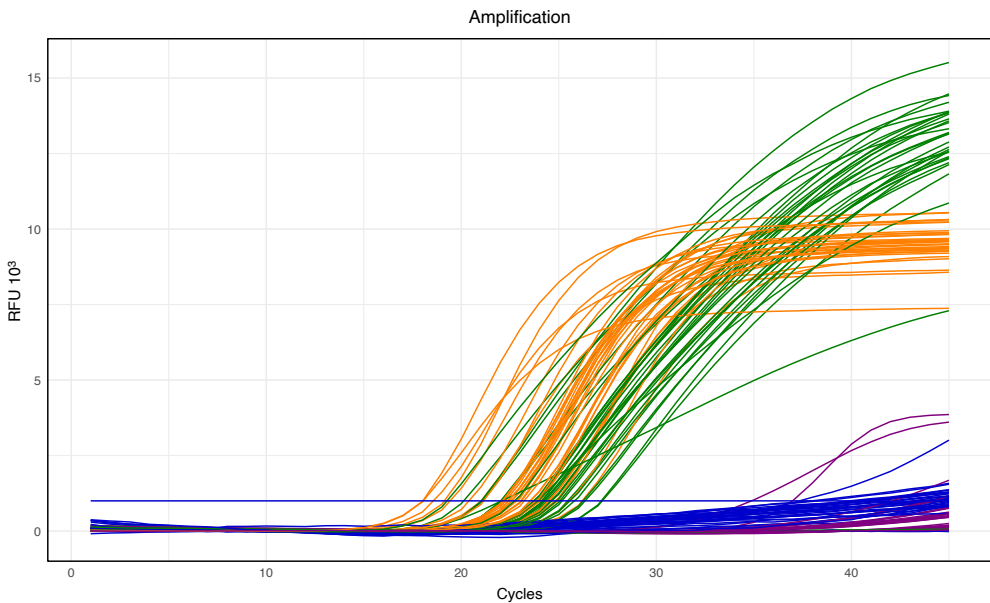
Fig. 22 Results of discriminated PCR runs: orange curves – positive samples detected in ROX channel; green curves – positive samples with L45R variant in HEX channel; blue curves – positive samples with E484K variant in FAM channel; purple curves – internal controls detected in Cy5 channel
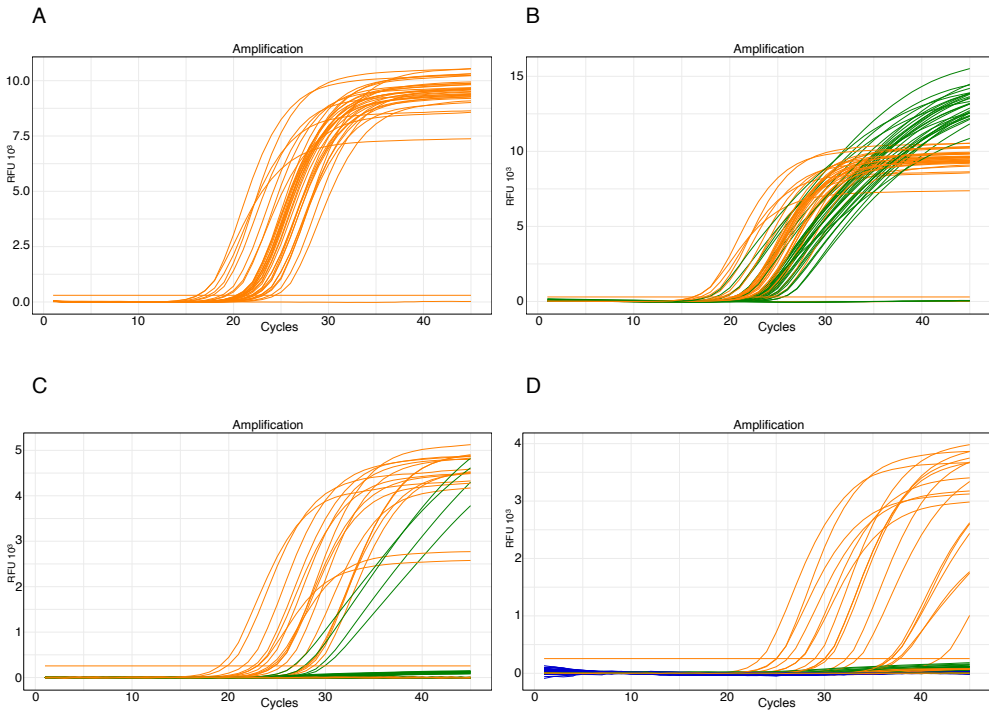
Fig. 23 Spectra of positive samples from two different dates: A – typical spectra of SARS-CoV-2-positive samples (ROX channel); B – green curves of Delta-positive samples from the beginning of year 2022; C – curves from the end of the January of 2022 when the Omicron was more common; D – samples suspected of Omicron variant

This data is provided both to physicians and patients. For doctors, the information about the variant is essential, as they can determine the subsequent treatment accordingly.

SEQUENCING OF SARS-COV-2

The extracted RNA was transcribed to cDNA using SuperScript IV (ThermoFisher). For sequencing of the S-protein, we used 7 overlapped amplicons (Tab. 12). The used amplification program is described in Tab. 13. These amplicons were sequenced using the version 3.1 Dye Terminator cycle sequencing kit with electrophoresis on an ABI 3500XL Avant Genetic Analyzer (both ThermoFisher Scientific; Waltham, MA, USA).

Tab. 12 Set of primers used for amplification of the S-protein of SARS-CoV-2

Name of primer set	Upper primer	Lower primer	Length
primer_Cov_s20720_D	10F	16R	718
primer_Cov_s20720_D	16F	22R	689
primer_Cov_s20720_D	22F	29R	795
primer_Cov_s20720_D	30F	36R	677
primer_Cov_s20720_D	36F	41R	583
primer_Cov_s20720_D	41F	46R	590
primer_Cov_s20720_D	46F	52R	708

Tab. 13 Program used for amplification of amplicons of the S-protein for sequencing

Amplification protocol			
Initial denaturation	95 °C	3 min	
Denaturation	95 °C	10 sec	35 cycles
Annealing and elongation	57 °C	1 min	

Next Generation Sequencing (NGS)

The NGS kit is based on a designed panel of 682 oligonucleotides allowing the preparation of any type and combination of PCR amplicons of the SARS-CoV-2 genomic sequence, including the possibility of PCR amplification of the entire genomic sequence. This method is designed to allow targeted genotyping of individual mutations, sequencing of selected regions of the genome using Sanger sequencing, or automated preparation of DNA libraries in 96-well plate format for multiplex sequencing on all types of Illumina, Pacific BioSciences, or Oxford Nanopore sequencers.

Sequencing of Total RNA

Total RNA isolated from positive samples were sequenced using the NGS method. The obtained results contained not only a sequence of whole genome of SARS-CoV-2 (Fig. 24 and 25), but also expressed human RNA from the nasal swab.

By analyzing the data obtained from the total RNA sequencing, we were able to prepare a map of RNA/proteins that are expressed in nasopharyngeal epithelial cells. We identified that one important protein involved in the immune response is expressed in these cells. Based on further experiments, we found that expression levels of this protein (SAA1) correspond to the subsequent course of the infectious disease. This finding led to filing of a patent application describing a method of prediction of severity of infectious diseases based on this marker.
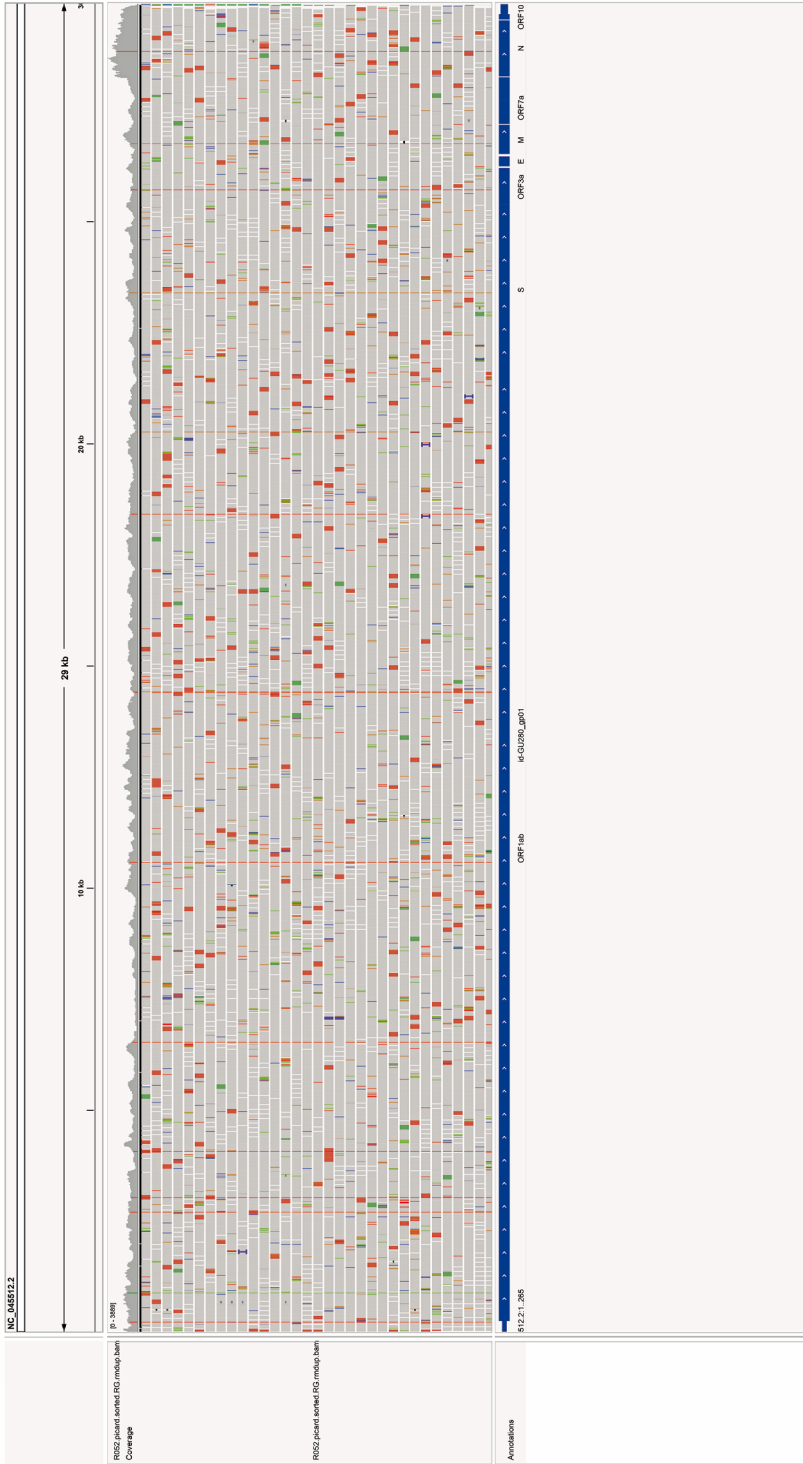
Fig. 24 Whole genome of one SARS-CoV-2 positive sample

Comparison of genotypes of 3 sequenced SARS-CoV-2 samples
Samples 52 and 53 were positive in December 2020
Sample 36 was positive in October 2020

Sample 52

Sample 53

Sample 36
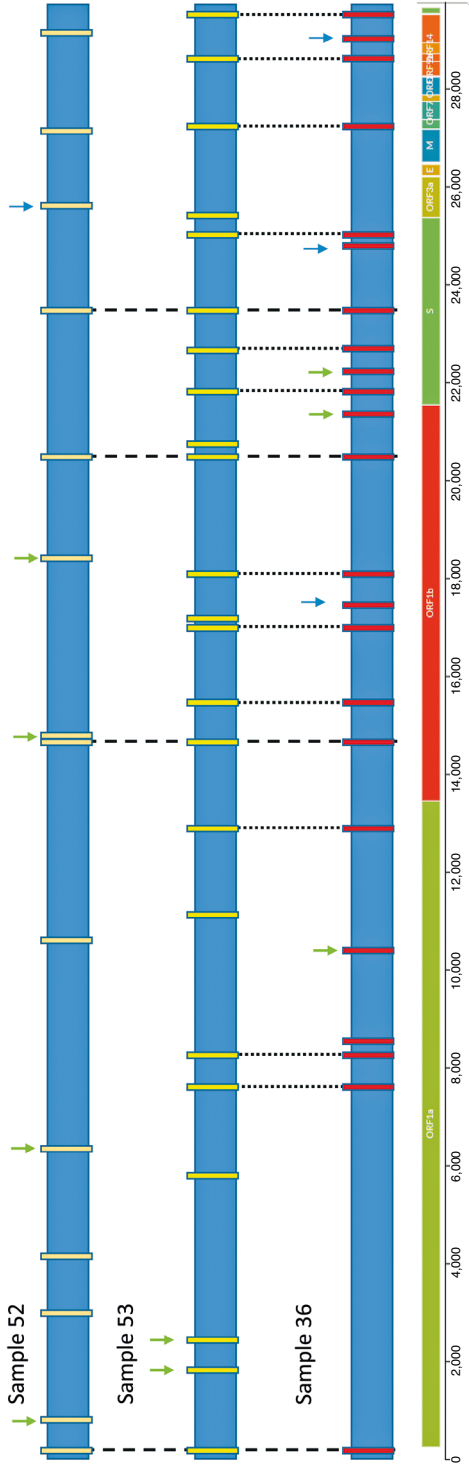
→ Variant is not present in GISAID database as a Czech Republic variant

→ Variant is not present in GISAID database

Fig. 25 Analysis of SARS-CoV-2 genome with variants

PCR Amplicon Sequencing

As the method of sequencing of total RNA is not useful for diagnostic laboratories, other simpler methods were developed.

Whole Genome Sequencing

We designed and optimized 6 PCR amplicon pairs (SC1–SC6) which enabled sequencing of selected regions or the entire SARS-CoV-2 genome using the Illumina platform.

S-protein Sequencing

We also designed and optimized 7 pairs of PCR amplicons (10F–52R) (Fig. 26) to sequence selected fragments or the entire S-protein gene region using targeted Sanger sequencing.
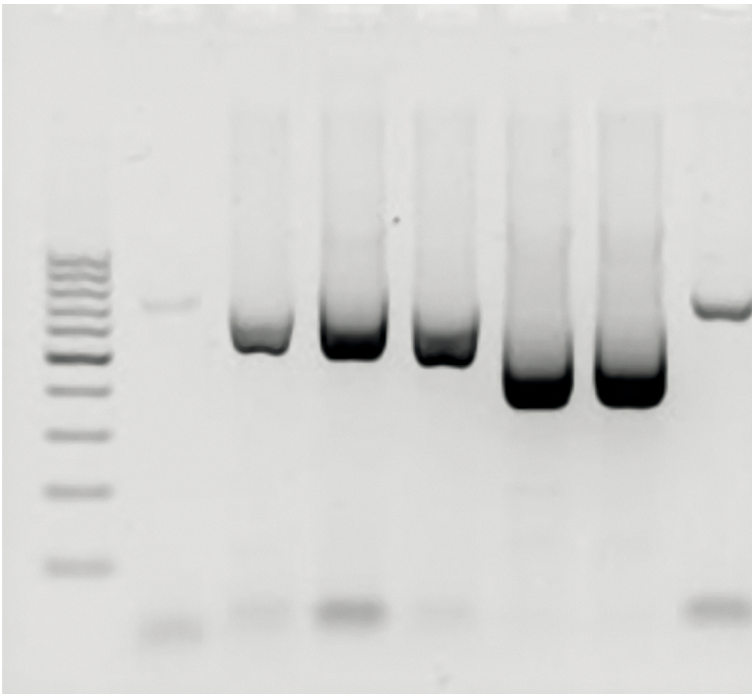
Fig. 26 PCR amplifications of S-protein. Agarose gel with 7 PCR amplicons of S-protein

MONITORING OF THE SARS-COV-2 INFECTION AT SCHOOLS DURING A PANDEMIC PEAK

To monitor the SARS-CoV-2 infection at schools, two experimental rounds of testing were conducted during spring 2021 with the aim to get a realistic picture of the prevalence of SARS-CoV-2 infection in children of different age groups in the Czech Republic at that time. The prevalence of infection and its evolution was correlated with the applied anti-epidemic measures. The data obtained were compared with the results of testing children in mainstream and predetermined schools of one region of the Czech Republic, which have different levels of risk in terms of infection and transmission within families.

In the first round, 313 asymptomatic children from elementary schools in three different regions of the Czech Republic were tested for the presence of the virus. In the second round, 762 asymptomatic school-aged children were tested for the presence of the virus. The children have been visiting 66 different schools within one region of the Czech Republic. The locations of the schools and regions are shown in Fig. 27.

In both rounds, the samples were collected and delivered into an accredited laboratory, where they were analyzed by RT-PCR with previous RNA extraction according to previously described protocols.

The data were then categorized by age and divided into nine sociological groups (Tab. 14). We present data from 1 September 2020, which was also the start of the 2020/2021 school year.

In another approach, C_T values characterizing the input concentration of viral RNA were collected, divided by age, and analyzed. The methodology used has remained the same over time, therefore, despite many factors influencing the quantification, the obtained data can be compared in a semi-quantitative manner. With this in mind and in such a robust dataset, it is possible to get an idea of the infectivity rate in different age groups (Fig. 28 and Tab. 15). As the C_T value increases, the concentration of SARS-CoV-2 RNA in the tested material decreases. Thus, this parameter is directly related to the level of infectivity of a given age group (Monod et al., 2021).
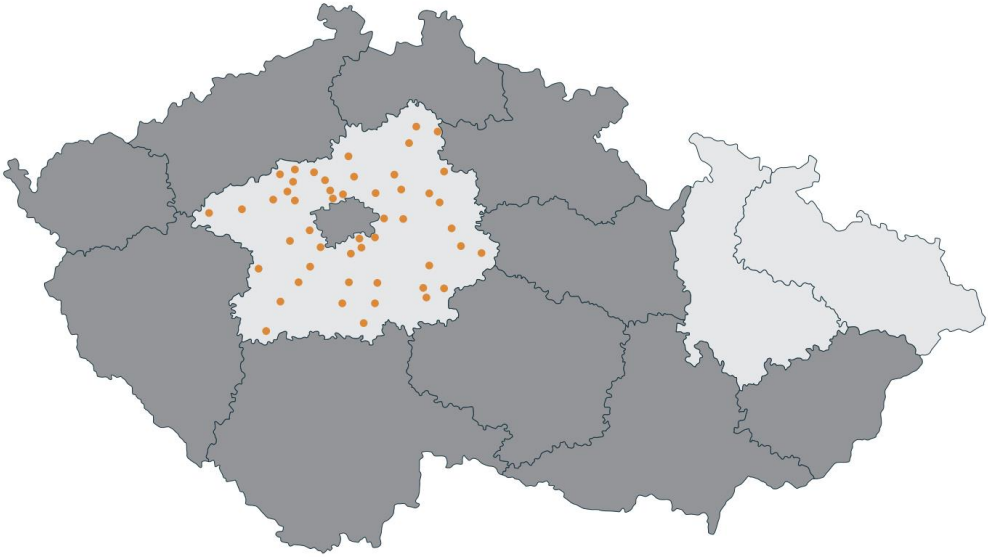
Fig. 27 Locations of schools and regions used for the study: orange dots represent the 66 schools participating in the second round of the experiment and the regions where the first round took place are marked in light grey

Tab. 14 Categorization by age to nine sociological groups

Category	Age	
1	0–2 years	Newborns and toddlers
2	3–5 years	Preschool children
3	6–8 years	Elementary school children I
4	9–13 years	Elementary school children II
5	14–15 years	Elementary school children III
6	16–18 years	Youth I / high schools
7	19–26 years	Youth II / universities
8	27–65 years	Adults
9	More than 66 years	Seniors
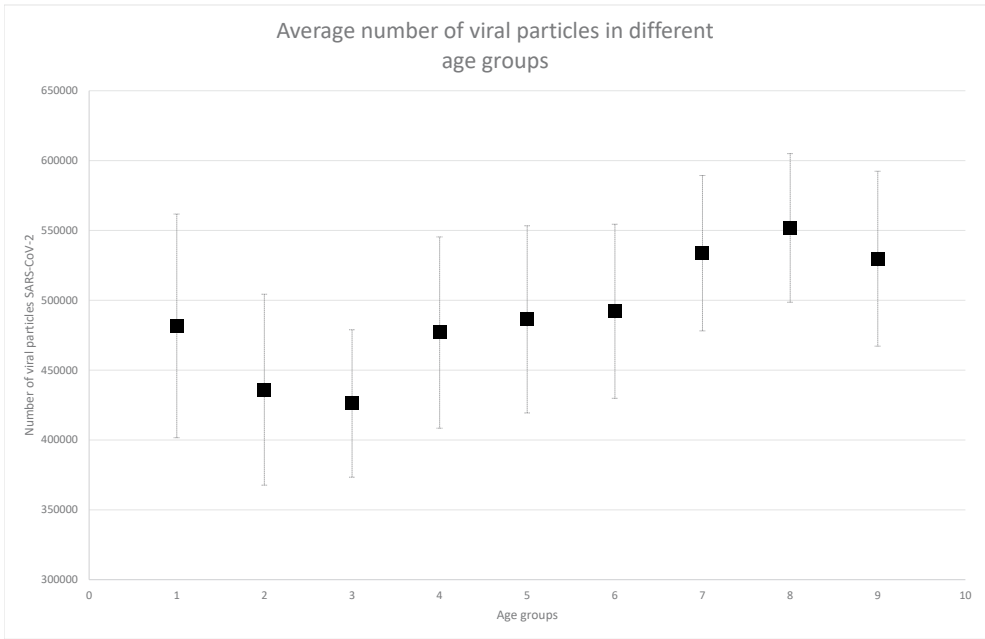
Fig. 28 Distribution of number of viral particles of SARS-CoV-2 in different age groups

Tab. 15 Distribution of number of viral particles of SARS-CoV-2 in different age groups

Age group	0–2 years	3–5 years	6–8 years	9–13 years	14–15 years	16–18 years	19–26 years	27–65 years	More than 66 years
Number of tests	956	5 045	5 598	5 999	3 171	2 187	12 825	79 852	12 923
Number of positive tests	332	846	992	1 515	889	550	2 712	22 008	4 108
Number of detected viral particles	482 000	436 000	426 000	477 000	486 000	492 000	534 000	552 000	530 000

In the second round, testing was accompanied by a questionnaire regarding COVID-19 history in the family, such as the number of cases or their severity. The questions are listed in Tab. 16 and were shared with parents of the tested children via the Google Questionnaire application. All the parents were contacted in seven days distance by teachers or school management. However, the analysis of the questionnaire data has not shown any solid pattern or relevant conclusions.

Tab. 16 Questionnaire for the second round with questions about family history in relation to the presence and course of COVID-19 in the families

1	Was your child (tested at school) been previously diagnosed as positive for COVID-19 by PCR? Yes – 2020 Yes – 2021 No
2	If your child was previously positive for COVID-19, what kind of symptoms did he/she have? No COVID-19 before Temperature Cough Headache Loss of smell and/or taste Diarrhea and/or vomiting Cold Rash Other
3	What is your child's health status 3 days after the testing at school?
4	How many adults live in the household?
5	How many children live in the household?
6	Have any of the adults in the shared household been previously diagnosed as positive for COVID-19 by PCR?
7	If any of the adults in the shared household were diagnosed as PCR positive, did they stay in the same household as the other members during the period of isolation/quarantine/illness?
8	Have any of the children (other than the child tested at school) from the shared household been previously diagnosed as positive for COVID-19 by PCR?
9	If any of the children (other than the child tested at school) from the shared household were previously diagnosed as positive for COVID-19 by PCR, did they stay in a shared household with other members during the period of isolation/quarantine/illness?
10	Has any member of the household been vaccinated against COVID-19?

Analysis of the age composition shows statistically significantly lower rates in individuals below 26 years of age in comparison with the adult population (27–65 years) (Tab. 17). The prevalence of infection in this group was not affected by the course of the epidemic in other age groups in the general public. An exception was the period after Christmas, probably caused by an infection transmission during family gatherings.

Tab. 17 Analysis of the age composition compared to the adult population (27–65 years)

Age group	OR	95% CI	P value
3–5 years	0.48	0.46–0.51	<0.0001
6–8 years	0.54	0.52–0.57	<0.0001
9–13 years	0.85	0.82-0.88	<0.0001
14–15 years	0.86	0.82-0.9	<0.0001
16–26 years	0.75	0.73-0.77	<0.0001

Even though the vast majority of children was tested on the basis of an indication, meaning either the child's current health problems or symptoms (cold, temperature, cough, headache, vomiting, diarrhea, loss of smell or taste) or an epidemiological indication, there was still a persistently low prevalence in the group of children aged 0–8 years.

The prevalence of infection increases in the group of children aged 9 to 15 years and partially in the group aged 15 to 18 years after school closure (Fig. 29 and Tab. 18). This observation suggests that infection transfers to children from their parents during the isolation of the children in their homes. The prevalence in other age categories increases with age. The epidemic in the category of people aged 19 to 26 has an interesting pattern. The prevalence in this group is consistently low, except for the autumn, comparable to the population of children aged 3 to 8 years. This observation is explained by the fact that young people at this age have less intensive contact with their families, work or study more from their own home, and are in a more restricted circle of peers, and thus are less exposed to infection.
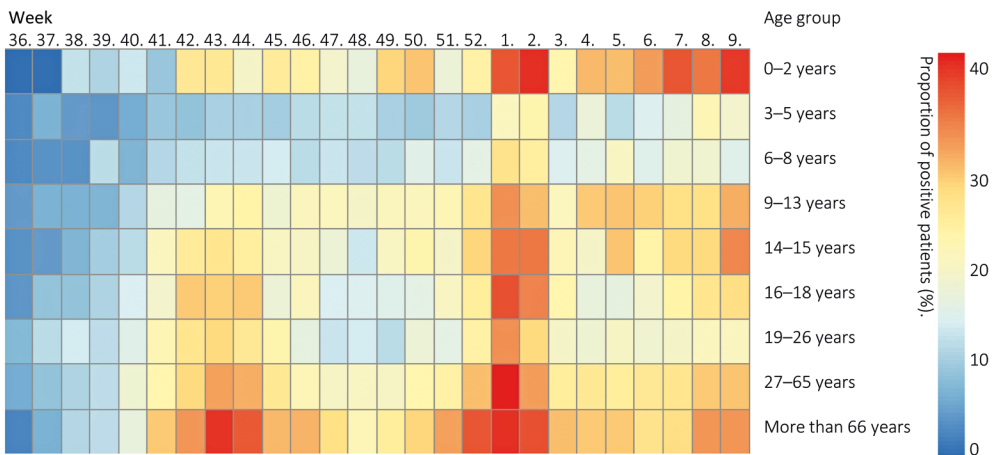
Fig. 29 Prevalence of positivity for SARS-CoV-2 in age groups by week in the Czech Republic between 1 September 2020 and 7 March 2021. The heatmap shows the status by weeks, the darker the color the higher the percentage of positive ones

Tab. 18 Timeline of anti-pandemic precautions in Czech Republic between 1 September 2020 and 7 March 2021

39th week 2020	Face masks in public transport
42nd week 2020	School closure, restriction of movement
44th week 2020	Curfew after 9 p.m.
47th week 2020	Opening of schools: 1.–2. grade at elementary schools
49th week 2020	Opening of schools: 3.–9. classes at elementary schools and for high school seniors
50th week 2020	Opening of schools, alternate teaching for high schools
51st week 2020	Free antigen testing
2nd week 2021	School closure, open only classes 1–2 of elementary schools and kindergartens
8th week 2021	Compulsory wearing of respirators, only predetermined schools open
9th week 2021	Lockdown

C$_T$ VALUES CALCULATED ON A WEEKLY BASIS MIGHT BE USED FOR EPIDEMIOLOGICAL PREDICTION

The C$_T$ value is in most cases not used in the diagnosis of COVID-19 disease and is not used to estimate viral load or even to monitor trends (Mušálková et al., 2023). There are several reasons for this.

Despite the relatively valuable information that the C$_T$ value holds, it is not used by clinicians. It allows us to quantify relative to some recognized housekeeping gene or at least evaluate the result in semi-quantitative terms. In practice, however, that is done very rarely or not at all, because information about viral load does not directly affect the treatment of patients. What would be of significantly greater importance, however, is to track viral load over time and monitor the evolution of the infection. By this approach we would be able to tell whether a given patient is before or after the main peak of infection. Unfortunately, all of these considerations are undermined by the non-standardized sample collection and the different technologies that there are in different laboratories.

However, C$_T$ values can also be used for other purposes. It appears that there is a direct link between viral load in a population and the prediction of an epidemic wave. In order to support this association and additionally have data with telling conclusions, it is necessary to throw out the big data due to the large bias.

We used data from the SPADIA lab to demonstrate the possibility of using a predictive model. The positive samples were kept with the C$_T$ value and we generated a graph on a weekly basis that tracked the viral load values in the population (represented by the average C$_T$ value of the positive samples) and the number of positive individuals or the ratio of positive versus negative individuals.

In this correlation, we used this number of samples to obtain the predictive matrix and the dependence between the viral load and an incoming wave. With increasing viral load in the population, the increase in the number of positive individuals and the arrival of the epidemic wave also started with a delay of 6–8 weeks.

Thus, this method is useful for predicting the arrival of an epidemic wave. However, it is directly dependent on the number of people tested and differences in technology across laboratories.

CONCLUSION

In different countries, pandemic management is approached differently. As it is a crisis situation with no clear perspective for solution, it is very difficult to provide a single correct view of the situation. What is apparent, however, is the fact that numerous people of different expertise are involved in the epidemic management, often with opposing views. This brings conflicts of opinion, which are reflected, among other things, in the mood of the people, and which also lead to problems in other areas of society – economics, sociology, business and services, etc.

One of the fundamental pillars of epidemic management is the detection of the SARS-CoV-2 virus and providing doctors with information regarding the diagnosis. If a diagnosis is prompt, chances for a successful treatment increase. Diagnosis is also a key tool for epidemiologists, allowing them to monitor the spread of the virus and its evolution. They can then predict the upcoming situation and act if necessary.

The data presented in this book are extremely robust and were collected by a large expert team. They were provided to state officials and institutions – the Ministry of Health, the Senate, the National Institute of Health, politicians – as well as the public during the entire epidemic.

The power of precision diagnostics, in addition to saving many lives, is in providing trends and data that can assist decisions about closing and opening schools, businesses, restaurants, and other public facilities. However, it is essential for the whole system of laboratory diagnostics to see also individual patients' results, rather than just the whole picture. Thorough analysis of obtained laboratory data and measured parameters can provide physicians with relevant information that can lead to a more effective treatment.

The purpose of this book was to provide a realistic view of the evolution of laboratory diagnostics of the SARS-CoV-2 virus over time, which can be used as a methodological framework for other parameters or detection of other pathogens in the future.

The methodologies of PCR, sequencing, pooling, and other techniques have developed to the point where they can be useful for testing many other pathogens that were previously untested or tested by insensitive and obsolete methods. In this respect, the SARS-CoV-2 epidemic had and continues to have a positive impact.

ACKNOWLEDGEMENTS

LIST OF ABBREVIATIONS

(–)ssRNA	minus single stranded RNA
(+)ssRNA	plus single stranded RNA
μL	microliter
ACE2	angiotensin converting enzyme 2
AlphaCoV	alphacoronavirus
BetaCoV	betacoronavirus
CD209L	C-type lectin
CDC	Centers for Disease Control and Prevention
CE-IVD	certified in vitro diagnostic
CoV	coronavirus
COVID-19	coronavirus disease 2019
C_T	cycle threshold
Cy5	cyanine5; fluorophore
DeltaCoV	deltacoronavirus
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotides
DPP4	dipeptidyl peptidase 4
dsDNA	double stranded DNA
dsRNA	double stranded RNA
E484K	substitution of glutamic acid to lysine at position 484
FAM	6-carboxyfluorescein; fluorophore
GammaCoV	gammacoronavirus
GISAID	Global Initiative on Sharing Avian Influenza Data
HBV	hepatitis B virus
HCV	hepatitis C virus
HEX	5-hexachlorofluorescein; fluorophore
IHIS	information system and government systems
L452R	substitution of leucine to arginine at position 452
LIS	laboratory information system
LOD	limit of detection
MERS-CoV	middle east respiratory syndrome coronavirus
mRNA	messenger RNA
NGS	next generation sequencing
nm	nanometer
nsp	non-structural protein

ORF	open reading frame
ORs	odds ratios
PCR	polymerase chain reaction
pp	polyprotein
RBD	receptor binding protein
RdRP	RNA-dependent RNA polymerase
RFU	relative fluorescence units
RNA	ribonucleic acid
ROX	rhodamine; fluorophore
RRAR/S	motif of aminoacids in SARS-CoV-2
RTC	replicase-transcriptase complex
RT-PCR	reverse transcription – polymerase chain reaction
RT-qPCR	real-time reverse-transcription polymerase chain reaction
SAA1	serum amyloid A1
SARS-CoV	severe acute respiratory syndrome coronavirus
ssDNA	single stranded DNA
STDs	sexually transmitted diseases
TMPRSS11D	transmembrane serine protease 11D
TMPRSS2	transmembrane serine protease 2
WHO	World Health Organization

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Web sites
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GISAID – https://www.gisaid.org/

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AUTHORS

Michal POHLUDKA

Michal Pohludka studied biochemistry and biotechnology at the Institute of Chemical Technology in Prague and subsequently completed his postgraduate studies in the field of molecular pathology at the 1st Faculty of Medicine at Charles University. Then, he spent more than a decade working for a global American company in various management positions in Central and Eastern Europe. His main focus was leading business and application activities in the field of clinical diagnostics and life sciences. Later, he founded his own consulting company.

With the arrival of the first wave of the COVID-19 epidemic, he put his professional activities on hold and went to help set up a newly established laboratory for PCR testing for the presence of SARS-CoV-2 in patient samples. There, he combined his previous professional and managerial experience. Within a few months, the laboratory became the largest in Central and Eastern Europe.

Together with representatives of Charles University, SPADIA LAB, a manufacturing company, and a development company, he founded the spin-off company GeneSpector. The aim was to create a complete solution for PCR testing and offer it, together with the know-how of the whole process, to Czech hospitals and laboratories to increase testing capacity and deliver patient results as quickly as possible.

During the COVID-19 epidemic, he set up or built one fifth of all Czech laboratories for SARS-CoV-2 PCR testing. This includes the complete sample pathway from arrival into the laboratory, through automated RNA isolation, PCR and its evaluation, and automatic reporting of results to information systems. Due to the size and complexity of the whole agenda, he decided to share his practical experience with both the professional and general public in the form of this book. The aim of this publication is also to show problems of the process and follow-up solutions, so he invited Lenka Piherová, application specialist and expert from the 1st Faculty of Medicine of Charles University, as a co-author.

Lenka PIHEROVÁ

Lenka Piherová studied biochemistry and biomedical engineering at the Institute of Chemical Technology in Prague and subsequently completed her postgraduate studies at the 1st Faculty of Medicine of Charles University in the field of molecular and cellular biology, genetics, and virology.

She has been studying genetic heart diseases for more than ten years and the core of her work is the analysis of genetic data with the aim to discover the cause of cardiomyopathies. She is an author and co-author of several scientific publications in this field.

When the COVID-19 epidemic started, she helped the General University Hospital in Prague with PCR testing for SARS-CoV-2. Her focus was on isolation of nucleic acids form swabs, speeding up testing, and discovery and optimization of a new isolation technique based on available chemicals and laboratory equipment. This solution became one of the pillars of the spin-off company GeneSpector.

Later, she became an application specialist, helping several laboratories to set up testing and solve problems that arose during testing. These experiences are included in this book.