Expression and characterization of SARS-CoV-2 proteins using recombinant vaccinia virus MVA-T7pol

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I. ABBREVIATIONS

ACE2	Angiotensin-converting enzyme 2
Ad26	Adenovirus type 26
Ad5	Adenovirus type 5
CAT	Chloramphenicol acetyltransferase
cDNA	Complementary DNA
CEF	Chicken embryo fibroblasts
ChAdOx1	Chimpanzee Adenovirus Oxford 1
CMIA	Chemiluminescent microparticle immunoassay
CoV	Coronavirus
COVID-19	Coronavirus infectious disease 2019
CTD	C-terminal domain
CVA	Chorioallantois Vaccine Ankara
DMV	Double membrane vesicle
E protein	Envelope protein
ELISA	Enzyme-linked immunosorbent type assays
EMC	Encephalomyocarditis
ER	Endoplasmic reticulum
ERGIC	ER-Golgi intermediate compartment
EV	Enveloped virion
FBS	Fetal bovine serum
FIP	Feline infectious peritonitis
FIPV	Feline infectious peritonitis virus

GGO	Ground-glass opacities
GOI	Genes of interest
HA	Hemagglutinin
HCoV	Human coronavirus
HR	Heptad repeat
IB	Infectious bronchitis
IBV	Infectious bronchitis virus
ICTV	International Committee of Taxonomy of Viruses
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IV	Immature virion
LFA	Lateral flow assay
M protein	Membrane protein
MCS	Multiple cloning site
MEM	Minimum Essential Medium
MOI	Multiplicity of infection
MV	Mature virion
MVA	Modified Vaccinia virus Ankara
N protein	Nucleocapsid protein
nAb	Neutralizing antibody
NSP	Non-structural protein
NTD	N-terminal domain
ORF	Open reading frame

RBD	Receptor binding domains
RdRp	RNA-dependent RNA polymerase
RIG-I	Retinoic-acid inducible gene-I
RT-PCR	Reverse transcription-polymerase chain reaction
S protein	Spike protein
SARS	Severe acute respiratory syndrome
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SNT	Sera neutralization test
TGEV	Transmissible gastroenteritis virus
tk	Thymidine kinase
UTR	Untranslated region
VOC	Variants of concern
WHO	World Health Organization
βCoV	*Betacoronavirus*

II. INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the causative agent of coronavirus infectious disease 2019 (COVID-19), was first detected in December 2019 in Wuhan (China) (Sun *et al.* 2020). Due to an efficient human-to-human transmission, SARS-CoV-2 spread throughout the world by travelers and community-based contacts (Q. Li *et al.* 2020), leading to a global pandemic within a few months. Most infections with SARS-CoV-2 will show asymptomatic or mild symptoms in the respiratory system. However, some patients will become seriously ill and require medical attention. For the better understanding of immunity directed against this new virus, the study for expression of SARS-CoV-2 antigens and the analysis of antibody responses directed against these antigens are crucial.

Modified Vaccinia virus Ankara (MVA), a highly attenuated vaccinia virus strain, has been developed as a safe and efficient vector system delivering bacterial and viral antigens. MVA-T7pol is a recombinant MVA containing the bacteriophage T7 RNA polymerase gene under the control of the natural vaccinia virus early/late promoter P7.5 in deletion II of the MVA genome (Sutter *et al.* 1995). MVA-T7pol is capable of producing high amounts of heterologous target proteins when used as an expression system, and in contrast to MVA vector viruses, there is no need for time-requiring constructions and isolations of recombinant viruses (Hebben *et al.* 2007; Pradeau-Aubreton *et al.* 2010).

This study describes the successful and efficient expression of selected SARS-CoV-2 proteins using the MVA-T7pol system to allow for a sophisticated immunoblot analysis of the humoral immune response against those antigens in COVID-19 patients.

III. LITERATURE REVIEW

1. A global pandemic—COVID-19

At the end of 2019, cases of pneumonia with unknown etiology were reported in Wuhan, China (Q. Li *et al.* 2020). At that time, it was not known that this disease would develop into a pandemic in the future and thus have a huge impact on human life worldwide. As more and more cases were reported worldwide, the World Health Organization (WHO) declared a Public Health Emergency of International Concern on Jan 30, 2020, and announced "COVID-19" as the official name of this new disease. The causative agent which causes the ongoing global pandemic is severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). This name was given by the International Committee on Taxonomy of Viruses (ICTV) because the virus is genetically related to the coronavirus that caused the 2003 SARS outbreak. As of March 2022, more than two years after the first outbreak of COVID-19, about 445 million global cases were confirmed, including 5 million deaths, making it one of the deadliest in history (JHU 2022).

1.1 History of coronavirus-related diseases

The COVID-19 pandemic has made a profound impact on various aspects of human life around the world. Prior to the COVID-19 outbreak, infections caused by other members of coronaviruses have historically posed challenges to veterinary medicine as well as human medicine. The history of coronavirus starts with the report of infectious bronchitis virus (IBV), which was

recognized as the causative agent of infectious bronchitis (IB) in 1937 (Beaudette 1937). It is notoriously difficult to control and has become one of the most important respiratory diseases in chickens. Nowadays, the disease is endemic in almost all regions of the world, especially the country that possesses a developed commercial poultry industry (Wit *et al.* 2011). For decades, new coronaviruses have been discovered continuously, and many of them are highly pathogenic and constantly affect the health of animals throughout the world, such as transmissible gastroenteritis virus (TGEV) and porcine epidemic diarrhea virus (PEDV). In 1946, an outbreak of infectious gastroenteritis in swine was reported (DOYLE & HUTCHINGS 1946). The causative virus was identified as TGEV and spread worldwide, causing diarrhea and vomiting in pigs of all ages (Valkó *et al.* 2018). PEDV was first isolated in Belgium in 1978 and is clinically similar to TGEV (Pensaert & Bouck 1978).

The first human coronavirus (HCoV), HCoV-229E, was identified in the 1960s from the nasal specimen of a patient with the common cold (Hamre & Procknow 1966). Later, a coronavirus isolated from the nasopharyngeal wash of a patient was found to be serologically distinct from HCoV-229E. It was reported in a publication in 1967 and named HCoV-OC43 (McIntosh *et al.* 1967). These two viruses, known to be circulating in the human population, were the focus of HCoV research in subsequent years until the outbreak of the highly pathogenic severe acute respiratory syndrome coronavirus (SARS-CoV-1) in 2002.

The first epidemic of the 21st century began with the emergence of a severe and readily transmissible disease: severe acute respiratory syndrome (SARS). It was first emerged in Guangdong Province of China in November 2002 and rapidly spread to 26 countries, resulting in more than 8000 cases and 774 deaths by July 2003 (Skowronski *et al.* 2005). Asian area was strongly affected and approximately 60% of the cases and 40% of the deaths were reported from mainland China (Feng *et al.* 2009). Major clinical manifestations include persistent fever, chills, dry cough, muscle aches, headache and occasionally diarrhea (HUI *et al.* 2003). The etiological agent was identified by cell culture in March 2003 and was namely SARS-CoV by WHO (Drosten *et al.* 2003). As many of the early cases of SARS in Guangdong took animal-related positions, it is thought to be a virus of animal origin with the genetic ability to cross species barriers and spread to humans through unknown intermediate hosts (He *et al.* 2003).

A decade later, Middle East respiratory syndrome (MERS), another highly contagious infectious respiratory syndrome in humans, emerged in Jiddah, Saudi Arabia (Zaki *et al.* 2012). It is an acute illness caused by a coronavirus called MERS-CoV, which attacks the respiratory system, especially the lung tissue and airways (Mackay & Arden 2015). Serological and nucleic acid-based evidence suggests that camels were direct sources of human infection and important for MERS-CoV transmission(Haagmans *et al.* 2014; Han *et al.* 2016; Hemida *et al.* 2013; Perera *et al.* 2013). While bats are suspected to be the evolutionary source of the virus (Anthony *et al.* 2017). By December 2021, more than 2580 confirmed MERS-CoV cases, including about 940 deaths,

have been detected in 27 countries since the disease was first identified (WHO 2021a).

1.2 Genomic and protein features of SARS-CoV-2

The word “corona” in coronaviruses (CoVs) is derived from the Latin word, meaning crown or halo, due to their wreath-like appearance when viewed under an electron microscope (Helmy *et al.* 2020). CoVs belong to the subfamily *Orthocoronavirinae* of the family *Coronaviridae*, the largest group in the order *Nidovirales*, according to the latest International Committee of Taxonomy of Viruses (ICTV) classification. The subfamily *Orthocoronavirinae* is further divided into four genera: alpha, beta, gamma, and delta. As shown in Table 1, SARS-CoV-2 belongs to the genus *Betacoronavirus* (βCoV).

SARS-CoV-2 is an enveloped, positive-sense, non-segmented, single-stranded RNA virus with a diameter of 50-200 nm and a genome size of about 30 kb (X. Xu *et al.* 2020). The virus genome consists of 5’ and 3’ untranslated regions (UTRs) and 15 open reading frames (ORFs) encoding probably 29 proteins (Figure 1) (Al-Qaaneh *et al.* 2021). The ORF1a and ORF1b gene are located at 5’ terminus of the viral genomic RNA, spanning about two-thirds of the virus genome, encoding for highly conserved polyproteins pp1a and pp1ab, which is proteolytically cleaved into 16 non-structural proteins (NSPs) (Malone *et al.* 2022; Simabuco *et al.* 2020). The remaining third of the genome encodes 4 structural proteins (spike, envelope, membrane, and nucleocapsid) and 9 accessory proteins (ORF 3a, 3b, 6, 7a, 7b, 8a, 8b, 9b, and 10), as shown in Figure 1.

The spike (S) protein is a large glycoprotein with 1273 amino acids in length and shares about 76% identify with SARS-CoV-1 (H. Xu *et al.* 2020). It is proteolytically cleaved into two subunits, S1 and S2, which are responsible for binding to the cell receptor and virus-cell fusion. A unique furin-like cleavage site was found to be involved in this process, which is absent in CoVs of the same clade (Coutard *et al.* 2020). However, it was recently reported that S proteins of some variants were predominantly in the un-cleaved form because of mutations in the furin cleavage site (Meng *et al.* 2021). A second cleavage site in S protein is located 130 residues from the N terminus of the S2 subunit (Segreto *et al.* 2021). The S1 subunit consists of two independent domains: the N-terminal domain (S1-NTD) and the C-terminal domain (S1-CTD), composing the receptor binding domains (RBD) which can directly interact with the host cell receptor angiotensin-converting enzyme 2 (ACE2) (J. Lan *et al.* 2020). The S2 subunit contains two heptad repeat domains (HR1 and HR2), mediating the virus-cell membrane fusion (Xia *et al.* 2020). The mechanism of SARS-CoV-2 replication is described in Figure 2.

The SARS-CoV-2 nucleocapsid (N) protein is the most abundant viral protein which interacts with the viral genome by capsulating the genomic material within the viral particles (Yoshimoto 2020). It is estimated that there are 1000 copies of N into each virion compared to only 100 copies of S (Bar-On *et al.* 2020). Given its abundant expression and genomic conservation, N has been commonly used as a serological marker of infection. A key function of N is to wrap the viral genome to protect coronaviruses from immune identification and degradation by host factors. A previous study has demonstrated that

SARS-CoV-2 N protein can significantly suppress INF-β production due to its ability to bind the retinoic-acid inducible gene-I (RIG-I), which could be one of the factors affecting the innate immune of the host (Oh & Shin 2021).

Envelope (E) and membrane (M) proteins, two other structural proteins of SARS-CoV-2, were reported to be conserved across members of the genus βCoV (Bianchi *et al.* 2020). Previous studies pointed out that E and M proteins of coronaviruses could be critical for viral entry, assembly and pathogenicity (Alsaadi & Jones 2019). M protein is regarded as a scaffolding platform to recruit other structural proteins (N, S, E proteins) and mediates their interactions (Schoeman & Fielding 2019). The SARS-CoV-2 E protein is a potential ion channel that was also demonstrated in other coronaviruses and blocking this channel can significantly reduce the pathogenicity of the virus, suggesting that the E protein could be an antiviral target (Tomar & Arkin 2020).

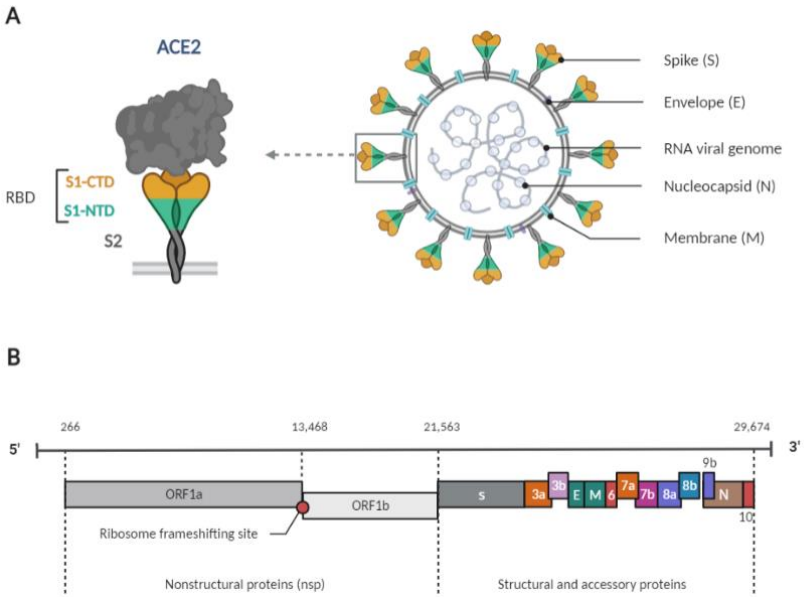

Figure 1 Schematic diagrams of the SARS-CoV-2. (A) Viral RNA and four structural proteins of SARS-CoV-2, including S, E, M, and N protein. The S protein is proteolytically cleaved into two subunits: S1 and S2. The S1 subunit consists of S1-NTD and S1-CTD, composing the RBD which can directly interact with the host cell receptor ACE2; (B) Genomic organization of SARS-CoV-2, including coding sequences for the non-structural proteins (ORF1a and ORF1b), the structural proteins (S, E, M, N) and 9 accessory proteins (ORF 3a, 3b, 6, 7a, 7b, 8a, 8b, 9b, and 10). The figure was created with BioRender.com.

The SARS-CoV-2 genome also contains some ORFs encoding accessory proteins that are less important in replication but have a role in pathogenesis. Their properties and mechanisms are not well explained until now. ORF3a is the largest accessory protein with 275 amino acids in length. Functionally, ORF3a acts as a crucial immune modulator and is responsible for the pathogenesis of SARS-CoV-2 via inducing apoptosis in cells (Ren *et al.* 2020). It was reported that ORF3a could self-assemble into oligomers and generate

ion channels to promote virus release, like SARS-CoV-1 (Kern *et al.* 2021). Anti-ORF3a antibodies are frequently observed in SARS-CoV-1 patient plasma (W. Lu *et al.* 2009). ORF3b, ORF6, ORF7a, ORF8 and ORF9b are potent factors to suppress interferon secreted by host cells (Redondo *et al.* 2021). The possible roles of ORF7b and ORF10 remain to be investigated.

Table 1 Classification of Coronaviridae based on the latest ICTV (October 2020).

Family	Subfamily	Genus	Subgenus	No. of species	Type species	Reservoir Host (Fan *et al.* 2019)
Coronaviridae	*Letovirinae*	*Alphaletovirus*	*Milecovirus*	1	*Microhyla letovirus 1*	Amphibians
	Orthocoronavirinae	*Alphacoronavirus*	*Colacovirus*	1	*Bat coronavirus CDPHE15*	Bats
			Decacovirus	2	*Bat coronavirus HKU10*	Bats
			Duvinacovirus	1	***Human coronavirus 229E***	**Humans**
			Luchacovirus	1	*Lucheng Rn rat coronavirus*	Rats
			Minacovirus	1	*Mink coronavirus 1*	Minks
			Minunacovirus	2	*Miniopetrus batcoronavirus 1*	Bats
			Mytacovirus	1	*Myotis ricketti alphacoronavirus Sax-2011*	Bats
			Nyctacovirus	2	*Pipistrellus kuhlii coronavirus 3398*	Bats
			Pedacovirus	2	*Porcine epidemic diarrhea virus*	Pigs
			Rhinacovirus	1	*Rhinolophus bat coronavirus HKU2*	Bats and pigs
			Setracovirus	2	***Human coronavirus NL63***	**Humans**
			Soracovirus	1	*Sorex 20raneus coronavirus T14*	Sorex araneus
			Sunacovirus	1	*Suncus murinus coronavirus X74*	Suncus murinus
			Tegacovirus	1	*Alphacoronavirus 1*	Porcines, canines
		Betacoronavirus	*Embecovirus*	5	***Human coronavirus HKU1*** ***Human coronavirus OC43***	**Humans**
			Hibecovirus	1	*Bat Hp-betacoronavirus Zhejiang2013*	Bats
			Merbecovirus	4	***Middle East respiratory syndrome-related coronavirus***	**Humans, camels, and bats**
			Nobecovirus	3	*Rousettus bat coronavirus HKU9*	Bats
			Sarbecovirus	1	***Severe acute respiratory syndrome-related coronavirus-1 and 2***	**Humans, bats**
		Deltacoronavirus	*Andecovirus*	1	*Wigeon coronavirus HKU20*	Birds
			Buldecovirus	5	*Bulbul coronavirus HKU11*	Birds
			Herdecovirus	1	*Night heron coronavirus HKU19*	Birds
		Gammacoronavirus	*Brangacovirus*	1	*Goose coronavirus CB17*	Birds
			Cegacovirus	1	*Beluga whale coronavirus SW1*	Whale
			Igacovirus	3	*Avian coronavirus*	Birds

HCoVs are in bold.

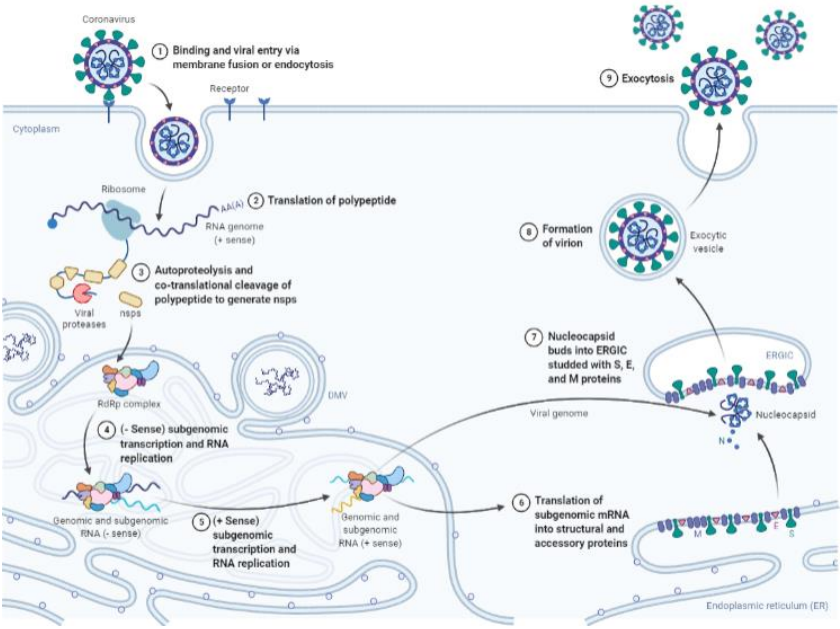

Figure 2 The replication of SARS-CoV-2 in host cells. The infection begins with the interaction between the viral S protein and the cellular receptor. The S1 subunit binds the ACE2 receptor and the S2 subunit is responsible for anchoring the S protein to the cell surface to promote membrane fusion. After fusion occurs, the RNA genome (ssRNA+) is released into the cytoplasm and used as the template to produce pp1a and pp1b polyproteins that are cleaved into 16 NSPs by viral proteases. Subsequently, RNA-dependent RNA polymerase (RdRp) is formed by NSP12 and drives the transcription of subgenomic mRNAs and viral genomic RNA replication. The RNAs are surrounded by a protective microenvironment created with double membrane vesicles (DMVs) to prevent them from being attacked by the host's immunity. Then the subgenomic mRNAs are translated into structural and accessory proteins at endoplasmic reticulum (ER) membranes, and translocated to the ER-Golgi intermediate compartment (ERGIC) where the positive-stranded genomic RNA binds the N protein and is assembled into the virion along with the S, E, and M proteins. Finally, the packed virion is secreted from the infected cell membrane by the exocytosis process (Simabuco *et al.* 2020; V'kovski *et al.* 2020). The figure was created with BioRender.com.

1.3 Clinical presentation and epidemiology of COVID-19

SARS-CoV-2 is an elusive virus that can affect most organ systems and cause different clinical manifestations in individuals. Usually, it has an estimated incubation period of 2 to 14 days. However, cases with an incubation period of 21, 24 or 27 days have been reported (Bai *et al.* 2020). The respiratory system is most commonly affected, inducing flu-like symptoms in patients: fever, wheeze, cough, fatigue, headache and chest pain, etc. Due to a compromised respiratory system, hypoxia and respiratory failure may aggravate the burden on the cardiovascular system (Clerkin *et al.* 2020). Diarrhea and vomiting are common gastrointestinal symptoms and have even been found in some cases without accompanying respiratory symptoms during disease progression (An *et al.* 2020). Other symptoms are manifested in the renal system (proteinuria and acute kidney injury) and neurological system (hyposmia, confusion and ageusia) (Cheng *et al.* 2020; Mao *et al.* 2020). The infection damages the organs and even develops into long-term sequelae (Logue *et al.* 2021). However, as large-scale testing has become available, there is growing evidence that many people infected with SARS-CoV-2 are asymptomatic but still transmit the virus to others (Gao *et al.* 2021).

COVID-19 was first reported in Wuhan and quickly spread to other provinces in China due to the Chinese New Year vacation in January 2020. Successively, the first cases of SARS-CoV-2 infection were confirmed in Thailand and Japan in mid-January 2020. By Jan 25, 2020, the number of confirmed cases had risen to 1320 in 10 countries (Australia, Japan, Singapore, France, the US, Viet Nam, South Korea, Thailand, Nepal, and China), as reported by WHO. After

only 2 months, more than 150 countries/territories have been affected, with about 16 thousand confirmed cases and 6 thousand deaths in total, at which point the WHO urgently declared SARS-CoV-2 as a pandemic. There have been at least 445 million reported infections and 5 million reported deaths caused by SARS-CoV-2 by March 2022 (JHU 2022). However, the situations varied in different countries/territories, with more than 100 million confirmed cases in the United States and the European Union, 30 million in India, 10 million in Russia, and less than 3 million in Australia and China (Figure 3).

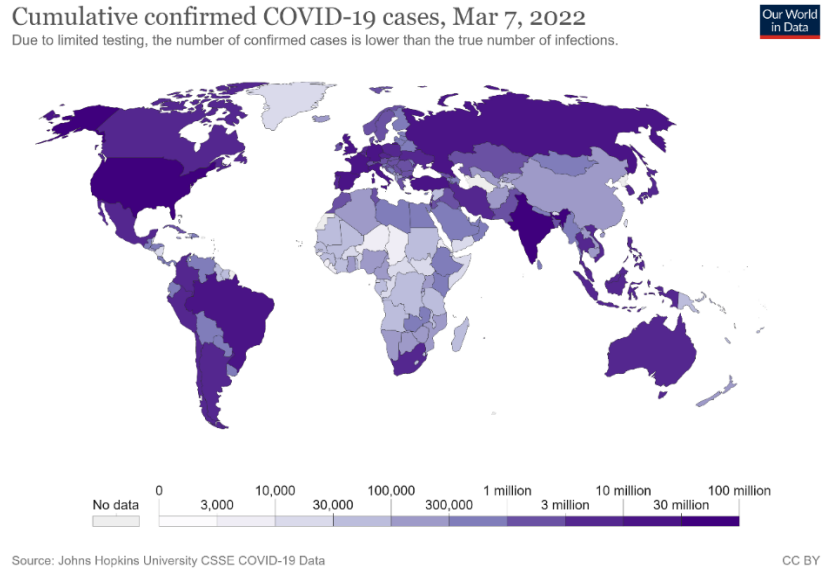

Figure 3 Cumulative confirmed COVID-19 cases by Jan 4, 2022 (Source: Our World; https://ourworldindata. Org/ covid-cases; with permission).

During the worldwide spread of SARS-CoV-2, new variants of the virus are constantly emerging. The definition of variants of concern (VOC) is proposed to describe those showing increased transmissibility, higher virulence, or failure in detecting by existing diagnostics. Among them, Alpha (B.1.1.7) was first isolated in the UK and drove the UK's second wave of COVID-19. Since then, Beta (B.1.351), Gamma (P.1), Delta (B.1.617.2) have been reported around the world, with clear evidence indicating a significant impact on transmissibility, severity and/or immunity during the course of the disease (Ramesh *et al.* 2021). SARS-CoV-2 Omicron (BA.1), the new variant emerging in late 2021, has been spread internationally. It can largely evade immunity from past infection or two vaccine doses, and the impact on pathogenicity and transmissibility remains unknown (Meng *et al.* 2021).

1.4 Diagnosis of SARS-CoV-2 Infection

The clinical manifestation of COVID-19 patients is multi-systemic and heterogeneous. Therefore, it is difficult to distinguish this disease from other infections only based on symptoms. Technologies and techniques play an important role in the diagnosis of COVID-19 diagnosis. The major diagnosis tools available so far were based on a) molecular tests, b) immunoassays, and c) radiological diagnosis.

1.4.1 Molecular tests

The method recommended by WHO for routine confirmation of infected cases and detection of SARS-CoV-2 is reverse transcription-polymerase chain reaction (RT-PCR). It has been considered as a gold standard for the diagnostic

of COVID-19, which has the ability to measure the genomic sequences of the specific virus directly. Two conserved regions in the SARS-CoV-2 genome are the targets of the most detections: the RdRP gene, located in the open reading frame ORF1ab, and the E gene (Udugama *et al.* 2020). Samples for testing can be collected at various sites of infection in the body, including the upper and lower respiratory tract aspirates, oropharyngeal, nasopharyngeal or nasal swabs, and bronchoalveolar lavage. Among them, Oropharyngeal and nasopharyngeal swabs were the most commonly used samples and some studies have found that the results of nasopharyngeal swabs are more reliable than those of oropharyngeal swabs (Wang *et al.* 2020). In a study conducted in China (Y. Xu *et al.* 2020), rectal swab samples from 8 infected children are positive, even though their nasopharyngeal swab samples tested negative.

1.4.2 Immunoassays

Immunoassays are based on the affinity reaction between the target antigen and a specific antibody (Born 1998). One set of immunoassays developed for the diagnosis of COVID-19 is the antigen test. Antigen tests allow for direct detection of viral components, such as viral structural proteins (N, S, E, M), without the requirement of special laboratory equipment (Yüce *et al.* 2021). It can be operated in enzyme-linked immunosorbent type assays (ELISA) format for better sensitivity, or on lateral flow assay (LFA) strips for rapid detection purposes which could return results in 15–20 min (Taleghani & Taghipour 2021). Antigen test detects the presence of viral antigens and therefore, like the PCR-based methods, reveals the patient's current infection,

not the past infection or recovery situation. Antigen tests usually are less sensitive compared to molecular tests according to a few studies (Ciotti *et al.* 2021; Mak *et al.* 2020a, 2020b).

Unlike molecular tests and antigen tests, serology tests look for the presence of antibodies produced by the immune system to detect viral infection indirectly. Two types of antibodies are commonly detected: immunoglobulin M (IgM) and immunoglobulin G (IgG). As IgM appears early in the course of infection, it is considered a marker of recent SARS-CoV-2 infection, after which IgG gradually becomes the most common and abundant in the serum as the disease progresses (Vidarsson *et al.* 2014).

Many companies have developed ELISA kits intended for the qualitative detection of IgG or IgM against the S protein in human serum or plasma (Yüce *et al.* 2021). The neutralization assay is a serological test utilized to detect the presence of antibodies responsible for defending cells from pathogens. As an alternative to cell-based neutralizing assays, the SARS-CoV-2 neutralizing antibody (nAb) ELISA kit is designed to measure the neutralizing antibodies against SARS-CoV-2 RBD (Sholukh *et al.* 2021). In addition, Abbott has launched an advancing serological test of COVID-19 named AdviseDx SARS-CoV-2 IgG II, which is based on a high throughput chemiluminescent microparticle immunoassay (CMIA) measuring the amount of IgG antibody against the S protein of SARS-CoV-2 in human serum or plasma (Bradley *et al.* 2021). It has been reported that the measurement of immunoglobulin A (IgA) also plays an important role in serological assessments of SARS-CoV-2, as the first interactions between the virus and immune system occur primarily on

the mucous membranes such as the nasopharynx and respiratory tracts (Russell *et al.* 2020). Euroimmun has developed an ELISA kit that provides semi-quantitative in vitro determination of IgA against S protein, with high specificity and sensitivity (Beavis *et al.* 2020).

1.4.3 Radiological diagnosis

A chest CT scan is a detailed specific chest X-ray that plays an important role in the clinical diagnosis of pneumonia and other respiratory diseases. The typical CT imaging presentation of COVID-19 is multifocal bilateral ground-glass opacities (GGO), with a peripheral and subpleural distribution (Y. Li *et al.* 2020). However, CT scans may have the disadvantage of low specificity because the manifestations of COVID-19 patients are similar to other viral pneumonia (Ai *et al.* 2020). They can be used as a complement to RT-PCR for the diagnosis of COVID-19 in order to make the results more accurate (Fang *et al.* 2020).

1.5 The control of COVID-19

1.5.1. Antiviral drugs

At the end of 2021, the Food and Drug Administration (FDA) has firstly authorized two oral antiviral drugs, Paxlovid and Molnupiravir, for the treatment of mild to moderate COVID-19 (WHO 2021b). Both showed a significant reduction in the risk of hospitalization and death associated with COVID-19, according to the study results released by their manufacturer (Mahase 2021a, 2021b). Paxlovid is a protease inhibitor antiviral therapy developed by Pfizer, made up of medicine called nirmatrelvir and the HIV drug

ritonavir. Molnupiravir was developed by Ridgeback Biotherapeutics and Merck, which can interfere with the replication of SARS-CoV-2 through the introduction of copying errors. Beyond that, some medications are recommended for patients who have been hospitalized with COVID-19, including dexamethasone (anti-inflammatory drugs), tocilizumab (a monoclonal antibody), remdesivir (an antiviral drug), and baricitinib (a rheumatoid arthritis drug) (Eastman *et al.* 2020; Favalli *et al.* 2020; Lammers *et al.* 2020; S.-H. Lan *et al.* 2020).

1.5.2 Vaccines

Soon after the emergence of the COVID-19 pandemic, multiple labs started the process of creating effective vaccines in order to achieve herd immunity worldwide. According to the COVID-19 vaccine tracker reported by WHO, to date, there are 140 vaccines under development in clinical phase trials while 194 in pre-clinical development, which are developed with different platforms such as protein subunit, inactivated virus, viral vector, DNA, RNA, and live attenuated virus (WHO 2022). The COVID-19 vaccines available in the market currently have been described in Table 2. Data from multiple studies showed a significant decrease in risk of infection of COVID-19 after two doses of the vaccines, with the efficacy of 67%-95%. As of March 2022, billions of doses of vaccines have been administered throughout the world. However, current vaccines may be less effective against infection of SARS-CoV-2 since Omicron was identified in South Africa and has been circulating around the world (Buchan *et al.* 2022).

Table 2 COVID-19 vaccines against SARS-CoV-2 available for use by March 2022 (Ahmad & Shabbiri 2022; Fiolet *et al.* 2021).

Name	Platform	Developer	Dose schedule
Comirnaty	mRNA	Pfizer/BioNTech	Day 0 + 21
Spikevax	mRNA	Moderna	Day 0 + 28
Nuvaxovid	Protein subunit	Novavax	Day 0 + 21
CoronaVac	Inactivated virus	Sinovac	Day 0 + 21
Convidecia	Viral vector (Ad5)	CanSino	Day 0
Vaxzevria	Viral vector(ChAdOx1)	AstraZeneca/ Oxford	Day 0 + 28
Janssen COVID-19 vaccine	Viral vector (Ad26)	Janssen Pharmaceuticals	Day 0
Sputnik V	Viral vector (Ad5, Ad26)	Gamaleya Research Institute	Day 0 + 21

Ad5, Adenovirus type 5; Ad26, Adenovirus type 26; ChAdOx1, Chimpanzee Adenovirus Oxford 1.

2. Modified Vaccinia virus Ankara (MVA) as an expression vector

2.1 MVA generation

MVA is an attenuated virus derived from the Chorioallantois Vaccinia Ankara (CVA) strain of the vaccinia virus. The attenuated strain was renamed MVA in 1968 after the 516th serial passage of CVA strain on primary chicken embryo fibroblasts (CEF) (Stickl & Hochstein-Mintzel 1971). It was tested as a vaccine candidate against smallpox and authorized in Germany in 1977 due to the data from clinical trials (Stickl *et al.* 1974). Until 1980, this first licensed MVA vaccine against smallpox was administered to more than 120,000 people without documentation of severe complications (Mahnel & Mayr 1994). Analysis of MVA genome revealed that, the long-term serial passages of CVA in CEF resulted in a genomic loss of approximately 30 kb (Meyer *et al.* 1991). Six large genomic deletions, totaling more than 24 kb in length, have been

identified, which are located in the left and right terminal regions of the genome (Figure 4). In addition, a multitude of shorter deletions, point mutations and insertions have occurred in the MVA genome, leading to gene truncation, fragmentation, or deletions of ORFs (Antoine *et al.* 1998). Genes affected by the genomic changes include host range gene K1L, genes encoding functional receptors for TNF, IFN-γ, etc., and genes encoding A-type inclusion body protein, and so on (Blanchard *et al.* 1998; Hermanson *et al.* 2012; Meyer *et al.* 1991).

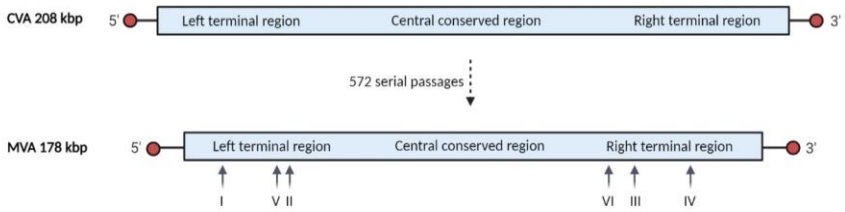

Figure 4 Major genomic change of MVA compared to CVA. The long-term serial passages of CVA in CEF resulted in a genomic loss of approximately 30kb. The locations of six major deletions (I–VI) of MVA relative to the CVA genome were described. The figure was created with biorender.com.

2.2 Viral morphology and life cycle

MVA, a large and complex enveloped virus, is a member of the *Orthopoxvirus* genus within the subfamily *Chordopoxvirinae* and the family *Poxviridae*. *Orthopoxviruses* illustrate the largest genus, including variola virus (now eradicated), vaccinia virus, monkeypox virus, camelpox virus and a range of other mammalian orthopoxviruses (Gubser *et al.* 2004). Within the same

genus, the viruses show genetical, antigenic, host range and morphologic similarity (Condit *et al.* 2006; Hatcher *et al.* 2015).

Poxviruses are brick-shaped, around 250 nm long and 360 nm wide, enveloped by at least one membrane. The core is biconcave in shape and associates with lateral bodies which fill the space between the core and outer membrane (Laliberte *et al.* 2011). The core encloses a linear, double-stranded DNA with 130-300 kb in length. Two distinct infectious virus particles exist: the mature virion (MV) which is the most abundant form with a single outer membrane, and the enveloped virion (EV) which is essentially an MV but additionally possesses a further outer lipid membrane and is antigenically distinct from MV (Hatcher *et al.* 2015; Senkevich *et al.* 2004).

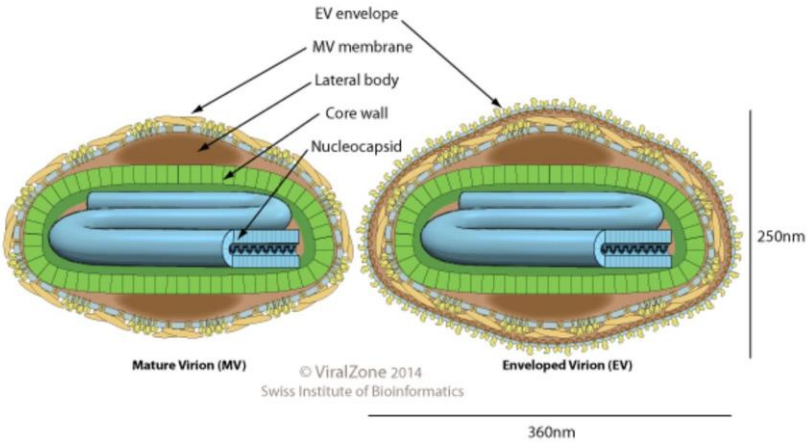

Figure 5 Poxvirus morphology (source: https://viralzone.expasy.org/149, SIB Swiss Institute of Bioinformatics, with permission).

Untypical for DNA viruses, MVA as well as all other members of the Poxviridae family, replicates in the cytoplasmic compartment of the cell (Minnigan & Moyer 1985). The replication cycle starts with the attachment of virus particles to the cell membrane, followed by the fusion or entry of the virus to deliver the core into the cellular cytoplasm (Laliberte & Moss 2010). Within this core, about 100 mRNAs are transcribed by the viral 'early' transcription machinery and extrude into the cytoplasm through pores for translation. Poxviral proteins translated from these early mRNAs are required for core uncoating, genome release, initiation of DNA replication and intermediate transcription (Jones *et al.* 1987; Mercer *et al.* 2012; Schramm & Locker 2005). After the initiation of DNA replication, the transcription and translation of intermediate and late stages occur. Virion assembly begins with the formation of crescent membranes which enlarge within the factories to form immature virions (IVs), and then condense into brick-shaped MVs. MVs can be transported through microtubules mediated trafficking and wrapped by an additional membrane derived from Golgi to form EVs (Moss 2015; Sodeik & Krijnse-Locker 2002). Then, the virions are released by cell lysis, exposed on the cell surface by exocytosis, or propelled into surrounding cells via actin tails (Smith & Law 2004).

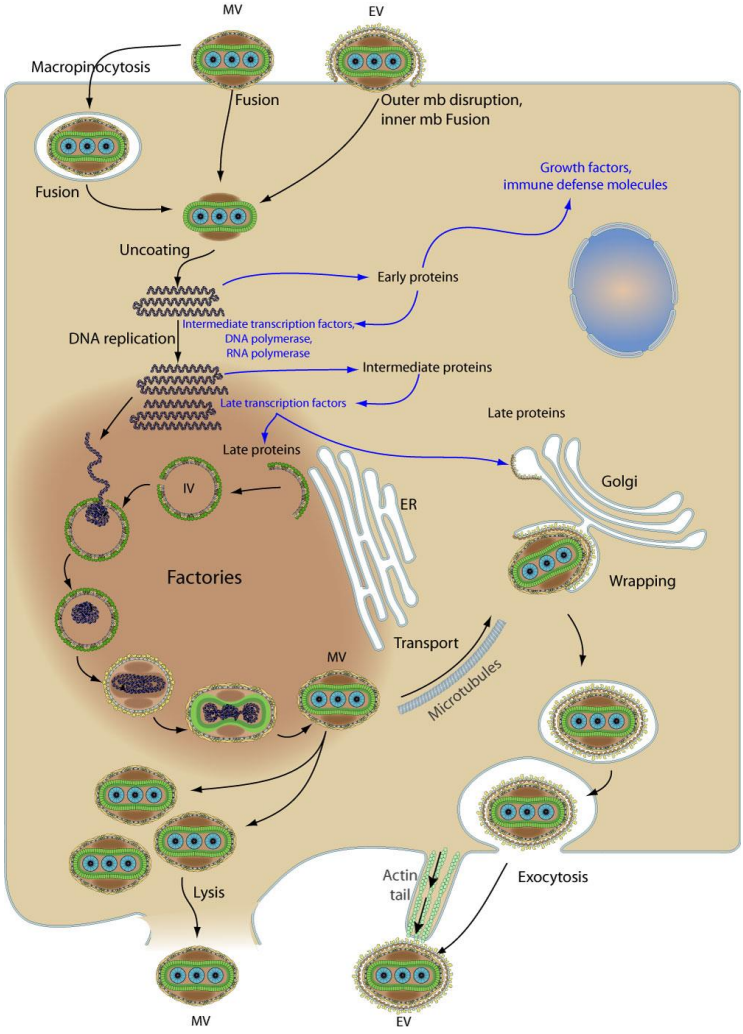

Figure 6 Poxvirus replication cycle (source: https://viralzone.expasy.org/4399, SIB Swiss Institute of Bioinformatics, with permission).

2.3 MVA as an expression vector

The first recombinant MVA was published in 1992. The sequences coding for the *E.coli* enzymes β-galactosidase and guanine phosphoribosyl transferase were inserted at the deletion III site of the MVA genome as the first heterologous genes, and were successfully expressed (Sutter & Moss 1992). The ability to efficiently express viral and recombinant genes encouraged the evaluation of MVA as an expression system. It has been studied as a viral vector for the construction of vaccines against various infections, including influenza (Altenburg *et al.* 2014), MERS (Song *et al.* 2013), SARS (Chen *et al.* 2005) and COVID-19 (Tscherne *et al.* 2021).

In contrast to these recombinant MVAs with stably inserted foreign genes which can be used as vaccine candidates, the MVA-T7pol expression system is a very suitable tool for transient expression of genes of interest (GOI) in cell culture. MVA-T7pol is a recombinant MVA containing the bacteriophage T7 RNA polymerase gene under the control of the natural vaccinia virus early/late promoter P7.5 in deletion II of the MVA genome. The plasmid pUCII LZ T7pol was constructed as a transfer vector to deliver the genes encoding T7 polymerase with promoter P7.5 and *LacZ* (a common reporter in *E.coli*) with promoter P11 to the MVA genome (Fig. 7). Flank 1 and flank 2 are homologous sequences required to integrate the genes into the site of deletion II of MVA genome. Following the generation of MVA-T7pol, synthesis of the T7 RNA polymerase in the cytoplasm of the MVA infected eukaryotic cells can be observed (Sutter *et al.* 1995).

Introduction of plasmids containing the GOI under the control of the T7 promoter into the cytoplasm of the cell and simultaneous infection with the

MVA-T7pol result in the transient expression of the GOI without the need of producing new recombinant viruses. The high efficiency of this expression system has been demonstrated (Sutter *et al.* 1995). The vaccinia virus/T7 RNA polymerase system has been used successfully to analyze the structure or function of viral proteins (Haß *et al.* 2014; Higuchi *et al.* 2016; Mu *et al.* 2021) and recently, the MVA-T7pol system was used to generate a recombinant vesicular stomatitis virus (VSV) in which the glycoprotein of VSV was replaced by the spike protein of SARS-CoV-2 (Yahalom-Ronen *et al.* 2020).

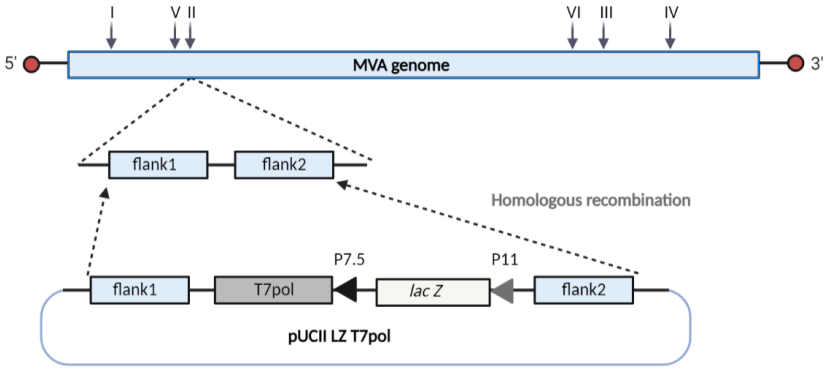

Figure 7 Schematic map of MVA-T7pol generation. Features of MVA with the major deletion sites I-VI are depicted in the diagram. Cassettes for the expression of the genes of T7 RNA polymerase and *E.coli lacZ* were inserted between the MVA flanks via homologous recombination. T7 RNA polymerase gene will be transcribed under the control of vaccinia virus early/late promoter P7.5. *E.coli lacZ* will be transcribed under the control of vaccinia virus late promoter P11. The figure was created with BioRender.com.

IV. MATERIALS AND METHODS

1. Sera samples

Human sera used in this study were kindly provided by the Bundeswehr Institute of Microbiology. The human sera panel included post-infection samples from individuals confirmed positive for SARS-CoV-2 infection in 2020. Negative control sera were obtained from individuals initially suspected for a COVID-19 infection, but negatively tested for SARS-CoV-2. Before use, all human sera were heat-inactivated for 15 min at 56°C.

2. Cells and viruses

CEF cells were isolated from 10-days-old chicken embryos (SPF eggs, VALO, Cuxhaven, Germany) and cultured in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, Taufkirchen, Germany), 1% MEM non-essential amino acid solution (Sigma-Aldrich, Taufkirchen, Germany) and 1% Penicillin–Streptomycin (Sigma-Aldrich, Taufkirchen, Germany). CEF cells were kept at 37 °C in a humidified 5% CO_2 atmosphere.

The recombinant MVA expressing the T7 polymerase (MVA-T7pol) (Figure 8A) and a recombinant MVA expressing the SARS-CoV-2 spike protein (MVA-SARS-2-S) were generated as described previously (Sutter *et al.* 1995; Tscherne *et al.* 2021).

3. Plasmid constructions

According to the manufacturer's recommendations, viral RNA of a SARS-CoV-2 isolate (strain MUC IMB-1) was purified using the QiaAmp Viral RNA extraction kit (Qiagen, Hilden, Germany). Complementary DNA (cDNA) was generated by the SuperScript VILO kit (Life Technologies, Darmstadt, Germany) and was used as a template to amplify the encoding sequence of 7 SARS-CoV-2 target proteins (N, M, E, ORF3a, ORF6, ORF7a, ORF8). A C-terminal hemagglutinin (HA) tag and restriction enzyme recognition sequences were added to each sequence by PCR (Table 3). The PCR products were purified and cloned into pOS6 (Sutter *et al.* 1995) under the control of the T7 promoter (Figure 8B). The colonies were numbered, picked and analyzed with PCR by the primers shown in Table 3. The PCR products were loaded on an agarose gel to determine their size. The positive colonies were cultured in the liquid medium. The plasmids were isolated and submitted for Sanger sequencing to confirm the sequence of the inserts.

Table 3 The sequence of oligonucleotides used for cloning the defined target regions.

Oligonucleotides	Sequence (5´→ 3´)	Amplicon size (bp)	Target protein
N-HA Forward	CAGCCCATGGGGTCTGATAATGGACCCCAAAATCAGCGAAATGCACCCCGCATTACG	1311	N-HA
N-HA Reverse	CTGCTGGTCGACTTATCAAGCGTAATCTGGAACATCGTATGGGTAGGCCTGAGTTGAGT		
E-HA Forward	CAGCCCATGGGGTACTCATTCGTTTCGGAAGAGACAGGTACGTTAATAGTTAATAGCGTA	273	E-HA
E-HA Reverse	CTCGAGTTATCAAGCGTAATCTGGAACATCGTATGGGTAGACCAGAAGATCAGGAACTCT		
M-HA Forward	CGACGAGGATCCATGGCAGATTCCAACGGTACTATTACCGTTGAAGAGCTTAAAAAGCTC	717	M-HA
M-HA Reverse	CTCGAGTTATCAAGCGTAATCTGGAACATCGTATGGGTACTGTACAAGCAAAGCAATATT		
ORF3a-HA Forward	CAGCCCATGGATTTGTTTATGAGAATCTTCACAATTGGAACTGTAACTTTGAAGCAAGGA	870	ORF3a-HA
ORF3a-HA Reverse	CTCGAGTTATCAAGCGTAATCTGGAACATCGTATGGGTACAAAGGCACGCTAGTAGTCGT		
ORF6-HA Forward	CAGCCCATGGGGTTTCATCTCGTTGACTTTCAGGTTACTATAGCAGAGATATTACTAATT	231	ORF6-HA
ORF6-HA Reverse	CTCGAGTTATCAAGCGTAATCTGGAACATCGTATGGGTAATCAATCTCCATTGGTTGCTC		
ORF7a-HA Forward	CAGCCCATGGGGAAAATTATTCTTTTCTTGGCACTGATAACACTCGCTACTTGTGAGCTT	411	ORF7a-HA
ORF7a-HA Reverse	CTCGAGTTATCAAGCGTAATCTGGAACATCGTATGGGTATTCTGTCTTTCTTTTGAGTGTG		
ORF8-HA Forward	CAGCCCATGGGGAAATTTCTTGTTTTCTTAGGAATCATCACAACTGTAGCTGCATTTCAC	411	ORF8-HA
ORF8-HA Reverse	CTCGAGTTATCAAGCGTAATCTGGAACATCGTATGGGTAGATGAAATCTAAAACAACACG		

The restriction enzyme sites are boxed (NcoI: CCATGG; SalI: GTCGAC; XhoI: CTCGAG; BamHI: GGATCC). The HA sequences are underlined.

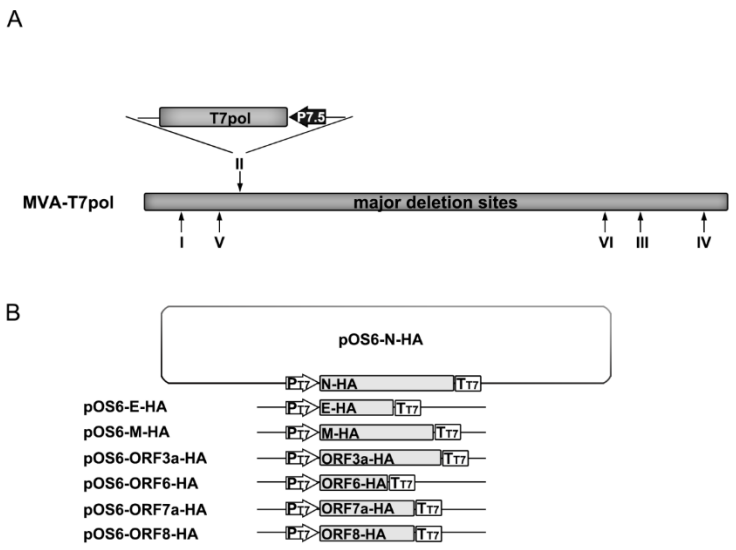

Figure 8 Schematic representation of the transient expression of SARS- CoV-2 proteins using recombinant MVA-T7pol. (A) The T7 polymerase gene under the control of the P7.5 early/late promoter was inserted into MVA deletion site II. (B) The plasmid vector pOS6 is designed to express proteins under the control of T7 promoter. SARS-CoV-2 gene sequences of N-HA, E-HA, M-HA, ORF3a-HA, ORF6-HA, ORF7a-HA and ORF8-HA, were cloned into pOS6. The transient protein expression of SARS-CoV-2 targets occurred when MVA-T7pol infected CEF cells were transfected with a recombinant pOS6. The transcription was initiated at the T7 promoter and stopped at the T7 terminator. P_{T7} , T7 promoter; T_{T7}, T7 terminator.

4. Recombinant protein expression

HA-tagged SARS-CoV-2 spike protein (S-HA) was obtained by infecting 80-90% confluent CEF cells with recombinant MVA-SARS-2-S at a multiplicity of infection (MOI) of 10. After 24h incubation, cells were harvested and lysed with 50 µl lysis buffer/well (1% Triton X-100, 25mM Tris, 1M NaCl) to extract the proteins.

To obtain the other 7 proteins, 6-well tissue culture plates with 80-90% confluent CEF cells were infected with MVA-T7pol at a MOI of 10 and transfected with 1 µg of recombinant pOS6 plasmid using X-tremeGENE HP DNA Transfection Reagent according to the manufacturer's recommendations (Roche Diagnostics, Penzberg, Germany). The lysates were quantified by BCA assay (Thermo Fisher Scientific, Planegg, Germany), aliquoted and stored at -80°C.

5. Immunoblot analysis of recombinant proteins

Successful expression of the eight SARS-CoV-2 target proteins was demonstrated by immunoblot analysis targeting the HA tag. Cell lysates were carefully thawed on ice, mixed with Laemmli sample buffer (Bio-Rad, Feldkirchen, Germany) and subsequently boiled for 5 min, with the exception of M-HA lysate (Lee et al., 2005). Samples were separated by SDS-PAGE and transferred onto nitrocellulose membranes (GE Healthcare, Freiburg, Germany) using a wet transfer system (Bio-Rad, Feldkirchen, Germany). The membranes were blocked with blocking buffer (5 % milk in PBST) for 1 h at room temperature. The membranes were probed with a monoclonal antibody directed against the HA-tag (1:8000 in blocking buffer; HA Tag mAb 2-2.2.14, Thermo Fisher Scientific, Germany) for 1 h at room temperature. The membranes were washed 3 times with PBST and incubated with a goat anti-mouse IgG/HRP (Agilent Dako, Glostrup, Denmark, 1:5000 in blocking buffer) for 1 h at room temperature. After washing 3 times with PBST, the membranes were covered by TrueBlue™ Peroxidase Substrate (SeraCare Life Sciences, Milford, USA).

6. SARS-CoV-2 antibody detection in patient sera by a systematic immunoblot analysis

Western blot analysis was performed to detect SARS-CoV-2 antibodies in different patient sera. Cell lysates were mixed with 4× Laemmli sample buffer and were boiled for 5 min at 95 °C. Electrophoresis of 35 µg protein/lane was performed at 80 V through the stacking gel, followed by separation of the proteins at 120 V for 70 min. The proteins were transferred onto a 4.5 cm×5 cm nitrocellulose membrane (GE Healthcare, Freiburg, Germany) at 100 V for 100 min at 4 °C using a wet transfer system (Bio-Rad, Feldkirchen, Germany). The membranes were blocked with blocking buffer (5 % milk in PBST) for 1 h at room temperature and incubated with human sera (1:200 dilution in 5 ml blocking buffer) at 4 °C for 16 h. After washing 3 times with PBST, the membranes were incubated with rabbit anti-human IgG/HRP (Agilent Dako, Glostrup, Denmark, 1:2000 in blocking buffer) for 1 h at room temperature. Membranes were washed with PBST and covered by 1 ml of TrueBlue™ Peroxidase Substrate (SeraCare Life Sciences, Milford, USA) for 5 min. The membranes were rinsed with dH_2O to stop the reaction.

7. Sera neutralization test (SNT)

NAb titers were determined at the Bundeswehr Institute of Microbiology as previously described (Haselmann *et al.* 2020). Briefly, SARS-CoV-2 (strain MUC IMB-1) was cultured in Vero E6 cells. Sera samples (duplicates), including positive and negative control samples, were serially diluted in 96-well tissue culture plates (Greiner bio-one, Frickenhausen, Germany) in

Minimal Essential Medium (MEM, plus Non-Essential Amino Acids Solution and Antibiotic-Antimycotic Solution; all Invitrogen, Thermo Fisher Scientific, Darmstadt, Germany) starting at 1:5 to a maximum of 1:640. 100 $TCID_{50}$ of the virus was pre-incubated with diluted sera samples for 1 h at 37 °C (5% CO_2) before Vero E6 cells ($1x10^4$ cells/50µl) were added to each well. After 72 h supernatants were discarded, cells were fixed (3% formalin/PBS) and stained with crystal violet (0.1%). The nAb titer corresponded to the reciprocal of the highest sera dilution showing complete inhibition of cytopathic effect (CPE). The result was considered invalid if the variation between duplicates was greater than one titer value. A virus retitration was performed on every plate.

8. ELISA

Anti-SARS-CoV-2 IgG and IgA ELISAs were performed at the Bundeswehr Institute of Microbiology according to the manufacturer's instructions (Euroimmun, Lübeck, Germany), and ratios were calculated correspondingly. Samples were evaluated as either not elevated (Ratio <0.8), indeterminate (0.8 ≤ Ration ≤ 1.1), or elevated (Ratio > 1.1) for both IgA and IgG, respectively, as suggested by the manufacturer.

V. OBJECTIVES

The worldwide spread of SARS-CoV-2 has a great impact in all aspects. The study for expression of SARS-CoV-2 antigens and the analysis of antibody responses directed against these antigens are crucial for the better understanding of immunity directed against this new virus. Therefore, using the expression potential of MVA vectors for COVID-19 proteins and the analysis of antibodies mounted against these proteins in patients, this work describes the following:

(i) Generation of recombinant proteins of SARS-CoV-2 by MVA-T7pol expression system

(ii) Detection and characterization of antibody responses against these SARS-CoV-2 proteins

VI. RESULTS

1. Construction of the transient T7pol expression vectors

Plasmid construction was performed to obtain the recombinant plasmids carrying gene sequences of SARS-CoV-2 proteins. Complementary DNA synthesized from viral RNA (strain MUC IMB-1) was used as the template to amplify the target genes of SARS-CoV-2 with an HA-tag sequence at 3'-end (N-HA, M-HA, E-HA, ORF3a-HA, ORF6-HA, ORF7a-HA and ORF8-HA). As shown in Figure 9, agarose gel electrophoresis showed the target bands with the expected size. The target gene was purified and inserted into pOS6 (Moss *et al.* 1990) under the control of T7 promoter (Fig. 8B). The resistance gene Amp^R on pOS6 was used for the selection of positive clones. Thymidine kinase (tk) gene, encephalomyocarditis (EMC) gene shown on the pOS6 map are not involved in this study.

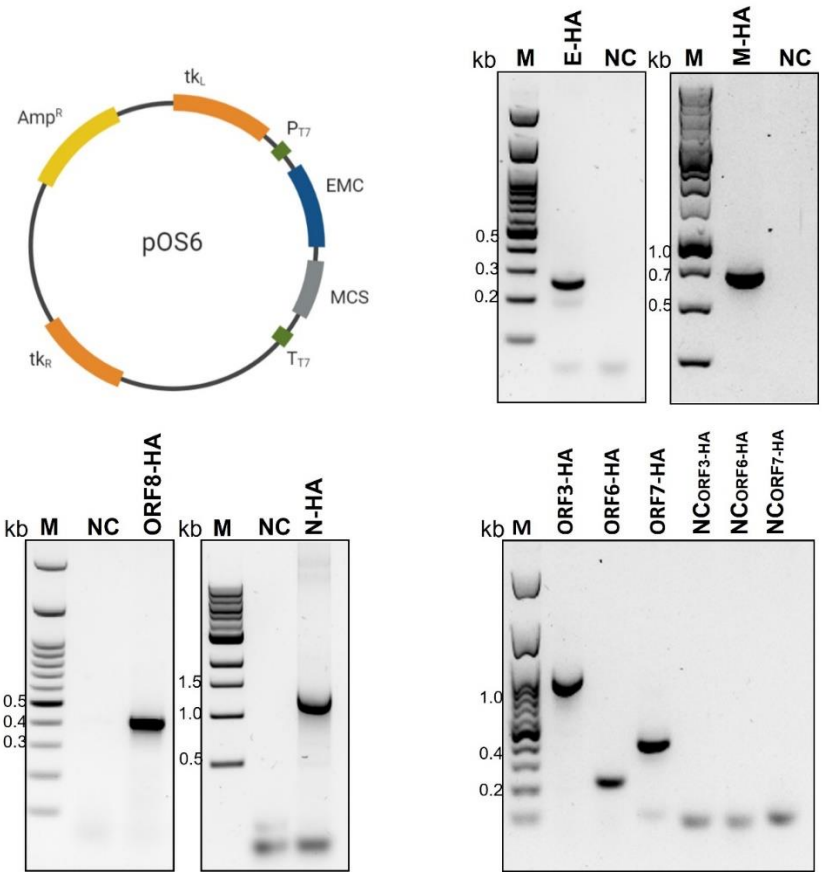

Figure 9 Amplification of SARS-CoV-2 target sequences by PCR. cDNA was generated by reverse transcription of total viral RNA and was used as a template to amplify the encoding sequence of 7 SARS-CoV-2 target proteins. A C-terminal HA tag and restriction enzyme recognition sequences were added to each sequence by PCR. The PCR products were then purified and inserted into MCS of pOS6. NC, non-template control; Amp^R, ampicillin resistant gene; tk_L (left) and tk_R (right), vaccinia virus TK gene segments; PT7, T7 promoter; EMC, encephalomyocarditis virus untranslated leader sequence; T_{T7}, T7 terminator; MCS, multiple cloning site.

Following the ligation of each insert into the linearized pOS6 vector, competent cells were transformed with ligation products. Colonies containing target constructs were identified by PCR with the specific primers shown in Table 3. A positive colony showed a band with expected size, while a negative colony showed no band or a band with unexpected size. The positive colonies showed in Figure 10 were cultured in the liquid medium, including pOS6-E-HA-2, pOS6-M-HA-1, pOS6-M-HA-2, pOS6-M-HA-3, pOS6-ORF3-HA-2, pOS6-ORF3-HA-3, pOS6-ORF6-HA-1, pOS6-ORF7-HA-1, pOS6-ORF8-HA-1, pOS6-N-HA-1. By Sanger sequencing, all inserts were confirmed with correct sequence and orientation (data not shown).

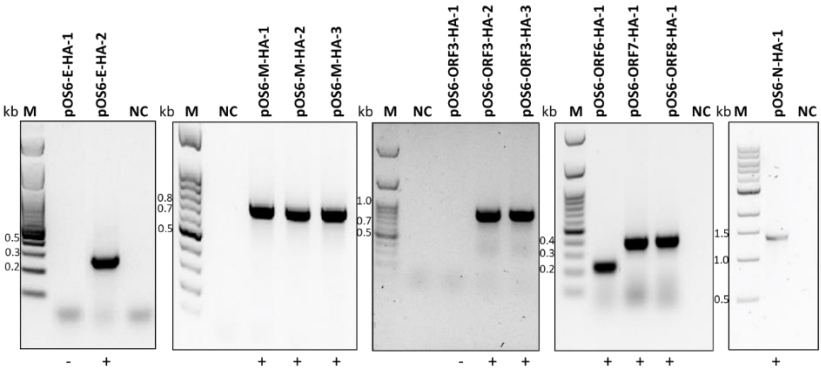

Figure 10 Positive colonies screening with PCR. The colonies were numbered, picked and analyzed with PCR by the primers shown in Table 3. The PCR products were loaded on an agarose gel to determine their size. A positive colony showed a band with expected size, while a negative colony showed no band or a band with unexpected size. The positive colonies were cultured in the liquid medium. The plasmids were isolated and submitted for Sanger sequencing to confirm the sequence of the inserts. –, negative colony; +, positive colony; NC, negative control without any colony.

2. Characterization of recombinant SARS-CoV-2 proteins

The transcription was initiated at the T7 promoter and stopped at the T7 terminator when MVA-T7 pol infected cells were transfected with recombinant plasmids carrying target genes of SARS-CoV-2. Transient expression of SARS-CoV-2 proteins was examined by western blot analysis using standard procedures. However, for the M protein, the method had to be modified, because it always showed a weak signal using standard procedures. The bands of M-HA (~25kDa) without boiling are always sharper than boiled ones, regardless of whether adding β-ME or not (Figure 11). It may imply that the boiling of the samples negatively affected the sensitive detection of the protein, independent of the use of a reducing agent.

HA-tagged SARS-CoV2 S protein was obtained by collecting the lysates from MVA-SARS-2-S infected cells. MVA-SARS-2-S was isolated in repetitive purification using fluorescent marker protein and showed genetic stability (Tscherne *et al.* 2021).

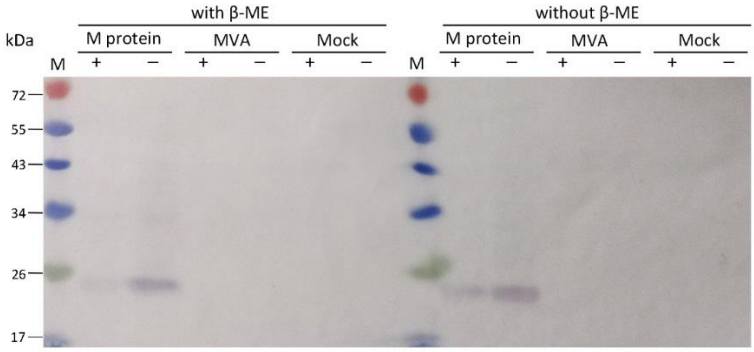

Figure 11 The conditions of sample treatment were explored for enhancing the signal of SARS-CoV-2 M protein. Cell lysates were harvested by lysis buffer and mixed with Laemmli sample buffer supplemented with or without β-ME. The samples were treated at 95°C or room temperature for 5 min. After electrophoresis and transfer, the specific proteins were identified by anti-HA tag antibody. The bands of M-HA (~25kDa) without boiling are always sharper than boiled ones, regardless of whether adding β-ME or not. The arrows may indicate the aggregates of M protein. +: the sample was incubated at 95°C; -: the sample was incubated at room temperature.

In the anti-HA immunoblot, specific bands with the expected size (N-HA ~47 kDa, E-HA ~12 kDa, M-HA ~25 kDa, ORF3a-HA ~34 kDa, ORF6-HA ~11 kDa, ORF7a-HA ~13 kDa and ORF8-HA ~17 kDa) of the SARS-CoV-2 proteins were detected (Figure 12A), indicating the efficient expression of the target proteins using the MVA-T7pol system. Moreover, the spike protein showed two prominent bands with molecular masses of about 190 kDa and 90~100 kDa, which is speculated to represent the glycosylated spike protein and the S2 cleavage product, respectively (Tscherne *et al.* 2021). The cleavage product seemed to be more prominent compared to full-length spike protein.

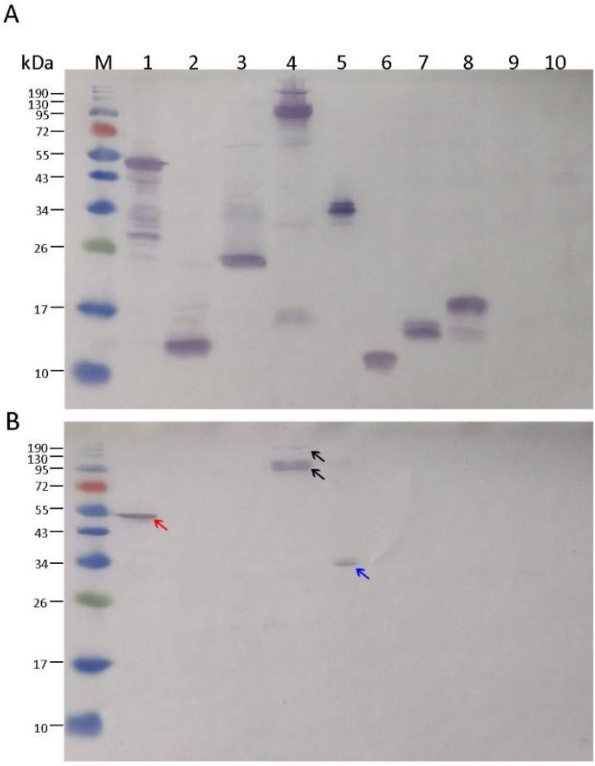

Figure 12 Analysis of SARS-CoV-2 proteins obtained by MVA T7pol expression system. (A) Lysates of N-HA (lane 1), E-HA (lane 2), M-HA (lane 3), S-HA (lane 4), ORF3a-HA (lane 5), ORF6-HA (lane 6), ORF7a-HA (lane 7) and ORF8-HA (lane 8) were separated by SDS-PAGE and analyzed by the western bot using the antibody against HA-tag. Lysates of non-recombinant MVA infected CEF cells (lane 9) and uninfected CEF cells (lane 10) served as control. (B) The lysates were analyzed by western blot using the patient sera (No. 1). Red arrow: N protein; black arrows: S protein and cleavage; blue arrow: ORF3a protein.

Next, human sera from a COVID-19 patient (No. 1, Table 4) with high titers of SARS-CoV-2 specific antibodies was used as the primary antibody for western

blot analysis (Figure 12B). Prominent bands in the samples of N and S proteins corresponded to the molecular masses shown in Figure 12A. The glycosylated full-length spike protein as well as the cleavage product were detected, respectively. Interestingly, also the ORF3a protein was detected. However, when sera of other COVID-19 patients were used, the ORF3a protein could not be detected (data not shown). No specific antibodies against the SARS-CoV-2 proteins M, E, ORF6, ORF7a and ORF8 could be detected in any patient sera.

Table 4 Comparison of western blot analysis with ELISA and SNT for the detection of SARS-CoV-2 specific antibody response.

Patient No.	Western blot[a]	IgG-ELISA	IgA-ELISA	SNT
1	N(++) S(++) ORF3a(+)	positive	positive	>80
2	N(++) S(++)	positive	positive	>80
3	N(++) S(+)	positive	positive	>80
4	N(+) S(+)	positive	positive	40
5	N(+) S(+)	positive	positive	40
6	N(+) S(+)	positive	positive	40
7	N(+) S(-)	negative	negative	negative
8	N(+) S(-)	negative	positive	negative
9	N(-) S(-)	negative	negative	negative

[a] -, negative signal; +, weak positive signal; ++, strong positive signal.

3. Detection of the humoral immune response against SARS-CoV-2

Next, sera were investigated with varying amounts of SARS-CoV-2 nAb, focusing on the presence of S and N specific antibodies in the western blot analysis. We classified 3 groups depending on the sera neutralization titer: group 1 (patient 1-3) >1:80, group 2 (patient 4-6) 1:40 and group 3 (patient 7-9) with negative SNT results (Table 4). Sera from groups 1 and 2 showed positive results in anti-SARS-CoV-2 IgG and IgA ELISAs as well. In group 3 samples were negative in both anti-SARS-CoV-2 ELISAs, except sample 8, which resulted positive in the anti-SARS-CoV-2 IgA ELISA.

As expected in all patients' sera from group 1 and 2, N and S specific antibodies could be detected by demonstrating corresponding specific bands. However, differences in the thickness and color saturation of the bands indicated that the used western blot system might be also useful for semi quantitative analysis, because signals induced by group 1 sera were more prominent compared to group 2 (Figure 13 and 14).

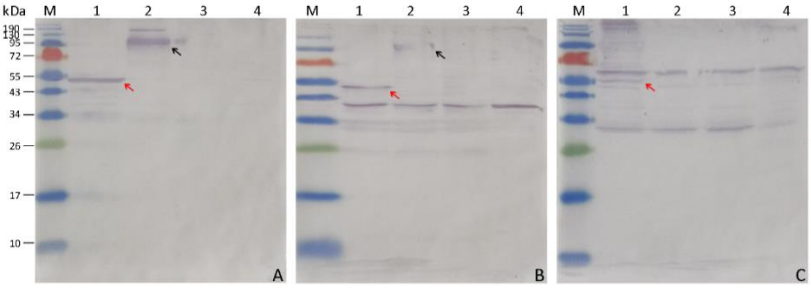

Figure 13 Analysis of SARS-CoV-2 IgG in patient sera using a systematic western blot. Cell lysates of N-HA (lane 1), S-HA (lane 2), non-recombinant MVA (lane 3), and uninfected cells (lane 4) were separated by SDS-PAGE followed by western blot analysis with patient sera. Red arrow: N protein; black arrows: S protein and cleavage. (A) Membrane incubated with sera from patient No.1, N(++) S(++); (B) Membrane incubated with sera from patient No.4, N(+) S(+); (C) Membrane incubated with sera from patient No.8, N(+) S(-). ++: strong positive signal; +: weak positive signal; -: negative signal.

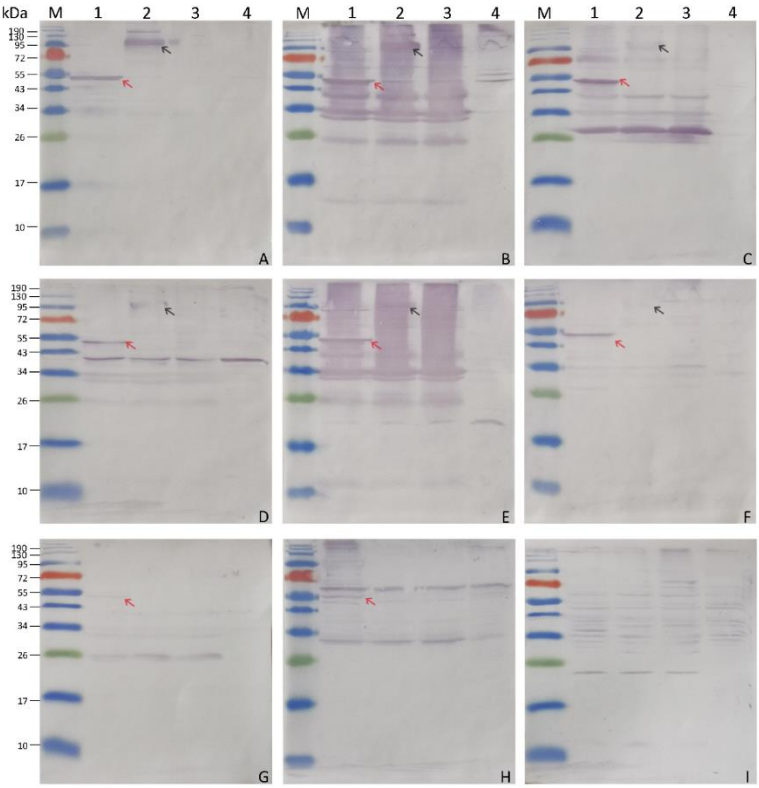

Figure 14 SARS-CoV-2 antibody detection in patient sera by a systematic western blot. Cell lysates of N-HA (lane 1), S-HA (lane 2), non-recombinant MVA (lane 3), and uninfected cells (lane 4) were separated by SDS-PAGE followed by western blot analysis with patient sera. The bands representing the presence of antibodies against N are marked by red arrows; the bands representing the presence of antibodies against S are marked by black arrows. (A) Patient No.1; (B) Patient No.2; (C) Patient No.3; (D) Patient No.4; (E) Patient No.5; (F) Patient No.6; (G) Patient No.7; (H) Patient No.8; (I) Patient No.9.

In sera from group 3, which were tested negative by SNT, no S specific antibodies could be detected. However, in two (patients 7 and 8) of the three samples, a weak but specific N signal was obtained (Table 4, Figures 13 and 14).

VII. DISCUSSION

1. COVID-19 proteins expression with MVA-T7pol system

The replication-deficient virus MVA-T7pol represents a powerful expression system for functional analysis of recombinant genes in the eukaryotic environment. In contrast to existing vaccinia virus/T7pol systems, the lack of viral replication in mammalian cells is an important safety advantage. Moreover, the MVA-T7pol system is able to produce high amounts of target proteins, and in contrast to recombinant MVAs, there is no need for time requiring construction and isolation of virus recombinants (Hebben *et al.* 2007; Pradeau-Aubreton *et al.* 2010).

The plasmid, pOS6, used for expressing COVID-19 genes under T7 promoter, is derived from pTM1 (Moss *et al.* 1990) which is a pUC derived plasmid containing T7 promoter, EMCV UTR, MCS, T7 terminator and tk genes leading to homologous recombination. A previous study reported that chloramphenicol acetyltransferase (CAT) gene cloned into pOS6 can be expressed efficiently in HeLa cells (Sutter *et al.* 1995). The promoters P7.5, PmH5, and P11 are very frequently used in recombinant MVA vectors. In MVA-T7pol system, we used the early and late P7.5 promoter which is able to express antigens during both the early and late stages of vaccinia virus (Alharbi 2018). Under the control of P7.5, transcription of T7 RNA polymerase is induced after viral entry and maintained during the complete cycle of MVA gene expression. Subsequently, T7 polymerase is synthesized and immediately identifies T7 promoter when recombinant plasmids were

transfected into cells (Sutter *et al.* 1995). These are beneficial for the rapid and efficient expression of foreign proteins.

In the present study, MVA-T7pol system was used for the generation of SARS-CoV-2 proteins. Besides the structural proteins of the coronavirus (N, E, M, S), the non-structural proteins encoded by ORF3a, ORF6, ORF7 and ORF8 were also investigated. To verify the correct expression of the virus proteins and because antibodies were not available for most of the investigated proteins, we used HA-tags added at the C-terminal part of proteins. Additionally, this allowed balancing the amount of used protein preparation in order to obtain comparable signals in the immune blot analysis. After 24 hours of infection/transfection, the target proteins could be successfully and easily detected by western blot. These results indicate that the recombinant vector MVA-T7pol can be used very efficiently for the expression of COVID-19 target proteins in CEF cells.

When analyzing the antigens expressed in this study, M protein always showed a weak signal when detected by western blot with boiling treatment. This may indicate that SARS-CoV-2 M protein represents a similar thermal aggregation characteristic as described for the SARS-CoV-1 M protein (Lee *et al.* 2005). For S protein, two detected bands migrating at molecular masses of about 190 kDa and 90~100 kDa were identified as full-length spike protein and cleavage, and their glycosylation was confirmed and demonstrated in the study (Tscherne *et al.* 2021).

2. Detection of the humoral immune response against SARS-CoV-2

Major targets of the anti-coronavirus immune response are the spike protein as well as the nucleocapsid protein. This has been shown also for SARS-CoV-1 (Meyer *et al.* 2014; Tan *et al.* 2004; Woo *et al.* 2004) as well as for SARS-CoV-2 (Brouwer *et al.* 2020; Camerini *et al.* 2021; Guo *et al.* 2020; Long *et al.* 2020).

In our experiments, N specific signals were always clearly visualized faster than other proteins when incubating with patient sera (data not shown). Moreover, in two of the sera (patients 7 and 8) N specific antibodies were detectable, but not S specific antibodies. This may indicate a higher sensitivity of N-based serological assays as others discussed it for SARS-CoV-1 (Leung *et al.* 2004) and SARS-CoV-2 (Burbelo *et al.* 2020) previously. For SARS-CoV-1 and other HCoVs it has been shown that anti-N antibodies appear earlier than anti-S antibodies (Meyer *et al.* 2014; Tan *et al.* 2004; Wu *et al.* 2004). The same has been described for COVID-19 patients (Alfego *et al.* 2021; Elslande *et al.* 2020). Some earlier studies pointed out that serological assays using the full-length N protein might demonstrate a higher rate of false-positive results, because SARS-CoV-2 N contains conserved regions similar to other human coronavirus N proteins (Okba *et al.* 2020; Yamaoka *et al.* 2020). In contrast, in a study performed by Elslande and colleagues, assays detecting anti-N antibodies demonstrated higher specificity (Elslande *et al.* 2020). However, because of the limited number of tested SNT negative sera, a conclusion regarding the specificity of our test system is not possible.

Results from SNT, quantifying neutralizing antibodies, correlated well with the ELISA results (S specific IgA and IgG) as well as with the results from the S specific western blot analysis. Therefore, detection of S-binding antibodies by immunoblot might have potential value as an indicator of a neutralizing Ab response, as discussed by others before (Dispinseri *et al.* 2021). Secretory IgA plays a crucial role in the immune defense of mucosal surfaces, which is the first point of entry of SARS-CoV-2 (Chao *et al.* 2020). The No. 8 sample with positive anti-S IgA and negative anti-S IgG may be due to the earlier appearance or lower specificity of SARS-CoV-2 IgA antibody (Jääskeläinen *et al.* 2020).

For SARS-CoV-1 as well as for SARS-CoV-2, it has been demonstrated that apart from the four structural proteins, accessory proteins might be incorporated into virions, inducing a specific immune response in patients (Hachim *et al.* 2020; Leung *et al.* 2004; Meyer *et al.* 2014; Neuman *et al.* 2008).

Zeng and colleagues reported that SARS-CoV-1 patients possess antibodies against ORF3a (Zeng *et al.* 2004), indicating that ORF3a is a minor structural protein on the surface of the viral envelope, and others considered here a potential target for vaccines or therapeutics (B. Lu *et al.* 2009). For SARS-CoV-2, it has been recently shown that the ORF3a promotes lysosomal exocytosis by promoting lysosomal targeting of the BORC-ARL8b complex and exocytosis-related SNARE proteins (Chen *et al.* 2021). Furthermore, Wang and colleagues demonstrated that ORF3a dampens IFN signaling via upregulating suppressor of cytokine signaling 1 (Wang *et al.* 2021). In our study, one COVID-19 patient clearly demonstrated ORF3a specific antibodies,

confirming the results of Camerini and colleagues, who detected SARS-CoV-2 ORF3a specific antibodies using a multi-coronavirus protein microarray (Camerini *et al.* 2021). However, because the antibodies were only detectable in a sera sample with the highest SNT titers, the value of this finding has to emerge.

3. The use of western blot for COVID-19 serology

Since the outbreak of COVID-19 pandemic, more and more assays have been developed for the diagnosis of SARS-CoV-2 infection in humans. This study describes the serological diagnosis of COVID-19 by western blot with recombinant proteins expressed with MVA-T7pol system. Serological assays based on recombinant antigens are widely used in laboratory diagnostics, especially for BSL3 viruses as SARS-CoV-1 and SARS-CoV-2. This has the advantage that no BSL3 containment is needed as it is for virus-based serological assays (Meyer *et al.* 2014). However, technologies for antibody detection like immunofluorescence assay (IFA) need to be processed in a BSL-3 laboratory and well-trained technicians.

Western blot is based on antigen-antibody response. Antibodies in the patient's sera will bind to specific viral proteins on the membrane, thus showing discrete bands depending on their molecular weight. When analyzing humoral immune responses in COVID-19 patients, the western blot technique helps to identify immunogenic antigens that may be used in vaccine development and serologic testing (Haveri *et al.* 2020).

In addition to many in-house-developed western blot systems, several companies are producing commercial kits for serological diagnosis of different infectious diseases. The important application area is that western blot was used in serological test to ensure the accuracy of an initial screening test for HIV infection and Lyme disease (Pavia & Wormser 2020). Due to its high level of sensitivity in identifying the key antigens, the value of this approach for COVID-19 diagnosis could also be the confirmation of a positive or a borderline-positive result from a screening test like ELISA and IFA. As most of the current vaccines are based on S antigen, simultaneous detection of antibodies against N and S protein may greatly help identify SARS-CoV-2 natural infection in vaccinated people. This also demonstrated the possibility of developing a chip for testing antibodies against multi-antigens based on western blot technology.

VIII. SUMMARY

SARS-CoV-2, the causative agent of COVID-19, was first detected in December 2019 in Wuhan (China) and spread throughout the world by travelers and community-based contacts, leading to a global pandemic within a few months. The research for the expression of the antigens and the analysis of humoral immunity is crucial for preventing the virus.

Within this study, the MVA-T7pol system was utilized for the generation and characterization of SARS-CoV-2 proteins. MVA-T7pol infected cells were transfected with the recombinant plasmids carrying target genes of SARS-CoV-2 with an HA-tag sequence at 3'-end (N-HA, M-HA, E-HA, ORF3a-HA, ORF6-HA, ORF7a-HA and ORF8-HA). In this process, T7 polymerase can be synthesized during MVA-T7 replicating in the host cell and binds the T7 promoter on the plasmids to start the transcription of SARS-CoV-2 target genes. The expression of SARS-CoV-2 proteins was examined by western blot analysis using standard procedures, and specific bands with the expected size of the SARS-CoV-2 proteins were detected clearly. The result indicated that the MVA-T7pol system may serve as a highly efficient and safe tool for COVID-19 protein expression.

Next, sera were investigated with varying amounts of SARS-CoV-2 nAb, focusing on the presence of S and N specific antibodies in the western blot analysis. For S specific humoral response, results from SNT, quantifying neutralizing antibodies, correlated well with the ELISA results (S specific IgG) and S specific western blot analysis. Therefore, detection of S-binding

antibodies by immunoblot might have potential value as an indicator of a neutralizing Ab response. N specific antibodies were detectable in two of the sera, but not S specific antibodies. This may indicate higher sensitivity of N-based serological assays.

This study initiated in response to COVID-19 may serve as an example for the rapid and efficient expression of antigens from an emerging virus, and the use of these antigens to analyze pathogen-specific immune responses.

IX. ZUSAMMENFASSUNG

SARS-CoV-2, der Erreger von COVID-19, wurde erstmals im Dezember 2019 in Wuhan (China) nachgewiesen. Durch die Infrastrukturen der globalisierten Welt verbreitete sich der Erreger schnell und effizient, was innerhalb weniger Monate zu einer Pandemie bisher unbekannter Dimension führte. Zur Bekämpfung von COVID-19, insbesondere zur Entwicklung effizienter Strategien zur Therapie- und Prophylaxe, ist die Erforschung der antigenetischen Strukturen von SARS-CoV-2 und die Analyse der durch das Virus induzierten Immunität von besonderer Bedeutung.

In dieser Arbeit wurde das MVA-T7pol-System für die Herstellung von SARS-CoV-2-Proteinen verwendet. Hierbei werden MVA-T7pol-infizierte Zellen mit rekombinanten Plasmiden transfiziert, die die HA-getaggten Zielgene von SARS-CoV-2 (N-HA, M-HA, E-HA, ORF3a-HA, ORF6-HA, ORF7a-HA und ORF8-HA) beinhalten. Während der Transkription und Replikation des MVA-T7pol in der Wirtszelle wird effizient die T7-Polymerase synthetisiert, welche den T7-Promotor vor den Zielgenen in der Plasmidsequenz erkennt, und somit die Transkription der SARS-CoV-2-Zielgene induziert. Die Expression von SARS-CoV-2-Proteinen wurde mittels standardisierten Western-Blot-Analysen untersucht. Spezifische Banden mit der erwarteten Größe der SARS-CoV-2-Proteine konnten in allen Fällen nachgewiesen, was die effiziente und sichere Expression von SARS-CoV-2-Proteinen durch das MVA-T7pol-System zeigte.

Anschließend wurden die exprimierten SARS-CoV-2-Antigene verwendet, um COVID-19-Patientenseren zu untersuchen. Diese Seren wurden zunächst

mittels Serumneutralisationstest und Antikörper-ELISA charakterisiert, dann die Immunantwort gegen die MVA-T7pol exprimierten SARS-CoV-2-Antigene mittels Immunoblot-Analyse untersucht. Hiermit konnten Antikörper gegen das Spikeprotein, das Nukleokapsidprotein und das ORF3a-Protein nachgewiesen werden, wobei S- und N-spezifischen Antikörper dominierten. Bei der S-spezifischen Immunantwort korrelierten die Ergebnisse des SNT gut mit den ELISA-Ergebnissen (S-spezifisches IgG) und der S-spezifischen Western-Blot-Analyse. Daher könnte der Nachweis von S-bindenden Antikörpern durch Immunoblot oder ELISA einen potenziellen Wert als Indikator für eine neutralisierende Antikörperreaktion haben. In zwei der Seren waren N-spezifische Antikörper nachweisbar, aber keine S-spezifischen Antikörper. Dies könnte auf eine höhere Empfindlichkeit von N-basierten serologischen Tests hinweisen.

In dieser Arbeit konnte ein effizientes Werkzeug (MVA-T7pol) zur heterologen Expression von SARS-CoV-2-Antigene genutzt werden, welche erste Einblicke in humorale Immunantwort gegen das SARS-CoV-2 ermöglichte. Die im Rahmen der COVID-19 Pandemie initiierte Studie kann als Beispiel dienen für die schnelle und effiziente Expression von Antigenen eines bisher unbekannten Virus und die Verwendung dieser Antigene zur Analyse erregerspezifischer Immunantworten.

X. REFERENCES

Ahmad, W. & Shabbiri, K. (2022) Two years of SARS-CoV-2 infection (2019–2021): structural biology, vaccination, and current global situation. *The Egyptian Journal of Internal Medicine* 34, 5

Ai, T., Yang, Z., Hou, H., *et al.* (2020) Correlation of Chest CT and RT-PCR Testing in Coronavirus Disease 2019 (COVID-19) in China: A Report of 1014 Cases. *Radiology* 296, 200642

Alfego, D., Sullivan, A., Poirier, B., *et al.* (2021) A population-based analysis of the longevity of SARS-CoV-2 antibody seropositivity in the United States. *EClinicalMedicine* 36, 100902

Alharbi, N.K. (2018) Poxviral promoters for improving the immunogenicity of MVA delivered vaccines. *Human Vaccines & Immunotherapeutics* 15, 203–209

Al-Qaaneh, A.M., Alshammari, T., Aldahhan, R., *et al.* (2021) Genome composition and genetic characterization of SARS-CoV-2. *Saudi Journal of Biological Sciences* 28, 1978–1989

Alsaadi, E.A.J. & Jones, I.M. (2019) Membrane binding proteins of coronaviruses. *Future Virology* 14, 275–286

Altenburg, A.F., Kreijtz, J.H.C.M., Vries, R.D. de, *et al.* (2014) Modified Vaccinia Virus Ankara (MVA) as Production Platform for Vaccines against Influenza and Other Viral Respiratory Diseases. *Viruses* 6, 2735–2761

An, P., Chen, H., Jiang, X., *et al.* (2020) Clinical Features of 2019 Novel Coronavirus Pneumonia Presented Gastrointestinal Symptoms But Without Fever Onset. *SSRN Electronic Journal*

Anthony, S.J., Gilardi, K., Menachery, V.D., *et al.* (2017) Further Evidence for Bats as the Evolutionary Source of Middle East Respiratory Syndrome Coronavirus. *MBio* 8, e00373-17

Antoine, G., Scheiflinger, F., Dorner, F., *et al.* (1998) The Complete Genomic Sequence of the Modified Vaccinia Ankara Strain: Comparison with Other Orthopoxviruses. *Virology* 244, 365–396

Bai, Y., Yao, L., Wei, T., *et al.* (2020) Presumed Asymptomatic Carrier Transmission of COVID-19. *JAMA* 323, 1406–1407

Bar-On, Y.M., Flamholz, A., Phillips, R., *et al.* (2020) SARS-CoV-2 (COVID-19) by the numbers. *ELife* 9, e57309

Beaudette, F.R. (1937) Infectious Laryngotracheitis. *Poultry Science* 16, 103–105

Beavis, K.G., Matushek, S.M., Abeleda, A.P.F., *et al.* (2020) Evaluation of the EUROIMMUN Anti-SARS-CoV-2 ELISA Assay for detection of IgA and IgG antibodies. *Journal of Clinical Virology* 129, 104468–104468

Bianchi, M., Benvenuto, D., Giovanetti, M., *et al.* (2020) Sars-CoV-2 Envelope and Membrane Proteins: Structural Differences Linked to Virus Characteristics? *BioMed Research International* 2020, 4389089

Blanchard, T.J., Alcami, A., Andrea, P., *et al.* (1998) Modified vaccinia virus Ankara undergoes limited replication in human cells and lacks several immunomodulatory proteins: implications for use as a human vaccine. *Journal of General Virology* 79, 1159–1167

Born, J.L. (1998) Analytical Chemistry Edited by R. Kellner, J.-M. Mermet, M. Otto, and H. M. Widmer. Wiley-VCH, New York. 1998. xxv + 916 pp. 21.5 × 30 cm. ISBN 3-527-286101. $84.95. *Journal of Medicinal Chemistry* 41, 4425–4425

Bradley, B.T., Bryan, A., Fink, S.L., *et al.* (2021) Anti-SARS-CoV-2 Antibody Levels Measured by the AdviseDx SARS-CoV-2 Assay Are Concordant with Previously Available Serologic Assays but Are Not Fully Predictive of Sterilizing Immunity. *Journal of Clinical Microbiology* 59, e00989-21

Brouwer, P.J.M., Caniels, T.G., Straten, K. van der, *et al.* (2020) Potent neutralizing antibodies from COVID-19 patients define multiple targets of vulnerability. *Science* 369, 643–650

Buchan, S.A., Chung, H., Brown, K.A., *et al.* (2022) Effectiveness of COVID-19 vaccines against Omicron or Delta infection. *MedRxiv* 2021.12.30.21268565

Burbelo, P.D., Riedo, F.X., Morishima, C., *et al.* (2020) Detection of Nucleocapsid Antibody to SARS-CoV-2 is More Sensitive than Antibody to Spike Protein in COVID-19 Patients. *MedRxiv* 2020.04.20.20071423

Camerini, D., Randall, A.Z., Trappl-Kimmons, K., *et al.* (2021) Mapping SARS-CoV-2 Antibody Epitopes in COVID-19 Patients with a Multi-Coronavirus Protein Microarray. *Microbiology Spectrum* 9, e01416-21

Chao, Y.X., Rötzschke, O. & Tan, E.-K. (2020) The role of IgA in COVID-19. *Brain, Behavior, and Immunity* 87, 182–183

Chen, D., Zheng, Q., Sun, L., *et al.* (2021) ORF3a of SARS-CoV-2 promotes lysosomal exocytosis-mediated viral egress. *Developmental Cell* 56, 3250-3263.e5

Chen, Z., Zhang, L., Qin, C., *et al.* (2005) Recombinant Modified Vaccinia Virus Ankara Expressing the Spike Glycoprotein of Severe Acute Respiratory Syndrome Coronavirus Induces Protective Neutralizing Antibodies Primarily Targeting the Receptor Binding Region. *Journal of Virology* 79, 2678–2688

Cheng, Y., Luo, R., Wang, K., *et al.* (2020) Kidney disease is associated with in-hospital death of patients with COVID-19. *Kidney International* 97, 829–838

Ciotti, M., Maurici, M., Pieri, M., *et al.* (2021) Performance of a rapid antigen test in the diagnosis of SARS-CoV-2 infection. *Journal of Medical Virology* 93, 2988–2991

Clerkin, K.J., Fried, J.A., Raikhelkar, J., *et al.* (2020) Coronavirus Disease 2019 (COVID-19) and Cardiovascular Disease. *Circulation* 141, 1648–1655

Condit, R.C., Moussatche, N. & Traktman, P. (2006) In A Nutshell: Structure and Assembly of the Vaccinia Virion. *Advances in Virus Research* 66, 31–124

Coutard, B., Valle, C., Lamballerie, X. de, *et al.* (2020) The spike glycoprotein of the new coronavirus 2019-nCoV contains a furin-like cleavage site absent in CoV of the same clade. *Antiviral Research* 176, 104742–104742

Dispinseri, S., Secchi, M., Pirillo, M.F., *et al.* (2021) Neutralizing antibody responses to SARS-CoV-2 in symptomatic COVID-19 is persistent and critical for survival. *Nature Communications* 12, 2670

DOYLE, L.P. & HUTCHINGS, L.M. (1946) A transmissible gastroenteritis in pigs. *Journal of the American Veterinary Medical Association* 108, 257–9

Drosten, C., Günther, S., Preiser, W., *et al.* (2003) Identification of a Novel Coronavirus in Patients with Severe Acute Respiratory Syndrome. *The New England Journal of Medicine* 348, 1967–1976

Eastman, R.T., Roth, J.S., Brimacombe, K.R., *et al.* (2020) Remdesivir: A Review of Its Discovery and Development Leading to Emergency Use Authorization for Treatment of COVID-19. *ACS Central Science* 6, 672–683

Elslande, J.V., Decru, B., Jonckheere, S., *et al.* (2020) Antibody response against SARS-CoV-2 spike protein and nucleoprotein evaluated by four automated immunoassays and three ELISAs. *Clinical Microbiology and Infection* 26, 1557.e1-1557.e7

Fan, Y., Zhao, K., Shi, Z.-L., *et al.* (2019) Bat Coronaviruses in China. *Viruses* 11, 210

Fang, Y., Zhang, H., Xie, J., *et al.* (2020) Sensitivity of Chest CT for COVID-19: Comparison to RT-PCR. *Radiology* 296, 200432

Favalli, E.G., Biggioggero, M., Maioli, G., *et al.* (2020) Baricitinib for COVID-19: a suitable treatment? *The Lancet. Infectious Diseases* 20, 1012–1013

Feng, D., Vlas, S.J.D., Fang, L., *et al.* (2009) The SARS epidemic in mainland China: bringing together all epidemiological data. *Tropical Medicine & International Health* 14, 4–13

Fiolet, T., Kherabi, Y., MacDonald, C.-J., *et al.* (2021) Comparing COVID-19 vaccines for their characteristics, efficacy and effectiveness against SARS-CoV-2 and variants of concern: a narrative review. *Clinical Microbiology and Infection*

Gao, Z., Xu, Y., Sun, C., *et al.* (2021) A systematic review of asymptomatic infections with COVID-19. *Journal of Microbiology, Immunology, and Infection* 54, 12–16

Gubser, C., Hué, S., Kellam, P., *et al.* (2004) Poxvirus genomes: a phylogenetic analysis. *Journal of General Virology* 85, 105–117

Guo, L., Ren, L., Yang, S., *et al.* (2020) Profiling Early Humoral Response to Diagnose Novel Coronavirus Disease (COVID-19). *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America* 71, ciaa310

Haagmans, B.L., Dhahiry, S.H.S.A., Reusken, C.B.E.M., *et al.* (2014) Middle East respiratory syndrome coronavirus in dromedary camels: an outbreak investigation. *The Lancet Infectious Diseases* 14, 140–145

Hachim, A., Kavian, N., Cohen, C.A., *et al.* (2020) ORF8 and ORF3b antibodies are accurate serological markers of early and late SARS-CoV-2 infection. *Nature Immunology* 21, 1293–1301

Han, H.-J., Yu, H. & Yu, X.-J. (2016) Evidence for zoonotic origins of Middle East respiratory syndrome coronavirus. *Journal of General Virology* 97, 274–280

Haselmann, V., Özçürümez, M.K., Klawonn, F., *et al.* (2020) Results of the first pilot external quality assessment (EQA) scheme for anti-SARS-CoV2-antibody testing. *Clinical Chemistry and Laboratory Medicine (CCLM)* 58, 2121–2130

Haß, M., Luttermann, C. & Meyers, G. (2014) Feline Calicivirus Can Tolerate Gross Changes of Its Minor Capsid Protein Expression Levels Induced by Changing Translation Reinitiation Frequency or Use of a Separate VP2-Coding mRNA. *PLoS ONE* 9, e102254

Hatcher, E.L., Wang, C. & Lefkowitz, E.J. (2015) Genome Variability and Gene Content in Chordopoxviruses: Dependence on Microsatellites. *Viruses* 7, 2126–2146

Haveri, A., Smura, T., Kuivanen, S., *et al.* (2020) Serological and molecular findings during SARS-CoV-2 infection: the first case study in Finland, January to February 2020. *Eurosurveillance* 25, 2000266

He, J., Xu, R., Yu, D., *et al.* (2003) [Severe acute respiratory syndrome in Guangdong Province of China: epidemiology and control measures]. *Zhonghua yu fang yi xue za zhi [Chinese journal of preventive medicine]* 37, 227–32

Hebben, M., Brants, J., Birck, C., *et al.* (2007) High level protein expression in mammalian cells using a safe viral vector: Modified vaccinia virus Ankara. *Protein Expression and Purification* 56, 269–278

Helmy, Y.A., Fawzy, M., Elaswad, A., *et al.* (2020) The COVID-19 Pandemic: A Comprehensive Review of Taxonomy, Genetics, Epidemiology, Diagnosis, Treatment, and Control. *Journal of Clinical Medicine* 9, 1225

Hemida, M.G., Perera, R.A., Wang, P., *et al.* (2013) Middle East Respiratory Syndrome (MERS) coronavirus seroprevalence in domestic livestock in Saudi Arabia, 2010 to 2013. *Eurosurveillance* 18

Hermanson, G., Chun, S., Felgner, J., *et al.* (2012) Measurement of antibody responses to Modified Vaccinia virus Ankara (MVA) and Dryvax® using proteome microarrays and development of recombinant protein ELISAs. *Vaccine* 30, 614–625

Higuchi, A., Toriniwa, H., Komiya, T., *et al.* (2016) Recombinant Measles AIK-C Vaccine Strain Expressing the prM-E Antigen of Japanese Encephalitis Virus. *PLoS ONE* 11, e0150213

HUI, D.S., WONG, P. & WANG, C. (2003) SARS: clinical features and diagnosis. *Respirology (Carlton, Vic.)* 8, S20–S24

Jääskeläinen, A.J., Kekäläinen, E., Kallio-Kokko, H., *et al.* (2020) Evaluation of commercial and automated SARS-CoV-2 IgG and IgA ELISAs using coronavirus disease (COVID-19) patient samples. *Eurosurveillance* 25, 2000603

JHU (2022) COVID-19 Dashboard by the Center for Systems Science and Engineering (CSSE) at Johns Hopkins University. *Available online at https://coronavirus.jhu.edu/map.html, updated on March 6, 2022.*

Jones, E.V., Puckett, C. & Moss, B. (1987) DNA-dependent RNA polymerase subunits encoded within the vaccinia virus genome. *Journal of virology* 61, 1765–71

Kern, D.M., Sorum, B., Mali, S.S., *et al.* (2021) Cryo-EM structure of the SARS-CoV-2 3a ion channel in lipid nanodiscs. *BioRxiv* 2020.06.17.156554

Laliberte, J.P. & Moss, B. (2010) Lipid Membranes in Poxvirus Replication. *Viruses* 2, 972–986

Laliberte, J.P., Weisberg, A.S. & Moss, B. (2011) The Membrane Fusion Step of Vaccinia Virus Entry Is Cooperatively Mediated by Multiple Viral Proteins and Host Cell Components. *PLoS Pathogens* 7, e1002446

Lammers, T., Sofias, A.M., Meel, R. van der, *et al.* (2020) Dexamethasone nanomedicines for COVID-19. *Nature Nanotechnology* 15, 622–624

Lan, J., Ge, J., Yu, J., *et al.* (2020) Structure of the SARS-CoV-2 spike receptor-binding domain bound to the ACE2 receptor. *Nature* 581, 215–220

Lan, S.-H., Lai, C.-C., Huang, H.-T., *et al.* (2020) Tocilizumab for severe COVID-19: a systematic review and meta-analysis. *International Journal of Antimicrobial Agents* 56, 106103–106103

Lee, Y.-N., Chen, L.-K., Ma, H.-C., *et al.* (2005) Thermal aggregation of SARS-CoV membrane protein. *Journal of Virological Methods* 129, 152–161

Leung, D.T.M., Hang, T.F.C., Hung, M.C., *et al.* (2004) Antibody Response of Patients with Severe Acute Respiratory Syndrome (SARS) Targets the Viral Nucleocapsid. *The Journal of Infectious Diseases* 190, 379–386

Li, Q., Guan, X., Wu, P., *et al.* (2020) Early Transmission Dynamics in Wuhan, China, of Novel Coronavirus–Infected Pneumonia. *New England Journal of Medicine*

Li, Y., Cao, J., Zhang, X., *et al.* (2020) Chest CT imaging characteristics of COVID-19 pneumonia in preschool children: a retrospective study. *BMC Pediatrics* 20, 227

Logue, J.K., Franko, N.M., McCulloch, D.J., *et al.* (2021) Sequelae in Adults at 6 Months After COVID-19 Infection. *JAMA Network Open* 4, e210830

Long, Q.-X., Liu, B.-Z., Deng, H.-J., *et al.* (2020) Antibody responses to SARS-CoV-2 in patients with COVID-19. *Nature Medicine* 26, 845–848

Lu, B., Tao, L., Wang, T., *et al.* (2009) Humoral and Cellular Immune Responses Induced by 3a DNA Vaccines against Severe Acute Respiratory Syndrome (SARS) or SARS-Like Coronavirus in Mice ▽ . *Clinical and Vaccine Immunology* 16, 73–77

Lu, W., Xu, K. & Sun, B. (2009) SARS Accessory Proteins ORF3a and 9b and Their Functional Analysis. *Molecular Biology of the SARS-Coronavirus* 167–175

Mackay, I.M. & Arden, K.E. (2015) MERS coronavirus: diagnostics, epidemiology and transmission. *Virology Journal* 12, 222

Mahase, E. (2021a) Covid-19: Pfizer's paxlovid is 89% effective in patients at risk of serious illness, company reports. *BMJ* 375, n2713

Mahase, E. (2021b) Covid-19: Molnupiravir reduces risk of hospital admission or death by 50% in patients at risk, MSD reports. *BMJ* 375, n2422

Mahnel, H. & Mayr, A. (1994) [Experiences with immunization against orthopox viruses of humans and animals using vaccine strain MVA]. *Berliner und Münchener tierärztliche Wochenschrift* 107, 253–6

Mak, G.C., Cheng, P.K., Lau, S.S., *et al.* (2020a) Evaluation of rapid antigen test for detection of SARS-CoV-2 virus. *Journal of Clinical Virology* 129, 104500–104500

Mak, G.C., Lau, S.S., Wong, K.K., *et al.* (2020b) Analytical sensitivity and clinical sensitivity of the three rapid antigen detection kits for detection of SARS-CoV-2 virus. *Journal of Clinical Virology* 133, 104684–104684

Malone, B., Urakova, N., Snijder, E.J., *et al.* (2022) Structures and functions of coronavirus replication–transcription complexes and their relevance for SARS-CoV-2 drug design. *Nature Reviews Molecular Cell Biology* 23, 21–39

Mao, L., Jin, H., Wang, M., *et al.* (2020) Neurologic Manifestations of Hospitalized Patients With Coronavirus Disease 2019 in Wuhan, China. *JAMA Neurology* 77, 683–690

McIntosh, K., Becker, W.B. & Chanock, R.M. (1967) Growth in suckling-mouse brain of "IBV-like" viruses from patients with upper respiratory tract disease. *Proceedings of the National Academy of Sciences* 58, 2268–2273

Meng, B., Ferreira, I.A.T.M., Abdullahi, A., *et al.* (2021) SARS-CoV-2 Omicron spike mediated immune escape, infectivity and cell-cell fusion. *BioRxiv* 2021.12.17.473248

Mercer, J., Snijder, B., Sacher, R., *et al.* (2012) RNAi Screening Reveals Proteasome- and Cullin3-Dependent Stages in Vaccinia Virus Infection. *Cell Reports* 2, 1036–1047

Meyer, B., Drosten, C. & Müller, M.A. (2014) Serological assays for emerging coronaviruses: Challenges and pitfalls. *Virus Research* 194, 175–183

Meyer, H., Sutter, G. & Mayr, A. (1991) Mapping of deletions in the genome of the highly attenuated vaccinia virus MVA and their influence on virulence. *Journal of General Virology* 72, 1031–1038

Minnigan, H. & Moyer, R.W. (1985) Intracellular location of rabbit poxvirus nucleic acid within infected cells as determined by in situ hybridization. *Journal of Virology* 55, 634–643

Moss, B. (2015) Poxvirus membrane biogenesis. *Virology* 479, 619–626

Moss, B., Elroy-Stein, O., Mizukami, T., *et al.* (1990) New mammalian expression vectors. *Nature* 348, 91–92

Mu, Y., Tews, B.A., Luttermann, C., *et al.* (2021) Interaction of Pestiviral E1 and E2 Sequences in Dimer Formation and Intracellular Retention. *International Journal of Molecular Sciences* 22, 7285

Neuman, B.W., Joseph, J.S., Saikatendu, K.S., *et al.* (2008) Proteomics Analysis Unravels the Functional Repertoire of Coronavirus Nonstructural Protein 3. *Journal of Virology* 82, 5279–5294

Oh, S.J. & Shin, O.S. (2021) SARS-CoV-2 Nucleocapsid Protein Targets RIG-I-Like Receptor Pathways to Inhibit the Induction of Interferon Response. *Cells* 10, 530

Okba, N.M.A., Müller, M.A., Li, W., *et al.* (2020) Severe Acute Respiratory Syndrome Coronavirus 2−Specific Antibody Responses in Coronavirus Disease Patients. *Emerging Infectious Diseases* 26, 1478–1488

Pavia, C.S. & Wormser, G.P. (2020) COVID-19: Is there a role for Western blots and skin testing for determining immunity and development of a vaccine? *Diagnostic Microbiology and Infectious Disease* 98, 115148

Pensaert, M.B. & Bouck, P. de (1978) A new coronavirus-like particle associated with diarrhea in swine. *Archives of Virology* 58, 243–247

Perera, R.A., Wang, P., Gomaa, M.R., *et al.* (2013) Seroepidemiology for MERS coronavirus using microneutralisation and pseudoparticle virus neutralisation assays reveal a high prevalence of antibody in dromedary camels in Egypt, June 2013. *Eurosurveillance* 18

Pradeau-Aubreton, K., Ruff, M., Garnier, J.-M., *et al.* (2010) Vectors for recombinational cloning and gene expression in mammalian cells using modified vaccinia virus Ankara. *Analytical Biochemistry* 404, 103–105

Ramesh, S., Govindarajulu, M., Parise, R.S., *et al.* (2021) Emerging SARS-CoV-2 Variants: A Review of Its Mutations, Its Implications and Vaccine Efficacy. *Vaccines* 9, 1195

Redondo, N., Zaldívar-López, S., Garrido, J.J., *et al.* (2021) SARS-CoV-2 Accessory Proteins in Viral Pathogenesis: Knowns and Unknowns. *Frontiers in Immunology* 12, 708264

Ren, Y., Shu, T., Wu, D., *et al.* (2020) The ORF3a protein of SARS-CoV-2 induces apoptosis in cells. *Cellular & Molecular Immunology* 17, 881–883

Russell, M.W., Moldoveanu, Z., Ogra, P.L., *et al.* (2020) Mucosal Immunity in COVID-19: A Neglected but Critical Aspect of SARS-CoV-2 Infection. *Frontiers in Immunology* 11, 611337

Schoeman, D. & Fielding, B.C. (2019) Coronavirus envelope protein: current knowledge. *Virology Journal* 16, 69

Schramm, B. & Locker, J.K. (2005) Cytoplasmic Organization of POXvirus DNA Replication. *Traffic* 6, 839–846

Segreto, R., Deigin, Y., McCairn, K., *et al.* (2021) Should we discount the laboratory origin of COVID-19? *Environmental Chemistry Letters* 19, 1–15

Senkevich, T.G., Ward, B.M. & Moss, B. (2004) Vaccinia Virus Entry into Cells Is Dependent on a Virion Surface Protein Encoded by the A28L Gene. *Journal of Virology* 78, 2357–2366

Sholukh, A.M., Fiore-Gartland, A., Ford, E.S., *et al.* (2021) Evaluation of cell-based and surrogate SARS-CoV-2 neutralization assays. *Journal of Clinical Microbiology* JCM0052721

Simabuco, F.M., Tamura, R.E., Pavan, I.C.B., *et al.* (2020) Molecular mechanisms and pharmacological interventions in the replication cycle of human coronaviruses. *Genetics and Molecular Biology* 44, e20200212

Skowronski, D.M., Astell, C., Brunham, R.C., *et al.* (2005) Severe Acute Respiratory Syndrome (SARS): A Year in Review. *Medicine* 56, 357–381

Smith, G.L. & Law, M. (2004) The exit of Vaccinia virus from infected cells. *Virus Research* 106, 189–197

Sodeik, B. & Krijnse-Locker, J. (2002) Assembly of vaccinia virus revisited: de novo membrane synthesis or acquisition from the host? *Trends in Microbiology* 10, 15–24

Song, F., Fux, R., Provacia, L.B., *et al.* (2013) Middle East Respiratory Syndrome Coronavirus Spike Protein Delivered by Modified Vaccinia Virus Ankara Efficiently Induces Virus-Neutralizing Antibodies. *Journal of Virology* 87, 11950–11954

Stickl, H., Hochstein-Mintzel, V., Mayr, A., *et al.* (1974) MVA-Stufenimpfung gegen Pocken. *DMW - Deutsche Medizinische Wochenschrift* 99, 2386–2392

Stickl, H. & Hochstein-Mintzel, V. (1971) [Intracutaneous smallpox vaccination with a weak pathogenic vaccinia virus ("MVA virus")]. *Münchener medizinische Wochenschrift (1950)* 113, 1149–53

Sun, P., Lu, X., Xu, C., *et al.* (2020) Understanding of COVID-19 based on current evidence. *Journal of Medical Virology* 92, 10.1002/jmv.25722

Sutter, G. & Moss, B. (1992) Nonreplicating vaccinia vector efficiently expresses recombinant genes. *Proceedings of the National Academy of Sciences* 89, 10847–10851

Sutter, G., Ohlmann, M. & Erfle, V. (1995) Non-replicating vaccinia vector efficiently expresses bacteriophage T7 RNA polymerase. *FEBS Letters* 371, 9–12

Taleghani, N. & Taghipour, F. (2021) Diagnosis of COVID-19 for controlling the pandemic: A review of the state-of-the-art. *Biosensors & Bioelectronics* 174, 112830–112830

Tan, Y.-J., Goh, P.-Y., Fielding, B.C., *et al.* (2004) Profiles of Antibody Responses against Severe AcuteRespiratory Syndrome Coronavirus Recombinant Proteins andTheir Potential Use as DiagnosticMarkers. *Clinical and Vaccine Immunology* 11, 362–371

Tomar, P.P.S. & Arkin, I.T. (2020) SARS-CoV-2 E protein is a potential ion channel that can be inhibited by Gliclazide and Memantine. *Biochemical and Biophysical Research Communications* 530, 10–14

Tscherne, A., Schwarz, J.H., Rohde, C., *et al.* (2021) Immunogenicity and efficacy of the COVID-19 candidate vector vaccine MVA-SARS-2-S in preclinical vaccination. *Proceedings of the National Academy of Sciences* 118, e2026207118

Udugama, B., Kadhiresan, P., Kozlowski, H.N., *et al.* (2020) Diagnosing COVID-19: The Disease and Tools for Detection. *ACS Nano* 14, acsnano.0c02624

Valkó, A., Bálint, Á., Bozsa, Á., *et al.* (2018) Prevalence of antibodies against transmissible gastroenteritis virus (TGEV) in Hungary. *Veterinary and Animal Science* 7, 100042

Vidarsson, G., Dekkers, G. & Rispens, T. (2014) IgG Subclasses and Allotypes: From Structure to Effector Functions. *Frontiers in Immunology* 5, 520

V'kovski, P., Kratzel, A., Steiner, S., *et al.* (2020) Coronavirus biology and replication: implications for SARS-CoV-2. *Nature Reviews. Microbiology* 19, 1–16

Wang, R., Yang, X., Chang, M., *et al.* (2021) ORF3a Protein of Severe Acute Respiratory Syndrome Coronavirus 2 Inhibits Interferon-Activated Janus Kinase/Signal Transducer and Activator of Transcription Signaling via Elevating Suppressor of Cytokine Signaling 1. *Frontiers in Microbiology* 12, 752597

Wang, Xiong, Tan, L., Wang, Xu, *et al.* (2020) Comparison of nasopharyngeal and oropharyngeal swabs for SARS-CoV-2 detection in 353 patients received tests with both specimens simultaneously. *International Journal of Infectious Diseases* 94, 107–109

WHO (2022) COVID-19 vaccine tracker and landscape. Available online at https://www.who.int/publications/m/item/draft-landscape-of-covid-19-candidate-vaccines, checked on 1/17/2022

WHO (2021a) Middle East respiratory syndrome. Available online at http://www.emro.who.int/health-topics/mers-cov/mers-outbreaks.html, checked on 12/31/2022

WHO (2021b) Coronavirus (COVID-19) Update: FDA Authorizes First Oral Antiviral for Treatment of COVID-19. Available online at https://www.fda.gov/news-events/press-announcements/coronavirus-covid-19-update-fda-authorizes-additional-oral-antiviral-treatment-covid-19-certain, checked on 1/17/2022.

Wit, J.J. (Sjaak) de, Cook, J.K.A. & Heijden, H.M.J.F. van der (2011) Infectious bronchitis virus variants: a review of the history, current situation and control measures. *Avian Pathology* 40, 223–235

Woo, P.C., Lau, S.K., Tsoi, H., *et al.* (2004) Relative rates of non-pneumonic SARS coronavirus infection and SARS coronavirus pneumonia. *The Lancet* 363, 841–845

Wu, H.-S., Hsieh, Y.-C., Su, I.-J., *et al.* (2004) Early detection of antibodies against various structural proteins of the SARS-associated coronavirus in SARS patients. *Journal of Biomedical Science* 11, 117–126

Xia, S., Zhu, Y., Liu, M., *et al.* (2020) Fusion mechanism of 2019-nCoV and fusion inhibitors targeting HR1 domain in spike protein. *Cellular and Molecular Immunology* 17, 765–767

Xu, H., Zhong, L., Deng, J., *et al.* (2020) High expression of ACE2 receptor of 2019-nCoV on the epithelial cells of oral mucosa. *International Journal of Oral Science* 12, 8

Xu, X., Chen, P., Wang, J., *et al.* (2020) Evolution of the novel coronavirus from the ongoing Wuhan outbreak and modeling of its spike protein for risk of human transmission. *Science China Life Sciences* 63, 457–460

Xu, Y., Li, X., Zhu, B., *et al.* (2020) Characteristics of pediatric SARS-CoV-2 infection and potential evidence for persistent fecal viral shedding. *Nature Medicine* 26, 502–505

Yahalom-Ronen, Y., Tamir, H., Melamed, S., *et al.* (2020) A single dose of recombinant VSV-ΔG-spike vaccine provides protection against SARS-CoV-2 challenge. *Nature Communications* 11, 6402

Yamaoka, Y., Jeremiah, S.S., Miyakawa, K., *et al.* (2020) Whole nucleocapsid protein of SARS-CoV-2 may cause false positive results in serological assays. *Clinical Infectious Diseases* 72, ciaa637-

Yoshimoto, F.K. (2020) The Proteins of Severe Acute Respiratory Syndrome Coronavirus-2 (SARS CoV-2 or n-COV19), the Cause of COVID-19. *The Protein Journal* 39, 198–216

Yüce, M., Filiztekin, E. & Özkaya, K.G. (2021) COVID-19 diagnosis —A review of current methods. *Biosensors and Bioelectronics* 172, 112752

Zaki, A.M., Boheemen, S. van, Bestebroer, T.M., *et al.* (2012) Isolation of a Novel Coronavirus from a Man with Pneumonia in Saudi Arabia. *The New England Journal of Medicine* 367, 1814–1820

Zeng, R., Yang, R.-F., Shi, M.-D., *et al.* (2004) Characterization of the 3a Protein of SARS-associated Coronavirus in Infected Vero E6 Cells and SARS Patients. *Journal of Molecular Biology* 341, 271–279

XI. APPENDIX

1. Consumables, reagents, chemicals

Description	Supplier
10x Tris/Glycine/SDS	Bio-Rad, Feldkirchen, Germany
4×Laemmli sample buffer	Bio-Rad, Feldkirchen, Germany
6-well flat bottom plate	Sarstedt, Nümbrecht, Germany
96-well tissue culture plates	Greiner bio-one, Frickenhausen, Germany
Cell culture flask (25/75/175 cm^2)	Sarstedt, Nümbrecht, Germany
Crystal violet	Sigma-Aldrich, Taufkirchen, Germany
GelRed	Biozol GmbH, Eching, Germany
Goat anti-mouse IgG/HRP	Agilent Dako, Glostrup, Denmark
MEM	Sigma-Aldrich, Taufkirchen, Germany
MEM non-essential amino acid solution	Sigma-Aldrich, Taufkirchen, Germany
Micro tubes 1,5ml	Sarstedt, Nümbrecht, Germany
NaCl	PanReac AppliChem, Darmstadt, Germany
Nitrocellulose membrane	GE Healthcare, Freiburg, Germany
Nonfat dried milk powder	PanReac AppliChem, Darmstadt, Germany
OneShot™Top10	Fisher Scientific, Waltham, USA
PBS	Thermo Fisher Scientific, Planegg, Germany

Penicillin–Streptomycin	Sigma-Aldrich, Taufkirchen, Germany
Rabbit anti-human IgG/HRP	Agilent Dako, Glostrup, Denmark
Serological pipette (5/10/25 ml)	Sarstedt, Nümbrecht, Germany
SFP eggs	VALO BioMedia GmbH, Cuxhaven, Germany
TAE buffer 50X	Fisher Scientific, Waltham, USA
Towbin buffer	Bio-Rad, Feldkirchen, Germany
Tris-Ultrapure	PanReac AppliChem, Darmstadt, Germany
Triton-X100	Sigma-Aldrich, Taufkirchen, Germany
TrueBlue™ Peroxidase Substrate	SeraCare Life Sciences, Milford, USA
Tween20	Sigma-Aldrich, Taufkirchen, Germany
X-tremeGENE HP DNA Transfection Reagent	Roche Diagnostics, Penzberg, Germany

2. Laboratory equipment and software

Description	Supplier
Adobe Reader	Adobe Systems, San Jose, USA
BioRender	BioRender, Toronto, USA
ChemiDocTMMP, Imaging System	Bio-Rad, Munich, Germany
DNASTAR Lasergene	DNASTAR, Inc., Madison, Wisconsin, USA
Eppendorf centrifuge 5810R	Eppendorf AG, Hamburg, Germany
Galaxy 170S incubator	New Brunswick (Eppendorf), Hamburg, Germany
Hanna Checker® pH meter	SIGMA-ALDRICH, St. Louis, USA

Image Lab 5.0 Software	Bio-Rad, Feldkirchen, Germany
Microplate reader Sunrise	Tecan Trading AG, Männedorf, Switzerland
Mini Protean Tetra cell SDS-Page System	Bio-Rad, Feldkirchen, Germany
Mini Vortex Mixer	Fisher Scientific, Waltham, USA
NanoDrop® ND-1000	PEQLAB Biotechnology GmbH, Erlangen, Germany
Trans Blot® Turbo™ Transfer System	Bio-Rad, Feldkirchen, Germany
Wet/Tank blotting system	Bio-Rad, Feldkirchen, Germany

3. Commercial kits

Description	Supplier
Anti-SARS-CoV-2 IgA ELISAs	Euroimmun, Lübeck, Germany
Anti-SARS-CoV-2 IgG ELISAs	Euroimmun, Lübeck, Germany
QiaAmp Viral RNA extraction kit	Qiagen, Hilden, Germany
SuperScript VILO kit	Life Technologies, Darmstadt, Germany

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