Population based studies on detection, prevalence and immunoreactivity of SARS-CoV-2 in Children

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Parastoo Kheiroddin

aus Sary, Iran

20.12.1988

im Jahr

2022

Acknowledged by the PhD committee and head of Regensburg Medical School

Supervisor: **Univ. Prof. Michael Kabesch**

Co-Supervisors: **Univ. Prof. Ralf Wagner**

Prof. Bianca Schaub

Prof. Christian Schultz

Examination Board:

1. **Univ. Prof. Wulf Schneider, UKR/UR, Chairman of the Examination Committee**
2. **Univ. Prof. Michael Kabesch, UKR/UR, (Supervisor), Examiner**
3. **Prof. Barbara Schmidt, UKR/UR, Examiner**
4. **Univ. Prof. Ralf Wagner, UKR/UR, (Co-Supervisor), Examiner**
5. **Univ. Prof. Sven Wellmann, UKR/UR, Substitute**

Day of public defense:

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Abbreviations

ACE2	angiotensin-converting enzyme 2
Ag	Antigen
AIM	activation-induced marker
APC	antigen-presenting cells
ARDS	acute respiratory distress syndrome
CD	Cluster of differentiation
CD	standard deviation
CDC	Centers for Disease Control
CHAMP	CHildhood Allergy and tolerance: Biomarkers and Predictors
COI	Cut Of Index
COKIBA	COronavirus Antibodies in KIds in Bavaria
COVID-19	Coronavirus Disease 2019
CT	computed tomography
Ct	cycle threshold
DAGS	Double-antigen sandwich
E	Envelope
ELISAs	enzyme-linked immunosorbent assays
ELISpot	enzyme-linked immunosorbent spot
FTD	Fast Track Diagnostics
GDPR	general data protection regulation
GI	Gastrointestinal
ICS	intracellular cytokine staining
ICTV	International Committee on Taxonomy of Viruses
ID	identity
IFN	interferon
Ig	Immunoglobulins
IL	Interleukins
IQR	interquartile range
ISRE	IFN-stimulated response element
IT	Information technology
Km	Kilometer
KUNO	KINDER-UNIKLINIK OSTBAYERN
KW	Calendar week
Lab	Laboratory

LFAs	lateral flow assays
M	Membrane
MCSF	macrophage colony-stimulating factor
MERS-CoV	Middle East Respiratory Syndrome Coronavirus
Min	Minute
MIP	Macrophage Inflammatory Proteins
MIS-C	Multisystem Inflammatory Syndrome in Children
Ml	Millilitre
N	Nucleocapsid
n	Sample size
n.a	Not available
NaCl	Sodium chloride
nm	nanometer
NP	Nucleoprotein
NPI	non-pharmacological interventions
ORFs	open reading frames
PCR	polymerase chain reaction
PIMS	Pediatric Inflammatory Multisystem Syndrome
PRRs	pathogen recognition receptors
PSO	post-symptom onset
P-value	probability value
RER	Rough Endoplasmic Reticulum
RKI	Robert Koch-Institut
RNA	Ribonucleic acid
RNP	ribonucleoprotein
RT-PCR	Real-time PCR
RT-qPCR	Quantitative reverse transcription PCR
S	Spike
s	second
SARS-CoV	Respiratory Syndrome Coronavirus
SARS-CoV-2	Severe Acute Respiratory Coronavirus 2
STACADO	Study to Avoid Outbreaks of Coronavirus At the DOmspatzen School
UTR	Untranslated Region
VOC	Variants of Concern
VUI	Variants under Investigation
WHO	World Health Organization
WICOVIR	Where Is the COrona VIRus?
µL	microliter

1 Introduction

1.1 Coronaviruses

Coronaviruses can cause infections in pulmonary ventilation and intestine in animals and humans (1). In 1930, the first member of the coronavirus family was discovered. However, at that time, this was mainly of interest to veterinarians since coronaviruses infect a variety of mammals and birds and were not considered highly pathogenic in humans (2)(3). In 2002 a Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) outbreak was reported in China, which caused progressive respiratory difficulties and could be passed from human to human; this report changed public attention to coronaviruses. (4)(5). Ten years later, in 2012, the Middle East Respiratory Syndrome Coronavirus (MERS-CoV) outbreak caused mortal respiratory illness and attracted public health attention again to this group of viruses (6). The novel Betacoronavirus was named Severe Acute Respiratory Coronavirus 2 (SARS-CoV-2), first described in Wuhan, China, in December 2019. It was reported by casing a severe disease which was named Coronavirus Disease 2019 (COVID-19) by the World Health Organization (WHO) (7)(8). In two years, hundreds of millions of people were infected since it was quickly transmitted. According to some observers, it is: 'the most crucial global health calamity of the 21st century so far and the greatest challenge that humankind faced since the 2nd World War' (9).

1.1.1 History and Epidemiology of SARS-CoV-2

In December of 2019, the first patients in China were reported to have symptoms of what later should be called COVID-19. Three weeks later, on December 31st, China alerted the WHO about a series of pneumonia cases, and one week after that, a new coronavirus was identified. WHO stated the Public Health Emergency of International Concern after the first human-to-human transmission case outside China by end of January 2020. The virus that caused COVID-19 was named SARS-CoV-2 by the International Committee on Taxonomy of Viruses (ICTV). It spread very fast, and at the beginning of March 2020, the WHO assessed that COVID-19 would officially be classified as a pandemic due to the rapid increase in the number of cases outside China (10).

It is estimated that by the end of February 2022 approximately 445 million people worldwide were infected with SARS-CoV-2, resulting in an estimated 6,016,728 deaths, based on a recent WHO report (10).

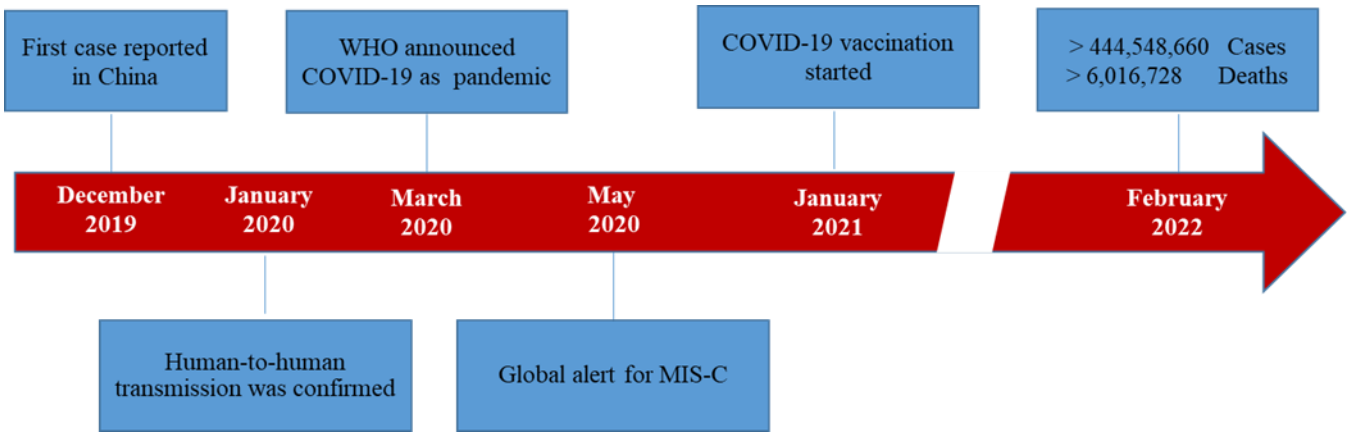

Figure 1: Timeline of the events in COVID-19 outbreak.
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1.1.2 SARS-CoV-2 virus and genome structure

SARS-CoV-2 is a giant envelope virus consisting of a phospholipid bilayer to form pleomorphic particles with a diameter of 80-120 nm (12). A coronavirus contains four structural proteins: spike (S) glycoprotein, nucleocapsid (N) protein, membrane (M) glycoprotein, and small envelope (E) protein (Figure 2) (13). The virus uses S glycoprotein for viral attachment, entry, and infection (14)(15).

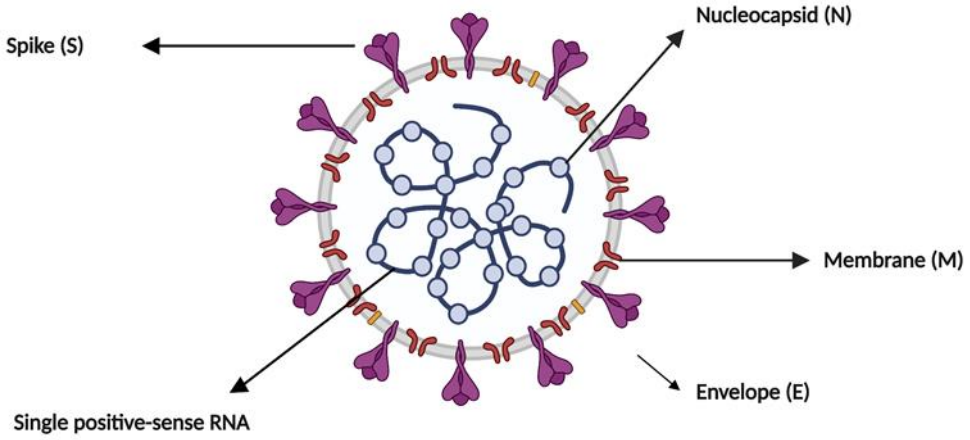

Figure 2: Structure of the SARS-CoV-2 virus. The typical 4 structural SARS-CoV-2 proteins S, N, M, and E. The genomic RNA is packed inside the particles by N protein.
Created with BioRender.com based on reference (14).

SARS-CoV-2 has a positive-sense single-strand Ribonucleic acid (RNA) genome, approximately 30 kilobases in size (16). The virus's genome starts with a 5`-leader-UTR (untranslated region)-replicate and ends with 3´-UTR-poly (A) tail. In addition, genes to code proteins including spike, envelope, membrane, nucleocapsid, multiple open reading frames (ORFs) (Figure 3).

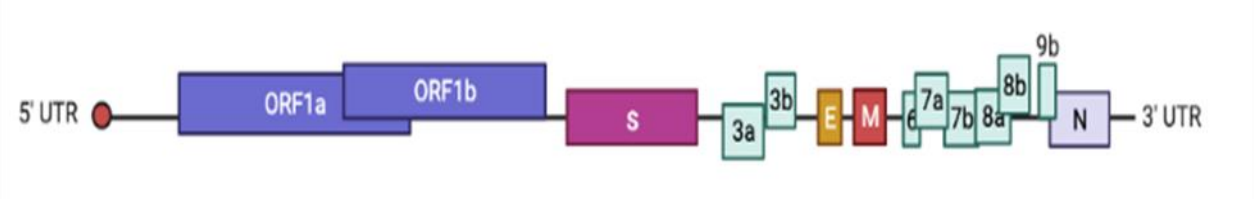

Figure 3: Genome structure of the SARS-CoV-2 virus. The virus genome, as shown here, starts with a 5´- cap structure, continues with the open reading frame 1a (ORF1a) and ORF1b (dark purple boxes), then the genes that code S (pink box), E (yellow box), M (red box), N (light purple box), and the genes for the accessory proteins in between (green boxes), and at the end is 3´polyadenylation.
Created with BioRender.com based on references (17)(18).

1.1.3 SARS-CoV-2 replication cycle

The SARS-CoV-2 viruses use the angiotensin-converting enzyme 2 (ACE2) receptors that are highly expressed in the lower respiratory tract and other organs such as the heart, kidneys, and gastrointestinal tract to support the process of cell entry and to establish the infection (19). The first step in the entering process is the attachment of the S glycoprotein to the ACE2 receptors in the host cells (Figure 4) (20). After the attachment, the virus can enter via two pathways. One is the Plasma membrane pathway, which is a cell-cell fusion for releasing the nucleocapsid-packed genomic RNA into the cytoplasm (Figure 4, step 1b, 2). The second pathway is Endosomal membrane (Figure 4,1a, 2) (21)(22). When the RNA is in the cytoplasm of the host cell (regardless of the pathway to enter), the host ribosome recognizes (Figure 4, step 3) this viral RNA and translates it to the RNA polymerase proteins, which can read the positive strand and generate single-stranded, negative-sense RNA (ssRNA-). RNA polymerase uses this ssRNA- to make more ssRNA+ strands (Figure 4, step 4) (14)(23). The viral structural proteins translate into the host Rough Endoplasmic Reticulum (RER) and then transfer to the Golgi complex for post-translational processing (Figure 4, steps 5, 6, 7). The viruses are then released via exocytosis from the host cells (Figure 4, steps 8, 9) (22)(23)(24).

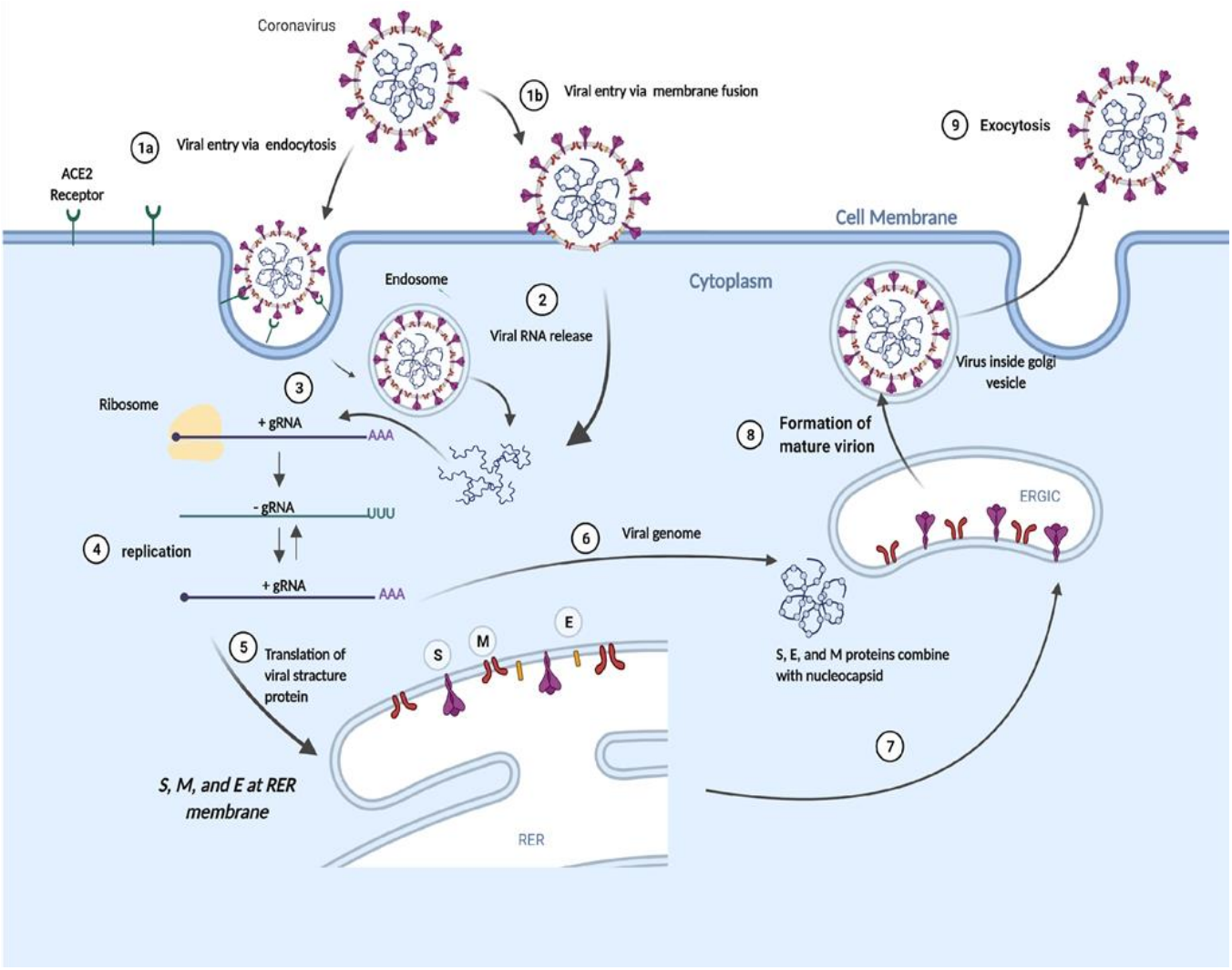

Figure 4: Schematic of the SARS-CoV-2 replication cycle. Please see text in SARS-CoV-2 replication cycle section above for description/explanation of the figure.
Created with BioRender.com based on reference (14).

1.1.4 SARS-CoV-2 variant development

Coronaviruses have the biggest genome size among RNA viruses with proof-reading capability (25). The genome of SARS-CoV-2 has 14 open reading frames (ORFs) and expresses 31 proteins from 11 protein-coding genes (26). SARS-CoV-2 has a low diversity due to the proof-reading activity and mutates at a low rate (27). The SARS-CoV mutation rate is 9.0 Å~ 10−7 in each replication cycle. Moreover, genetic recombination is common in the replication of the virus (28).

Virus mutations affect diagnosis, treatment, and vaccine development (29). Those mutations that improve the efficiency in transmission, replication, and infection spread faster worldwide.

Different countries reported a total of more than 3,215,645 SARS-CoV-2 genomic sequences analyzed from December 2019 to October 30, 2021 (25). To evaluate the impact of possible effects of variants, factors such as improvement in transmission, illness severity, higher mortality, higher risk of long-COVID, undetectability by diagnostic tests, lower sensitivity to neutralizing antibodies, and a higher chance of infecting vaccinated individuals are assessed (30). WHO divided SARS-CoV-2 variants considering the risk to global public health based on significant genomic changes into two groups: Variants of Concern (VOC) and Variants under Investigation (VUI). WHO has determined five Variants of Concern (VOC) by June 2022 as they have an impact on the epidemiological situation of the disease, including Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1 or B.1.1.28.1), Delta (B.1.617.2), and Omicron (B.1.1.529) (26)(27)(28).

1.1.5 Pathogenesis of SARS-CoV-2 infection

COVID-19 disease, based on increasing severity, could be divided into three phases (Figure 5): the first 1–5 days are the asymptomatic phase. In this phase, in the upper respiratory tract, the virus multiplies. During this time, innate immune cells do not cause major hindrances. The second phase comes with common symptoms of COVID-19, such as fever, dry cough, pharyngitis, shortness of breath, joint pain, and tiredness, starting within 3–14 days after the initial virus encounter. During this time, the nosocomial transmission of infection can enhance the chances of community spread (29). The third phase starts by triggering the innate immune reaction when the virus starts to move via the airways to the lower respiratory tract. Patients at this stage start to have a stronger pro-inflammatory response that leads to viral sepsis accompanied by other complications, including pulmonary edema, Acute Respiratory Distress Syndrome (ARDS), different organ failures, and death (30)(31).

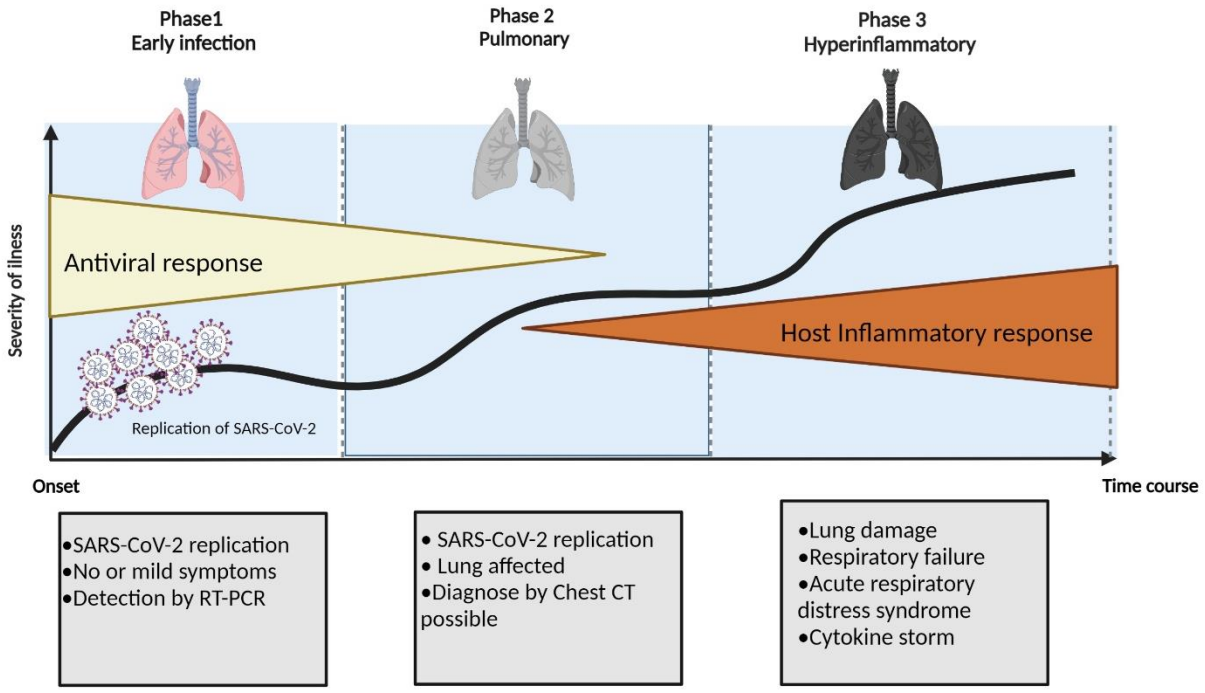

Figure 5: Schematic representation of the clinical course of classic COVID-19. The disease progression over time is divided into three pathological phases: an early infection phase, a pulmonary phase, and a hyper inflammation phase.
Created with BioRender.com based on reference (32)(33) .

SARS-CoV-2 follows the path to reach the lungs via the naso-oral cavity, so the main symptom of COVID-19 patients, especially in severe cases with alveolar edema, is impairment of the oxygen-carbon dioxide exchange, which causes a high risk of respiratory failure (34). Furthermore, there is a high risk that severely ill COVID-19 patients suffer from long-term lung injury and fibrosis because of pulmonary microthrombosis (35).

Post-COVID syndrome is characterized by persisting for more than 12 weeks signs and symptoms that develop during or after SARS-CoV-2 infection, which are not explained by an alternative diagnosis. Post-COVID cases suffer from mostly complex symptoms with fatigue, exhaustion (fatigue), exercise intolerance, shortness of breath, pain, neurocognitive deficits, circulatory or sleep disorders, and a decreased quality of life (36).

Especially at the beginning of the pandemic, it was thought that children were not affected by the comorbidity and mortality associated with COVID-19 and that they developed milder symptoms. However, starting in April 2020, pediatricians reported severe complications among children after mild COVID-19. In May 2020, the possibility of the connection of critical illness in children and SARS-CoV-2 infection alerted the Centers for Disease Control (CDC) and Prevention's Health Alert Network to issue a global warning for Multisystem Inflammatory Syndrome in Children (MIS-C) which is also referred to and now commonly known as Pediatric Inflammatory Multisystem Syndrome (PIMS) (37)(38). It is suggested to be a post/delayed-infectious disease characterized by symptoms including fever, inflammation, and multiorgan dysfunction that frequently affects the gastrointestinal (GI), cardiac, respiratory, and neurologic systems (39)(40)(41).

1.1.6 Immune response in SARS-CoV-2 infection

After SARS-CoV-2 infection, various components of the innate and adaptive immune systems participate in the fighting. Once the host immune system recognizes the virus, it evokes the innate or adaptive immune response. By evaluating severe cases, enormous inflammatory responses, including a massive cytokine expression, which needs the involvement of a vast range of immune cells (such as macrophages and neutrophils), have been characterized (Figure 6) (42). The first step of the immune system to identify the virus is taken by pathogen recognition receptors (PRRs), which are present on immune cells. It is mainly Toll-like receptors 3, 7, and 8, which lead to enhanced interferon (IFN) production (43). Interferon (IFN) type I not only activates the innate immune response but also induces the effective adaptive immune response against viral infection (44). IFN-stimulated response element (ISRE) controls the JAK-STAT pathway activation, which is initiated by IFN type I. IFN type I accumulation can cut off viral replication and has an important role as an immune modulator to boost phagocytosis of antigens by macrophage. Therefore, blocking the activity of IFNs, revision the expression of macrophages, or disturbing the signaling pathway of JAK-STAT has impact on the survival of the virus (44)(45).

Antiviral immunity is not only by innate immunity, but also the adaptive immune response plays a critical role. T cell activity relies on the presence of APC (antigen-presenting cells). CD4+ helper T cells make the adaptive immune system response easier by helping the CD8+ cytotoxic T cells as well as humoral immunity. The cluster of molecules, including granzymes, perforin, and IFN-g, which are essential in destroying virus-infected cells, is secreted by CD8+ cells. While the neutralizing antibody, which is produced by humoral immune response and B-cells, protects the body from re-infection (44). B lymphocyte development starts in the bone marrow and migrates to peripheral immune organs where they can recognize antigens. The activation of B cells happens by facing foreign antigens. They go through clonal expansion and differentiation into plasma cells for antibody generation or memory B cells (46). Antibodies, also called immunoglobulins (Ig), are categorized into five different Ig classes: IgM, IgD, IgG, IgA, and IgE. Each Ig consists of a combination of constant regions and antigen-binding sites. IgM, IgA, and IgG generate after SARS-CoV-2 infection and target the viral spike (S) and nucleoprotein (NP). IgM is detectable before IgG, peaking two to five weeks after starting symptoms and declining over a three to five week period post-symptom onset (PSO). After three to seven weeks of developing symptoms, an IgG peak appears and persists for at least eight weeks (Figure 7) (47)(48).

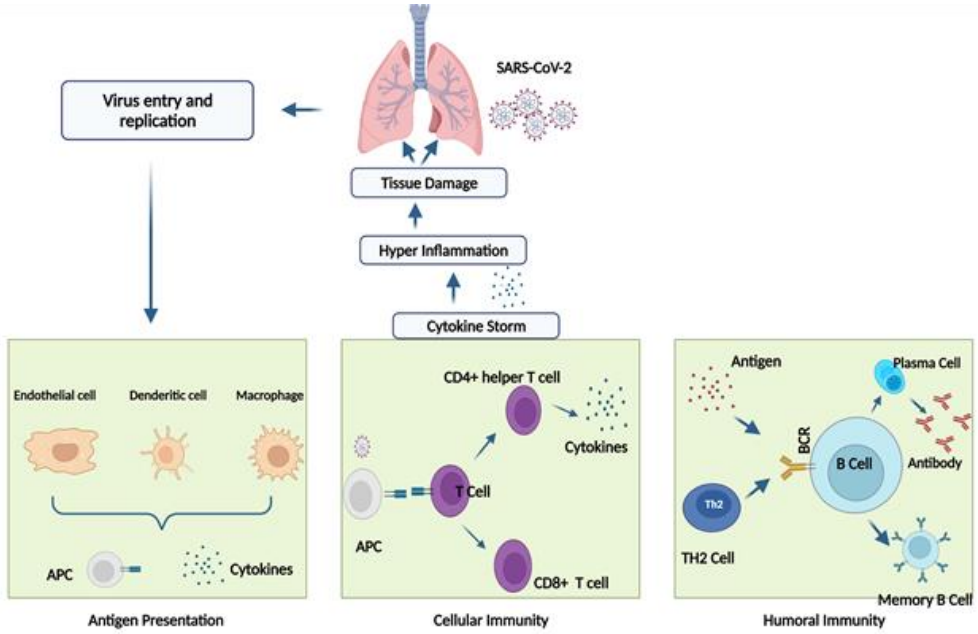

Figure 6: Schematic of the immunopathogenesis of COVID-19 infection.
Created with BioRender.com based on reference (44)(49)

There is an association between the disease severity and high levels of cytokines such as IFN-g, MIP-1a, interleukins (especially IL-1, IL-2, IL-4, IL-7, IL-10, IL-12, and IL-13), macrophage colony-stimulating factor (MCSF), and TNF-a in COVID-19 patients (50). The critical moment in the pathogenesis of COVID-19 is the "cytokine storm," which induces inflammation that results in lung injury and other complications such as acute respiratory distress syndrome (ARDS), pneumonitis, respiratory failure, organ failure, and high risk of death (Figure 6) (44) (50).

1.1.7 Necessity for SARS-CoV-2 detection tests

Countries affected by the virus applied strict restrictions to social and economic life, such as social distancing, closing schools and business activities, sometimes even preventing people from leaving their homes with full lockdowns to be able to contain the spread of the virus. However, these restrictions cause dramatic economic problems, lack of physical activity, stress, and severe psychological and developmental effects, especially on children (29). While children were not the priority of testing due to limitation in the resources in the beginning of the pandemic, they were suspected to be crucial for spreading the infection.

To control and prevent the spread of infection without massive interference with daily lives of all those uninfected, identifying who is infected and who is not is essential. Thus, a testing system should achieve three main goals:

a) Early identification of infected to stop infection chains and prevent local outbreaks.

b) Detection of those who have developed immunity and can safely return to the public.

c) Monitor the mutations.

Different diagnostics techniques for SARS-CoV-2 are introduced so far as follows:

i. Detection of viral RNA: even if the virus is present in extremely small amounts, RT-qPCR can detect the viral RNA. Therefore, RT-qPCR is the most sensitive test to detect SARS-

CoV-2. RT-qPCR can detect the viral RNA even if only ~100 copies of viral RNA per milliliter is available in the sample. RT-qPCR Ct value is representing the viral genetic material concentration in a patient sample; lower Ct values (high viral load) are more likely to indicate acute disease and high infectivity (51).

ii. Detection of viral antigens: Antigen tests target the SARS-CoV-2 nucleocapsid protein present on the outer surface of the Coronavirus. The test is working with immunodiagnostic technique; lateral flow assays (LFAs). The sensitivity of this test is lower than RT-qPCR tests and cannot detect positives reliably in the early phase of infection (52).

iii. Detection of host antibodies: like infections with other pathogens, SARS-CoV-2 infection elicits the development of IgM and IgG antibodies, which are detectable by serological techniques, such as enzyme-linked immunosorbent assays (ELISAs). In SARS-CoV-2 infections, IgM and IgG antibodies can arise nearly simultaneously in serum if tested within 2 to 3 weeks after starting symptoms (Figure 7) (53).

iv. Cellular immunity test: antibodies and T cell responses mediate long-term protection from viral infection. The T-cell response to the virus is generally measured in the blood around two weeks after initial infection. T cell reactions against SARS-CoV-2 can be detected over a longer period than antibody titers. There are molecular (next-generation sequencing) and cellular (enzyme-linked immunosorbent spot (ELISpot), activation-induced marker (AIM), and intracellular cytokine staining (ICS)) technologies for the evaluation of T cell responses to SARS-CoV-2. ELISpot is used to measure the duration of sustained T cell responses to SARS-CoV-2 (54).

v. Sequencing test: to identify the new variants of SARS-CoV-2, sequencing plays an essential role in understanding the genetic development and spread of SARS-CoV-2 in the context of the COVID-19 pandemic (55).

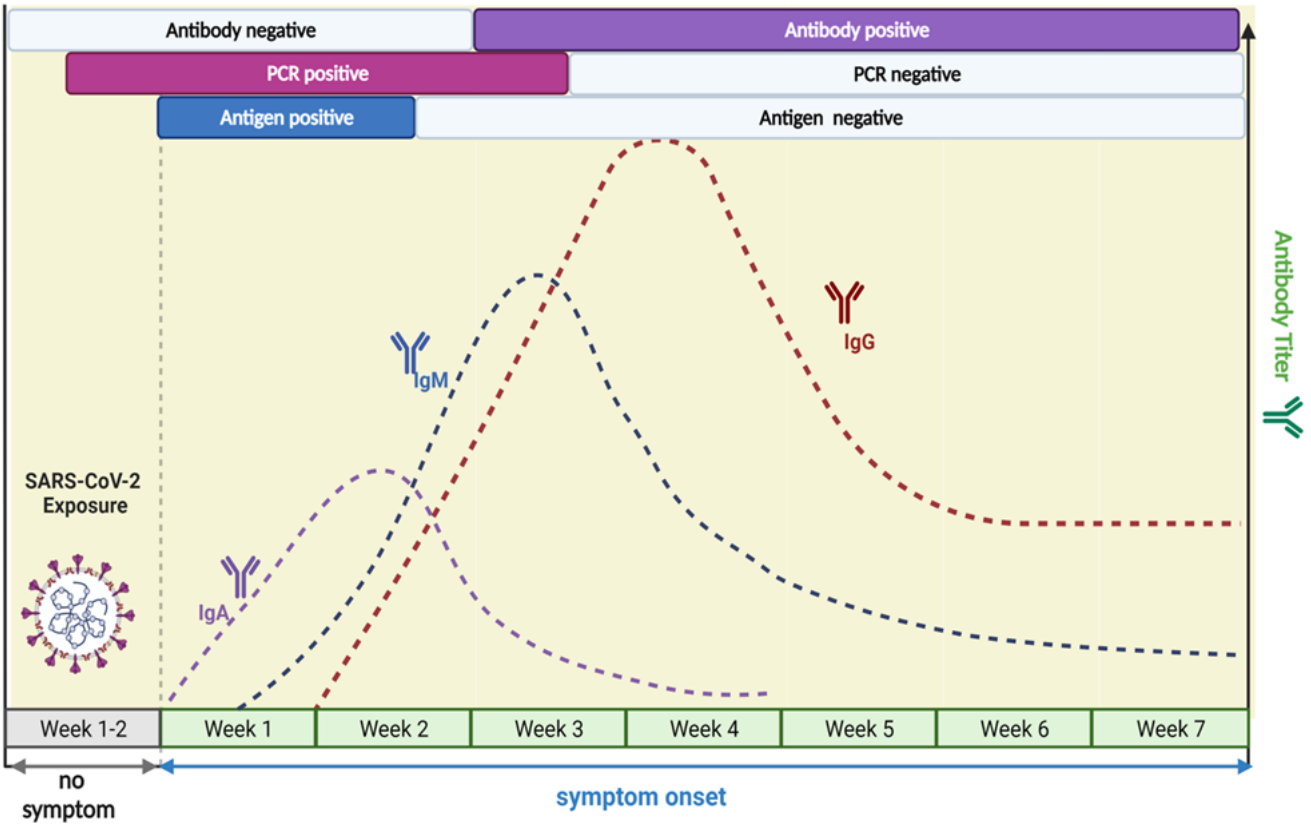

Figure 7: Schematic of antibody generation and detection after COVID-19 infection
Created with BioRender.com based on reference (53)

1.2 Aims of this thesis

Despite the global interest and concern about COVID-19, data on children remained limited throughout the pandemic, and data on pediatric patients with COVID-19 were lacking from the beginning of the pandemic. The main aim of this thesis was to address some of the pressing questions of the time:

- To understand the prevalence of SAS-CoV-2 infection in children after the first pandemic wave.

- To explore if we develop a children specific-test system to:

    1) Test how many children are affected by SARS-CoV-2?
    2) Apply that test system systematically children specific-test system?
    3) Bring children back to school and keep them safe despite the pandemic and lack of vaccination possibilities.

2 Materials and methods

2.1 Study cohorts

During the pandemic, we collected different cohorts to address specific questions in the course of the pandemic. We used different techniques in each cohort as it shown in Table1.

Table 1: Different methods in each cohort

Cohort / Technique	Antigen test	single PCR test	Pool PCR test	Antibody test	Participants	Number of tests
COKIBA	×	×	×	✓	Children	n=2,934 blood
Comparing antigen and PCR testing	✓	✓	✓	×	Adults: Anonymized leftover samples (Patients and students)	Swabs n=311 Gargle n=309
STACADO	×	✓	✓	×	Children and Adults	n=864 pools
WICOVIR	✓	✓	✓	×	Children Adults	n= 16,245 pools
St. Hedwig's hospital staff	✓	✓	✓	×	Adults	n= 697 pools

2.1.1 COKIBA (COronavirus Antibodies in KIds in BAvaria) study cohort

In a cross-sectional design, we investigated children (51.32% male and 48.37% female) from three distinct regions of southeast Germany to assess the true prevalence of SARS-CoV-2 infections in areas with very differently reported infection rates by antibody testing. We established a network of pediatricians who volunteered to take part in the study and focused on three areas/counties within Bavaria with very high (Tirschenreuth county), moderate (Rosenheim), and average infection rates (Regensburg) as indicated by positive PCR tests per 100,000 inhabitants according to the Robert Koch Institute, the German center for disease prevention. The assessment and sample collection took place from May 22nd to July 22nd, 2020. Overall, n=2,934 children aged 1–17 years participated in the study. All the blood samples from this cohort were tested with two SARS-CoV-2 antibody tests.

2.1.2 Comparing the sensitivity and specificity of antigen tests and RT- qPCR by using Gargle and Swab samples

In total, 309 gargle samples and 311 nasal swabs were collected for routine testing in hospitals of the Order of St. John in Regensburg and Straubing, Germany, from October 2020 until April 2021. Two nasal swabs were collected by medical personnel. One nasal swab was transferred immediately to the extraction buffer from a STANDARD™ F COVID-19 Ag FIA kit (SD BIOSENSOR Inc., Suwon, Korea) to be tested with an antigen test, and a second nasal swab from the same patient was transported to the laboratory for RT-PCR testing on the same day for quality control. Gargle samples were provided by patients and medical students by gargling for approximately 30s with 10 mL of sterile water (Ampuwa). The recovered gargle fluid of approximately 10 mL was then transferred to a 250 mL container, and antigen against SARS-CoV-2 was analyzed in each gargle sample immediately after sampling. The remaining sample fluid was kept for quality control and RT-qPCR analysis. All samples analyzed here were leftovers from routine testing and were anonymized before the analysis in this study was performed. We used this cohort to develop the testing system and compare the first antigen and PCR and the second single and pool gargle PCR tests.

2.1.3 STACADO (Study to Avoid Outbreaks of Coronavirus At the DOmspatzen School) study cohort

Students of the Regensburg Domspatzen boarding school, with a world-famous boys' choir dating back to the year 975, were aged 10 to 21 at the time of the study. During the 2020/2021 school year, n=282 students attended school (n=265 in the school year 2021/2022), while n=138 staff members worked at the school during this time. Participation in all study procedures was voluntary; informed consent was obtained from parents, students, and staff who were willing to participate in the study.

We asked participants to gargle; in the first phase of the school testing (STACADO study), gargling was performed with Saline solution and was changed soon to distilled water due to better acceptance by students. Participants were gargling twice a week at home first thing in the morning

for 30–60 s to achieve maximal recovery of virus from throat rinsing. Participants brought their tube sample into school in a zip-lock bag.

During the study's first phase, school personnel labeled the individual samples with a unique ID, registered, collected, and transferred them to the laboratory. In the laboratory, single samples were pooled, and laboratory personnel kept tracking participants' IDs in each pool. During phase 1, each pool contained only five participants due to capacity limitation for depooling of positive pools. In the case of a positive pool, the school was informed, and choir rehearsal in the afternoon was canceled for that group. Laboratory personnel did individual testing of the positive pool by using the single leftover samples from the participants in that pool.

The results were automatically sent out via emails to the school's managers based on the sample ID from the laboratory via a secure, established software developed by our partner MaganaMed. This cohort was used as a pilot cohort to establish the logistic process and develop a workfellow for the pool PCR testing system at school and in the beginning the PCR tests were performed by the Synlab laboratories in Weiden.

2.1.4 WICOVIR (Where Is the COrona VIRus?) study cohort

In the 2nd phase of school testing (WICOVIR testing), participants gargled with 5-6 ml of tap water twice a week at home first thing in the morning for 30–60 s and divided the gargle samples into two falcon tubes. In the schools, one tube was emptied by the participants into a pooling container that was positioned in a pooling station (usually in front of classrooms). Pool participants were defined by the schools and usually contained the pupils of one class and the school staff (teachers) attached to that class. A video documenting the pooling procedure is also available on the study website (www.we-care.de/WICOVIR). Only the number of participants in a pool was transmitted for data protection reasons.

After pooling, the pooling containers were sealed and transported to the laboratory within 1 h. Transport of samples was organized through schools and voluntary helpers, or, if that was not possible, through courier service or study personnel. A drive-through to make sample delivery easy for volunteers was established outside the laboratory. Due to the high number of pools, the

maximum number was n=655 pools per day, we made fixed delivery time points for schools. We divided the schools based on their distance to our test center into three different groups:

- Group 1: they had to deliver the pools to the test center by 8.30, so the laboratory could start the first run by 9 o'clock, and the results were sent out by 11:00.
- Group 2: they had to deliver the pools to the test center by 10:00, so the laboratory could start the second run by 10.30 o'clock, and the results were sent out by 12:30.
- Group 3: the samples from Cham were transferred to the test center by 11.30, so the laboratory could start the third by 12:00, and the results were sent out by 14:00.

However, in the case of the positive pools, more runs were needed. The delivery time points for each group and the processing time of the samples are shown in detail in Figure 1.

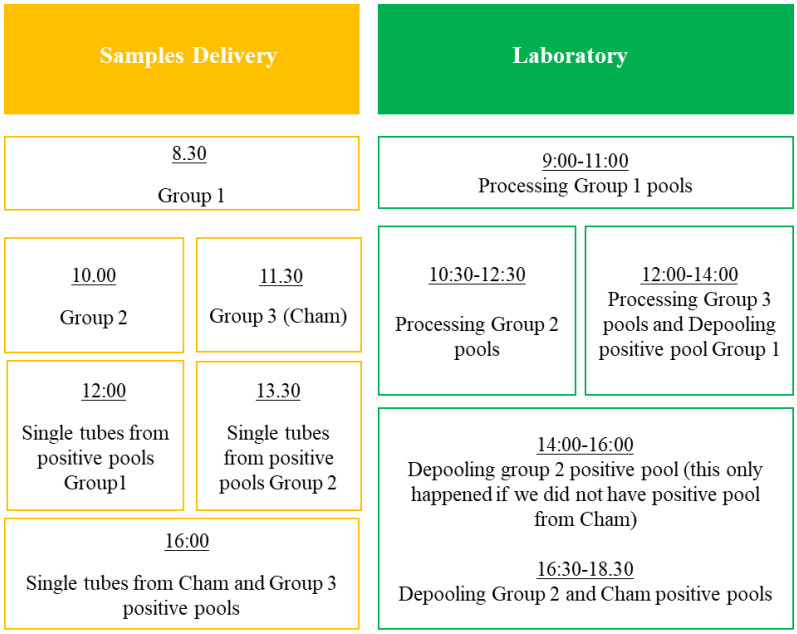

Figure 8: Time points of sample delivery and sample processing in the laboratory

In total, during the 17 school weeks of the study, we tested n=92 schools (located in Regensburg city and the adjacent counties) with n=16,245 pools and n=237,093 tests. In this cohort, students from first grade (6 years of age) to grade 12 (17 years of age) and the kindergarten children (the youngest was 3 years old) participated.

Figure 2 shows the course of the test and pool numbers in Regensburg and counties. During the holidays (KW13 and KW14 = Easter holidays; KW21 and KW22 = Whitsunday holidays), very

few tests were carried out. Students and the school staff participated voluntarily in the study. For reasons of anonymization, communication with study participants in the course of the study was through the schools only. Gargling and pooling were performed as explained in the STACADO cohort.

The exclusion criteria included a positive SARS-CoV-2 test result within two months prior to participation (to avoid positive results in RT-qPCR testing due to prolonged viral RNA shedding not indicating infectivity).

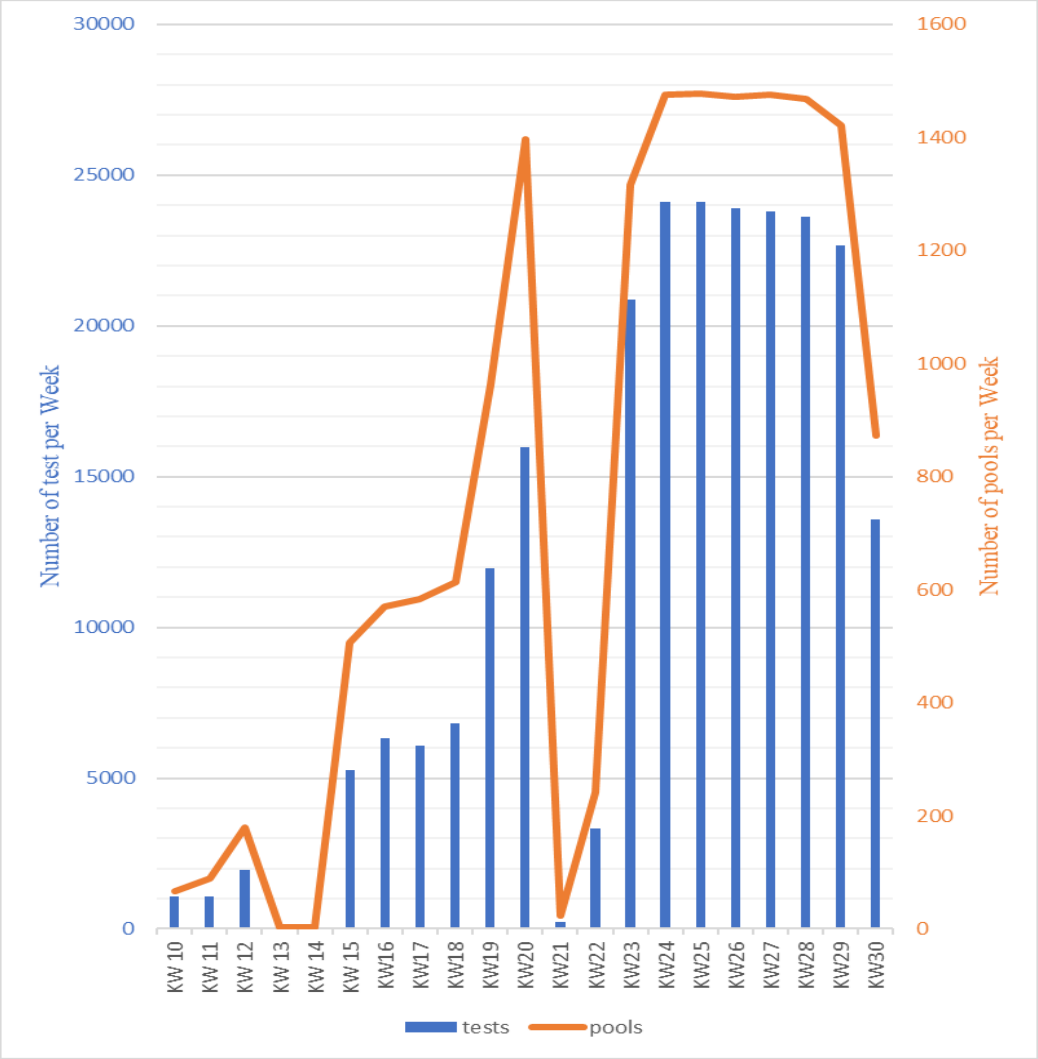

Figure 9: Development of pool numbers and participant numbers from Regensburg and counties

2.1.5 St. Hedwig's hospital staff cohort

The study was performed at St. Hedwig's hospital, which harbors the KUNO University Children's Hospital and the University Maternity Hospital, approximately n=650 regular staff members (and 70 medical/nursing students) participated in the testing over 10 weeks between December 2021 and March 2022. The gargling process was the same as mentioned above in WICOVIR study. In brief, all participants gargled with approximately 6 mL of tap water at home twice or three times per week for approximately 30-60s and divided into two screw-cap tubes. In the hospital, one tube was emptied by the participant into a pooling container positioned in a pooling station, and the other (back-up) was kept by participants in case of a positive pool result. The maximum number of participants accepted for one pool was 20 (later reduced to 10); consecutive staff members attended the pooling station as they entered the hospital. Pooling was supervised by a coworker who linked the barcode of the staff member to the pool barcode in our COVID hospital COVIDA software (MaganaMed GmbH, Regensburg).

We made a testing plan in the way that all early pools until 8:00 a.m. were tested with Cepheid (referred to as "early tests"). For those that entered the hospital later at regular work times between 7:30 and 8:45 a.m., we collected all and started the first run with Allshang/Bio-Rad by 9.30 latest. In the case of a positive early tests depooling and individual testing were done with the first run of Allshang/Bio-Rad, and results were sent out latest by 11:00 o'clock.

The second Allshang/Bio-Rad run started at 12:00 p.m. with additional pools of latecomers and single samples from the positive pools from the first run; results were sent out latest by 14:00 o'clock. PCRs for pools and de-pooling in the afternoon were performed on the Cepheid system again.

All participants in this cohort were tested by pool/single PCR and for one week with antigen tests. All data were anonymized before the analysis in this study was performed.

Ethics statement

All experiments were carried out in accordance with the principles espoused in the Declaration of Helsinki. Al the studies were approved by the Ethics Committee of the University of Regensburg:
STACADO file-number: 20-1953-101.
WICOVIR hospital (also anonymized) file-number: 21-2240-101, 21-2240_2-101.
CoKiBa file-number: 20-1865-101.

2.2 Materials and methods per technique

We used different methods to detect the SARS-CoV-2 virus or the antibodies; the material and methods per technique are described below.

2.2.1 RT-qPCR technique

2.2.1.1 RT-qPCR reagents

Table 2: RT-qPCR Reagents

Product	Supplier
2x Luna® Universal Probe One-Step Reaction Mix (E3007E)	New England BioLabs
20x Luna® WarmStart® RT Enzyme Mix (E3007E)	New England BioLabs
Nuclease-free water	New England BioLabs
Premixed N2 *(N gene)*, ORF1b*(Orf1b)* and RP2 *(RPP30)* assay primers and probe, each containing 2 primers (6.7 µM) and 1 probe (1.7 µM)	Eurofins Genomics

Table 3: Primers and Probes Sequences

Name (target)	Primer/probe ID	Primer	Supplier
N2 *(N gene)*	N2-F N2-R N2-P	TTACAAACATTGGCCGCAAA GCGCGACATTCCGAAGAA **FAM**-ACAATTTGCCCCCAGCGCTTCAG- **BHQ1**	Eurofins Genomics
ORF1b *(Orf1b)*	ORF1b-F ORF1b-R ORF1b-P	TGGGGTTTTACAGGTAACCT AACACGCTTAACAAAGCACTC **TexasRed-** TAGTTGTGATGCAATCATGACTAG- **BHQ2**	Eurofins Genomics
RP2 *(RPP30)*	RP-F RP-R RP-P	AGATTTGGACCTGCGAGCG GCAACAACTGAATAGCCAAGGT **HEX**–TTCTGACCTGAAGGCTCTGCGCG– **BHQ1**	Eurofins Genomics

2.2.1.2 RT-qPCR method setup

To detect SARS-CoV-2 with BIORAD Real-Time PCR System (CFX96; Bio-Rad, Hercules, California, USA) we established the sensitive triplex RT-qPCR test using triplex PCR to detect 2 genes of SARS-CoV-2 (ORF1b and N2 gene) and one human gene as an internal control (Rnase P gene) table 3 shows primers and probes sequences. The master mix base preparation on table 4 and the PCR protocol is shown in table 4.

Table 4: Reaction preparation

Reagent	Amount
Nuclease-free water	1.3 µl
2x Luna® Universal Probe One-Step Mix	10.0 µl
Respective Forward primer Respective Reverse primer Respective Probe	0.1 µl 0.1 µl 0.05 µl
20x Luna® WarmStart® RT Enzyme Mix	1.0µl
Sample (RNA)	7.0µl

Table 5: RT-qPCR Protocol for BIO RAD CFX96 Touch Real-Time PCR

Cycles	Temperature	Time
1	55°C	10min
1	95°C	2min
42 cycles	95°C	10 sec
	57°C	30 sec

To establish the PCR setup, we used already confirmed positive RNA samples provided by the institute of laboratory medicine, microbiology and hygiene, hospital of the Order of St. John, Regensburg, Germany. In this diagnostic laboratory, they used one-step RT-qPCR with the LightCycler® Multiplex RNA Virus Master (target E gene) using a Light Cycler 480 II instrument (Roche Diagnostics, Mannheim, Germany). Table 6 is the data comparison of different positive RNAs on these two cyclers.

Table 6: Comparison of different RNA sample RT-qPCR Ct values on BIO RAD and ROCHE cyclers

Sample Number	BIO RAD			ROCHE
	Ct:N2	Ct:ORF1b	Ct:RP (Human gene)	Ct: E gene
1	18.93	18.16	28.27	20.07
2	24.34	24.33	32.38	25.04
3	28.28	28.2	26	29
4	33.81	34.74	30	34
5	36.42	38.71	39.95	35.81

2.2.2 RNA Isolation technique

2.2.2.1 RNA Isolation reagents

Table 7: RNA Isolation Reagents

Product	Supplier
GTT lysis buffer	MagnifiQ™ RNA Set (1920)
MQBB binding beads	MagnifiQ™ RNA Set (1920)
Isopropanol	MagnifiQ™ RNA Set (1920)
A1W wash buffer	MagnifiQ™ RNA Set (1920)
Ultrapure water	MagnifiQ™ RNA Set (1920)
Ethanol (96-99%)	Merk

Table 8: MQBS binding suspension reagents

Reagent	volume per sample
Isopropanol	300µl
	+
MQBB binding beads	20µl
	=
MQBS binding suspension	320µl

Table 9: RNA Isolation Preparation

Plate name	Reagent	Volume per well
Samples	GTT	400µl
Samples	MQBB binding suspension	320µl
Wash 1	A1W	600µl
Wash 2	80% ethanol	600µl
Wash 3	80% ethanol	600µl
Elution	Ultrapure water	150µl

2.2.2.2 RNA Isolation method setup

To setup the RNA isolation with our Auto-Pure96 system (Hangzhou Allsheng Instruments, Shanghai, China) the MagnifiQ™ RNA buffer kit (A&A Biotechnology, Gdansk, Poland) was used with the capacity of isolation RNA from 96 pools in 27 minutes (table 10).

Table 10: RNA Isolation Protocol for Auto-Pure 96 (Allsheng) machine

Step	Name	Plate	Mix Time (Min)	Mix Amp (%)	Wait Time (Min)	Volume (µl)	Mix Speed (1-10)	Temp (°C)
1	-Load-	1						
2	Bind	2	8.0	80	0	900	3	OFF
3	Wash1	4	1.0	80	0	600	3	OFF
4	Wash2	5	1.0	80	0	600	3	OFF
5	Wash3	6	1.0	80	2.5	600	3	OFF
6	Elution	8	5.0	80	0	150	3	OFF
7	-Unload-	1						

We used positive gargle samples from the institute of laboratory medicine, microbiology and hygiene, hospital of the Order of St. John, Regensburg, Germany. We ran the same samples on our Allsheng, and established an innotrain system (BEXS 12s extraction system in combination with the inno-train BEXS 12) in the diagnostic laboratory. After that, both groups of RNA were run on our BIO RAD RT-qPCR machine to compare the Ct values, as is shown in table 11. To check if there is any contamination from one well to another, we put water in between the positive samples.

Table 11: Comparison of RT-qPCR Ct values of RNA samples isolated by different methods

Sample Number	Allsheng Isolation			Innotrain Isolation		
	Ct:N2	Ct:ORF1b	Ct:RP	Ct:N2	Ct:ORF1b	Ct:RP
1	27.27	29.14	29.51	28.32	26.42	28.83
2	N/A	N/A	N/A	N/A	N/A	N/A
3	31.40	31.26	34.45	29.98	20.28	35.04
4	N/A	N/A	N/A	N/A	N/A	N/A
5	32.55	32.60	34.01	32.65	32.80	34.73

As a last establishing test, we run different dilutions of the SARS-CoV-2 RNA with specific copy numbers through our established RNA isolation Alshenng system and BIO RAD RT-qPCR system (Table12).

Table 12: Ct values of SARS-CoV-2 RNA samples with copy number isolated

Dilution		Copie (C/ml)	Allsheng Isolation/ BioRad PCR Ct N2	Allsheng Isolation/ BioRad PCR Ct ORF1b
RV (original)	1	10.000.000	25.44	25.94
Dilution 1	1-1	5.000.000	26.01	25.74
Dilution 2	1-2	1.000.000	28.13	27.97
Dilution 3	1-3	100.000	30.84	30.95
Dilution 4	1-4	10.000	35.17	34.64
Dilution 5	1-5	1.000	35.67	35.79
RV (original)	2	1.000.000	28.02	27.86
Dilution 1	2-1	500.000	29.68	28.91
Dilution 2	2-2	100.000	32.30	32.4
Dilution 3	2-3	10.000	33.88	34.06
Dilution 4	2-4	1.000	38.14	34.27
Dilution 5	2-5	100	0	0

2.2.3 Cepheid, Real time PCR technique

2.2.3.1 Cepheid test reagents

Table 13: Cepheid test Reagents

Product	Supplier
Xpert Xpress SARS-CoV-2	Cepheid (Catalog number) XPRSARS-COV2-10

1.2.3.2 Cepheid test method

Cepheid is an automated molecular test for the fast qualitative detection of SARS-CoV-2. Qualitative test system: the Xpert Xpress™ SARS-CoV-2 assay (cartridge system) on a GeneXpert instrument (Cepheid, Sunnyvale, CA, USA). The test is ready to use; only 300 µL of the sample needs to be added to the cartridge,

then can provide rapid detection of the SARS-CoV-2 in approximately 45 minutes. The cartridge system includes an extraction step and amplification targeting the E- and N2-genes. The machine has four channels that can analyze n=4 samples simultaneously.

2.2.4 Antigen test technique

1.2.4.1 Antigen test material

Table 14: Antigen test Reagents

Product	Supplier
Extraction buffer tube	SD BIOSENSOR (REF F-NCOV-01G)
Filter cap	SD BIOSENSOR (REF F-NCOV-01G)
Test device(individually in a foil pouch with desiccant)	SD BIOSENSOR (REF F-NCOV-01G)

1.2.4.2 Antigen test method

Gargle and swab samples were tested for the SARS-CoV-2 antigen right after sampling. Either the nasal swab or 150 µl of gargle sample was transferred into an extraction buffer tube provided with the STANDARD™ F COVID-19 Ag FIA kit (SD BIOSENSOR Inc., Suwon, Korea), followed by treatment according to the manufacturer's instructions. Briefly, after closing the buffer tube with the provided nozzle cap, the tube was squeezed 10 times to mix the sample with the extraction buffer. Then, we applied 4 drops of the extracted specimen to the well of the respective test cassette. After 15 min of incubation at room temperature, the test cassette was loaded into the analyzer (SD BIOSENSOR), and the COI (as a numerical representation of the measured fluorescence signal) was calculated automatically by the analyzer. A COI-1.0 represents a positive result for SARS-CoV-2 nucleoproteins, according to the manufacturer.

2.2.5 Antibody test technique

2.2.5.1 Antibody test reagent

Table 15: Antibody test Reagents

Product	Supplier
Elecsys Anti-SARS-CoV-2 (ACOV2)	Roche (REF 092030951900)

Blood samples (2.7 ml) were collected in S-Monovette (serum tube) from all participants. After serum separation, the serum samples were tested for response to SARS-CoV-2 with two different test kits:

1. The commercially available, licensed qualitative Elecsys Anti-SARS-CoV-2 (Roche Diagnostics, Rotkreuz, Switzerland; https://diagnostics.roche.com) with a sensitivity of 99.5% and a specificity of 99.8%, according to the manufacturer. It is directed against the N-protein and can detect IgA, IgM, and IgG (without differentiation between them); the cutoff value is 1.0. The Elecsys Anti-SARS-CoV-2 immunoassay was run on the fully-automated cobas® 6800/8800 Systems and performed according to the manufacturer's instructions. Briefly, the first step is incubation for 9 minutes of the samples with a mix of biotinylated and ruthenylated nucleocapsid (N) antigens. Then, in the second step takes another 9 minutes, the DAGS complexes bind to the solid phase via interaction of biotin and streptavidin. The last step is the measurement; microparticles are magnetically captured onto the surface of the electrode on the measuring cell, the sample and reagent mixture is transferred to the measuring cell. Unbound substances are subsequently removed. Electrochemiluminescence is then induced by applying a voltage and measured with a photomultiplier. The signal yield increases with the antibody titer. The test has a threshold value of 1.0. All samples with a value < 1.0 were considered negative.
2. A validated and published in-house ELISA in the group of Prof. Ralf Wagner, with a sensitivity of 96% and a specificity of 99.3%, as previously reported (56)

2.3 Data transfer and software development

To assess the acceptance of the CoKiBa antibody tests, we designed an anonymous online survey applying our previously described 'Qnome' database and questionnaire system (www.qnome.eu, MaganaMed GmbH, Regensburg). Qnome was updated through our previews CHAMP study in the way that we generated unique IDs for the biological specimen. Each study participant has his/her own patient ID, and each biosample from that participant has an individual ID sticker on sample collection materials (tubes). These IDs are prelinked in Qnome, assuring that we can connect every participant with their respective biosamples. All data were collected in an online survey using self-administered parental questionnaires. All acquired data was fully anonymized and only accessible at an individual level to the participant using an individual code on the Qnome platform (www.qnome.eu). The parents entered clinical data in an online survey. That way, anonymization of data on the level of the dataset was achieved while the test values were directly accessible to parents.

A critical area in testing is the automated, fast, and safe forwarding of results from the laboratory to the tested person or the schools. A browser-based software tool was developed in the STACADO study and improved and updated in the WICOVIR study by IT cooperator partner MaganaMed GmbH, Regensburg, according to our specifications. The software was used to keep track of barcoded pools, pool results, pool dissolving, and allowing for automated correspondence of test results and summary statistics of test results, irrespective of the laboratory software in the participating test centers. The software was implemented in Javascript (front-end) and typescript (back-end). Data were stored on a PostgreSQL relational database management system. The database and application were hosted at an ISO27001 certified data center in Germany. A general data protection regulation (GDPR) compliant data protection concept was implemented and approved by the data protection officer. The software only handled pool IDs and alphanumeric sample IDs (unique, pseudonymized), but no personal information on participants.

During the Hospital testing, we used our COVIDA software, which we also developed in cooperation with our IT cooperator partner MaganaMed GmbH, in part based on the WICOVIR software. By using COVIDA software, we could link the individual barcode of the hospital staff

member to the pool barcode, so everyone could check his or her pool test result online by using his or her barcode.

2.4 Statistics

Data from the gargle pool tests are presented using descriptive statistics. Normally distributed data are presented as mean with standard deviation (SD) and non-parametric data are presented as the median and interquartile range (IQR). Uncensored data were compared using a Wilcoxon test, and in case of censored values, a generalized Wilcoxon test was applied using the 'survival' package in R statistics. Permutation tests were performed to calculate differences in infection rates between SARS-CoV-2-naı̈ve and immunized staff by using the 'coin' package in R statistics, version 4.1.2. A P-value <0.05 was considered statistically significant.

Regarding the antibody test descriptive statistics were calculated using frequencies (percentages) for categorical data and median (interquartile range) for metric data. Participants' characteristics and symptoms are presented stratified by antibody response. Differences between groups were analyzed using X^2-tests for categorical variables and t-test for independent groups, respectively. All analyses were performed using SPSS.23.

3 Results

At the beginning of the pandemic children were not tested due to limited PCR testing resources. Therefore, the question at that time was, how could we test children efficiently to understand how many children are affected by SARS-CoV-2 infection and what role children truly play in the pandemic. In that context primary questions were: 1. To understand the prevalence of SAS-CoV-2 infection in children after the first pandemic wave. 2. To figure out how we could establish a children-friendly and fast testing system (without losing sensitivity) and 3. To figure out if optimized testing could help to prevent infection in children. In addition, we wanted to know if systematic testing (and targeted isolation of positive children) would allow children to go back to school safely while they had no opportunity yet to get vaccinated.

3.1 Evaluating the prevalence of SARS-CoV-2 infected children after the first SARS-CoV-2 pandemic wave in Bavaria

After the first peak of the Corona pandemic, while data on children remained limited throughout the pandemic due to limited PCR testing resources, we screened a large number of children in rather severely affected areas of Bavaria (Figure 10). The study aimed to have an overall picture of the infection rate in children while the schools, kindergartens, and nurseries were closed.

Blood was taken, and the serum samples were separated from all participants. By the use of two different test assays, specific antibody response to SARS-CoV-2 was evaluated. One method was Elecsys Anti-SARS-CoV-2 assay (Roche Diagnostics, Rotkreuz, Switzerland). It is directed against the N-protein and can detect IgA, IgM, and IgG (without differentiation between them); the cutoff value is 1.0; we did this part. The second test was an in-house ELISA developed and done by the group of Prof. Wagner; this test targets the S-protein of SARS-CoV-2 and quantifies total IgG. The results of this section are already published (57).

In total, n=2,934 children participated in the study, n=2,906 were tested successfully with at least one of the two applied antibody tests, and 2,832 (96.5%) had also entered necessary study data into the Qnome online tool. The Qnome tool that we use in this study has been adapted through our previous work (for our CHAMP project) and made it possible to connect the blood tube ID to the questionnaires and give the parents access to the result of their own child.

Among them, n=161 participants were identified positively by at least one antibody test; concordance of 83.9 % (both tests positive, n=135), and discordance of 16.1 %. Overall, n=158 were ELISA positive, and n=139 were ELECSYS positive.

Tirschenreuth (with 1,638 positive PCR tests/100,000 inhabitants) had the highest incidence at that time then, followed by Rosenheim, with 1,111 positive PCR tests/100,000 inhabitants, and Regensburg, with 586 positive PCR tests/100,000 inhabitants. We observed a correlation between the incidence of the general population of the regions and the prevalence of SARS-CoV-2 antibodies in children (figure 10). From the county of Tirschenreuth, 13.1% of children were antibody positive. At the same time, in two other regions, the rate was less than one-fourth of Tirschenreuth; in Rosenheim, 3.7% of tested children were positive, and the positive rate in children in Regensburg was 3%.

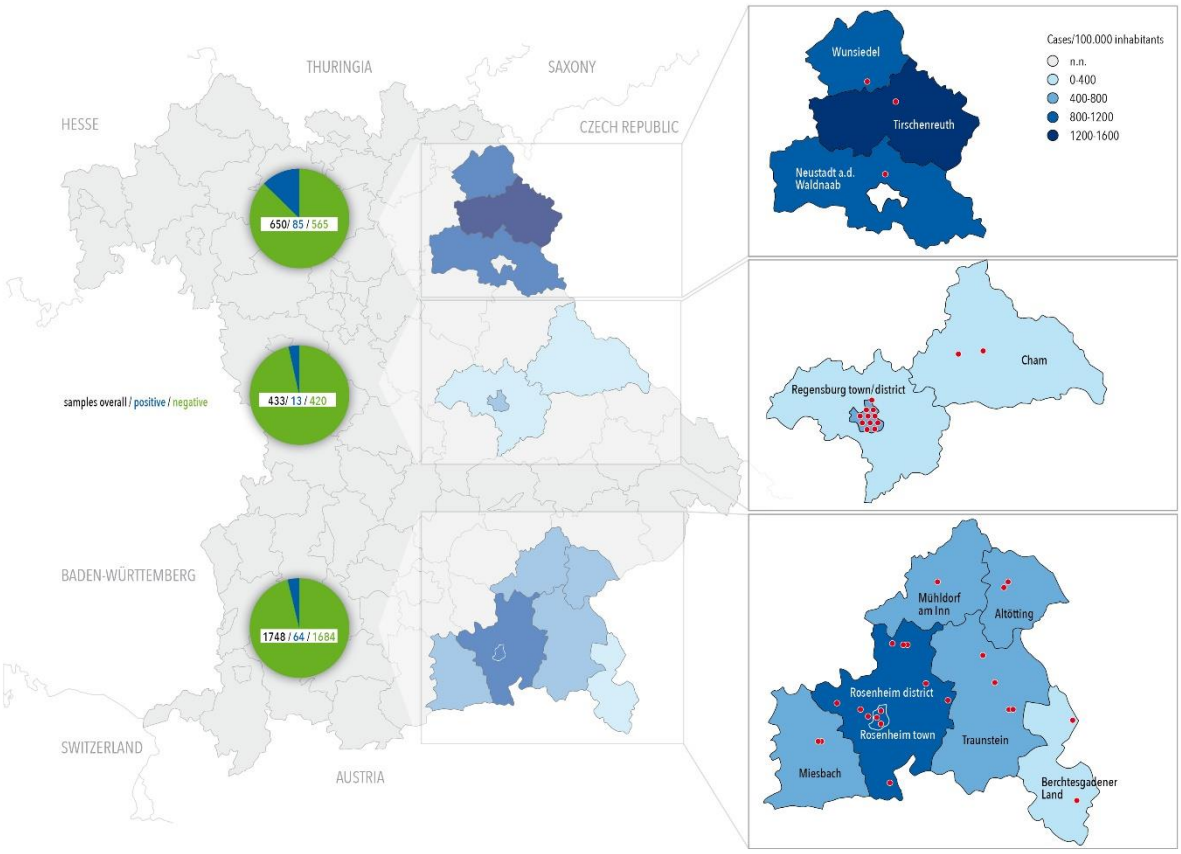

Figure 10: Bavaria map with the location of centers in the study (red dots) and COVID-19 prevalence until July 2020 (color-coded by county). Numbers for overall, negatively, and positively tested children are given in the circle chart (including also non-randomly tested children, e.g., siblings). This figure is from our publication (57) and was designed by Michael Kabesch with help from Birgit Kulawik.

Based on the questionnaire data from this population, only n=263 children had previously tested the SARS-CoV-2 PCR test. n=21 out of 263 had positive test results, while only n=15 individuals had positive antibody responses (71.4%), while in six subjects, no antibody response in any of the two tests could be found.

We extracted symptom data from the questionnaire (Table 16). We figured out that symptoms are a very poor tool to identify SARS-CoV-2 positive children since very few are differences between antibody positive and negative children. Among those symptoms, only loss of smell would be specific symptoms to distinguish COVID-19 from other common viral infections in children. Thus, it emphasizes the necessity of testing to identify SARS-CoV-2 positives.

Table 16: Symptoms of study participants after antibody measurement

Symptoms	Negative AB test (n=2670)	Positive AB test (n=161)	*p*
No symptoms, % (N)	30.1% (804)	23.5 (38)	.072
Runny nose, % (N)	42.5% (1135)	32.7 (53)	.014*
Sore throat, % (N)	28.2% (753)	18.5% (30)	.007*
Headache, % (N)	24.3% (648)	24.1% (39)	.955
Dizziness, % (N)	6.5 % (173)	4.9% (8)	.436
Exhaustion/ fatigue, % (N)	24.0 % (640)	25.3% (41)	.699
Muscle aches, % (N)	14% (373)	16% (26)	.460
Inflammation of the eyes, % (N)	4.4% (117)	3.1% (5)	.430
Loss of smell, % (N)	1% (27)	4.9% (8)	<.001*
Loss of taste, % (N)	2.4% (64)	6.8% (11)	.001*
Shortness of breath, % (N)	5.1 % (137)	3.7% (6)	.420
Coughing, % (N)	41% (1096)	30.9% (50)	.010*
Fever, % (N)	37.6% (1004)	38.3% (62)	.865
Chills, % (N)	7.3% (194)	3.7% (6)	.086
Rash, % (N)	5.3% (142)	2.5% (4)	.111
Diarrhea, % (N)	16.5% (441)	13% (21)	.235
Nausea, % (N)	11.4% (304)	9.9% (16)	.556
Loss of appetite /difficulty feeding, % (N)	11.2 % (298)	5.6% (9)	.026*
Other symptoms, % (N)	2.5 (66)	2.5 (4)	.998

Notes: * $p < .05$; chi² test

3.2 Developing a testing system

To be able to test many children frequently, we needed to develop a testing system that is highly cost-efficient, specific and sensitive, painless, and acceptable to children.

3.2.1 Comparing the sensitivity and specificity of antigen tests and RT- qPCR by using Gargle and Swab samples

First, we aimed to study if, instead of nasal swabs, we could use gargle samples, which are more children-friendly, in combination with antigen tests, which promised rapid results with little technical investment. Thus, we compared the sensitivity and specificity of a fluorescence-based antigen-test (STANDARD™ F COVID-19 Ag FIA kit (SD BIOSENSOR Inc., Suwon-si, Korea) with those of RT-qPCR, first by using nasopharyngeal swabs and then by using gargle samples. The results of this section are already published (58).

For that purpose, we used the SD BIOSENSOR FIA test, one of the most sensitive antigen tests available on the market. For comparisons, we also used two other widely used antigen tests CLINITES Rapid COVID-19 Antigen Test (SIEMENS Healthineers., Houston, TX, USA) and NADAL COVID-19 Ag test (Ref.243103N-20, nal von minden., Moers, Germany). As is shown in Table 17, the SD BIOSENSOR FIA test was indeed the most sensitive of these antigen tests to identify predefined SARS-CoV-2 positive samples in our basic experiments.

Table 17: Detection limits of different antigen-test kits in comparison to RT-qPCR positive gargle samples

Sample Number	Ct value by RT-qPCR	SD BIOSENSOR /COI	CLINITES	NADAL
1	20.1	Positive/2.59	Positive	Negative
2	21,8	Positive/2.03	Negative	Negative
3	22,6	Positive/2.11	Positive	Negative
4	24,1	Negative	Negative	Negative
5	26,1	Negative	Negative	Negative
6	28,3	Negative	Negative	Negative
7	30,5	Negative	Negative	Negative
8	32,6	Negative	Negative	Negative

To compare the sensitivity and specificity of the SD BIOSENSOR FIA antigen test and RT- qPCR, we established a collection of leftovers from n=311 nasal swabs and n=309 gargle samples. Out of these, 47 swab samples and 64 gargle samples were determined to be positive by standard and validated RT-qPCR. On all of these samples, we used the SD BIOSENSOR FIA antigen test.

Figure 11 represents the correlation between Ct values from RT-qPCR (left side) and COI values from antigen tests (right side). Positive samples that were false negative on antigen tests are shown in red. The false-negative rate was much higher in gargle samples than in swabs; 16.18% (50 out of 309) of gargle RT-qPCR positive, and 1.92% (6 out of 311) of swab RT-qPCR positive samples were negative on the antigen test.

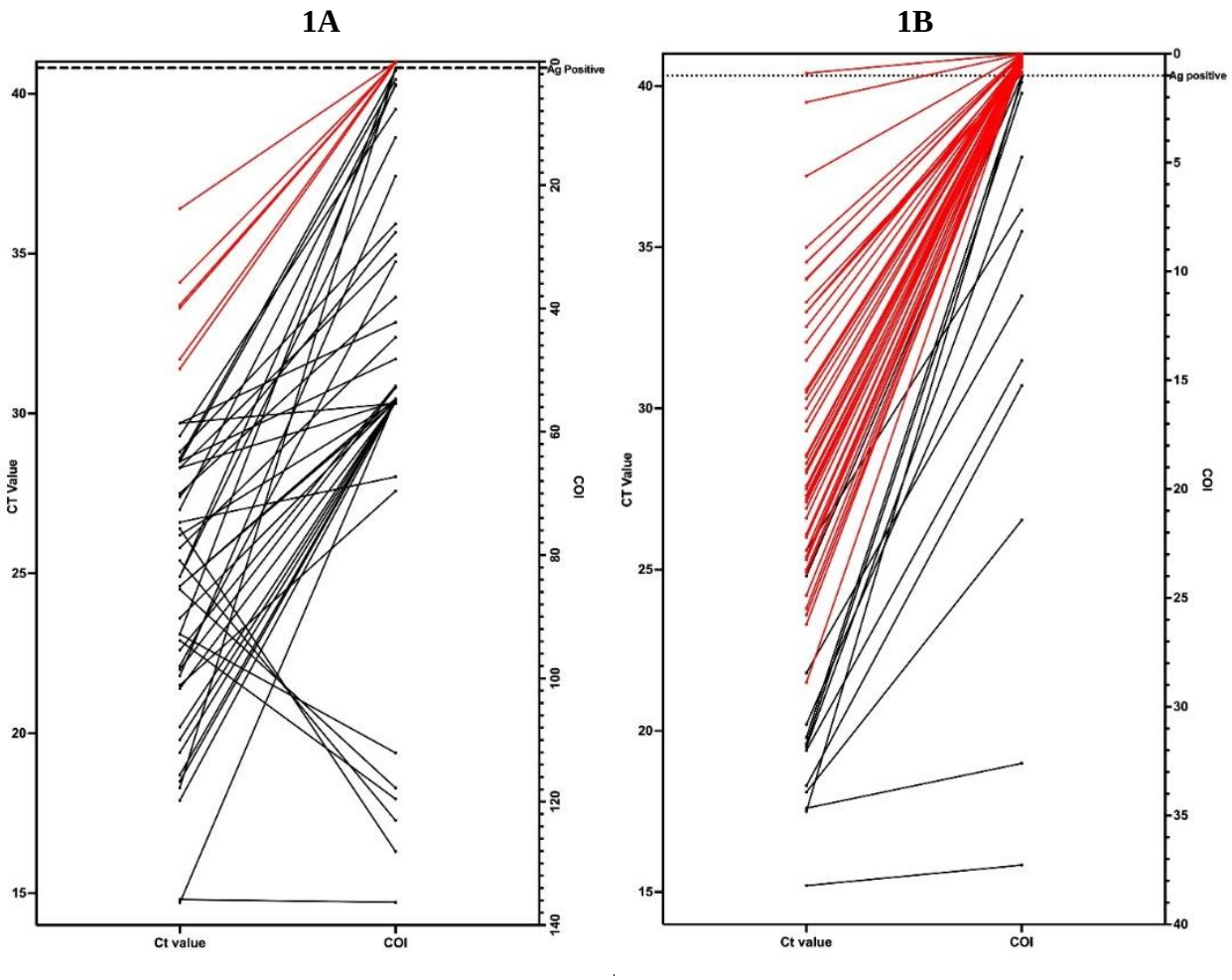

Figure 11: Correlation between Ct values from RT-qPCR (left side) and COI values from antigen tests (right side). Positive samples that were negative on antigen tests are shown in red. (A) (on the left) shows the results in 47 PCR-positive swab samples. (B) (the right one) shows the results in 64 PCR-positive gargle samples. This figure is from our publication (58) and was designed by Paratoo Kheiroddin.

The Ct value of the positive samples with their antigen COI results from figure 11 are shown in Table 18, 2A (gargle samples) and 2B (swab samples).

Table 18: Comparisons of RT-qPCR and antigen test results with (2A) positive gargle and (2B) swab samples. Samples are sorted based on viral load according to PCR results (Ct-values).

2A **2B**

(Positive gargle samples result with antigen test) **(Positive swab samples result with antigen test)**

Number	PCR Ct	Ag test result	COI	Number	PCR Ct	Ag test result	COI
1	**15.2**	**Positive**	**37.28**	1	**14.7**	**Positive**	**54.75**
2	**17.5**	**Positive**	**1.13**	2	**14.8**	**Positive**	**136.29**
3	**17.6**	**Positive**	**32.6**	3	**17.9**	**Positive**	**55.11**
4	**18.1**	**Positive**	**21.43**	4	**18.3**	**Positive**	**1.18**
5	**18.3**	**Positive**	**15.25**	5	**18.5**	**Positive**	**54.87**
6	**19.4**	**Positive**	**14.1**	6	**18.7**	**Positive**	**54.84**
7	**19.5**	**Positive**	**4.74**	7	**19.4**	**Positive**	**55.27**
8	**19.6**	**Positive**	**1.81**	8	**19.8**	**Positive**	**55.04**
9	**19.6**	**Positive**	**1.81**	9	**20.2**	**Positive**	**54.9**
10	**19.8**	**Positive**	**1.3**	10	**21.4**	**Positive**	**55.23**
11	**20.2**	**Positive**	**8.16**	11	**21.5**	**Positive**	**69.62**
12	21.5	negative	0.39	12	**21.8**	**Positive**	**18.56**
13	**21.8**	**Positive**	**11.13**	13	**22**	**Positive**	**52.84**
14	23.3	negative	0.04	14	**22.1**	**Positive**	**32.42**
15	23.6	negative	0.51	15	**22.6**	**Positive**	**52.58**
16	23.6	negative	0.54	16	**22.9**	**Positive**	**119.54**
17	23.8	negative	0.16	17	**23.1**	**Positive**	**112.08**
18	24.2	negative	0.46	18	**23.1**	**Positive**	**1.11**
19	**24.8**	**Positive**	**1.03**	19	**23.6**	**Positive**	**54.97**
20	24.9	negative	0.09	20	**24.5**	**Positive**	**117.76**
21	25	negative	0.62	21	**24.6**	**Positive**	**54.63**
22	25	negative	0.62	22	**24.6**	**Positive**	**55.14**
23	25.3	negative	0.39	23	**24.9**	**Positive**	**3.69**
24	25.4	negative	0	24	**24.9**	**Positive**	**12.3**
25	**25.6**	**Positive**	**7.18**	25	**25.4**	**Positive**	**123.01**
26	25.6	negative	0.25	26	**25.8**	**Positive**	**44.68**
27	26	negative	0	27	**26.2**	**Positive**	**55.13**

28	26.6	negative	0	28	**26.4**	**Positive**	**128.04**
29	26.6	negative	0.03	29	**26.6**	**Positive**	**67.3**
30	26.9	negative	0.01	30	**27**	**Positive**	**3.91**
31	27.1	negative	0.41	31	**27.4**	**Positive**	**27.68**
32	27.2	negative	0.07	32	**27.5**	**Positive**	**38.18**
33	27.3	negative	0.33	33	**28.3**	**Positive**	**31.29**
34	27.3	negative	0.31	34	**28.3**	**Positive**	**55.14**
35	27.5	negative	0.1	35	**28.5**	**Positive**	**2.86**
36	27.6	negative	0.29	36	**28.5**	**Positive**	**48.2**
37	28	negative	0.31	37	**28.6**	**Positive**	**1.45**
38	26.1	negative	0.04	38	**28.8**	**Positive**	**26.31**
39	28.1	negative	0.07	39	**29.3**	**Positive**	**7.72**
40	28.3	negative	0.08	40	**29.7**	**Positive**	**55.4**
41	28.5	negative	0.18	41	**29.7**	**Positive**	**42.26**
42	28.56	negative	0.17	42	31.7	negative	0.01
43	29.3	negative	0.09	43	36.4	negative	0.05
44	29.3	negative	0.05	44	33.3	negative	0.04
45	29.6	negative	0.16	45	31.4	negative	0.18
46	30	negative	0.13	46	34.1	negative	0.2
47	30.3	negative	0.13	47	33.4	negative	0.02
48	30.5	negative	0.13				
49	30.6	negative	0.02				
50	30.6	negative	0				
51	31.5	negative	0.25				
52	32.06	negative	0.25				
53	32.54	negative	0.15				
54	33	negative	0.3				
55	33	negative	0.61				
56	33	negative	0.83				
57	33.3	negative	0.95				
58	34	negative	0				
59	34.03	negative	0.09				
60	34.54	negative	0.05				
61	35	negative	0.02				
62	37.21	negative	0.02				
63	39.5	negative	0.01				
64	40.4	negative	0				

Regarding the negative samples, we had n=220 antigen-negative results from both swab and gargle samples, which we confirmed as negative samples by RT-qPCR. However, the false positive rate, when the antigen-test was positive and RT-qPCR was negative, was higher in swab samples, 11.57% (36 out of 311), than in gargle samples, 8.09% (25 out of 309).

Based on these experiments, we concluded that antigen-test sensitivity was higher in swab samples (83.92%) than in gargle samples (75.73%). Antigen-test sensitivity for PCR-positive samples up to Ct values 30 (15-20(n=8), 20-25 (n=17), and 25-30(n=16)) was 100% in swab samples. While for positive gargle samples, 100% sensitivity was only observed in samples with Ct values 15-20 (n=10). The sensitivity dropped dramatically as the Ct value increased in gargle samples. In the group of PCR positive gargle samples with Ct values 20-25 and 25-30, antigen-test efficiency went down to 25% (n=12) and 4.1% (n=24) respectively. Thus, in our setting, even high-performing fluorescence-based antigen tests did not detect SARS-CoV-2 in samples with Ct values above 30 in any of the specimen, neither in swab nor in gargle samples.

We examined if the dilution effect of gargle samples may be the reason for the reduced sensitivity of the test. Therefore, we used a lower Cut-off Index (COI) in the SD BIOSENSOR test for gargle samples, as shown in Table 19. We got reasonable higher sensitivity and unreasonably lower specificity after using lower COI in positive gargle samples.

Table 19: Effect of lowering the COI from 1 to 0.1 on sensitivity and specificity of detecting PCR-positive and negative gargle samples

Sensitivity: efficiency of detecting positive gargle samples (n=64)	COI	Specificity: rate of false positive of negative samples (n=176)
65,63%	0,1	59,66%
51,56%	0,2	39,20%
45,31%	0,3	26,70%
35,94%	0,4	21,59%
32,81%	0,5	19,32%
29,69%	0,6	12,50%
25,00%	0,7	8,52%
25,00%	0,8	6,82%
23,44%	0,9	3,41%
21,88%	1	0,00%

Simply combining the gargle procedure with current antigen tests failed. Based on our experimental data, the antigen test can only reliably detect positive swab samples when the Ct value is lower than 30, and the threshold is even reduced to Ct values < 20 if gargle samples were used. Indeed, our results show that antigen tests are not sensitive enough, not with swabs but definitely not with gargle samples, to detect SARS-CoV-2 positive individuals early with a low virus load (relating to Ct values above 30). Therefore, we concluded that antigen tests would not be helpful for preventive testing and other strategies had to be developed.

3.2.2 Sensitivity and specificity of pooling gargle samples to detect SARS-CoV-2 using RT-qPCR

Next, we focused on the combination of gargle samples and pool PCR tests. By pooling, we would reduce the cost of RT-qPCR tests and increase PCR capacity for mass tests. The question was if we would significantly lose sensitivity by this procedure. To test for this possibility, we did RNA isolation and RT-qPCR of positive gargle samples with different virus loads as a single sample and diluted the same sample in a pool of 20 negative individuals (pool of 21) (table 20), and performed RT-qPCR for two different SARS-CoV-2 specific genes on these samples.

Table 20: RT-qPCR Ct values of 2 SARS-CoV-2 signature genes in a single gargle sample (left panel) and dilution of that sample in a pool of 21 individuals (right panel)

Sample Number	Ct values of the Single sample		Ct values of the Same sample in pool of 21	
	N2	ORF1b	N2	ORF1b
1	22,89	22,88	26,91	26,93
2	25,32	25,8	30,24	30,3
3	26,31	26,75	29,7	29,62
4	27,98	2786	31,85	31,45
5	31,21	30,54	34,24	33,93
6	32,85	32,01	35,36	36,5
7	33.04	31.82	34.64	34.92
8	34.63	33.70	36.60	34.21
9	35,80	35,85	_	_

Indeed, our result shows that we lose sensitivity by pooling but not to the degree that would hinder the application for mass testing. By using 21 gargle samples in a pool, we could still reliably detect single positive samples up to Ct values of 35 in that pool. Based on these proof of concept experiments we were confident that by using this procedure, we could now significantly increase detection capacity compared to a single PCR test. In a pandemic situation, faster detection of positive individuals may be crucial. Thus, using gargle pools in combination with RT-qPCR may be the key to successfully breaking infection chains.

3.3 Applying pool gargle testing at schools to detect SARS-CoV-2

So far, our results had shown that the gargle pool PCR is a reliable method to detect positive samples up to Ct 35, and Ct value 35 in our PCR settings translates to 1.000-copy numbers per milliliter as we already reported in the material and methods section. Thus, this limit is sufficient to detect infections so early that viral load is still so low that infection transmission is very unlikely. Next, we asked whether this gargle pool PCR test system could be applied in practice at schools and could detect positives early enough to separate the potential positive individual early, and by that if it would be possible to avoid SARS-CoV-2 outbreaks at schools in a real-life situation.

3.3.1 Pilot study to investigate the setup of school testing to prevent outbreaks: STACADO (Study to Avoid Outbreaks of Coronavirus At the DOmspatzen School)

To explore the feasibility of gargle pool PCR testing in the school setting and assess the possibility of preventing outbreaks by repeated mass testing, we performed a pilot project at the Domspatzen school, a world-famous boys' choir dating back to the year 975. The special risk at that school is the choir singing which was associated with a high super-spreading risk early in the pandemic. To maximize the prevention of outbreaks of SARS-CoV-2 despite ongoing choir activity, we reasoned that early isolation of positive choir members before spreading the virus, would add to the already

established non-pharmacological interventions (NPIs) which seemed reasonable and achievable by such a sensitive test regimen based on gargle pool testing.

Domspatzen was a perfect partner as due to choir singing, the need of the school for further safety interventions was great and acceptance of the study was estimated to be high. A close health cooperation between the University children's hospital and the school had already been established previously and thus, access to the school was relatively easy. The 1st phase of the study was a 16-week evaluation period. We mainly focused on the establishment of the logistic process of establishing a workflow and developing turnaround times of the test result to isolate the potential positives from the rest of the class early enough to prevent the spreading of the virus.

The gargling and pooling process is explained in detail in the Material and Method section as it was developed in that pilot phase of the study. Briefly, we asked participants to gargle; gargling was performed with the Saline solution first and was changed soon to distilled water due to the request and better acceptance by students; participants gargled on two days per week in the morning before attending school (Paper under submission).

During the pilot study, the individual samples were registered and collected in the school and transported (app. 2 hours) to the partnering laboratory in Weiden, where samples were pooled; each pool contained only 5 participants due to capacity-limitation for depooling of positive pools. In case of a positive pool, the school was informed that choir rehearsal in the afternoon was canceled for that group. Regarding depooling of the positive pool, laboratory personnel did depooling by using the single leftover samples from the participants in the positive pool.

With the partner MaganaMed we developed and established software (based on software we had developed earlier to manage study samples in our CHAMP asthma project) that retrieved laboratory results automatically from the laboratory via a secure connection and distributed results via emails to the school's managers based on the school ID of the samples.

During this time, our focus was to develop the workflow, and since we did not have the equipment for massive testing available in our own laboratory, the PCR tests were performed in the Synlab laboratories in Weiden, Oberpfalz. In that phase, we had to use the pooling system as developed by Synlab, which was limited to 5 samples, and pooling was performed in the laboratory in Weiden.

In total, n=2,148 samples were tested in n=864 pools along with 56 additional single PCR tests (for quality control of ambivalent primary results) from September 2020 to March 2021. None of the gargle pool tests was positive. However, one positive student was identified outside pool testing during that time: A 14-year-old choir boy was infected, most likely by a family member, directly after testing negative in the STACADO pool PCR. Initially, he developed only mild symptoms not suggestive of COVID-19, and thus, he attended one choir practice and participated in the choral at Regensburg cathedral the following day. A day later, he had full symptoms and tested positive for SARS-CoV-2 by PCR. Due to the setup of regular testing in the choir, choir boys were tested repeatedly within a time span of 14 days after the event. None of the 50 directly exposed choir members became infected.

Through this study, we established a gargling procedure for students at school, sample collection, registration, and transportation workflow, and the software for rapid and safe data transfer. However, we also saw the limitations, including the slow speed and limited capacity of the testing procedure with a local commercial partner, which was not scalable to larger masses. Sample registration and transport were not robust enough and were too cumbersome. We needed improvements to be able to apply the testing system to a bigger scale and in more schools.

3.3.2 Rollout of preventive school testing by gargle pool RT-qPCR: The WICOVIR project (Where Is the Corona Virus?)

First, it was crucial to optimize fast and reliable RNA isolation and PCR testing system with a higher capacity for pooling and depooling tests than the one we used in the pilot study in collaboration with a commercial laboratory.

To establish such a testing system, we first tried to adapt the protocol from our partner laboratory in Erlangen that was based on direct lysis pool qPCR. In their protocol, they lyse the samples, break the viral protein capsid by heating, and use it as a template for the qRT-PCR reaction, which detects N1 and N2 genes of the SARS-CoV-2 virus. After first trials with this protocol using existing equipment in our laboratory in Regensburg, we found that their method did not work reliably with

our (rather old) existing equipment in Regensburg. Thus, we next contacted further research groups working on pool PCR test setups. Finally, we exchanged protocols and visited two further laboratories that were in the process of establishing massive COVID testing in Austria and were leading the field internationally at that time.

In the group of Johannes Zuber at the Vienna BIOCENTER, they used both RNA isolation and lyses method to have the viral material and then ran them as templates for the qRT-PCR, detecting UTR and ORF10 genes of SARS-CoV-2. The author of the thesis visited the group for two days. However, their protocols for sampling, collecting, processing, and reporting results were not applicable to our aim; they also pooled the samples in the laboratory via laboratory personnel, and the maximum participant in each pool was n=10. In addition, their method needed a long time of sample processing, from the time they had the samples in the laboratory until they had the RNA ready was around 2.30 hours, and then 1.30 hours for the PCR. Therefore, the shortest time they could identify the positive pool was 4.30 hours after receiving the samples. Thus, their protocol did not work with our aim since we wanted to inform the school about the positive pools until the students were still at school and could do the deepoling on the same day.

Next, we visited the group of Daniel Wallerstorfer at NOVOGENIA GmBH (Eugendorf, Austria). There, they also pooled the samples in the laboratory. RNA isolation was done by Auto-Pure96 Nucleic Acid Purification, that we found very fit with our setting and aim. Then they used the Fast Track Diagnostics (FTD) SARS-CoV-2 assay that could detect the N and Orf1ab genes of the virus. After an overview of the partner's protocols and testing system based on our purpose, which is testing students at school and considering the global supply-chain disruptions during the pandemic, we designed and established our own testing system that worked perfectly with our aim.

In the first step, we designed the triplex PCR to detect two SARS-CoV-2 genes (ORF1b and N2 gene) and one human gene (Rnase P gene) as an internal control to ensure that the testing system worked fine in negative samples (all the primers and probes sequences and PCR protocol information are available in Method section). RT-PCR-based SARS-CoV-2 RNA detection was performed on the BIORAD Real-Time PCR.

It turned out that the most challenging part of the test system was establishing a fast and reliable RNA isolation system for massive testing. As we figured it out after visiting NOVOGENIA, Auto-Pure96 Nucleic Acid Purification System (Hangzhou Allsheng Instruments, Shanghai, China) was the best option for our purpose. However, we needed to adjust the machine isolation program and finalize the best final volume elution buffer to avoid contamination from one well to the next well on the plates (the final protocol is shown in the Method section). This machine works semi-automated, and the preparation does not take long; as we found out later during the studies, it is also possible to prepare all the washing and final plates in advance, seal them and use them for one week. Combining the Allsheng extraction robot with the MagnifiQ™RNA buffer kit (A&A Biotechnology, Gdansk, Poland) made it possible to isolate RNA from the 96-well plate in 27 minutes.

After establishing the RNA isolation and PCR system, we had to make some adaptations in the laboratory processing to handle the high number of samples (maximum number of n=655 pools (of up to 21 samples per pool= 13.755 tested individuals per day) and process them in the shortest possible time, as the registration of samples in the laboratory information system was now the speed limiting part in testing.

As the first step in the laboratory, 1ml of the pool was transferred to the matrix tube to handle that high number of samples in a semi-automated way. This transferring step also prevents cross-contamination since the transferring was performed one by one, then the matrix tubes were put in the 96 well plate; by having that transferring of pool samples to the isolation plate was possible to be done once by using a pipetting robot (INTEGRA,VIAFLO 96-Channel) (Figure 12, step 2). Next, the barcode of the pool and the matrix tube were connected via the WICOVIR software for sample tracing and automated reporting of results to the school by the software. By connecting the tube and pool ID quickly with the software, we did not need to label the matrix tubes; simply by scanning the matrix tube, we could identify the pool ID, which saved a lot of time. Also, in the WICOVIR software, the matrix tube location on the 96-well plate was registered automatically based on the scanning order; this reduces the risk of losing track of tubes in case of any changes in the location of the tubes by accident later in the laboratory.

After that, all the samples in the 96-well plate format underwent RNA extraction and RT-qPCR test, as shown in Figure 12. With all these adaptations and improvements, we managed to process the samples very fast; the whole process, from having the pools in the laboratory to sending the result out, took around 2.30 hours.

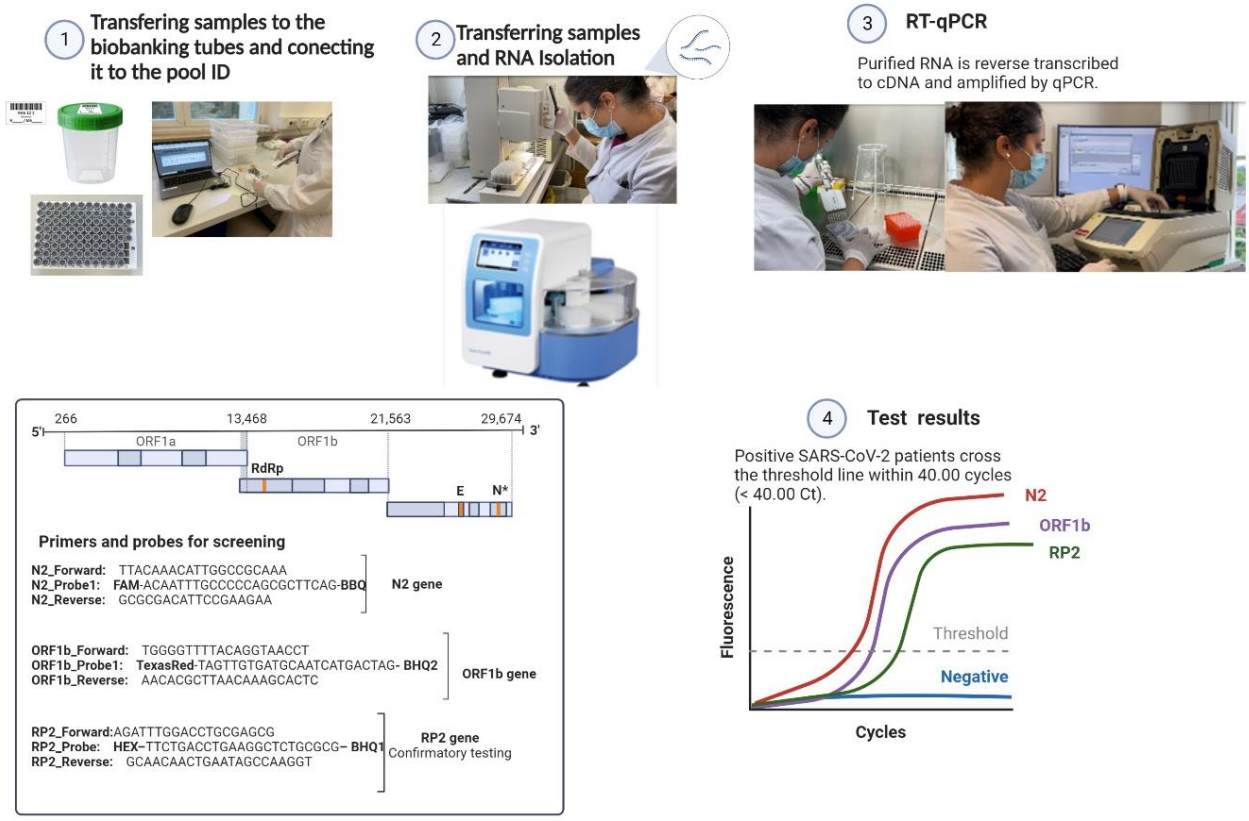

Figure 12: Laboratory workflow and testing process. The figure is showing sample processing as it explained in detail above.
Created with BioRender.com

In parallel, based on our RNA isolation and PCR setting, we further improved the workflow and logistics process to have the pool results faster and be able to do deepoling by the same day. During this study phase, we asked the schools to prepare the pools and only transferred the pools to the laboratory, as shown in Figure 13. If a pool was positive, the school provided us the single samples of the participant in that pool.

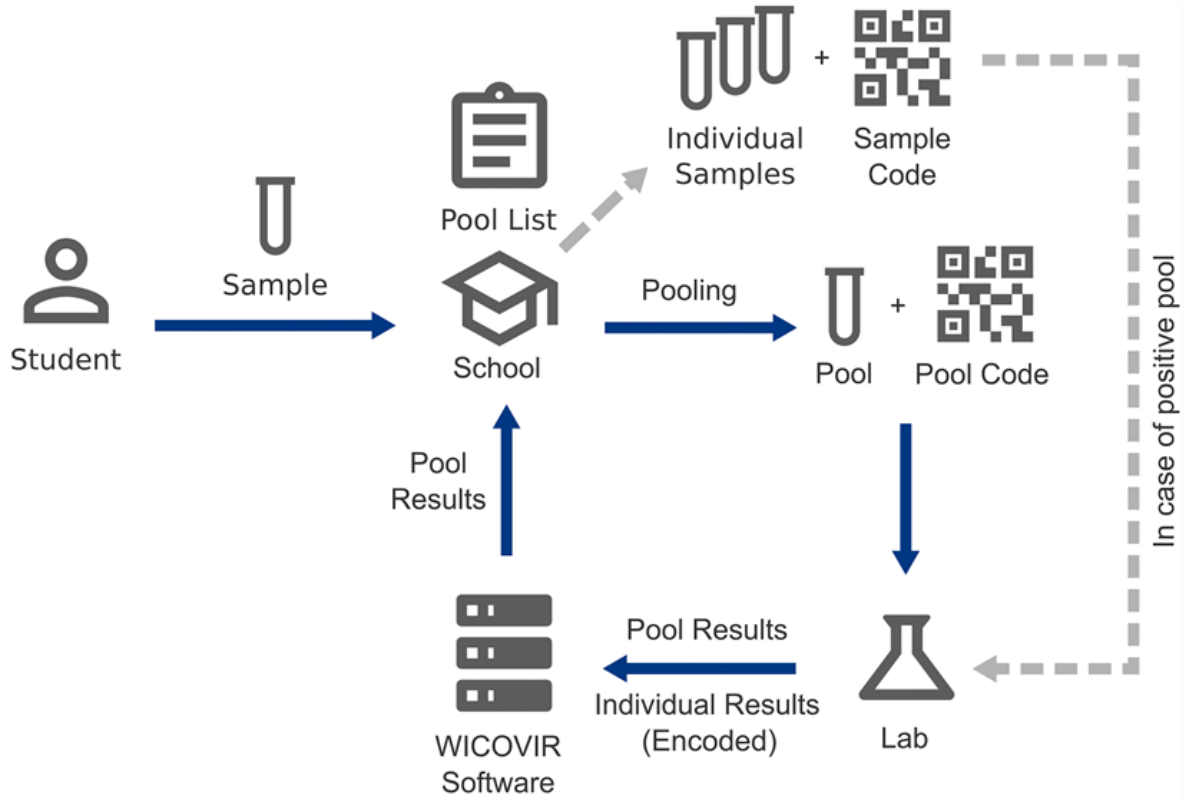

Figure 13: Sample/data flow and data protection. At schools, samples were pooled. Pools were barcoded and sent to the laboratory. Pools were registered in the software by laboratory personnel, and later the results were sent to schools via software. In the case of a positive pool, individual samples were collected, labeled, and sent to the laboratory for pseudonym testing. This figure is from our publication (59) and was designed by Philipp Pagel.

Pooling at schools, which was an idea from the project partners in Erlangen, massively saved extra work time for laboratory personnel when we tested many schools. In addition, we did not need space to keep that amount of single backup tubes in the laboratory, and it did not produce huge plastic waste after each testing day since the personal tubes stayed with the individual, were washed at home, and did not need to be discarded. However and foremost, it was the handling time in the laboratory that could massively be reduced by pooling in schools by the individuals themselves. Thus, this allowed much shorter turn-around times for test results.

Participants gargled with 5-6 ml of tap water twice a week at home and divided the gargle samples into two falcon tubes. Then, students emptied one tube into a pooling container in a pooling station

(usually in front of classrooms); the other (backup) was retrieved from schools and tested only in the case of a positive pool result. Pool participants were defined by the schools and usually contained the pupils of one class and the school staff (teachers) attached to that class (Figure 14). Transport of samples and drive-through organization of sample delivery to the laboratory are described in detail in the Method section.

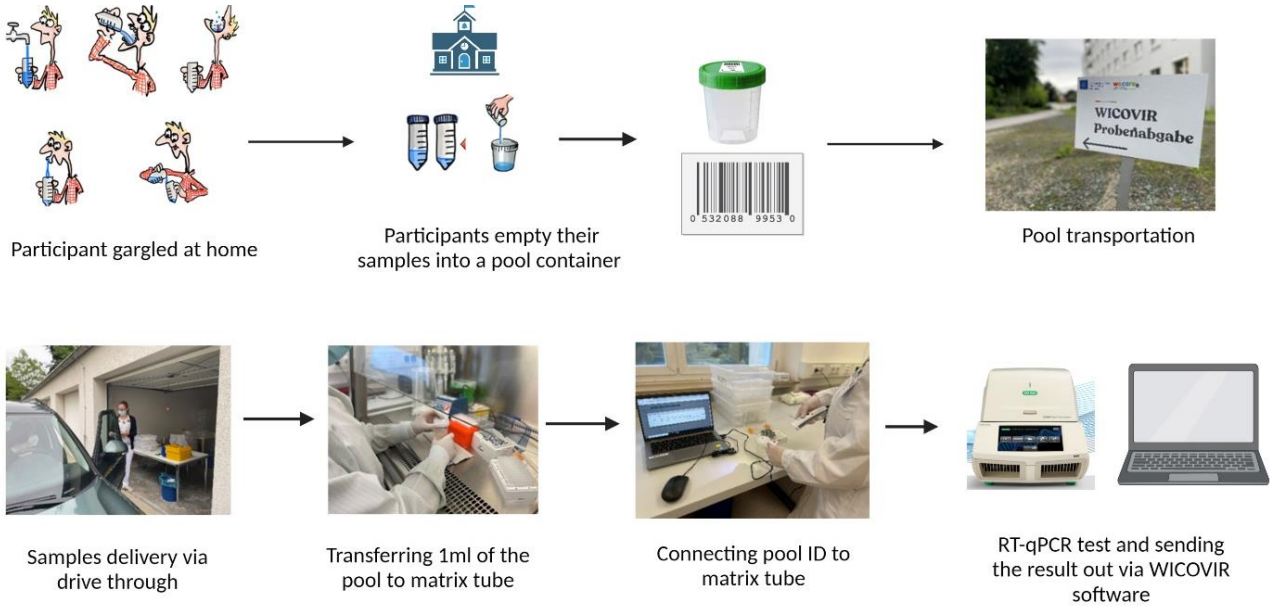

Figure 14: **Sample collection, pool preparation, and transportation**. Participants gargle at home and empty their samples in the pooling container at school. Schools make the list of the participants in each pool, label them, and transfer them to the laboratory via the drive-through organization. In the laboratory, 1 ml of the pool is transferred to a matrix tube, and the pool ID is connected to the tube via the WICOVIR software. After test processing, the result is sent out via WICOVIR software.

We asked all schools that performed both gargle pool PCR (WICOVIR testing) and self-administered antigen testing to give anonymous feedback in an online questionnaire on their experience. Significant differences in acceptance, handling, and overall ratings were observed; gargling was received significantly better than antigen testing resulting in an overall "school grade" of 1.5 for gargle pool RT-qPCR tests compared to 4.1 for antigen tests (grades 1–6, where 1 is best).

After establishing this fast testing system for massive screening of SARS-CoV-2 positives in n=54 schools in Regensburg, we explored how the system could be implemented safely and quickly in all different settings, including in remote rural areas if it should be used in all of Bavaria. We evaluated that if the system could be set up successfully in a remote and large county, it would be robust enough for all Bavarian counties.

The county of Cham (128.094 inhabitants as of 31.12.2020, 1.527 km2), located 65.4 kilometers away from Regensburg, had a seven-day incident exceeding 200 COVID-19 cases per 100.000 inhabitants in March 2021, which was one of the highest at that time in Bavaria and all of Germany, participated in the project. From Cham, n=38 schools, including n=4300 students and teachers, participated in our study in the format of n=215 pools and tested twice per week. In the beginning, the pools were sent to NOVOGENIA GmBH (Eugendorf, Austria), and we only performed the depooling test in the case of the positive pool, while from June 4th, the PCR testing of all the pools and depooling of the positive pools were performed exclusively by us.

In addition to the Cham that we tested, other counties/cities also participated in the study, including:

1. Schwandorf county: n=4 schools participated in the study. Pooling and depooling tests were performed in the Kneissler laboratory in Burglengenfeld.
2. Tirschenreuth county: n=8 schools participated. Pooling and depooling tests were performed by Scheiber laboratory, located within the county in Waldsassen.
3. Nuremberg city: n=13 schools participated. Pooling and depooling tests were performed by DATEV laboratory, located in Nuremberg.

Each laboratory used specific methods and setups to prepare the viral material (RNA isolation or lyses) and RT-qPCR test. We designed the ring experiment twice during the study with real gargle pools with blinded samples of predetermined positive and negative pools: We evaluated different techniques in different laboratories and confirmed if the results were comparable. The ring test was

designed so that all laboratories performed the test on the same day; the result is shown in Table 21, which is already published (60).

We show here how the WIOCIVR protocol can be successfully implemented within four weeks in rural and urban regions with minimal effort, making use of preexisting logistical structures and laboratory testing facilities or creating new regional collaborations.

Table 21: **Results of ring experiments in participating laboratories**

Blinded sample		Regensburg/ Cham	Kneissler/ Schwandorf	Scheiber/ Tirschenreuth	DATEV/ City of Nuremberg
Positive gargle sample	Results test 1	Positive CT values: N2 gene: 31.64 ORF1b gene: 31.59	Positive CT value: N gene: 39.3	Positive CT values: E gene: 35.01 RdRP gene: 35.12	Positive CT value: N1 gene:33.62
Negative gargle sample		negative	negative	negative	negative
Positive gargle sample	Results test 2	Positive CT values: N2 gene: 32.36 ORF1b gene: 31.9	Positive CT values: N gene: 34.87 S gene:33.14	Positive CT values: E gene: 33.6 RdRP gene: 36.7	Positive CT value: N1 gene: 35.18
Negative gargle sample		negative	negative	negative	negative

Besides all the advantages of our WICOVIR setup, including easy setup, reliability, high sensitivity, highly acceptable by students and school managers, high capacity for running samples (94 pools per run, of up to 21 samples per pool means =1,974 individuals), quick and fast, and low plastic needs for sampling, it is also very cost-efficient. For regular massive screening price of the test per individual plays a critical role. We proved that we have found that gargle pool testing can be provided at an overall cost (including transport, personal, equipment, and consumables) of <1 EUR per person tested. We calculated the RNA isolation and PCR price per plate in detail in Table 22, which costs around 4.44€ per pool and calculating with an average of 21 individuals per pool

in the school setting, laboratory material costs of approximately 20 Eurocents per tested individual.

Table 22: WICOVIR laboratory cost per plate

	Item	Company	total price	price per plate
1	Luna Probe One-Step RT-qOCR Kit #E3007E	New England Biolabs GmbH	1,738.00 €	79.00 €
2	Primer probes	Eurofins	700,20 €	23.34 €
3	MagnifiQ RNA Set (1920) #602-1920-S (isolation kit)	Hamann Laborautomation	4,032.00 €	161.28 €
4	Pure Ethanol per 500ml		68.10 €	34.05 €
5	1.4 ml Matrix tubes with barcode 1 pack (960 tubes)	Micronic	264.40 €	26.44 €
6	Screw Caps for externally threaded tubes 96-well format - Grey(MP53820) 1 pack (960 screw caps)	Micronic	211.00 €	21.10 €
7	Filter tip PP, premium surface, 0,1-10µl, super slim, transparent, Article no (07-613-8300) price per 1000	nerbeplus	56.00 €	5.60 €
8	Low retention filter tip, 1000 µl XL, Biosphere ® plus, transparent, , price per 1000	nerbeplus	56.00 €	5.60 €
9	Filter tip PP, premium surface, 100µl,200 super slim, transparent, Article no (07-613-8300) price per 1000	nerbeplus	56.00 €	5.60 €
10	Hard-Shell 96W Low Skrtd Wht/Clr Pkg of 50 white shell/clear well PCR plate rigid 2-component design	BIO RAD	270.00 €	5.40 €
11	MICROSEAL B ADHES SEAL,100/PK	BIO RAD	200.00 €	2.00 €
12	Combitips® advanced, PCR clean, 5,0 mL, blau, farblose Spitzen, 100 Stück	Eppendorf	115.00 €	1.15 €
13	Combitips® advanced, PCR clean, 50 mL, blau, farblose Spitzen, 100 Stück	Eppendorf	119.00 €	3.19 €
14	300 µl GRIPTIP, Filter 5 Racks of 96 Tips, V96	Integra	74.00 €	44.40 €
	total per Plate			**418.15 €**

3.3.2.1 Results of the application of WICOVIR testing at Schools.

Overall, during the 17 school-weeks of the WICOVIR study the testing system was applied in n=92 schools (located in the Regensburg and Cham). We tested the students and teachers of these schools in the format of n=16,245 pools and with n=237,093 tests. In total, we identified n=21 positive pools in schools, with an average Ct value of 34.5. The average Ct value of the single tested was 31. The highest Ct value that we could detect was single Ct:36 in the pool of n=20. Table 23 presents more detailed information on the positive pools and participants. Some parts of the results from this study are already published (59) (60).

Towards the end of the project, we also had children in the study who tested positive for the Delta variant. It was possible to identify these children early with the pool PCR test, so that no further infections occurred.

Table 23: Ct value of positive pools and the positive individual from the pool (located in the Regensburg and Cham)

ID	status	Ct Pool	pool size	Ct single	Antigen test	comment
KW8-1	student	33	15	32	not available	COVID19 residue
KW15-1	student	36	14	31	same day, negative	family positive
KW15-2	student	36	20	36	next day, negative	
KW15-3	student	39	9	36	same day, negative	
KW16-1	student	34	8	36	same day, negative	2 further students identified by contact tracing
KW16-2	student	32	14	27	same day, negative	
KW16-3	student	35	6	28	n.a.	whole family identified as positive (5 members)
KW17-1	school staff	33	12	32	same day, positive (after pool result)	
KW17-2	student	35	15	34	not available	COVID19 residue
KW17-3	student	35	13	33	not available	
KW-19-1	student	34	8	27	n.a.	whole family identified as positive (5 members)
KW20-1	student	34	9	26	same day, positive	whole family identified as positive (5 members)
KW20-2	student	26	4	24	n.a.	whole family identified as positive (3 members)
KW20-3	student	36	13	29	same day, negative	
KW20-4	student	39	10	36	n.a.	COVID19 residue
KW22-1	student	36	?	25	n.a.	sibbling positve
KW24-1	student	35	13	31	n.a.	potentially residue from old case (April)
KW26-1	student	39	18	36	n.a.	residue from old case (May)
KW28-1	student	33	13	30		Delta Variant, no other infections reported
KW28-2	student	33	17	28		delta Variant one other infection
KW29-1	student	33	20	29	n.a.	Alpha two other student infected, family unaffected (vaccinated)
	average	**34.5**		**31**		

Besides the positive students that we found via pool testing, we had two cases that we could not detect via pool testing. In one of the cases, with the help of the Cham Health Department, we could track the sample and confirm that the Ct of the sample at that time point was over our threshold:

i. In the Cham, one child took part in the pool PCR test, and this pool was evaluated as a negative pool. A day later, a child developed symptoms, and a new PCR test resulted in a Ct value of 26. Since the reserve samples for this child were still available, and we were able to retest them in close cooperation with the Cham Health Department. It was possible to show that the individual Ct value of this child was a Ct value of 37 on the day of the pool test, which is just above the safe detection threshold of the pool PCR test. Nevertheless, there was no infection in this class group, which can be explained by the fact that a virus load below the low detection limit of the pool test does not lead to infection, even with delta variants.

ii. A second case occurred towards the end of the project in a high school in Regensburg. Two students had taken part in the pool PCR test, then showed symptoms several days later. This occurred on the weekend, so they were tested by the health department test center, and they were positive. Unfortunately, due to a lack of information from the Regensburg Health Department, the exact infection situation could not be tracked. However, the time between a positive individual test and the pool PCR test was long. It is quite conceivable that the students were still negative in the pool PCR test and then became symptomatic before they would have been identified in the next pool PCR testing on Monday. Afterward, the rest of the class remained negative. However, on Friday, before they were tested, these two students had close contact with a student from another class. This student was then found positive in the pool PCR test the next Monday.

We also had one case of transmission of the virus from one to other students in the same class. This case happened when the boarding school student independently switched from pool PCR testing to

antigen testing (after this was legally possible due to the test regulation by the Ministry of Education). One student did an antigen test by himself on Sunday evening, which was negative. The student attended class the following Monday, and a day later (Tuesday), he was symptomatic and was positive for SARS-CoV2 in the individual PCR test. In this case, there was an infection in his class in close contact, which we identified by the WICOVIR pool PCR test. That student, with a positive PCR but no symptoms at the time of detection, was isolated from the rest of the class early enough, and all other students in the class continued to be tested. There was no further infection in this class.

3.4 Applying pool gargle testing at hospital to avoid Outbreaks of SARS-CoV-2 during the Omicron wave

Finally, we explored if our test system could be applicable to a hospital setting, if it would be robust through high incidence phases of the pandemic, and if it would benefit beyond standard antigen testing, which was the minimum standard for hospital testing as introduced by law as of autumn of 2021.

We applied our high sensitivity and specificity testing system to test the staff of our large university pediatric and maternity hospital to avoid mass illness and simultaneous illness in specific areas of the hospital to prevent ward lock-down and keep it safe for patients and employees even during the high incidence phase of the omicron wave. Most patients in this setting were still unvaccinated at the end of 2021, and thus, SARS-CoV-2- naïve and especially vulnerable to nosocomial infection with the Omicron variant.

We assessed how our test system could address specific challenges in testing hospital staff. Different from teachers and students, hospital staff works in shifts, is not organized in classes, cannot go into quarantine easily, and needs results even faster to provide safe service to patients. To overcome this challenge, the staff members were included at random in the pools (according to their arrival at the hospital). Therefore, a positive pool always represented members from different departments and units (even though a few members of the same unit may have been in the same pool). In addition, we assessed if pool testing can still be applied efficiently with high numbers of positive results to be expected, as was the case during the Omicron wave. The results of this section are already published (61).

For that, we analyzed a 10-week testing period at our children's hospital St. Hedwig's. We asked all participants to gargle roughly 30-60s with about 6 ml of tap water at home two or three times (based on the incidence) per week and divided into two screw-cap tubes (WICOVIR standard). In the hospital, the first thing after arrival, participants went to the pooling station and emptied their samples by themselves into a pooling container positioned, and the second tube (backup) was kept by participants in case of a positive pool result. The maximum number of participants accepted for one pool was 20 at the beginning and later reduced to 10 when the incidence was beyond 3000 infections per 100,000 individuals to avoid exceeding the testing capacity due to the high number

of single samples for deepoling the positive pools. By using COVIDA software (MaganaMed GmbH, Regensburg), the barcode of the staff member was linked to the pool barcode by an individual who was supervising the pool; the software was developed in a way that did not allow to add of more than 20 individual barcodes to the pool ID as we requested.

Overall, we performed n=8793 systematic tests translating to n=697 pool PCR runs; five pools were false positive (0.7%) during the ten weeks of study. Furthermore, we did n=852 PCR runs for depooling. During the study, by regular pool testing, we detected n=65 asymptomatic SARS-CoV-2 positive staff members, and n=97 staff members detected positive by single/individual PCR tests since they developed symptoms (Figure 15).

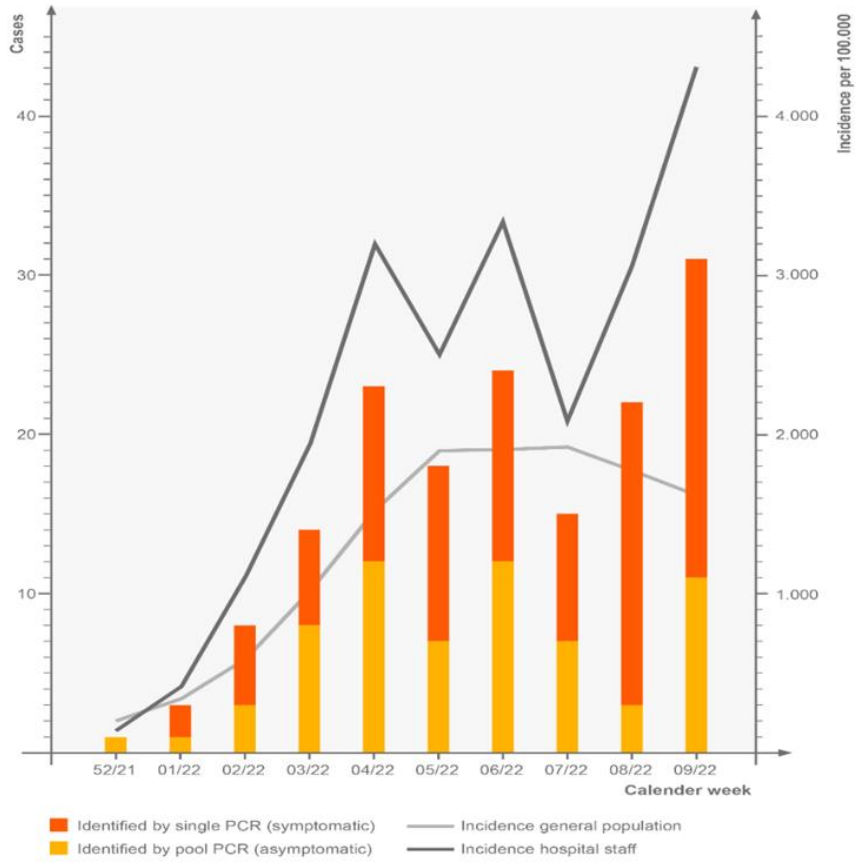

Figure 15: Weekly numbers of individuals positively tested for SARS CoV-2 by pool testing (asymptomatic) and single PCR (symptomatic) plotted against the incidence in the general population. This figure is from our publication (61) and was designed by Michael Kabesch with help from Birgit Kulawik.

Figure 16 compares the Ct value of individual backup samples analyzed in the depooling process when a pool was positive (asymptomatic pool participant) to an individual sample when a person became symptomatic (symptomatic staff member). The Ct values of the SARS-CoV-2 positive staff members identified by regular pool testing were significantly higher compared with symptomatic positive staff members detected by a single PCR test [median (IQR): 31.5 (26.4 – 33.6) vs. 26.3 (22.1 – 30.2); $p<0.001$] (Figure 16).

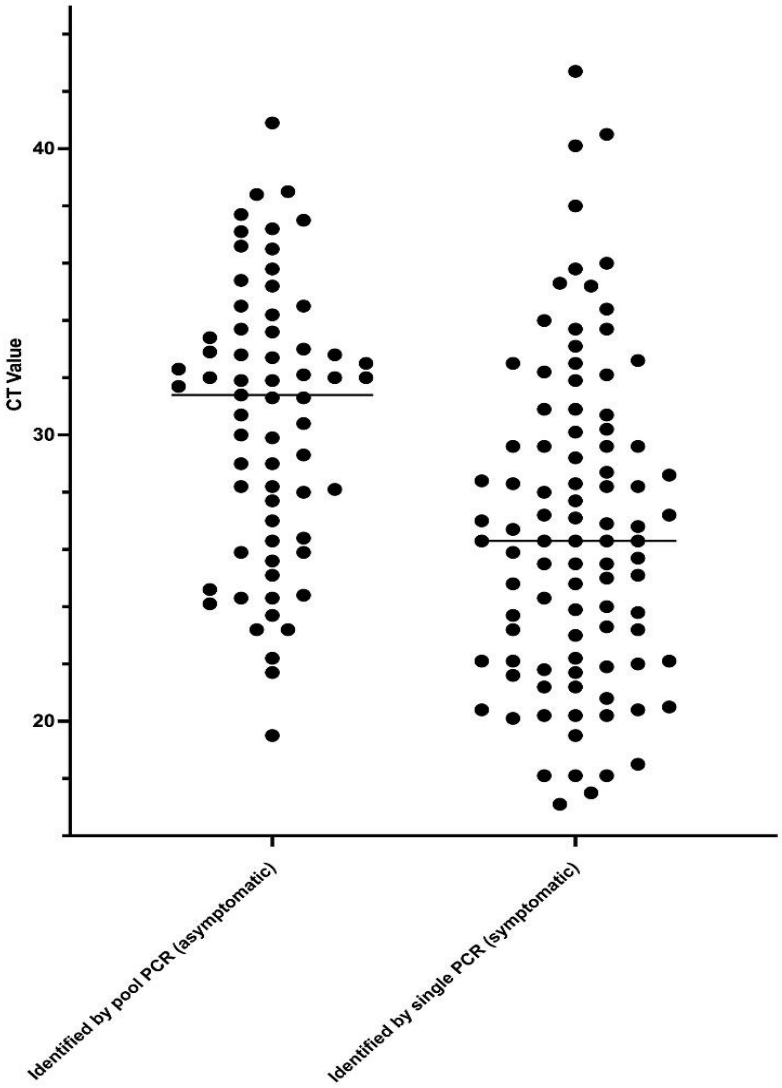

Figure 16: Ct values and median values of individuals positively tested for SARS CoV-2 by pool testing (asymptomatic) and single PCR (symptomatic). This figure is from our publication (61) and was designed by Paratoo Kheiroddin.

We could not perform gargle pool testing for one week (due to the Omicron infection of the author of the thesis during weeks 9-10 of the study). Interestingly, during that time, the Ct value of the PCR test of those that became symptomatic decreased by 2 PCR cycles, representing a higher viral load of samples at the time of detection.

During the study period, we neither observed an outbreak in a specific section of the hospital nor an increase in nosocomial infections in patients.

We compared the incidence of SARS-CoV-2 positive staff members of St. Hedwig's Children's hospital that we were identifying through the study with the weekly incidence of the general Bavarian population. The incidence in our hospital staff was higher than in the general population by an average factor of 1.5 to 2 fold (Table 24 and Figure 15).

Table 24: Comparison of the incidences per week between hospital staff (by PCR testing) and the general population (as reported to the health authorities by unsystematic testing)

Calender week	Identified by pool PCR (asymptomatic)	Identified by single PCR (symptomatic)	Incidence hospital staff	Incidence general population
52/21	1	0	138	200
1/22	1	2	417	341
2/22	3	5	1111	591
3/22	8	6	1944	1019
4/22	12	11	3194	1522
5/22	7	11	2500	1897
6/22	12	12	3333	1904
7/22	7	8	2083	1920
8/22	3	19	3055	1773
9/22	11	20	4306	1617

4 Discussion

In the project leading to this thesis, first, we evaluated the prevalence of COVID19 in the first wave in 2020 retrospectively by measuring children's antibody levels across three differently affected regions of Bavaria when prospective PCR testing was unavailable to children. Correlating strongly with regional differences in the prevalence of SARS-CoV-2 infection in the general population, we next explored the possibility of establishing a prospective test system for children to allow for preventive testing in schools. This led to the development of a gargle pool PCR system for high throughput performance, which is robust, has detected all variants so far, is effective in high incidence, and is applicable in different settings with slight adaptation in logistics, including schools, hospitals, and companies, in urban and even remote rural areas. We proved gargle pool PCR to be superior to antigen tests for the purpose of preventive testing in sensitivity, specificity, acceptance, and cost. Applying the test system, we could demonstrate that children are no drivers of the pandemic and that schools can be kept free of transmissions (up to delta variant) or slow down transmission (Omicron variant).

4.1 Evaluating the prevalence of SARS-CoV-2 infected children after the first pandemic wave in Bavaria

The only way to have an overview of the number of affected children after the first pandemic wave in Germany at a time when testing children in the acute phase of the diseases was not performed due to limited PCR testing resources, was to assess the antibody response in a large number of children. However, shortly after the first wave of the pandemic, this was only possible by establishing the logistics of massive testing, so pre-existing structures such as collection sets, proper collecting data, and reporting result tools were developed in advance.

Our Qnome data software (https://qnome.eu) was developed in cooperation with our IT partner MaganaMed, based on the approach that all study-relevant documentation (including questionnaires, biosample collection protocols, and Standard Operating Procedures (SOPs) for each individual project is processed and safely stored in it. We had adapted and improved the Qnome tool through our previous work (for our CHAMP project) in the way that we generated

unique IDs for the biological specimen. Each study participant has his/her own patient ID, and each biosample from that participant has an individual ID sticker on sample collection materials (tubes). These IDs are prelinked in Qnome, assuring that we can connect every participant with their respective biosamples. In the CoKiBa study, by using Qnome we could connect the blood tube ID to the questionnaires and extract the questionnaire's data in real-time so that we could give the parents access to the result of their own child.

By having this system ready just when the pandemic started, we could react very fast after the first wave of the pandemic and performed SARS-CoV-2 antibody measurements by using two different methods on n=2832 children in three regions in Bavaria with relatively high, moderate, and average overall incidence of COVID-19 in the CoKiBa Study. One most significant challenge in this study was that we analyzed the samples while the sampling collection was still running and reported the result in the shortest time to the parents anonymously, which could only be done via our online Qnome tool.

We observed that n=161 children had at least one positive test result. We saw minor differences in test results which could be due to two different targets of the antibody tests that our project partner (Wagner laboratory) and we used, one directed against the N-protein (Roche ELECSYS, n=139 positives) and another one targeting S-protein (Wagner-ELISA, n=158). We saw a correlation between positive antibody rate in children and regional incidence; children in Tirschenreuth with the highest incidence in Germany at that time (1,638 positive PCR tests/100,000 inhabitants) had positive antibody response 3–4 times more often than in the two other test regions (Regensburg 586 positive, and Rosenheim 1,111 positives PCR tests/100,000 inhabitants).

In our study, positive test results in children for SARS-CoV-2 antibodies correlated strongly with the massive differences in prevalence between the tested regions (the Tirschenreuth hot spot). Our set-up and thus our findings are different from a study by Hippich et al. (62). They tested children (1-18 years) between April and July 2020 for SARS-CoV-2 antibodies all across Bavaria and found a general prevalence of 0.87%, while our numbers are mainly driven by testing in strongly affected regions.

Our study showed a correlation between age and positive antibody responses, with more positive SARS-CoV-2 tests in older children. Younger children were less affected (4.9%) than older children (5.7%) and youth, who showed the strongest point prevalence in our testing. (7.3%).

Based on the questionnaire data, only n=263 children tested with SARS-CoV-2 PCR previously, and n=21 had a positive PCR results. Interestingly, around 30% of positive PCR tests did not show antibody responses in our tests. This is a higher percentage than observed in a study by Sorg *et al.*, where only 0.5% of seronegative participants had previous SARS-CoV-2 infections (63). Our interpretation of this data is the rate of SARS-CoV-2 infection may even be higher than we report since not all infected children may have developed the antibody.

This study comprehensively investigated the SARS-CoV-2 antibody levels in children approximately two months after the first COVID-19 peak and showed COVID rate was very similar to the general (adult) population. It became clear very fast in the pandemic that children were not affected strongly by COVID-19. However, disturbing reports, first from the UK, Italy, and the US, emerged that children suffered from what is now called PIMS, an exclusively pediatric immune multisystemic syndrome, which developed some weeks after the infection leading to severe and even deadly diseases. In addition, long-term effects (what is now called long/post-COVID) were observed in children as early as August 2020 from our group.

As part of the German national strategy to fight the pandemic, schools, kindergartens, and nurseries went into lockdown very early on in the pandemic in Germany. In contrast to other parts of society, they remained in lockdown much longer and repeatedly. At the same time, it became clear that vaccines would be available to adults only early in 2021 and not to children due to safety concerns. In this situation, we reasoned that preventive testing would probably be the only way to keep children safe from massive infection but at the same time allow them to go back to normal life, however, at the end of 2020. No such testing system was on the horizon. Only recently, it has been reported that it is possible to increase the efficiency of lockdown by reducing the infected number by 60% if it is combined with mass testing (64). We tried to achieve that testing would replace lockdowns for children.

4.2 Establishment of a systematically testing system to prevent SARS-CoV-2 outbreak:

Developing an easy, child-friendly sample collection system that is widely accepted by project-participants

To offer testing to unaffected or asymptomatic individuals repeatedly, sampling needs to be convenient, without pain, and simple to collect. This becomes even more critical when offering frequent testing to children when sampling needs to be especially harmless and simple to be acceptable to the tested child and not to forget, their parents. Otherwise, testing will be rejected by the participants and result in low-quality samples or low participation rates due to the difficulty or unpleasantness of the sampling process.

Collecting samples that are comfortable for participants to provide in massive screening was a critical step for us, similar to other studies (65). Gargle samples are easy and painless to offer and can be performed reliably by test subjects without the involvement of health professionals. Anyone who can brush their teeth can provide a gargle sample. We showed that gargling is so simple that even first graders up from the age of 5 years and even younger Kindergarten-children (yet unpublished WICOVIR data) can perform it at home without jeopardizing quality. Sampling first thing in the morning may even be advantageous for recovering a virus-enriched material due to reduced airway clearance during the night (66). However, gargling at home without supervision always carries the risk that no gargling liquid is provided; this was more important when we were testing young students in the school in the context of testing system. Overcoming this issue in the individual test was easy since we always had human gene as a control; in the pool testing, we asked the responsible persons on the pool stations to check samples before emptying them into the pool since plain water is distinguishable from a gargled sample.

First, we asked students in our STACADO pilot school testing at Domspatzen to gargle with Saline, which was reported in different studies as a cheap alternative viral transport medium for nucleic acid testing of SARS-CoV-2 (67)(68). However, we got clear feedback from students that repeated gargling with Saline is unpleasant. To make it more comfortable and acceptable for the participants,

we changed to sterile water (Ampuwa) first, which was much better accepted. However, one had to provide this water in containers, and thus, a huge logistic setup (similar to Saline) would have been needed. Therefore, we searched for other alternatives for simplification. After additional tests to exclude that it would interfere with the chemistry of downstream laboratory protocols for RNA isolation and PCR, we finally introduced gargling with tap water, which is cheap and easily accessible to everyone as well as environment-friendly as it is not packed in plastic and does not need to be transported producing CO2. Furthermore, letting their children use tap water raised the least concern among parents, which all other media did. Overall, using tap water gargle samples to screen for SARS-CoV-2 has proven to work excellently in our and other studies (69).

To explore the acceptance of our water gargling-based testing system in practice and different settings, we always performed surveys in schools and workplace setting such as the hospital. As mandatory antigen tests were introduced by the government in these places in 2021 while the gargle pool testing model project was running, this was an opportunity to compare the acceptance of both methods.

In the Domspatzen school (STACADO project), we performed an online survey, developed together with students in a citizen science project, on the acceptance of both testing methods in participating teachers/school staff and students. The gargling system was viewed as significantly more effective and acceptable also by the participants.

During the school testing, In the WICOVIR project, we asked headmasters and teachers responsible for school hygiene in all our participating schools to give feedback on the performance, acceptability, and applicability of both the gargle procedure and the antigen testing in schools. In total n=71 schools participated in the anonymous survey. Our gargle testing rated significantly better in acceptance, handling, and overall evaluation of the gargling method with the responsible school authorities.

During the hospital testing and invited all hospital staff to participate in an online survey. Our gargle pool PCR was not only viewed as superior in safety over antigen-testing by the hospital personnel, but staff members also preferred the gargle pool testing over self-testing by nasal swabs at home. The higher acceptance of testing using gargle samples was also reported by Kocagoz *et*

al. (70). Based on our own test results and the review of the literature; we conclude that gargling is superior to nasal (or mouth/throat) swabs in acceptance of the method.

Finding the right method to detect the virus in gargle samples in a high throughput, mass-testing setup

Thus, the next question was if and how gargle samples can be combined with downstream methods of virus isolation and detection with a sufficient detection rate to apply in preventive testing. First, we needed to ensure that the antigen test worked at all in our hands and with our samples. Our experimental data showed that positive swab samples analyzed with Biosensor Inc., which is one of the most sensitive antigen tests available on the market, as also shown in our comparisons (58), antigen testing could reliably detect low virus loads comparable to a Ct value of 30 in our PCR setting. The detection limit was reported to be similar but lower in other studies. Lindner *et al*. reported the Ct value lower than 27 (71), and it was lower than the Ct value of 25 in the Yamayoshi *et al*. study (72).

We saw the detection limit was reduced to Ct values of 20 when we used the gargle samples with the same antigen test. Although sample concentration is higher in swabs than in gargle, but cannot be the only reason, we got reasonable higher sensitivity and unreasonably lower specificity after using lower COI in positive gargle samples. We came to the point that not only dilution but also gargle samples may be changed the chemistry of the test, resulting in this dramatic detection limit change.

Overall, in our preparatory result, Biosensor antigen-test sensitivity was 84% (swab samples) and 76% (gargle samples). The sensitivity of the Biosensor rapid antigen test with swab samples in comparison to qRT-PCR was reported to be lower in other studies, 74% and 65% in Lindner *et al*. and Jegerlehner *et al*. studies, respectively (71)(73). Even with the swab as testing material, the false positive and negative rates were unacceptable to us for mass screening, considering the high numbers of children that would either be undetected or end up in unjustified isolation. Indeed, this was later also observed in the field.

During our WICOVIR study, we identified n=8 SARS-CoV-2 positive students by our PCR-based WICOVIR testing system (Average Ct value 31.6) that had performed self-administered antigen testing simultaneously. Only n=2 (25%) also had a positive antigen test at that time, which showed that the sensitivity of the antigen test was poor at the early stage of the infection.

Confirmation that antigen tests would not be helpful for preventive testing also came from our Hospital study, when we had a one-week replacement of the regular gargle pool PCR testing by antigen tests due to the sick leave of the laboratory team. Remarkably, at this time, many more staff members went to PCR testing with symptoms and showed higher virus load (lower Ct) when tested by PCR. Therefore, our interpretation of this situation is that the antigen test was not sensitive enough to detect positive cases in the time between infection and symptoms. Therefore, more positive and potentially infectious staff members remained undetected. This is also reflected by the lower Ct value found when staff members were finally tested by PCR after developing symptomatic. Overall, this may have led to infection among hospital staff, as also infection rates increased /peaked exactly in this and the following week amongst hospital staff. Coste *et al*. reported a similar result; the sensitivity of the SD Biosensor test for samples from asymptomatic COVID-19 patients was 28–33 % (74).

We observed in our study that, despite the advantages in the processing of the antigen tests, such as being faster and easier to perform, no need for trained personnel and equipped laboratory, and the possibility of applying at home, they are much less sensitive and specific than RT-qPCR. The same was also reported in another study by Liotti *et al*. concluding that although SD Biosensor antigen needs a few minutes and fundamentally less laboratory effort to results, it has reliable sensitivity only for samples with Ct values lower than 25 means most commonly; asymptomatic patients tests negative with it (75). Frequent population testing to detect the positives in the early infection phase is essential in controlling the pandemic (76). However, this is only achieved by repeatedly testing the population with a sensitive testing regime. Therefore, we explored if we could combine the advantages of gargling with the high performance of qRT-PCR.

Solving the challenges of gargle pool RT-qPCR: Sensitivity and Depooling

It was common knowledge, even at the beginning of the pandemic, that gargle samples are a reliable source of material to detect virus RNA with single qRT-PCR protocols (70). Our initial experiments and other studies showed that small patches of 1-5 gargle samples in a pool format are also a reliable source of SARS-CoV-2 detection (77)(78). However, the big concern was that the test sensitivity might be lower due to dilution effects caused by the gargle liquid. Indeed, others showed that when gargle solution is compared to swabs, about 1 - 1.5 log levels reduction in the viral load (copies / mL) due to dilution effects are observed (79-81). However, this effect was much less dramatic than previously estimated and can be countered by developing especially sensitive extraction and detection tools in isolation and PCR setup, which we did.

We started with a pool of five in cooperation with Synlab in our pilot school testing study (STACADO). After establishing our own RT-qPCR test, we increased the pool size to 21 and even further. We were aware of the detection limit in our study since initial data show that positive gargle samples with Ct value up to 35 (means 1.000 copy/mL) were reliably detectable within the pools of 21 in our RT-qPCR. During our WICOVIR study, we noticed our real threshold was even higher than Ct 35 since we could detect the positive hospital personnel with the Ct value of 38 in the pool with 20 participants at the actual state.

At a certain point, the number of pooling participants' criteria is not the detection limit of the PCR as other centers (Novogenia, personal information) experimented successfully with pools of up to 500 individuals. The major challenge is the deepoling process and turns–around the time of depooling; and result reporting in the case of a positive pool, which depends on the positive pool rate, which can be predicted from the general incidence. As we observed during our WICOVIR study with Alpha (and Beta) variants mainly, while we were testing in a total of n=92 schools, the maximum positive pools we had was n=4 pools per week. In the Omicron wave and increasing incidence testing only in one hospital, we had n=6 positive pools per day, so we needed to decrease the pool size to n=10.

In the pilot school-testing (STACADO project), the pooling was performed in the Synlab laboratory. Based on their capacity and experience, they made a pool of 5, and since they had the single leftover samples, they could perform the depooling without waiting for the single tube collection and transportation. However, in the WICOVIR study, the pooling was performed in the school, so in the case of the positive pool, we had to wait for single samples. In the first week of the pilot phase of WICOVIR, while we only tested Domspatzen (located in the city and close to the central laboratory) with five pools, we faced challenges in terms of individual sample collection and delivery time to the laboratory when we got one pool positive. This resulted in very late evening work and brought stress to the laboratory team and the parents of the children in the positive pool.

One way to speed up that process is automatizing the data transfer, which we tackled with our partner MaganaMed. In the laboratory process, this involves sample labeling, for which we took responsibility and introduced some innovative measures in sample handling and registration. During the school testing, the WICOVIR browser-based software tool made it possible to send results automated, fast, and safe from the laboratory to the tested schools. As soon as the school got the result based on the pool ID and their list of participants, they collected, labeled, and transferred the single samples of the individuals in the pool. For that purpose, a study related to currier service (with a drive-through at the main laboratory) was implemented.

In the laboratory, 1 ml sample from these pre-labeled tubes was transferred to the matrix tube. Quickly the ID of the sample and the matrix tube were connected via the software. In addition, to save time since, in the WICOVIR software, the matrix tube location on the 96-well plate was registered automatically based on the scanning order, it was always possible to track back and find the ID of the sample even if by accident, the location of the tubes were changed.

To process the high number of pools (maximum n=655 pools per day), we divided the schools into different groups based on their distance to the laboratory. We had three fixed time points to process the samples (as shown in Figure 8 in the Material and Method section) and the fourth run only in case of a positive pool from the third run. We made the timeline in a way that it was possible to add the single samples of the positive pool to the next runs to avoid extra testing runs. Our last run was the samples from Cham; we provided the health ministry of Cham with the necessary equipment, and one-laboratory personnel from us went there on the days of the testing to do sample

transferring from the pool to the matrix tube and ID connection. However, due to the distance and transfer, the testing result was ready later in the afternoon when the students were not at school anymore. To overcome this issue and be able to perform depooling in the case of the positive pool in the same evening, we asked the student to leave their second tube in the school so the school manager could collect, label, and send them to us. With this organization, we could perform depooling by the same evening.

The timing of depooling became even more critical when we tested hospital staff since we needed to do it fast enough not to disrupt hospital service. Again, this was facilitated by using self-developed software (COVIDA) and generating a list of all participants in that positive pool displaying the contact details of that person in the hospital. Members of the test team (usually 2-3, according to demand, usually secretaries) called the 20 individuals in a pool, and within usually 10 (maximum 20) minutes, samples were retrieved. When more than one pool was positive (in the high incidence phase), we had smaller pools with fewer participants (n=10), and thus, retrieval was even faster; decreasing the pool size made it possible to fit the single samples with the next pool-testing run since it still fitted the 96 sample number per run. By reducing the size of the pools, it was possible to perform the depooling of the late positive pools on the Cepheid system, which had four channels. We made smaller pools (n=3, n=3, n=4) of the positive pool and tested them on Cepheid; in 45 minutes, we had the result and already could inform the negative ones and performed the individual testing to identify the positive person. This system is fast but expensive with limited capacity, so we only used it in case of one late positive pool. Notably, the staff members were included randomly in the pools (according to their arrival in the hospital); this was a difference in logistic strategy from school testing, where school classes are recommended to be tested together. Therefore, a positive pool always represented members from different departments and units. Consequently, no department had to shut down completely if a pool tested positive.

Moreover, during the Omicron wave and high incidence, we asked all the hospital personnel to inform us if they had any symptoms, did not feel well, and if they suspected to be positive due to any contact. Those got the single test and did not participate in the pool to prevent unnecessary

depooling. In addition, positive people were not allowed to participate in the pool testing for two weeks after their first negative test to avoid a false positive pool.

Roll-out of gargle pool testing in different settings

We showed that our repeated gargle pool RT-qPCR WICOVIR testing setup could be applied quickly in different settings. After establishing our testing system for massive screening of SARS-CoV-2 infections in schools located close to our laboratory in Regensburg, we showed that by using pre-existing logistic structures and laboratory testing facilities, our robust and simple system could be implemented safely and quickly in all different settings, including in remote rural areas. In total n=12 schools from Schwandorf and Tirschenreuth counties participated in the study by organizing their own laboratories. They worked with pre-existing PCR protocols, which were not as sensitive as ours, which we showed by ring testing experiments (60). However, they all worked sufficiently for the purpose. In addition, all elementary schools from Cham County participated and were tested by central but remote labratories (first NOVOGENIA, later Regensburg). WICOVIR was also used and performed in companies, including restaurants; these data are still in the publication process.

We also show our test system could be applicable to the hospital setting with high acceptance and robustness through high incidence phases of the pandemic during the Omicron wave with few adaptions, such as reducing the pool size to n=10 participants in each pool.

What can be achieved with a functional mass screening test in a pandemic?

In the first part of the WICOVIR school study, as it has been published so far (22.2.2021-30.7.2021), the average Ct value for a positive pool was 34, and the average of the individual positive sample in that pool was 31. Thus, it seems reasonable that the positive individual was detected early enough to prevent passing on the infection in the school environment,

By the end of the third week of repetitive WICOVIR testing two times per week, we observed a significant decrease in the rate of positive children in that cohort from 0.042 to 0.012 ($p = 0.008$).

The time of our WICOVIR study was, in total, 23 school weeks. However, we tested students for 19 weeks (4 weeks of school vacation). During this time, we had n=7 weeks of zero COVID cases. We observed the highest positive pools either after the vacation time or during the weeks that we had new school to participate in our study; for example, during week 19, we had n=1294 pools, and among them, one was positive, while in week 20, the pool numbers raised to n=1784, and respectively we detected five positive pools in that week. This suggests certain effectiveness of frequently testing to control the virus circulation.

Further inquiries in more detail in the county of Cham, where all primary school children (n = 4,200) in 38 schools regularly participated in WICOVIR by default, indicated that no SARS-CoV-2 infection was detected in study participants outside the WICOVIR testing and no transmission occurred in the schools during the testing period (Publication currently in revision with Journal of Disaster Medicine, as of 31.6.2022).

When comparing the incidence of the counties participating in the WICOVIR tests (incidence of 100–250 per week), we found that children in schools were positive less often than expected (1 out of every 5,600 tests) while at the same time, children and youth seem to contribute to the disproportionally strong overall incidence according to RKI data, simply because they were systematically tested in the schools as the only group in the general population.

This leads to the conclusion that they are infected anywhere but in the schools, e.g., in close contact with positive family members, relatives, and friends outside the schools. Altogether, our data and the data from the group of Sweeney-Reed (82)(83) suggest that with a proper testing concept in place, schools are a safe place for children in times of pandemic. The overall data on WICOVIR is not yet published, but approximately 1 million tests have been performed so far in schools, Kindergartens, public services, and companies and even spin-offs were established. Our data and concepts provided the background for the decision of the Bavarian state to establish regular gargle pool-based testing instead of antigen testing in elementary schools in the autumn of 2021. However, it needs to be considered that all these school tests took place when the dominant variant was still Alpha Beta or Delta. What worked then may not work with the Omicron variant.

During the Omicron wave, we focused on testing of St. Hedwig's Children's hospital personnel with the WICOVIR test system. The aim was to explore if the test system could withstand Omicron and slow down the infection rate of hospital staff so that wards did not have to close due to missing/ sick personnel. Due to the high sensitivity of the test system, we identified asymptomatic SARS-CoV-2 positive staff members via pool PCR testing significantly earlier, with the higher Ct value median was 31, in comparison with individual samples when a person became symptomatic Ct value median was 26. During the study period, while we performed n=8793 systematic tests translating to n=697 pool PCR, we had five false positive pools (0.7%). We compared the weekly incidence of the general Bavarian population (Figure 15) with the incidence of St. Hedwig's Children's Hospital, SARS-CoV-2 positive staff members identified by our twice-per-week regular testing system. The incidence in our hospital staff was higher than in the general population by an average factor of 1.5 to 2 fold. While vaccination rates of our staff are much higher than in the general population, the number of detected infections in our hospital was much higher than reported for the general population. This might be explained by the fact that antigen-test-based detection of SARS-CoV-2 is mainly used for the general population, which, based on the data we presented, has much less sensitivity to detect positives, especially when there are newly infected and the Ct value is higher. Moreover, due to the possibility of home testing, some positive cases may not be reported and were not included in the incidence. On the other hand, because of the high case numbers and delays in reporting the results the incidence in the general population might have been underestimated. Overall, we (based on our hospital testing) and others have concluded that the dark number of positive tests in the general population is as high as a factor of 2 to 3.

Advantages of gargle pool testing and costs

Pooling gargle samples helped overcome the most significant challenges of massive screening of SARS-CoV-2 positives during the pandemic by increasing test capacity, saving resources, and having more results in a shorter time than a single test. Making pools from the swab samples need to be done by laboratory personnel. While gargle samples offer a more effortless and safer option for pooling, since every individual can pour their sample into the pooling container, which also reduces the chance of contamination and mixing samples; using gargle samples does not bring

extra work for lab personnel, especially during the pandemic that the diagnostic labs are overload by samples.

Another essential factor for frequently testing is cost and resources. Although individual RT-qPCR testing is the most accurate method, it is still the most expensive diagnostic procedure, while with pooling; it is possible to reduce the cost. Pooling liquid gargle samples is efficient in terms of cost. We calculated that by pooling gargle samples, the cost for RNA isolation and PCR price per pool is around 4.4€; considering the other cost, including transport, personnel, equipment, and consumables, it would still be <1 EUR per person tested.

The limitations of such a gargle pool test system are the machines, laboratory space, and consumables needed and, as a key factor, experienced staff to run the tests. While machines can be ordered in advance and represent an investment of approximately 100,000€, consumables were a limiting factor throughout the pandemic.

Due to the pandemic, the procurement of consumables has become a previously unimaginable difficulty. This is a critical factor because of the large quantities necessary for large-scale testing. Here we see an advantage of using gargle pool tubes relative to all other types of tests that rely on disposable test systems such as swabs: Children's personalized pool test tubes can be reused by washing them out. The individual tubes are always used by the same child, and therefore, there is no risk of hygiene. We normally replaced the tubes every 6-8 weeks, or when one of these tubes broke, or in the case that they were collected and used for depooling.

Not only plastics but also the availability and accessibility of many regular laboratory items and reagents such as probes and primers, filter tips, and molecular-based ethanol were also challenging during the pandemic due to shortages in supply chains and high demands. Therefore, it was also crucial for the processes in the laboratory to use as few single-use consumables as possible; by pooling samples, we significantly reduced the usage of laboratory material for testing. Overall, we see this point in the supply chain and supply of consumables for a Germany-wide rollout as one of the key sticking points where such testing can fail.

What is the role of testing at this stage of this pandemic (or other future pandemics)?

Our aim in establishing the WICOVIR testing system was to react quickly to the pandemic when there was no large-scale testing available, and at the time that due to test resources limitations, children were not the priority of getting tests. Children were suffering physiologically and mentally through the pandemic and the lockdown; they were considered the main transmitters of the virus and were forced to stay home since the schools were closed. WICOVIR water gargle pool RT-qPCR testing was developed to safely bring the children back to school and avoid the uncontrolled spreading of the virus and outbreaks when vaccination for children was unavailable yet.

This aim achieved in 2021, while during our study time (up to summer vacation 2021), the dominant variant was still Alpha; the first Delta variant among the positive student was identified in July. At this time, with our sensitive, fast, and reliable testing system, we could achieve zero COVID situation in approximately 40% of our testing weeks, no outbreak, and no transmission occurred in the schools during the study time. Nevertheless, this infection tracking was only possible during the dominance of variants with lower infection potential than Omicron.

With the emerging of new variants like Omicron that even vaccinated individuals were infected several times with, testing cannot achieve a zero COVID situation as in an open society as we observed in hospital testing even by increasing the testing time to three times per week. Nevertheless, it can still slow down infection and prevent mass illness and simultaneous sickness in specific areas, which may still be necessary for critical infrastructures such as hospitals to prevent departments or units from going into lockdown due to a lack of available personnel and make it safe for patients.

By overcoming all the challenges in method-development and logistics, our WICOVIR testing proved to be sensitive, quick, applicable in different settings, well acceptable by participants, and a cost-efficient screening system for massive testing on a large scale. Using in-house RT-qPCR, WICOVIR is easily adaptable for new variants or another pandemic. For further pandemics, with having such a proper massive testing system to identify infected people from the beginning while

the incidence is still low, it would be possible to contribute to the prevention of the outbreaks and avoid several lockdowns until the effective vaccine and treatment develop, representing a big improvement to what we experienced in the first two years of the COVID-19 pandemic.

5 Summary

Despite the global interest and concern about COVID-19, real data on children remained limited throughout the pandemic. Early in the COVID-19 pandemic, children and adolescents were thought to be the main transmitters of the disease. In Germany, schools and childcare centers were closed very early during the pandemic, which led to considerable disruption of regular school operations, which impaired many children's development and quality of life.

In the first study of the project, we evaluated infection rates of children retrospectively in 3 regions of Bavaria, which were a hotspot (Tirschenreuth), affected moderately (the pre-alpine region around Rosenheim) and at average (Regensburg region) according to available PCR test data from adults during the first COVID-19 wave. As children were not tested at that time due to the lack of PCR test availability, the only way to understand the epidemiology of SARS-CoV-2 infection in children was to evaluate the immune reaction of a large number of children. Infection rates determined by seroconcersation ranged between 3% and 13% and were very similar to the numbers in adults, as shown later. Children had no higher transfection rate, but lockdown measures hit them extremely hard and longer than the rest of the society. While they had mild acute COVID-19, they showed long time effects such as PIMS early on and had no chance to get vaccinated early on. Thus, we hypothesized that only with a large-scale and sensitive testing system it would be possible to bring the children safely back to school in a timely limited zero-COVID approach. This was achieved in the further studies published within this project.

To develop an effective, applicable mass-testing setup to prevent outbreaks, different challenges in each step, including sampling process and logistics, the efficiency of the methods with different variants, reporting time, applicability, and implementable of the system in various settings had to be overcome. We developed a user-friendly gargle test system using feedback from children and simplifying the method using tap water. The broad acceptance of the procedure was evaluated by surveys. We solved data transfer issues in collaboration with IT partners using a software tool we had recently developed for the CHAMP project. We optimized and streamlined RNA extraction

methods and developed sensitive and robust PCR test systems to be able to generate screening results in app. two hours on a high throughput scale. We optimized and automated sample handling and found a new way of sample registration, reducing sample-handling time in the laboratory enormously.

We showed that our test system is superior to antigen testing and performs well in all different kinds of settings so that it could be rolled out for mass testing in schools and even in other applications. Within the WICOVIR study, we observed that with the proper RT-qPCR testing system in place, testing twice a week, already after the first three weeks of testing, the rate of positive children in that cohort decreased significantly ($p = 0.008$). On average, positive pools from our school testing study showed an average Ct value of 34.5 and an average individual CT value of 31 (range 24-36). When antigen tests were performed concomitantly, only 25% of positive individuals were detected at the same time, confirming the superiority of gargle pol PCR to antigen tests also in the field.

During the Omicron wave, while the incidence was rising dramatically from 200 to 3000 positive individuals per 100.000 inhabitants, we showed that a gargle pool testing system can still work and perform much better in detecting positives than antigen testing. Our data suggests that only with the continuous and sensitive gargle pool test system in place the closing of complete wards due to mass illness of staff could be avoided.

After overcoming all the challenges, our ready-to-use testing system could be adapted quickly for new kinds of variants or further pandemics helping to slow down the spread of any kind of respiratory virus, avoiding outbreaks and lockdowns until proper defense strategies such as vaccines are in place.

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9 Curriculum Vitae

PARASTOO KHEIRODDIN

Regensburg,Germany

+49 176 4715 6393

kheiroddinpa@gmail.com

Education

University Hospital Regensburg, *Regensburg, Germany, 2022*
Doctor of Philosophy (Dr. rer. physiol)
Dissertation title: Population based studies on detection, prevalence and immunoreactivity of SARS-CoV-2 in Children**.**

_____

Tabriz University of Medical Sciences, *Tabriz, Iran , 2015*
Master of Science: **Human Genetics**
Dissertation title: Molecular analysis of mutations in the RET proto-oncogene in patients with medullary thyroid carcinoma in Azarbaijan- Iran**.**

_____

Shahid Chamran University of Ahvaz, *Ahvaz, Iran, 2011*
Bachelor of Science: **Cellular and Molecular Biology**

Experience

Researcher, 02/2019 - Current
University Hospital Regensburg, Regensburg
Topic: Clinical study to find asthma remission biomarkers in children, using Omics analysis.

Research Assistant, 10/2016 - 01/2019
Hannover Medical School (MHH), Hannover
Topic: In vivo study to find the contribution of PPAR-gamma deletion in Hepatic Stellate Cells to liver fibrosis and cancer.
Research assistant, 04/2012 - 11/2012
Aban Hospital, Tehran, Iran

Research assistant, 07/2011 - 12/2011

Novin Medical Genetic Laboratory. Sari, Sari, Iran

Intership, 07/2010 - 09/2010
Novin Medical Genetic Laboratory, Sari, Iran

Core Qualifications

Cellular and Molecular Biology and Biotechnology:DNA/RNA purification, Gel electrophoresis, Polymerase chain reaction technique (PCR), Real-time PCR, RFLP assay, sequencing, Library preparation, Primer design, Sanger sequencing, Genotyping, Microscopy: light and fluorescence

Protein Chemistry: Standard protein purification techniques, Protein staining, Western blot, SDS page, ELISA
Cell Culture techniques
Animal testing: Mouse handling, injection and operation

Certifications

- Good Scientific Practice and Statistics, Hannover Medical School, Hannover, Germany, 2016
- FELASA B Certification, Laboratory Animal Science, Hannover Medical School, Hannover, Germany, 2016
- Method Course, Analysis of Genomic Data, University Regensburg, Germany, 2020

PUBLICATIONS

- Thurner L, Kesselc C, Fadled N, . . ., **Kheiroddine P** and et al. **IL-1-Ra-antibodies in myocarditis following SARS-CoV-2 vaccination**. New England Journal of Medicine, August 2022.
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- **Kheiroddin,P.,** Sakhinia,E., Hashemi,Z.R., and et al. **RET gene analysis in patients with medullary thyroid carcinoma in specific population of Iran,** European Human Genetics Conference. Barcelona, Spain.2016.
- **Kheiroddin,P**., Sakhinia,E., Hashemi,Z.R., and et al. **RET Gene Analysis in patients with Medullary Thyroid Carcinoma.** Accepted for publication. Clinical Laboratory Journal, Germany. 2016.

Languages

Farsi: First Language

English: C2
Proficient

Germn: B1
Intermediate

References

Michael Kabesch: Prof. Dr. med. Univ. University Children's Hospital Regensburg (KUNO), Regensburg, Germany Email: Michael.Kabesch@barmherzige-regensburg.de

Publications

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Results of WICOVIR Gargle Pool PCR Testing in German Schools Based on the First 100,000 Tests

Parastoo Kheiroddin[†], Patricia Schöberl[1,2†], Michael Althammer[1], Ezgi Cibali[1,3], Thea Würfel[1], Hannah Wein[1], Birgit Kulawik[1,2], Heike Buntrock-Döpke[1,2], Eva Weigl[1,2], Silvia Gran[1,2], Magdalena Gründl[4], Jana Langguth[5], Benedikt Lampl[5], Guido Judex[6], Jakob Niggel[7], Philipp Pagel[7], Thomas Schratzenstaller[8,9], Wulf Schneider-Brachert[10], Susanne Gastiger[11], Mona Bodenschatz[11], Maike Konrad[11], Artem Levchuk[11], Cornelius Roth[11,12], David Schöner[12], Florian Schneebauer[13], René Rohrmanstorfer[13], Marcus P. Dekens[14], Susanne Brandstetter[2,15], Johannes Zuber[14,16], Daniel Wallerstorfer[13], Andreas Burkovski[11], Andreas Ambrosch[3], Thomas Wagner[17] and Michael Kabesch[1,2] on behalf of the WICOVIR Study Group*

[1] Department of Pediatric Pneumology and Allergy, University Children's Hospital Regensburg (KUNO) at the Hospital St. Hedwig of the Order of St. John and the University of Regensburg, Regensburg, Germany, [2] Science and Innovation Campus Regensburg (WECARE) at the Hospital St. Hedwig of the Order of St. John, Regensburg, Germany, [3] Institute of Laboratory Medicine, Microbiology and Hygiene, Hospital of the Order of St. John, Regensburg, Germany, [4] Public Health Institute Cham, Cham, Germany, [5] Public Health Department Regensburg, Regensburg, Germany, [6] Pediatric Office Judex, Regensburg, Germany, [7] Maganamed Limited, Regensburg, Germany, [8] Medical Device Lab, Ostbayerische Technische Hochschule Regensburg, Regensburg, Germany, [9] Regensburg Center for Biomedical Engineering, University and OTH Regensburg, Regensburg, Germany, [10] Department of Infection Prevention and Infectious Diseases, University Hospital Regensburg, Regensburg, Germany, [11] Microbiology Division, Department of Biology, Friedrich-Alexander-University Erlangen-Nuremberg, Erlangen, Germany, [12] DATEV eG, Nürnberg, Germany, [13] NOVOGENIA Limited, Eugendorf, Austria, [14] Research Institute of Molecular Pathology, Vienna BioCenter, Vienna, Austria, [15] University Children's Hospital Regensburg (KUNO) at the Hospital St. Hedwig of the Order of St. John and the University of Regensburg, Regensburg, Germany, [16] Medical University of Vienna, Vienna BioCenter, Vienna, Austria, [17] Intego GmbH, Erlangen, Germany

Background: Opening schools and keeping children safe from SARS-CoV-2 infections at the same time is urgently needed to protect children from direct and indirect consequences of the COVID-19 pandemic. To achieve this goal, a safe, efficient, and cost-effective SARS-CoV-2 testing system for schools in addition to standard hygiene measures is necessary.

Methods: We implemented the screening WICOVIR concept for schools in the southeast of Germany, which is based on gargling at home, pooling of samples in schools, and assessment of SARS-CoV-2 by pool rRT-PCR, performed decentralized in numerous participating laboratories. Depooling was performed if pools were positive, and results were transmitted with software specifically developed for the project within a day. Here, we report the results after the first 13 weeks in the project.

Findings: We developed and implemented the proof-of-concept test system within a pilot phase of 7 weeks based on almost 17,000 participants. After 6 weeks in the main phase of the project, we performed >100,000 tests in total, analyzed in 7,896 pools, identifying 19 cases in >100 participating schools. On average, positive children showed an individual CT value of 31 when identified in the pools. Up to 30 samples were pooled (mean 13) in general, based on school classes and attached school staff. All

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Clara Prats,
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***Correspondence:**
Michael Kabesch
michael.kabesch@barmherzige-regensburg.de

[†] These authors have contributed equally to this work and share first authorship

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three participating laboratories detected positive samples reliably with their previously established rRT-PCR standard protocols. When self-administered antigen tests were performed concomitantly in positive cases, only one of these eight tests was positive, and when antigen tests performed after positive pool rRT-PCR results were already known were included, 3 out of 11 truly positive tests were also identified by antigen testing. After 3 weeks of repetitive WICOVIR testing twice weekly, the detection rate of positive children in that cohort decreased significantly from 0.042 to 0.012 ($p = 0.008$).

Interpretation: Repeated gargle pool rRT-PCR testing can be implemented quickly in schools. It is an effective, valid, and well-received test system for schools, superior to antigen tests in sensitivity, acceptance, and costs.

Keywords: children, COVID-19, Germany, PCR, pooling, gargle, schools, pandemic

INTRODUCTION

Children and youth are still severely affected by the COVID-19 pandemic, even though the acute phase of the disease is mostly mild in the young (1). They are over-proportionally affected by secondary consequences of the pandemic such as social deprivation, lack of physical activity, decrease in economic status, and dysconnectivity, especially in rural communities (2, 3), and in countries like Germany, where closing of schools was not perceived as the last option in fighting the pandemic but as the first (4, 5).

Consequently, severe psychological and developmental impairments have now become obvious (6). On the other hand, SARS-CoV-2 infections may also lead to major health problems in children in the long run (7, 8): Pediatric Inflammatory Multiorgan Syndrome (PIMS) is a severe, potentially deadly consequence of COVID-19, affecting only the young (9). Children are also affected by post-COVID syndrome (PCS). Therefore, it is of the utmost importance to balance the needs of children to attend school and have a chance for social development despite the pandemic, with the proper protection to minimize the risk of SARS-CoV-2 infection in the school environment (10). In the current state of the pandemic, such concepts cannot wait but need to be implemented now (11).

We gained experience in a proof-of-concept study, which started in the summer of 2020, on how testing of school children can be achieved and contribute to safety in schools (12) in addition to already existing non-pharmaceutical interventions such as wearing face-masks, maintaining social distance, disinfecting hands, and increasing ventilation in rooms, all of which were implemented in German schools in the autumn of 2020. Based on this experience, we developed a safe, efficient, and cost-effective SARS-CoV-2 testing system for schools: WICOVIR (Where Is the COrona VIRus?). Here, we present the concept and provide the first data based on >100,000 tests. Due to the introduction of compulsory antigen testing in schools in Bavaria starting on April 12 (week 15), 2021, we had the opportunity to compare self-administered point of care (PoC) antigen tests to gargle pool rRT-PCR tests for 6 weeks.

MATERIALS AND METHODS

Study Design and Population

The objective of this proof-of-concept study was to show that regular gargle pool rRT-PCR testing is safe, efficient, and cost-effective in all school environments, including students from first grade (~6 years of age) to grade 12 (~17 years of age) of all German school forms. Here, we report on our experience after 11 full school weeks (and 2 weeks of vacation) of testing. After achieving approval from the Bavarian State-Ministry for Education and Cultural Affairs (February 26, 2021), and funding from the Bavarian State-Ministry for Health and Medical Care (March 26, 2021), we started the pilot phase, which lasted for 5 full school weeks and 2 vacation weeks to build up the test system and which was followed by 6 weeks of the main study phase of regular testing after Easter vacation. We invited all schools in counties close to the two original study centers in Erlangen and Regensburg to participate in the study through internet platforms, print media, and personal information (**Figure 1**). Interested schools were asked to participate in two introductory webinars taking place twice weekly, where the study design was explained (**Figure 2**). Detailed information material was developed for the study, specifically addressing the information needs of children, youth, parents, and school staff. These were made publicly available through the study website (www.we-care.de/WICOVIR).

Schools that participated had to agree to study terms, e.g., to comply with hygiene standards and study protocols and a data protection contract had to be signed. Through participating schools, informed consent was obtained from parents, school children, and staff who volunteered to participate in the project. The prerequisite for participation was informed consent and school attendance; the exclusion criteria included a positive SARS-CoV-2 test result within 2 months prior to participation (to avoid positive results in rRT-PCR testing due to prolonged viral RNA shedding not indicating infectivity).

Due to the specific conditions during the third wave of the pandemic, we distributed study information by digital channels/website, FAQs, emails, and phone calls to address all questions of participants. The participation in the study was voluntary. For reasons of anonymization, communication with

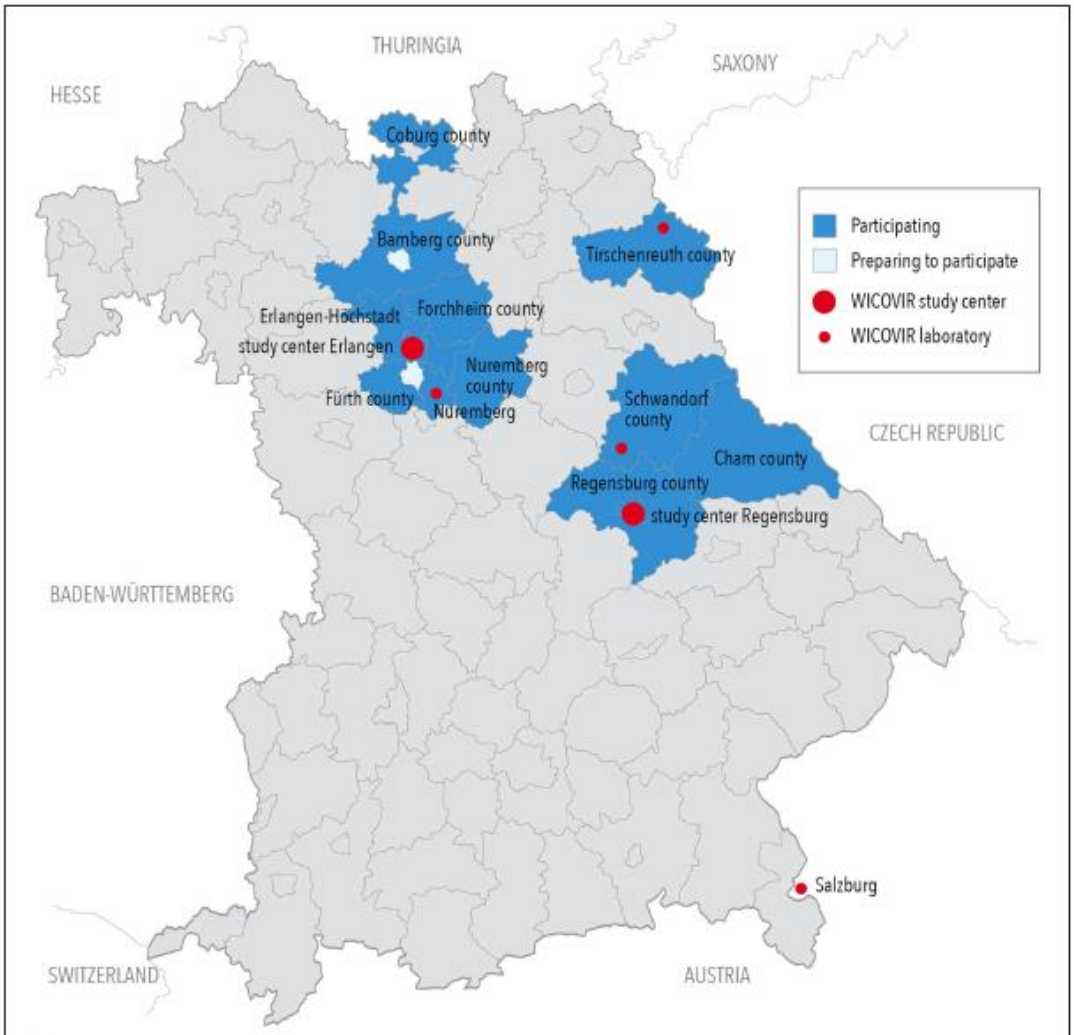

FIGURE 1 | Map of Bavarian counties participating in WICOVIR as of May 15, 2021.

study participants in the course of the study was through the schools only. We trained teachers and school staff in study procedures through on-site initiation visits. Transport of samples was organized through schools and voluntary helpers, or, if that was not possible, through a courier service or study personal. A drive-through to make sample delivery easy for volunteers was established outside the laboratory. We also established a network of primary care pediatricians who volunteered to support schools in all questions concerning the study and infection protection in case of positive results. The study was approved by the Ethics Committee of the University of Regensburg (file-number: 21-2240-101).

Data Collection and Management

The data protection principle of the study was to collect as little data from participants as possible. No personal or medical data of participants were collected in the pooling study. Only the schools kept track on-site of who participated in a specific pool. Those records were deleted within 24 h and were only needed to resolve positive pools. A browser-based software tool

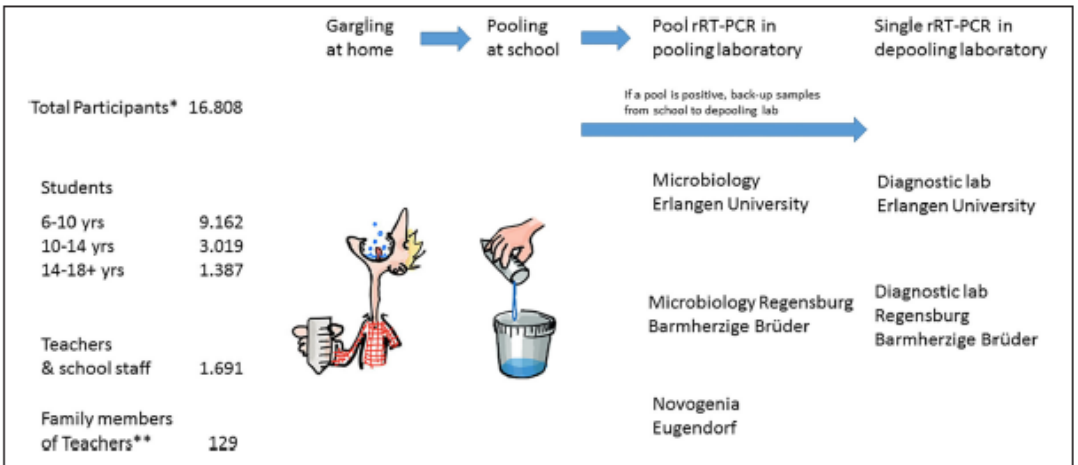

FIGURE 2 | Study design and numbers of the WICOVIR study as of May 12, 2021. *Participant numbers change over time, as new schools were included and some schools/individuals may have dropped out while others joined. **In pilot the phase, we explored teacher pools (as no clearance for student tests was yet available) and allowed family members of teachers to participate in these teacher pools.

was developed for the study by MaganaMed GmbH to keep track of barcoded pools, pool results, pool dissolving, and to allow for automated correspondence of test results and summary statistics of test results, irrespective of the laboratory software in the participating test centers. The software only handled pool IDs and alphanumeric sample IDs (unique, pseudonymized), but no personal information on participants. All identifying information was exclusively handled by participants, schools, diagnostic labs, and health authorities, respectively (**Figure 3**). Additional information on the software is available upon request from the authors or from the company (https://maganamed.com).

Gargle Procedures

The feasibility of gargling (throat washings) for SARS-CoV-2 detection has been shown previously (13). Even though the diagnostic sensitivity is slightly lower when compared to nasopharyngeal swabs, the absence of invasiveness of gargling is a decisive advantage, especially in our setup of repetitive testing in children. In this study, all participants gargled with ~6 ml of tap water at home twice or three times per week, first thing in the morning (before brushing teeth and breakfast) for ~30–60 s to achieve maximal recovery of virus from throat rinsing. Feasibility of the gargling procedure in the school setting was tested previously in the STACADO study and reported elsewhere (12). A video providing exact guidance and documentation of the gargling procedure is available online at www.we-care.de/WICOVIR. Gargle recovery fluid was collected by the participant in a screw-cap tube and divided into a second screw cap tube in approximately equal amounts (2–3 ml each). Both tubes were brought into school in a zip-lock bag. One was for pooling and the other one (back-up) was retrieved from schools and tested only in the case of a positive pool result.

Pooling Procedures

In the schools, one tube was emptied by the participant into a pooling container that was positioned in a pooling station. Pool participants were defined by the schools and usually contained the pupils of one class and the school staff (teachers) attached to that class. The maximum number of participants accepted for one pool was 30. In the Erlangen study site, we explored testing in pools of teachers with their attached families in a small set-up including 129 family members in teacher-centered pools. The pooling station was specifically designed by the Medical Device Lab of OTH Regensburg (Ostbayerische Technische Hochschule Regensburg) for the purpose of this study according to exact hygiene specifications developed to avoid splash contamination. Prototypes were provided by the technical workshop of the University of Regensburg. Pooling stations were manufactured according to our specifications and donated to the study unconditionally by local industry (Krones AG, Regensburg, Germany). A video documenting the pooling procedure is also available at the study website (www.we-care.de/WICOVIR). In brief, pooling took place under the supervision of a teacher in classes, and schools defined and documented participants of their pools in-house. Only the number of participants in a pool was transmitted for data protection reasons. Every sample contributing to a pool was defined as a test sample. After pooling, the pooling containers were sealed and transported to the laboratory within 1 h.

Depooling Procedure

The second tube (back-up tube) with gargle fluid was kept with the students/at school and was only retrieved in the rare event of a positive pool. In that case, back-up tubes of all participants in a positive pool (according to the documentation of the school) were barcoded with a unique identifier at the school so that

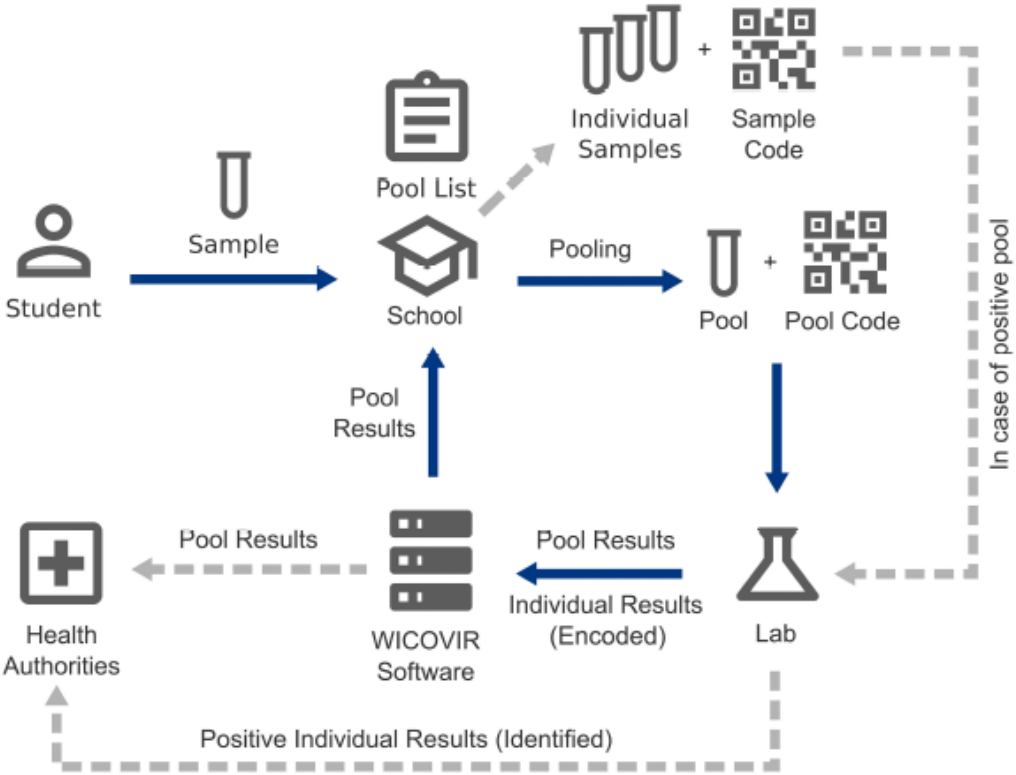

FIGURE 3 | Sample/data flow and data protection. Student samples were pooled in the participating schools, who kept analog records of pool participants and registered pools in the software. Pools were barcoded and sent to the lab. Pool test results were documented in the software by the lab and schools received results through the software. In the case of a positive pool, individual samples pertaining to a positive pool were sent to the lab and individual results were entered into the software in a pseudonymized fashion. Health authorities had access to the pseudonymized data in the software and received personalized results directly from the labs through official reporting software.

only pseudonymized samples were transported to the medical laboratory which provided the individual medical testing of samples by PCR procedures certified for medical testing. In cases defined as urgent by the public health authorities, schools were requested to provide clear names to the laboratory immediately in accordance with the infection protection act. For all samples in the Regensburg region, depooling was achieved within 12 h after pool samples entered the laboratory; for all other cases, this was achieved by at least the next day.

SARS-CoV-2 Pool rRT-PCR Testing

To test gargle pools, we applied previously described (14, 15) as well as recently optimized methods. As WICOVIR is a proof-of-concept study for the rollout of a pool test system in the state of Bavaria, we allowed for different, site-specific rRT-PCR methods, to test if already existing laboratories could be integrated in a large-scale rollout. Individual gargle samples of known virus content were used to determine detection limits in different pool sizes with the different methods. All test methods were able to detect a positive sample with a set cycle threshold (CT value of 32 in a pool of 30 samples). We performed conformation tests between sites and laboratories. Specifically, we continuously tested positive pools in different labs in ring experiments (data available on request). Analytical methods for pool rRT-PCR of the different laboratories are shown in **Table 1** and given in detail in the **Supplementary Material** section.

Online Survey on Acceptance of Test Regimes in Schools

To assess the acceptance of the WICOVIR gargle pool rRT-PCR and self-administered antigen tests, we designed an anonymous online survey applying our previously reported qnome database and questionnaire system (www.qnome.eu). The questionnaire

TABLE 1 | Comparison of PCR test methods in WICOVIR laboratories in this study phase.

Test steps	Regensburg	Eugendorf/Salzburg	Erlangen
Sputolysis	–	Ascorbic acid	Ascorbic acid
RNA isolation	RNA extraction: MagNA Pure DNA/RNA kits (Roche) BEXS Ready Viral DNA/RNA kits (Inno-train) MagnifiQ RNA buffer kit (A&A Biotechnology)	RNA extraction: MagnifiQ RNA buffer kit (A&A Biotechnology)	Lysis: Tris(2-carboxyethyl)phosphine hydrochloride (TCEP HCl) (Sigma-Aldrich)
qPCR master mix	LightCycler® Multiplex RNA Virus Master (Roche)	FTD™ SARS-CoV-2 (Siemens Healthineers)	2 × Luna Probe One-Step Reaction Mix (NEB)
Targeted genes	E gene of SARS-CoV-2	N gene and ORF1ab region of SARS-CoV-2	N1 region of the N-gene of SARS-CoV-2
Extraction control	Equine arteritis virus (EAV)	Equine arteritis virus (EAV)	RNAse P
PCR cycler	Light Cycler 480 II (Roche)	Quantstudio 5 (Thermo Fischer Scientific)	qTOWER^3 G (AnalytikJena)
Confirmation method	Xpert Xpress™ SARS-CoV-2 assay targeting E and N2	Initial assay already targets 2 genes	N1 and N2 regions of the N-gene of SARS-CoV-2

consisted of 15 questions, could be used freely, and is available upon request. All school heads of participating schools ($n = 96$) at the time point of the survey (week 3 of the main phase) were invited to fill out the questionnaire, as both the WICOVIR testing and the antigen PoC tests were performed concomitantly in these schools, allowing for direct comparisons of the procedures.

Statistical Analyses

Data from the gargle pool tests are presented using descriptive statistics. For analyzing the difference between proportions of positive tests between different phases of the study, statistical tests that considered dependent groups could not be performed (as individuals with repeated measurements were included but anonymized); thus, we performed the non-parametric Mann–Whitney *U*-test. Differences in the data from the online survey assessing indicators of acceptance were analyzed using a *t*-test for dependent groups for metric indicators and McNemar tests for dichotomous and dichotomized indicators. All analyses were performed using SPSS.23.

RESULTS

We performed 23,582 tests pertaining to 1,621 pools in the school setting in the pilot phase of the study and the adjacent vacation (**Figure 4** and **Supplementary Table 1**, upper panel) to establish all study procedures, test feasibility, and acceptance of methods. The pilot phase lasted until students returned to schools after Easter vacation and the main study phase started on April 12. In the main phase, 114 schools participated. In total, 16,808 individuals participated, and of these, 14,988 were students of different age groups (**Figure 2**) The main study phase, which has lasted 6 weeks so far, started with three laboratories that provided regular pool testing (Erlangen, Regensburg, and a diagnostic laboratory in Eugendorf, as capacities in Regensburg could not be ramped up fast enough to cover the demand in the initial phase). Depooling using the back-up samples was performed in Erlangen and Regensburg (for pools tested in Regensburg and Eugendorf). In the main phase, we performed 77,763 tests in 6,274 pools, with an average of 12,800 tests per week and an average pool size of 13, respectively (**Supplementary Table 1**).

Within the pilot phase, we identified four positive pools, and 16 positive pools were found in the main study phase (**Table 2**). The average CT value of a positive pool was 34 (range 26–39), and it contained a mean of 14 tested individuals (range 4–26), which corresponded to an average CT value of 31 (range 24–37) in the back-up sample of individual positive pool participants. In these 20 positive pools, we detected a total of 19 novel infections. In the Regensburg study center, three already known cases of previous SARS-CoV-2 infections in children who still underwent testing as requested by the study protocol were identified. In the Erlangen study center, where also relatives living in the same household were invited to take part in the testing, two positive pools showed two positive individuals each. Also in Erlangen, one pool could not be resolved successfully as not all back-up samples could be retrieved reliably in the pilot phase of the study. Of those that were found to be positive, all but two were students. Overall, we found a positive rate of 1:400 in pools, respectively, translating to one newly identified positive individual every 5,600 tests.

In schools that participated in the pilot phase, voluntary participation rates of students were between 95 and 98%. Compulsory antigen testing was introduced in Bavarian Schools on April 12, 2021; however, children participating in the WICOVIR project were allowed to continue the WICOVIR test regime by law under the condition that they perform one antigen test per week (usually at the first day of the week present at school) to assure that the WICOVIR procedure was safe. That gave us the unique and unexpected opportunity to compare sensitivity of compulsory self-antigen tests to WICOVIR gargle pool rRT-PCR testing at a large scale: On every Monday morning from calendar weeks 15–20 (main phase), all children participating in WICOVIR testing that day also had to perform antigen tests concomitantly (leading to a total of ~25,000 concomitant tests). Out of eight antigen tests that were done in schools on the same morning that gargling was also performed,

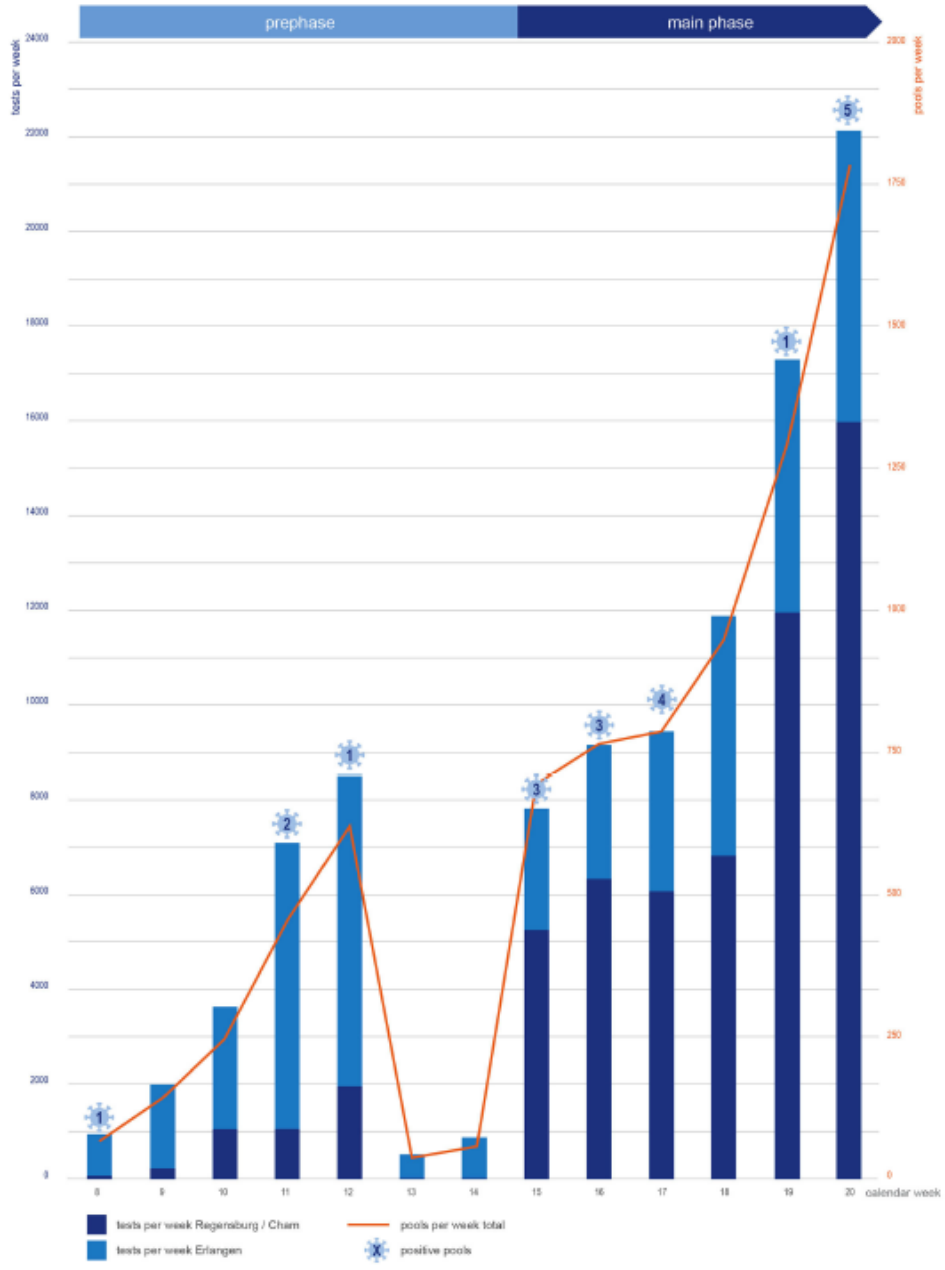

FIGURE 4 | Development of test and pool numbers in the pilot and main phase of the WICOVIR project and positive results of testings per week.

TABLE 2 | Characteristics of positive pools and positive individuals in the WICOVIR project.

ID	Status	Pool size	CT Pool	CT single	Antigen test	Comment
1	Student	15	33	32	Not available	COVID-19 residue
2	Student*	26	32	37	Not available	
3	Teacher's husband*	26	32	30	Same day, positive (after pool result)	
4	Student	8	34	30	Not available	
5	?	7	34	?	Unknown	Not all single samples retrieved
6	Student	14	36	31	Same day, negative	
7	Student	20	36	36	Next day, negative	
8	Student	9	39	36	Same day, negative	
9	Student	8	34	36	Same day, negative	
10	Student	14	32	27	Same day, negative	
11	Student	6	35	28	Not available	
12	School staff	12	33	32	Same day, positive (after pool result)	
13	Student	15	35	34	Not available	COVID-19 residue
14	Student	13	35	33	Not available	
15	Student+	20	34	27	Same day, negative	
16	Student+	20	34	32	Same day, negative	
17	Student	8	34	27	Same day, negative	
18	Student	9	34	26	Same day, positive	
19	Student	4	26	24	Not available	
20	Student	13	36	29	Same day, negative	
21	Student	10	39	36	Not available	COVID-19 residue
22	Student	20	33	31	Not available	

** and + mark positive individuals from the same positive pool.*

all but one showed negative results as did one antigen test performed the day after the positive PCR result (**Table 2**). Twice, antigen tests showed a positive result when applied for confirmation after pooling and depooling had already identified a positive individual. Based on these data, we calculated sensitivity for the early stage of the infection in the school setting of self-administered antigen tests to be 12.5% (1/8) to 27.3% (3/11) compared to the truly positive results by pool rRT-PCR tests. We cannot calculate the sensitivity and specificity of gargle pool rRT-PCR in this setting, as no more comparable sensitive testing was performed to define sensitivity and no positive cases outside the WICOVIR testing were reported to be found.

Three weeks into the main phase of the project, we noticed a decrease of positive results (weeks 15–17: 0.042% vs. weeks 18–20: 0.012%; $p = 0.008$). Interestingly, positive cases also in the last 3 weeks were restricted to children who joined the testing system for the first time within the 2 weeks before.

Three weeks after the WICOVIR main phase and the compulsory self-administered antigen testing had started, we invited all schools that performed both concomitantly to give anonymous feedback in an online questionnaire on their experience ($n = 71$ of 96 invited school heads responded). Significant differences in acceptance, handling, and overall ratings were observed for both procedures (**Figure 5**). Overall, gargling was received significantly better than antigen testing, resulting in an overall "school grade" of 1.5 for gargle pool rRT-PCR tests compared to 4.1 for antigen tests (grades 1–6, where 1 is best).

DISCUSSION

Repeated gargle pool rRT-PCR testing can be implemented quickly in schools as shown in our WICOVIR project. It is an effective, valid, and well-received test system for schools to detect SARS-CoV-2 infections in a rather early phase with high CT values. According to our data, it is superior to antigen tests in sensitivity and acceptance.

Repeated testing of large parts of a population is thought to be a major public health tool against the COVID-19 pandemic (16). Testing becomes especially important, when other measures of protection (such as vaccination) are not available or not feasible (such as complete social isolation) for a population, as is the case (and will be for quite some time) for children. Models estimate that, in theory, testing 75% of a population twice a week with a fast turnaround of reliable test results and immediate protection measures will break infection chains and contribute, together with other measures, to a "no-COVID situation" within 4–6 weeks (16). To achieve high testing frequency, testing needs to be extremely cost-efficient and easily scalable. Gargle pools do not require additional staff for swabbing. Furthermore, pooling in schools helps to drastically reduce the number of samples to be handled in the laboratory (by a factor of >10).

While being the most accurate method, individual rRT-PCR testing is still the most expensive diagnostic procedure. PoC antigen tests are cheaper, are much less sensitive, and require professional swabbing. No costs for swabbing occur in the case of self-applied antigen tests, but since those tests are restricted

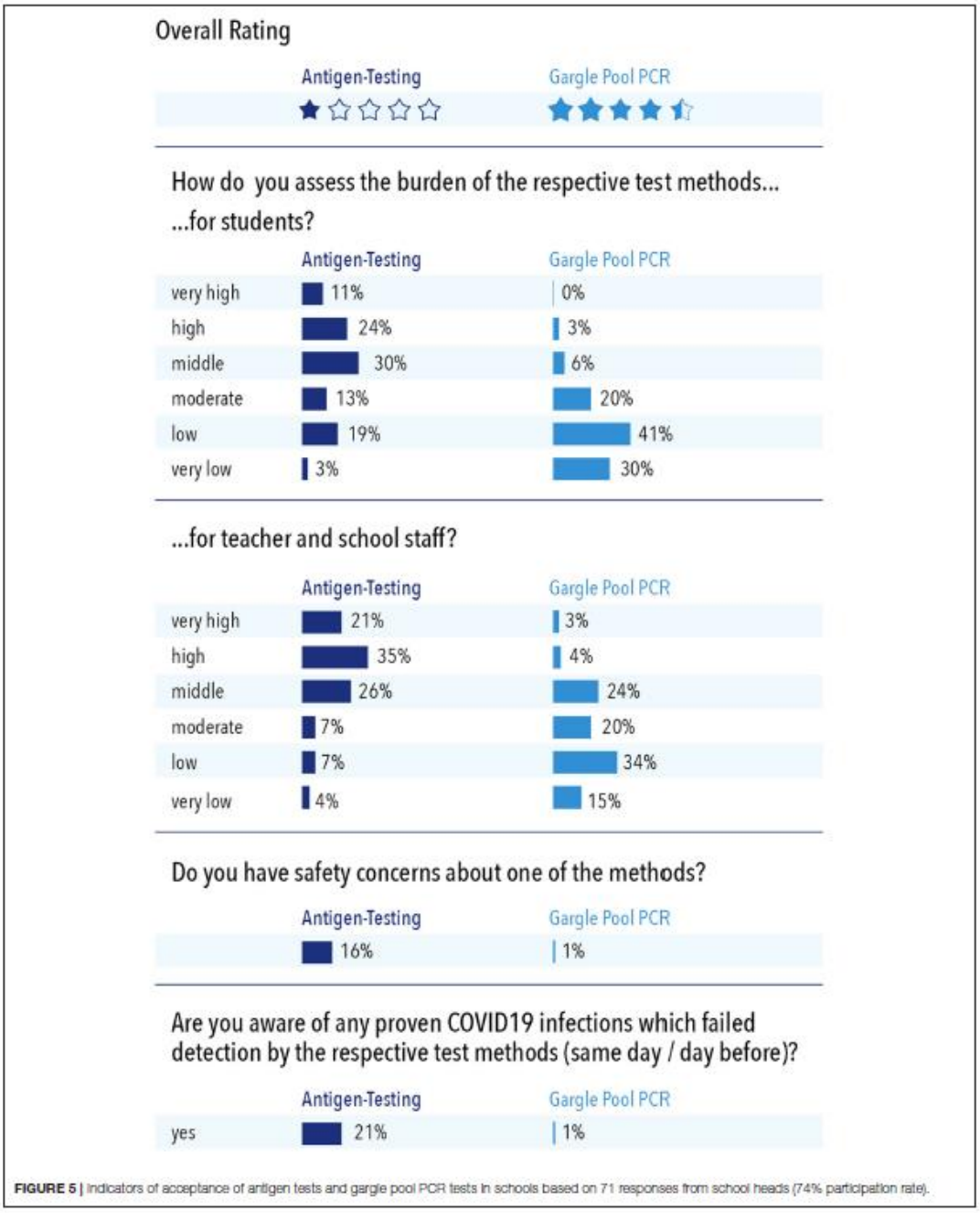

FIGURE 5 | Indicators of acceptance of antigen tests and gargle pool PCR tests in schools based on 71 responses from school heads (74% participation rate).

to sample collection from the front part of the nose by the children themselves, sensitivity of those tests may be diminished in comparison to professional swabbing. Gargle pool testing considerably reduces costs for an individual test, mainly for three reasons: (1) Depending on the pool size, pooling itself reduces costs by a factor of 10–30. At no point in our study did we find any indication that, in a realistic set-up, pool sizes of up to 30 participants would limit detection sensitivity. (2) Gargling does not require any staff for swabbing, which drastically reduces costs for sample collection (for German PoC tests, two-thirds of the PoC costs come from sample collection). (3) From a formal point of view, gargle pools are considered to be a preemptive public health test, but have no individual medical diagnostics. As a consequence, they may be performed outside of medical labs. Thus, we have found that gargle pool testing can be provided at an overall cost (including transport, personal, equipment, and consumables) of <1 EUR per person tested.

To offer a widespread testing of school children, testing needs to be simple but sensitive, acceptable for the tested child and their parents, readily available, and easily accessible. All current standard test systems are lacking one or another quality needed for such a broad test regimen. As we aimed to establish such a system in schools, we first addressed the questions of test acceptability. We had already gained experience with gargle rRT-PCR tests, which were introduced as the testing standard in our university children's hospital for clinical practice and study purposes in March 2020. When compared to nasopharyngeal swabs, only a slight decrease in sensitivity was observed for gargle samples (13). We found a high acceptance rate of these tests in our STACADO and STACAMA studies in children (12). The youngest children that can perform gargle maneuvers in our clinical setting were 3 years old, and as a general rule, children who can brush their teeth themselves can also gargle. In those studies, children gave very clear feedback, that they (and in some cases even the parents) rejected gargling with physiological NaCl (0.9%). Therefore, we introduced gargling with distilled water and later with tap water (or still mineral water), neither of which interfered with SARS-CoV-2 PCR testing. In the STACADO setting, we had started with gargling at school but quickly it became obvious that the procedure was so simple that it could be performed at home without losing quality with the advantage that the yield of potential virus material was expected to be higher when the specimen was sampled first thing in the morning due to reduced airway clearance during the night (17). Aspiration risk with such low quantities as 6 ml of water is neglectable. Thus, gargling is a safe, painless, easy-to-perform, and robust method to collect repeated samples in children.

However, gargling at home has the disadvantage that samples are not collected under supervision and study procedures may not have been performed perfectly in the home setting. Thus, this is a limitation of the procedure. When the samples are pooled in the school, it can usually be determined easily if gargle fluid is in the tube (in comparison to clear water) and if the amount of the gargle sample is as expected. When we performed quality control in random pools, all tested single samples contained human RNA as an indicator that gargle fluid had been collected. However, it is expected in this test system, like in all others except a professional swab taken by trained medical personal, that a perfect probe cannot be guaranteed. Furthermore, the acceptance of the tests according to the results from our online survey with school heads and by the families according to voluntary participation rates of 95–98%, was surprisingly good, suggesting that gargling is a feasible procedure in children.

rRT-PCR pool tests were established for SARS-CoV-2 testing early on in the pandemic (18) and further developed by members of our consortium (14) as well as compared systematically to other techniques (15). In our study, the average CT value for a positive pool was 34 and that of the individual positive sample in that pool was 31. Thus, in most cases the positive individual was detected so early that passing on the infection in the school environment with hygiene concepts in place was rather unlikely based on what we know currently and what we observed in WICOVIR.

The challenge in a school setting is the timely performance of the pooling and the subsequent testing that provides a great challenge to routine laboratories together with the organization of depooling in the case of a positive pool and the communication of results when pools are used. We have solved all these issues in WICOVIR. Pooling is performed in the schools using pooling stations to speed up the process (and to reuse the gargle tubes, overcoming the issue of limited supply of plastic ware, and reducing the plastic waste in the pandemic). We keep personal data of participants only in schools and no personal data go to the lab with the pooling container. Pool testing is thus anonymous but can direct true individual testing to where the virus is to be found, saving resources as recently published (19). The drawback of this anonymous testing in WICOVIR is that we cannot evaluate population characteristics of the total test population, except for those few that tested positive.

Our results show how superior in sensitivity gargle pool PCR testing is compared to antigen tests. Only gargle pool rRT-PCR detected nine true positive cases in ~25,000 tests when both gargle pool rRT-PCR testing and self-administered antigen tests were applied the same morning compared to one positive antigen test. At this stage, gargle pool rRT-PCR testing as applied here did not show false-positive or false-negative test results to the best of our knowledge. However, with increasing number of tests, we expect to also find rare cases of false results with this system as with any other testing.

Furthermore, two antigen tests, performed after the positive PCR result was already available, were positive. It has to be noted that antigen tests are specifically not designed to detect early SARS-CoV-2 infection (20, 21). This difference becomes especially obvious if testing is performed repetitively, when in most cases gargle pool testing can prevent infection cascades in schools while antigen tests cannot. According to the health authorities in the County of Cham, where all primary school children ($\sim n = 4{,}200$) in 38 schools participated regularly in WICOVIR by default, no SARS-CoV-2 infection was detected in study participants outside the WICOVIR testing, suggesting a very high sensitivity of the pool rRT-PCR performed in the study.

Overall, we detected 19 novel infections by our school test system. In the fourth and fifth week of the main phase, only 1 of the more than 27,000 tests within those 2 weeks was

positive, suggesting that repeated tests make the group more safe, especially as the one positive individual during that period had just joined the test system with a first (positive) test. However, also the general incidence of SARS-CoV-2 infections in the participating counties dropped at the same time. When compared to the incidence of the counties participating in the tests (incidence of 100–250 per week), we found that children in schools were positive less often than expected (1 out of every 5,600 tests) while at the same time, children and youth seem to contribute to the disproportionally strong overall incidence according to RKI data[1]. This leads to the conclusion that they get infected anywhere but in the schools, e.g., in close contact with positive family members, relatives, and friends outside the schools. Accordingly, no indication for a large number in school children was found.

Prior to our studies, we were unsure whether a high SARS-CoV-2 infection rate in the general population would limit pool sizes and increase costs. From a practical point of view, this has never been problematic in any of our regions under observation, even with an incidence of up to 250 new infections per 100 k people in 1 week. One reason for this may be that individuals tested in our settings have typically been non-symptomatic, which is different from other testing set-ups such as emergency sites at hospitals or local testing centers. Specifically, school children and students with symptoms were requested by a directive of the ministry, implemented in February 2021, to present to the local pediatrician, stay at home, and not attend school before tested negatively.

We conclude after >100,000 tests that gargle pool rRT-PCR testing is an easy, sensitive, and robust test system for schools. Especially as children in primary school will not be vaccinated any time soon, such a smart and suitable test system for children that can be implemented easily is urgently needed and shall be rolled out immediately. Our data show that with a proper testing concept in place, schools are a safe place for children in times of the pandemic.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethical Committee of the University of Regensburg. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

MKa, AA, AB, DS, and TWa contributed to conception and design of the study. PS established and supervised the field work, which was performed by PS, BK, HB-D, EW, and SGr. AB, AA, and DW organized and supervised the laboratory work. PP, AB, MD, and JZ developed novel methods. PP, MA, EC, TWü, HW, SGa, MB, MKo, FS, AL, CR, and RR performed laboratory work. MG, JL, and BL performed contact tracing and public health measurements. JN and PP provided IT support and developed the software for the project. GJ organized and supervised support of local pediatricians. WS-B supplied the hygiene concept and performed sequencing. TS developed the pool testing devices. SB performed the statistical analysis. PK wrote the first draft of the manuscript. AA, AB, DW, and TWa wrote sections of the manuscript. MKa wrote the final version of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fped.2021.721518/full#supplementary-material

[1]https://experience.arcgis.com/experience/478220a4c454480e823b17327b2bf1d4

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Conflict of Interest: TWa has invested in a company that performs PCR pool tests for companies. RR, FS, and DW are employed by NOVOGENIA, a commercial PCR test laboratory. JN and PP are employed by Maganamed GmbH, a commercial software company. DATEV eG is a software company which provides a non-profit test center for the project.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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How to Implement Safe, Efficient and Cost-Effective SARS-CoV-2 Testing in Urban and Rural Schools within One Month

Parastoo Kheiroddin [1,†], Magdalena Gründl [2,†], Michael Althammer [1,†], Patricia Schöberl [1,3], Linda Plail [3], Ezgi Cibali [4], Kristina Schraml [5], Josef Scheiber [5], Claudia Kiesewetter [6], Andreas Kneissler [6], Christopher Bartl [7], Daniel Wallerstorfer [7], Julien-Tyron Barth [8], Shabnam Rajabi [8], Cornelius Roth [8], Andreea Matei [8], Christin Fetz [8], Heike Buntrock-Döpke [1,3], Susanne Gastiger [9], Mona Bodenschatz [9], Maike Konrad [9], Jakob Niggel [10], Philipp Pagel [10], Guido Judex [11], Michael Hubmann [12], Catherine M. Sweeney-Reed [13], Andreas Ambrosch [4], Thomas Wagner [14], Andreas Burkovski [9] and Michael Kabesch [1,3,*]

[1] Department of Pediatric Pneumology and Allergy, University Children's Hospital Regensburg (KUNO) at the Hospital St. Hedwig of the Order of St. John and the University of Regensburg, 93049 Regensburg, Germany; Parastoo.Kheiroddin@klinik.uni-regensburg.de (P.K.); michi.althammer1998@gmail.com (M.A.); Patricia.Schoeberl@barmherzige-regensburg.de (P.S.); Heike.Buntrock-Doepke@barmherzige-regensburg.de (H.B.-D.)
[2] Public Health Institute Cham, 93413 Cham, Germany; Magdalena.Gruendl@stmgp.bayern.de
[3] Science and Innovation Campus Regensburg (WECARE) at the Hospital St. Hedwig of the Order of St. John, 93049 Regensburg, Germany; Linda.Plail@barmherzige-regensburg.de
[4] Institute of Laboratory Medicine, Microbiology and Hygiene, Hospital of the Order of St. John, 93049 Regensburg, Germany; Ezgi.Cibali@barmherzige-regensburg.de (E.C.); Andreas.Ambrosch@barmherzige-regensburg.de (A.A.)
[5] BioVariance GmbH Diagnostics, 95652 Waldsassen, Germany; kristina.schraml@biovariance.com (K.S.); josef.scheiber@biovariance.com (J.S.)
[6] Labor Kneissler GmbH & Co KG, 93133 Burglengenfeld, Germany; Claudia.Kiesewetter@labor-kneissler.de (C.K.); Andreas.Kneissler@labor-kneissler.de (A.K.)
[7] NOVOGENIA Limited, 5301 Eugendorf, Austria; christopher.bartl@novogenia.com (C.B.); daniel.wallerstorfer@novogenia.com (D.W.)
[8] DATEV eG, 90429 Nuremberg, Germany; Julien-Tyron.Barth@DATEV.de (J.-T.B.); Shabnam.Rajabi@DATEV.de (S.R.); Cornelius.roth@DATEV.de (C.R.); Andrees.Matei@DATEV.de (A.M.); Christin.Fetz@DATEV.de (C.F.)
[9] Department of Biology, Microbiology Division, Friedrich-Alexander-University Erlangen-Nuremberg, 91054 Erlangen, Germany; susanne.gastiger@fau.de (S.G.); mona.bodenschatz@fau.de (M.B.); maike.konrad@fau.de (M.K.); andreas.burkovski@fau.de (A.B.)
[10] Maganamed Limited, 93053 Regensburg, Germany; jakob.niggel@maganamed.com (J.N.); philipp.pagel@maganamed.com (P.P.)
[11] Pediatric Office Judex, 93049 Regensburg, Germany; gjudex@web.de
[12] Pediatric Office Hubmann, 90513 Zirndorf, Germany; dr.hubmann@me.com
[13] Department of Neurology, Center for Behavioral Brain Sciences, Otto von Guericke University Magdeburg, 39106 Magdeburg, Germany; catherine.sweeney-reed@med.ovgu.de
[14] Intego GmbH, 91058 Erlangen, Germany; thomas.wagner@intego.de
* Correspondence: michael.kabesch@barmherzige-regensburg.de; Tel.: +49-941-369-5801; Fax: +49-941-369-5802
† Contributed equally.

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Abstract: (1) Background: With vaccination and new variants of SARS-CoV-2 on the horizon, efficient testing in schools may enable prevention of mass infection outbreaks, keeping schools safe places and buying time until decisions on feasibility and the necessity of vaccination in children and youth are made. We established, in the course of the WICOVIR (Where Is the COrona VIRus) study, that gargle-based pool-PCR testing offers a feasible, efficient, and safe testing system for schools in Germany when applied by central university laboratories. (2) Objectives: We evaluated whether this approach can be implemented in different rural and urban settings. (3) Methods: We assessed the arrangements required for successful implementation of the WICOVIR approach in a variety of

settings in terms of transport logistics, data transfer and pre-existing laboratory set-up, as well as the time required to establish the set-up. (4) Results: We found that once regulatory issues have been overcome, all challenges pertaining to logistics, data transfer, and laboratory testing on different platforms can be solved within one month. Pooling and depooling of samples down to the individual test result were achievable within one working day in all settings. Local involvement of the community and decentralized set-ups were keys for success. (5) Conclusion: The WICOVIR gargle-based pool-PCR system is so robust and simple that it can be implemented within one month in all settings now or in future pandemics.

Keywords: school testing; monitoring; surveillance; SARS-CoV-2; COVID-19; gargle; pool-PCR; children; rural; urban; implementation

1. Introduction

School-based testing has become an important concept for keeping children safe at school during the Coronavirus disease (COVID-19) pandemic, especially as provision of vaccination to children is associated with considerable delay [1]. Antigen tests have been used widely to test adults for severe acute respiratory virus (SARS-CoV-2), and they have also been introduced into schools, with children performing the test themselves in classrooms. Recent data show that antigen tests cannot reliably detect new SARS-CoV-2 infections at an early stage [2,3], and that testing in the school environment is also cumbersome [4,5]. We and others have shown that gargle-based pool Polymerase Chain Reaction (PCR testing can provide a safe, efficient, and cost-effective alternative, [6–9] and thus the WICOVIR (Where Is the COrona VIRus?) study was initiated in March 2021 in the south of Germany [10].

Previously, we had gained experience in a proof-of-concept study, which began in the summer of 2020, of how testing of school children can be established successfully in selected urban schools [6]. Implementation of a PCR-based test system in rural regions was perceived as a major challenge or even obstacle for a broad, countrywide rollout. Here we provide evidence that the WICOVIR concept, which is based on gargle-based pool PCR testing, can be successfully implemented in a grassroots-approach in rural and urban counties within four weeks.

2. Materials and Methods

The WICOVIR project was designed in January 2021, approved by the ethics committee of the University of Regensburg in March 2021 (file number 21-2240_2-101), proposed to the Bavarian State Ministry of Health in February, and funded at the end of March 2021. All study procedures and protocols are available online (www.we-care.de/wicovir) and have been published in detail elsewhere [10]. Here we present findings following the implementation of the test system in four different Bavarian counties (Figure 1).

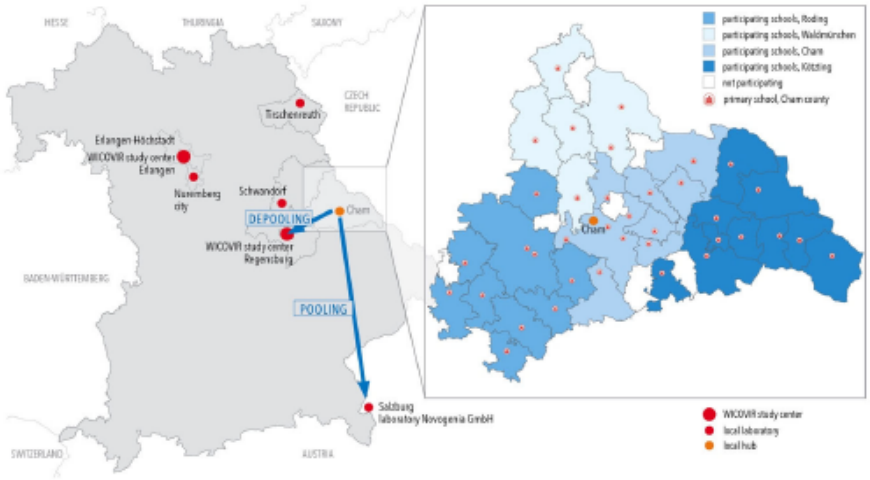

Figure 1. Map of participating counties of Eastern Bavaria and location of test centers. Map of participating counties of Bavaria. A zoom-in of the county of Cham exemplifies test-center and school locations and organization of routes in sectors for collecting samples. For Cham, pools were transported to the test center in Eugendorf near Salzburg, Austria, and for depooling to the test center in Regensburg, Bavaria, in the beginning of the project. In all other counties, local test centers were used or established within the county.

2.1. Local and Regional SARS-CoV-2 Testing Setup

For the county of Cham, Novogenia performed pool tests and the hospital laboratory in Regensburg performed depooling. In the Novogenia GmbH laboratory in Eugendorf/Salzburg, ribonucleic acid (RNA) was extracted using the MagnifiQ™ RNA buffer kit (A&A Biotechnology, Gdansk, Poland) using an Auto-Pure96 Nucleic Acid Purification System (Hangzhou Allsheng Instruments, Shanghai, China) according to the manufacturer's protocol. Realtime (RT)-PCR-based SARS-CoV-2 RNA detection was performed using a Quantstudio 5 Real-Time PCR System (Thermo Fischer Scientific, Waltham, MA, USA) using the single-well dual target (open reading frame (ORF)1ab and nucleocapsid (N) gene) Fast Track Diagnostics (FTD) SARS-CoV-2 assay using a total volume of 10 µL for the reaction according to the manufacturer's instructions. For depooling, samples were sent to Regensburg once a positive pool was identified by Novogenia. Depooling was performed as part of the study protocol either with the one-step RT-qPCR with the LightCycler® Multiplex RNA Virus Master (target E gene) using a Light Cycler 480 II Instrument (Roche Diagnostics, Mannheim, Germany) using a GeneXpert instrument (Cepheid, Sunnyvale, CA, USA).

For the county of Schwandorf, the Kneissler laboratory in Burglengenfeld performed pool testing and depooling. RNA was extracted using the foodproof Magnetic Preparation Kit VI (Biotecon, Potsdam, Germany) using either an AutoPure96 Nucleic Acid Purification System (Hangzhou Allsheng Instruments, Shanghai , China) or a RoboPrep96 (Biotecon, Potsdam, Germany), according to the manufacturer's instructions. The RT-PCR was performed using the SARS-CoV-2 Complete Kit (Kylt, Emstek, Germany) using either a LightCycler96 (Roche, Basel, Switzerland), Stratagene Mx3005p (Agilent Technologies, Santa Clara, CA, USA) or an AriaMx Cycler (Agilent Technologies, Santa Clara, CA, USA), according to the manufacturer's instructions. The detection was based on a single well, dual target assay, which detects the N- and S-genes of the SARS-CoV-2 virus.

For Tirschenreuth county, the Scheiber laboratory, located within the county in Waldsassen, performed pool-PCR and depoooling. RNA was extracted using the Echo-LUTION Viral RNA/DNA Swab Kit (BioECHO, Cologne, Germany) according to the manufacturer's protocol. RT-PCR-based SARS-CoV-2-RNA detection was performed using a CXF 96 or CFX Opus 96 (BioRad, Munich, Germany) using the VirQ Rapid SARS-CoV-2 kit, targeting the RNA-dependent RNA polymerase (RdRP) and envelope (E)genes (BAG Diagnostics, Lich, Germany) in a total volume of 20 µL according to the manufacturer's instruction.

For the city of Nuremberg, the DATEV laboratory, located in the city itself, performed pool-PCR and depooling. Sputolysis of the pooled samples was achieved by addition of ascorbic acid to produce a final concentration of 62.5 mM (Roth, Karlsruhe, Germany). Afterwards, an 18 µL aliquot of the pool was mixed with 2 µL of TCEP (Tris(2-carboxyethyl)phosphine hydrochloride (25 mM, Sigma-Aldrich, Munich, Germany)) and heated at 95° C for 5 min. 2 µL of the inactivated sample were added to the PCR reaction mix (10 µL of 2 × Luna Probe One-Step Reaction Mix (New England Biolabs (NEB), Frankfurt/Main, Germany), 1 µL of 20 × Luna Warm Start RT Enzyme Mix (NEB), 1.5 µL Primer/Probe Set (Integrated DNA Technologies), 0.5 µL UDG (NEB), and 5 µL RNase-free H20). The samples were then transferred into a MIC PCR Cycler (Biozym Scientific GmbH, Hess. Oldendorf, Germany), running the RT-PCR program of 55 °C for 15 min, followed by 95 °C for 2 min, and finishing with 45 cycles of 95 °C for 10 sec and 55 °C for 45 sec.

For all locations except Regensburg, depooling was not necessary during the reported period of time but would have been performed according to the standard operating procedures of established, clinically accredited routine laboratories, determined by the respective public health officers.

2.2. Ring Experiments

To investigate whether existing equipment and test-setups in each laboratory could all be used successfully for detection of positive gargle-based pools and provide comparable results, a ring experiment with blinded samples of predetermined positive and negative gargle-based pool samples was designed. Samples were delivered to the laboratories 24 h after a positive pool was initially detected, and ring testing sample work-up and PCR were performed 48 h after gargling in all laboratories on the same day.

2.3. Data Management

Commercial and routine laboratories in this study used internationally recognized standards such as Health Level 7 (HL7) and lab data transfer (LDT) as well as custom comma separated value (CSV) tables for data transfer. The WICOVIR software was implemented in Javascript (frontend) and typescript (backend), respectively. Data were stored on a PostgreSQL relational database management system. The database and application were hosted at an ISO27001 certified data center in Germany. Encrypted back-ups were generated several times per day and stored off-site in a separate data center of another host provider in Germany. A general data protection regulation (GDPR)-compliant data protection concept was implemented and approved by the data protection officer in charge.

3. Results

3.1. General Study Set-up, Logistics, and Laboratory Testing

After receiving ethical approval, a positive funding decision, clearance of data protection issues, and the approval of the Ministry of Education to perform tests in the schools within this project, we established a website to enable broad communication of study-related issues to the public. We also set up weekly webinars for the heads of the school to transfer information necessary for implementation of the testing program in local schools

in an efficient and timely manner. Beyond the initial study areas in Regensburg and Erlangen, three counties and one city area decided to participate in the early phase of the project (Table 1).

Table 1. Characteristics of the four study regions.

County/City Name	Cham	Schwandorf	Tirschenreuth	Nuremberg
Inhabitants *	128.094	148.477	71.696	515.543
County/City area *	1.527 km²	1.458 km²	1.084 km²	186 km²
Set-up				
Decision to participate	14.03.21	23.03.2021	07.05.2021	08.03.2021
First regular test	12.04.21	03.05.2021	10.05.2021	15.04.2021
Schools				
Number of schools	38	4	8	13
Forms of schools	All primary schools	Selection of schools from among all school forms	Selection of schools from among all school forms	All interested schools
Laboratories				
Name	Novogenia/ Regensburg	Kneissler	Scheiber	DATEV
Location	outside county	within county	within county	within city
Result summary **				
Weekly pools (approx.)	430	36	200	225
Participants (approx.)	4300	800	2300	2100
Positive individual tests	6	0	0	0

* from https://www.statistikdaten.bayern.de/genesis/online, last assessed 26.09.2021; ** as of 23 July 2021.

For these counties and city, we offered local events to promote the study procedures to school directors and local authorities on-site. In the rural counties, a regional organizational team, which usually included the school board, the local health officer, and the county authority), was established in a timely manner. Our central study team supported the regional teams in the ordering of equipment and training of teachers. Local teams organized transport of samples from schools to the laboratories involved. Participating laboratories adapted study procedures to their pre-existing, standard set-up and performed confirmation tests (Table 2).

Table 2. Peripheral laboratories and test procedures in participating laboratories.

Test Steps:	Novogenia/ Cham	Kneissler/ Schwandorf	Scheiber/ Tirschenreuth	DATEV/ Nuremberg
Sputolysis	ascorbic acid	ascorbic acid	_	ascorbic acid
RNA isolation	MagnifiQ RNA buffer kit (A&A Biotechnology)	Foodproof magnetic preparation Kit VI (Biotecon)	EchoLUTION Viral RNA/DNA Swab (BioECHO)	Lysis: TCEP (Tris(2-carboxyethyl) phosphine hydrochloride
qPCR master mix	FTD™ SARS-CoV-2 (Siemens Healthineers)	SARS-CoV-2 Complete Kit (Kylt)	VirQ Rapid SARS-CoV-2 (BAG Diagnostics)	2× Luna Probe One-Step Reaction Mix (NEB)
Targeted genes	N gene and ORF1ab region of SARS-CoV-2	N gene and S gene of SARS-CoV-2	RdRP gene and E gene of SARS-CoV-2	N1 region of the N gene of SARS-CoV-2

Extraction control	equine arteritis virus (EAV)	human ß-actin gene	RNase P	RNase P
PCR cycler	Quantstudio 5 (Thermo Fischer Scientific)	LightCycler96 (Roche), Stratagene Mx3005p (Agilent Technologies), AriaMx (Agilent Technologies)	CFX 96 (BioRad), CFX Opus 96 (Bio-Rad)	MIC Magnetic Induction Cycler (Biozym)
Confirmation method	Initial assay already targets 2 genes	Initial assay already targets 2 genes	Initial assay already targets 2 genes	N1 and N2 regions of the N gene of SARS-CoV-2

Despite differences in the set-ups, all laboratories were able to detect positive and negative gargle-based pool samples in ring experiments correctly, although the CT values differed (Table 3).

Table 3. Results of ring experiments in participating laboratories.

Blinded Sample		Regensburg/ Cham *	Kneissler/ Schwandorf	Scheiber/ Tirschenreuth	DATEV/ City of Nuremberg
Positive gargle sample	Results test1	Positive CT values: N2 gene: 31.64 ORF1b gene: 31.59	Positive CT value: N gene: 39.3	Positive CT values: E gene: 35.01 RdRP gene: 35.12	Positive CT value: N1 gene:33.62
Negative gargle sample		negative	negative	negative	negative
Positive gargle sample	Results test2	Positive CT values: N2 gene: 32.36 ORF1b gene: 31.9	Positive CT values: N gene: 34.87 S gene:33.14	Positive CT values: E gene: 33.6 RdRP gene: 36.7	Positive CT value: N1 gene: 35.18
Negative gargle sample		negative	negative	negative	negative

* Novogenia was no longer performing tests in the project at the time of the ring experiments. CT: cycle threshold.

3.2. *IT Solutions for Data Transfer*

Meanwhile, the IT team implemented four different ways of transfering laboratory results into the WICOVIR software electronically, according to the needs of the individual participating laboratories (Figure 2).

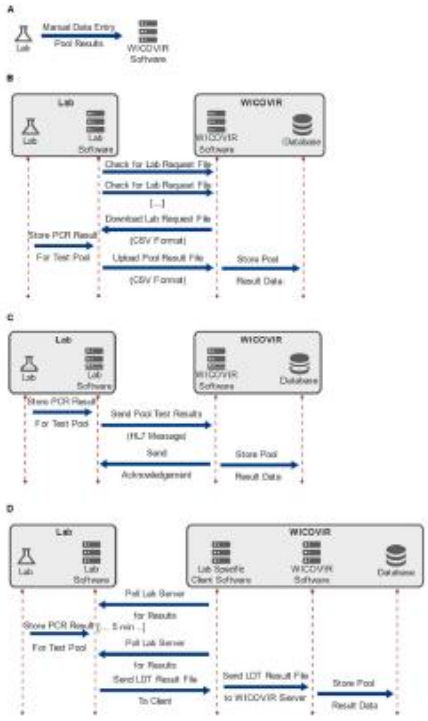

Figure 2. Interfacing with laboratories. Data transfer concepts for the implementation of WICOVIR according to different laboratory set-ups. Four separate interfaces for electronic data exchange between labs and the WICOVIR software have been established. They use different levels of standardization with respect to data formats and protocols ranging from manual entry **(A)** over exchange of CSV files **(B)** to HL7 **(C)** and LDT **(D)**.

First, we implemented an easy to use and flexible data entry system, which allowed manual individual data entry. Furthermore, data exchange using a file exchange protocol based on CSV files was created. In this scenario, the laboratory software would periodically check for the presence of a request file on the WICOVIR server. Once found, the request file is downloaded and parsed and the file itself contains the sample IDs corresponding to the test pools requested. In turn, the laboratory software then uploads the results files to the WICOVIR server, where they are parsed and the values stored to the database. For laboratories that support standardized solutions, such as HL7 or LDT, this data transfer approach was provided. The standardized nature of these formats and protocols helped in the rapid development of these interfaces. However, the need to address the details of each interface and the complexity of these standards diminished to some extent the initial advantage offered. Overall, implementation of four separate interfaces was, however, successfully accomplished by a small team, and a fully operational state was achieved within four weeks, in close collaboration with IT staff from the individual laboratories.

3.3. *Implementation in Different Rural Settings*

On 14 March 2021, the Eastern Bavarian County of Cham (128.094 inhabitants as of 31.12.2020, 1.527 km²), which had a seven-day incidence exceeding 200 COVID-19 cases per 100.000 inhabitants at the time, decided to participate in the project. A first pilot test, which included all children willing to participate in the primary schools of the county, was performed successfully on 22 March 2021 with samples received by the laboratory at 9:30 a.m. and pool testing results available at 1:30 p.m. Within the following two weeks, all necessary preparations were made for a regular testing scheme. We note that ethical approval was granted for the inclusion of all children attending the participating schools. After the Easter vacation, all 38 primary schools in the county participated in WICOVIR on a regular basis (Figure 1). Samples were collected from all schools throughout the county by drivers of the local road service teams and processed by the study team in a laboratory of the local hospital in the county. Pools were unpacked from containers, and 1 mL of the pooled solution was transferred into a microtube (Micronic) under a laboratory hood, then scanned into the WICOVIR software system. The workflow was established with three workers, with one person unpacking, one pipetting, and one registering, so that up to 220 pools were prepared for automated analysis within 75 min at Novogenia GmbH laboratory in Eugendorf/Salzburg and, subsequently, also in the central WICOVIR laboratory in Regensburg.

Pooling results were available the same evening, and in the case of a positive pool, depooling was performed in the WICOVIR laboratory in Regensburg the next morning. The local public health office informed the schools and families about positive results on the evening of the test, and a quarantine was implemented until the positive individual was identified the following morning. Within the first four weeks after implementation of the testing program, 15,724 tests were performed, identifying three positive cases. Three weeks into the project, testing was further extended to an additional 3000 children returning to primary schools after lockdown, and a further three positive cases were detected.

The rural County of Tirschenreuth was a COVID-19 hotspot in the first wave of the pandemic in Germany in the spring of 2020, and showed a high incidence of infections again early in the second wave in the winter of 2020-2021. However, through intensive voluntary testing introduced early on in 2021 and strict infection protection measures, as well as the participation in scientific evaluation of the previous breakouts, Tirschenreuth achieved a continuously low incidence of new COVID-19 cases early in 2021. Participation in the WICOVIR project was thus a logical next step. Overall, eight schools, with 2280 students and teachers, participated in the project. The program was initiated on 22 April 2021, and after clearing administrative issues and financing, regular testing started on 10 May 2021. For testing in Tirschenreuth county, a local laboratory was available, substantially reducing transport time to less than one hour. WICOVIR was also set up in the rural county of Schwandorf, in which testing was implemented in a selection of primary and secondary schools using a laboratory in the county. Due to restrictions in financing, however, only seven schools were able to participate in the testing there.

3.4. *Implementation in an Urban Setting*

In the city of Nuremberg, a large company took the initiative to establish their own testing laboratory in close cooperation with the WICOVIR Erlangen central laboratory. A laboratory container was established on the company site for testing of employees, and resources were donated by the company for school testing. The container was delivered to the site on 06 April 2021. The first laboratory employees started on 12 April 2021, and after a test phase under the supervision of the Erlangen laboratory, regular testing commenced on 15 April 2021. Within the first three months, 1738 pools, including 18,671 individuals, were tested. The number of schools in which testing took place increased to 16 over this time period.

4. Discussion

After establishment of WICOVIR facilities for testing for SARS-CoV-2 infection in schools in two large, centrally located scientific laboratories in Erlangen and Regensburg, we show here how the WIOCIVR protocol can be successfully implemented within four weeks in rural and urban regions with minimal effort, making use of pre-existing logistical structures and laboratory testing facilities or by creating new regional collaborations. Quality of testing and comparability of test results were assured through ring experiments. A common databank structure allowed for a shared but anonymous analysis of study results and quality control in real time. Close collaboration with public health officials on site, the school administration, and local authorities were keys for success.

The overall technical feasibility of gargle-based pool testing in a pandemic has been shown repeatedly [10]. In a previous feasibility study, we established that implementing an algorithm that required participating families to remember to access a web-based app on a regular basis, to enable selection of participants for each testing round, based on an algorithm to maximize the probability of detecting a positive case, was a rate-limiting step [7]. The inclusion of all pupils attending the participating schools in the WICOVIR program, made possible by the reduction in testing costs, enabled implementation of a successful monitoring program, in which chains of infection were broken before the emergence of symptoms [10]. The question remained, however, whether such an approach could be implemented outside of a clearly defined study setting, such as on a university campus or within a small test area, and, if applicable, which resources and set-ups would be necessary for a rapid implementation.

Overall, we found that regulatory issues were the most cumbersome and time-consuming aspect to be overcome in the process of establishing such a test program. Once all data protection issues were cleared centrally with the Ministry of Education, all other steps in the implementation process on site were comparatively easy. Whenever local authorities and school boards were convinced that pool testing offered an advantage for schools and pupils, their support was overwhelming. Major concerns at the start were the reliability of gargling at home, additional restraints/workload for the schools, time until test results are communicated, and logistics (supply and transport).

We addressed all general issues extensively in our previous publication, and we showed that these did not depend on the region where the test system was implemented [10]. We demonstrated that the test system is safe, as it protected participating schools from outbreaks; efficient, as it successfully led to the identification of a positive individual within a day; and cost-effective, as it reduced the costs per tested participant to less than €1 in the study setting. In contrast, the issues of documentation and communication of results may be affected by the respective regional laboratory set-ups. However, our IT team developed solutions for all situations we encountered in the participating laboratories, and these are applicable to almost every other laboratory set-up [6,7,10]. Once the software was established, information flow pertaining to schools, health officers, and test participants was swift. Depooling results were always communicated to health authorities and participants within a day. To make timely use of the test data by health officers is important in rendering the test system successful in preventing disease spread. Rapid testing without a similarly swift reaction of health officials to contain transmission is of no value.

Supply logistics with test equipment (plastic ware) were organized centrally to reduce costs and guarantee the availability of plastic ware in times of material shortages and quadrupling prices, which were relevant even in an industrialized country such as Germany. The insufficiency of supplies emerged as a substantial limiting factor for testing, and resource-sparing procedures are thus key to the success of such a testing system. In our system, tubes for gargling were personalized and reused by the test participants and laboratory procedures were designed to use a maximum of only two pipette tips per pool sample.

Transport logistics of the gargle-based pool samples in large counties presented a major concern at the beginning of the project. A range of solutions were developed to solve this issue. For example, in Cham County, which is a large region, the schools were grouped into four routes, with a driver for each. The driver picked up samples in the morning, and the first batch was delivered to a central collection point in the county (laboratory of the local hospital) by approximately 8:30 a.m. Pipetting could then start, while samples from farther, more remote schools, were delivered later (Figure 1, small graph). In Cham, drivers were recruited from the county workforce, but in other instances, members of the families whose children attended the schools volunteered to provide this transportation. The lengths of individual routes were limited in order to avoid delays in the processing of the samples. Transport logistics are sensitive to external disruption such as the traffic situation or weather conditions and require adequate human resources and detailed planning.

At the central collection point in the laboratory of the local hospital, pool-samples were registered in the documentation system and pipetted into matrix-tubes for automated processing in the testing laboratory, dramatically reducing the volume for transport (by a factor of 100-fold) and allowing a swift handling of the samples in the testing laboratory. (As samples had already been registered in the test system, no further documentation was required in the testing laboratory.) For this step, a laminar air flow, a vortex, a pipette, and a documentation system (barcode scanner and laptop) were needed. On average, 200 pools were transferred into matrix-tubes and documented within 75 min, before they were sent on to the testing laboratory. This transfer step relies on manual work and is a rate-limiting step in the automatization of the process.

In our opinion, having a test laboratory within the county or region offers a crucial advantage, as it reduces transport time (and thus time until results are available to participants). In addition, performing PCR testing locally was perceived by the study team, in communication with the schools and families, to strengthen the feeling of self-empowerment within the community in fighting the pandemic, resulting in the establishment of a partnership based on trust between the test laboratory and the local community. However, as shown in the set-up for Cham, such a local laboratory is not a prerequisite for a successful implementation. Nonetheless, when depooling is necessary for positive pools, long transport times may lead to the availability of depooling results only on the following day, which can critically delay identification of positive individuals and the timely implementation of protective measures to break an infection chain.

We conclude that even without a local laboratory in the county, testing in schools using the WICOVIR protocol can be achieved successfully within less than four weeks, and logistical challenges can be overcome. With the WICOVIR setup, testing in schools is easy to set up, reliable, and low in cost, leading to timely results which were generally available on the same day or within a maximum of 24 hours.

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Gargle pool PCR testing in a hospital during medium and high SARS-CoV-2 incidence

P. Kheiroddin [a], V.D. Gaertner [b], P. Schöberl [a,c], E. Fischer [a], J. Niggel [d], P. Pagel [d], B.M.J. Lampl [e,f], A. Ambrosch [g], M. Kabesch [a,c,*]

[a] University Children's Hospital Regensburg (KUNO), St. Hedwig's Hospital of the Order of St. John and the University of Regensburg, Regensburg, Germany
[b] Newborn Research Zurich, University Hospital and University of Zurich, Zurich, Switzerland
[c] Science and Innovation Campus Regensburg (WECARE), St. Hedwig's Hospital of the Order of St. John, Regensburg, Germany
[d] Maganamed Limited, Regensburg, Germany
[e] Department of Public Health, City and County of Regensburg, Regensburg, Germany
[f] Department of Epidemiology and Preventive Medicine, Faculty of Medicine, University of Regensburg, Germany
[g] Institute of Laboratory Medicine, Microbiology and Hygiene, St. Hedwig's Hospital of the Order of St. John, Regensburg, Germany

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SUMMARY

Background: Hospitals need to be protected from SARS-CoV-2 infections to protect vulnerable patients. Thus, a safe, efficient, and cost-effective SARS-CoV-2 testing system for hospitals, in addition to standard hygiene measures and vaccination of staff, is necessary. Here we report on the feasibility and performance of a pool real-time reverse-transcriptase polymerase-chain-reaction (rRT-PCR) test system at, medium and high incidence.

Methods: We implemented a testing concept based on gargling at home and pooling of samples in the hospital before PCR testing in the laboratory. We used two PCR systems (point of care and standard 96-well plate system) to adapt to challenges in the hospital setting and respond to a rising incidence in the Omicron wave.

Findings: During our 10-week study period, we performed 697 pool PCRs (8793 tests in total) and identified 65 asymptomatic staff members by pool PCR and 94 symptomatic staff members by positive individual PCR. Virus loads in those detected by pool testing were significantly lower ($P<0.001$). The test system remained workable even during the peak of the Omicron wave and no outbreaks occurred in any specific area of the hospital during the study period. Unvaccinated individuals were over-represented in the positively tested (37% vs 22% positive tests, $P=0.04$). The test procedure was well accepted by a majority of the hospital staff (84%).

Conclusion: Repeated gargle pool rRT-PCR testing can be implemented quickly in hospitals and is an effective, easily adaptable and well-accepted test system for hospitals, even during phases with very high infection rates.

* Corresponding author. Address: University Children's Hospital Regensburg (KUNO), St. Hedwig's Hospital of the order of St. John, Steinmetzstr. 1-3, D-93049 Regensburg, Germany. Tel.: +49 941 369 5801; fax: +49 941 369 5802.
E-mail address: michael.kabesch@barmherzige-regensburg.de (M. Kabesch).

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Introduction

Hospitals must be safe for patients and staff despite COVID-19, which they were not in the beginning of the pandemic [1,2]. To achieve this goal, vaccination of staff members is a central strategy, but as the occurrence of new virus variants show, vaccination alone is insufficient. In addition, general non-pharmaceutical interventions such as wearing face-masks, keeping social distance, disinfecting hands, and increasing ventilation in rooms are still necessary and useful to contain the spreading of the virus [3]. A sufficient testing regime is thought to be the third pillar in the strategy against the virus.

In the WICOVIR (Where Is the Corona VIRus?) project, we showed previously that a gargle pool real-time reverse-transcriptase polymerase-chain-reaction (rRT-PCR) test system is a safe, efficient, cost-effective, and accurate way to test large numbers of students and teachers in a school setting [1], which can be implemented quickly and easily [4]. We now report on the application of this system to test the staff of a large university pediatric and maternity hospital. Most patients in this setting were still unvaccinated at the end of 2021 and were thus especially vulnerable to nosocomial infection with the Omicron variant. We assessed how the WICOVIR test system can address specific challenges in testing hospital staff. Different to teachers and students, hospital staff work in shifts, cannot be quarantined easily and need results even sooner. Also, we assessed whether pool testing can still be applied efficiently with high numbers of positive results to be expected as was the case during the Omicron wave.

Materials and methods

Study design

The objective of this proof-of-concept study was to explore whether regular gargle pool rRT-PCR testing is safe, efficient and feasible in a hospital environment. The study was approved by the Ethics Committee of the University of Regensburg (file-number: 21-2240-101). Regular and mandatory WICOVIR testing in the hospital started on 20th December 2021, when testing of hospital staff at least twice per week (depending on vaccination status and previous infection history) became mandatory by German law in order to be allowed entry to the hospital premises. The weekly incidence of SARS-CoV-2 infections per 100,000 inhabitants for Bavaria was retrieved from the official website of the Bavarian public health office (Landesamt für Gesundheit und Lebensmittelsicherheit, LGL) [5].

A browser-based software tool developed with MaganaMed GmbH (Regensburg, Germany) for the study was used to keep track of barcoded pools, pool results, pool dissolving (de-pooling) and to allow for automated correspondence of test results and summary statistics of test results as previously described [1]. Immunization data (SARS-CoV-2 vaccination history and past infections were collected from all staff members. To comply with the prerequisites of the federal infection protection act, test documentation was combined with a database query to match the immunization status of each individual staff member with the necessary test frequency. Additional information on the software is available upon request from the authors or from the company (https://maganamed.com).

Gargle, pooling and de-pooling procedures

The general feasibility of gargling (throat washings) for SARS-CoV-2 detection [6] and the specific WICOVIR procedure have been described previously [1]. Even though the diagnostic sensitivity is slightly lower when compared with nasopharyngeal swabs, the absence of invasiveness of gargling is a decisive advantage for the acceptance of repetitive testing. In brief, all participants gargled with approximately 6 mL of tap water at home twice or three times per week for approximately 30–60 s to achieve maximal recovery of virus from throat rinsing. A video providing exact guidance and documentation of the gargling procedure is available online at www.we-care.de/WICOVIR. Gargle recovery fluid was collected by the participant in a screw-cap tube and divided into a second screw-cap tube in to approximately equal amounts (2–3 mL each). Both tubes were brought into the hospital in a zip-lock bag. One was for pooling and the other (back-up) was retrieved from staff members and tested only in case of a positive pool result.

In the hospital, one tube was emptied by the participant into a pooling container positioned in a pooling station. The maximum number of participants accepted for one pool was 20 (later reduced to 10) consecutive staff members were attending the pooling station as they entered the hospital. Pooling was supervised by an individual who linked the barcode of the staff member to the pool barcode in our COVID hospital COVIDA software (MaganaMed GmbH, Regensburg). A video documenting the pooling procedure in general is available at www.we-care.de/WICOVIR.

All test procedures were handled by a 50% laboratory worker, a 50% student for support sample handling, and a 50% medical assistant for organizing pools and recall of backup samples. In the event of a positive pool, the COVIDA software immediately generated a list of participants in the positive pool and provided contact details. Pool participants were contacted by the test team and the backup tube with gargle fluid was retrieved usually within 10–20 min from each participant. Individual testing of participant in positive pools was performed immediately. Thus, de-pooling was achieved within 3–4 h after a positive pool was detected. Results from negative pools could be retrieved online using the barcode of the respective pool, which was known to the participants of such a pool.

SARS-CoV-2 pool rRT-PCR testing

We used two set-ups to process gargle pool samples for rRT-PCR: (i) the point of care (PoC) GX-VI-4 module of the GeneXpert instrument (Cepheid, Sunnyvale, CA, USA) as previously described [7] and (ii) a combination of RNA isolation by the Auto-Pure96 Nucleic Acid Purification System (Hangzhou Allsheng Instruments, Shanghai, China) and subsequent PCR on a Bio-Rad real-time PCR system (CFX96; Bio-Rad, Hercules, CA, USA) as previously described [1,4]. Briefly, the GX-VI-4 module of the GeneXpert instrument allows the use of four cartridges of predefined mastermix concomitantly detecting SARS-CoV-2 E and N2 genes. Feasibility for pooling has been shown elsewhere [3]. The Allshang/Bio-Rad system has a capacity of 96 samples per run. Briefly, RNA is extracted from both single and pool samples using the MagnifiQ™ RNA buffer kit (A&A Biotechnology, Gdansk, Poland) on the Auto-Pure96

Nucleic Acid Purification System according to the manufacturer protocol. RT-PCR-based SARS-CoV-2 RNA detection was performed on a Bio-Rad real-time PCR system using the single-well dual target (ORF1b and N2 gene). We ensured that both systems detected both the Delta and the Omicron variants with high specificity and sensitivity using RNA from sequenced samples as references.

Online survey on acceptance of test regime

To assess the acceptance of the WICOVIR gargle pool rRT-PCR by hospital staff, we designed an anonymous online survey applying our previously reported 'qnome' database and questionnaire system (www.qnome.eu). The questionnaire consisted of seven questions and is available upon request.

Statistical analyses

Data from the gargle pool tests are presented using descriptive statistics. Normally distributed data are presented as mean with standard deviation (SD) and non-parametric data are presented as the median and interquartile range (IQR). Uncensored data were compared using a Wilcoxon test, and in case of censored values, a generalized Wilcoxon test was applied using the 'survival' package in R statistics. Permutation tests were performed to calculate differences in infection rates between SARS-CoV-2-naïve and immunized staff by using the 'coin' package in R statistics, version 4.1.2. A P-value <0.05 was considered statistically significant.

Results

The study was performed at the St. Hedwig's hospital which houses the KUNO University Children's Hospital and the University Maternity Hospital, totaling approximately 650 regular staff members (and 70 students) over 10 weeks between December 2021 and March 2022 (Figure 1). During a pre-test phase in the autumn of 2021, we implemented a Cepheid PoC rRT-PCR system to allow for rapid diagnosis of influenza, respiratory syncytial virus (RSV) and COVID-19 cases by multiplex PCR in our large emergency department at the hospital. Subsequently, we explored the possibility of using that system for pool PCR testing of our staff members. From October we offered a free and voluntary gargle pool PCR test service to our hospital staff, symptomatic or asymptomatic. In December of 2021, regular testing became mandatory for all hospital staff to be allowed to enter hospital premises by federal law. Detailed regulation on who was to be tested, how often and by which test system (antigen tests or PCR) were officially published (Supplementary Table S1). In brief, all staff members had to be tested at least twice a week, and test strategies had to be documented. We used the WICOVIR software for the documentation of all testing procedures and combined it with the COVIDA software which held all information (e.g., SARS-CoV-2 vaccination status and infection history) needed to regulate hospital entry and to determine necessary test frequencies by algorithm. All staff members received a personalized barcode linked to that software to enter the hospital through a gate with a barcode scanner. The same barcode was used to link the test samples to sample pools and PCR results. That way, participants of a positive pool could be identified immediately and called back to provide their back-up sample for de-pooling and single PCR testing.

During the medium-incidence phase of the project (incidence of 200 positive PCR tests per 100,000 inhabitants in Bavaria in the last week of December 2021) the size of the gargle test pool was set at 20 staff members and all PCR tests were conducted with the PoC system, which has the capacity to analyze four samples in parallel in 45 min. In case of a positive pool result, backup samples were retrieved immediately, which took approximately 10–20 min and the pool of 20 was dissolved into four pools of five which ran on a PoC system again. That way, 15 of 20 staff members knew that they were negative within 45 min after the positive pool was detected and could continue to work without any restrictions, while the last five samples from the positive pool in the second run were now tested individually. Thus, it took approximately 2 h to identify the positive sample. This set-up was feasible as long as no more than two positive pools occurred per test day.

When incidence rose to 1522 positive PCR tests per 100,000 inhabitants in Bavaria in week 4 of 2022 due to the Omicron wave (high incidence), leading to three or more positive pools per day in our hospital, we reduced the pool size to $N = 10$ participants per pool and increased the test interval to three tests per week and activated the Allshang/Bio-Rad system in addition to the Cepheid test system. Thus, we could combine the flexibility of testing with increased capacity. All pools until 8:00 a.m. were now tested with Cepheid (early tests) while the Allshang/Bio-Rad system was used to handle the large number of staff members who entered the hospital at regular work times between 7:30 and 8:45 a.m. During this second round of pool tests, all positive pool tests from the early test round were de-pooled running single samples individually. Results were ready by 10.45 a.m. and a second run for dissolving positive pools from the second round and additional pools of latecomers were run at 12:00 p.m., with results available at 13:45 p.m. latest. PCRs for pools and de-pooling in the afternoon were performed on the PoC Cepheid system again. Thus, the time for receiving results increased to a maximum of 5 h while the average was less than 3 h.

Overall, we performed 8793 systematic tests during the study period translating to 697 pool PCR runs. Of these, five pools were false positive (0.7%). Additionally, 852 PCR runs were necessary for de-pooling. During the study period of 10 weeks, we identified 65 asymptomatic staff members to be positive by pool testing and 97 staff members became symptomatic and were tested positive by single/individual PCR tests (Figure 1). In general, Ct values of staff members identified by regular pool testing were significantly higher compared with individual PCR tests of symptomatic staff members (median (IQR): 31.5 (26.4–33.6) vs 26.3 (22.1–30.2); $P<0.001$). In a great majority of cases, these values were beyond the detection limit of antigen tests (Figure 2). Of note, gargle pool tests could not be performed for one week due to an Omicron infection of laboratory personnel (week 9–10 of the study). During that time, virus loads of tests performed when individuals became symptomatic increased by two PCR cycles (Ct values were representing two exponential steps difference). During the study period, we neither observed an outbreak in a specific section of the hospital nor an increase in nosocomial infections in patients but many random infections in the staff. The small group of unvaccinated staff members were over-represented in the positively tested (37.1% positive tests in

SARS-CoV-2-naïve staff vs 21.9% in staff with at least one vaccination or infection; P=0.04; Figure 3).

We compared the incidence of staff members identified to be SARS-CoV-2 positive by our test regime to the weekly incidence for the general Bavarian population in the age range (18–60 years) most similar to our hospital staff as provided by the Bavarian public health office (Supplementary Table S2; Figure 1). For every week, the incidence in our hospital staff surpassed the incidence in the general population by an average factor of 1.5- to 2-fold.

At the end of the study period, we asked hospital staff to answer an anonymous online questionnaire about their opinion

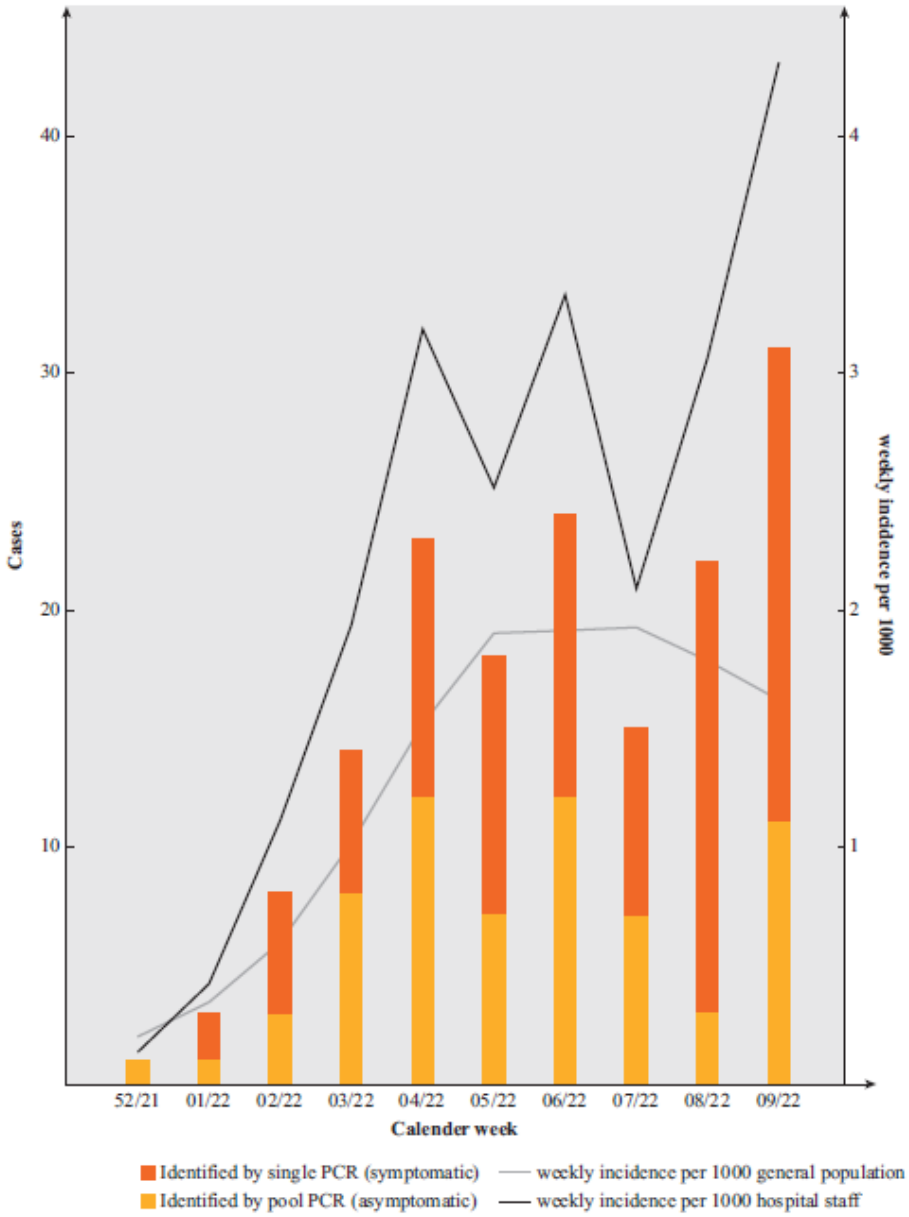

Figure 1. Weekly numbers of individuals positively tested for SARS CoV-2 by pool testing (asymptomatic) and single PCR (symptomatic) plotted against the incidence in the general population. The numbers for general population of Regensburg city and county were taken from official reports by the Bavarian Public Health office (LGL) [5].

on the implementation, safety and convenience of the gargle pool rRT-PCR test system. Approximately 1/3 of the staff members (202/650) from all areas of the hospital (doctors (N = 43), nursing staff (N = 96), administration and scientific offices (N = 33) and midwives and supportive services (N = 30)) participated in the questionnaire. Overall, 75% rated the implementation as 'good' or 'very good' (22% 'fair' or 'sufficient', 3% 'insufficient') and only a minority (13%) experienced waiting times (mean: 3 min). An overwhelming majority rated the gargle pool PCR system superior in safety for staff and patients when compared with antigen-based tests (90% vs 10%) and when asked for the preference of a test system, 84% selected the gargle pool PCR system over any antigen-based test system.

Discussion

Repeated gargle pool rRT-PCR testing can be implemented quickly and with high acceptance in hospitals and adapted easily even to massive increases in incidence. Due to the high

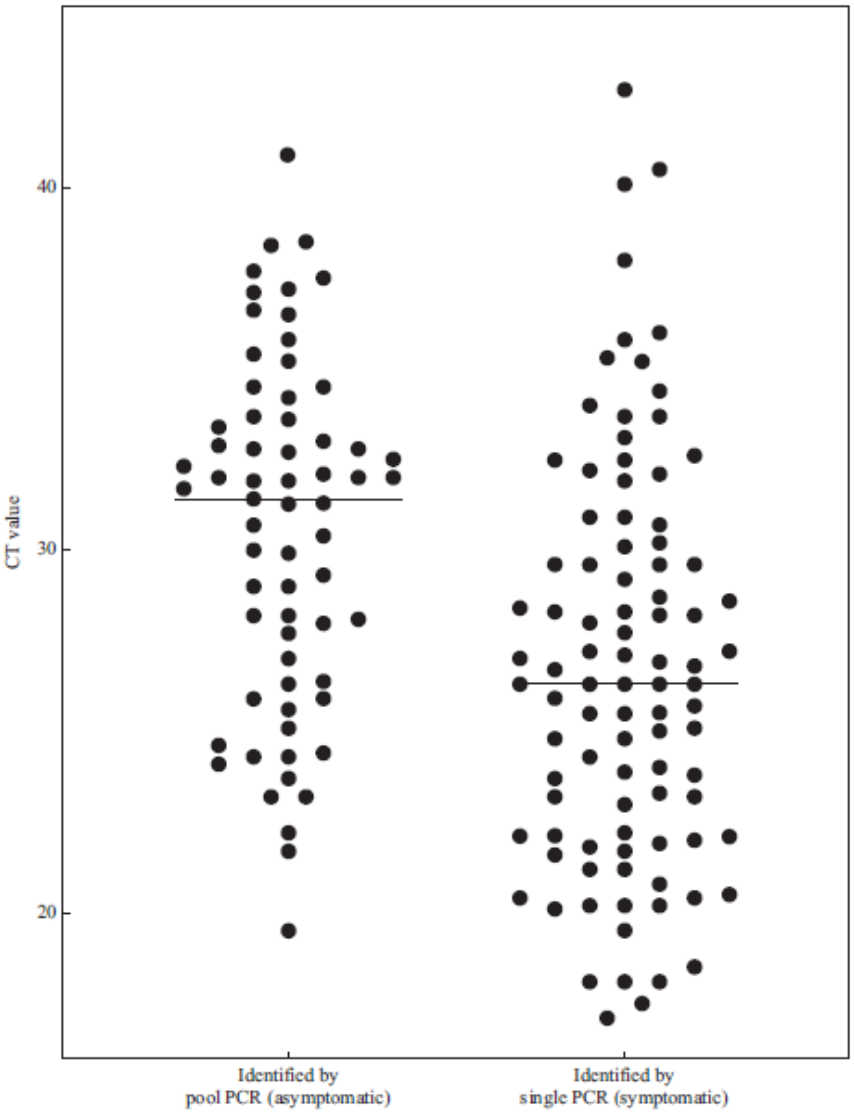

Figure 2. Ct values and median value of individuals positively tested for SARS CoV-2 by pool testing (asymptomatic) and single PCR (symptomatic).

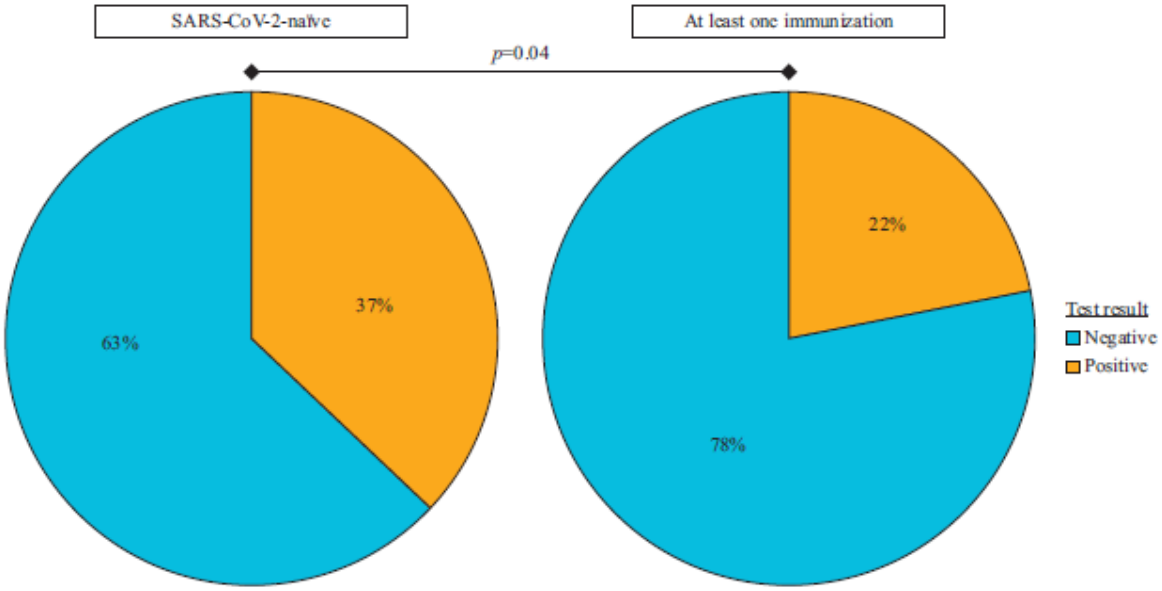

Figure 3. Percentage of individuals positively tested for SARS CoV-2 by immunization status.

sensitivity of the PCR test system, positive staff members could be removed from hospital service early enough to avoid infection chains in the hospital.

Our proof-of-concept study aimed to describe the technical, digital and logistical set-up of a gargle pool rRT-PCR testing system in a medium-sized hospital during the onset of the Omicron wave in Germany, and we were able to show the feasibility and acceptance of such an approach. Furthermore, it gave a detailed and accurate picture of Omicron infection-dynamics in hospital staff during that time.

The aim of any testing in a hospital setting is to avoid infection of patients and other staff members. Ideally and theoretically, nobody with a potential infection should work in the hospital. Realistically, this cannot be achieved without major interference with hospital services and the availability of staff. A regular hospital testing scheme in addition to high vaccination/immunization rates is therefore a more feasible approach to that end. The specific challenge of testing in a hospital environment is the need for very high accuracy (which can only be achieved by PCR testing) and the need for transmitting many test results very fast (which is difficult to achieve by PCR testing). Gargle pool PCR test systems can help to overcome limitations in PCR testing rates [1,8,9] and with intelligent software, data transmission of results can be speeded up as we have shown in the WICOVIR project for schools [1].

Our WICOVIR test system, as described in detail elsewhere [1], is based on gargling at home and pooling samples on entering the institution, in this case the hospital. Thus, pooling logistics in the laboratory are not necessary, pre-analytic sample handling is dramatically reduced and time is gained, which is key to successful hospital testing. When a pool was found positive, samples were retrieved immediately from all staff members pertaining to that pool. This was facilitated by the software COVIDA which was based largely on pre-developed software from WICOVIR [1] but adapted and expanded specifically for the hospital test set-up. During the time of de-pooling, members of such a pool were asked to follow strict hygiene measures and to avoid direct patient contact wherever possible. As de-pooling was so fast, this never disrupted hospital service. Importantly, members of single departments and units were not tested in pools clustering the respective department, unit or ward but by random order. Thus, if a pool was tested positive, no single department, unit or ward had to shut down completely. This was a fundamental change in strategy from school testing, where school classes are recommended to be tested together due to logistics [4].

The limitations of such a gargle pool test system are the machines and consumables needed and, as a key factor, experienced staff to run the tests. While machines can be ordered in advance and represent an investment of approximately €100,000, consumables were a limiting factor throughout the pandemic. Shortages in supply chains especially in the PoC test system threatened to shut down operations and forced us to adapt procedures. However, it was the combination of a fast and individualizable PoC system (Cepheid) and a high-throughput 'workhorse' system (Allshang/Bio-Rad) which proved ideal for the challenges of the hospital setting. Conversely, the technical expertise required to run the Allshang/Bio-Rad system is substantial. Furthermore, infection of technical personnel needs to be considered and thus, a backup test system with antigen-tests was put in place and had to be activated in week 9 of the project, when lab workers were not available due to Omicron infection. During that time, staff members had to perform self-tests at least twice a week for regular screening testing without symptoms and only one staff member went to PCR testing without symptoms due to a positive antigen test. Interestingly, during that same time more

symptomatic infections were recorded, and Ct-values of the symptomatic tests decreased substantially, indicating a higher virus load at the time infections were detected. Our interpretation of this observation is that the antigen test was not sensitive enough to detect positive cases in the time interval between infection and symptoms. Therefore, more staff members remained undetected while already positive and potentially infectious. This is also reflected by the higher virus loads found when staff members were finally tested when they were symptomatic. While this is not surprising, our study is one of the few that provides actual (but limited) data for that observation.

When the infection numbers rose to unprecedented heights in week 4 of January 2022, due to the Omicron wave, we were not sure whether the pooling system would withstand and allow us to handle such a high number of positive pools to be processed in time. This was always the major argument of opponents to PCR pooling tests. However, with two adjustments to our system, namely adding two runs of the Allshang/Bio-Rad system and decreasing the pool size from 20 to 10 participants when the (true) incidence was beyond 3000 infections per 100,000 individuals, the turn-around time for results was still very acceptable within a 4-h frame. To optimize pool size for our set-up, we developed a pool size calculator: expected incidence, plate- and laboratory personal capacity, as well as requested turn-around time of results were taken into account.

For a good performance of the testing procedure, its acceptance by the hospital staff was imperative. To investigate this, we invited all hospital staff to participate in an online survey. A participation rate of approximately 30% was achieved and can be considered representative, also according to the distribution of participants over employment groups. Interestingly, gargle pool PCR was not only viewed as superior in safety over antigen-testing by the staff, but staff members also preferred the gargle pool testing over self-tests by nasal swabs at home. The reason for this may be that swabbing the nose every two to three days is indeed unpleasant in the long run and more invasive than gargling. This should be considered for the acceptance of future test strategies for hospitals and nursing homes for the elderly, where testing regimes are considered to be needed for the future.

Applying PCR-based tests allowed very precise detection of SARS-CoV-2 infections in our staff. Patients were tested routinely on hospital referral and on a regular basis while they were in-patients. This allowed for a comprehensive picture of infection dynamics in our hospital during the study period which coincided with the beginning of the Omicron wave in Germany. We also compared numbers of positive tests in our staff with officially reported infection rates (Supplementary Table S2). We had full information on the vaccination status and infection history of our staff to plot against infections. While vaccination rates of our staff were much higher than in the general population, the number of detected infections in our hospital were much higher than reported for the general population. This might be explained partly by the fact that detection of SARS-CoV-2 for the wider population is now primarily based on antigen PoC tests, which are inferior to PCR in terms of diagnostic validity, and because a concept of closely knitted, sensitive testing in a defined cohort and setting such as medical staff in a hospital can detect cases more effectively. Conversely, the incidence on the population level might have been underestimated due to delays in reporting because of the high case numbers. Furthermore, the vaccine efficacy in terms of protection against infection (estimated by the Farrington method) might be overestimated due to misclassification concerning vaccination status. Therefore, we conclude that publically reported infection rates underestimate the true number of infections by approximately a factor of 2. In our cohort, the relative risk of getting infected by Omicron was higher in unvaccinated staff members. However, these numbers need to be interpreted with caution, as we only studied a small cohort. Interestingly, outbreaks and nosocomial infections may be avoided in a hospital setting, even in times of high infection rates, when non-pharmaceutical interventions are complemented with vaccination and a truly functional test regime. Our analysis of infection chains revealed that the vast majority of infections of our staff members occurred in the private setting or during the private contact of staff members (e.g., during breaks).

We conclude that repeated gargle pool rRT-PCR testing can be implemented quickly in hospitals and is an effective, easily adaptable and well-accepted test system for hospitals, withstanding even very high infection rates. Our data show that with a proper testing concept in place, hospitals can be a safe place for patients and staff members even during a pandemic.

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Author Contributions

P.K., M.K. and A.A. contributed to conception and design of the study. P.S. established and supervised test procedures, which were performed by P.K., P.S., E.F. and M.K. A.A. supervised the labwork. B.M.J.L. analysed public health measurements. J.N. and P.P. provided IT support and developed the software for the project. V.D.G. performed the statistical analysis. M.K. and P.K. wrote the first draft of the manuscript, V.D.G. provided specific section of the text. M.K. wrote the final version of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

Conflict of interest statement

Maganamed GmbH is a commercial software company and Jakob Niggel and Philip Pagel are employed by Maganamed GmbH. All other authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhin.2022.05.018.

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SARS-CoV-2 Detection Limits in Swab and Gargle Samples by Comparing Antigen and RT-qPCR Testing

Parastoo Kheiroddin [1,*], Natascha Borchers [1], Ezgi Cibali [2], Thea Würfel [1], Sarah Nowosadtko [1], Michael Kabesch [1,3,†] and Andreas Ambrosch [2,†]

[1] Department of Pediatric Pneumology and Allergy, University Children's Hospital Regensburg (KUNO) at the Hospital St. Hedwig of the Order of St. John and the University of Regensburg, 93049 Regensburg, Germany; natascha.borchers@klinik.uni-regensburg.de (N.B.); thea.wuerfel@stud.uni-regensburg.de (T.W.); sarah.nowosadtko@stud.uni-regensburg.de (S.N.); michael.kabesch@barmherzige-regensburg.de (M.K.)

[2] Institute of Laboratory Medicine, Microbiology and Hygiene, Hospital of the Order of St. John, 93049 Regensburg, Germany; ezgi.cibali@barmherzige-regensburg.de (E.C.); andreas.ambrosch@barmherzige-regensburg.de (A.A.)

[3] Science and Innovation Campus Regensburg (WECARE) at the Hospital St. Hedwig of the Order of St. John, 93049 Regensburg, Germany

* Correspondence: parastoo.kheiroddin@klinik.uni-regensburg.de; Tel.: +49-941-943-5845

† These authors contributed equally to this work.

Abstract: Background: Antigen tests for SARS-CoV-2 testing are rapid and inexpensive but usually have lower sensitivity than RT-qPCR and are only validated for nasopharyngeal/throat swabs; the latter are considered the gold standard in terms of material collection but are not tolerated by patients with frequent sampling. The present study, therefore, investigates the extent to which SARS-CoV-2 antigen testing is comparable to RT-qPCR from an easily obtained gargle solution compared to nasopharyngeal swabs. **Methods:** The performance of a high-quality POC fluorescence immune antigen test in single nasal swab samples and gargle samples compared to RT-qPCR was investigated (total n = 620 samples (gargle samples = 309, and nasal swabs = 311)). **Findings:** In our setting, the detection of SARS-CoV2 with an antigen test was reliable up to a Ct value of 30 for single nasal swab samples and was reduced to Ct:20 for single gargle samples. The overall antigen-test sensitivity is 83.92% (swab samples) and 75.72% (gargle samples). **Interpretation:** Antigen tests showed reliable results up to a detection limit of Ct: 30 with only nasal swab samples but not gargle samples. If the use of gargle samples is preferred due to their advantages, such as painless testing, easy handling, and the lack of a need to involve trained personnel for sample taking, reliable results can only be achieved with RT-qPCR.

Keywords: COVID-19; SARS-CoV-2; Germany; pool gargle; RT-qPCR; antigen test

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1. Introduction

At the beginning of the COVID-19 pandemic, only material from nasopharyngeal swabs was recommended for virus detection by the WHO [1]. Later, gargle samples were also shown to be a suitable source for testing; using gargle samples brings different benefits: (a) Its collection is neither painful nor unpleasant and is easy to perform, thus increasing test acceptance [2]; (b) it is safer for healthcare personnel since gargling can be performed without contact by the test persons themselves; (c) when pooling samples to increase test capacity and save resources, gargle samples offer an easier and safer option, since every individual can throw their sample into the pooling container, which also reduces the chance of contamination and mixing samples [3–5]. A disadvantage of gargle samples may be that the test sensitivity is lower due to dilution effects caused by the gargle amount [6–8].

Although RT-qPCR is the gold standard for detecting SARS-CoV-2 [9], it requires special equipment, skilled laboratory personnel with a background in molecular biology, and

at least 3 h of processing time, which are not feasible in all settings, thus limiting its application. In contrast, antigen tests, which are mainly based on fluorescence immunoassays and the detection of a specific nucleocapsid protein derived from SARS-CoV-2 [10], are faster, cheaper, easier to perform, and robust in almost any situation. Numerous antigen and RT-qPCR tests are now on the market, and the most common tests require nasopharyngeal swabs [11].

Here, we evaluate if one could combine the convenience of gargling with the simple testing procedure of antigen tests. Thus, we compared the sensitivity and specificity of a fluorescence-based antigen-test (STANDARD™ F COVID-19 Ag FIA kit (SD BIOSENSOR Inc., Suwon-si, Korea) with those of RT-qPCR, first using nasopharyngeal swabs and, in a second step, gargle samples. Finally, we tested how the gargle samples performed with a selection of other antigen tests and with RT-qPCR pool testing.

2. Methods

2.1. Study Cohort and Sampling

In total, 309 gargle samples and 311 nasal swabs were collected for routine testing in hospitals of the Order of St. John in Regensburg and Straubing, Germany, from October 2019 until April 2020, when the original variant and the Alpha Variant of SARS-CoV-2 were predominant in Germany. Two nasal swabs were collected concomitantly by medical personnel in the emergency room. One nasal swab was transferred immediately to the extraction buffer from a STANDARD™ F COVID-19 Ag FIA kit (SD BIOSENSOR Inc., Suwon, Korea) to be tested with an antigen test, and a second nasal swab from the same patient was transported to the laboratory for RT-PCR testing on the same day for quality control.

Gargle samples were provided by patients and medical students by gargling for approximately 30 s with 10 mL of sterile water (Ampuwa). The recovered gargle fluid of approximately 10 mL was then transferred to a 250 mL container, and antigen against SARS-CoV-2 was analyzed in each gargle sample immediately after sampling. The remaining sample fluid was kept for quality control and RT-qPCR analysis. All samples analyzed here were leftovers from routine testing and were anonymized before the analysis in this study was performed.

2.2. SARS-CoV-2 Antigen Testing Procedures

Gargle and swab samples were tested for SARS-CoV-2 antigen right after sampling. Either the nasal swab or 150 µL of gargle sample was transferred into an extraction buffer tube provided with the STANDARD™ F COVID-19 Ag FIA kit (SD BIOSENSOR Inc., Suwon, Korea), followed by treatment according to the manufacturer's instructions. Briefly, after closing the buffer tube with the provided nozzle cap, the tube was squeezed 10 times to mix the sample with the extraction buffer. Then, we applied 4 drops of the extracted specimen to the well of the respective test cassette. After 15 min of incubation at room temperature, the test cassette was loaded into the analyzer (SD BIOSENSOR), and the COI as a numerical representation of the measured fluorescence signal was calculated automatically by the analyzer. A COI $\geq$ 1.0 represents a positive result for SARS-CoV-2 nucleoproteins, according to the manufacturer.

To better evaluate our data from the antigen testing of the gargle, we checked our results against two other antigen tests—one with a similar (76%) and one with a lower sensitivity (36%) [10]: Eight positive PCR gargle samples with increasing Ct values were tested with the CLINITES Rapid COVID-19 Antigen Test (SIEMENS Healthineers., Houston, TX, USA)—a test with similar sensitivity—and with the NADAL COVID-19 Ag test (Ref.243103N-20, nal von minden., Moers, Germany). For both tests, the sample and buffer were mixed at a ratio of 1:1. For the CLINITES, the waiting time was 1 min, and then 4 drops were added, followed by 15 min of incubation time. For the NADAL kit, the waiting time was 2 min after mixing the sample and buffer, and then 2 drops were added into the sample well, followed by an incubation of 15 min.

2.3. RT-qPCR Testing Procedures

To detect SARS-CoV-2 genomic RNA in gargling samples, we performed RNA extraction as a first step by using BEXS Ready Viral DNA/RNA kits (Inno-train Diagnostik, Kronberg, Germany). We added a fixed amount (10 µL per sample) of a 70-base-pair fragment of Equine Arteritis Virus (EAV, TIB Molbiol, Berlin, Germany) as an extraction control to each sample. Then, we conducted one-step RT-qPCR with the LightCycler® Multiplex RNA Virus Master (target E gene) on a Light Cycler 480 II Instrument (Roche Diagnostics). A positive sample was confirmed with a second qualitative test system: the Xpert Xpress™ SARS-CoV-2 assay (cartridge system including an extraction step and amplification targeting the E- and N2-genes) on a GeneXpert instrument (Cepheid, Sunnyvale, CA, USA).

To investigate the detectability of individual positive gargle samples in a standardized gargle pool in our PCR setting, we added 1 mL of each positive sample with different Ct values 20 mL of a negative gargle pool of 20 participants. Then, RNA was extracted from both single and pool samples by using the MagnifiQ™ RNA buffer kit (A&A Biotechnology, Gdansk, Poland)) on an Auto-Pure96 Nucleic Acid Purification System (Hangzhou Allsheng Instruments, Shanghai, China) according to the manufacturer's protocol. RT-PCR-based SARS-CoV-2 RNA detection was performed on a BIORAD Real-Time PCR System using the single-well dual target (ORF1b and N2 gene). Further information regarding the primer and probe sequences is available in Supplementary Table S1.

To show that the BIORAD and Light Cycler 480 II Instrument (Roche Diagnostics) have very similar sensitivities and specificities for detecting SARS-CoV-2, we ran identical samples on both systems (Supplementary Table S2).

3. Results

We performed antigen tests and RT-qPCR tests by using the LightCycler® Multiplex RNA Virus Master (target E gene—TibMolBiol) on 311 nasal swabs and 309 gargle samples (total $n = 620$). Out of these, 47 swab samples (Figure 1A) and 64 gargle samples (Figure 1B) were determined to be positive by RT-qPCR (duplicate testing in two test systems) with constant Ct values below 40.

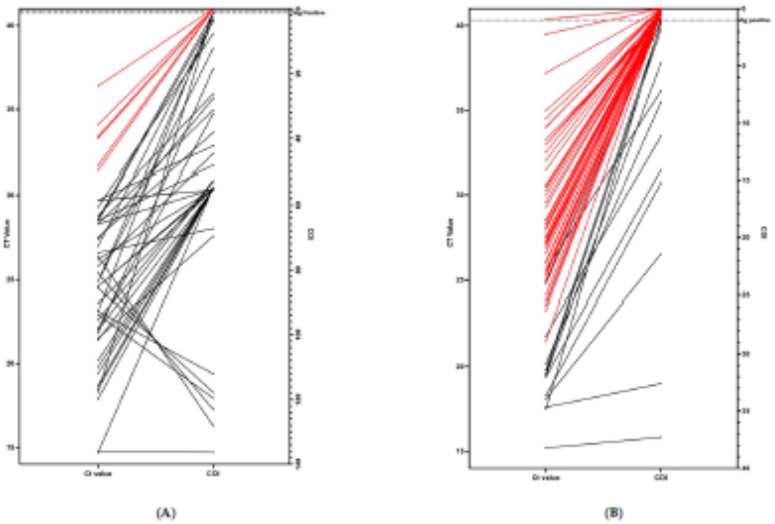

Figure 1. Correlation between Ct values from RT-qPCR (left side) and COI values from antigen tests (right side). Positive samples that were negative on antigen tests are shown in red. (**A**) (on the left) shows the results in 47 PCR-positive swab samples. (**B**) (right one) shows the results in 64 PCR-positive gargle samples.

The false-negative rate of the antigen test—when the antigen test was negative but the RT-qPCR test was positive—was high in gargle samples at 16.18% (50 out of 309), while it was only 1.92% (6 out of 311) in the swab samples. Regarding the false positive rate—when the antigen test was positive, but the RT-qPCR test was negative—it was higher in the swab samples at 11.57% (36 out of 311) than in gargle samples at 8.09% (25 out of 309). In general, the sensitivity of the antigen test was higher in the swab samples (83.92%) than the gargle samples (75.73%). The antigen test's sensitivity for PCR-positive samples with Ct values of 15–20 was 100% in both swab (n = 8) and gargle (n = 10) samples. In the group of PCR-positive samples with Ct values of 20–25 and 25–30, the antigen test's efficiency stayed at 100% for the swab samples (n = 17, n = 16), but dropped to 25% (Ct 20–25, n = 12) and 4.1% (Ct 25–30, n = 24) for the gargle samples. Our results show that the antigen test did not detect SARS-CoV-2 in samples with Ct values above 30 in either swab or gargle samples. We had n = 220 antigen-negative results from each of the swab and gargle samples, which we could confirm as negative samples with RT-qPCR.

The other two antigen tests (CLINITEST/NADAL) were tested as indicators for our selected SD BIOSENSOR FIA test; neither of the other two test kits was more appropriate (Table 1). Furthermore, we explored the possibility that a simple dilution effect introduced by using gargle samples may have reduced the sensitivity. Therefore, we calculated the effect of lowering the positive cutoff index in the SD BIOSENSOR test for gargle samples on the sensitivity and specificity of the test results, as shown in Table 2. That procedure did not significantly increase the sensitivity, while the specificity was massively decreased at the same time.

Table 1. Comparison of different antigen test kits with positive gargle samples.

Sample Number	Ct value by RT-PCR (E Gene)	SD BIOSENSOR/COI	CLINITES	NADAL
1	20.1	Positive/2.59	Positive	Negative
2	21.8	Positive/2.03	Negative	Negative
3	22.6	Positive/2.11	Positive	Negative
4	24.1	Negative	Negative	Negative
5	26.1	Negative	Negative	Negative
6	28.3	Negative	Negative	Negative
7	30.5	Negative	Negative	Negative
8	32.6	Negative	Negative	Negative

Table 2. Comparison of the efficiency of detecting positive gargle samples and the rate of false positives for negative gargle samples by changing the COI.

Sensitivity: Efficiency of Detecting Positive Gargle Samples (n = 64)	COI	Specificity: Rate of False Positives for Negative Samples (n = 176)
65.63%	0.1	59.66%
51.56%	0.2	39.20%
45.31%	0.3	26.70%
35.94%	0.4	21.59%
32.81%	0.5	19.32%
29.69%	0.6	12.50%
25.00%	0.7	8.52%
25.00%	0.8	6.82%
23.44%	0.9	3.41%
21.88%	1	0.00%

Based on these test results, we determined that gargle samples cannot easily be used with currently available antigen tests that are licensed for the use with swab samples. To explore if gargle samples could be pooled for PCR testing to bring the costs of testing down to areas similar to those of antigen testing and to explore if pooled gargle samples in combination with PCR testing would have similar or higher detection limits compared to those of antigen tests, we tested if single positive gargle samples of Ct values between 32 and 38 could be detected by PCR testing in standardized gargle pools of 20 + 1 samples. Therefore, we added 1 mL of positive gargle fluid to 20 mL of pooled gargle fluid from negative individuals and performed RNA extraction followed by RT-qPCR, as described for single-sample PCR (Table 3). Indeed, even when pools of 21 were used, positive gargle samples with a set Ct value of up to 35 could be detected in the pools.

Table 3. Comparison of different RT-qPCR Ct values of the single samples and in the pool of 21 individuals.

Number	Single Sample		Same Sample in Pool of 20	
1	N2	ORF1b	N2	ORF1b
2	22.89	22.88	26.91	26.93
3	25.32	25.8	30.24	30.3
4	26.31	26.75	29.7	29.62
5	27.98	27.86	31.85	31.45
6	31.21	30.54	34.24	33.93
7	32.85	32.01	35.36	36.5
8	33.04	31.82	34.64	34.92
9	34.63	33.70	36.60	34.21
10	35.80	35.85	_	_

4. Discussion

Our data show that swab samples containing viral loads correlating to Ct values of up 30 (in our PCR testing setup) can reliably be detected with high-quality antigen testing. Using 10 mL of gargle samples with the same antigen test reduces the detection limit to Ct values of 20. When gargle samples were analyzed in pools of 21 by PCR, single positive samples with Ct values of up to 35 were reliably detected within the pools.

For our experiment, we used a well-established antigen test that is based on fluorescence detection and provides a numerical output for measurements. That the antigen test by Biosensor Inc. gives reliable results was shown previously in several reports: In a study published in the spring of 2021 on 359 nasopharyngeal swab samples from Italy, the antigen test showed good sensitivity for samples with Ct values lower than 25 [11]. In a further study, the STANDARD Q COVID-19 antigen test showed the sensitivity of 74.4% in 289 swab samples, in which 31 out of 39 positives were detected with the antigen test, and all positive samples had RT-qPCR Ct values lower than 27 [12].

Using gargle samples instead of swabs reduces the detection limit of the antigen test when applied reliably in swab samples by 2^{10} fold. Dilution alone does not seem to be a reasonable explanation for this dramatic change, even though nucleoprotein antigens of SARS-CoV-2 are expected to be more concentrated in a nasal swab than in 10 mL of gargle fluid. If dilution would be the main factor, lowering the COI for determining a positive result could be a possible strategy for adjusting the detection limit. However, lowering the detection limit does not lead to a reasonable increase in sensitivity without an unreasonable decrease in specificity, as shown in Table 2. Therefore, we conclude that the chemistry of antigen tests would have to be specifically designed to work with gargle samples. This is not a specific feature of the test that we used here, but a common feature of antigen tests on the market that are designed to be used with nasal swabs, as we showed in our

experiments. When different specimens were compared for antigen testing in another study, no positive gargle samples ($n = 7$, RT-qPCR Ct value: 26.3–36) were detected by the antigen test, which confirms our data showing that gargle samples with Ct values higher than 20 are not detectable with standard antigen tests. In that study, antigen tests showed a 44.4% sensitivity overall using a nasopharyngeal swab (8 out of 18 samples), and all positive samples found with the antigen test had Ct values lower than 25 [13]. Another study performed in Switzerland among hospitalized patients showed that the sensitivity of the SD Biosensor test for samples from asymptomatic COVID-19 patients was 28–33%, while it dropped to 25% in patients with 5 days of COVID-19 symptoms [14].

Taken together, our study seems to be the largest comparison of gargle versus nasal swab samples analyzed concomitantly with antigen tests and Rt-q PCR.

Simply combining the gargle procedure with current antigen tests failed. Still, it would be desirable to use gargle samples for the reasons mentioned above: It is a painless, safe, and well-accepted procedure that is especially suitable for children and repeated testing. The advantage of combining it with antigen testing would have been the simplicity of antigen testing in the field, the independence from laboratory logistics, and, not to be neglected, the attractive price of antigen tests compared to RT-qPCR. Thus, we investigated if gargle samples could be combined with pool PCR testing as an alternative.

Indeed, our results show that, by using 21 gargle samples in a pool, we are not only able to detect positive samples reliably and with costs comparable to those of antigen testing but can also do so with a significantly increased detection limit. We calculated the costs of our pool PCR to be less than EUR 1 per participating sample, taking material, personnel costs, and logistics into account (data available upon request from the authors). In our setting presented here and in a study on school testing that was recently published [3], we showed that the detection limit for pool testing is a Ct value of 35, increasing detection by a factor of 2^5 compared to antigen testing. In a pandemic situation, faster detection of positive individuals may be crucial; thus, using gargle pools in combination with RT-qPCR may be the key to successfully breaking infection chains without increasing costs. In situations where laboratory accessibility is limited, the combination of nasal swabs and a high-quality antigen test may be a feasible option.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/covid2060056/s1, Table S1: ORF1b and N2 gene primers and probes sequences information, Table S2: Comparison of sensitivity and specificity of two RT-qPCR settings (ROCHE, and BIORAD).

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the regional Ethics Committee of the University of Regensburg.

Informed Consent Statement: Patient consent was waived since SARS diagnostic was carried out as part of routine diagnostics in the context of medical care of patients during pandemic.

Conflicts of Interest: All authors declare that they have no competing financial or personal interests.

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ORIGINAL RESEARCH
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Symptoms, SARS-CoV-2 Antibodies, and Neutralization Capacity in a Cross Sectional-Population of German Children

Otto Laub[1†], Georg Leipold[2†], Antoaneta A. Toncheva[3†], David Peterhoff[4,5†], Sebastian Einhauser[4†], Patrick Neckermann[4], Natascha Borchers[3], Elisangela Santos-Valente[3], Parastoo Kheiroddin[3], Heike Buntrock-Döpke[3,6], Sarah Laub[1], Patricia Schöberl[3,6], Andrea Schweiger-Kabesch[3], Dominik Ewald[7], Michael Horn[8], Jakob Niggel[9], Andreas Ambrosch[10], Klaus Überla[11], Stephan Gerling[3], Susanne Brandstetter[3,6], Ralf Wagner[4,5†], Michael Kabesch[3,6†] and Corona Virus Antibodies in Children from Bavaria (CoKiBa) Study Group*

[1] Pediatric Office Laub, Rosenheim, Germany, [2] Pediatric Office Dr. Leipold, Regensburg, Germany, [3] University Children's Hospital Regensburg (KUNO) at the Hospital St. Hedwig of the Order of St. John, University of Regensburg, Regensburg, Germany, [4] Institute of Medical Microbiology and Hygiene, Molecular Microbiology (Virology), University of Regensburg, Regensburg, Germany, [5] Institute of Clinical Microbiology and Hygiene, University Hospital, Regensburg, Germany, [6] Member of the Research and Development Campus Regensburg (WECARE) at the Hospital St. Hedwig of the Order of St. John, Regensburg, Germany, [7] Pediatric Office Dr. Heuschmann & Dr. Ewald, Regenstauf, Germany, [8] Pediatric Office Dr. Horn, Schönau, Germany, [9] Maganamed Limited, Regensburg, Germany, [10] Institute of Laboratory Medicine, Microbiology and Hygiene, Hospital of the Order of St. John, Regensburg, Germany, [11] Institute of Clinical and Molecular Virology, FAU Erlangen-Nuremberg and Universitätsklinikum Erlangen, Erlangen, Germany

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***Correspondence:**
Michael Kabesch
michael.kabesch
@barmherzige-regensburg.de

[†] These authors have contributed equally to this work

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Background: Children and youth are affected rather mildly in the acute phase of COVID-19 and thus, SARS-CoV-2 infection infection may easily be overlooked. In the light of current discussions on the vaccinations of children it seems necessary to better identify children who are immune against SARS-CoV-2 due to a previous infection and to better understand COVID-19 related immune reactions in children.

Methods: In a cross-sectional design, children aged 1–17 were recruited through primary care pediatricians for the study (a) randomly, if they had an appointment for a regular health check-up or (b) if parents and children volunteered and actively wanted to participate in the study. Symptoms were recorded and two antibody tests were performed in parallel directed against S (in house test) and N (Roche Elecsys) viral proteins. In children with antibody response in either test, neutralization activity was determined.

Results: We identified antibodies against SARS-CoV-2 in 162 of 2,832 eligible children (5.7%) between end of May and end of July 2020 in three, in part strongly affected regions of Bavaria in the first wave of the pandemic. Approximately 60% of antibody positive children ($n = 97$) showed high levels (>97th percentile) of antibodies against N-protein, and for the S-protein, similar results were found. Sufficient neutralizing activity was detected for only 135 antibody positive children (86%), irrespective of age and sex. Initial COVID-19 symptoms were unspecific in children except for the loss of smell and taste and unrelated to antibody responses or neutralization capacity. Approximately 30%

of PCR positive children did not show seroconversion in our small subsample in which PCR tests were performed.

Conclusions: Symptoms of SARS-CoV-2 infections are unspecific in children and antibody responses show a dichotomous structure with strong responses in many and no detectable antibodies in PCR positive children and missing neutralization activity in a relevant proportion of the young population.

Keywords: antibody, neutralizing, COVID-19, SARS-CoV-2, children

INTRODUCTION

Early in the COVID-19 pandemic, children and adolescents were thought to be important transmitters of the disease but were also believed to be only mildly affected (1). Later, evidence increased that children are not major spreaders (2–4). However, a pediatric multiorgan immune syndrome in children and youths was reported (5), occurring weeks to months after the SARS-CoV-2 infection, also in children with mild or no symptoms in the initial phase of the disease. Recent studies linked PIMS to the presence of antibodies to SARS-CoV-2 and some authors suggested that high levels of antibodies against SARS-CoV-2 may in fact contribute to the occurrence of the full-fledged syndrome (6). These observations indicate that immune reactions to SARS-CoV-2 exposure may differ, at least in strength, between children and adults.

When vaccination for SARS-CoV-2 was first administered to adults, stronger systemic vaccination reactions to the vaccine were reported in younger individuals (7). In some of our cases, high antibody levels were already observed directly after vaccination when these symptoms occurred (own observation), suggesting a possibility that these individuals may have had an unnoticed SARS-CoV-2 infection previously. With vaccination of children against SARS-CoV-2 in sight, it is important to better identify those that were already infected and to improve our understanding of SARS-CoV-2 related immune responses in children overall.

In many children allegedly mild or inapparent infections occurred and PCR testing was performed rarely. Therefore, we screened a large number of children in rather severely affected areas of Bavaria (Southern Germany) for symptoms as well as overall and neutralizing antibody levels against SARS-CoV-2 in the first pandemic wave in spring of 2020, in a population-based approach.

METHODS

Study Design and Population

In a cross-sectional design we investigated children from three distinct regions of South East Germany to assess the true prevalence of SARS-CoV-2 infections in areas with very differently reported infection rates by antibody testing. We established a network of pediatricians who volunteered to take part in the study and focused on three areas/counties within Bavaria with very high, moderate, and average infection rates as indicated by positive PCR tests per 100,000 inhabitants according to the Robert Koch Institute, the German center for disease prevention (**Figure 1**). The assessment and sample collection took place in three study areas: Tirschenreuth; Regensburg city and county; and Oberbayern/ alpine region from May 22nd to July 22nd, 2020. In areas where the number of willful study participants exceeded the capacity of local pediatricians, a study team supported sample collections.

Invitation to participate for children aged 1–14 years was based on two approaches: (a) All children of that age group who were scheduled for a prevention program visit in 2020 with the respective pediatrician were invited to participate (random selection) and (b) all children of families who actively wanted to participate were also tested (own intention to participate). In approach (b), also siblings older than 14 years were allowed to participate in the study, as for ethical reasons, children older than 14 could not be excluded from antibody testing if families presented them together with younger siblings for testing. The study was approved by the Ethics Committee of the University of Regensburg (file-number: 20-1865-101).

Data Collection and Management

All data were collected in an online survey using self-administered parental questionnaires. The questionnaires can be obtained upon request from the authors. All acquired data was fully anonymized and only accessible at an individual level to the participant using an individual code on the Qnome platform (www.qnome.eu) as previously described in detail (8). Clinical data was entered by the parents in an online survey. That way, anonymization of data on the level of the dataset was achieved while the test values were directly accessible to parents.

SARS-CoV-2 Antibody Tests

Blood was taken from all participants by venipuncture. Specific antibody response to SARS-CoV-2 was evaluated by the use of two different test kits: the commercially available, licensed qualitative Elecsys Anti-SARS-CoV-2 (Roche Diagnostics, Rotkreuz, Switzerland; https://diagnostics.roche.com) with a sensitivity of 99.5% and a specificity of 99.8%, according to the manufacturer; and a validated and published in-house ELISA with a sensitivity of 96% and a specificity of 99.3% as previously reported (9). The Elecsys Anti-SARS-CoV-2 assay does not discriminate between the antibody type(s) present and can detect IgA, IgM, and IgG. The test is based on a recombinant nucleocapsid (N) antigen and has a cutoff value of 1.0 (S/Co). The

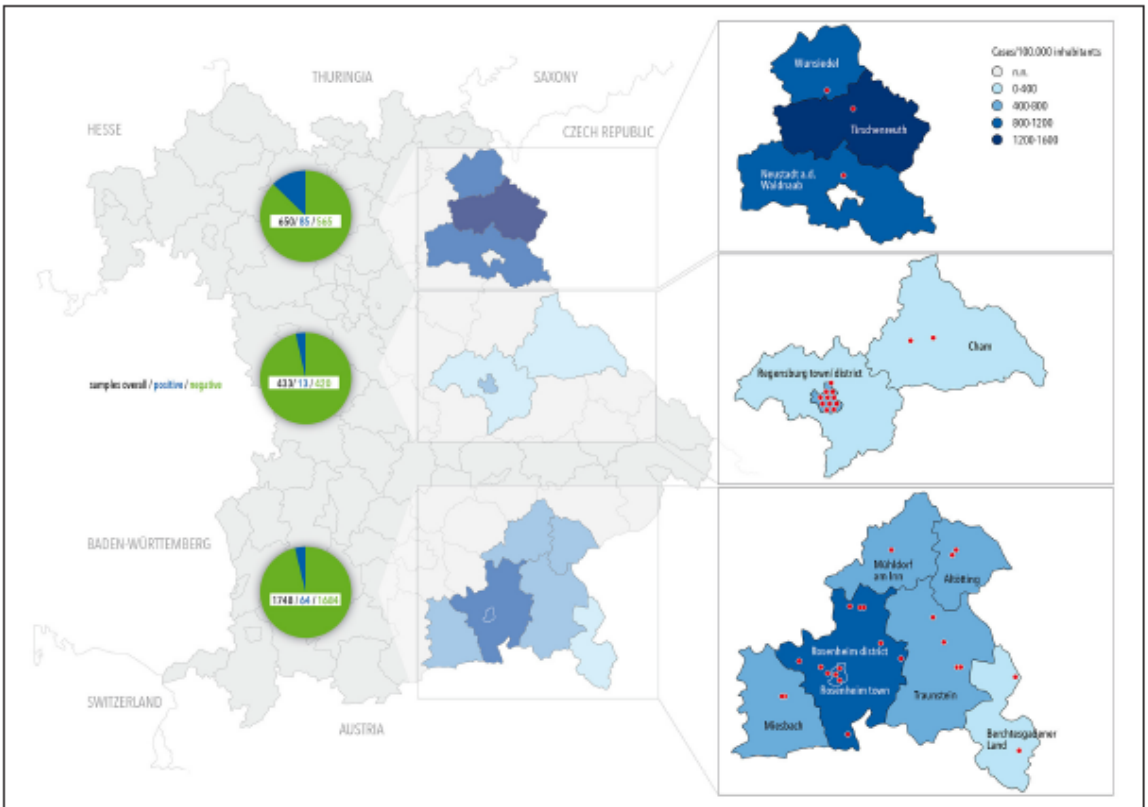

FIGURE 1 | Map of Bavaria with location of centers contributing to the survey (red dots) and COVID-19 prevalence until July 2020 (color coded by county). Number for overall, negatively and positively tested children are given in the circle chart.

in-house ELISA is based on SARS-CoV-2 S-protein's receptor-binding domain, quantifies total IgG and has a cutoff value of 1.0 (S/Co). The detected reactivity correlates with the SARS-CoV-2 neutralization titer as described previously (9). All samples with S/Co <1.0 were considered negative.

SARS-CoV-2 Neutralization Test

Neutralizing antibodies were evaluated by titration of sera against SARS-CoV-2 pseudotyped Vesicular Stomatitis Virus (VSV). The test is based on VSV-ΔG*FLuc pseudotyped with SARS-CoV-2-Spike-ΔER, which correlates with SARS-CoV-2 neutralization as described previously (10, 11). Pseudoviral titers were determined by limited dilution and fluorescence microscopy. For all samples, a fixed inoculum of 25,000 ffu was neutralized for 1 h and luciferase activity was determined 20 h post infection of HEK293T-ACE2^{+}-cells. IC50 values were fitted using the algorithm: 'log (inhibitor) vs. normalized response'. Data were analyzed and Spearman's correlations (R) were calculated in GraphPad Prism 8 software (GraphPad Software, San Diego, USA).

Statistical Analyses

Descriptive statistics were calculated using frequencies (percentages) for categorical data and median (interquartile range) for metric data. Participants' characteristics and symptoms are presented stratified by antibody response. Differences between groups were analyzed using χ^2-tests for categorical variables and *t*-test for independent groups, respectively. All analyses were performed using SPSS.23.

RESULTS

Overall, 2,934 children participated in the study of whom 2,906 were tested successfully with at least one of the two applied antibody tests and 2,832 (96.5%) had also entered necessary study data in the online tool. Demographic data of the children participating in the study are given in **Table 1** and locations of test-centers across counties are depicted in **Figure 1**.

Overall, 161 participants were classified seropositive with any test*, of which 158 were ELISA positive and 139 showed a positive ELECSYS signal, yielding a total concordance of 83.9 % (n = 135 positive in both tests) and a total discordance of 16.1 %

TABLE 1 | Characteristics of study participants stratified for antibody (AB) test result.

General characteristics	Negative AB test (*N* = 2,670)	Positive AB test (*N* = 162)	*p*
Study participation due to...			
random selection (health check-up), % (N)	66.0 (1,763)	32.1 (52)	
own intention to participate, % (N)	34.0 (907)	67.9 (110)	<0.001*
Sex (male), % (N)	51.7 (1,380)	50.6 (82)	0.792
Age (years) (Md, IQR)	7 (4.0–10.0)	8 (4.7–11.0)	0.070
	(range 0–17)	(range 0–16)	
Any chronic disease, % (N)	12.3 (329)	9.3 (15)	0.247
Does your child usually attend...			
Nursery, % (N)	6.1 (163)	4.9 (8)	
Kindergarten, % (N)	27.5 (733)	23.5 (38)	
Elementary school, % (N)	30.3 (809)	29.0 (47)	
Secondary school (Mittelschule), % (N)	4.9 (130)	9.9 (16)	
Secondary school (Realschule), % (N)	8.5 (227)	11.1 (18)	
Grammar school, % (N)	11.0 (295)	9.9 (16)	
School for special needs, % (N)	0.6 (17)	1.2 (2)	
None of them, % (N)	11.1 (296)	10.5 (17)	0.138
SARS-CoV-2 PCR testing, % (N)	8.8 (234)	17.9 (29)	<.001*
Positive SARS-CoV-2 PCR test, % (N)	0.2 (6)	9.3 (15)	<0.001*
Hospitalization due to COVID-19, % (N)	0.2 (6)	1.2 (2)	0.019*
Household member COVID-19, % (N)	6.0 (161)	47.5 (77)	<0.001*
Any symptom, % (N)	70.1 (1,871)	76.5 (124)	0.080

*$p < 0.05$; chi² test, t-test for independent groups. IQR, interquartile range. Md, median.

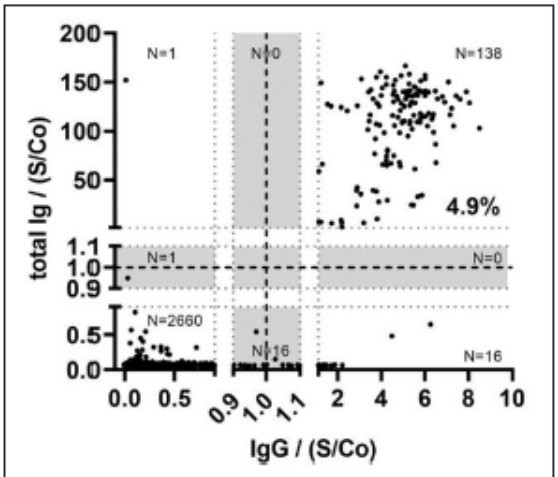

FIGURE 2 | Comparison between the N protein directed Elecsys Anti-SARS-CoV-2 assay (total Ig) and the S protein directed in-house SARS-CoV-2 assay detecting IgG (IgG) in the total study population (*N* = 2,832). Strong dotted lines represent the assay cutoff values, ±10% borderline intervals (gray areas). Signal-to-cutoff (S/Co) ratios are given for both assays.

(n = 23 ELECSYS-positive/ELISA-negative; n = 3 ELECSYS-positive/ELISA) (**Figure 2**). A positive result in at least one of the two tests defined a positive case.

Strong regional differences were observed in the prevalence of SARS-CoV-2 antibodies in children (**Figure 1**). Overall, children in the heavily affected county of Tirschenreuth (with 1,638 positive PCR tests/100,000 inhabitants when the survey was performed) had positive antibody response 3–4 times more often than in the two other test regions, with 586 positive PCR tests/100,000 inhabitants in Regensburg and 1,111 positive PCR tests/100,000 inhabitants in Rosenheim (September 2020). When only those children randomly selected [approach (a)] and only one child (the youngest) per family were included in the analysis, 7.2% of tested children where positive in Tirschenreuth, 3.1% in Regensburg and 1.8% in Oberbayern/Alpine region. In those who participated on their own intention, e.g., due to symptoms that may have been related to COVID-19 or suspected contact to a COVID-19 patient [approach (b)], 15.9% were found positive in Tirschenreuth, 2.3% in Regensburg and 7.8% in Oberbayern/Alpine region, again taking only one child per family into account.

The older the children, the more positive SARS-CoV-2 tests were found, with 4.9% positive in the 0–6 year-olds (n = 1,299), 5.7% in the 7–10 year-olds (n = 849) and 7.3% positive in the 11–17 year-olds (n = 684). Children with chronic diseases tended to be slightly less often positive (4.3% of 344) than those without chronic diseases (5.9% of 2,488). Within the study population, only 263 children had already received a SARS-CoV-2 PCR test previously and 21 had a positive test result. Of these, 15 individuals showed elevated antibody responses (71.4%) while in 6 subjects no antibody response in any of the two tests could

be found. Two hundred and thirty-eight children lived in a household with a positively tested family member and of these, 32.4% developed antibodies against SARS-CoV-2. Thus, living with a SARS-CoV-2 positive family member is the single most prominent association with a SARS-CoV-2 infection in children in our study population. We assessed symptoms potentially related with SARS-CoV-2 infections in our study population but found very few specific features (other than the loss of smell and taste) which would allow to discriminate COVID-19 from common viral infections in children (**Table 2**).

Despite the good level of concordance (83.9%) between the occurrence of N-protein specific (Roche Elecsys) and S-protein specific antibodies (in house ELISA), N-specific titers (ELECSYS) did not correlate with our in-house S-protein ELISA in the overall analysis (**Figure 2**). Considering this obvious discordance regarding N- and S-protein specific antibody titers, the positive population in any test with sufficient material for further testing ($n = 161$) was analyzed for neutralizing antibodies (nAbs).

In the following neutralizing activity was detected for $n = 135$ participants, providing a total concordance of 95.7 % ($n = 133$ positive; $n = 21$ negative) and a discordance of 4.3 % ($n = 2$ N-seropositive/neutralization-negative; $n = 5$ N-seronegative/neutralization-positive) of the Elecsys result with the presence of nAbs. For comparison, the ELISA showed 83.2 % concordance ($n = 133$ positive; $n = 1$ negative) and 16.8 % discordance ($n = 25$ S-seropositive/neutralization-negative; $n = 2$ S-seronegative/Neutralization-positive) with the result of the neutralization assay (**Figure 3** and **Supplementary Table 1**). As internal control, $n = 81$ randomly chosen negative sera (matching the age and sex distribution of the positive population) were tested for the presence of neutralizing antibodies, of which none exhibited a positive result yielding a specificity of 100% (**Supplementary Figure 1**).

Correlating (Spearman) the quantitative results of the three assays showed a significant correlation for each pair, while the ELISA correlated best ($R = 0.62$) with the IC-50 of the neutralization assay, the quantitative readout of the Elecsys showed inferior correlation with both the ELISA ($R = 0.46$) and the neutralization ($R = 0.50$). This was not surprising, as the manufacturer doesn't recommend any quantitative readout of the ELECSYS assay. Furthermore, no significant effects could be found on any of the three (quantitative) test results regarding age or sex of the participants (**Supplementary Figure 2**). Neither antibody levels nor neutralization capacity did correlate with any of the classical symptoms named in **Table 2** (detailed analysis in **Supplementary Figure 3**).

DISCUSSION

In our study, performed in regions of Germany with a relatively high incidence of COVID-19 in adults in the first phase of the pandemic, approximately 6% of tested children were positive for SARS-CoV-2 antibodies in two tests directed against the N- and S-proteins of the virus. Symptoms of COVID-19 were found to be rather unspecific in children while antibody response was strong in most cases. SARS-CoV-2 neutralization capacity was independent of age, sex or symptoms in those children with antibodies and absent in those without antibodies.

This study showed an unexpected high prevalence rate of SARS-CoV-2 infections in children in Germany in the first wave, comparable to similar studies in Germany (12). The antibodies

TABLE 2 | Symptoms of study participants after antibody measurement: stratified for antibody (AB) test result.

Symptoms	Negative AB test ($N = 2,670$)	Positive AB test ($N = 162$)	p
No symptoms, % (N)	30.1 (804)	23.5 (38)	0.072
Runny nose, % (N)	42.5 (1,135)	32.7 (53)	0.014*
Sore throat, % (N)	28.2 (753)	18.5 (30)	0.007*
Headache, % (N)	24.3 (648)	24.1 (39)	0.955
Dizziness, % (N)	6.5 (173)	4.9 (8)	0.436
Exhaustion/ fatigue, % (N)	24.0 (640)	25.3 (41)	0.699
Muscle aches, % (N)	14.0 (373)	16.0 (26)	0.460
Inflammation of the eyes, % (N)	4.4 (117)	3.1 (5)	0.430
Loss of smell, % (N)	1.0 (27)	4.9 (8)	<0.001*
Loss of taste, % (N)	2.4 (64)	6.8 (11)	0.001*
Shortness of breath, % (N)	5.1 (137)	3.7 (6)	0.420
Coughing, % (N)	41.0 (1,096)	30.9 (50)	0.010*
Fever, % (N)	37.6 (1,004)	38.3 (62)	0.865
Chills, % (N)	7.3 (194)	3.7 (6)	0.086
Rash, % (N)	5.3 (142)	2.5 (4)	0.111
Diarrhea, % (N)	16.5 (441)	13.0 (21)	0.235
Nausea, % (N)	11.4 (304)	9.9 (16)	0.556
Loss of appetite/difficulty feeding, % (N)	11.2 (298)	5.6 (9)	0.026*
Other symptoms, % (N)	2.5 (66)	2.5 (4)	0.998

*$p < 0.05$; chi² test.

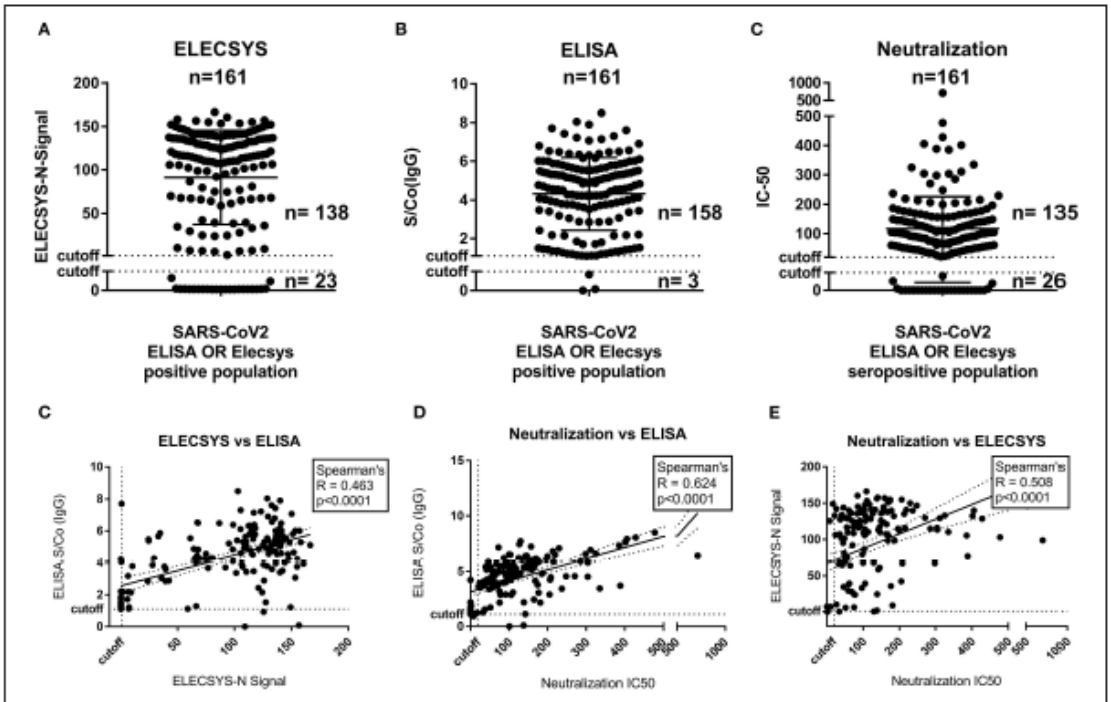

FIGURE 3 | N- and S-protein specific binding antibody titer and neutralization capacity analysis of in any test positive children ($n = 161$). **(A)** Distribution of N-specific antibody signal (Elecsys, S/Co). **(B)** Distribution of S_{RBD}-protein ELISA binding antibody titers (S/Co). **(C)** Distribution of Neutralization titers (IC50). **(D)** Correlation of N-specific antibody signal (Elecsys, S/Co) with S_{RBD}-protein ELISA binding antibody titers (S/Co). **(E)** Correlation of N-specific antibody signal (Elecsys, S/Co) with S_{RBD}-protein ELISA binding antibody titers (S/Co).

in our study were determined approximately 2 months after the peak of the first pandemic wave. Despite the closing of schools, kindergartens, and nurseries very early on in the pandemic in Germany, a surprisingly high number of children showed antibodies in our study. One possible explanation for that could be that many parents who participated in the study suspected a coronavirus infection in their children due to symptoms or outbreaks in their community. Indeed, children were explicitly not tested in the beginning of the pandemic when PCR test capacities were limited. Thus, the study may have addressed an unmet need of parents to get their children tested, which was further supported by the observation that participation in the study was overwhelming.

About 70% of the positive children showed S/Co >100 in the ELECSYS test, a value approximately representing the 97th percentile of all previously available test values (provided by Roche, personal communication). We are aware that the assay is not registered for quantitative readout, nevertheless the measures give an indication for a strong antibody response in children. Compared to the 70% of seropositive children with a mild to asymptomatic course of the initial SARS-CoV-2 infection only 21% of seropositive adults with mild symptoms showed such high values in one of our studies conducted at the same time (13). A similar observation was made for the S protein based in-house ELISA test, where also high values were observed in more than half of the positively tested children. These data may suggest that children mount stronger antibody responses to SARS-CoV-2 than adults on a regular basis.

We used two different antibody tests, one directed against the N-protein and one targeting the S-protein, which explains the slight differences and discordance in test results. With two capable antibodies used for testing at the same time, we have good confidence that we were able to catch all truly seropositive children after SARS-CoV-2 infection. Interestingly, in those few cases where children were initially positive in PCR testing, approximately 30% did not show antibody responses in our tests. This is a higher percentage than observed in our studies in adults (13). Furthermore, approximately 15% of antibody positive children showed no neutralization capacity.

Taken together, it seems that children show a somewhat dichotomous response to SARS-CoV-2 in terms of antibody generation and neutralization. While a great majority mounts exceptionally high antibody responses, a significant subgroup shows no antibodies after infection or no neutralization

capacities. Both, strong-responders and non-responders, represent larger fractions of the population than in our adult study populations (13). It could be speculated that strong antibody responses may contribute to the milder acute course of the initial infection observed in children, but in adults, high levels of S-specific (and neutralizing) antibodies seem to be connected to severe courses of COVID-19 (14). On the other hand, considering the lower neutralizing antibody levels in a substantial group of children, a lower protection from reinfection is much more probable, as neutralizing antibody levels were found to be highly predictive to prevent future (symptomatic) infection (15).

Our study indicates that very few symptoms are specific for COVID-19 in children. On the other hand, only 23% of children with detectable SARS-CoV-2 antibodies were free of symptoms in the weeks before the antibody test. Interestingly, even children as young as 6 years of age were able to indicate loss of smell and taste—the only specific symptom for COVID-19 we could identify in children. It is currently debated, if a loss of smell and taste is also a feature of future mutants of SARS-CoV-2, as data for the SARS-CoV-2 delta variant suggest otherwise. Thus, screening for SARS-CoV-2 infections in children by symptoms does not seem to be useful.

A large number of children acquired antibodies against SARS-CoV-2 when family members had developed COVID-19. Therefore, we suggest that children confronted with COVID-19 in the household should systematically be screened for SARS-CoV-2 antibody responses e.g., 4 weeks after the diagnosis in the index case, thereby not missing out on potential childhood SARS-CoV-2 infections despite of mild or absent symptoms in children. Especially with new, more contagious virus variants, infections in families become even more relevant.

Based on our results we propose to screen children from households with COVID-19 cases on a regular basis for SARS-CoV-2 antibodies as well as children from areas with high prevalence of COVID-19, if any symptoms suggestive for COVID-19 occur. Alternatively, prospective PCR based test systems in schools seem to be reasonable and feasible (16). Therefore, we would recommend longitudinal antibody testing as well as vaccination; if found to be safe; for children to ensure full protection from future disease.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the University of Regensburg (file-number: 20-1865-101). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

OL, GL, SG, KÜ, RW, and MK were responsible for the study design. OL, GL, HB-D, SL, AS-K, DE, MH, JN, and MK performed the data collection. AT, DP, SE, PN, NB, ES-V, PK, PS, RW, and AA carried out the laboratory analysis and the data interpretation. Data Analysis was performed by DP, SE, PN, SB, RW, and MK. OL, GL, DP, SE, SB, RW, and MK wrote this manuscript. All authors contributed to the article and approved the submitted version.

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THE COKIBA STUDY GROUP ARE (IN ALPHABETICAL ORDER)

Bettina Aichholzer, Georg Mair, Michaela Wruk, Imke Reischl, Paediatric Office, Dr. Aichholzer, Dr. Mair, Dr. Wruk, Reischl, Bad Endorf, Germany;
David Antos, Paediatric Office Dr. Antos, Traunstein, Germany
Stephan von Koskull, Christian Becker, Paediatric Office Dr. von Koskul, Becker, Bad Aibling, Germany
Elisabeth Beer, Hubert Schirmer, Paediatric Office Dr. Beer, Dr. Schirmer, Marktredwitz, Germany
Georg Birkinger, Paediatric Office Dr. Birkinger, Wasserburg am Inn, Germany
Andreas Blueml, Paediatric Office Blueml, Trostberg, Germany
Mona Castrop, Paediatric Office Dr. Castrop, Regensburg, Germany
Jost Dieckerhoff, Paediatric Office Dr. Dieckerhoff, Rosenheim, Germany
Renate Eichhorn, Paediatric Office Dr. Eichhorn, Regensburg, Germany
Dominik Ewald, Paediatric Office Dr. Heuschmann & Dr. Ewald, Regensburg/Regenstauf, Germany
Gudrun Fleck, Alfred Heihoff, Paediatric Office Dr. Fleck, Dr. Heihoff, Regensburg, Germany

Jürgen Geuder, Paediatric Office Dr. Geuder, Freilassing, Germany
Jens Grombach, Paediatric Office Dr. Grombach, Neuoetting, Germany
Peter Gutdeutsch, Florian Segerer, Paediatric Office Dr. Gutdeutsch, Dr. Segerer, Regensburg, Germany
Thomas Habash, Sonja Habash, Paediatric Office Dres. Habash, Cham, Germany
Susanne Harner, University Childrens'Hospital Regensburg (KUNO) at the Hospital St. Hedwig of the Order of St. John University of Regensburg, Regensburg, Germany
Christoph Herbst, Paediatric Office Dr. Herbst, Edling, Germany
Daniela Heuschmann, Paediatric Office Dr. Heuschmann & Dr. Ewald, Regensburg/Regenstauf, Germany
Meike Hofmann, Paediatric Office Hofmann, Mitterteich, Germany
Michael Horn, Paediatric Office Dr. Horn, Schoenau am Chiemsee, Germany
Birgit Jork-Kaeferlein, Monika Schwarz, Reinhard Hopfner, Paediatric Office Dr. Jork-Kaeferlein, Dr. Schwarz, Dr. Hopfner, Prien, Germany
Guido Judex, Bastian Baumgartner, Monika Corbacioglu, Sabrina Lindner, Bettina Meinel, Alena Bauer, Hannes Löw, Annamaria Szulagyi-Kovacs, Paediatric Office Dr. Judex, Dr. Baumgartner, Dr. Corbacioglu, Dr. Lindner, Dr. Meinel, Bauer, Löw, Szulagyi-Kovacs, Regensburg, Germany
Michael Kabesch, University Childrens'Hospital Regensburg (KUNO) at the Hospital St. Hedwig of the Order of St. John University of Regensburg, Regensburg, Germany
Annegret Klein, Paediatric Office Dr. Klein, Oberaudorf, Germany
Cosima Koering, Paediatric Office Dr. Koering, Altoetting, Germany
Niclas Landvogt, Claudia Soehngen, Karin Rasp, Gudrun Schick-Niedermeier, Paediatric Office Dr. Landvogt, Dr. Soehngen, Traunreut, Germany
Marinus Laub, Paediatric Office Laub, Rosenheim, Germany
Otto Laub, Paediatric Office Laub, Rosenheim, Germany
Georg Leipold, Petra Schmid-Seibold, Paediatric Office Dr. Leipold Schmid-Seibold, Regensburg, Germany
Johannes Pawlak, Michaela Reitz, Paediatric Office Dr. Pawlak, Dr. Reitz, Rosenheim, Germany
Georg Puchner, Paediatric Office Dr. Puchner, Regensburg, Germany
Christiane Razeghi, Stefan Razeghi, Paediatric Office Dres. Razeghi, Miesbach, Germany
Christine Rehe, Klaus Rehe, Paediatric Office Dres. Rehe, Kolbermoor, Germany
Matthias Scheffel, Ludwig Kaesbauer, Paediatric Office Dr. Scheffel, Dr. Kaesbauer, Muehldorf, Germany
Roland Schmid, Michael Strobelt, Paediatric Office Dr. Schmid, Dr. Strobelt, Bruckmuehl, Germany
Nina Schoetzau, Paediatric Office Dr. Schoetzau, Miesbach, Germany
Marko Senjor, Paediatric Office Dr. Senjor, Wasserburg, Germany
Michael Sperlich, Paediatric Office Dr. Sperlich, Ampfing, Germany
Guenter Theuerer, Guenter Steidle, Paediatric Office Dr. Theurer, Dr. Steidle, Traunstein, Germany
German Tretter, Paediatric Office Dr. Tretter, Altenstadt an der Waldnaab, Germany
Victor von Arnim, Paediatric Office Dr. von Arnim, Roding, Germany
Marlene Volz-Fleckenstein, Paediatric Office Dr. Volz-Fleckenstein, Regensburg, Germany

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fped.2021.678937/full#supplementary-material

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Conflict of Interest: JN was employed by the company Maganamed Limited.

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