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CLINICAL MICROBIOLOGY IN THE TIME OF COVID-19:

EVALUATION AND APPLICATION OF SARS-COV-2 LABORATORY DIAGNOSTICS

MAARIT AHAVA

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"Es la vida, más que la muerte, la que no tiene límites"
(It is life, more than death, that has no limits)

Gabriel García Márquez,
El Amor en los Tiempos del Cólera
Love in the Time of Cholera

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This dissertation is based on the following original publications, which shall be referred to in the text by their Roman numerals. Some unpublished data are also presented. The copyright holders gave permission to reprint the publications.

I. Kortela E*, Kirjavainen V*, **Ahava MJ***, Jokiranta ST*, But A, Lindahl A, Jääskeläinen AE, Jääskeläinen AJ, Järvinen A, Jokela P, Kallio-Kokko H, Loginov R, Mannonen L, Ruotsalainen E, Sironen T, Vapalahti O, Lappalainen M, Kreivi HR, Jarva H, Kurkela S, Kekäläinen E.: Real-life clinical sensitivity of SARS-CoV-2 RT-PCR test in symptomatic patients. PLoS One. 2021;16: e0251661.
*Equal contribution

II. Jääskeläinen AE, **Ahava MJ**, Jokela P, Szirovicza L, Pohjala S, Vapalahti O, Lappalainen M, Hepojoki J, Kurkela S.: Evaluation of three rapid lateral flow antigen detection tests for the diagnosis of SARS-CoV-2 infection. J Clin Virol. 2021;137:104785.

III. **Ahava MJ**, Kurkela S, Kuivanen S, Lappalainen M, Jarva H, Jääskeläinen AJ.: Detection of SARS-CoV-2 nucleocapsid antigen from serum can aid in timing of COVID-19 infection. J Virol Methods 2022;302:114469.

IV. **Ahava MJ**, Kortela E, Forsblom E, Pätäri-Sampo A, Friberg N, Meretoja A, Kivivuori S-M, Lappalainen M, Kurkela S, Järvinen A, Jarva H: Incidence, microbial etiology and outcome of severe bacterial infections in hospitalized patients with COVID-19 – a population-based registry study. Manuscript submitted

V. **Ahava, MJ**, Jarva, H, Jääskeläinen, AJ, Lappalainen, M, Vapalahti O, Kurkela, S. Rapid increase in SARS-CoV-2 seroprevalence during the emergence of Omicron variant, Finland. Eur J Clin Microbiol Infect Dis 2022. Epub ahead of print.

ABBREVIATIONS

ACE2	Angiotensin-converting enzyme 2
AMR	Antimicrobial resistance
CI	Confidence interval
CLIA	Chemiluminescence immunoassay
COVID-19	Coronavirus disease 2019
Ct	Cycle threshold
E	Envelope
ELISA	Enzyme-linked immunosorbent assay
ESBL	Extended spectrum beta-lactamase
GISAID	Global Initiative on Sharing All Influenza Data
hCOV	Human coronavirus
ICU	Intensive care unit
MALDI-TOF	Matrix assisted laser desorption ionization time-of-flight mass spectrometry
MERS-CoV	Middle East respiratory syndrome coronavirus
N	Nucleocapsid
NAAT	Nucleic acid amplification test
OR	Odds ratio
ORF	Open reading frame
RNA	Ribonucleic acid
ROC	Receiver operating characteristic
RT-PCR	Reverse transcription polymerase chain reaction
S	Spike
SARS-CoV	Severe acute respiratory syndrome coronavirus
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2

ABSTRACT

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the causative agent of coronavirus disease 2019 (COVID-19). SARS-CoV-2 is a large, enveloped, positive-sense, single-stranded RNA virus that is readily transmissible. Symptom severity varies, and the infection can be fatal, especially in vulnerable populations.

During the COVID-19 pandemic, clinical diagnostic microbiology has played a key role in the WHO-recommended "test, trace and isolate" strategy to contain the pandemic. Diagnostic laboratories have provided epidemiological insight through serosurveys and sequencing, and laboratories have helped clinicians and the public understand and make interpretations of complex test methods, procedures and results.

As the pandemic has progressed, the laboratory methods used for viral RNA, antigen and antibody detection have developed quickly. This thesis evaluates the clinical and analytical performance of RT-PCR and antigen tests for the detection of SARS-CoV-2 and discusses their utility and limitations. It also describes the frequency of severe bacterial infections complicating COVID-19 and reports on sero-surveillance regarding the emergence of the Omicron variant.

We assessed the clinical sensitivity of SARS-CoV-2 RT-PCR in comparison with clinical suspicion of SARS-CoV-2 infection that was graded retrospectively. We found that despite the high analytical sensitivity of RT-PCR, its performance against a reference standard based on clinical criteria was only moderate.

The evaluation of three commercially available rapid antigen tests was performed by comparing the results to those of RT-PCR and virus culture. As expected, the antigen tests were less sensitive than RT-PCR.

We also evaluated a novel diagnostic method for nucleocapsid antigen detection in serum samples. The test sensitivity was high if the sample was taken less than two weeks after symptom onset but declined rapidly after that timepoint. These results suggest that N antigen detection from serum samples can be useful in diagnosing acute COVID-19.

We described the frequency, severity, etiology and timing of bacterial infections in hospitalized COVID-19 patients. Bloodstream infections and culture-verified pneumonias were identified in 40/585 (6.5%) cases, and the impact on patient outcomes in our cohort was not statistically significant. This result may inform recommendations on empirical antibiotic treatment and encourage the withholding of routine antibiotic initiation.

Lastly, the impact of a novel variant of concern, namely, Omicron, in the Greater Helsinki area was estimated through a serosurvey of IgG-class antibodies conducted on samples sent for routine diagnostic purposes. Vaccination coverage of

at least one dose across all age groups was 79% at the time of the study. During the sixteen-week study period, which coincided with Omicron becoming the dominant variant in Finland, the seroprevalence of antibodies against nucleocapsid antigen rose from under 10% to over 30% in age groups under 45 years old.

The results presented in this thesis underline the need for continuous microbiological surveillance of emerging infectious diseases as well as the importance of studies on diagnostic accuracy.

TIIVISTELMÄ

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) aiheuttaa Coronavirus disease 2019 eli COVID-19 -tautia. SARS-CoV-2 on kookas, vaipallinen, positiivissäikeinen yksijuosteinen RNA-virus, joka tarttuu verrattain helposti. Oirekuvien kirjo on laaja ja COVID-19 voi eritoten iäkkäiden ja rokottamattomien keskuudessa olla kohtalokas.

Pandemian aikana kliinisillä, diagnostisilla laboratorioilla on ollut ennennäkemättömän tärkeä rooli osana WHO:n suosittamaa testaamisen, jäljittämisen ja tautitapausten eristämisen -strategiaa. Lisäksi laboratoriot ovat tuottaneet tietoa epidemian kulusta vasta-aineseurannalla ja virusmuunnosten tunnistamiseksi tehdyillä testeillä. Laboratorioiden työ on myös auttanut niin kliinikkoja kuin maallikkojakin tulkitsemaan diagnostisten testien toimintaperiaatteita ja tuloksia.

Pandemian edetessä menetelmät, joilla havaitaan SARS-CoV-2 viruksen perimäainesta eli RNA:ta, viruksen antigeeneja tai virusta vastaan muodostuneita vasta-aineita, ovat kehittyneet nopeasti. Tämä on edellyttänyt suuria ponnistuksia menetelmien kehittämiseksi, koestamiseksi ja käyttöönottamiseksi. Tässä väitöskirjassa esitellään tutkimustuloksia RT-PCR- ja antigeenitestien kliinisestä ja analyyttisestä suorituskyvystä sekä arvioidaan testien käyttökelpoisuutta ja rajoitteita. Lisäksi tarkastellaan vakavien bakteeri-infektioiden yleisyyttä sairaalahoitoisilla COVID-19-potilailla sekä Omicron-variantin ilmaantumisen vaikutusta vasta-aine-esiintyvyyteen.

Arvioimme SARS-CoV-2 nukleiinihappo-osoitusmenetelmien kliinistä herkkyyttä verraten testiä kliinisin kriteerein määriteltyyn epäilyyn infektiosta. Havaitsimme, että pandemian alkumetreillä, huolimatta nukleiinihappo-osoituksen korkeasta analyyttisestä herkkyydestä, testin suorituskyky verrattuna kliiniseen epäilyyn on parhaimmillaankin vain kohtalainen.

Kolmen kaupallisen CE-IVD-merkityn pika-antigeenitestin vertailussa totesimme antigeenitestien herkkyyden RT-PCR-testejä matalammaksi..

Uusi nukleokapsidiantigeenin seerumista osoittamiseen tarkoitettu menetelmä oli hyvin herkkä analysoitaessa näytteitä, jotka oli otettu kahden viikon sisällä oireiden alkamisesta. Pian tämän aikapisteen jälkeen herkkyys laski.

Kuvasimme COVID-19-potilaiden bakteeri-infektioiden yleisyyden, vakavuuden ja aiheuttajakirjon. Veriviljelypositiivisia ja mikrobiologisesti varmennettuja keuhkokuumeita todettiin vain 40/585 (6.8%) potilaalla. Havainnollamme on merkitystä empiirisen antibioottihoidon aloituksen kannalta ja se voi rohkaista antibioottihoidosta pidättäytymiseen.

Arvioimme Omicron-muunnoksen ilmaantumisen vaikutusta määrittämällä vasta-aineita satunnaisotannalla valituista seeruminäytteistä. Tutkimusjakso käsitti 16 viikkoa vuodenvaihteessa 2021–2022. Rokotuskattavuus vähintään yhden rokoteannoksen saaneiden henkilöiden osalta oli tutkimusjakson aikana 79 %. Nukleokapsidia vastaan muodostuneiden vasta-aineiden esiintyvyys nousi nopeasti etenkin alle 45-vuotiaiden ikäryhmissä: alle 10 %:sta yli 30 %:iin.

Tuloksemme korostavat mikrobiologisen seurannan ja diagnostisten testien suorituskyvyn arvioinnin tärkeyttä.

1. INTRODUCTION

The novel coronavirus, later named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), emerged in Wuhan, China, in late 2019[1,2]. The clinical manifestation of SARS-CoV-2 infection was named coronavirus disease 2019 (COVID-19) by the World Health Organization (WHO)[3]. The exact pathway of emergence, the zoonotic origin and the possible intermediate hosts of SARS-CoV-2 are still a matter of ongoing debate to some extent, because the immediate precursor has not yet been identified[4]. Bats are suspected to be the original reservoir[5], pangolins and raccoon dogs have been suggested as possible intermediate hosts, and strong evidence corroborates zoonotic spillover into humans[6]. Other theories, such as the origin being a research laboratory, have been explored[7] and widely circulated in the media[8–10], but thus far, the evidence points by far most convincingly toward zoonotic transmission from bats via intermediate hosts. Evidence of geographical clustering as well as sequencing results from environmental samples point toward the Huanan Seafood Market as the specific location[11].

The first cases of novel atypical pneumonia were detected by local physicians in Wuhan in December 2019, and the WHO was notified on the 31st of December[12]. Information on human-to-human transmission was publicized in January[13]. At this point, the spread to several provinces inside China was evident, and rapid global spread was retrospectively confirmed[14]. In the United States, a study found SARS-CoV-2 reactive antibodies in blood donations from December 2019 to January 2020[15].

On the 30th of January, 2020, the WHO announced a public health emergency of international concern. Only just over one month later, on March 11th, a pandemic was declared[16]. Public health interventions and research efforts to contain the spread, prevent fatalities, and find effective preventive measures, treatments and vaccines have made overwhelming leaps at a historic pace. The number of publications, the amount of international collaboration and the short timeline in which several effective vaccines were available exceeded expectations[17]. However, hindsight also reveals gross miscalculations, missed opportunities and sometimes even blatant negligence of human health[18–21].

Laboratory methods involved in virological diagnostics have been used to an unprecedented extent during the pandemic. The aim of this thesis was to evaluate the microbiological methods deployed in the diagnosis of SARS-CoV-2 infection and to use laboratory diagnostics and laboratory information systems as tools for epidemiological investigations.

2. REVIEW OF THE LITERATURE

2.1. Virology of coronaviruses

Coronaviruses are enveloped, positive-sense, single-stranded RNA viruses with large genomes of approximately 30 000 base pairs[22]. The name "corona" comes from the spike-decorated exterior of the virus particle, which gives the particle the appearance of a star's corona in an image captured with an electron microscope[23].

2.1.1. Taxonomy

SARS-CoV-2 belongs to the order *Nidovirales*, suborder *Coronavirineae*, family *Coronaviridae*, subfamily *Orthocoronavirinae,* and genus *Betacoronavirus*[2]. The other genera within the subfamily are *Alphacoronavirus, Gammacoronavirus, and Deltacoronavirus,* of which alphacoronaviruses also include coronaviruses that infect humans. The closest relative of SARS-CoV-2 among human coronaviruses is severe acute respiratory syndrome coronavirus (SARS-CoV), which also belongs to the subgenus *Sarbecovirus* and to the species for *severe acute respiratory syndrome-related coronavirus*. SARS-CoV caused a pandemic scare in 2002-2003[24,25].

In addition to SARS-CoV and SARS-CoV-2, a third coronavirus that causes severe disease manifestations has emerged in recent decades: Middle East respiratory syndrome coronavirus (MERS-CoV), which belongs to the *Merbecovirus* subgenus and was first identified in 2012; it is still a cause of local epidemics[26,27]. Dromedary camels are the only zoonotic reservoir of MERS-CoV identified to date[28]. Figure 1 depicts the relation of SARS-CoV, MERS-CoV and SARS-CoV-2 to zoonotic coronaviruses.

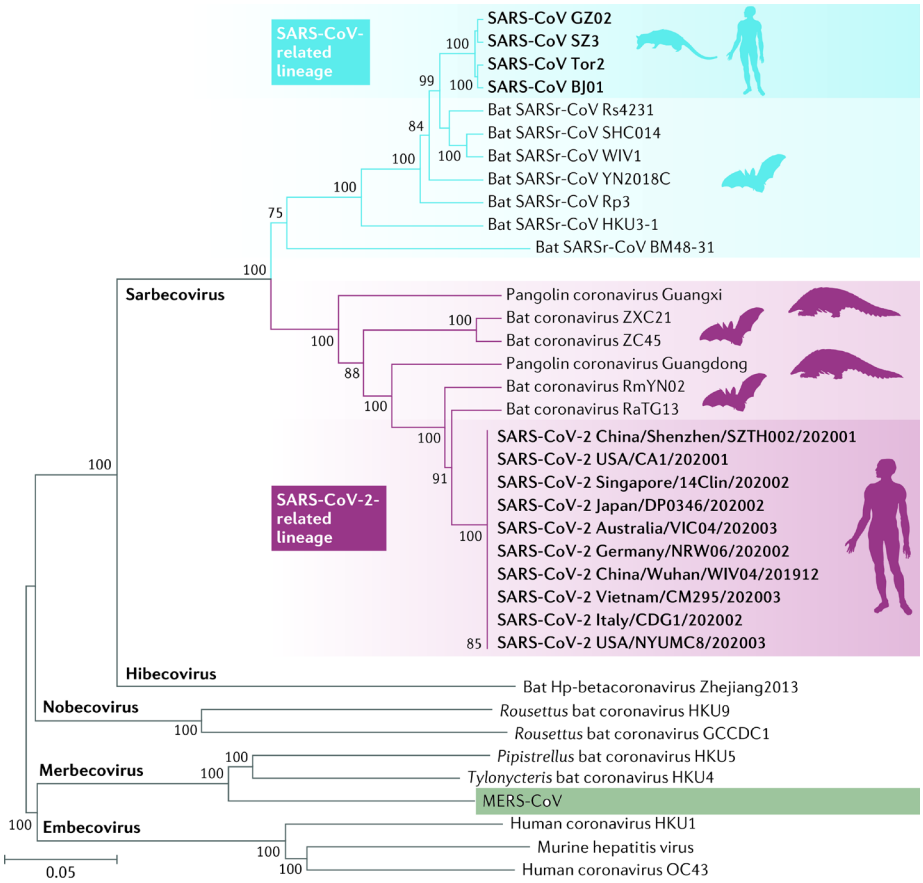

Figure 1. Placement of select SARS-CoV and SARS-CoV-2 strains within the *Betacoronavirus* phylogeny. Modified from Hu, B., Guo, H., Zhou, P. et al. Nat Rev Microbiol 2021.[29]

Other coronaviruses known to infect humans are betacoronaviruses: HCoV HKU1, and HCoV OC43 (see subgenus *Embecovirus* in Figure 1) and alphacoronaviruses HCoV 229E and HCoV NL63[30–33]. These so-called seasonal coronaviruses reportedly cause mostly mild illness, although their range of disease manifestations can be broad[34–36]. Some theories suggest that at the time of their transition to humans from other hosts, they may have been the cause of large epidemics, with severe disease manifestations appearing more frequently and resulting in high fatality rates[37].

2.1.2. Genome and proteome

SARS-CoV-2 shares 79% of its genetic identity with SARS-CoV, 88% with bat-SL-CoVZC45 and 96% with the closest coronavirus identified in bat RATG13[38,39]. The most striking differences from close relatives are in the *S* gene coding structural spike (S) protein[5]. Coronavirus S protein is a trimeric class I fusion protein and

consists of subunits S1 and S2[40]. SARS-CoV-2 has a polybasic S1/S2 cleavage site, setting it apart from related coronaviruses that have monobasic cleavage sites[41]. In SARS-CoV, the S protein is found in both upright and lying down conformations and has a very tightly packed down position toward the neighboring protomer. In contrast, SARS-CoV-2 S is mostly situated in a more inward-facing lying down position, which is thought to provide an advantage in immune evasion and stability while not being ideal for receptor binding[42].

Other structural proteins of SARS-CoV-2 are the envelope (E), membrane (M), and nucleocapsid (N). For diagnostic purposes, the *E* gene, which shares high nucleotide sequence homology with the *E* genes of related coronaviruses, coding E protein has been widely used in reverse transcription polymerase chain reaction (RT-PCR) methods as well as the *N* gene. The placement of genes encoding structural proteins is shown in Figure 2. Both *N* and *E* are a part of the three-target method that was the first to be recommended by the WHO, with the third target being the *RdRp* gene, which encodes the RNA-dependent RNA polymerase[43]. The N and S proteins are the targets of commercially available antigen tests[44,45].

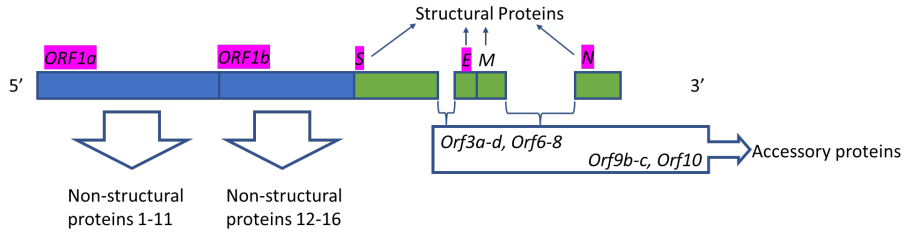

Figure 2. SARS-CoV-2 genome. Common targets of diagnostic RT-PCR highlighted in pink.

The open reading frame gene *orf1ab* encodes two polyproteins, which are further cleaved into sixteen nonstructural proteins, and it is directly translated after fusion. The nonstructural proteins have a role in immune evasion, transcription and replication, including a fine-tuned proofreading mechanism to maintain the integrity of the large genome[46–51].

2.1.3. Receptor binding and replication

Coronavirus cell entry depends on effective binding to host cell receptors. SARS-CoV-2 binds to angiotensin-converting enzyme 2 (ACE2)[52]. ACE2 is a protein found both in soluble form and embedded in the human cellular membrane, and it has an important role in the maintenance and regulation of blood pressure through the renin-angiotensin-aldosterone system[53]. In the cell membrane, it also functions as a receptor for SARS-CoV-2 and other viruses[52,54]. In addition to the direct interaction between the host cell receptor and the S protein protruding from the

viral particle, virus entry is mediated by host proteases, and in the case of SARS-CoV-2, these include transmembrane serine protease TMPRRS2, cathepsin L, and furine[39,55]. In addition to human ACE2, SARS-CoV-2 binds to a multitude of animal ACE2 receptors: pig, ferret, cat, dog, rhesus macaque, and hamster ACE-2. Some of these findings have significance as potential viral reservoirs with close contact with humans and some are important animal models[5,56–58].

To enter the target cell, S1 binds to ACE2, and TMPRSS2 cleaves the protein exposing the S2 domain, which is responsible for fusion with the cell membrane[52]. TMPRRS2 has been proposed as a mediator in the development of serious forms of coronavirus infection, given that NL63 and the Omicron variant of SARS-CoV use TMPRSS2 less efficiently[59]. The cell entry process is facilitated by neuropilin-1. The effect of neuropilin-1 is notable in the cells of the upper respiratory tract, which are highly relevant in terms of SARS-CoV-2 tissue penetration, infectivity and transmissibility[60–62]. A schematic illustration of the SARS-CoV-2 virion and cell entry is presented in Figure 3.

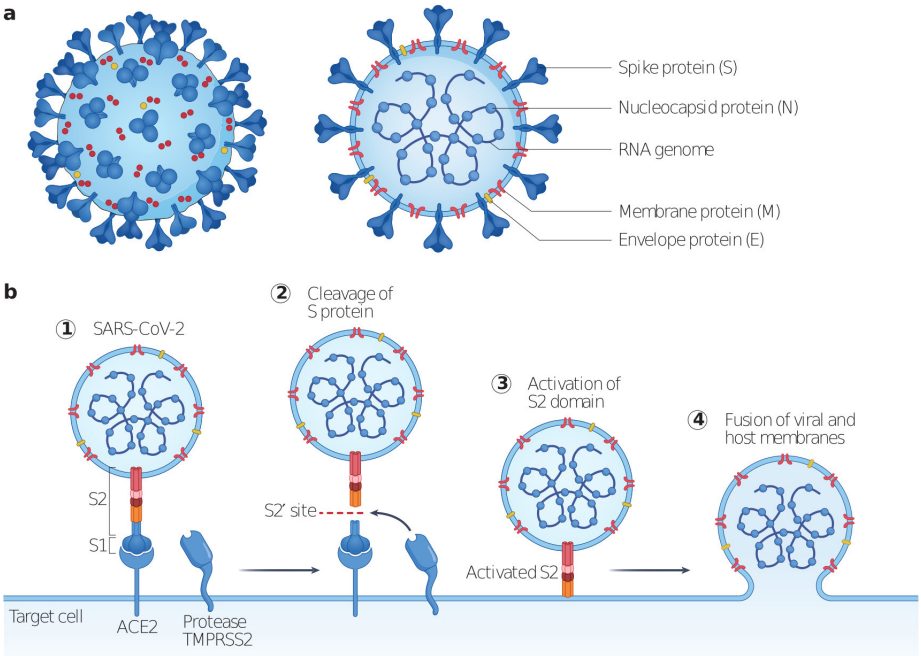

Figure 3. a) The structure of the viral particle and b) the phases of receptor binding and fusion. Modified from Lamers, M.M., Haagmans, B.L. Nat Rev Microbiol 2022. [63]

Following entry, viral protein production begins with replicase proteins. Transcription takes place inside double membrane vesicles, and in addition to double-stranded RNA, a discontinuous viral transcription process leads to the production of subgenomic negative-sense RNAs [64,65]. Discontinuous viral

transcription is presented in Figure 4. For diagnostic purposes, the detection of subgenomic RNA and its relative abundance compared to genomic RNA has been used to assess active replication in cells [66], but further studies have shown the persistence of subgenomic RNA in diagnostic samples beyond the point at which infectious or culturable virus was present [67,68].

Viral particles are assembled within the endoplasmic reticulum, Golgi apparatus and intermediate complex, and the particles are released by exocytosis in secretory vesicles.

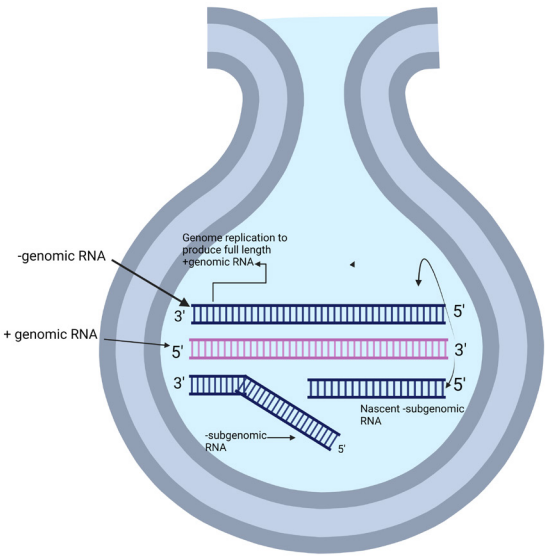

Figure 4. Synthesis of negative-sense subgenomic RNA by discontinuous transcription. Created with BioRender.com.

2.2. Zoonotic transmission of coronaviruses

Recent history has shown a trend toward a higher risk of emerging infectious diseases (e.g., HIV, SARS-CoV, MERS-CoV, SARS-CoV-2, swine influenza, and Zika virus [1,24,26,69–73]), re-emerging infectious diseases (tuberculosis, malaria, measles, and yellow fever[74–77]) and devastating epidemics caused by these diseases. The role of diseases transmitted to naïve human populations from other animals; that is, diseases caused by zoonoses in this development is dramatic due to the lack of immunity in humans[78]. This development is also interconnected to the themes of climate change, overpopulation, international travel, urbanization, deforestation and growing socioeconomic inequality[79–84].

Geographically, the most diverse reservoirs of betacoronaviruses in zoonotic hosts are in Asia[85], whereas Central and South America are dominated by

alphacoronaviruses[86]. Both of these areas are populous and have swiftly growing economies, and the boundary between wildlife and humans is becoming more strained and immediate. This strain creates more opportunities for disease transmission[87].

The role of bats in the zoonotic transmission of SARS-CoV-2 is considered likely, but not definite, because SARS-CoV-2 or an immediate precursor has not been detected in bats thus far. *Rhinolophus affinis*, the intermediate horseshoe bat, is a reservoir for several SARS-related coronaviruses, most notably RATG13, a close relative of SARS-CoV-2[5]. However, due to the infrequent close contact between humans and bats, the presence of an intermediate host seems more than plausible. Several mammals, especially those sold at the Huanan market, have been proposed. The pangolin was an early suspect, since SARS-CoV-2-related coronaviruses were detected in Malayan pangolins[88,89].

The two other coronaviruses that have crossed over into humans in recent decades, SARS-CoV and MERS-CoV, did so through intermediate hosts. Palm civets were the source of SARS-CoV spillover into humans, and MERS-CoV circulated in dromedary camels for approximately 30 years before infections were detected in humans [90,91].

2.3. Diagnostic methods for SARS-CoV-2

The rapid evolution of different diagnostic platforms and methods, both commercial and laboratory-developed, was necessary as the pandemic began. Laboratories around the world were overwhelmed with unprecedented volumes of samples and high demands for both diagnostic tests for individuals as well as laboratory-based surveillance of the epidemic [92,93]. At the time of writing, in June 2022, 11 million SARS-CoV-2 tests had been performed by the Finnish health care system. Approximately 10 million of these are RT-PCR tests, and 0.8 million are antigen tests. By the end of May 2022, the number of COVID-19 cases diagnosed in Finland was 1 097 810, and the number of deaths attributed to COVID-19 was 4 586. [94]

The first published laboratory-developed test for COVID-19 diagnosis to receive WHO recommendation was created by Corman et al[43]. This real-time RT-PCR test was designed without viral genomic nucleic acids but was based on previous knowledge of SARS-CoV and the SARS-CoV-2 nucleic acid sequence provided by Zhang et al. to GenBank[95]. SARS-CoV-2 sequences added to the Global Initiative on Sharing All Influenza Data (GISAID) were used to confirm successful primer design[96]. Evaluations of different diagnostic methods are important for laboratories providing diagnostic services as well as for clinicians interpreting the results while the epidemic progresses, and information on the limitations of the methods is needed [97,98].

Laboratory confirmation of COVID-19 mostly takes place during acute infection, when RT-PCR and rapid antigen tests are most likely to be positive. Serological assays can be used to assess the possibility of a previous infection. Figure 5 depicts the typical timeframes of test positivity for RT-PCR, rapid antigen tests and antibody detection assays.

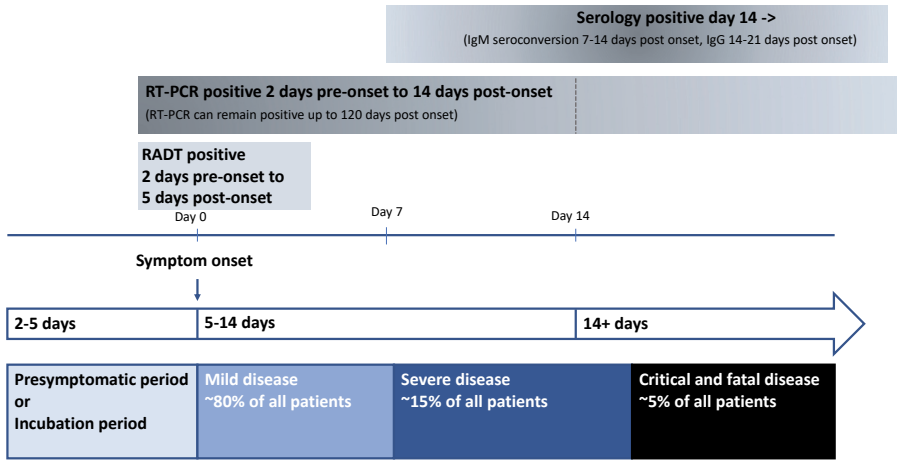

Figure 5. Timeline of test positivity and progression of disease severity.

Prior to the analysis of all the diagnostic samples, the preanalytical steps, such as deciding to order a test, selecting the appropriate test, and collecting and transporting the sample, must be working well to ensure that the sample quality meets the standards for a successful analysis, while the postanalytical phases, such as reporting the results and actions following the results, are, in the end, the most essential part of efficient laboratory method utilization, as presented in the brain-to-brain loop concept introduced by Lundberg[99,100]. This concept naturally applies to the diagnosis of SARS-CoV-2 infection as well[101,102].

2.3.1. Nucleic acid amplification tests

PCR testing represents the current gold standard in most clinically relevant areas of diagnostic virology, especially concerning the diagnosis of acute infections[103–105]. PCR testing is based on copying the genetic material in the sample with a template, using nucleotides with triphosphate groups and target-specific primers. The reaction is prompted by polymerase enzyme and cyclical temperature changes. Cycles of copying result in the exponential amplification of the target, making the end product easily detectable even when the initial amount of genetic material of interest in the sample is minimal[106].

PCR is a method with high analytical sensitivity and specificity and a relatively rapid turnaround time, especially when performed in large batches in high-throughput automated platforms. The high sensitivity comes at the cost of some drawbacks, including the risk of contamination[107–109].

PCR inherently has high analytical sensitivity, scalability for large sample volumes, endless potential for modification in the case of new causative agents and an abundance of available commercial platforms and reagents, making it a very valuable diagnostic method. RT-PCR tests quickly became the mainstay of COVID-19 diagnosis[110–112]. Laboratories were faced with extraordinary sample volumes while simultaneously struggling with material shortages, when even the most mundane laboratory equipment, such as pipette tips and plastic tubes, were becoming scarce or even impossible to obtain due to the immense surge in demand globally[113]. To respond to the high demand, some laboratories expanded their testing capacity by sample pooling[114].

Other nucleic acid amplification test methods employed in SARS-CoV-2 diagnostics include loop-mediated isothermal amplification (LAMP), transcription-mediated amplification (TMA), nicking enzyme amplification (NEAR), recombinase polymerase amplification (RPA) and clustered regularly interspaced short palindromic repeat (CRISPR-Cas) systems [115–119]. All these methods are based on isothermal amplification as opposed to the cyclic temperature changes in RT-PCR. TMA is a method that was created with the purpose of directly amplifying RNA, while the NEAR, RPA and LAMP methods require a reverse transcription step to then be followed by the amplification of complementary DNA. The commercial SARS-CoV-2 detection methods involving isothermal amplification currently in use and under development are concentrated mostly on the potential use of isothermal amplification in rapid diagnostic tests and in point-of-care settings [120,121].

2.3.2. Rapid antigen tests

In addition to PCR, antigen tests have long had an important role in the diagnostic laboratory and have become a part of COVID-19 testing strategies as well[122–127]. Their advantages usually lie in their ease of use and short turnaround time for individual samples[128,129].

Rapid lateral flow tests represent the fastest and most simplistic form of antigen tests, usually involving only 4-5 steps, very few reagents and a test cassette. They are usually convenient to perform even in point-of-care settings, and in the context of the COVID-19 pandemic, they have been widely used by patients themselves once these tests have become available in pharmacies and through other distributors[130]. Antigen testing usually does not involve any amplification of the target molecule, which limits its ability to detect low concentrations of a target[126,131,132]. In addition to antigen tests intended for upper respiratory tract

samples, some methods for other sample materials, such as plasma and serum have been developed, and based on preliminary evaluations, the sensitivity of these tests appears to be fairly high[133,134].

2.3.3. Serology tests

Serological assays have been employed for epidemiological surveys, screening of travelers, retrospective identification of cases after acute infection and diagnosing suspected cases of long COVID[15,135,136]. Serological diagnostics of COVID-19 are often based on enzyme-linked immunoassays (ELISAs), chemiluminescence immunoassays (CLIAs) or immunochromatography, and the performance of these tests varies considerably[137–140]. In research use, more labor-intensive methods, including neutralization tests, are used, but for a multitude of reasons, e.g., inadequate sensitivity and slow turnaround time, they are not suitable for diagnostic purposes[141].

Seroconversion usually occurs 7-21 days after infection/symptom onset: IgG and IgA class antibodies usually appear slightly earlier after two weeks[142]. Neutralizing antibodies in COVID-19 have been studied extensively to understand their role in host defense and to identify possible drug and vaccine targets[66,141,143–146]. Importantly, a correlation between neutralizing antibodies and protective immunity has been established[147,148].

2.4. Clinical picture and treatment of COVID-19

COVID-19 has a variety of disease manifestations: it can affect the upper respiratory tract, lungs, gastrointestinal tract, and nervous system and causes olfactory dysfunction in some patients[149–155]. Symptom severity ranges from asymptomatic to life-threatening[13,156,157]. The wide range of possible symptoms complicates diagnosis based on symptoms. Different variants have been reported to cause different ranges of symptoms and affect morbidity and mortality[158–160]. Table 1 lists four variants of concern that either became dominant variants at some point (Alpha, Delta, or Omicron) or raised significant concern due to an increase in severe disease manifestations in the case of the Beta variant.

Table 1. Variants of concern, location and time of detection and severity as compared with previous or simultaneously circulating strains

Variant	Country where first detected	First detected	First detected case in Finland	Severity
Alpha	Great Britain	September 2020	December 2020	No increase in severe cases compared to wild-type[161]
Beta	South Africa	September 2020	December 2020	More severe cases compared with Alpha and wild-type virus
Delta	India	October 2020	March 2021	More severe cases compared with Alpha
Omicron	Botswana, South Africa	November 2021	November 2021	Less hospitalization compared with Delta

Demographic factors affect the likelihood of specific clinical manifestations and disease severity. The most pronounced factors worsening the prognosis were being of male sex, advanced age and having comorbidities[151,162,163].

The more severe forms of COVID-19 can involve multiorgan failure. Critically ill patients often require mechanical ventilation, and mortality is high: a study conducted in Italy early in the pandemic reported a mortality rate of 27 per 1000 patient-days in intensive care units (ICUs)[164]. Thromboembolic complications, often seen in severely ill patients, tend to prolong hospital stays and increase mortality, and myocardial and cerebrovascular infarction especially increase the risk of long-term consequences of COVID-19[165,166].

An unfavorable inflammatory response is thought to be a driving force in the development of the more severe forms of COVID-19[167–171]. Auto-interferon antibodies have a role in severe disease, and a rapid interferon response is associated with better control of viral replication[172–174]. Autoantibodies against cytokines can lead to the neutralization of the target cytokine, which disturbs the immune response, and this sequence of events is suggested to be the mechanism through which autoantibodies against type I interferon increase the risk of developing severe COVID-19[172,175]. In addition to an inadequate type I interferon response, excessive interleukin-6 and tumor necrosis factor alpha have also been identified as drivers for severe disease[174]. These findings have guided the search for effective immunomodulatory therapies, such as interleukin-6 inhibitors[176].

2.4.1. Coinfections and secondary infections

Coinfections are defined as simultaneous infections, while secondary infections are acquired during or after the course of the primary infection[177]. Often, the practical definition of a coinfection is an infection that is diagnosed simultaneously with the main infectious agent, whereas secondary infections are diagnosed later. It can be difficult to determine which microbe was factually encountered first

by the host or if the process was simultaneous. To circumvent this problem, coinfections can alternatively be divided into community-acquired and nosocomial infections, based on the environment where the infection was likely acquired. This terminology, even if at times imprecise, can be more informative than others, since the nature of co and secondary infections is such that they cannot be categorized reliably in most cases [178].

The incidence of bacterial infections in COVID-19 patients has been reported to vary widely, ranging from 0-45%[179,180]. Studies on coinfections often fail to report the timing and type of bacterial complications, whether the coinfections are community acquired or nosocomial. Another limitation is that the information on the microbiological methods in use is often incomplete[181,182]. Understanding the burden of bacterial coinfections in COVID-19 patients is important, because viral infections in general are fertile breeding grounds for bacterial infections, and bacterial pneumonia has been an important cause of death during pandemic influenzas, particularly during the pre-antibiotic era[183,184]. Nevertheless, more recently, in the A(H1N1) swine influenza pandemic of 2009, the incidence of bacterial pneumonia was reported to be approximately 4% in hospitalized patients and 32.1% in critically ill patients[185,186].

The incidence of viral coinfections during COVID-19 is thought to be low, but as is the case with bacterial infections, estimates on their frequency vary: a retrospective observational study of 989 patients found seven patients (0.6%) to have a viral coinfection[187], while a study that reanalyzed 4,259 SARS-CoV-2-positive respiratory tract samples for a set of bacterial and viral pathogens with nucleic acid amplification testing found 2% had positive findings in the viral panel alongside SARS-CoV-2[188].

Findings indicative of fungal infection during COVID-19, especially concerning *Aspergillus* species, are a matter of ongoing debate about what constitutes proof of an infection and when the findings should be interpreted as colonization[189–193]. Mucormycosis has been reported mostly in India in patients with diabetes mellitus who have received corticosteroid therapy due to COVID-19 infection[194,195].

2.4.2. Treatment of COVID-19

COVID-19 treatment has evolved quickly through large international collaborations to organize randomized, controlled studies. One such effort is the RECOVERY trial, which is currently evaluating empagliflozin, sotrovimab, molnupiravir and paxlovid and comparing high-dose corticosteroids to standard regimens[196]. Another international collaboration is the Solidarity trial currently evaluating artesunate, imatinib, and infliximab[197].

Many treatment options showing early promise have not succeeded in large, rigorous clinical trials, including ivermectin and hydroxychloroquine[198,199].

On the other hand, receiving dexamethasone decreased 28-day mortality for patients receiving invasive mechanical ventilation or supplemental oxygen but not for patients who did not require respiratory support[200]. Tocilizumab, a monoclonal antibody targeting interleukin-6-receptor, was found to be beneficial for patients with hypoxia and signs of systemic inflammation, and the effect was independent of the need for supplemental oxygen and corticosteroid treatment[201]. For patients who had COVID-19 infection but had not produced antibodies against SARS-CoV-2, administering a combination of two non-competing monoclonal antibodies, casirivimab and imdevimab, resulted in lower 28-day mortality, but the effect was not observed in patients who displayed spontaneous antibody development[202]. Notably, monoclonal antibodies should be administered with consideration of local variant susceptibility[203]. In the US, early administration of convalescent plasma is recommended for outpatients in high-risk groups[204]. Improved outcomes were not observed for hospitalized patients, suggesting that at the point at which clinical deterioration is evident, the window for convalescent plasma treatment is already closed[205]. This observation underlines the need for the early identification of high-risk patients.

In addition to pharmaceutical solutions, other treatment options have been optimized: being placed in the prone position has been shown to benefit patients receiving mechanical ventilation[206]. ICU protocols provide information on the specifics of mechanical ventilation and extracorporeal membrane oxygenation (ECMO) treatment as well as on the patient groups for whom these treatments are most beneficial[207,208].

2.5. COVID-19 vaccination

After the pandemic began, vaccine candidates soon underwent development. Most of the attention and efforts were centered around the S protein. This approach was at least partly due to its homology to the SARS-CoV S protein, which is highly conserved, and raising expectations that even during viral evolution, a vaccine targeting the S protein was unlikely to lose too much of its efficacy[209,210]. Trials of the adenovirus vector vaccine against MERS-CoV have been successful, positioning the adenovirus platform as a prime candidate for SARS-CoV-2 vaccine development[211]. Similarly, messenger RNA (mRNA) vaccine technology has reached a point in which it has the potential to be rapidly adaptable to novel pathogens[212,213]. Combining promising platform technologies and knowledge of SARS-CoV neutralizing antibodies targeting the S1 receptor binding domain, which made S an appealing target, enabled the first clinical trial to be under way in March 2020[214,215].

The aforementioned two novel vaccine types, mRNA vaccines and adenovirus vector vaccines, are now the most widely distributed COVID-19 vaccines and have

provided very good protection against severe disease [216–219]. The current vaccines do not prevent infection, and protection weakens over time, so the circulation of SARS-CoV-2 is bound to continue, and future boosters are likely to be needed[220]. The development of vaccines that could provide sterilizing immunity is also actively being explored. One of the approaches that aim to achieve sterilizing immunity is the intranasal administration of vaccines. This approach is believed to provide more robust mucosal immunity with more efficacy in preventing infection[221]. Preclinical studies have demonstrated high IgA class antibody titers in addition to a strong IgG response[222–225]. The clinical efficacy of this approach is still under evaluation, and concerns about rapidly waning mucosal immunity must be addressed[226,227].

2.6. COVID-19 pandemic

The word "pandemic" was initially derived from the Greek words "pan" (all) and "demos" (people). It has had a variety of slightly different and sometimes vague meanings throughout history, but in modern texts and current scientific literature, it can be understood as an epidemic affecting a substantial proportion of the world and its inhabitants[228,229].

The origin of the current COVID-19 pandemic is the city of Wuhan in Hubei province, China. More specifically, the first identified cases were associated with a wet market selling seafood, live poultry and mammals[1]. Person-to-person transmission was confirmed in family clusters [13,230,231]. Wuhan was placed under lockdown on the 23rd of January[232].

On January 30th, the WHO announced a public health emergency of international concern, and on March 11th, a COVID-19 pandemic was declared.[16]

Rapid international spread followed the celebration of the Chinese New Year and winter holidays[233–235]. Countries affected heavily during the spring of 2020 included Italy, Spain, the UK and the US, despite strict control measures and the partial cessation of international travel[236–239].

Variants of concern shaped the recent history of this pandemic in sometimes unexpected ways. Alpha/B.1.1.7 was the first variant to exhibit a clear advantage in terms of increased transmissibility. The emergence of Alpha/B.1.1.7. was also found to be associated with worse patient outcomes in comparison with previously circulating variants[240–243]. Beta/B.1.351 was detected first in South Africa[244]. Gamma/P.1. emergence in Brazil was associated with high infection rates in late 2020 and early 2021 despite high previous seroprevalence, indicating an immune evasion advantage of the variant[245], with additional evidence of mutations that were potentially beneficial for evasion[246,247]. Previously, highly transmissible variants were soon replaced by Delta/B.1.617.2 and most recently Omicron/B.1.1.529 with superior transmissibility and immune evasion potential[159,248–252].

It is clear from the epidemiological evidence that not only the transmissibility and other attributes of the variant affect the success of a given variant. Actions and movement of people, as well as the stringency or relaxation of control measures, have a significant role in the selection and expansion of certain variants[253–256]. The complexity of virological and societal effects warrants continuous monitoring of the epidemic with a diverse set of methods to understand the attributes and evolution of the pandemic. Among other methods, mathematical modeling has gained an important role in predicting future waves and in guiding restrictions, hospital and laboratory preparedness and other public health measures[114,257–261].

3. AIMS OF THE STUDY

The aim of this study was to evaluate the microbiological methods used in the laboratory diagnosis of COVID-19 patients and to apply them in relevant settings for epidemiological purposes. The specific aims of the study were as follows:

- To assess the real-life clinical sensitivity of SARS-CoV-2 RT-PCR methods and determine if the clinical sensitivity is lower than the analytical sensitivity.
- To assess the performance of SARS-CoV-2 rapid lateral flow antigen tests in comparison with RT-PCR and virus culture.
- To evaluate the analytical performance of a serum SARS-CoV-2 N antigen assay and to assess the utility of the assay in relation to symptom onset: Can positivity be interpreted as a signal of acute SARS-CoV-2 infection?
- To determine the frequency of microbiologically confirmed severe bacterial infections in hospitalized COVID-19 patients and to assess whether severe infections have a deleterious effect on patient outcomes.
- To characterize the exposure to SARS-CoV-2 during the emergence of the Omicron variant in Finland through a serosurvey: Was there an increase in seroprevalence in the weeks following Omicron introduction?

4. MATERIALS AND METHODS

Figure 6 depicts the timeframes during which the samples and data were collected for the studies described in this thesis.

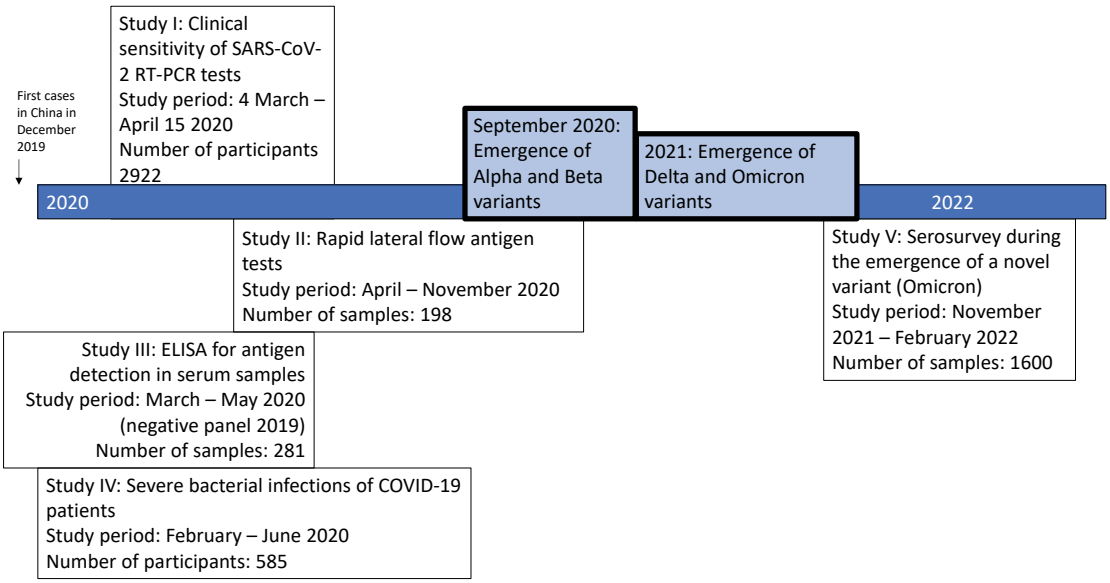

Figure 6. Timeline of the studies. Studies I-IV were conducted early in the pandemic, prior to the emergence of variants of concern, with the exception of study II, which overlapped with the global emergence of Alpha and Beta; the first cases in Finland were identified in December 2020, after the study period. Study V was conducted during the emergence of Omicron.

4.1. Registry Data

4.1.1. Study design and participant selection for evaluating clinical sensitivity (I)

The evaluation of the clinical sensitivity of the RT-PCR tests used in the HUS Diagnostic Center HUSLAB was conducted retrospectively through the inspection of medical records. The HUS Diagnostic Center, HUSLAB (later HUSLAB) is a publicly funded diagnostic laboratory providing diagnostic services to primary health care clinics and hospitals in the Helsinki capital region and surrounding areas, reaching a patient population of approximately 1.7 million. The aim of this

study was to compare the sensitivity of SARS-CoV-2 RT-PCR to high clinical suspicion of SARS-CoV-2 infection.

In this study, the outpatients and inpatients were evaluated separately, because it seemed plausible that the pretest probability was different for patients with mild respiratory tract symptoms who would be treated as outpatients and those who ended up hospitalized. We deduced this pattern based on the fact that COVID-19 has a wide range of presentations and symptom severity. In the outpatients, there was a higher probability that the experienced symptoms were actually due to another cause, such as seasonal allergies, whereas patients with severe symptoms were more likely to be SARS-CoV-2-positive[13,180]. Soon after the epidemic reached Finland, the incidence of influenza dropped considerably, and COVID-19 became the reasonable presumptive diagnosis for severely or critically ill patients with symptoms compatible with viral pneumonia[262,263].

During the study period, between the 4th of March and the 15th of April 2020, which was early in the pandemic, the testing criteria and strategies were updated frequently. However, the criteria mostly involved symptomatic patients, often with relevant exposure, such as travel history to affected areas or contact with a confirmed case, risk factors for severe disease or being a health care professional.

We sampled the outpatient cohort from a manually recorded line list that included all outpatients sampled for SARS-CoV-2 during the study period. From these lists, systematic, quasi-random sampling was performed by including every fifth patient. Patients under the age of 18 were excluded, as were patients who were not residents of the Helsinki Uusimaa district. The electronic patient records of the included outpatients were assessed.

Inpatients experiencing fever or respiratory symptoms, including difficulty breathing or symptoms of the gastrointestinal tract, were considered suspected COVID-19 cases and were treated in designated COVID-19 cohort wards. Suspected patients were treated in individual rooms. The cohort wards included 6 ICUs and 11 non-ICU wards. All the patients treated in these wards during the study period were included. The exclusion criteria were being aged below 18 years or having a SARS-CoV-2 RT-PCR performed in laboratories other than HUSLAB.

To evaluate the clinical sensitivity of the RT-PCR tests, a reference standard based on clinical criteria was created. We decided to retrospectively grade the patients, based on clinical details, into four groups:

1) SARS-CoV-2 "Not suspected"; patient was deemed not compatible with COVID-19 by the physician in charge, or another acute illness explaining the symptoms was diagnosed.
2) SARS-CoV-2 "Not excluded"; COVID-19 diagnosis was neither confirmed nor excluded, and no other diagnosis was reached.
3) "High suspicion" of SARS-CoV-2; in the patient records, COVID-19 was noted as suspected, or one of the following criteria was met:
   i. respiratory symptoms and/or fever and/or compatible findings on radiological imaging and travel history to epidemic regions, which at the time were Austria (Tirol area), northern Italy, Spain, Iran, South Korea, and China;
   ii. respiratory symptoms and fever and findings compatible with infection in radiological imaging during April; or
   iii. respiratory symptoms or symptoms of the gastrointestinal tract or fever or findings compatible with infection in radiological imaging and contact with a laboratory-confirmed SARS-CoV-2-positive individual within a 14-day period before the onset of symptoms and
4) "Laboratory confirmed"; a patient who had a positive result for SARS-CoV-2 by RT-PCR.

Depending on the availability of sampling swabs, upper respiratory tract testing was performed with either nasopharyngeal or oropharyngeal samples. Other routinely analyzed sample types were tracheal aspirates, bronchoalveolar lavage samples and sputum as well as sinus and lung biopsies. All the samples were inactivated with MagNA Pure Lysis/Binding Buffer (Roche Diagnostics, Mannheim, Germany) prior to analysis to ensure adequate biosafety.

4.1.2. Quality registry and laboratory information system data (IV)

For the study evaluating the frequency of bacterial infections among hospitalized COVID-19 patients, laboratory information system data on microbiological tests and findings were collected manually. The patients included in this evaluation were hospitalized in the specialized health care center in the Greater Helsinki area between February 27th and June 21st 2020 and were recorded in the COVID-19 quality registry of the Helsinki University Hospital. This registry contains extensive clinical details, including the patient sex, age, comorbidities, duration of hospitalization, symptoms, complications and treatment outcome[264,265]. In addition to the laboratory information system and quality registry data, the complete duration of antibiotic treatment during hospitalization was recorded for each patient.

The incidence of bacterial infections in hospitalized COVID-19 patients was determined through a systematic retrospective inspection of the HUSLAB

laboratory information system. Data were collected on all the microbiological sample types. The date of sampling, time elapsed between positive SARS-CoV-2 RT-PCR and sampling, time elapsed between hospitalization and sampling, and findings as well as their significance were recorded. For bacterial sample detection, the resistance genes and unusual phenotypic susceptibility patterns were recorded. Culture-verified pneumonias and blood culture-verified blood stream infections were categorized as severe bacterial infections. When a culture was collected ≤48 hours after hospital admission, the positive finding was interpreted as a community-acquired bacterial infection, and when the positive culture was collected >48 hours after hospital admission, the bacterial infection was considered nosocomial.

Data from the population-based quality registry were used to identify risk factors associated with severe bacterial infections as well as to assess the prognosis of patients with severe bacterial infections. The quality registry covered all hospitalized SARS-CoV-2 laboratory-confirmed patients in the Greater Helsinki area.

Blood cultures were incubated in a Biomérieux BacT/Alert™ incubator, positive samples were cultured on nonselective culture media, and identification was performed with matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF), specifically a Biomérieux Vitek™ MS MALDI-TOF assisted with biochemical methods such as oxidase, catalase, and latex agglutination. One blood culture set included four bottles, of which two were aerobic and two were anaerobic. All the blood culture findings were considered significant with the exception of normal skin flora, such as coagulase-negative staphylococci, growth in a single bottle.

The sample types collected for respiratory tract culture were sputum, tracheal aspirates, bronchoalveolar lavage, pleural fluid and samples collected from tracheostomy tubes. These samples were cultured on both selective and nonselective culture media to maximize the yield of significant pathogens. Their species-level identification and antimicrobial susceptibility were reported for credible pathogens.

Pneumococcal or *Legionella* urinary antigens were tested with an Alere BinaxNOW™ *Streptococcus pneumoniae* Antigen Card and an Alere BinaxNOW™ *Legionella* Urinary Antigen Card, respectively. Two different viral PCR panels for viruses other than SARS-CoV-2 were used for COVID-19 patients in our cohort: triplex nucleic acid amplification test (NAAT), namely Cepheid GeneXpert Xpert™ Xpress Flu/RSV, and multiplex NAAT Seegene Allplex™ respiratory panel 1-3.

Urinary tract cultures were performed on chromogenic media. Identification was performed with MALDI-TOF assisted with biochemical methods.

Data regarding the microbiological tests requested after hospitalization were collected for a follow-up period lasting until December 2020, constituting follow-up periods of 154-286 days for individual patients.

4.2. Samples

4.2.1. Respiratory samples for rapid antigen test evaluation (II)

Rapid antigen tests on respiratory samples were evaluated with three sets of archived upper respiratory tract swab samples from symptomatic patients. The first set of positive samples was selected based on the cycle threshold (Ct) values to assess the analytical sensitivity of the rapid assays against RT-PCR and included 62 samples. Positive samples were grouped as follows: Ct <25, Ct 25-29.99, and Ct ≥30. Performance that could be generalized to represent the intended use in the real-life outpatient setting was evaluated with a second set of positive samples from outpatient clinics by systemically selecting all positive samples from the 18th of November 2020 backward until 96 samples were reached. The Ct values of these samples ranged from 10.74–32.49. These samples are considered to reflect the patient population, and the sample quality is such that they are most likely to be eligible for point-of-care rapid antigen testing in a real-life scenario: the upper respiratory tract samples of outpatients. The specificity was assessed with 40 RT-PCR negative samples.

4.2.2. Samples for serum antigen test evaluation (III)

An antigen test on the serum samples was evaluated with three sets of archived serum samples. Specificity was assessed with a negative panel of 155 serum samples. *Aspergillus* antigen-positive samples (11 samples) were collected in 2020, and the remaining 144 samples were collected prior to 2020 before the circulation of SARS-CoV-2 in Finland and included specimens positive for a variety of antigens and autoantibodies described in detail in III.

A set of 70 samples from SARS-CoV-2-positive patients was also analyzed with a microneutralization test. Another set had 2-4 sequential samples from selected individuals collected at different time points, for a total of 56 samples from 27 individuals.

4.2.3. Serum samples for the serosurvey during the emergence of a novel variant (V)

During each week beginning on the 15th of November 2021 and ending on the 6th of March 2022, 100 HIV-negative serum samples were randomly selected for the serosurvey from samples sent for HIV screening to HUS Diagnostic center HUSLAB. The total number of analyzed samples from the study period was 1 600.

4.3. Laboratory methods

4.3.1. RT-PCR (I)

SARS-CoV-2 diagnostics in HUSLAB are performed on several different platforms to both maximize the capacity and to ensure continuous diagnostic services. The RT-PCR methods used in the studies were as follows:

The laboratory developed a test that is based on Corman et al.[43] and targets the *N* gene.

Cobas™ SARS-CoV-2 test using the automated Roche Cobas 6800™ platform targeting the *orf1ab* and *E* genes (Roche Diagnostics, Mannheim, Germany).

Amplidiag™ test targeting the *orf1ab* and *N* genes (Mobidiag, Espoo, Finland).

These methods were all found to be adequately sensitive and highly specific in a previous analytical evaluation performed in our laboratory. In that evaluation, the Cobas™ test showed the highest sensitivity in a dilution series of positive patient samples, which is consistent with the limit of detection reported by the manufacturer of 0.009 $TCID_{50}$/mL[266]. Positive and negative percent agreement values for the three tests were calculated by using a consensus result as the reference value, and the consensus result was defined as the result obtained with at least two of the studied methods. The positive percent agreement of the Cobas™ test was 100%, while it had the lowest negative percent agreement of 89.4%, reflecting higher sensitivity compared to the laboratory-developed test and Amplidiag™ test [267].

These three RT-PCR tests were evaluated in study I. Different test platforms were not assessed separately. The laboratory developed test was also used as a reference standard in study II. For studies III and IV, the laboratory confirmation of SARS-CoV-2-positive patients was based on results obtained from these three platforms.

4.3.2. Rapid lateral flow antigen tests (II)

The antigen tests evaluated were Panbio™ (Abbott Diagnostic GmbH, Jena, Germany), Quidel Sofia™ SARS FIA (Quidel, San Diego, California), and Standard Q™ COVID-19 Ag tests (SD Biosensor, Republic of Korea). All the evaluated tests target the N antigen. The tests were performed according to the manufacturer's instructions except for the use of frozen samples in saline instead of fresh samples. The tests were performed in a centralized laboratory by specifically trained health care personnel as opposed to point-of-care testing.

Panbio™ and Standard Q™ represent traditional rapid lateral flow "stick tests" in which the lines on the test strip are interpreted visually, whereas the Sofia™ test requires a separate instrument (Sofia 2™) to assess the fluorescence of the control and test lines. All the evaluated tests are CE IVD-marked.

4.3.3. Enzyme-linked immunosorbent assay for antigen detection in serum samples (III)

The Salocor SARS-CoV-2 Antigen Quantitative Assay Kit™ (Salofa Ltd, Salo, Finland) was the assay evaluated in study III and is intended to quantify the N antigen in serum and plasma samples. It is based on a double antibody sandwich method. The method is described in III: Supplement.

A standard curve with binomial fitting based on the calibrator absorbance values with known SARS-CoV-2 N protein concentrations (pg/ml) was used to calculate the concentration values of the samples. Concentration values ≥2.97 pg/ml were interpreted as positive as per the manufacturer's instructions.

4.3.4. Virus culture (II)

Virus culture on Vero E6 cells expressing TMPRSS2 transcript variant 2 cDNA (GenBank accession number NM_005656.4) was deployed as an additional reference test for the rapid lateral flow antigen tests. Cytopathic effect was scored from non-observable to extensive cell death, and infection was confirmed by RT-PCR. Virus culture was performed for 59 RT-PCR-positive samples used in the evaluation of rapid antigen tests.

4.3.5. Serological assays (III, V)

The ELISA and CLIA tests used in the serum antigen test evaluation were Euroimmun SARS-CoV-2 IgG (S1 antigen) and Abbott Architect™ SARS-CoV-2 IgG (N antigen) tests, respectively.

In the serosurvey study, the Abbott Alinity i™ SARS-CoV-2 IgG CLIA test was used to detect anti-S1 and anti-N antibodies. The serosurvey samples were analyzed for N antibodies to determine the proportion of people who had evidence of previous infection. S1 antibodies are present in the immunized population and in patients who have had a SARS-CoV-2 infection. Naïve populations, who are without previous infection and are not vaccinated, test negative in both assays.

In the microneutralization test for samples in study III, Vero E6 cells and the SARS-CoV-2/Finland/1/2020 strain were used. Neutralization was assessed by cytopathic effect. The 50% endpoint of the serum dilution that inhibited SARS-CoV-2 infection in 2 out of 3 parallel wells was considered the neutralization endpoint titer. The method is described in detail in Jääskeläinen et al[137].

4.4. Statistics (I, II, III, IV, V)

Statistical analyses were performed using the SPSS statistical program package, version 25 (IBM SPSS Inc., Chicago, Illinois), GraphPad Prism 8.0.1 software (San Diego, California) and R software (R Core Team (2021). R: A language and environment for statistical computing, R Foundation for Statistical Computing, Vienna, Austria).

4.4.1. Sensitivity and specificity (II, III)

The analytical sensitivity for studies II and III was calculated by dividing the number of true positives that the test was able to identify by the number of true positives determined by RT-PCR used as the reference standard. The specificity was calculated by dividing the number of true negatives that the test correctly identified as negative by the number of true negatives as determined by the reference standard. Clopper-Pearson confidence intervals (95%) were calculated for sensitivity and specificity estimates.

For the rapid antigen tests (II), McNemar's test was used to assess the concordance, and Cohen's kappa coefficient was used to assess the agreement between different antigen tests.

For the serum antigen test (III), a receiver operating characteristic (ROC) curve was graphed for serum samples collected within 14 days of symptom onset to illustrate the diagnostic abilities of the SARS-CoV-2 serum N antigen test at different cutoff values and to validate the cutoff value stated by the manufacturer.

4.4.2. Clinical sensitivity of SARS-CoV-2 RT-PCR tests (I)

A statistician conducted or supervised all the statistical analyses in the study concerning the clinical sensitivity of SARS-CoV-2 RT-PCR (I). P values below 0.05 were considered statistically significant.

The sample size for the outpatient sample in study I was calculated assuming 70% sensitivity for the RT-PCR based on previous publications[97,268] and 10% prevalence in tested populations since the median positivity rate at the time of the study in the Greater Helsinki area was 9.6%. The minimal statistical power was set at 80%, and the type I error was set at <0.05. Two different sample size estimates based on[269,270] were calculated: 1550 and 1600 patients, respectively.

Comparisons between groups based on clinical suspicions of COVID-19 were performed separately for inpatients and outpatients. Demographic and clinical details were compared to determine whether the groups were sufficiently comparable and to detect possible confounding factors. For categorical factors,

Pearson's chi-squared test was used with Yate's correction or Fisher's exact test as appropriate. Age distributions were compared using Mann-Whitney's U test.

To evaluate the RT-PCR test sensitivity based on repeat testing, all the patients who had at least one positive RT-PCR result from a single symptomatic episode within 14 days of symptom onset were included. First, negative samples were considered false negatives, and first positive results were considered true positives.

To estimate clinical sensitivity, patients categorized as "high suspicion" were considered false negatives.

Calculations of the 95% confidence intervals (CI) for sensitivity were performed with the Wilson score method.

4.4.3. Multivariable analysis (IV)

The effect of severe bacterial infections was assessed using multivariable analysis (V). Comparisons of categorical variables were performed with Pearson's X^2 test. The Shapiro-Wilk test was used to test continuous variables for distribution normality, and normally distributed variables were reported as the mean with standard deviation; the values that were not normally distributed were reported as the median with interquartile range. Exceptions were the length of stay parameters, which were reported using both the mean and the median.

Odds ratios (ORs) with 95% CIs were calculated. Variables with missing data (BMI, smoking, alcohol, or illegal drugs) were not considered for inclusion in the multivariable model. The tests were two-tailed, and $p<0.05$ was considered statistically significant.

5. RESULTS AND DISCUSSION

During many periods of the pandemic, diagnostic laboratories have struggled with a high demand for testing combined with a scarcity of reagents and laboratory equipment as well as staff shortages. This difficulty also affected the choice of tests evaluated in this thesis: the methods were selected based on availability, cost and the information available on their performance. The studies in this thesis were designed to add to the available evidence on RT-PCR and antigen test performance and to provide information on a novel method of antigen detection in serum samples as well as to characterize the epidemiological situation through laboratory-based surveillance of microbiologically confirmed bacterial infections and a seroprevalence study. Table 2 summarizes the research questions addressed in each study and their key findings.

Table 2. Summary of the studies, research questions and main findings

Study	Research question	Hypothesis	Main findings
I: Clinical sensitivity of SARS-CoV-2 RT-PCR tests	What is the real-life clinical sensitivity of SARS-CoV-2 RT-PCR tests?	Clinical sensitivity is lower than analytical sensitivity	Clinical sensitivity 47.3%, Analytical sensitivity 89.9%
II: Rapid lateral flow antigen tests	How do SARS-CoV-2 RADTs perform compared to RT-PCR?	RADT sensitivity is lower than RT-PCR	The evaluated antigen tests detected 80–83% of the cases detected with RT-PCR
III: ELISA for antigen detection in serum samples	Could antigen detection from serum samples be used as a diagnostic tool in acute SARS-CoV-2 infection?	During acute infection, N antigen can be detected in serum	Within two weeks of symptom onset the test detected 95.6% of RT-PCR positive cases, making it a promising tool for diagnosing acute infection
IV: Severe bacterial infections of COVID-19 patients	How common are bacterial infections among hospitalized COVID-19 patients and what is the impact on prognosis?	Frequency of severe bacterial infections would be approximately 10%. Severe bacterial infections prolong hospital stays and increase case-fatality rates significantly.	Frequency of severe bacterial infections was 6.5%. Severe bacterial infections prolonged hospital stays significantly, but the increase in case-fatality rates was not statistically significant.
V: Serosurvey during the emergence of a novel variant	Is there an increase in anti-N antibody prevalence in the Helsinki area following the introduction of Omicron in Finland?	Proportion of anti-N antibody-positive samples will rise from under 10% to a proportion of approximately 20–50%.	Proportion of anti-N antibodies at the end of the study period was 28.2%. Sharpest increase was observed in age group <30.

RADT: Rapid antigen detection test

5.1. Clinical sensitivity of SARS-CoV-2 RT-PCR tests (I)

Altogether, 3008 individuals were eligible for this study. The excluded patients and grading are presented in detail in flow charts (I: Figures 1 and 2) and are summarized in Figure 7.

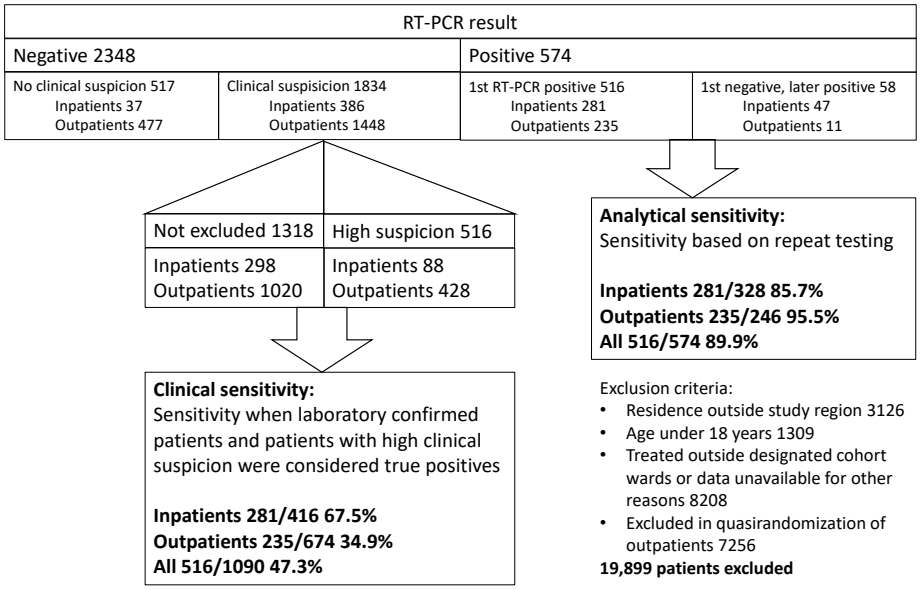

Figure 7. RT-PCR results and clinical suspicion grading of patients included in study I. Patients with negative RT-PCR results were grouped into "no clinical suspicion", "not excluded" and "high suspicion", based on their clinical grading. The sensitivity was calculated for inpatients and outpatients separately. The clinical sensitivity of RT-PCR was assessed by including patients with high clinical suspicion in the denominator. The RT-PCR-confirmed cases were used to calculate the analytical sensitivity.

The demographic differences between the groups were minor, which was expected when taking into account the epidemiological situation and characteristics of COVID-19. Transmission at the time of the study in Finland was low, and men and the elderly were more susceptible to severe disease[149,179,271]. The inpatients in our study were older than the outpatients (median age 66 vs. 43 years old), and males were slightly overrepresented in the inpatient group (54.8%), while in the outpatient group, 69.1% of the participants were females. The RT-PCR-confirmed group (group 4 in the grading system) was more likely to have a fever (91.5% vs. 76.1%, p value <0.001) and gastrointestinal symptoms (25.0% vs. 7.8%, p value <0.001) and more likely to be admitted to the ICU (47.3% vs. 36.4%, p value 0.03) than the high suspicion group (group 3, in the grading system) (I: Tables 2A and 2B).

For the inpatient cohort, based on repeated testing of the same patients, the analytical sensitivity was 85.7% (281/328, 95% CI 81.5-89.1%). It is important to note that sensitivity estimates based on repeat testing assume that patients who subsequently test positive after negative tests have been false negatives in previous testing time points, which may not be the case. The clinical sensitivity calculated based on high clinical suspicion was 67.5% (281/416, 95% CI 62.9-71.9%). When both the repeat testing and high suspicion groups were included in the analysis, the clinical sensitivities for nasopharyngeal and oropharyngeal swab samples were 71.2% (195/274, 95% CI 65.5-76.2%) and 66.7% (36/54, 95% CI 53.4-77.8%), respectively. The difference was not statistically significant (p value: 0.51), and the confidence intervals overlapped. In the inpatient group, the sample type was unknown for 84 patients who belonged to the repeat testing or high clinical suspicion group, yielding a 56.0% (47/84) sensitivity. It is possible that some samples in the group of unknown sample material were nasal or oral samples, which might explain the lower sensitivity.

In the outpatient cohort, sensitivity based on repeated testing of the same individuals was 95.5% (235/246, 95% CI 92.2-97.5%). It is noteworthy that at this point, repeat testing in the outpatient population was rather unusual since the testing capacity was limited. Repeat testing was likely to be performed only under special circumstances and in cases in which the clinical suspicion was especially high, and diagnosis confirmation was considered relevant. Repeat testing of outpatients was also performed when there was a new exposure, which may in some cases have reflected a separate disease episode, causing bias.

In the outpatient cohort, the sensitivity calculated based on the grading of clinical suspicion was 34.9% (235/674, 95% CI 31.4-38.5%). The low sensitivity can be at least partially explained by the fact that clinical suspicion grading is not expected to be highly specific, but issues related to sample collection are likely to contribute as well and are a crucial part of the successful implementation of diagnostic tests. Preanalytical factors such as the sampling equipment, transport media, distance to analytical laboratories, transport conditions, timing of sampling, sampling technique and location all play an important role in the overall performance of the tests, and during the pandemic, preanalytical laboratories and other sampling locations have been under extraordinary strain.

Sensitivity differences between sample types did not reach statistical significance in the outpatient group either; the sensitivity for nasopharyngeal samples was 33.6% (154/458, 95% CI 29.4-38.1%), and for oropharyngeal samples, it was 40.2% (39/97, 31.0-50.2%) (p value: 0.22). The result is somewhat contradictory to the widely accepted notion that nasopharyngeal samples generally perform better than oropharyngeal samples[272], but to investigate this point further, a more detailed study design would be necessary, possibly involving simultaneous nasopharyngeal and oropharyngeal sampling from suspected cases in a serial

manner. The study period coincided with a swab shortage, which may have resulted in the performance of sampling using suboptimal swabs.

When both inpatients and outpatients were included in the same analysis, the sensitivity was 89.9% (516/574, 95% CI 88.2-92.1%) when repeat testing was used as a standard for confirmation and 47.3% (516/1090, 95% CI 44.4-50.3%) when high clinical suspicion was considered diagnostic.

When the time between symptom onset and sampling was assessed (I: Figure 3 and Supplement Table 4), no statistically significant difference was found in our patient material. However, the analytical sensitivity in our sample was the highest, at over 90%, when sampling was performed 1-4 days after symptom onset. The overwhelming majority of samples the laboratory confirmed and the high clinical suspicion groups with information on symptom onset (971/1010, 96.1%) were collected within two weeks after symptom onset when the sensitivity of RT-PCR can be expected to be high. When samples were collected 7-14 days after symptom onset, the analytical sensitivity was still 89.0%. Data on symptom onset were available for only a portion of patients, and the reliability of the reported symptom onset could be low due to recall bias.

5.1.1. Implications for clinicians

Molecular testing currently represents the most practical alternative for the laboratory diagnostics of acute viral infections and is rapidly gaining more of a foothold in other fields of clinical microbiology, such as the diagnosis of bacterial and parasitic infections[273–276], the detection of antimicrobial resistance (AMR) [277,278] and sequencing-based epidemic surveillance[279–281].

To understand the real-world value of a given laboratory test, careful evaluations of sensitivity and specificity must be conducted. These evaluations will ideally account for more than just the analytical performance of the test: in effect, preanalytical factors such as the sampling technique, timing and transport media will contribute in a nonnegligible way to the overall diagnostic performance and thus the value of the test[97,282–284]. Setting up studies to reflect real-life situations and the real-life performance of a diagnostic test realistically is challenging, even more poignantly so when a perfect gold-standard test does not exist, as is the case for RT-PCR[285]. The aim of our study was to address this issue by using a robust clinical evaluation as the standard against which the RT-PCR methods used for SARS-CoV-2 diagnosis were compared. From the clinician's point of view, this approach can be especially useful, because it considers the full spectrum and scale of the clinical presentation as well as the clinicians' evaluation of an individual patient.

Our study found a stark contrast between the high analytical sensitivity of SARS-CoV-2 RT-PCR tests and their relatively low clinical sensitivity. The difference was even more pronounced in the outpatient cohort than among

inpatients, which is partly explained by the very few and selected outpatients who were subjected to repeat testing.

An issue related to the high analytical sensitivity of RT-PCR is patients who remain RT-PCR-positive for prolonged periods of time even in the absence of viable virus, which makes a distinction between prolonged RT-PCR positivity and true reinfection with SARS-CoV-2 problematic[286,287]. Exploring this aspect of sensitivity was outside the scope of our study.

The results of our evaluation provide a more austere view of the overall performance and the caveats surrounding the interpretation of test results for health care personnel and the general public. It is crucial that the preanalytical practice be optimized to support test performance and that clinical decision-making is not informed only by one isolated test result but by the applicability and reliability of that result. Our results provide useful tools for clinicians to understand the possible shortcomings of SARS-CoV-2 RT-PCR and the associated sampling process as a whole. A realistic view of test sensitivity is needed for adequate biosafety measures, cohort purposes, and of course, the efficient and timely diagnosis and patient care of individual patients.

5.1.2. Implications for epidemic control

Rapid, reliable testing is one of the cornerstones of well-organized and timed infection control measures such as contact tracing, quarantining and cohorting[288,289]. In clinical decision-making, the possibility of a false negative RT-PCR test is a conundrum that clinicians, especially in the emergency department, cohort wards and those working with vulnerable populations such as immunocompromised patients, face regularly. Clinical grading systems, radiological criteria and biochemical markers are constantly under development to aid in decision making[149,150,157,268,290–294]. In challenging clinical scenarios, interpreting microbiological test results solely on the basis of their analytical sensitivity can be outright misleading. Forming a holistic picture with the test's clinical performance, predictive value in a given patient population and the clinical picture of an individual patient can improve decisions on repeat testing, further differential diagnostics and ultimately improve patient care. Furthermore, in epidemiological surveillance, case number estimates based on RT-PCR results should be supplemented with information from other methods, such as seroprevalence studies. This is necessary to account for the cases missed by RT-PCR and for the asymptomatic and mild cases with a lower likelihood of being sampled at all.

5.1.3. Timing of sampling

No definite conclusions on the optimal sampling time frame can be made based on our evaluation, but consistent with previous publications[66,112,295], sampling

early after symptom onset using our material within four days seemed to lead to higher sensitivity. It must be noted that in our study, samples from patients sampled under one day after symptom onset yielded a lower sensitivity (78.4%, 95% CI 62.8-88.6%), which could be indicative that the replication is reaching its peak later during the course of the disease. This finding needs to be interpreted with caution, and simultaneously acknowledging that RT-PCR test positivity in no way equals infectivity and in turn RT-PCR test negativity does not equal true negativity or noninfectivity[97,284,296,297]. Quantitative RT-PCR and viral load analysis can address questions of infectivity with precision[298,299], but a meta-analysis on viral kinetics and infectivity suggests that repeat testing or the determination of viral loads in individual cases is often unnecessary and that a standard isolation time of 10 days is a sufficient precaution for hospitalized patients with non-severe disease[300]. At the time of writing, July 2022, the recommendation for SARS-CoV-2 outpatients in Finland is to avoid contacts for a minimum of five days and at least two asymptomatic days, but it must be noted that the recommendations of health officials evolve as the epidemiological situation changes and when new information becomes available[301].

A limitation to the estimate of analytical sensitivity based on repeated testing is that at the time of collection of the first sample, the patient may not have a detectable viral load yet or even an infection, i.e., making them a true negative at that timepoint. This result may lead to an underestimation of the analytical sensitivity. Suboptimal sampling technique is another issue that may affect the sensitivity estimate.

New variants can have mutations that affect the RT-PCR target genes, causing the RT-PCR to miss cases, underlining the need to include several targets to maintain high sensitivity even in the case of mutations.

Overall, the results of this study must be interpreted in light of the development of diagnostic methods after the early stages of the pandemic, and acknowledgment of the criteria used for assessing the clinical suspicion was based on the information and evidence available during those early stages. Additionally, the study only reflects the performance of the RT-PCR methods used in the study.

5.1.4. Preanalytical factors

The impact of preanalytical factors on test performance and the reliability of results can hardly be stressed enough[101,102,302–304]. The first step in any process involving laboratory diagnostics is choosing the correct test for the patient at an appropriate time point, requiring active dialog between the laboratory and the clinicians requesting the tests.

After the decision to test, a multitude of other factors come into play: the correct anatomical sampling location, correct sampling technique and suitable transport media. Once the sample is collected, logistics must be organized in

a way that ensures biosafety and provides results rapidly and efficiently, which can be challenging in settings with long distances to health care providers and centralized laboratories.

Several reports during the pandemic on test sensitivity and specificity regarding different sample materials have established nasopharyngeal or lower respiratory tract samples as the RT-PCR sample material of choice in most situations[66,110] due to the high sensitivity and relative convenience of sample collection. Other sample types include nasal, oral, oropharyngeal, saliva, gargle, fecal, and urine[13,110,272,305–318]. In select populations, for example, children, some trade-off in sensitivity may be advisable to enable the collection of samples in a noninvasive and comfortable way[319].

The main takeaway from our results is that when interpreting SARS-CoV-2 RT-PCR results, one should consider the possibility of a false negative result. The clinical sensitivity based on our grading of clinical suspicion of SARS-CoV-2 infection was lower than the analytical sensitivity based on repeat testing, at 47.3% and 89.9%, respectively, when both inpatients and outpatients were included. Repeat testing should be performed with a relatively low threshold when symptoms persist and no other cause is found, especially in hospital settings in which the impact of a false negative result can be very detrimental to the control of nosocomial SARS-CoV-2 infections and when appropriate care might be delayed if the accurate diagnosis is not reached in time.

5.2. Rapid lateral flow antigen tests (II)

The sensitivity of the rapid lateral flow antigen tests was determined against the laboratory-developed RT-PCR used in the HUS Diagnostic Center HUSLAB to detect the *N* gene. This approach was one of the three tests included in the evaluation of the clinical sensitivity of RT-PCR described in study I.

In total, 198 respiratory tract swab samples were taken, but due to availability issues for the evaluated tests, the number of samples analyzed with each of the antigen tests differed. Performance was also assessed in comparison with virus culture for a subset of 59 samples.

For the Panbio™ test, the overall agreement with the RT-PCR was 86.32%, for Standard Q™ it was 84.85%, and for Quidel Sofia™ it was 84.57%. The sensitivity values of each evaluated test are outlined in Table 3.

Table 3. Sensitivity values of the three rapid lateral flow antigen tests in comparison with RT-PCR (II)

	Quidel Sofia™ SARS FIA (Quidel), sensitivity	**Standard Q™ COVID-19 Ag (SD Biosensor), sensitivity**	**Panbio™ (Abbott), sensitivity**
All	80% (119/148)	81% (128/158)	83% (123/152)
Ct<25	99% (88/89)	99% (96/97)	98% (90/92)
Ct 25–29.99	82% (116/123)	69% (120/130)	76% (116/126)
Ct≥30	12% (3/25)	31% (8/26)	38% (10/26)

All the samples in the negative panel (n=40) tested negative according to all the evaluated rapid lateral flow antigen tests, yielding a specificity of 100% for all the evaluated tests. All the tests also gave a valid result for all the samples.

5.2.1. Appropriate application in clinical settings

Antigen tests have clear advantages in some respects even if they fall short of the high analytical performance of RT-PCR. The possibility for rapid self-testing and point-of-care testing as well as availability for frequent retesting are some of the main appeals of rapid antigen tests[320,321]. Commercially available antigen tests have shown a wide variety of performance results according to one large, thorough evaluation of 122 tests reporting sensitivities ranging between 0-80%, clearly indicating wide differences in quality between manufacturers and the need for independent evaluations of test performance[322].

Some aspects of the advantages of antigen testing remain debated, such as using antigen test positivity as a marker for infectivity [323]. This issue should also be considered in the context of highly variable test performance. Some studies have demonstrated a reasonable correlation between antigen tests and virus culture[324–326]. This finding was demonstrated in our study as well; see study II: Table 2. However, virus culture itself is, at best, a proxy for viable, infectious viruses, and as a demanding and complex laboratory method, it is subject to error[327–330].

Antigen testing has been adopted for routine use in several clinical laboratories and point-of-care testing facilities and as a self-testing tool for the general public[130]. When a population has partial immunity, the disease mostly causes mild manifestations, and there is no need to perform RT-PCR on all symptomatic individuals. Self-testing can be a meaningful way to contain the spread while keeping the workload of laboratories performing RT-PCR testing more manageable. To achieve a situation in which the laymen using these tests are aware of their performance and limitations, the results of independent, clinical evaluations must be accessible and communicated along with the test package instructions. During the pandemic, communicating research to a wider

audience has become a continuous trust-building exercise between the scientific community, public health authorities, publishers, the media, and the public. Efforts to be clear, understandable, and honest about uncertainties surrounding research and scientific discovery and to be forthcoming about outright mistakes are mandatory to create a true dialog that benefits both the advancement of science and the adoption of applications. Communicating issues surrounding diagnostic methods is a part of this equation[331–333].

5.2.2. Choosing rapid testing methods and strategies

For rapid testing in large emergency departments and hospitals, it seems expedient to adopt rapid, RT-PCR-based point-of-care testing methods to provide the necessary sensitivity for the hospital environment and patient population [334]. In settings in which testing and care are less centralized, especially in low population density areas such as Finnish Lapland, rapid antigen testing may be logistically superior and facilitate efficient contact tracing [335]. When screening large populations, especially asymptomatic populations, it is important that the test performance in the population in question is defined well enough to be able to draw conclusions about the results and their impact on epidemic control, and recent evidence suggests that mass testing of asymptomatic individuals with rapid antigen testing is not efficient in reducing transmission[336,337]. It is also noteworthy that while an individual rapid antigen test is performed quickly, analyzing large sample volumes with these methods is time-consuming and labor-intensive.

Large-scale self-performed home testing with rapid antigen tests has also presented new challenges for surveillance. In Finland, cases diagnosed with home tests are not usually systematically recorded, which makes real-time surveillance more challenging since laboratory-confirmed RT-PCR positive cases currently represent only a fraction of the overall cases. In the UK and in Greece, surveillance of self-testing results has already been set up, and exploring possible reporting tools for self-testing results is a step that more countries are likely to take in the future[338,339].

In our evaluation, the antigen tests were able to identify 80-83% of the cases identified with RT-PCR, which confirms that the evaluated rapid antigen tests are less sensitive than RT-PCR but perform at an acceptable level, especially if testing can be repeated on several days, as is currently recommended for self-testing in Finland[340].

5.3. Enzyme-linked immunosorbent assay for antigen detection in serum samples (III)

An ELISA test for SARS-CoV-2 N antigen detection in serum samples was evaluated for its sensitivity at different timepoints in relation to symptom onset and positive RT-PCR tests. Altogether 126 serum samples from RT-PCR-confirmed SARS-CoV-2 cases were used, 24 of which were collected on the same day as the positive respiratory tract sample for RT-PCR and 58 of which had information on symptom onset.

In comparison with the 24 simultaneously collected upper respiratory tract samples for RT-PCR, the Salocor™ assay for antigen detection in serum samples reached a sensitivity of 91.7% (95% Clopper Pearson CI 73.0-99.0%) in comparison with the RT-PCR tests described and evaluated in study I.

In the negative panel, 145 of the 148 samples tested negative in the Salocor™ N antigen test, yielding a specificity of 98.0%. All three samples that tested positive were collected prior to the beginning of the pandemic, so they can be reliably assumed to be false positives. Infection with seasonal coronaviruses and further possible cross reactivity cannot be ruled out in these cases since no simultaneous upper respiratory tract samples are available for retrospective analysis. Two of the false positive samples gave low positive results (4.0 and 5.5 pg/mL), but interestingly, one of the false positive samples with a high positive result (84.5 pg/mL) was from a patient who had myocarditis. Another recent evaluation of the N antigenemia assay identified one false positive result in a patient with myocardial infarction [341]. These findings may warrant further evaluation of the assay's specificity in populations with elevated myocardial enzymes.

In the samples for which the date of symptom onset was available, serum samples collected within 7 days of symptom onset reached a sensitivity of 96.2% (95% CI 80.4-99.9%). Samples collected at 8-14 days post-symptom onset had a sensitivity of 91.7% (95% CI 73.0-99.9), whereas samples collected at 15-21 days post-onset had a 50% sensitivity (95% CI 11.8-88.2%) and over 21 days 28.6% (3.7-71.0%).

For samples collected within 14 days post-symptom onset, an ROC analysis was performed (Figure 8 and Table 4). The cutoff value assigned by the manufacturer (2.97 pg/mL) was validated to be optimal in terms of high sensitivity (93%) without compromising specificity (98%).

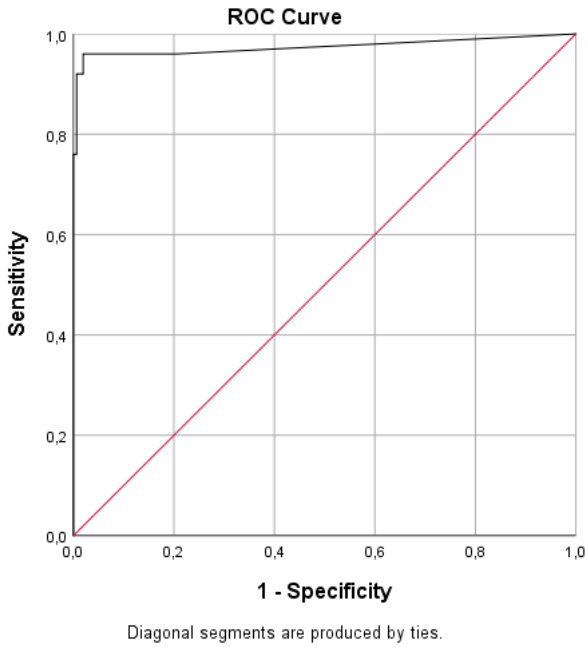

Figure 8. ROC curve for samples collected during the first two weeks after symptom onset. The area under the curve was 0.972, demonstrating excellent diagnostic accuracy for samples collected within this timeframe.

Table 4. Coordinates of the receiver operating curve (Figure 8)

Cutoff	Sensitivity	1-Specificity
-1.0000	1.000	1.000
0.0050	0.956	0.209
0.0550	0.956	0.203
0.1500	0.956	0.189
0.2500	0.956	0.182
0.3500	0.956	0.155
0.4500	0.956	0.142
0.5500	0.956	0.122
0.6500	0.956	0.101
0.7500	0.956	0.088
0.8500	0.956	0.081
0.9500	0.956	0.074
1.1500	0.956	0.068
1.4000	0.956	0.061
1.5500	0.956	0.054
1.6500	0.956	0.047
1.8000	0.956	0.034
2.0000	0.956	0.027
2.5407	0.956	0.020
3.4907	0.933	0.020
4.7500	0.933	0.014
6.4996	0.933	0.007
9.5467	0.911	0.007
13.3633	0.889	0.007
28.4162	0.867	0.007
43.1000	0.844	0.007
45.5754	0.822	0.007
65.5754	0.800	0.007
120.3843	0.800	0.000
160.6219	0.778	0.000
169.1851	0.756	0.000
176.6975	0.733	0.000
181.0000	0.000	0.000

5.3.1. Potential for practical application

A SARS-CoV N antigen assay for serum samples was previously developed and found to be sensitive early after disease onset[342]. However, since the SARS epidemic in 2002-2003 was contained quickly, no further diagnostic utility was assessed.

One avenue for the application of SARS-CoV-2 N antigen detection from serum samples includes the screening of large populations in hospital settings, where serum and plasma samples from patients are readily available. Based on our results, the N antigen assay could also possibly be used for evaluating prolonged RT-PCR positivity to distinguish prolonged viral shedding from reinfection, and it could provide aid in interpreting results from recently vaccinated populations with inconclusive serology results.

The predictive value for disease severity has also been assessed, and the results are promising[134]. Further studies are required to confirm the association. This information is especially interesting, because it is increasingly clear that specific treatment interventions are most effective when administered early in the disease course. Some treatments are only beneficial when administered early for patients who have a specifically high risk for the development of severe disease, as is the case for treatment with convalescent plasma, and methods to identify these patients are urgently needed[204].

5.3.2. Temporal relation of symptom onset and test positivity

In our sample material, the sensitivity appeared to decrease rapidly as the time from symptom onset exceeded two weeks. It must be noted, however, that our sample size is small, and especially in the smallest subgroups, samples collected 15-21 days and over 21 days after symptom onset, the confidence intervals are very wide and overlap.

The ability of our study to ascertain detailed and complete kinetics of antigenemia is limited, but it nevertheless frames a possible timeline and provides a starting point for more detailed assessments.

5.4. Severe bacterial infections of COVID-19 patients (IV)

The frequency of severe bacterial infections, including culture-verified blood stream infections and pneumonias, was 6.5% (40/585 patients) in our cohort during the first wave of the pandemic in spring 2020. Seven (17.5%) of these patients had a bloodstream infection, 28 patients (70%) had a significant finding in respiratory tract culture, and five patients (12.5%) had both a bloodstream infection and a respiratory tract culture finding.

The etiology of severe bacterial infections was diverse: gram-negative organisms dominated the respiratory tract findings while gram-positive organisms were more common in the blood cultures. No severe fungal infections were detected.

The results of the respiratory tract cultures are presented in Figure 9. The proportion of samples with gram-negative organisms appears to increase consistently until 30 days after hospital admission.

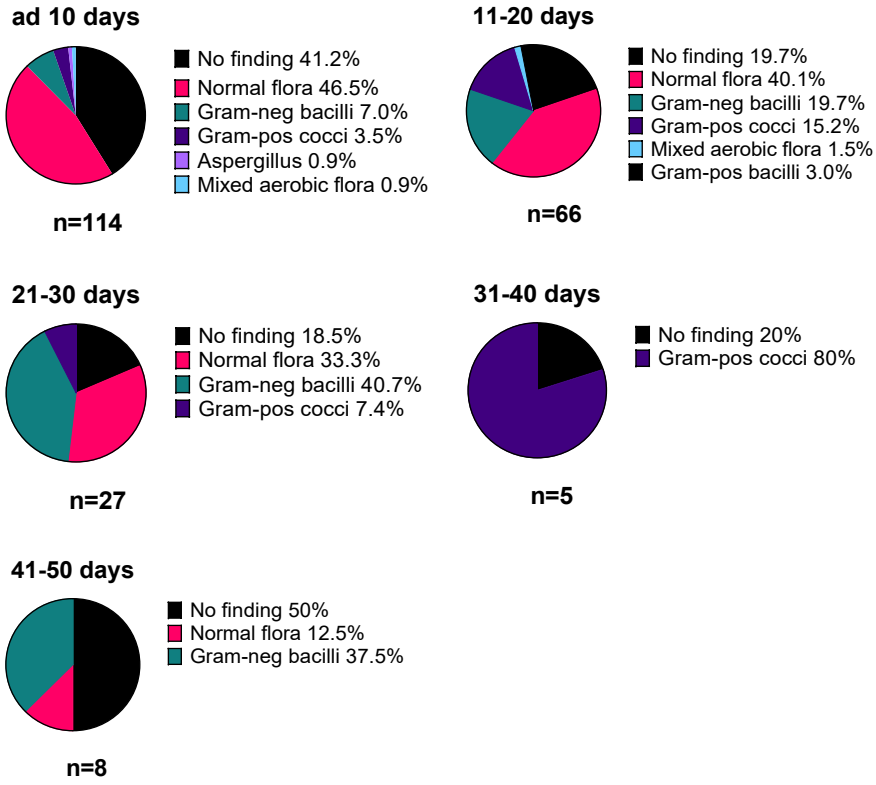

Figure 9. Respiratory tract cultures. Time between hospital admission and culture sampling, n=total number of samples collected within the timeframe.

The most common findings of the respiratory tract cultures were *Staphylococcus aureus* (9 strains), *Pseudomonas aeruginosa* (7 strains), *Escherichia coli* (4 extended spectrum beta lactamase (ESBL)-producing strains and one susceptible strain), and *Klebsiella pneumoniae* (3 susceptible strains and one ESBL strain). The extensive list of findings is presented in IV: Table 3.

Fifteen patients in our cohort had at least one positive blood culture bottle. Three were considered skin contaminants. The skin contaminants were *Corynebacterium amycolatum, Staphylococcus epidermidis* and *Staphylococcus*

hominis. Twelve out of the 453 (2.6%) patients who had at least one set of blood cultures drawn had a significant bloodstream infection.

The most common significant findings in blood culture were *Staphylococcus aureus* (3 strains, 3 patients) and *Streptococcus pneumoniae* (2 strains, 2 patients). Patients with a bloodstream infection received between 3 and 91 days (median 21.5 days) of antibiotic treatment.

Six out of the twelve significant findings in blood culture were classified as community-acquired infections. The causative agents in this group were *Bacillus cereus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus hominis*, *Streptococcus dysgalactiae*, group G and *Streptococcus pneumoniae*.

In nosocomial infections, the causative agents of bloodstream infections were *Serratia marcescens* and *Staphylococcus aureus* in three cases and *Staphylococcus epidermidis* and *Streptococcus pneumoniae* in three cases.

For 6/12 of the patients with a bloodstream infection, the focus of the infection was clinically considered to be pneumonia, and five of these patients also had the same finding in respiratory tract culture as in blood culture.

All the recovered *Staphylococcus aureus* strains in our study were methicillin-susceptible. The proportion of ESBL-producing Enterobacterales out of all the Enterobacterales was 5/12 (41.7%). No carbapenemase-producing Enterobacteriaceae or vancomycin-resistant enterococci strains were detected.

5.4.1. Treatment outcomes

The 28-day case fatality rate of patients with severe bacterial infection was 5/40 (13%), whereas for patients without severe bacterial infection, it was 57/545 (10%) (p value 0.686).

At 90 days after hospital admission, 68/585 (11.6%) of all the patients had died, and 517/585 (88.4%) survived, with 6/40 (15%) fatalities in the severe bacterial infection group and 62/545 (11.4%) in the no severe bacterial infection group (p value 0.490).

Antibiotic treatment was initiated in 522/585 (89%) cases. High antibiotic coverage was consistent with the recommendations in place at that time.

5.4.2. Other diagnostic tests for patients with respiratory symptoms

Pneumococcal or *Legionella* urinary antigens were tested for 126/585 (21.5%) and 37/585 (6.3%) patients, respectively, and all tested negative. Viral NAAT panels for viruses other than SARS-CoV-2 were requested for 260 patients (258 patients had the triplex NAAT for influenza A, influenza B and respiratory syncytial virus requested, 1 patient had the more extensive panel requested and 1 patient had both panels requested) for respiratory samples. No additional viral infections were found in the NAATs.

In our cohort, screening for influenza and RSV was performed extensively, with 260/585 (44.4%) patients sampled at least once. Despite this level of sampling, no coinfections with these viruses were detected. At the time of the study, during the first wave of SARS-CoV-2 in Finland, the attack rates of influenza and RSV dropped, which was attributed to the nationwide lockdown that began on March 17th, 2020[343]. Some studies have found much higher incidences of viral infection, often using more aggressive testing strategies [344] or multiplex PCR with a wide range of different viral targets [345,346], resulting in a wide array of findings, some of which could be innocent bystanders instead of actually contributing to symptoms.

In contrast with the frequent testing of viral pathogens, pneumococcal urinary antigen tests were taken only from approximately every fifth patient, and only 6.3% of patients were tested for *Legionella pneumophila* urinary antigen. This testing level could lead to the underestimation of community-acquired coinfections caused by these pathogens. In Finland, *Legionella* infections are likely to be underdiagnosed based on EU-level epidemiological data [347]. Since sampling for these tests is noninvasive and the tests are easy to perform, more active testing might be advisable. Especially since in the current situation due to vaccine-induced immunity, as well as natural immunity for many patients, a positive SARS-CoV-2 RT-PCR test should not be interpreted as the definitive diagnosis of a severely ill patient, but their bacterial etiology should be considered alongside viral infections.

As nonpharmaceutical interventions such as recommendations on wearing masks, working remotely and social distancing are relaxed, the circulation of other infectious agents is increasing, and simultaneous infection with several agents becomes more probable [348–350].

Nine patients had respiratory tract samples taken for fungal culture, and 5 patients were sampled more than once during their hospital stay. Three patients had growing samples, and *Candida albicans* was expected to be dominant and was found in the samples from all three of these patients. One patient also had *Candida glabrata* growth in the same sample.

5.4.3. Urine culture

The data presented in this chapter are unpublished results (Maarit J. Ahava et al.).

We identified 29/585 (5.0%) patients who had possible infections based on monomicrobial growth in urine culture. The findings are listed in Table 5. Some of these findings have low uropathogenic potential and possibly represent colonizing flora. Some patients had more than one bacterial species growing at different times, resulting in a total of 33 culture-positive samples. Altogether, 178/585 (30.4%) patients had at least one urine sample collected for culture during hospitalization for COVID-19. Some patients were sampled more than once, and the total number of collected samples was 226.

Table 5. The findings in urinary tract culture.

Finding	N
Escherichia coli	9
Escherichia coli ESBL	2
Enterococcus faecalis	3
Enterococcus faecium	4
Enterococcus gallinarum	1
Klebsiella pneumoniae	3
Lactobacillus species	1
Proteus mirabilis	1
Providencia rettgeri	1
Pseudomonas aeruginosa	4
Serratia marcescens	1
Staphylococcus epidermidis	1
Staphylococcus lugdunensis	1
Unidentified coccus	1

Over 30% of hospitalized SARS-CoV-2 patients had at least one sample taken for urine culture. *Escherichia coli* was, as expected, the most common finding and was detected in the samples of 10/30 (33.3%) patients. One of the patients with an *E. coli* urinary tract infection had an ESBL-producing strain, which may have been selected due to empirical antibiotic treatment administered as part of the COVID-19 treatment protocol.

5.4.4. Risk of severe bacterial infections

The risk of severe bacterial infections in our cohort was low. This result is at least partially attributable to the ceftriaxone treatment initiated for most hospitalized patients at the time. Additionally, the low incidence of AMR in Finland has probably contributed to the relatively good outcomes of severely ill COVID-19 patients with severe bacterial infections[351].

Some studies have described much higher incidences of community-acquired bacterial infections in hospitalized COVID-19 patients of up to 30-60%[346,352,353]. Other large-scale studies on bacterial infections have more conservative estimates, ranging between 1 and 10%[187,354–356]. Substantial differences in incidence estimates may be due to geographical differences, variety in testing methods and study design.

Due to constraints from the small number of severe bacterial infections, it is difficult to draw conclusions on the differences between community-acquired and severe nosocomial bacterial infections since no meaningful statistical analysis can be performed. Additionally, since a prolonged hospital stay naturally exposes

the patient to a higher possibility of nosocomial infection, it is challenging to draw conclusions on the causal relationship between length of hospital stay and nosocomial infections.

5.4.5. Findings after hospitalization

The data presented in this chapter are unpublished results (Maarit J. Ahava et al.).

Only one of the 517 discharged patients was sampled for influenza and RSV after their COVID-19 hospitalization. The viral triplex NAAT was taken 9 days after the patient was discharged and 22 days after the positive SARS-CoV-2 test, and it was positive for RSV.

Subsequent hospitalization was a common occurrence based on the number of blood cultures taken after hospitalization; 42 (42/517, 8% of surviving patients) patients had blood cultures drawn after discharge. Of these patients, 6 had positive blood cultures during subsequent hospitalizations. One of these patients had a severe bacterial infection during their COVID-19 hospitalization. The five other patients did not have a severe bacterial infection complicating their COVID-19 infection.

Fecal samples for *Clostridioides difficile* toxin PCR were collected from 13 patients, and 4 of them had a positive finding. Patients with positive *Cl. difficile* PCRs had received 0, 7, 11 and 44 days of antibiotic treatment during their COVID-19 hospitalization. To compare, 42 patients were tested during hospitalization, and none of them had positive *Cl. difficile* toxin PCR.

Findings after hospitalization in our cohort were infrequent. The retrospective, observational nature of the study must be considered when interpreting these results, since no systematic follow-up testing was performed on these patients. Microbiological testing was based on the needs of an individual patient and the clinical evaluation performed by the physician treating them.

Despite these limitations, some interesting findings emerged. Even though only one sample was taken for viral PCR-targeting pathogens other than SARS-CoV-2, it proved to be positive for RSV. The four patients who were diagnosed with a *Cl. difficile* infection after COVID-19 hospitalization could be considered to have infectious sequelae affecting them well beyond their recovery from COVID-19. Additionally, 14/517 (2.7%) patients were sampled for *Cl. difficile* toxin PCR, indicating that at least 2.7% of the patients suffered from diarrhea after hospitalization.

The blood culture positivity rate of 6/42 (14.3%) was quite high, meaning that 6/517 (1.2%) surviving patients had a blood culture-positive bacterial infection during subsequent hospital stays. This finding might reflect the fact that people who are hospitalized for COVID-19 are in many ways often vulnerable to infection: increased age, comorbidities, such as type II diabetes, and high body mass index

are all risk factors for severe forms of COVID-19 and for increased morbidity in general.

5.4.6. Role of antibiotic treatment for COVID-19 patients

In our cohort, in accordance with the guidelines of that time period, most patients (89%) received empirical antibiotic therapy. The local treatment guidelines at the time of the study recommended the initiation of ceftriaxone treatment for hospitalized COVID-19 patients, but the guideline was updated to recommend a more sparing use of antibiotics. Administration of a broad-spectrum empirical antibiotic therapy is very likely to have reduced the risk of nosocomial bacterial infection but must be evaluated both for the possible risks and harm caused. The use of antibiotics results in a selection toward more antimicrobial resistance. This resistance can have devastating effects on individual patients both immediately and in the longer term. The population-level effects of AMR are also significant [357,358]. The side effects of antimicrobials should be considered: skin reactions and gastrointestinal disturbances are the most common ones, and diarrhea caused by *Cl. difficile* is a challenging, and often costly, disease to treat[359–361]. A clear causal relationship cannot be drawn for the patients in our cohort who had positive *Cl. difficile* PCR results after their hospitalization, but it is possible that a portion of these infections were caused by the broad-spectrum antibiotics administered during the previous hospital stay.

Early identification of patients with severe bacterial infections remains a challenge, because the clinical picture of severe COVID-19 can resemble that of bacterial infection[356]. However, the conclusion of our study was that antimicrobial treatment of COVID-19 patients should be reserved for situations in which evidence of bacterial infection is present, and in those cases, the choice of antibiotics should be guided by local susceptibility patterns and preferably by determining the causative agent and the susceptibility of the strain in question.

5.5. Serosurvey during the emergence of a novel variant (V)

During the pandemic, the detection of SARS-CoV-2 antibodies has had limited relevance in the diagnoses of individual patients, because the development of detectable antibody levels takes days or even weeks[143]. A symptomatic episode may already be over once serological diagnosis is possible. Additionally, positive antibody results obtained during an acute episode may in fact be evidence of a previous infection and not be related to the current episode, and serial samples to assess seroconversion are rarely available.

There is notable inter-assay variability in antibody test performance and large variation between individuals in terms of antibody kinetics and the strength of

seroresponses[137,140,145,362]. Both of these issues further complicate the usability of antibody testing in most clinical scenarios.

While practical applications for the testing of individuals are limited, the opportunities to evaluate population-level exposure to SARS-CoV-2 with antibody assays are vast. Serosurvey data can provide vital information for monitoring the pandemic and assist in public health decision-making. They provide information on vaccine coverage and can aid in assessing the number of infections that have not been detected with RT-PCR and in individuals who have not undergone RT-PCR testing.

Late in 2021, Omicron became the primary variant in the Helsinki area[363]. During a study period of 16 weeks, we randomly selected one hundred samples per week, sent them for routine diagnostic purposes, namely, HIV screening, and analyzed the samples for SARS-CoV-2 antibodies against N and S1 antigens. The generalizability of these results is limited by the fact that while our random sample selection provides a relatively representative sample of the population of the geographical area in question, HIV screening is more often performed in young adults and working-age adults, introducing excessive kurtosis to the age distribution in our study. Therefore, our sample is less reflective of transmission in children and elderly individuals. Both these extremes of the age spectrum can have a significant role in the population-level dynamics of the pandemic.

We found that the seroprevalence of N antibodies rose quickly, especially in the population under 30 years of age. During the first week of the study period, 2/50 (4.0%) of this age group were N antibody-positive, whereas the proportion at the end of the study period was 10/28 (35.7%). The N antibody-positive group represents both those who had undergone the infection and those who had not been vaccinated as well as those who had undergone the infection and were vaccinated. The rate of N antibody positivity per week for different age groups is detailed in Table 6. The baseline seroprevalence of N antibodies, including all age groups on week 46, was 7%. At baseline, the lowest seroprevalence was observed in the youngest subgroup of study subjects under the age of 30.

At the same time point, the proportion of those who had been vaccinated but had not had the infection (S1 positive and N negative) in all age groups was 76%. This subgroup peaked on calendar week 49/2021 when the proportion was 92%. On week 9/2022, at the end of the study period, the proportion of this subgroup was 69%, likely indicating that those with vaccine-induced immunity also experienced SARS-CoV-2 infections during Omicron emergence.

During the study period, a new subgroup of N antibody-positive samples that were negative for the S1 antibody emerged and accounted for 1-4% of samples per week on calendar weeks 2-9/2022.

V: Figure 2 shows the evolution of different subgroups of serological results over the study period and shows the increasing trend in N- and S1-positive samples over calendar weeks 1-6/2022. Samples positive for the N antigen but negative for S1

first start appearing on calendar week 2 and peak on calendar week 9/2022. The proportions of both the N- and S1-negative (naïve: neither vaccinated nor infected) and N-negative, S1-positive (vaccinated population) populations diminished during the study period.

Table 6. Number (N pos) and proportion (% pos) of N antibody-positive samples for each week in different age groups (<30 years, 30-44 years, ≥45 years). The sharpest increase in N antibody positivity is observed in those under 30, but toward the end of the study period, the rise seems to halt.

	<30			30–44			≥45		
Calendar week	**N**	**N pos**	**% pos**	**N**	**N pos**	**% pos**	**N**	**N pos**	**% pos**
46/2021	50	2	4.0	37	4	10.8	13	1	7.7
47/2021	40	2	5.0	29	2	6.9	31	2	6.5
48/2021	36	2	5.6	31	2	6.5	33	2	6.1
49/2021	25	0	0.0	36	1	2.8	39	0	0.0
50/2021	34	0	0.0	24	0	0.0	42	1	2.4
51/2021	42	6	14.3	42	1	2.4	16	0	0.0
52/2021	30	1	3.3	46	3	6.5	24	0	0.0
1/2022	39	6	15.4	32	3	9.4	29	1	3.4
2/2022	46	9	19.6	39	4	10.3	15	2	13.3
3/2022	34	8	23.5	37	9	24.3	29	3	10.3
4/2022	51	12	23.5	31	7	22.6	18	4	22.2
5/2022	46	20	43.5	36	6	16.7	18	1	5.6
6/2022	41	20	48.8	41	12	29.3	18	2	11.1
7/2022	35	9	25.7	38	9	23.7	27	4	14.8
8/2022	39	14	35.9	40	13	32.5	21	3	14.3
9/2022	28	10	35.7	41	14	34.1	31	3	9.7
All weeks	616	121	19.6	580	90	15.5	404	29	7.2

The increase in N antibody seroprevalence among both the naïve and vaccinated populations reflects the rapid spread of the Omicron variant. Omicron has been shown to infect even those fully vaccinated and those with protection through previous infection readily [159,248,251].

While the group that was N antibody-positive and S1 antibody-negative is only a small proportion of those who have had a natural infection, they possibly represent the group with the most recent natural infection. It is also possible that Omicron infection results in delayed anti-S1 antibody production.

S1 seroconversion usually takes place 2-3 weeks after infection. Our data do not distinguish very recent and previous infections from one another. Additionally, natural infection in the naïve versus vaccinated population could not be distinguished. Furthermore, the data did not include serial samples from the same individuals to determine the timeframe during which the infection was

acquired. However, the rise in N antibody prevalence was so marked that it is very likely to reflect a very high infection rate in late 2021 and early 2022.

5.6. Strengths and limitations

The strength of all the studies discussed in this thesis is the wide availability of representative samples since a comprehensive sample archive from a large diagnostic laboratory was available as material for the studies. Additionally, the laboratory information system includes data on a large population, since the HUS Diagnostic Center serves the Greater Helsinki area, providing services to a population of 1.7 million. The HUS Diagnostic Center was among the first laboratories to establish SARS-CoV-2 diagnostics in Finland and to also perform large-scale evaluations of the methods [137,267,334]. The sample archives provided an excellent opportunity to assess the possible cross-reactivity of the assays, to characterize sensitivity with multiple different approaches and to study epidemiological parameters. Despite being a large laboratory in terms of sample volumes, HUSLAB provides most of its services only to a limited geographical area, which limits the applicability of our results to other countries and, in some cases, to other areas of Finland.

The limitations of the RT-PCR's clinical sensitivity study (I) include the inherent difficulty in creating a clinical grading system that is well balanced and comprehensive. This issue was especially challenging at the beginning of the pandemic, when the full spectrum of COVID-19 disease manifestations and transmission dynamics were only just beginning to unfold. Researchers collecting the clinical data were not blinded to the RT-PCR results, which may have introduced bias. Our approach did not address the issues surrounding persistent RT-PCR positivity connected to the high analytical sensitivity of RT-PCR, which has complicated the diagnosis of possible reinfections.

The evaluation of rapid antigen tests (II) was performed with frozen samples instead of fresh samples, which might limit its ability to reflect the test performance and could lead to underestimating sensitivity.

The limitations also include missing clinical details for some samples and patients, especially the symptom onset date for II and the symptom severity for III. Cross reactivity for seasonal coronaviruses could not be ruled out in II and III. This possibility will be an especially important issue to address, since the circulation of seasonal coronaviruses has increased and will increase further as societies open and restrictions are lifted. In many scenarios in the future, it will be important to differentiate SARS-CoV-2 from related coronaviruses.

Due to the retrospective and observational nature of the study of bacterial infections in hospitalized COVID-19 patients (IV), no systematic sampling was performed, which may have led to us missing some infections. Additionally,

clinically and radiologically diagnosed bacterial pneumonias were not included, which leads to an underestimation of pneumonia frequency, since respiratory tract cultures are not routinely collected. For findings in urine culture, no detailed evaluation of patient records was performed to determine the clinical significance of each finding, which limits the conclusions that can be drawn regarding the risk of urinary tract infection in hospitalized COVID-19 patients. The study period was during the first wave of the pandemic in early 2020, which limits the applicability of our results to the current situation, in which immunomodulatory treatments for COVID-19 have become part of routine treatment protocols and can affect patient susceptibility to nosocomial infections[364].

The serosurvey (V) was limited by the unavailability of respiratory tract specimens for the exact timing of the infections as well as a lack of clinical details regarding symptoms. Simultaneous upper respiratory tract samples could have been used to confirm the rapid spread of Omicron, possibly supplemented with genotyping of the virus as well. Additionally, neutralization testing of the samples would have provided interesting information. The vaccination status of the individuals with serological signs of infection was unfortunately unknown, as were other clinical details, such as comorbidities and symptomatic episodes at the time of the study and prior to the study, which limits our ability to draw conclusions about the effect of vaccine-induced immunity or natural immunity from previous infections.

6. CONCLUDING REMARKS

Our results on diagnostic methods of acute COVID-19 infection confirm some assumptions regarding the performance characteristics of RT-PCR and the rapid antigen testing of respiratory tract samples. First, it is clear that preanalytical factors alongside the performance characteristics of individual test protocols affect RT-PCR sensitivity and must be acknowledged in clinical decision-making. Our results raise further questions on how tests such as RT-PCR could be evaluated with more nuance when an absolute gold standard is not available.

Commercial antigen tests were found to have slightly varying sensitivity, which underlines the need for an independent evaluation of these tests to assure high quality and reliability of the results. The results of the performance evaluations of antigen tests should also inform recommendations on retesting and confirmatory RT-PCR testing.

We also assessed a new method that, based on our results, could prove useful for the diagnosis of acute infection alongside RT-PCR and respiratory tract sample antigen testing: ELISA to detect the presence of the N antigen in serum samples. More detailed evaluations are needed to determine the indications for serum antigen testing and to identify the patient populations for whom it is most useful and practical. The possibility of a correlation between antigenemia and progression to severe disease should be explored. The clinical importance of these findings could provide much-needed help in patient management. Other areas of interest are antigenemia's possible role in the pathophysiology of the disease.

The low frequency of severe bacterial infections among hospitalized COVID-19 patients warrants further research to determine the underlying mechanisms that make COVID-19 patients seemingly less susceptible to bacterial infections than, for instance, influenza patients[183,184,365]. The immunomodulatory treatments in current use should be evaluated for their potential to increase the risk of bacterial complications.

Our serosurvey results underline the need to be able to react promptly when new outbreaks and new variants of concern emerge since even in populations with high vaccine coverage, the spread can be exceedingly swift. It is possible that in future instances, a variant could cause substantially more severe disease in addition to being highly infective[366,367].

SARS-CoV-2 remains a permanent companion of humankind, at least for the foreseeable future. Epidemiological vigilance and continuous re-evaluation of the performance of diagnostic assays is necessary to detect anomalies and to provide high-quality diagnostic services to benefit patient care.

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