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ABSTRACT

Various diagnostic methods are used in infection diagnostics. The key parameters for these tests are specificity and sensitivity, as well as usability and time to results. The general trend in infection testing has been towards tests based on gene amplification, such as polymerase chain reaction (PCR). The testing is often central laboratory-based, where obtaining the results can take several hours or days after collecting the sample. In managing acute infections, such as influenza, the time to results is critical for proper medication and isolation. Gene amplification methods can be analytically highly specific and sensitive, but may not indicate the current state of the disease. Methods multiplying genetic material can react to past infections or contamination, complicating or misleading the diagnosis. During the COVID-19 pandemic, utilization of rapid antigen tests experienced a renaissance. These tests are based on the ability of microbe-specific antibodies to bind to structural parts of microbes. The detected parts are produced only in the acute phase of the disease when the microbe is replicating. During the pandemic, these rapid on-site tests enabled quick diagnosis, treatment, and early isolation measures.

This thesis studied the applicability of antibody-based two-photon excitation fluorometry (separation-free mariPOC technology) for the detection of microbial antigens from feces and urine, and the utility of rapid antigen detection as part of diagnostic process. In **Study I**, sample pretreatment methods and antigen tests were developed for the mariPOC platform. Feces and urine showed elevated fluorescence levels. However, the methodology has a unique property to compensate matrix effects enabling accurate results. Special features of these matrices were taken into account in the immunoassay, instrumentation, and data algorithm designs. As a result, feces and urine proved well suited for the separation-free method.

In **Studies II** and **III**, *Clostridioides difficile* and SARS-CoV-2 antigen tests were developed, respectively, and the performance and usability were evaluated in comparison to routine practices. According to the results, the sensitivity of the tests met their intended purpose, and the specificity was state-of-the-art. It is concluded that microbial antigens are clinically accurate markers of ongoing infection and hence suited for the frontline management of infectious diseases. Antigen tests would be useful in the control of the spread of infectious diseases during epidemics and/or pandemics.

KEYWORDS: Infectious disease, acute infection, antigen detection, stool

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TIIVISTELMÄ

Infektiodiagnostiikassa käytetään erilaisia testejä, joiden keskeisiä suorituskyky-parametreja ovat spesifisyys ja herkkyys sekä käytettävyys ja aika näytteenotosta tuloksen raportointiin. Infektiodiagnostiikassa on ollut trendi siirtyä geenimonistus-menetelmiin, kuten PCR, koska ne ovat analyyttisesti herkkiä. Nämä menetelmät eivät kuitenkaan aina kerro taudin senhetkisestä tilasta, koska mikrobien geneettistä materiaalia voi olla elimistössä viikkoja infektion jälkeen tai mikrobi ei aiheuta tautia. Geenimonistustestit voivat siten monimutkaistaa diagnosoimista. Vaikka testit itsessään voivat olla nopeita ja soveltua vieritestaukseen, testit suoritetaan usein keskuslaboratoriossa. Tällöin tuloksen raportointiin näytteenotosta voi kestää päiviä. Akuuteissa infektioissa, kuten influenssassa, tuloksen raportointinopeus näytteenotosta on kriittinen hoidon ja eristystoimien aloittamisen kannalta. COVID-19-pandemian myötä antigeenipikatestit kokivat kunnianpalautuksen. Nämä testit perustuvat spesifisten vasta-aineiden kykyyn sitoa näytteessä olevien mikrobiantigeenejä eli mikrobien osia ja kykyyn tuottaa luettava signaali nopeasti. Pikatestit mahdollistavat nopean diagnoosin, mikä mahdollistaa lääkehoidon aloittamisen ja toimet leviämisen ehkäisemiseksi taudin alkuvaiheessa.

Tässä väitöskirjassa tutkittiin vasta-aineisiin perustuvan pesuvapaan, kaksoisfotonivirittеisen fluoresenssimääritystekniikan soveltuvuutta mikrobien osoittamiseen ulosteesta ja virtsasta sekä nopean antigeeniosoituksen merkitystä osana diagnoosiprosessia. Osatyössä I kehitettiin mariPOC-mitta-alustaan uloste- ja virtsanäytteiden esikäsittelymenetelmiä ja antigeenitestejä. Näytteiden todettiin nostavan fluoresenssisignaalitasoja mittauksessa näytekohtaisesti. Mittatekniikalla pystyttiin kompensoimaan näytematriisien vaikutukset fluoresenssiin. Tulosten mukaan uloste ja virtsa soveltuivat näytemateriaaliksi erotusvapaassa kaksois-fotonivirittеisessä fluorometriassa. Osatöissä II ja III arvioitiin kehitettyjen *Clostridioides difficile*- ja SARS-CoV-2-testien suorituskykyä ja käytettävyyttä verrattuna muihin testeihin ja testauskäytänteisiin. Tulosten mukaan testien herkkyys vastasi käyttötarkoitustaan ja spesifisyys lähestyi 100%:a. Tulosten mukaan mikrobiantigeenit ovat hyviä merkkiaineita akuutin infektion tunnista-miseen ja niiden käyttöä tulisi lisätä akuutin (ensivaiheen) taudin tunnistamisessa. Tämä tehostaisi sairauksien diagnosointia ja hoitoa sekä edesauttaisi tartuntatautien kontrolloinnissa epidemia- ja pandemiatilanteissa.

AVAINSANAT: Infektiotauti, akuutti infektio, antigeeniosoitus, uloste

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Abbreviations

CDI	*Clostridioides difficile* infection
CE	Conformité européenne (European Conformity)
CFU	Colony-forming unit
CI	Confidence interval
COVID-19	Coronavirus disease 2019
CRP	C-reactive protein
Ct	Cycle threshold
DFA	Direct fluorescence assay
DNA	Deoxyribonucleic acid
EDAC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
ELISA	Enzyme-linked immunosorbent assay
EN	European norm
ESCMID	European Society of Clinical Microbiology and Infectious Diseases
EU	European Union
FDA	Food and Drug Administration, United States
FFA	Focus forming assay
g	G-force
G	Genogroup (norovirus); Serotype (rotavirus)
GII.4	Genogroup II, genotype 4, norovirus
GDH	Glutamate dehydrogenase
HNL	Human neutrophil lipocalin
IgG	Immunoglobulin G
IgM	Immunoglobulin M
ISO	International Organization for Standardization
IVD	In vitro diagnostics
LoD	Limit of detection
MEIA	Membrane enzyme immunoassay
mRNA	Messenger ribonucleic acid
MxA	Myxovirus resistance protein A
N	Nucleocapsid, viral protein

NAAT	Nucleic acid amplification test
NPV	Negative predictive value
OD	Optical density
PCR	Polymerase chain reaction
PCT	Procalcitonin
PFU	Plaque-forming units
POC	Point of care
PPV	Positive predictive value
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals (EU regulation)
RNA	Ribonucleic acid
RTI	Respiratory tract infection
RT-qPCR	Real-time quantitative polymerase chain reaction
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
$TCID_{50}$	50% tissue culture infectious dose
TEM	Transmission electron microscopy
TPX	Two-photon excitation
WHO	World Health Organization

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I Koskinen Juha M., Soukka Jori, Meltola Niko, Koskinen Janne O.. Microbial identification from feces and urine in one step by two-photon excitation assay technique. *J Immunol Methods*, 2018; 460: 113-118. https://doi.org/10.1016/j.jim.2018.06.017.

II Savolainen Roosa*, Koskinen Juha M.*, Koskinen Janne O., Kaukoranta Suvi-Sirkku. Prospective evaluation of the mariPOC test for the detection of *Clostridioides difficile* GDH and toxins A/B. *J Clin Microbiol*, 2020; 58(4): e01872-19.
https://doi.org/10.1128/jcm.01872-19 (*Equal contribution).

III Koskinen Juha M.*, Antikainen Petri*, Hotakainen Kristina, Haveri Anu, Ikonen Niina, Savolainen-Kopra Carita, Sundström Kati, Koskinen Janne O.. Clinical validation of automated and rapid mariPOC SARS-CoV-2 antigen test. *Sci Rep*, 2021; 11: 20363.
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(*Equal contribution).

1 Introduction

Acute infections are a constant threat to our well-being. The most common acute infections are flu-like illness and gastroenteritis caused either by viruses or bacteria, such as influenza, SARS-CoV-2, norovirus or *Campylobacter*. Our living environment, daily habits and social contacts define whether we confront these acute infections from zero to several times a year. The severity of the symptoms varies from asymptomatic to life-threatening conditions. Respiratory tract infections (RTIs) and diarrheal diseases cause morbidity and are among the top ten causes of mortality worldwide, especially in countries with low levels of healthcare. According to the World Health Organization (WHO), these diseases cause three million annual deaths globally (WHO, 2020a; GBD 2019 Diseases and Injuries Collaborators, 2020).

While many microbial infections resolve without medication, some need medication and/or hospitalization. In some cases, such as urinary tract infection and acute otitis media, antibiotic medication is used in clinical practice without prior microbial specific diagnostics. This is justified as the causative agent in urinary tract infections, almost without exception, is bacteria (Laupland et al., 2007), and in otitis, the causative agent is often bacteria (Ruohola et al., 2006; Schilder et al., 2017). RTIs are also commonly medicated empirically with antibiotics without prior microbial-specific diagnostics, although the root cause of infection is often a virus (Koskinen, 2008; Meyers et al., 2018) against which antibiotics have no effect. The misuse of antimicrobials has been recognized as contributing to antimicrobial resistance (Holmes et al., 2016), which has increased intensively among bacteria in recent decades and is recognized as one of the top ten global public health threats by the WHO (WHO, 2019). In addition, suboptimal rapid diagnostics may be a driver for antimicrobial resistance through inappropriate antibiotic use (Holmes et al., 2016). This calls for optimal rapid diagnostic measures for optimal antimicrobial use and in the control of antimicrobial resistance.

During the past decades, diagnostic demand and development have focused on analytical sensitivity and specificity of test methods (Brendish et al., 2015). This has been accomplished through the introduction of nucleic acid amplification tests

(NAATs), more specifically, usually polymerase chain reaction (PCR) (Mullis & Faloona, 1987**)**. Simultaneously, point of care (POC) and rapid acute infectious disease diagnostics have been a hot topic in scientific studies and discussions. Indeed, diagnostic tests have evolved towards POC and rapidity. Many tests provide results within a few hours or less, even in minutes (Brendish et al., 2015). However, in practice, the trend in how diagnostics is utilized has been the opposite in general. Diagnostics has become more and more centralized into vast central laboratories in pursuit of optimizing unit economy and controlling quality. Focusing on these targets has ignored the importance and benefits of rapidity in obtaining test results and the clinical specificity of test methods. While antigen testing, e.g., lateral flow assay, is commonly considered insensitive compared to PCR tests (Brendish et al., 2015), the specificity of the modern antigen tests reaches the level of PCR (Dinnes et al., 2022; Hayden et al., 2024). The dogma that antigen testing in general is insensitive is based on misconception that antigen and PCR tests should correlate 100% in sensitivity. There is a reason for the difference in apparent sensitivity between PCR and antigen detection (Mina et al., 2020; Mina et al., 2021; Mina & Andersen, 2021). The reason is explained and discussed in this thesis.

The aim of this thesis was to develop and evaluate diagnostic tools to support clinicians in their efforts to manage and control acute infections. The significance of detecting microbial antigens during acute phase infection as part of diagnostic and decision-making processes was studied with an automated multianalyte antigen detection test. The thesis scope is in upper RTIs and gastrointestinal infections, and on developing methodology for their diagnostic testing. Owing to the COVID-19 pandemic, research related to coronaviruses and diagnostic methods burst, and new research approaches were taken for RTI diagnostics to assess the clinical significance of the diagnostic test result. Thus, SARS-CoV-2 diagnostics is used here as the main model for discussing the research evidence on the clinical significance of different methods and markers for acute infection. In **Study I**, the suitability of urine and stool as sample matrices in the ArcDia two-photon excitation (TPX) assay technique was studied and developed. **Study II** and **Study III** focused on the development and evaluation of novel diagnostic methods based on the mariPOC platform (ArcDia TPX assay technique), as well as the clinical accuracy and usability of antigen detection. The knowledge gathered here will be useful to develop practices in microbial diagnostics and infectious disease management. In large parts, the knowledge is not limited to the assay technology applied here.

2 Review of the Literature

2.1 Importance of acute infections and rapid diagnostics

Infectious diseases have a significant impact on our lives throughout our lifetime. The most common infectious diseases are respiratory tract and gastrointestinal infections. Lower respiratory infection was the 2nd (0.4 million) and 6th (0.5 million) cause of mortality in low-income and high-income countries in 2019, respectively. Diarrheal diseases were the 5th (0.3 million) cause of mortality in low-income countries, while being much less frequent in high-income countries. When combined, these are the leading causes of mortality in low-income countries. Despite the fact that countries with undeveloped healthcare suffer the most; mortality due to these diseases has greatly declined during the last decades (WHO, 2020a; GBD 2019 Diseases and Injuries Collaborators, 2020). In 2021, during the COVID-19 pandemic, lower respiratory infection was the 5th (2.2 million) leading cause of mortality, while COVID-19 was the 2nd (8.8 million) (WHO, 2024). Nevertheless, besides mortality and morbidity, these diseases still cause remarkable absence from school or work, affecting productivity and well-being. Well-being may be heavily affected by complicated medical conditions, such as pneumonia and colitis.

In 2019, the WHO announced the top ten threats to global health, all of which were directly or indirectly related to infectious diseases (WHO, 2019). According to the WHO, influenza or other emerging pathogens with pandemic potential pose a direct threat to our health, while some threats may increase vulnerability to infectious diseases. Emerging antimicrobial resistance is one of the most important concerns. One of the threats was realized soon after when the COVID-19 pandemic started in early 2020 (WHO, 2020b), causing shock reactions. Shock reactions and fear of the novel virus were expected (Ropeik, 2004). As a consequence of shock reactions, the WHO has recognized threat management policies ever since (WHO, 2021).

Rapid diagnostics providing clinically relevant results is essential in identifying a pathogen causing acute infection symptoms in the early phase of an infection. By identifying the pathogen, appropriate medication and isolation measures can be

implemented on time. Promptly initiated medication may reduce further complications, such as pneumonia or colitis, and expensive nursing (Muthuri et al., Brendish et al., 2015; WHO, 2025). The use of rapid diagnostics also reduces the unnecessary use of antibiotics (Brendish et al., 2015; Holmes et al., 2016). Although clinical decisions are heavily guided by diagnostics, only a few percent of healthcare budgets are currently dedicated to diagnostics (WHO, 2025), and the majority of outpatients are diagnosed and treated empirically without microbial-specific diagnostics.

2.2 Acute infection

2.2.1 Clinical course and diagnostics

Bacterial and viral infections begin with the exposure of a person's host cells to infectious material. Successful multiplication or replication often leads to an immune response, which starts to limit the infection. Even in the presence of past immunity or vaccine introduced immunity, the immune response may not be able to restrain the infection completely. Thus, the immune system may not be able to prevent the transmission of the pathogen from person to person. Depending on the nature of the pathogen and the individual, the infection may target different cells and tissues or organs. Tissue tropism has implications for choosing the optimal location for sampling to obtain a diagnostic specimen. While pathogens are usually eventually cleared by the immune system, antimicrobials (antibiotics and/or antivirals) are often required or at least beneficial to limit disease severity.

The time from infection onset to pathogen detection may have a significant impact on the treatment efficacy and disease outcome. The earlier the medication is initiated, the better the efficacy in limiting the severity of the disease. Therefore, rapid, sensitive, and specific diagnostics from samples collected from the appropriate site and at the appropriate time are important for early pathogen detection (Heinonen et al., 2010; Zasowski et al., 2020; Mattila et al., 2021; Hammond et al., 2022). The acute phase of the infection, when the pathogen is replicating and the individual is contagious, is followed by the convalescent phase. The individual may still have symptoms due to inflammatory responses, but is no longer contagious to others (Carrat et al., 2008; Atmar et al., 2008; Killingley et al., 2022).

In clinical practice, symptoms of acute infection are the main reason to perform diagnostics as part of the diagnosis. However, in the case of epidemic management, frequent testing of asymptomatic individuals can be preferred. In such testing, the absolute analytical sensitivity of acute RTI testing is secondary to frequency (how often) and turnaround time of testing for detecting infectious individuals and

containing the spread of a viral disease transmitted through human-to-human route (Larremore et al., 2021). An individual is most contagious when the viral load is high during the onset of an acute infection. Figure 1 (adapted from Mina et al., 2020) explains how viral load and positivity of different test methods develop over the course of an infection. Understanding this helps to choose the optimal diagnostic methods based on the infection phase and the testing indication.

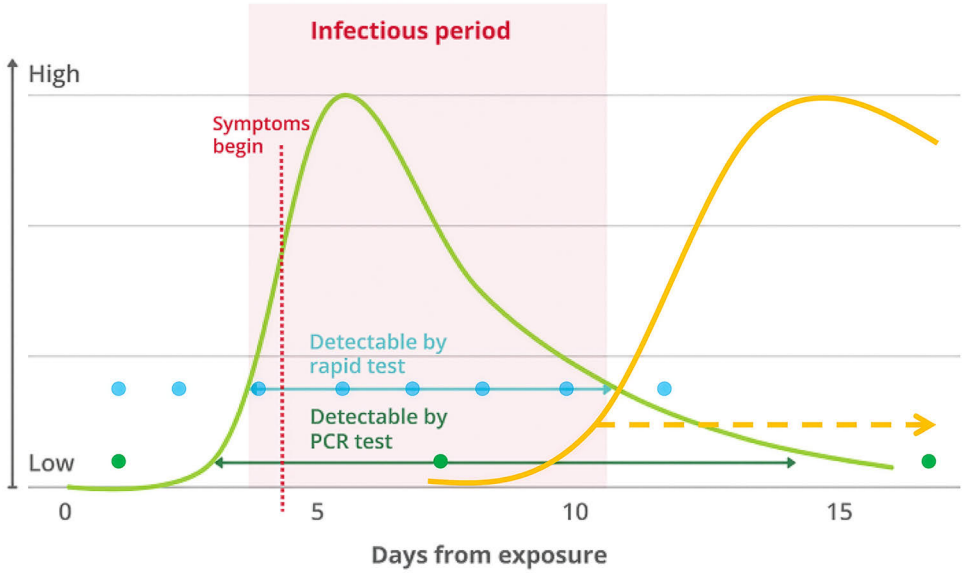

Figure 1. Schematic figure describing the course of an acute viral infection and the principles for serial testing and the choice of optimal diagnostic method. The Y-axis is to schematize the development of viral load (curved light green line) and virus-specific IgG level (curved orange line) since exposure to an infectious material. Blue and dark green lines show the most probable time windows for positive test results for antigen and PCR tests, respectively. Dots represent testing time points in serial testing. The infectious period is the time window when viable viruses can be detected by viral culture (pale pink background color). Virus-specific IgG becomes detectable when the infectious period begins to end (dashed orange line). Adapted from Atmar et al., 2008; Mina et al., 2020; Basile et al., 2021; Larremore et al., 2021; Colazo Salbetti et al., 2023; Soni et al., 2023; Mina et al., 2021; Mina & Andersen, 2021.

When the viral load is high, infected individuals can be detected even with tests that have relatively low analytical sensitivity, where the positive result is indicated by a signal visible to the naked eye. As the viral load increases rapidly already in the asymptomatic phase, frequent testing with rapid antigen tests increases the likelihood of detecting cases compared to less frequently testing with highly sensitive NAATs. This means that so-called serial testing, i.e., screening of individuals to identify and isolate contagious persons, is more effective when using

a less sensitive test with a shorter testing interval than using a highly sensitive test with a longer interval and/or longer result time (Figure 1). The serial testing model by Larremore et al. (2021) was verified in a real-life setting by Soni et al. (2023). When infectious individuals were detected as early as possible, the probability of transmitting the disease further decreased when prompt prevention acts were implemented.

2.2.2 Symptoms

Acute respiratory tract and gastrointestinal infection symptoms vary from asymptomatic to severe or even lethal complications (Carrat et al., 2008; Atmar et al., 2008; Killingley et al., 2022; GBD 2019 Diseases and Injuries Collaborators, 2020). Typical signs for RTIs include sneezing, runny nose, cough, sore throat, or discomfort in the sinuses, while gastroenteritis is distinguished as having diarrhea and/or vomiting. Both RTIs and gastrointestinal infections may also cause similar symptoms, such as fever, fatigue, muscle pain, headache, chills, or loss of appetite. Therefore, initial symptoms may not be indicative of the infection focus. Most commonly, an acute infection is noticed when rapidly evolving symptoms appear within a few days of being infected. Symptoms of self-limiting illness of viral infection usually resolve within a few days or up to two weeks. In RTI, couch, fatigue, and lung issues may persist for weeks (Heikkinen & Järvinen, 2003; Carrat et al., 2008; Atmar et al., 2008; Killingley et al., 2022). Full recovery from infection may also take weeks, especially in terms of physical stamina and chronic fatigue syndrome, such as in long COVID (Davis et al., 2023). The causative agent is mostly impossible to identify solely based on symptoms (a general inflammation marker) and/or by relying on the epidemiological situation. This is because multiple pathogens causing similar symptoms may circulate at the same time. Sometimes it is even difficult to distinguish respiratory tract infection from gastrointestinal infection or allergy, or a bacterial infection from a viral infection (Heikkinen & Järvinen, 2003; Dykewicz et al., 2020). Although most of the cases are asymptomatic or with mild symptoms, these diseases do cause indisposition, significant hospitalization, and mortality worldwide. Advanced age is a major risk factor for severe acute infectious disease (Verity et al., 2020).

2.2.3 Viral shedding

In order to choose the right diagnostic method for acute infection for the right purpose, it is important to understand when viral replication starts, how and when possible antimicrobial treatment works, how viruses spread, and what is the time window when an individual may spread the virus. This is important in order to

assess the significance of a diagnostic test result in terms of diagnosis and prevention of viral spread. The incubation period of an acute infection can be considered as the time from contact with infectious material to the emergence of a detectable microbial load or to the onset of symptoms. The contagious phase is the time during when an individual sheds viable microbes with the potential to spread infectious material and infect other individuals (Kirby et al., 2023). Experimental studies where methods to detect viable viruses were utilized, and viral incubation time and contagious phase were well established based on study design with known inoculation or transmission moment, are summarized in Table 1 for influenza and SARS-CoV-2. These experimental studies demonstrated that the incubation period for detectable viral replication and symptom onset is less than 5 days and most often only 1 to 2 days. Observational and modeling studies based on contact tracing suggest similar or, more often, longer incubation times from several days to even weeks (Lauer et al., 2020; Zhu et al., 2021; Wu et al., 2022). Viral replication and the infectious period last less than two weeks in immunocompetent individuals. Surprisingly, viral dynamics for most immunocompromised individuals seem to be similar to those of immunocompetent individuals, although prolonged viral shedding may occur among immunocompromised individuals (Berengua et al., 2022; Utzon et al., 2023). Viral shedding period in the challenge studies in Table 1 was similar to studies describing naturally acquired infections (Wölfel et al., 2020; Singanayagam et al., 2020).

Table 1. Incubation period from day 0 until virus detection.

Pathogen	Host species	Method	Specimen	Incubation period (days)	Infectious phase until (days)	Ref
SARS-CoV-2	Human	Culture (FFA)	Nose (MT) & Throat	≤5 median 2.5	≤10	Killingley 2022
	Hamster	Culture	Nasal wash	≤1-2	2-5	Sia 2020
Influenza	Human	Culture	Nasal / nasopharynx	≤4 average 2	7-10	Carrat 2008
	Ferret	Culture	Nasal wash / OPS	≤3	4¤ - 7	Inagaki 2016
	Ferret	Plaque assay	Nasal wash	≤1-2	≤6	Roberts 2012

¤ Time when other ferrets were infected.
FFA = Focus formation assay
OPS = oropharyngeal swab
MT = mid-turbinate.

The review by Carrat et al. (2008) compiles the incubation times for influenza from human challenge studies starting from inoculation day 0:

- Viral shedding increased sharply between half a day and a day after challenge and consistently peaked on day 2, ranging from 1 to 4 days.
- The duration of viral shedding was 4.8 days on average and did not go beyond day 10.
- The total symptom scores increased on day 1 and peaked on day 3. Systemic symptoms peaked on day 2. Highest symptom scores were from 2 to 6 days after inoculation.
- Viral shedding preceded illness by 1 day.
- The frequency of symptomatic infection was 70%.

Different specimen sampling locations for obtaining mucosal excreta, e.g., nasal, nasopharyngeal, and throat, might affect the viral shedding times reported in the literature. Nevertheless, when assessing the first day of viral shedding, all other specimen types other than the one that is optimal for the disease only underestimate the start of viral shedding. In contrast, when studying an imperfect specimen type, the contagious phase could actually be longer. In the SARS-CoV-2 human challenge study by Killingley et al. (2022) viral load was 1 to 2 logs lower in the throat than in the nose as assessed by real-time quantitative PCR (RT-qPCR) and focus formation assay (FFA). Also, Kleiboeker et al. (2020) reported a median viral ribonucleic acid (RNA) concentration 20 times higher in the nasopharynx than in the oropharynx. Maximum viral RNA loads were 900 times higher in the nasopharynx than in the oropharynx. Oropharyngeal swab specimens had 1 to 2 logs lower median load of viral RNA than any specimen type from the nose (nasal or nasopharyngeal swab or aspirate). Using RT-qPCR as the detection method, oropharyngeal swab sampling has been reported to have a detection rate sensitivity of 21 to 27% when compared with nasopharyngeal sampling (Wang H. et al., 2020; Wang X. et al., 2020). The sensitivity of oral, anterior nasal, and nasopharyngeal sampling for an antigen test was 18%, 63% and 73%, respectively, in comparison to RT-qPCR with a cycle threshold (Ct) cut-off of 35 (Wölfl-Duchek et al., 2022). Although SARS-CoV-2 has been shown to infect the throat and salivary glands (Huang et al., 2021), the presence of the viral material in the throat and saliva is maybe rather due to the secretion of nasopharyngeal excreta into the throat. In addition, viral shedding might differ between humans and animals.

The results obtained in humans by Carrat et al. (2008) are well in line with the animal model studies of ferrets. In the study by Inagaki et al. (2016), ferrets shed viruses at day 3 or earlier from inoculation. Although the ferrets shed a low amount of viable viruses until day 7, the infection was transferred, and other ferrets were

infected only until day 4 during the peak of viral shedding. The ferrets were able to infect other ferrets through direct contact only when antigens were detectable. The ferrets did not infect other ferrets after antigens were undetectable, although viable viruses were present based on viral culture. Viral RNA was still detectable for days, even though viral culture was negative and ferrets were incapable of infecting other individuals. Inagaki et al. (2016) concluded that "the antigen-detection test estimated the infectious period with comparable, if not better, accuracy than culture" and that "PCR...is not an appropriate method for indicating infectivity". Roberts et al. (2012) had more frequent sampling for ferrets than Inagaki et al. (2016), showing that incubation time for viral shedding and peaking was 1 to 2 days or possibly even less, while viral shedding lasted at most 6 days. Transmission of viral infection did not happen between co-housed ferrets between 16 and 20 hours after inoculation, but transmission occurred between 24 and 28 hours. Viral load of the transmission-infected ferrets peaked at day 3. They also noticed that transmission of the influenza virus occurred already before fever. For naturally acquired seasonal and pandemic influenza virus infections, viable viruses have been reported to be detectable and peak earliest 1 to 2 days before onset of symptoms (Ip et al., 2016). Seemingly, antigens are detectable already in the early phase of an infection when viral transmission may occur, and the antigen-test positivity does not necessitate symptoms.

In the SARS-CoV-2 human challenge study by Killingley et al. (2022), the median time to detect viable virus in viral culture was 2.5 days, and at the latest on day 5 after inoculation. Viable virus was detectable in 50% and 90% of the infections on days 2 and 4, respectively. Only 53% (18/34) of inoculated volunteers, without evidence for previous infection or vaccination, had PCR and culture (FFA) confirmed infection. This suggests that half of the test subjects were not actually infected, although wild-type SARS-CoV-2 was intranasally pipetted at a dose of 10 defined by the 50% tissue culture infectious dose ($TCID_{50}$) method. Even with RT-qPCR, viral RNA was not detectable one day after inoculation in most cases that eventually became positive by viral culture, but viral RNA and antigens were often detected at day 2. The short incubation time of one to four days for viral shedding is explained by a cell model study where extensive coronavirus RNA transcription occurred already in 6 to 8 hours after infecting the cells (Hofmann et al., 1990). This suggests that the difference in the time windows when viral RNA and antigens are first detected after being infected is, in practice, negligible (Figure 1). However, the detection of exposure to viral RNA may distort the conclusions on viral shedding or clinical diagnosis. Such exposure may be due to environmental contamination, for example, through close contact with an infectious individual (Colaneri et al., 2020; Santarpia et al., 2020; Oksanen et al., 2022; Tan et al., 2023) or being in close proximity to the administration of an

intranasal live attenuated vaccine (Curran et al., 2012). Furthermore, an RT-qPCR study suggests similar SARS-CoV-2 load in both children and adults (Polese-Bonatto et al., 2021).

Similar incubation time for SARS-CoV-2 was reported by Sia et al. (2020) from a challenge study utilizing golden hamsters. Viable SARS-CoV-2 was detectable after 1 to 2 days following inoculation or direct contact with other hamsters. Viral load peaked between the 2nd and the 5th day. Viral transmission between the hamsters occurred only when viable virus was isolated in viral culture from the donors. Also, a study utilizing NAAT detection solely has shown SARS-CoV-2 RNA load peaking after 2 days from inoculation of ferrets (Ciurkiewicz et al., 2022).

Carrat et al. 2008 concluded that viable influenza is excreted mostly for 5 days and up to 9 days from the onset of the symptoms. For SARS-CoV-2, around 50% of infected individuals were positive in viral culture until day 7 (Singanayagam et al., 2020; Keske et al., 2023), and up to day 14 has been reported in non-severe cases (Keske et al., 2023). An infectious period of such length is likely common for viruses causing acute respiratory tract infections or gastroenteritis, as the declining viral shedding reflects the successful response of the immune system.

From the diagnostic point of view, viral shedding and the course of infection of viral gastroenteritis are somewhat similar to RTIs, as shown by Atmar et al. (2008) in a study where adult volunteers were infected with norovirus. Two-thirds of the individuals developed viral gastroenteritis, with symptoms appearing within one to four days after inoculation. One-third developed diarrhea and vomiting, while another third had only vomiting. Others had no symptoms of gastroenteritis, being asymptomatic despite being shown to be infected. Antigens were detectable as early as the second day and as late as the tenth day from inoculation. Interestingly, norovirus was replicating in all inoculated individuals, and all had high antigen load in their stool samples regardless of whether they were symptomatic or asymptomatic. RT-qPCR was positive as early as 18 hours after inoculation. The median RT-qPCR-positivity time was 28 days, while lasting up to 56 days from the inoculation. The positivity of the tests was almost the same regardless of the symptoms. For the most part, stool was solid when the viral concentration was the highest between days two and five, suggesting the form of the stool sample is irrelevant for diagnostics. A norovirus culture study showed that even stool samples with high genomic count obtained by RT-qPCR are not necessarily infectious (Costantini et al., 2018).

Viral shedding was discussed above, mostly based on sampling individuals. In order to estimate the transmission route between individuals, the infectious microbe should also be detected in the material shed by an infectious individual before it reaches a recipient. The animal model studies showed that individuals

were able to infect others through direct contact and airborne droplets (Roberts et al., 2012; Inagaki et al., 2016; Sia et al., 2020). A lot of concern has been focused on viral shedding and transmission through aerosols. Whereas indirect evidence, such as artificially generated aerosols, detection of genomic material, and computational modeling suggest transmission through aerosols (Vuorinen et al., 2020; Salmenjoki et al., 2021; Oksanen et al., 2022a; Oksanen et al., 2022b), experimental direct evidence with infectivity studies argues against transmission through aerosols, or at least the probability for aerosol-transmission is negligibly low (Dudley, 1924; Brankston et al., 2007; Herfst et al., 2012; Colaneri et al., 2020; Santarpia et al., 2020; van Doremalen et al., 2020; Kim et al., 2020; Chu et al., 2021; Comber et al., 2021; Kutter et al., 2021; Port et al., 2021; Sharma et al., 2022; Oksanen et al., 2022a; Tan et al., 2023).

2.3 Markers of acute infection

Microbial infection is often recognized based on symptoms, which may overlap between different pathogens (Heikkinen & Järvinen, 2003; Dykewicz et al., 2020). In the case of chronic disease pathogens, such as HIV and hepatitis, the disease may go undetected for a long time as the initial symptoms are not evident or specific. Microbe-specific diagnostics give the cause for the disease, while cellular markers may provide information about the level of inflammation that the microbial invasion is causing, and possibly hints at whether the microbe is bacterial or viral (Pfäfflin & Schleicher, 2008; Reinhart et al, 2012). Microbial culture is a classical method to detect the infection causing agent (Costantini et al., 2018; Basile et al., 2021), but it might be labor intensive, having a long turnaround time. To simplify microbial detection, the present, recent, or past presence of microbial infection can be recognized with methods that detect directly parts of the microbes or indirectly adaptive immune responses. Serological diagnostics help to detect infections otherwise difficult to detect, such as Epstein–Barr virus infection, and provide information about the infection phase, e.g., primary or secondary infection (Hedman & Rousseau, 1989; Koskinen et al., 2006).

Detection of bystander microbes or traces, e.g., genetic material, of past and cleared infection may mislead the diagnosis of acute infection, leading to overdiagnosis (Atmar et al., 2008; Polage et al., 2015; Cevik et al., 2020; Mina et al., 2021; Killingley et al., 2022). Possibly, it may lead to another disease-causing agent to go undetected by further tests (Krutova et al., 2019). Similarly, a test with poor detection capability may miss cases. Thus, there is a need for suitable diagnostic markers of acute phase infections that help physicians in their clinical decision-making for diagnosis and optimal medication.

2.3.1 Inflammation markers

Microbial infection induces inflammation and immune responses, which can be used in diagnosis. Several inflammation markers, such as C-reactive protein (CRP), procalcitonin (PCT), and cytokines, have been used as markers for infection, even if their induction may not be specific for a single pathogen. These inflammation markers are used to estimate the intensity of inflammation and somewhat the nature of the disease-causing microbe (bacterial versus viral). Analysis of leukocyte count is a well-settled marker of inflammation that is not specific for infection. Although leukocyte count most often increases during inflammation normal or reduced leukocyte count does not always rule out severe inflammation (Pfäfflin & Schleicher, 2008; Reinhart et al, 2012).

CRP is perhaps the most utilized and clinically the most important acute-phase biomarker for inflammation and infection. The intense increase in CRP concentration in the blood circulation suggests harsh inflammation. Following the initiation of a triggering event, the CRP level may rise heavily from a normal level within hours. CRP is considered to be very sensitive for the detection of inflammation activity but lacks specificity for any certain disease, infection, or condition (Pfäfflin & Schleicher, 2008; Reinhart et al, 2012; Sproston & Ashworth, 2018; Aulin et al., 2021). As the CRP level may increase rapidly, recent studies suggest that CRP velocity could differentiate between bacterial and viral infection better than CRP level alone. It can be thought that the CRP response would be more intense in the case of bacterial infection. CRP velocity is defined as the admission CRP level divided by the time from symptom onset (Paran et al., 2009; Bernstein et al., 2021; Largman-Chalamish et al, 2022). Although CRP may correlate better with bacterial than viral infection, it does not necessarily predict it at the individual patient level.

Similarly to CRP, PCT level may increase harshly during inflammations related to many different conditions. PCT is a sensitive marker for inflammation and is thought to be somewhat more specific for bacterial infections than CRP, although that is case dependent (Pfäfflin & Schleicher, 2008; Reinhart et al, 2012; Aulin et al., 2021). A systematic review from 2007 suggests that PCT is not a reliable marker to differentiate sepsis from non-infectious causes of systemic inflammatory response syndrome in critically ill patients (Tang et al., 2007). In contrast to using only one biomarker, the utilization of multiple biomarker combinations and algorithms may differentiate between viral and bacterial infections or even between bacterial species. Additionally, these biomarkers can guide management of the use of antibiotics (Houten et al, 2017; Neeser et al., 2019; Aulin et al., 2021).

Maybe the most promising marker to differentiate between viral and bacterial infection by ruling in viral infection is the myxovirus resistance protein A (MxA).

The expression of MxA was shown in inflammatory dermatoses with and without known viral etiology, such as papillomavirus and psoriasis, respectively, but not with bacterial origin (Fäh et al., 1995). The role of certain viruses in psoriasis has been described (Zhou & Yao, 2022), while an unknown viral etiology might be present. This means that the MxA might be increased due to undetected viruses, and the causality with psoriasis is uncertain.

MxA expression is significantly increased in viral respiratory and gastrointestinal infections compared to the control group. Interestingly, MxA expression was detected similarly in respiratory syncytial virus and *Streptococcus pneumoniae* infections, but this might be somewhat explained by viral infection preceding the detection of *S. pneumoniae* (Engelmann et al., 2015). However, the expression of MxA levels heavily overlaps with bacterial and viral infections, although there is a statistically significant difference among the populations (Halminen et al., 1997; Engelmann et al., 2015; Rhedin et al., 2022; Piri et al., 2022; Metz et al., 2023). Thus, the diagnostic power at the individual level is not superior to differentiate viral infection from bacterial infection, but together with all other clinical information, may strengthen the diagnosis and, for example, the decision to withhold from prescribing antibiotics. Of note is that the transition from utilizing high negative predictive value (NPV) testing, i.e., NAAT, instead of high positive predictive value (PPV) testing, i.e., viral culture and antigen detection, may have negatively affected the studies reporting the differentiation power of the MxA detection. The reason for this is that the MxA is probably increased only during the acute phase of an infection when the virus is replicating, and NAAT is not specific for that phase. Human neutrophil lipocalin (HNL) has been studied to correlate better with bacterial than viral etiology, but the diagnostic power at the individual level was low (Venge et al., 2015a; Venge et al., 2015b; Venge et al., 2017).

Intestinal ailments may arise from inflammatory or non-inflammatory reasons. Irritable bowel syndrome (IBS) is a condition in which the intestines are dysfunctional due to non-inflammatory reasons, while inflammatory bowel disease (IBD) is a condition in which the intestines are inflamed due to infectious or non-infectious disease. Calprotectin is a biomarker, the concentration of which in feces is elevated in inflammatory bowel conditions that can be of infectious or non-infectious origin. Calprotectin concentration in stool is at a normal level in IBS but is elevated in IBD (Chang et al., 2014; Nemakayala & Cash, 2019). *Clostridioides difficile* infection (CDI) is an example of a bacterial infectious bowel disease causing mild to severe inflammation where fecal calprotectin is elevated and correlates with disease-causing fecal toxin positivity and disease severity (Swale et al., 2014; Rao et al., 2016; Peretz et al., 2016; Kim et al., 2017; Barbut et al., 2017). Fecal calprotectin is also helpful in monitoring the efficacy of recovery

from bowel inflammation, such as in the case of CDI treatment (Konturek et al., 2016). Fecal calprotectin has also been suggested to differentiate between viral and bacterial gastroenteritis, and other causes of intestinal inflammation, as calprotectin was much lower in viral infections than in infections caused by bacteria or other causes of inflammation. In the diagnosis of bacterial infection, the sensitivity and specificity were 88.9% and 76.0%, respectively (Duman et al., 2015).

2.3.2 Pathogen culture

Visual detection of pathogen growth is a classical marker for acute bacterial infection. In a plate culture, bacteria form colonies, which are easy to count as colony-forming units (CFU). Concentration of bacteria cultured in medium can be determined based on the optical density (OD) at 600 nm, where OD 1.0 is roughly 1.0×10^9 bact/ml. Viral culture is the reference *in vitro* method for assessing infectiousness and infectivity (Costantini et al., 2018; Basile et al., 2021) if the material in question contains viable viruses able to infect cells in optimal laboratory conditions (Manzulli et al., 2021). Viral culture may not be optimized for all viruses, but at least for SARS-CoV-2 and influenza well-established viral cultures can detect very low amounts of viable viruses (Singanayagam et al., 2020; La Scola et al., 2020; Manzulli et al., 2021; Keske et al., 2023; Jaafar et al., 2020).

As viruses are not necessarily easy to reproduce *ex vivo*, there are several indicative methods to detect the presence and quantity of viruses. Plaque-forming units (PFU) indicate viral quantity as the number of infected cells on a fixed monolayer of host cells. When viable viruses infect a cell, the cell is disturbed, and a plaque is formed. Based on the sample dilution, PFU per specimen can be calculated. Focus forming assay (FFA) is a variation of the plaque assay where the infected cells are identified prior to cell lysis by immunostaining utilizing fluorescently labeled specific antibodies to show the presence of viral antigens. In the $TCID_{50}$ (50% tissue culture infectious dose) assay, the sample is diluted until the endpoint dilution kills 50% of infected host cells. Transmission electron microscopy (TEM) has also been sometimes used directly to detect intact viruses or bacteria on fixed slides. The number of microbes per area can be calculated. Agglutination assays usually indicate intact viruses or bacteria. However, agglutination may also occur in the presence of antigens only. The methods above are not microbe-specific; thus, the multiplied microorganisms should be verified using microbe-specific detection methods in the case of polymicrobial samples, such as patient samples.

2.3.3 Microbial antigens and nucleic acids

Each viable and functional microbe has a genome and proteins in its structure. Additionally, enveloped viruses and bacteria contain carbohydrates and lipids in their structure. Basically, the detection of genetic material or microbial antigens is method to indirectly show the presence of a pathogenic organism. Fundamentally, also microbial metabolites can also utilized. NAAT methods are used to detect genetic material in a sample after *ex vivo* amplification when even the lowest traces can be detected. Antigen detection methods are designed to detect directly from a sample *in vivo* expression of genes, the translated microbial specific proteins, and/or carbohydrates when those are multiplied during microbial invasion. When the detection is targeted against abundantly expressed antigens, the approach relies on the natural amplification of genes into phenotype. For example, when one genomic gene of influenza per virus particle, is translated into a thousand or more proteins per virus particle this evens out the analytical sensitivity difference between gene detection and antigen detection. In the case of influenza, this corresponds to about 10 RT-qPCR cycles, and in the case of *Streptococcus pyogenes*, more than 15 cycles (Vakkila et al., 2015).

Before the revolution of PCR (Mullis & Faloona, 1987**)** based methods at the beginning of this millenium diagnostics of acute infections was done based on culture methods, direct fluorescence assay (DFA) (Coons et al., 1942), sandwich immunoassays, such as enzyme linked immunosorbent assay (ELISA) (Engvall & Perlmann, 1971) and rapid lateral flow assays. PCR methods became more and more popular as they were analytically very specific and analytically much more sensitive than antigen detection-based methods. In comparison to antigen detection methods, PCR methods provided many more findings. Basically, the sensitivity became the most widely used parameter to assess which method is the best, although the conclusions were drawn without clinical assessment of patient cases and without control groups (Novak-Weekley et al., 2010; DiMaio et al., 2012; Li et al., 2012; Charrel et al., 2013; Salez et al., 2013; Brendish et al., 2015).

While the direct detection of antigen represents the naturally occurring antigen concentration in the sample, the RT-qPCR Ct value has also been used as a surrogate marker of viral load. Often the studies fail to acknowledge that Ct value is a surrogate marker, and conclusions about virus titer are made directly based on the Ct value. The limitation of using Ct values as a surrogate marker is that they do not correlate well with viable viral concentration or titer in clinical samples. The higher the Ct values are, the less likely the samples contain viable viral particles, on average. However, there can be even an inverse correlation for positive findings between RT-qPCR and viral culture when the Ct value increases (Phillips et al., 2009; Jaafar et al. 2020; Kim et al., 2021). Therefore, it has been suggested that Ct values should not be used for assessing the clinical relevance of the finding (Evans

et al., 2021). The patient process usually includes only one test (sampling) point which makes it convenient not to miss a case, even though the sampling would have been done after the acute phase of the infection (Figure 1). In RTI testing, sensitive NAAT testing is also more robust for poor sampling than antigen detection when the peak of viral load has passed. In addition, specificity of antigen detection methods has been criticized due to varying performances.

There is a need for both the NAAT and antigen testing, and their benefits should be combined, like in the case of CDI diagnostics. CDI is the most commonly diagnosed cause of antibiotic and healthcare-associated infectious diarrhea, and several microbe-specific markers, each having special characteristics, are used in CDI diagnostics. CDI is a disease for which diagnostics are recommended to utilize both detecting the genetic material and expressed proteins. According to the European diagnostic guidance, CDI diagnostics benefits from both the high sensitivity of NAAT, which provides high NPV, and antigen tests, which provides high clinical PPV (Crobach et al., 2016). Traditionally, the most sensitive test has been toxigenic culture, which can be considered as a genotypic method as it does not provide information whether the specimen contains in vivo expressed proteins. In the toxigenic culture, the presence of a toxigenic strain is either verified by NAAT or by the detection of proteins expressed during cell culture ex vivo. *C. difficile* has toxigenic and non-toxigenic strains, and asymptomatic carriage of toxigenic strains is common. Colonized toxigenic *C. difficile* bacteria may overwhelm beneficial microbiota in the intestine after antibiotic treatment. The disease emerges when intestinal cells are damaged in the presence of toxin A and/or B proteins produced by the bacteria. Toxin A and B are cytotoxins expressed by toxigenic *C. difficile* strains, causing cell death in the intestine and potentially leading to severe colitis (Sullivan et al., 1982). All *C. difficile* strains also produce a *C. difficile*-specific surface protein glutamate dehydrogenase (GDH), which is basically wholly conserved (Carman et al., 2012), in large quantities. The direct cell cytotoxicity assay has been traditionally considered a gold standard for the detection of toxin A/B in stool by causing cytopathic effect on cells, while toxigenic culture has been regarded as the most sensitive method for the detection of viable toxigenic strains. Due to the abundance of GDH, the GDH antigen test or NAAT has a similar NPV, around 99%, for CDI. However, because *C. difficile* may be carried in the intestines without clinical disease, NAAT (gene detection) has only 50% PPV for CDI. The great strength of toxin A/B testing (protein detection) is that it has a much higher 95-99% PPV for CDI. Thus, highly sensitive GDH antigen test and NAAT are used to support ruling out the disease, while the highly specific toxin A/B antigen test is used to rule in the disease. Similar to toxigenic culture, NAAT provides information about carriage of a toxigenic strain when toxins are not detectable (Crobach et al., 2016).

The information is important in ruling in or out carriage of a toxigenic strain for cohorting purposes and hygiene measures. See more about the detection of antigens and nucleic acids in section 2.7.

2.3.4 Serological responses

Serology is a classical way to study recent/acute or past infection and refers to the diagnostic identification of antibodies in the serum. The immune system responds to invading pathogens and their antigens by producing IgM and IgG antibodies that are typically markers for acute and past or primary and secondary infections, respectively. Antibodies, mainly IgM, are detectable earliest after some days and typically a few weeks from the onset of infection and symptoms, thus, not usually suited for acute phase diagnostics. Microbe-specific IgG antibodies are mostly detectable after 14 days, usually after the symptomatic acute phase of an infection. Primary and secondary infections can be differentiated either with paired serum samples by monitoring the increase in antibody levels, class change from IgM dominated response to IgG response, or by using the antibody avidity index by defining the affinity (avidity) of the antibodies (Hedman & Rousseau, 1989; Koskinen et al., 2006). Serology is the method of choice to detect past infection (in case of chronic or immune symptoms caused by past infection) or to demonstrate immune status arising from vaccination or past infection.

2.4 Samples for diagnosing acute infections

Traditionally, the sample matrix for direct pathogen detection of RTI has been nasopharyngeal secretions, which have been obtained from the nasopharynx with a nasopharyngeal swab or by aspiration. For pharyngitis, the matrix is oropharyngeal secretions sampled from the oropharynx using a swab. Stool has been the specimen for gastroenteritis. Serum has been used for serological detection of antibody responses and biomarkers. Intrinsically, whole blood is the matrix for sepsis diagnostics. These matrices represent the location where the specific pathogens and analytes are located during infections. Especially, advancement in highly sensitive NAAT methods that multiply target genes has contributed to studying alternative specimen types that are less invasive or easier to obtain. For qualitative testing, even low amounts of target in a specimen are enough to give a high detection rate similarly to the most optimal specimen type. For methods detecting the analyte directly, e.g., microbial culture and antigen testing, lower microbe quantity may affect the detection rate dramatically (Basso et al., 2021; Lindner et al., 2021; Hagbom et al., 2022; Jegerlehner et al., 2022; Ren et al., 2022;).

2.4.1 Respiratory secretions and saliva

For upper RTIs, a nasopharyngeal swab from one nostril taken by a healthcare professional has been the standard sample type. Along with the COVID-19 pandemic, nasal sampling from both nostrils with the same swab became standard for self-testing and as part of healthcare professional testing. Nasal swab sampling from both nostrils performed well when compared with nasopharyngeal swab sampling. However, the term nasal sampling was uniformly used for samples obtained from anterior nasal and nasal mid-turbinate, although there is a significant difference in the depth of sampling (Lindner et al., 2021). Alternative sample types were also studied and used, such as throat swab and saliva. Nasopharyngeal aspirate and nasal wash were widely used before the 2010s but are uncommon nowadays.

As respiratory pathogens, such as influenza, SARS-CoV-2 and *S. pneumoniae*, infect our nasal respiratory epithelia, nasopharyngeal secretions are an obvious specimen type for testing. The gel-like nasal discharge, having a cleaning function, consists mostly of water, saline electrolytes and glycoproteins, such as mucins. It also contains detached epithelial cells and colonized microbes. Although nasopharyngeal secretions functions as a barrier for invading pathogenic micro-organism, these can sometimes cross the barrier and cause an infection. During infection, the pathogen load in nasal discharge can be extremely high. Nasal secretion contains antimicrobial proteins of humoral, e.g., lysozyme and complement, and cellular defense mechanisms, e.g., neutrophils and macrophages. The nasal secretion also contains substances, such as kallikrein, kinins, eosinophilic proteins, and protease inhibitors. Many of the proteins are involved in inflammatory response and in allergic rhinitis (Beule, 2010; Tomazic et al., 2020). Sputum seems to be somewhat similar to nasopharyngeal secretion, as sputum is partly postnasal drip, containing carbohydrates, proteins, glycoproteins, albumin and lysozyme (Ryley & Brogan, 1968). Sputum may be thicker than nasopharyngeal secretion, making it more difficult to extract target analytes from the mucus. Nevertheless, sputum may contain high quantities of viral material (Wölfel et al., 2020). These specimen matrices contain various compounds with active functions that may interact with diagnostic test reagents. When present, blood in the specimen brings additional adsorption and fluorescence properties to the sample matrix.

Saliva is a slightly alkaline electrolyte solution that contains, for example, mucus, amylase, lysozymes lingual lipase, white blood cells, epithelial cells and secretory IgA. Proteolytic activity of saliva may negatively impact diagnostic testing (Humphrey et al., 2001; Thomadaki et al., 2011). In addition, saliva may have factors that inhibit immunoassays (Mitchell et al., 2009). As a specimen for RTI tests, saliva has been shown qualitatively to perform similarly to

nasopharyngeal and nasal samples in RT-qPCR (Huang et al., 2021; Schwob et al., 2023; Fougère et al., 2021; Bastos et al., 2021). However, the antigen load and viable virus quantity in saliva seem to be up to a hundred times lower compared to nasopharyngeal and nasal specimens. Thus, saliva can be a challenging specimen for antigen tests (Hagbom et al., 2022; Jegerlehner et al., 2022). Chemiluminescent assays with higher sensitivity compared to lateral flow assays have shown performing better with saliva than lateral flow antigen tests (Ren et al., 2022; Basso et al., 2021). Although SARS-CoV-2 infects the throat area, such as salivary glands, and may lead to loss of taste (Huang et al., 2021), antigen and viable virus load in throat specimens are lower compared to nasopharyngeal and nasal specimens (Wang H. et al., 2020; Wang X. et al., 2020; Killingley et al., 2022). Thus, the selected specimen and sampling type affect the apparent sensitivity of an antigen test more than NAAT.

2.4.2 Feces and urine

Feces and urine are the waste products of our digestive tract, excreted by two separate routes. Both of the matrices are complex and contain similar substances, such as inorganic salts, urobilinoids (decomposition products of hemoglobin), and albumin (Athar et al., 1999) that might interfere with fluorescence measurement of such methods by attenuating or enhancing fluorescence. The matrices also contain substances that can potentially interfere, e.g., inhibit or cause unspecific binding, with the bioaffinity reactions of immunoassays (Yolken & Stopa, 1979). *Legionella pneumophila* serotype 1 and *Streptococcus pneumoniae* are RTI and pneumonia causing bacteria, which are diagnosed from urine with antigen tests, such as rapid lateral flow tests (Jørgensen et al., 2015), by detecting bacteria-specific polysaccharide antigens. Genetic material of RTI and gastroenteritis viruses can be detected from feces without viable viruses (Costantini et al., 2018; Wölfel et al., 2020; Sia et al., 2020). As a matter of fact, feces have inactivation activity against membrane viruses (Kurmi et al., 2013). Separation-free (wash-free, homogeneous) assay format brings benefits in the format of simple assay protocols and automation, and therefore, has been a target for technology development since the 1980s. However, it also means that the problematic sample matrix cannot be washed away before completing the immunoassay reaction and fluorescence readout. Sometimes getting a stool sample may be complicated due to medical or time reasons. Then, a rectal swab may be an alternative choice for stool, at least for NAAT (Goldfarb et al., 2014).

2.4.3 Vomit

Both gastroenteritis and RTI may cause abdominal discomfort, leading to vomiting. Vomit is a cocktail of partly digested food and drinks combined with hydrochloric acid and digestive enzymes. Although vomiting is a symptom, it does not mean that the cause is in the chyme. For example, norovirus can cause only vomiting without diarrhea (Atmar et al., 2008), but it does not still mean that the viral replication is primarily taking place in the stomach. Fundamentally, pathogens causing gastroenteritis inhabit the intestines where they multiply and reproduce. Pathogenic material is then passed along with the stool and sometimes vomit. Although norovirus RNA is frequently detected from vomit in similar genomic equivalents as from stool, only some of the cases show viral replication (Hagbom et al., 2021). *Helicobacter pylori*, which causes gastritis and stomach ulcers, colonizes the stomach mucous. Although the bacterium is especially lurking and hiding in the stomach mucosa, stool antigen testing is a very common and useful way to indicate the presence of the bacteria in the stomach rather than analyzing chyme. Gastric acid has a low pH of two, which rapidly inactivates enveloped viruses with membrane but not non-enveloped viruses without membrane (Darnell et al., 2004; Zhou et al., 2017). Enveloped viruses may retain viability better during fed-state gastric fluid with a higher pH 3 and above (Darnell et al., 2004; Zhou et al., 2017; Chin et al., 2020). An *in vitro* study that used artificially produced mucus suggested that mucus may also protect enveloped viruses from inactivation by acidic and bile-pancreatic juice (Hirose et al., 2017). Artificially simulated fed-state gastric and intestinal fluid also protected MERS-coronavirus (Zhou et al., 2017), although no viable SARS-CoV-2 has been isolated from stool (Wölfel et al., 2020) or the feces of infected golden hamsters (Sia et al., 2020).

2.4.4 Blood and serum

In addition to the detection of antibodies, blood can be used to identify viremia by detecting infectious viruses or parts of the viruses, antigens or genetic material. The presence of viral antigens, or genetic material in the bloodstream may suggest more severe symptoms during infection (Hemming et al., 2014). Traditionally, serum has been the matrix of choice for serological laboratory assays, as potentially interfering factors, such as clotting factors and blood cells, have been removed. The demand for rapid and POC tests has increasingly shifted the development toward the direct use of whole blood, for example, finger-prick blood. Blood, including serum and plasma, may contain many interfering agents that need to be considered in assay design. Common inherent interfering agents include, for example, blood cells, heterophile antibodies, anti-animal antibodies, autoantibodies, rheumatoid factor, and complement components (Selby, 1999). In

diagnostic testing of acute infections, such as RTI and gastrointestinal infections, blood and serum are rarely used nowadays since other specimen types are more relevant and less invasive.

2.5 Two-photon excitation fluorometry

Two-photon excitation fluorometry, which utilizes micrometer-sized polymer microparticles as a solid phase carrier for the detection of bioaffinity reactions, is a technique that combines optical microscopy and fluorescence measurement in separation-free analysis. In the bioaffinity reaction, fluorophore-labeled binders concentrate on the surface of the microparticle in proportion to the concentration of the specific analyte. The fluorophore is excited with two simultaneous infrared photons. The excitation energy is then released as the emission of a higher-energy photon at a visible wavelength. Thereby, the emission fluorescence can be differentiated from the excitation fluorescence (Hänninen et al., 2000; Meltola et al., 2004).). The technique is also known as the ArcDia TPX assay technique.

In the TPX technique, the bioaffinity assay and signal generation are performed inside microvolume reaction chambers without physically separating the bound and the unbound fractions of target analytes and the reagents. The separation is brought about by optical phenomena. Fluorescent brightness of individual microparticles is measured, one by one, by scanning through the transparent bottom of the reaction chamber with a focused laser beam (1064 nm). The beam is deflected using piezo-driven mirrors. A microparticle entering the focus backscatters the excitation light, and the microparticle is pushed by optical forces through the focus, which is similar in size to the microparticle. The two-photon excited fluorescence brightness of the particles is measured continuously. When the backscattering of exciting light exceeds a preset threshold (a particle is in the focus), the fluorescence is recorded for a microparticle. The fluorescence is also measured from the solution phase when there is no particle in the focus. The ratio of apparent brightness of the microparticle to the solution signal, reflecting unbound tracer and sample matrix, depends on the degree of bioaffinity binding. In the absence of binding, the ratio is close to unity. Data reduction algorithms calculate the mean brightness of the particles and the solution phase (Hänninen et al., 2000; Koskinen et al., 2007). In applications that require quantitative diagnostic results, the signal intensity is compared to a standard curve for obtaining the concentration of the sample (Koskinen et al., 2004).

The technique has shown wide applicability. The technique has been applied for the detection of biomolecules in different sample matrices, such as the detection of mucosal antigens (Koskinen et al., 2007), serum antigens (Hänninen et al., 2000; Koskinen et al., 2004), and antibodies (Koskinen et al., 2006), antibody avidity

(Smolander et al., 2010), and nucleic acid sequences (Meltola et al., 2005; Vaarno et al., 2004). The technique has also been shown to enable phenotypic species identification and antimicrobial susceptibility testing directly from patient samples, containing commensal flora, in hours (Koskinen et al., 2008; Stenholm et al., 2013). This unique feature of the technology is promising for the future of diagnostics as the antimicrobial resistance problem gets even worse and treatment decisions need to be more routinely backed up by information regarding the susceptibility to available antimicrobials. So far, the technology has been commercially available under the brand name mariPOC with particular focus on diagnostic testing of acute infections by antigen detection. The choice to apply antigen detection, while most other new product initiatives applied NAAT at the time, was based on the available scientific evidence about phenotypic testing in 2008 (Koskinen, 2008). Ever since, a growing amount of data has accumulated to support the phenotype testing approach.

2.6 Development and regulatory aspects of diagnostic tests

Several aspects need to be taken into account when developing diagnostic tests for acute infections from a scientific design point of view. The key aspect in a diagnostic test is the target marker molecule that is to be detected. In the case of acute infections, the target should provide information about an ongoing infection. If the marker is detectable outside the acute infection, the clinical indication might be misleading. The marker should optimally be found in all strains of the same species or even within the genus, depending on the diagnostic purposes. However, differentiation between species, strains, or even variants may be desired when there is a clinical benefit. The detectable marker should be conserved in a way that the test performance is not prone to variations that happen due to evolutionary reasons. The marker is preferred to be abundant in the organism, making it easier to reach good clinical sensitivity. The test should not be prone to unspecific binding due to the sample matrix, nor be interfered with by common agents potentially present in the specimen, and not to cross-react with other microbes potentially also present in the sample. The effects of interfering agents are often minimized by utilizing non-specific proteins, e.g., bovine serum albumin, immunoglobulins mimicking the reagent antibody and enzyme inhibitors. Affinity binders raised against recombinantly produced analytes may not detect the native target from a clinical sample. Therefore, cultured or real clinical samples should be available early on in the development path of new tests and its raw material affinity binders. In addition to the scientific validity, the test design and development process has to take into account a growing amount of harmonized industry standards, international

guidance and regulatory demands. Scientific principles for obtaining clinical performance figures for tests are described, for example, by Leeflang & Allerberger, 2019a and Leeflang & Allerberger, 2019b.

The main purpose of legislation is to ensure that the products are safe for the individual who is to be tested and for the user of the test. Safety comes through usability design and risk management. In Europe, the tests need to meet the requirements of Regulation (EU) 2017/746 of the European Parliament and of the Council of 5 April 2017 on in vitro diagnostic (IVD) medical devices. According to the regulation, the diagnostic test shall be designed for its intended purpose. The test shall be designed to provide information about the pathological state of the individual to be tested. A pathological state is a physical condition that is caused by a disease. In this context, the pathological state can be considered to indicate whether the individual has, for example, an ongoing infection that may affect the physical condition of the individual. The test shall also be designed for its function. Such functions can be, for example, screening, monitoring, diagnosis or aid to diagnosis, or prognosis. Furthermore, the function can be either to rule in or rule out the cause of the disease or infection. Tests with high positive or negative predictive value are meant for ruling in or out a disease or cause of the disease, respectively. The regulation emphasizes that the indication and specifications for the test shall follow the state of the art in medicine. The state of the art should represent the clinical performance of the device in terms of the intended purpose and function in relation to the pathological status of the patient.

A diagnostic test can be a commercial IVD CE-marked test or an in-house test (laboratory-developed test). For commercial tests, the manufacturer assures that the test fulfils rules and regulations, and provides performance figures. Most often, the commercial test needs to be validated in a diagnostic laboratory prior to use. In-house tests are not necessarily evaluated by a second party or geographically for different populations. Along with Regulation (EU) 2017/746, both the commercial and in-house tests fall under the regulation. The regulation is intended to minimize risks and health hazards for the patient and user.

The utilization of chemicals in the products shall be designed to comply with the European regulation (EC) No 1907/2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH). Table 2 shows the relevant standards related to the development of *in vitro* diagnostic tests for the detection of acute infectious diseases in question. ISO 13485 standard is the internationally recognized standard for quality management systems in the design and manufacture of medical devices. The other standards guide on a more specific subject.

Table 2. Standards to be applied in the design and development of the tests.

Purpose	Standard
Quality management	ISO 13485 Medical devices — Quality management systems — Requirements for regulatory purposes
Risk management	ISO 14971 Medical devices - Application of risk management to medical devices
Usability	IEC 62366 Medical devices - Application of usability engineering to medical devices
Quality control	EN 13975 Sampling procedures used for acceptance testing of in vitro diagnostic medical devices - Statistical aspects
Test reagent stability	EN ISO 23640 In vitro diagnostic medical devices - Evaluation of stability of in vitro diagnostic reagents
Clinical performance	EN 13612Performance evaluation of in vitro diagnostic medical devices ISO 20916 In vitro diagnostic medical devices — Clinical performance studies using specimens from human subjects — Good study practice
Product labelling and product information	EN ISO 15223-1 Medical devices - Symbols to be used with medical device labels, labelling and information to be supplied - Part 1: General requirements EN ISO 18113-1 In vitro diagnostic medical devices - Information supplied by the manufacturer (labelling) - Part 1: Terms, definitions and general requirements (ISO 18113-1:2009) EN ISO 18113-2 In vitro diagnostic medical devices - Information supplied by the manufacturer (labelling) - Part 2: In vitro diagnostic reagents for professional use (ISO 18113-2:2009) EN ISO 18113-3 In vitro diagnostic medical devices - Information supplied by the manufacturer (labelling) - Part 3: In vitro diagnostic instruments for professional use
Software	EN 62304+A1: Medical device software – Software life cycle processes

2.7 Diagnostic validity for pathological state

The Regulation (EU) 2017/746 states that an IVD test used for detecting infectious disease shall provide information about what is the pathological state that the test provides information on, and that the intended use shall be specified. In order to meet the requirement, test results for acute infectious disease should be interpreted as its indication is defined. The shift from antigen detection to NAAT methods took place with the fact that NAAT methods are analytically much more sensitive (Brendish et al., 2015), even though low copy number findings may lack clinical relevance. Although NAATs are generally analytically more sensitive than antigen detection, NAAT methods have been used beyond their limits. The question remains whether test results represent the state of ongoing infection. Many diagnostic laboratories report, for the clinicians, pathogen-positive results with RT-

qPCR Ct values higher than 35 or even up to Ct 40 to 50, although for example, for rotavirus, one genome per reaction result was obtained at Ct 39 (Zeng et al., 2008). Theoretically, an RT-qPCR test designed and performing properly (>90% multiplying efficacy per cycle) should be able to detect with 95% CI (Confidence Interval) about the initial 5-10 copies per reaction. Such a low copy number will become detectable after about 33-35 amplification cycles. This is the theoretical limit of detection for a typical qPCR test. Thus, amplifications detected beyond Ct 35 most probably reflect other than specific gene detection. Ct values approaching and higher than 40 would mean less than one initial viral genome per reaction. Commercial RT-qPCR methods often claim 95% CI sensitivity at around Ct 35 and ten copies per reaction for repeatable detection. For a high performing antigen test, the positive agreement with RT-qPCR approaches 100% for those samples close to Ct 30 and below (Routsias et al., 2021). As the theoretical LoD of RT-qPCR is around 33-35, the difference in analytical sensitivity is only about 10- to 30-fold (2^5) in favor of PCR (equivalent to 5 Ct values), not a thousand-fold.

The following example, using rotavirus as a model analyte, describes the difference between antigen detection and RT-qPCR in relation to symptom severity in a viral intestinal infection. By combining the results from studies by Phillips et al. (2009) and Kang et al. (2004), it can be concluded that more severe disease and antigen positivity correlated with low RT-qPCR Ct value (<25), while high Ct values (>30) correlate with less severe disease or with no symptoms. Above Ct 30, a similar frequency of Ct values was obtained from infectious intestinal disease cases and healthy controls. Severity of rotavirus infections is highly correlated with Ct values. Patients with Vesikari severity score 10-15, 3-9, and 0 had Ct <21, Ct 15 to 36, and Ct >28, respectively (Figure 2). Phillips et al. (2009) suggested a Ct value cut-off in the range of 25–28 for attributing illness to rotavirus infectious intestinal disease in patients of all ages. Similarly, for SARS-CoV-2, Ct cut-offs of approximately 23 to 28 and 28 to 36 apply for infectious individuals and for unlikely- or non-infectious individuals, respectively (La Scola et al., 2020; Singanayagam et al., 2020; Jaafar et al., 2020; Pickering et al., 2021; Basile et al., 2021; Lopera et al., 2022; Stohr et al., 2022; Kirby et al., 2023). The justification for using results with high Ct values could be that these cases represent subclinical or masked infections. However, the data suggest that when a viral infection is in the acute phase, infectious viruses can be isolated, and the Ct values are low, even for asymptomatic cases. This excludes the possibility that high Ct values in the healthy control group represent asymptomatic acute phase subclinical infections (Atmar et al., 2008; Jaafar et al., 2020; Kim et al., 2021; Killingley et al., 2022).

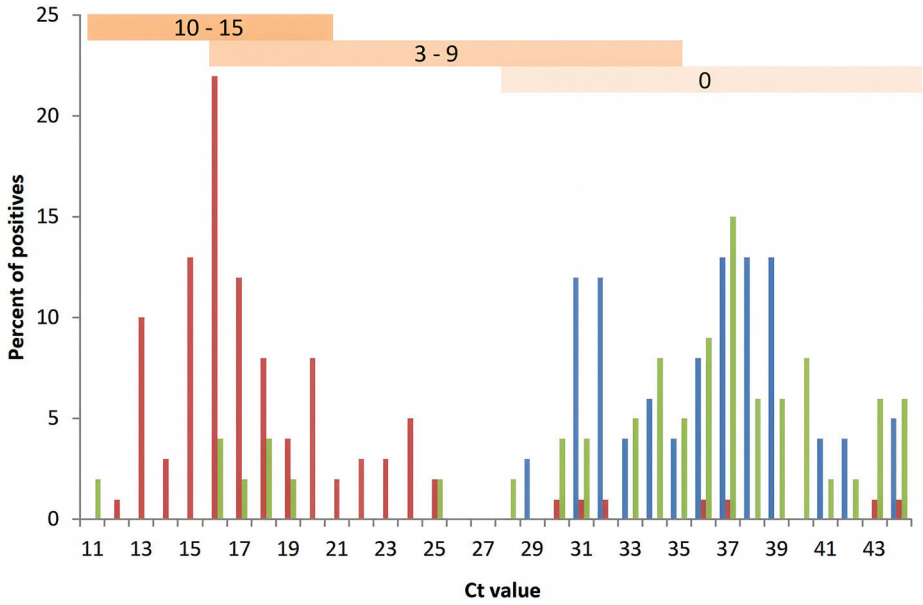

Figure 2. Schematic figure about the causality between antigen and genetic material detection in disease severity. Orange horizontal bars show infectious intestinal disease severity in the Vesikari score. Vertical bars show the percentage of positive cases by RT-qPCR Ct value. Red and blue bars show antigen-positive and antigen-negative infectious intestinal disease cases, respectively. Green bars are the healthy control group for which RT-qPCR Ct values were obtained. The figure was derived by combining the results from studies by Phillips et al. (2009) and Kang et al. (2004).

As NAAT methods are analytically very sensitive, they do not necessarily indicate acute infection (Advani et al., 2012; Inagaki et al., 2016). The reasons are many including the view that they can detect genetic material as contamination, which may originate from multiple sources and/or ways (Curran et al., 2012) or as traces of long term shedding from past infection (Atmar et al., 2008; Mina et al., 2020; Singanayagam et al., 2020; Wölfel et al., 2020; Killingley et al., 2022; Lopera et al., 2022; Colazo Salbetti et al., 2023), cf. crime scene investigations, where biological traces may merely suggest that someone was present (Sousan et al., 2022; Goray et al., 2024). Thus, NAAT is suitable for detecting recent infection after the clearance of viral shedding, in cases where viral culture and antigen detection tests yield negative results. NAAT usually has a high NPV (ruling out the presence of microorganisms) but often a low clinical PPV (ruling in an acute disease).

Traditionally, NAAT methods directly detect the presence of parts of the genomic sequence that do not indicate acute infection. Bocavirus 1 DNA is frequently detected from nasopharyngeal secretion of children, but in half of the cases, this arises from past infection and/or persistence of genomic material

(Jalving et al., 2023). Christensen et al. (2013) showed that acute bocavirus 1 infection can be recognized more accurately by detecting the virus-derived messenger RNA (mRNA), which is a transcriptional intermediate produced during viral replication. This suggests that bocavirus antigen detection correlates with mRNA transcription and acute infection better than the detection of DNA by RT-qPCR (Kols et al., 2019; Bruning et al., 2016). Also, an endonuclease treatment of samples prior to RT-qPCR seems to correlate with active viral infection by removing DNA persistence while retaining genomic material inside a virion intact (Rayamajhi Thapa et al., 2025).

Viral antigen detection tests for acute RTI often target the viral nucleocapsid (N) protein. At least for coronaviruses, the expression of N-protein is the key pathogenicity factor (Wada et al., 2018), and it is essential for the coronavirus replication and transcription of the viral RNA (Almazán et al., 2004; Zúñiga et al., 2010). Without the accumulation of the N-protein, the coronaviral mRNA is degraded by the nonsense-mediated decay pathway of eukaryotic cells (Wada et al., 2018). Alexandersen et al. (2020) concluded that the detection of RNA is not an indicator of actively replicating SARS-CoV-2. Their data suggest that virion and subgenomic RNAs are stable in cellular double-membrane vesicles and, therefore, can be detected long after the acute infection. Moreover, there are convincing data that antigen detection correlates better with viral culture, which is the reference method for infectivity and presence of active infection, than NAAT (Pekosz et al., 2021; Rusanen et al., 2021; Pickering et al., 2021; Kirby et al., 2023; Lopera et al., 2022). Furthermore, Zhang et al. (2021) found that parts of the reverse-transcribed SARS-CoV-2 RNA can integrate *ex vivo* into the human genome without the ability to yield infectious viruses and suggested that this could explain at least partly the long-term RNA shedding. However, in vivo evidence remains to be shown.

Reagents of antigen tests or the clinical sample may possibly be contaminated too, but the needed contamination level is high and probably needs direct contact with microbial material. Due to the analytical sensitivity of NAATs, they are prone to contamination. A study of SARS-CoV-2 primer–probe sets from four major European suppliers found a significant level of contamination in the reagents. False positives as low as RT-qPCR Ct 17 were obtained (Wernike et al., 2020). Low levels of SARS-CoV-2 RNA contamination have also been found from surfaces and air in rooms where mildly ill individuals stayed without notable viable virus being isolated (Santarpia et al., 2020; Zhou et al., 2021). It has also been shown that environmental contamination may result in positive test results in PCR among individuals sampled in the same area where intranasal influenza vaccine dosing was done (Curran et al., 2012). These data suggest that individuals present near symptomatic patients can be contaminated by RNA without being infected with

viable virus. Thus, methods detecting the viral RNA by amplification are prone to clinically insignificant positive results, especially when a significant part of the population has been infected recently. Many studies describe how to utilize RT-qPCR Ct cut-offs to increase the clinical usefulness at the cohort level. Although Ct values correlate at the population level, the use of Ct values may not be practical at individual level (Evans et al., 2021), and in any case, clinicians have not been trained to interpret Ct values. In contrast, many studies have shown that the presence of viral antigens in the samples correlates with the presence of viable virus (Pekosz et al., 2021; Rusanen et al., 2021; Pickering et al., 2021; Kirby et al., 2023; Lopera et al., 2022).

Detection of genetic material from stool may not have causality for intestinal disease. Along with the beginning of COVID-19, the SARS-CoV-2 was related to RTI (Zhou et al., 2020) and gastroenteritis (Xiao et al., 2020). However, as the pandemic continued, it became obvious that SARS-CoV-2 is not a gastrointestinal pathogen (Wu et al., 2020). It has been previously shown with other coronaviruses that they do not cause gastroenteritis (Paloniemi, 2016). Although receptors for SARS-CoV-2 can be found from the intestines and the viral RNA can be detected in stool (Xiao et al., 2020), viable viruses are not normally present in stool (Kim et al., 2020; Wölfel et al., 2020; Sia et al., 2020). In addition, viral RNA is not always detected in stool or urine during acute SARS-CoV-2 infection (Pan et al., 2020). Isolation of the influenza A virus from stool has been described. Although the virus is replicating *ex vivo* in intestinal cells, the isolated virus was rather passing through the intestines than replicating *in vivo* in intestinal cells, although replicating *ex vivo* in intestinal cells (Al Khatib et al., 2021). When considering the viability of enveloped RTI viruses in feces, for example, the H5N1 virus spiked into chicken feces was inactivated within 24 hours at chicken body temperature (42 °C). This indicates the harsh conditions of the stool for enveloped viruses and that their replication in the gastrointestinal tract is unlikely (Kurmi et al., 2013). Moreover, despite bocavirus being a non-enveloped virus like norovirus, and its DNA is often detected in stool, it causes solely RTI, not gastroenteritis (Paloniemi, 2016).

Antibiotic-associated diarrhea caused by *Clostridioides difficile* is a peculiar disease to diagnose. *C. difficile* has toxigenic and non-toxigenic strains, which both may colonize and hide in a sporadic form in the large intestine, mainly the colon. The use of antibiotics negatively impacts gut microbiota that may contribute to evasion of toxigenic *C. difficile* and expression of exotoxins A and B, causing disruption in the colon cells and functionality, leading to diarrhea and colitis. As in many cases of diagnostics of acute infections, the testing of CDI shifted from culture and antigen methods to gene detection methods in the 2010s. The shift from phenotypic to genotypic testing has led to overdiagnosis (Polage et al., 2015) and

almost doubled the reported CDI cases in the southeastern United States (Ilieş et al., 2020). Concern about overdiagnosis resulted in the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) Study Group for *Clostridioides difficile* issuing an updated diagnostic guidance in 2016. The recommended algorithm for CDI testing begins with a highly sensitive test, i.e., a GDH antigen test or a NAAT, and is followed by a clinically highly specific test, i.e., an antigen test for free toxins A and B. Alternatively, GDH and free toxins A/B can be tested simultaneously as the first step. The guidance highlights the use of the toxin A/B test in the assessment of CDI (Crobach et al., 2016). In order to simplify the process and to avoid the use of a two-step algorithm including antigen testing, other plausible approaches to reduce overdiagnosis and overtreatment, such as testing ordering practices (Khuvis et al., 2023) and toxin prediction algorithm (Hogan et al., 2022) have been suggested.

3 Aims

The aim of this thesis was to study the applicability and performance of the TPX assay technique for the detection of biomolecules, mainly viral and bacterial specific antigens from urine and fecal samples. Further, the aim was to develop new tests for the mariPOC test system and to evaluate their performance and usability in clinical practice.

The specific research aims were:

I To develop an easy and safe sample pretreatment protocol for urine and fecal/stool samples, and to study and develop the applicability of the sample matrices to the two-photon excitation assay technique.

II To develop multianalyte antigen tests for acute gastroenteritis and *Clostridioides difficile* in the automated mariPOC test system, and to evaluate their clinical performance and usability.

III To develop a rapid SARS-CoV-2 antigen detection test in the automated mariPOC test system and evaluate its clinical performance.

4 Summary of Materials and Methods

4.1 mariPOC test system and ArcDia two-photon excitation assay technique (III)

The feasibility of the separation-free TPX assay technique (see section 2.5) for the detection of respiratory tract infections was established in the thesis work by Koskinen (2008). Based on the work, an automated digital laboratory platform for the rapid multianalyte testing of acute infectious diseases (mariPOC test system) was developed. To meet the diagnostic demand for COVID-19 tests during the pandemic, a test detecting SARS-CoV-2 was developed and described for the platform in **Study III**. In the assay technique, target antigens are captured from a sample with specific monoclonal antibodies onto the surface of a solid-phase carrier of polystyrene microparticles (**III**, Fig. 1b). When fluorescent monoclonal antibody conjugates (tracer) (Meltola et al., 2004) bind to the captured antigens, three-component immunocomplexes are formed directly and quantitatively in proportion to the concentration of the analyte in the sample (**III**; Fig. 1b). Data reduction algorithms calculate the mean brightness of the particles and the solution phase, and compare it to a preset cut-off to determine the qualitative or quantitative result reported to the user on the graphical user interface.

The mariPOC test's operational steps, subsequent to sampling and sample treatment, are placing the sample tube into the analyzer for automated analysis and objective fluorescent result reading shown on a graphical user interface. The analyzer aspirates the sample through a pierceable cap and dispenses, through a resealing multilayer cover, 20 µL aliquots into the reaction chambers (one per tested analyte) containing dried test reagents. The test reagents are "packed" in dry format in different configurations inside the cartridge wells. The configuration package consists of a 384-well microtiter plate and a plate sealing cover to protect the test reagents prior to use and prevent excessive evaporation of sample solution during analysis. The wells have a transparent bottom, which allows optical fluorescence measurement from the wells. One well contains one test reagent for one sample, making each reaction independent of others. Thus, after closing the sample tube cap, the whole analysis is executed without opening any containers containing potentially infectious sample (**III**; Fig. 1a). The system has sophisticated autoverification functions to assess the technical reliability of analyses, and the results can be transferred automatically to the laboratory information system and/or as anonymized epidemiological data (Gunell et al., 2016) into the mariCloud service. The hands-on time is one minute per sample, and

the analyzer works in continuous feed and walk-away mode. The current throughput of one analyzer is up to 300 single analyte tests or 100 multianalyte tests in 24 h. The mariPOC user interface provides quantitative information about the pathogen loads in the sample for the user. Quantification is presented as the Ψ (psi) value, which is a signal strength value and a multiple of the cut-off signal level.

4.2 Development of antigen detection tests

Commercial mariPOC tests were developed under a certified quality management system to fulfil the quality requirements for regulatory purposes for medical devices based on European norm (EN) and International Organization for Standardization (ISO) 13485 to fulfil the requirements of the European Parliament directive 98/79/EC and Commission Decision 2010/227/EU prior to Regulation (EU) 2017/746. The standards in Table 2 were utilized, when applicable, in the design and development of the tests.

4.2.1 Test reagents (I)

Analyte-specific antibodies (ArcDia International Ltd, Finland) were coated onto monodisperse, carboxyl-modified microparticles by using passive coating and EDAC [1-ethyl-3-(3-dimethylaminopropyl)carbodiimide] fixation. The analyte-specific tracer antibodies were prepared by conjugating the antibody with a succinimidyl ester of the fluorescent labeling reagent (Meltola et al., 2004) by using methods described previously (Waris et al., 2002). Relevant optimizations of microparticle count and tracer concentration in the reaction were done. The test reagents were dried on 384-well plate wells as described previously (Koskinen et al., 2005). The plate's reaction cuvettes were covered with hermetic sealing as described by Koskinen et al. (2014) (Figure 3). The mariPOC CDI test was designed to target *C. difficile*-specific GDH and toxin A/B proteins. The mariPOC SARS-CoV-2 test was designed to target the conserved nucleocapsid protein (Frank et al., 2022), which is often the target in the case of membrane RTI viruses (Waris et al., 1988; Koskinen et al., 2007; Frank et al., 2022). The test reagents were challenged for other microbes and with substances potentially present in the stool or the nasal cavity.

In terms of this thesis, a test reagent is the dried reagent detecting one target, such as SARS-CoV-2 nucleocapsid protein or toxin A/B proteins. As the test reagents can be individual products, they can also be called tests, although they are often part of a multianalyte test. The multianalyte mariPOC Gastro test (test reagent configuration) utilizes test reagents for adenovirus, rotavirus, norovirus

GII.4, norovirus GI, and Campylobacter. The mariPOC Gastro+CDI test combines the CDI and Gastro tests for the same sample.

Figure 3. Test reagents dried in individual wells inside the test plate covered with hermetical sealing (test configuration package).

4.2.2 Sample pretreatment for feces and urine (I)

In the proof-of-concept **Study I**, stool was suspended into the mariPOC RTI sample buffer (B02, ArcDia Int.) using an optimal 20-fold dilution and vigorous vortexing for 15 seconds. The suspension was centrifuged for 5 min at 1000 × g to remove solid and particulate material. Urine was diluted into the RTI sample buffer and vortexed without any other treatment.

In the IVD CE mariPOC products, the fecal specimen was collected by dipping the dry flocked swab (552C, Copan Italy, Italy) into the stool, moving it rigorously for 5 to 10 seconds. The optimum specimen amount for firm stool is shown in Figure 4. The swab absorbed around 150 µL of diarrheal stool. The sample was then suspended in 2.6 mL of the mariPOC Gastro sample buffer (B06, ArcDia Int.) by vigorous vortexing for 15 seconds. The suspension was filtrated through a multilayer syringe filter (A-46, ArcDia Int.) having a 0.2 µm final pore size in order to remove all possibly interfering solid and particulate material (Figure 5).

Figure 4. Optimum amount of stool in the swab.

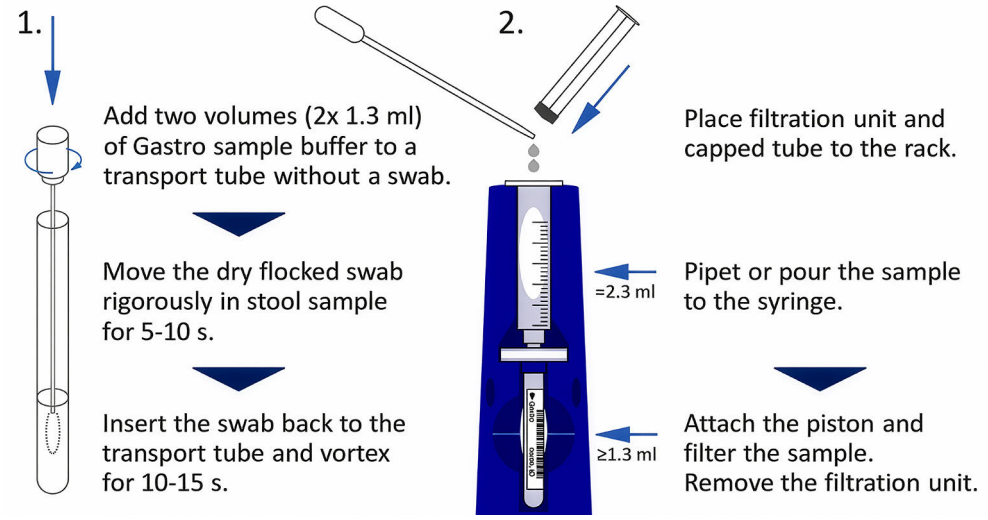

Figure 5. Sample pretreatment process for stool specimen.

4.3 Suitability of urine and stool for separation-free immunoassay utilizing two-photon excitation fluorometry (I)

In **Study I**, the suitability of urine and stool for separation-free two-photon fluorometry was studied by analyzing dilution series of pretreated specimens in the TPX assay. Briefly, test reagents detecting *Legionella pneumophila* serotype 1 and adenovirus were used in the case of urine and stool, respectively. The assay analysis was studied for both functionality of the immunoassay and the two-photon excitation fluorometry in the presence of urine and stool matrix, in the presence of specific antigen, and without.

4.4 Clinical validation of mariPOC tests

4.4.1 CDI test (II)

In **Study II**, the clinical performance of the CDI test, consisting of the GDH and toxin A/B test reagents, was evaluated in a prospective study by analyzing 337 fecal specimens with the mariPOC Gastro+CDI test (test plate product code 2027M, ArcDia Int.) in the clinical laboratory of Vaasa Central Hospital in Finland from May to September 2017. The stools were left-over samples from routine diagnostics, whose test was the GenomEra *C. difficile* PCR assay (Abacus Diagnostica Oy, Finland). Of these specimens, 157 were also tested with the TechLab C. diff Quik Chek Complete (Alere Inc.; now Abbott) membrane enzyme

immunoassay (MEIA). The clinical performance of the CDI test was evaluated for the detection of expressed proteins and toxigenic strains. Also, the detection of *C. Difficile* ribotypes and toxinotypes and *Clostridium* species were studied with the CDI test. Pure cultures of bacterial strains with known toxin profiles and unified bacterial concentrations based on the optical density of the stock suspension were analyzed.

4.4.2 SARS-CoV-2 test (III)

In **Study III**, the mariPOC SARS-CoV-2 test (test plate product code 1204S, ArcDia Int.) was validated for clinical use. Analytical and clinical performance of the test was evaluated following principles that are generally applied for in vitro diagnostic medical devices. Briefly, the analytical sensitivity of the test (Limit of Detection, LoD) was determined as the lowest concentration giving at least 19 positives out of 20 replicates (≥ 95% positivity). Analytical specificity was studied by challenging the test reagent against relevant microbes commonly found in the nasal cavity. The test reagent was also challenged with possible interfering agents. Clinical specificity was validated by analyzing 205 freshly sampled nasopharyngeal swabs. Clinical sensitivity was validated by analyzing 58 frozen RT-qPCR-positive nasopharyngeal samples from two specimen cohorts. Sensitivity of the SARS-CoV-2 test was categorized into different Ct values and compared with published studies utilizing viral culture.

4.5 Semi-quantitative antigen detection in clinical studies

4.5.1 Stool consistency in CDI diagnostics (II)

As part of **Study II**, the correlation between antigen load, as antigen concentration in stool, and stool consistency was studied. Antigen concentrations, as Ψ values, measured from the stools were plotted against the stool consistency. The CDI suspicion stools were collected from symptomatic patients as part of routine diagnostics in Vaasa Central Hospital, Finland, during May to September 2017. There were 38 GDH and 30 toxin A/B positive samples. Consistency of the stool samples was categorized as watery, loose, or solid.

4.5.2 Follow-up of coronavirus infections (III)

Individuals suspected of respiratory tract infection were sampled and tested in order to follow up on the acute phase of infections. Individuals self-sampled

nasopharyngeal swabs (553C, Copan Italy) during the course of infections after having obtained informed consent. The sampling was done to the posterior nasopharyngeal wall by swirling the swab in the nasopharynx at a depth of 8–12 cm. The swabs were stored at +2 to +8°C for a few days or frozen at -20 °C for longer times if not analyzed immediately after sampling. The specimens were processed following the manufacturer's instructions by suspending the swabs in 1.3 mL of the mariPOC RTI sample buffer and vigorous vortexing for 15 seconds. The samples were analyzed with the mariPOC Respi+ test (test plate product code 1194M, ArcDia Int.), including the test reagent for SARS-CoV-2 developed as part of the **Study III**. The Respi + test also included test reagents for coronavirus OC43, influenza A and B viruses, respiratory syncytial virus, parainfluenza virus 1 to 3, metapneumovirus, adenovirus, and *Streptococcus pneumoniae*.

5 Summary of Results

5.1 Development of antigen detection tests

5.1.1 Test reagents (I)

In **Study II** and **Study III,** test reagents for the detection of *C. difficile* GDH and toxin A/B from stool samples and SARS-CoV-2 from nasopharyngeal excretions were developed, respectively. Antibody-coated microparticle count, which provides optimal antigen capture area and practical frequency of the microparticles for the fluorescent measurement of the assay reaction, was defined in a proof-of-concept (**Study I**). While a nanomolar tracer concentration was used in the proof-of-concept study and for the SARS-CoV-2 test reagent, up to one-fold higher concentration was used in the actual study for the *C. difficile* test reagents.

Stool may contain microbial antigens in extremely high concentrations. In a separation-free immunoassay, such a concentration may cause a hook effect, leading to a falsely low or negative test result. Therefore, a higher tracer concentration was used to increase the test reagent capacity and to minimize the high dose hook effect. Using a higher tracer concentration had no effect on analytical sensitivity because the reagent-based fluorescence merged with the inherently rather high fluorescence background from the stool matrix.

Test reagents were dried into a 384-well microtiter plate without affecting the immunological activity of the antibodies. Immunoassay activities of the dried reagents were 90 to 110% when compared to the reagents before drying, and were similar for all the developed test reagents. The plates were covered with a multilayer mariPOC sealing tape. The test reagents showed no cross-reactions with the studied microbes. Studied substances, potentially present in stool or in the nasal cavity, had no interference with the test reagents, except for medical carbon. Medical carbon, added to the stool, caused fluorescence inhibition that led to rejection of the analysis result by autoverification of the mariPOC test system.

5.1.2 Sample pretreatment for feces and urine (I, II)

Urine does not contain particulate material, per se. Therefore, only diluting the urine with a sample buffer was enough. Diluted urine gave clear yellow solutions with less intense color than undiluted urine. Stool contains particulate material, which needs to be removed. Centrifugation of stool suspensions for 5 min at 1000 × g gave clear yellow-brownish solutions, removing most of the solid material (**I**), but did not remove enough particulate material from all of the samples. After centrifugation, around one-tenth of the stool samples had particulate material that caused so-called excess triggering in the TPX measurement. Triggering means small flashes of particulate material in the optical focal point that may lead to unsuccessful analysis as the actual particles are masked by the particles arising from the sample (Figure 6). Basically, it means that small particles are constantly detected, interfering with actual particle measurement. In addition, solution background fluorescence cannot be defined reliably because backscattering exceeds all the time the threshold for particle measurement. If the exogenous particles are similar to test reagent microparticles in size, they are potentially measured similarly to reagent particles and interfering with the analysis. Even centrifugation with higher force at 10,000 × g did not remove the issue. Filtration of stool suspensions through a multilayer syringe filter having a 0.2 µm final pore size removed all possibly interfering solid and particular material in the case of each sample. This enabled a low failure rate of 1.4% for the analysis of stool samples (**II**).

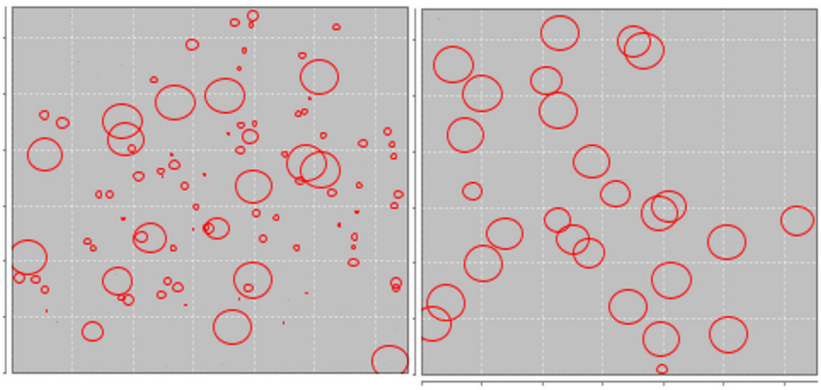

Figure 6. Particle position in the well in the square measurement area of an ArcDia TPX assay. The width of the circle is relative to the time that the particle has been trapped in the focal point by optical forces. Small circles (left) are triggers that are small particles that interfere with the trapping of the test reagent solid-phase carrier microparticles. On the right is a particle measurement without triggering.

5.2 Suitability of urine and stool for separation-free immunoassay utilizing two-photon excitation fluorometry

5.2.1 Effect of stool and urine matrix on two-photon excitation fluorescence measurement (I)

The effect of stool and urine matrices on the fluorescence detection and microparticle trapping in the separation-free TPX assay was studied. Dilution series from both matrices were prepared in the sample buffer. Diluted urine or stool supernatant was mixed with the test reagent mixture. Fluorescence from the reaction wells was measured kinetically without any separation of unbound and bound reagent fractions or potentially interfering matrix.

When test reagent specific analytes were not present in the sample, stool supernatant and urine elevated both immunocomplex specific microparticle and solution fluorescence signals as a function of matrix concentration. When the solution fluorescence (F_S) was subtracted from the microparticle fluorescence F_{MP} signal obtained from the same well, the subtraction ($F_{MP} - F_S$) was around zero, and the subtracted fluorescence remained clearly below the cut-off in all analyte-free urine and stool dilutions. The cut-off values were determined as three times the standard deviation of reactions with the highest matrix concentration (two times dilution) of the analyte-free urine or stool (**I**; Fig. 1). Based on hundreds of stool samples analyzed in **Study II**, stool and reagent matrix (solution phase) fluorescence were typically in the thousands of counts per second (in TPX units) while clinical cut-offs for subtracted specific signal were in hundreds counts per second. After the filtration of stool samples, the matrices had no effect on the microparticle trapping.

5.2.2 Specific antigen detection in the presence of urine or stool matrix (I)

The effect of urine and stool matrices on the separation-free immunoassay was studied in recovery assays. Analyte-free samples were spiked with specific antigen, and a dilution series was prepared for analyte-positive samples. The recovery of carbohydrate antigen from urine was around 200% and 100% when urine was diluted 1:1 and 1:32, respectively. This unexpected recovery was repeatable while the possibility for unspecific binding was excluded. The recovery of protein antigen from stool was 85%, 88% and 99% when the stool matrix was diluted by a factor of 10, 20, and 80, respectively. Optimal sample dilution and the ability to detect infection-originated antigens in urine and stool were studied using reference

test positive samples in a dilution series. The signal changes ($F_{MP} - F_S$) with respect to sample dilution were linear. The highest specific fluorescence signals were observed with the lowest urine dilutions. Therefore, there was no indication of general immunoassay inhibition. The dilution series for positive stool samples showed a typical hook-effect in separation-free assays (**I**; Fig. 2). Further optimizations showed that around 17-fold dilution was practical for all forms of stools, providing the highest sensitivity in the assays.

5.3 Clinical validation of mariPOC tests

5.3.1 CDI test (II)

In **Study II**, clinical performance of the CDI test was evaluated at Vaasa Central Hospital in Finland in 2017. The median age of the patients was 74 (13–98) years. Performance of the CDI test was evaluated against the TechLab C. diff Quik Chek Complete (Alere Inc.; now Abbott) membrane enzyme immunoassay (MEIA) and GenomEra *C. difficile* PCR assay (Abacus Diagnostica Oy, Finland). Because of the complexity of the diagnostic algorithms and possible alternative approaches to diagnose CDI (Crobach et al., 2016), the CDI test was evaluated for the detection of detectable proteins and for the detection of the presence of toxigenic strains.

In total, 157 specimens were tested with the mariPOC, TechLab, and GenomEra in order to compare the two phenotypic methods. In detecting GDH, the sensitivity of the mariPOC was slightly lower (95.2%) than that of TechLab (100.0%), but no toxin-positive or toxigenic cases were missed by the mariPOC test, as the missed GDH cases were negative by PCR. The mariPOC test found all toxin A/B-positive samples in this cohort (100.0%), while the TechLab test found 87.1%. According to the PCR results, 86% (36/42) of *C. difficile* findings by GDH detection were toxigenic strains (**II**; Table 1).

In total, 337 specimens were tested with the mariPOC and GenomEra in order to compare the methods for the detection of toxigenic *C. difficile*. For the detection of toxigenic *C. difficile* strains, the sensitivities of the mariPOC and TechLab GDH tests and GenomEra PCR were 94.7%, 94.7%, and 97.4%, respectively. When comparing phenotypic and genotypic methods in order to exclude colonization with a toxigenic strain, NPVs obtained with the mariPOC and TechLab GDH tests were 99.3% and 98.3%, respectively, which were slightly lower than the 99.7% NPV obtained by GenomEra PCR. Compared with toxin B gene detection, the sensitivities of the mariPOC and TechLab toxin A/B tests were 81.6% (31/38) and 71.1% (27/38), respectively. The mariPOC test and GenomEra PCR had the highest analytical PPV (100.0%) for toxigenic *C. difficile* (**II**; Table 2). The specificities of the mariPOC and TechLab tests presented in the table are against

toxin gene detection and do not represent true analytical specificities for GDH because toxigenic PCR cannot be used to rule out GDH positivity. In the whole cohort, the true analytical specificities of the mariPOC toxin A/B and GDH tests were 100.0% (306/306) and 98.3% (290/295), respectively, when specimens positive by PCR only were regarded as being GDH and/or toxin A/B negative.

The GDH test reagent detected all studied ribotypes, and the toxin A/B test reagent correctly detected all studied toxinotypes. The GDH test reagent did not react with *Clostridium* species other than *C. difficile*, but the toxin A/B test reagent detected the toxins produced by *Clostridium sordellii*, as both species share similar toxins. In addition to the species in the original **Study II**, the test reagents did not react with *Clostridium clostridioforme* or *Clostridium orbiscindens* (*Flavonifractor plautii*), which were tested later on.

5.3.2 SARS-CoV-2 test (III)

Clinical performance of the mariPOC SARS-CoV-2 test was evaluated in **Study III** with three specimen cohorts. Firstly, the 100% (203/203) clinical specificity was verified by analyzing freshly sampled nasopharyngeal swab specimens in Pori, Finland, in February 2021. Secondly, clinical sensitivity was verified by analyzing two sets of RT-qPCR-positive nasopharyngeal swab samples. Thirteen consecutively positive samples were collected from patients visiting primary healthcare COVID-19 drive-in stations in the Helsinki capital area of Finland from March to April 2020. Forty-five samples with known RT-qPCR Ct values (16 to 34) were received from the frozen specimen library of the Finnish Institute of Health and Welfare, Helsinki, Finland.

The sensitivity of the test was 100.0% (13/13) in cohort 1, where the nasopharyngeal swab specimens were suspended directly into the sample buffer or first into saline. In cohort 2, the sensitivity was 84.4% (38/45) when the nasopharyngeal swabs were initially suspended in undefined transport media and further diluted with the sample buffer (**III**; Table 2). This dilution diluted the specimens 4 to 20 times (2 to 4.3 PCR Ct units) more than the recommended sample pretreatment. This lowered the apparent sensitivity. Despite the small specimen count in cohort 1, both cohorts had similar statistical reliability based on 95% confidence intervals.

Sensitivity of the test and cumulative positivity rates of viral culture in comparison to different RT-qPCR Ct values were categorized (**III**; Table 4). The distribution of the Ct values in comparison to the mariPOC positivity were plotted (**III**; Fig. 2). The test showed 100% (31/31) positivity rate compared to the RT-qPCR for Ct values ≤ 28. Above the Ct value 28, the positivity rate of the mariPOC

declined as typical for an antigen test, reaching 91.9% (34/37) with Ct values ≤ 30. The lowest detected Ct value was 33.24.

The LoD was 2.7 $TCID_{50}$ per test in a 20 µL reaction volume for gamma-irradiation-inactivated culture supernatant (strain USA-WA1/2020). The LoD equals 1690 genome equivalents per test. The LoD, as RT-qPCR Ct value, was 33 for UV-inactivated SARS-CoV-2 culture supernatant. The test gave a negative result with seasonal coronaviruses (OC43, 229E, or NL63) and other tested microbes, but it gave a positive test result for the recombinant N-protein of SARS-CoV-1. Possible interfering agents in the nasal cavity had no effect on the test reagent.

5.4 Semi-quantitative antigen detection for clinical studies

5.4.1 Stool consistency in CDI diagnostics (II)

The diagnostic guidance for CDI by ESCMID recommends testing only unformed stool samples that take the shape of a container (Crobach et al., 2016). The correlation between the consistency of stool and *C. difficile* GDH and toxin A/B concentrations was studied as part of **Study II**. High GDH and toxin A/B concentrations were detected irrespective of the consistency of the stool. Statistical differences were not observed between different stool consistencies and analyte concentrations (t-test, all >0.1). Interestingly, the two watery samples that had high toxin concentrations had only low or moderate concentrations of GDH (Figure 7).

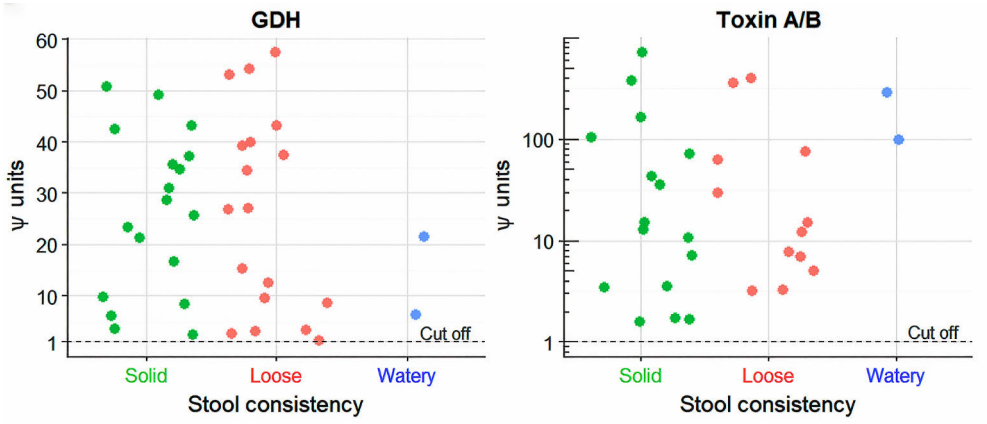

Figure 7. GDH (left) and toxin A/B (right) concentrations shown as the mariPOC Ψ unit in solid, loose and watery stool samples. Ψ is a multiple of the cut-off.

5.4.2 Follow-up of coronavirus infections (III)

In **Study III**, the developed SARS-CoV-2 test reagent as part of the multianalyte mariPOC Respi+ test was used in case studies to follow the viral antigen load in the nasopharynx during infections in Finland. Sampling was started already before the onset of the symptoms when infection was suspected because of exposure to an infectious family member or as part of infection management testing. The primary focus of the study was on SARS-CoV-2, but also coronavirus OC43 infections were encountered. The results show that coronavirus antigen load peaks rapidly already in the presymptomatic phase and at the latest during the first symptomatic days. Then the viral antigen load starts to decline within a week until becoming negative at the latest within two weeks (Figure 8). In these cases, the positive test result on the presymptomatic day prevented further infections at ground zero as the individual was expelled. The SARS-CoV-2 positive individual had received two doses of the SARS-CoV-2 Wuhan strain vaccines, and the omicron strain was prevalent at the time, suggesting escape previous immunity (Figure 8B).

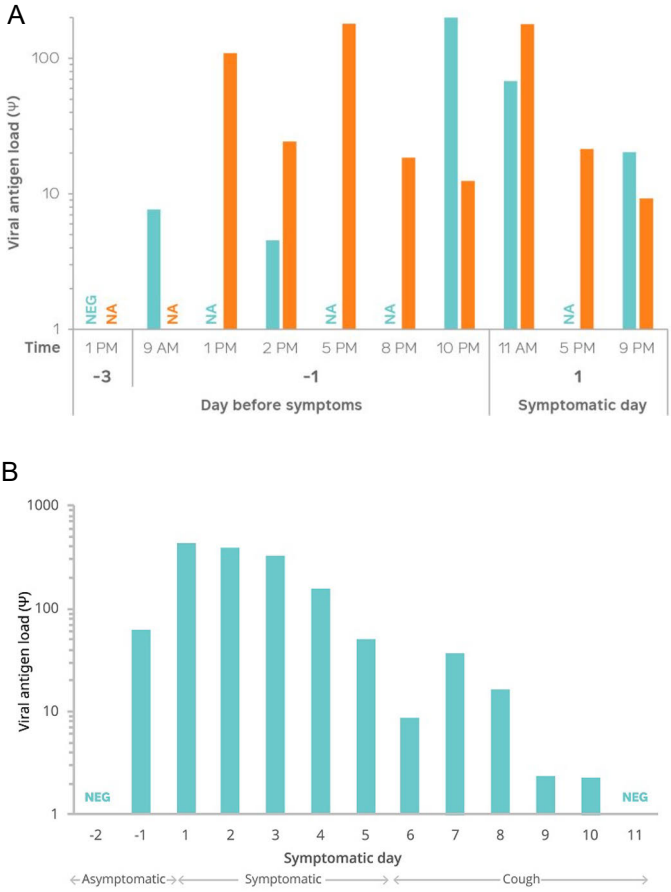

Figure 8. A) Viral antigen load (Ψ) at the beginning of coronavirus OC43 infection for two individuals (turquoise and amber) in late 2021. B) Viral antigen load on each day during the acute phase of SARS-CoV-2 infection around New Year's Eve 2021. Antigen load is shown as the mariPOC Ψ value, which is a multiple of the cut-off. NA, no sample was available at that time point. NEG, negative test result.

6 Discussion

6.1 Antigen detection from feces and urine with two-photon excitation assay technique (I, II)

The aim of **Study I** was to show the proof-of-concept and applicability of the two-photon excitation fluorometry assay technique for the detection of biomolecules from urine and feces. The methodology has a unique property to compensate for matrix effects, enabling the differentiation of a specific fluorescence signal from elevated background fluorescence. As hypothesized, stool and urine contained substances that elevated fluorescence levels in the TPX technique. However, the methodology's unique property to compensate for matrix effects enabled successful analyses. **Study II** showed that the developed protocol provided reliable and accurate results in comparison to routine clinical laboratory methods. In addition, the product development of the developed commercial IVD CE-marked tests was audited for compliance with the ISO 13485 standard by Lloyd's Register Group Limited.

By the time the CDI test was developed (**Study II**), antigen detection and culture were still the most utilized diagnostic methods for CDI. However, diagnostic methods started to rapidly shift towards gene detection, which provides much higher analytical sensitivity (Brendish et al., 2015). Higher sensitivity was driven and desired even though it led to lower clinical specificity in indicating the pathological stage of the disease (Kang et al., 2004; Atmar et al., 2008; Prill et al., 2012; Polage et al., 2015; Cevik et al., 2020; Singanayagam et al., 2020).

Clinical performance and usability of the mariPOC CDI test were evaluated in Finland in **Study II**. The test was further evaluated in an independent study in the Czech Republic by Krutova et al. (2019). Both of the studies utilized the mariPOC Gastro+CDI combination test in accordance with the diagnostic guidance by the ESCMID Study Group for *C. difficile* (Crobach et al., 2016). The studies validated that the TPX assay technique can provide accurate results from stool samples. The tests detected cases similarly to other antigen detection tests routinely used in diagnostic laboratories at the evaluation time. Despite the complex and challenging stool matrix, the specificity of the tests was very high. Specificity for toxin A/B detection in the two studies was 100.0% and 99.2%. Specificity for the GDH test

was 95.7% and 95.2%. The cause for lower specificity for the GDH test is not fully known. Reasonable immunoassay signals suggested true specific detection of GDH, but other tests were negative. Nevertheless, from a diagnostic point-of-view the specificity of the GDH test can be lower because, in any case, GDH is present in both the toxigenic and non-toxigenic strains of *C. difficile*. In GDH detection, high sensitivity is needed to screen and catch possible CDI cases for further evaluation about of toxin production or demonstrating the presence of a toxigenic strain. The GDH test had similar sensitivity to RT-qPCR (97.3%) and toxigenic culture (96.4%). The toxin A/B test had higher sensitivity when compared to another toxin detecting test. The mariPOC toxin A/B test gave 14.8% to 20.0% more true positive findings. In comparison to toxigenic culture and RT-qPCR, the sensitivity of the toxin A/B test was 66.7% and 83.8%, respectively. The sensitivities and specificities were the state of the art when compared to other antigen detection tests widely reviewed by Crobach et al. (2016).

In contrast to GDH detection, high specificity is important for the toxin A/B detection, as true positivity has high PPV for CDI. False positive toxin A/B result or not evaluating toxin A/B expression, when utilizing solely gene detection methods, may lead to great overdiagnosis (Polage et al., 2015). The detection of toxins produced by *C. sordellii* is common among *C. difficile* toxin A/B tests on the market. *C. sordellii* produces hemorrhagic toxin and lethal toxin, which have a high homology with *C. difficile* toxin A and toxin B, respectively. Cross-reaction with *C. sordellii* toxins is a known property of toxin A/B antigen tests. However, cross-reactivity studies showed that the developed GDH test does not detect *C. sordellii*. Both the GDH and the toxin A/B tests must be positive to confirm the positivity for CDI. GDH-negative but toxin A/B positive test result may suggest *C. sordellii* infection. When assessing CDI management, the Gastro+CDI test had 99% NPV and 98% PPV for CDI while additionally providing alternative causative pathogens for non-CDI cases as recommended by the European guidance (Crobach et al., 2016). Toxigenic culture and toxin gene detection by NAAT reflect the presence of a toxigenic strain potentially causing disease, while the presence of toxin A/B antigen in stool reflects the disease causing agents. Thus, the phenotypic toxin A/B antigen test or cell cytotoxicity assay should not even agree 100% with the toxigenic methods.

Specificity of the mariPOC Gastro test for viral infections was ≥99.5%. Specificity of the *Campylobacter* test was 99.7%. Some samples that were positive for *Campylobacter* in the Gastro test but negative by culture were also positive with an RT-qPCR (Krutova et al., 2019). Routinely used campylobacter plate cultures are specifically detecting only *C. jejuni* and *C. coli*. The mariPOC campylobacter test reagent detected also *C. hyoilei* and *C. upsaliensis*, and, therefore, can give a positive result for other *Campylobacter* species than *C. jejuni*

and *C. coli*. Multiple *Campylobacter* species are associated with gastroenteritis (Man, 2011).

The diagnostic guidance for CDI by ESCMID recommends testing only unformed stool samples that take the shape of a container (Crobach et al., 2016). This is mainly because of the risk of detecting genetic material in bacterial carriage, and is not well documented for toxin A/B antigen detection. As part of **Study II**, the correlation between the consistency of stool and *C. difficile* GDH and toxin A/B concentrations was studied. High GDH and toxin A/B concentrations were detected irrespective of the consistency of the stool (Figure 8). The result encourages further studies to assess the diagnostic value of GDH and toxin A/B quantification, and the significance of stool consistency in CDI management.

The capability of the TPX technique to detect antigens in stools was applied to study whether SARS-CoV-2 antigen is present in fecal samples of subjects in the acute disease phase. According to the results, the virus was not detected in stool samples, not even when the virus was peaking in the nasopharynx in the first days of the infection. An antigen spiked freshly into stool was detected, verifying the validity of the assay method for stool specimens. The result is in line with the studies that were unable to detect viable SARS-CoV-2 from stool samples (Kim et al., 2020; Wölfel et al., 2020; Sia et al., 2020). These data strengthen the evidence that the virus is not associated with gastrointestinal infection (Wu et al., 2020), although the presence of viral RNA and N-protein has been detected from gastrointestinal tissues by RT-qPCR and immunofluorescent staining, respectively (Xiao et al., 2020).

6.2 Performance and utility of SARS-CoV-2 test (III)

Before the COVID-19 pandemic, the first commercial IVD CE-marked rapid antigen test for coronavirus OC43 was developed for the mariPOC platform. The test was used to study the coronavirus infection course and antigen load during the acute phase (Bruning et al., 2018, Figure 8). The coronavirus OC43 test and the infection course studies formed the basis for the quick development of the mariPOC SARS-CoV-2 test as part of **Study III** to respond to the emerged RTI pandemic.

During the initial phase of the COVID-19 pandemic, there was much concern about the antigen test's ability to detect positive cases with sufficient sensitivity and against new variants, although genetic sequences (targets of NAATs) are more prone for variation than amino acid sequences. Over the course of the pandemic, the worries were shown to be unfounded. The mariPOC SARS-CoV-2 test detects a conserved epitope from the N-protein because it detected also the SARS-CoV-1

as well making the test robust against emerging (spike protein) variants. The test showed similar sensitivity across SARS-CoV-2 variants (Gunell et al., 2022; Krutova et al., 2022). In general, the N-proteins of viruses are highly conserved. Particularly, in the case of SARS-CoV-2, deep mutational scanning suggests that variation in the N-protein does not affect antigen tests' ability to detect current and emerging SARS-CoV-2 variants (Frank et al., 2022).

As the test is basically an immunometric assay, the antigen detection reaction by antibodies follows reaction kinetics according to the law of mass action. The massive demand for SARS-CoV-2 diagnostics, together with the development of techniques to obtain higher-affinity antibodies during the last decade, provided specific antibodies with unprecedented affinities. This enabled the development of antigen detection tests with sensitivity close to RT-qPCR LoD. The LoD of the test was as low as 2.7 $TCID_{50}$/test in a 20 µL reaction volume, corresponding to Ct 33, at the immunoreaction equilibrium. Sensitivity around Ct 33, ranging from Ct 27 to 34, has been reported for some other rapid antigen tests (Routsias et al., 2021).

Specificity of the mariPOC SARS-CoV-2 antigen test, among the three evaluation studies, was a superior 99.94% (1737/1738) (**III**; Gunell et al., 2022; Krutova et al., 2022). Among published peer-reviewed studies, the average specificity of commercial SARS-CoV-2 antigen test worldwide has been reported to be 99.1% and 99.7% for symptomatic and asymptomatic individuals, respectively (Dinnes et al., 2022). Specificity of the test was clearly over 98%, which was the European Commission's minimum requirement for specificity (European Commission, 2021). This is a reasonable requirement in order to minimize false positive results. In contrast to the demands for antigen tests, there were no requirements for NAATs to show the clinical significance of the high Ct value results. Due to the high specificity of the mariPOC tests, a positive result is a strong indication of etiology, and there is no need to verify the positivity, for example, by NAAT. As a matter of fact, the positive NAAT cases should be evaluated for the presence of antigens to show actively replicating viruses and the acute phase of the particular infection. This is actually recommended by some commercial NAAT manufacturers (bioMérieux, 2021). Otherwise, a positive NAAT result may lead to a false diagnosis that may have negative consequences when heavy quarantine acts are implemented (Surkova, 2020) or another etiological agent may go undetected as the diagnostic triage was stopped. Likewise, a single diagnostic test result alone should not lead to a diagnosis without clinical assessment. The diagnosis of acute infectious diseases is not absolute, and there remains a possibility of false diagnosis.

Overall sensitivity of the mariPOC SARS-CoV-2 antigen test ranged from 71.5% to 93.2% when compared with RT-qPCR up to Ct 40. This is expected, as NAAT provides positive results that are not indicating the acute phase of an

infection and the presence of viable viruses able to infect cells. The presence of viable viruses in the specimen starts to decline rapidly in samples with higher Ct values than 25 (La Scola et al., 2020; Singanayagam et al., 2020; Jaafar et al., 2020). The European Commission had a minimum requirement for sensitivity of ≥90% for samples with Ct ≤25 (European Commission, 2021). The mariPOC sensitivity was 99%, on average, for samples with Ct 25 and below, which was clearly above the minimum requirement (**III**; Gunell et al., 2022; Krutova et al., 2022). As antigen detection is highly dependent on high quality sampling, poor sampling may reduce the apparent sensitivity significantly.

Antigen detection methods detect directly the concentration of antigen in the sample whereas NAAT multiplies the target sequence. The genome multiplying reaction may be affected by inhibition and other factors. Therefore, Ct values correlate with viral concentration at the cohort level but do not provide reliability for individual samples (Evans et al., 2021). Above Ct 33, which is about the theoretical LoD for RT-qPCR, the recovery of viable virus is random (La Scola et al., 2020; Singanayagam et al., 2020; Jaafar et al., 2020). The mariPOC SARS-CoV-2 test was able to detect single test subject cases where Ct was around 33 (**III**, Krutova et al., 2022), which is in line with the determined LoD of Ct 33. Antigens were detected in 93.6% and 88.3%, on average, of the RT-qPCR positive cases up to Ct 30 and 33, respectively (**III**; Gunell et al., 2022; Krutova et al., 2022). These are similar figures to those described for many other antigen tests (Dinnes at al., 2022). Reported sensitivities for the same test can vary significantly, as the obtained sensitivity of the antigen test is often more affected by the study setting and patient cohort than the test's actual sensitivity. For example, NAAT, which is often used as the comparison method, can detect traces of past infections, and then the antigen test should not even be positive, as there are no actively replicating viruses left (Cevik et al., 2020; Singanayagam et al., 2020; Albert et al., 2021; Mina et al., 2021; Mina & Andersen, 2021).

The state of the art SARS-CoV-2 test reagent can be utilized in a single-analyte test or as part of a multianalyte syndromic test. The multianalyte tests help to differentiate between SARS-CoV-2 and other viruses, such as influenza. In comparison to other antigen detection tests, such as lateral flow assays, the closed tube mariPOC test and the design of operational steps minimize specimen handling and possible exposure of the user to infectious material. Objective result read-out and LIS connectability minimize manual work and human errors, which can account for 3.7% of manual entries (Mays & Mathias, 2019), and provide flexibility and efficiency for the diagnostic process. During the pandemic over the counter tests for RTI became available from grocery stores. This was a huge change from previous practices, where RTI diagnostics were done by professionals in specialized laboratories. With sophisticated autoverification and automation, the

mariPOC test system is an independent, self-standing digital laboratory, and such digital test systems are paving the way for decentralized diagnostics.

6.3 Microbial antigens as markers of acute infection (II, III)

In this thesis, in vitro diagnostic tests were developed to provide information about the acute phase of the infection. In **Study II**, it was shown that the developed CDI test provides accurate information on whether an individual has clinical CDI or not in accordance with the European diagnostic guidance (Crobach et al., 2016). **Study III** showed that the performance of the developed SARS-CoV-2 test was the state of the art, with the link between antigen positivity and the acute phase (Figure 8). Through the review and analysis of scientific literature, the test positivity is strongly linked to active viral replication.

Based on the results and scientific literature, there is clear evidence that antigen positivity is a marker for acute infection and, vice versa, antigen negativity suggests that there is no significant viral expression. Antigen load, correlating with viable viral load, is high during the acute phase of the infection when an individual is the most infectious (Figure 8, Albert et al., 2021; Pekosz et al., 2021; Rusanen et al., 2021; Lopera et al., 2022). For this reason, there is no need for extreme sensitivity to detect these cases. Testing often with a less sensitive test (serial testing) rather than only once with a highly sensitive test will help to detect asymptomatic infectious individuals before the onset of symptoms and to contain the spread of the disease in the most efficient manner (Mina et al., 2020; Larremore, 2021; Soni et al., 2023). The same principle probably applies, depending on the case, for at least certain bacterial infections, and at least for C. *difficile*.

During the last decades, the diagnosis of acute infectious diseases has greatly shifted from phenotypic to genotypic methods. This has led to an increased number of extra findings, due to higher analytical sensitivity and prolonged shedding of genetic material as discussed in section 2.7. Usually, these do not lead to significant overtreatment (overdiagnosis) as the diseases are mostly self-healing and there are antimicrobials only for a few viruses. After all, the great majority of acute infections are treated, or left untreated, at the primary care level without any pathogen-specific diagnostic measures. Antibiotics are still often used just in case, despite the viral origin of the infection. Nevertheless, increased detection of infections by NAAT may mislead causality assessment and disease burden estimation. In addition, interpreting the results from low PPV multiplex NAAT with multiple simultaneous positive findings can be complex and may, therefore, have little effect on antibiotic prescribing in children, where bacterial infections are

common (Mattila et al., 2022). Metaphorically, in cancer, this kind of shift, where diagnosis is based only on genetic information without gene expression, could lead to overwhelming overdiagnosis and heavy unnecessary treatments, which are costly and even could be harmful to the patient's health. Unfortunately, many pathogens still lack antigen tests or, especially, highly performing tests. Thus, further development in the field of antigen diagnostics is needed to exploit the full potential of the methodology.

6.4 Role of rapid antigen detection in clinical microbiology

High-throughput automated NAAT analyzers have enabled and driven infectious disease diagnostics towards centralization. Centralized laboratory service can provide efficient specimen testing and optimized unit economics. However, this does not necessarily always enable efficient outpatient and infection management, especially during a pandemic or epidemic, when samples are shipped from surrounding residential areas. Although a high throughput central laboratory test would analyze samples within a few hours or minutes, obtaining a test result from sampling to result reporting to the clinician or the patient may take days. Delay is caused by logistics, both that of the physical sample and the results, and scheduled batch analyses. Sometimes, the information is outdated by the time the result is obtained. In general, a specimen provides information about the condition at the time of sampling. The condition might have changed when the test result is available. Delay may cause less impactful measures in the prevention of the spread of the disease or unnecessary quarantine/isolation acts. Certainly, delayed results are linked with suboptimal specific drug use, such as unnecessary use of or late prescription of antimicrobials. These lead to higher morbidity and mortality (Muthuri et al., 2014) and negative side effects. Delay may cause uncertainty and discomfort for the individual being tested, especially when the consequences of the test result heavily affects their life and near future plans.

Clinical diagnosis guidelines and practices of acute infections evolve tardily according to advancements in diagnostic methods. The laboratory diagnostic field is becoming more and more regulated. This will slow the introduction and implementation of new methods or even block methods that do not tolerate high regulation costs in the niche market. Heavy regulation may lead to a situation where the lack of diagnostics causes more harm than less controlled diagnostic products would. Depending on the characteristics of the pathogen and disease, a false test result has more or less impact on the outcome of the patient or individual, although most infections are handled without microbe-specific diagnostics. In the United States, a less demanding "emergency use authorization" regulation was

implemented for SARS-CoV-2 diagnostic tests at the beginning of the COVID-19 pandemic. The whole pandemic was successfully gone through with these loosened regulation without any major issues. At the same time, Europe in particular tightened the IVD regulation.

Rapid antigen tests provide timely, accurate results about the infection course (Mina et al., 2020). The reason why, for example, for SARS-CoV-2, antigen positivity indicates acute infection lies in the viral replication cycle. For coronaviruses, the prior expression and accumulation of the N-protein is essential for viral mRNA transcription (Wada et al., 2018). This means that N-protein load must increase prior to viral replication and therefore antigens should be well present in the beginning of the infection. A common assumption in clinical microbiology is that NAAT would detect viral infections well before antigen tests, and antigen detection is not sensitive enough. However, scientific evidence suggests otherwise (Mina et al., 2020; Mina et al., 2021; Mina & Andersen, 2021). The assumption favoring NAAT is based on mostly on the fact that RT-qPCR finds high Ct value cases. There is sparse clinical inspection of individual cases or published scientific literature on whether this detection has clinical significance. There is clear scientific evidence that viral load in the nasopharynx peaks prior to the onset of symptoms and is the highest during the first days of infection. Studies have shown that viral load starts to peak rapidly in 12 to 48 hours after being infected in RTI (Table 1) and gastroenteritis (Atmar at al., 2008). Symptoms developed most likely 12 to 24 hours after a viral load increase. This is reasonable as the symptoms are caused by the immune system reacting to the viral invasion in human cells and tissues. The high antigen load one day prior to symptoms supports the rapid onset of viral peaking from infection onset (Figure 8). Also, RT-qPCR Ct values suggest high viral load one day before symptom onset (Pan et al., 2020). These days pose the greatest risk for spreading the infection and, therefore, the most important window for rapid testing and infection control measures (Sohn et al., 2020). As the immune system clears the viruses, the viral loads start to decline after a few days until cleared within one or two weeks, which is the time for virus-specific immunoglobulin levels to become detectable (Figure 1). In summary, based on the scientific evidence, an individual with symptomatic disease is most infectious from the presymptomatic phase (one day prior to symptom onset) and the first symptomatic days when viable viruses and antigens are detectable until the clearance of the virus (Figure 8; Atmar et al., 2008; Carrat et al, 2008; Singanayagam et al., 2020; Basile et al., 2021; Killingley et al., 2022).

7 Conclusions

In this thesis, antigen tests utilizing two-photon excitation fluorometry were developed. The tests were implemented in the mariPOC test system (IVD CE), and their performance and usability were evaluated. **Study I** showed the applicability of separation-free two-photon excitation fluorometry for the detection of microbial antigens from feces and urine. **Study II** and **Study III** showed that the performance and usability of the developed tests were state of the art, with reasonable sensitivity, high accuracy, and specificity in comparison to other IVD tests and routine practices. A significant finding of this thesis is how precisely a specific fluorescence can be distinguished from the background fluorescence, which can be several times higher than the specific signal itself, with two-photon excitation fluorometry. Automated and rapid antigen detection, suitable for decentralized settings, allowed central laboratory-level analyses and clinically relevant results. The developed applications and test reagents are currently utilized in clinical diagnostics around the world. As a result of the high sensitivity and strong scientific evidence, the negative result of the mariPOC antigen detection is used to release COVID-19 patients from isolation. The developed tests provide tools not only for diagnostic testing but also for scientific studies assessing antigen load in the sample, which correlates reasonably well with viral infectivity.

More clinical studies are needed to evaluate the significance and optimization of different sampling methods and testing practices. Based on scientific literature and personal unpublished data, nasal swabs have shown good performance when compared to nasopharyngeal sampling in antigen test. Although saliva and throat swab samples may perform well in NAATs, those are suboptimal for antigen tests. The suitability of rectal swabs in gastroenteritis and CDI diagnostics by antigen tests remains a question. Although the developed toxin A/B test performed very well, the cell cytotoxicity neutralization assay may still provide higher sensitivity to detect the toxins. The neutralization assay requires laboratory skills and does not provide quick results. Current CDI guidance is against repeated testing (Crobach et al., 2016), although toxin A/B detection could possibly be used to evaluate treatment effectiveness and/or the course of the infection, similarly to, for example, coronavirus infections (Figure 8).

Based on the literary summary and follow-up of acute infections (Figure 8), it is reasonable to conclude that antigens are specific biomarkers of ongoing acute respiratory tract infection or gastroenteritis, and the benefits of their detection should be utilized to their full potential. In pandemic conditions, screening of asymptomatic individuals frequently enough every second or third day with antigen testing further prevents viral spread without the need for long quarantine measures of exposed individuals (Leng et al., 2021). Frequent testing with reasonably sensitive (low cost) tests has a greater impact on infection management than infrequent testing with slow and extremely sensitive (high cost) tests, as it finds the infections earliest (Mina et al., 2020; Larremore et al., 2021; Soni et al., 2023). While antigen detection has a high positive predictive value for ruling in an infection, it also has a high negative predictive value for ruling out infectivity (Basile et al., 2021; Pekosz et al., 2021; Rusanen et al., 2021; Pickering et al., 2021; Lopera et al., 2022; Kirby et al., 2023). NAATs remain the most sensitive tests with the highest negative predictive value for infection and the ability to detect recent infections. Spread of RTIs can be managed better when it is understood that it is people who spread the virus, not miasma, as was thought in the Middle Ages, through nasopharyngeal excretion from our noses and throats. The nasal cavity is extensively harbored with infectious viruses during the acute phase of the infection (Figure 8; Carrat et al., 2008; Killingley et al., 2022). The studies discussed in section 2.2.3 suggest that the route of viral RTI transmission occurs rather through direct contact with infectious material (picking up / wiping nose) and droplets through air (sneezing and coughing) than by indirect contact or through aerosols. As the detection of antigens correlates well with infectivity, the utility of antigen tests to assess infection transmission should be further studied.

Utilization of multianalyte antigen testing with short sample-to-answer time may enhance the management of infectious diseases, especially by recognizing contagious individuals. It might be impossible to stop the spread of acute infectious diseases at the population level, but utilizing proper management measures, the severity of the diseases can be lowered with early medication, and economical and social impacts can be minimized. Accurate diagnosis and having a real-time epidemiological picture available mean that individuals belonging to risk groups can be better protected from infections. Optimal diagnostics should lead to accurate diagnosis, which could optimize the use of antimicrobials and help in pandemic and infectious disease management. By doing so, radical measures, such as closing society, which have harsh countereffects, can be avoided.

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