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In this issue

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Foreword: Current laboratory aspects of COVID-19

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FOREWORD

At the time of writing this foreword for the "COVID-19" thematic issue of the eJIFCC, over half a billion Coronavirus Disease 2019 (COVID-19) positive cases and more than 6.3 million COVID-19 related deaths have been recorded worldwide emphasizing the global impact of this pandemic. We have already witnessed initial waves of the infection, and the vaccination campaigns in most countries have topped the near maximum number of immunized individuals, who opted to receive at least one dose of vaccine. On the other hand, we cannot predict recurrence of dramatic increases in the number of new (severe) cases in the future.

Since the beginning of the pandemic, huge efforts have been made for the development of effective diagnostic tools and strategies 1) to identify and isolate SARS-CoV-2-infected patients to control the pandemic, 2) to limit the risk of contamination, 3) to perform differential diagnosis between COVID-19 and other viral infections, such as seasonal flu, and 4) to treat patients effectively with any respiratory symptoms

to avoid serious consequences. Many biomedical companies and research laboratories have been working hard to develop competent and approved methods for the rapid detection of SARS-CoV-2 ribonucleic acid (RNA), antigens and antibodies [1].

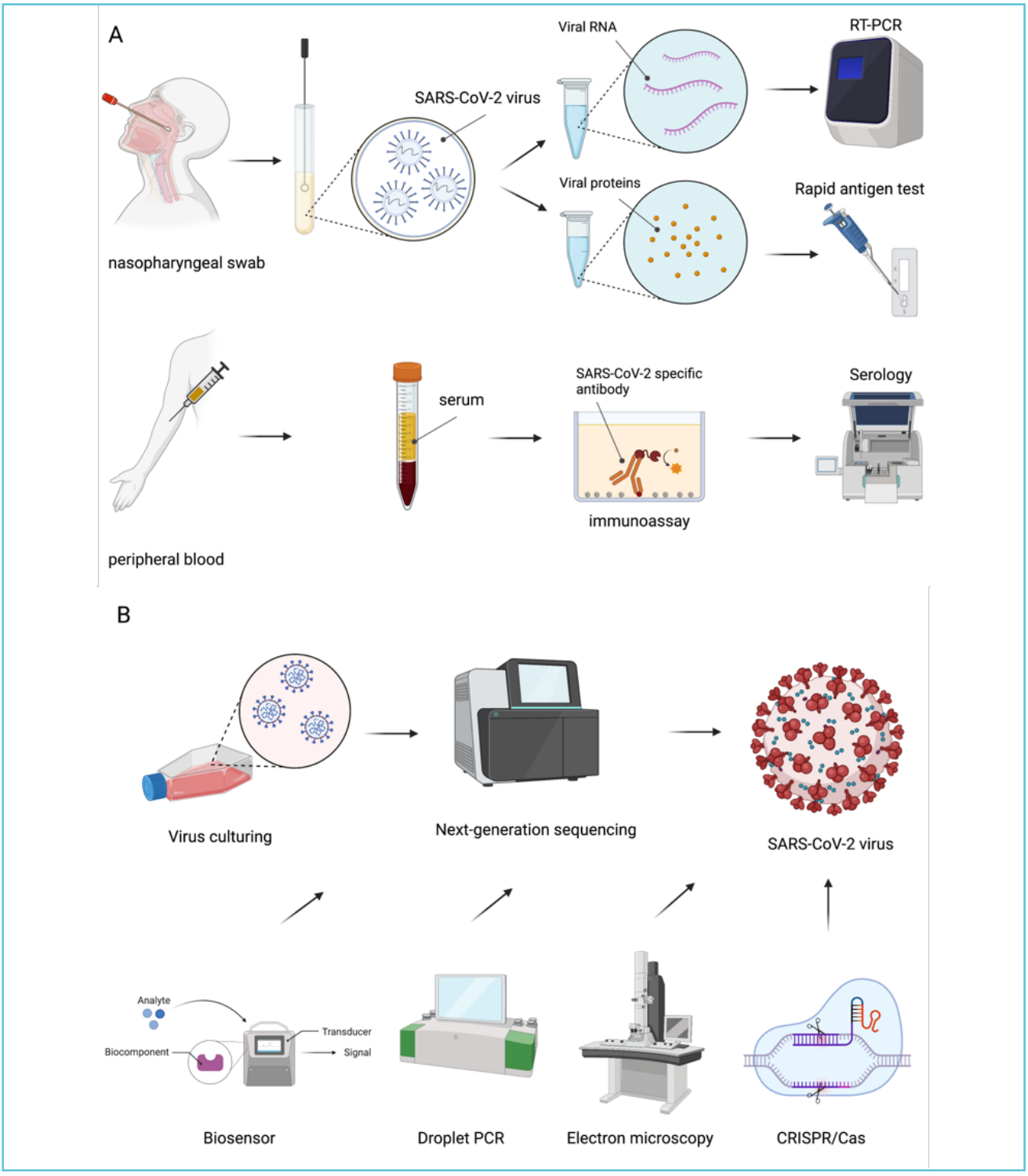
The efficacy of diagnostic laboratory tests to detect SARS-CoV-2 infection strongly depends on the timing of the testing [2]. Both molecular and serology tests are not useful in the early period (i.e., in the first week) of the supposed infection, because the virus is still in its incubation phase without sufficient levels of viral RNA, proteins or induced antibodies in the circulation. Therefore, the moment of the suspected infection, symptoms, medical history, and detailed physical examination need to be considered before testing to achieve the highest sensitivity of laboratory examinations. In the case of molecular tests, the highest chance for positivity can be reached after two weeks of the onset of infection, but we must know that there are no commercially available diagnostic tools with a sensitivity of 100%, especially in those asymptomatic cases the viral load is low. Repeated nasopharyngeal swabs on 2-3 consecutive days may be effective in overcoming the window period of SARS-CoV-2 incubation. Nonetheless, the positivity ratio is reduced over time due to the elimination of the virus and the remission of the disease [3]. Regarding serological tests, SARS-CoV-2 specific IgM and IgG antibodies become detectable after one month following the presumed infection, but the level of IgG remains elevated for at least 6-8 months in most cases [4]. Finally, increased level of viral proteins in nasopharyngeal swabs can be measured for the early diagnosis of COVID-19 infection by rapid antigen test, however, RT-PCR should be performed in those suspicious cases when this test was negative [1]. Approved, regularly used laboratory techniques for the diagnosis of SARS-CoV-2 infection are depicted in Figure 1A.

Besides conventional laboratory methods, additional diagnostic tools have also become available in this field. Virus culturing and next-generation sequencing (NGS) methods have been applied to identify the novel coronavirus and to characterize its molecular structure [1]. Currently, droplet digital PCR, clusters of regularly interspaced short palindromic repeats/Cas (CRISPR/Cas)-based methods, electron microscopy, biosensor, etc., can support the diagnosis of COVID-19 infection, which are under validation in routine laboratory and research settings [1]. These other diagnostic methods and the still research-related tools are depicted in Figure 1B.

Recently, the importance of clinical laboratory tests has also emerged 1) to manage the hospitalization of patients with COVID-19 related disorders, 2) to distinguish severe and non-severe clinical states and 3) to predict the outcome of the disease. For the aforementioned purposes huge amount of clinical data has accumulated and is elegantly summarized by Tomo et al [5]. Several serum and plasma biomarkers have been identified as independent risk factors to assess disease severity and to predict unfavorable outcome of COVID-19, such as elevated activity of total lactate dehydrogenase (LDH) isoenzymes [6], high soluble ACE2 activity [7] and increased D-dimer [8]. In addition, serological tests aid in the evaluation of the humoral response following different types of vaccines [9], but they can also estimate the incidence of SARS-CoV-2 infection in those patients with newly diagnosed malignancy and under anti-cancer therapy [10].

This current thematic issue of the eJIFCC is constituted by a series of manuscripts submitted from various parts of the world and provides an overview on various laboratory aspects of COVID-19. The topics of the manuscripts range from recent clinical data from the laboratory considerations for reporting cycle threshold value in RT-PCR tests via the detailed analysis of routinely available laboratory parameters in

Figure 1 **Routinely available, approved diagnostic methods (A) and other laboratory tools with, as yet, research-related techniques (B) in the diagnosis of SARS-CoV-2 infection**



The figure was created using BioRender.com.

hospitalized COVID-19 patients as new prognostic biomarkers up to the comparison of different serological assays for the evaluation of humoral immune response. Based on the findings of previously published scientific literature and those presented in this thematic issue, it can very well be emphasized that there are no effective diagnostic procedures and therapeutic interventions without the 24/7 active role of routine clinical laboratories worldwide.

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Call for manuscript submissions for a thematic eJIFCC issue on "Pharmacogenetics and personalized therapy"

Guest editors for the thematic issue:
Ron H. N. van Schaik and Sanja Stankovic

Pharmacogenetics (PGx) is one of the core elements of personalized medicine. PGx information reduces the likelihood of adverse drug reactions, minimize the potential risk of toxicity and optimizes therapeutic benefits. The concept of PGx-guided therapy integrates DNA profiling and data analysis using bioinformatic tools, leading to result interpretation and decision-making to offer the best available therapy. Dosage adjustments according to the patient's genotype for some drugs have already been implemented into clinical guidelines and drug labels contains PGx information, enabling safer and cost-effective treatment.

With an eJIFCC issue dedicated to this pharmacogenetics, we would like to offer new insights into the highly attractive field and its clinical relevance in most areas of medicine, trying to answer where we are now and where we should be heading into the implementation of PGx in clinical practice. We invite you to submit a paper on ***"Pharmacogenetics and Personalized Therapy"*** to be published in this thematic issue. Submitted papers will be peer reviewed according to the regular procedure of the eJIFCC Journal.

Important deadlines

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- Deadline for submission of the manuscript: **September 15, 2022**

Potential types of articles

- Original Article
- Critical Reviews
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Manuscripts need to be submitted by e-mail

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In this issue: Current laboratory aspects of COVID-19

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Laboratory considerations for reporting cycle threshold value in COVID-19

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ABSTRACT

The Coronavirus Disease 2019 (COVID-19) pandemic is caused by the SARS-CoV-2 RNA virus. Nucleic acid amplification testing (NAAT) is the mainstay to confirm infection. A large number of reverse transcriptase polymerase chain reaction (RT-PCR) assays are currently available for qualitatively assessing SARS-CoV-2 infection. Although these assays show variation in cycle threshold values (Ct), advocacy for reporting Ct values (in addition to the qualitative result) is tabled to guide patient clinical management decisions. This article provides critical commentary on qualitative RT-PCR laboratory and clinical considerations for Ct value reporting. Factors contributing to Ct variation are discussed by considering relevant viral life-cycle factors, patient factors and the laboratory total testing processes that contribute to the Ct variation and mitigate against the reporting of Ct values by qualitative NAAT.

1. INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is an enveloped RNA Betacoronavirus identified amongst patients with pneumonia in Wuhan City in China in December 2019 (1). SARS-CoV-2 is responsible for causing coronavirus disease 2019 (COVID-19), categorised as a pandemic by the World Health Organisation (2). Current statistics estimate that the pandemic has resulted in 518,368,648 globally confirmed cases, with global mortality estimated at 6,266,459 (3), with new variants of concern continuously emerging to date (4).

Nucleic acid amplification tests (NAAT) testing is an essential tool in detecting SARS-CoV-2 viral RNA in infected persons and is the reference standard for diagnosing infection and screening for viral variants of concern. The qualitative reverse transcriptase polymerase chain reaction (RT-PCR) molecular methodology is commonly employed to identify viral infection and is considered the gold standard for diagnosing positive cases of COVID-19. RT-PCR amplifies genomic structural and non-structural targets of SARS-CoV-2. The method is highly sensitive and specific at identifying viral gene targets. The various NAAT assays utilise automated and manual sample steps to improve analysis throughput. The proliferation of new assays shows variable assay characteristics and regulatory subscription (5).

RT-PCR tests for viral RNA detection can be reported qualitatively (positive or negative or equivocal) or quantitatively using the cycle threshold (Ct) value. Some assays also use a semi-quantitative output, for example, stratifying positive results as high or medium or low. The Ct value is the measurable number of output cycles that describe DNA amplification of the viral nucleic acid target (with background assay noise removed) (6) and thus functions as a cut-off point to identify positive viral nucleic acid present in the sample. The amplification is detected in the exponential phase, with no limitation of reagents, and the viral cDNA doubles with each PCR cycle. A threshold value can be manually or automatically inserted in the analysis of the result to identify the point at which exponential amplification is achieved. In the context of SARS-CoV-2, it identifies the presence of viral RNA for particular gene targets present in the viral RNA. Not all molecular techniques utilised to quantify SARS-CoV-2 produce Ct values; however, the RT-PCR method is the most ubiquitous assay methodology utilised in diagnosing SARS-CoV-2 infection and generates a Ct value. RT-PCR does not distinguish between detecting viable live virus shed in the sample from viral fragments of non-viable (non-infectious) virus present in the sample.

The reporting of the Ct value to indicate a proxy measure of the amount of virus (viral load) in qualitative RT-PCR analysis for SARS-CoV-2 for the diagnosis and care of patients infected with SARS-CoV-2 is controversial, with the majority of leading international guidelines recommending against reporting Ct values. This review will discuss the viral dynamics of SARS-CoV-2, the general design of the qualitative RT-PCR assay used to measure SARS-CoV-2 infection (highlighting important laboratory factors relevant to the interpretation of Ct values), and then examine the potential clinical and laboratory factors that impact the Ct value and its interpretation.

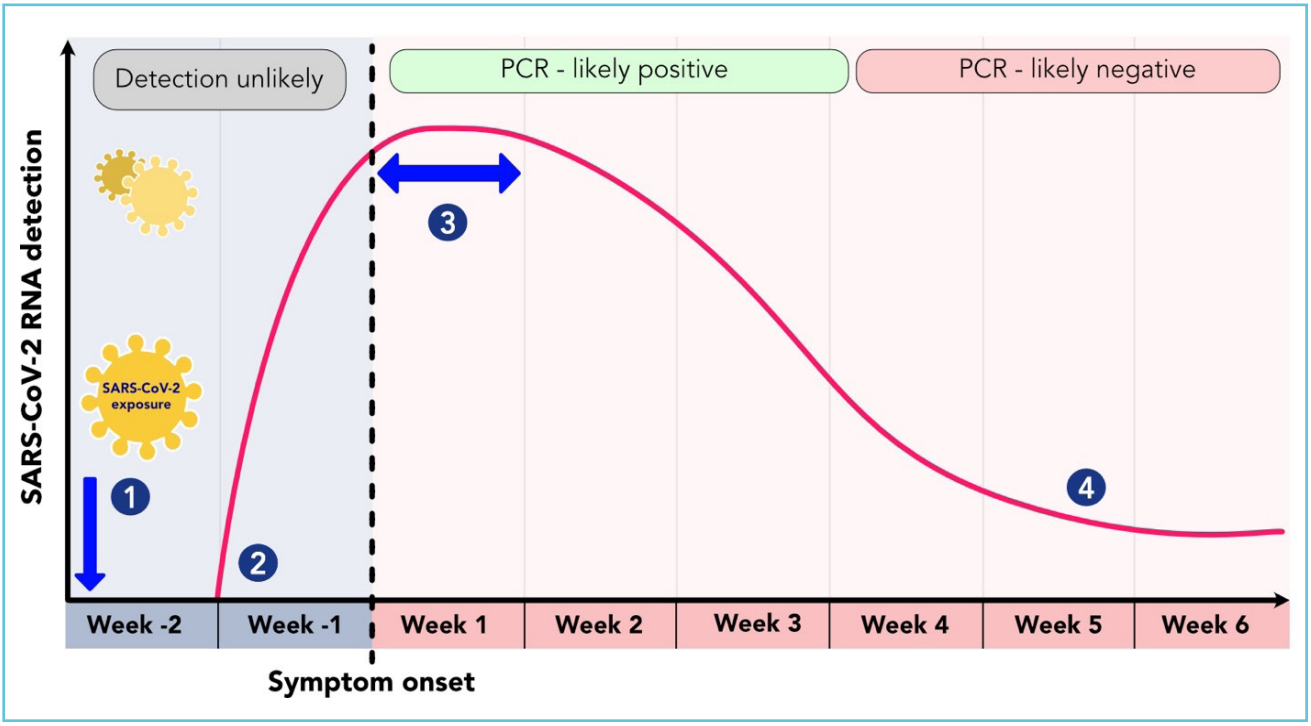
2. SARS-COV-2 VIRUS

SARS-CoV-2 is a single-stranded enveloped RNA virus belonging to the genus Coronavirus and the family Coronaviridae. The SARS-CoV-2 virion ranges in size from 70-90 nm, as evidenced by ultrastructural studies of virus-infected cells. The virus's genome is ± 30kb (26-32 kb) and compromises 6-11 open reading frames (ORF), which encode 9680 amino acid polyprotein. ORF one

constitutes 67% of the genome and encodes 16 non-structural proteins (nsps) compared to the remainder of accessory and structural proteins. The nsps include two viral cysteine proteases, including papain-like protease (nsp3), chymotrypsin-like, 3C-like, or main protease (nsp5), RNA-dependent RNA polymerase (nsp12), and helicase (nsp13). The four structural proteins include spike surface glycoprotein (S), membrane, nucleocapsid protein (N), envelope (E) and accessory proteins like ORFs (2,7). Specific structural and non-structural genes form RNA targets for molecular-based nucleic acid tests to identify SARS-CoV-2 viral infection in humans. Different molecular-based assays amplify single or multiple gene targets for SARS-CoV-2.

The life cycle of SARS-CoV-2 has been reviewed in human infection and is incompletely understood. In Figure 1, a summary of generalised findings in nasopharyngeal swab PCR is indicated (8-10). Systematic reviews and metanalysis indicate that the mean incubation is five to six days (range: 2-14 days). A definitive Ct cut-point that defines potential infectivity is unknown. Infectiousness factors include viral strain and the varying degrees of infectivity based on the characteristics of the virus variant of concern properties.

Figure 1 **Generalised schema of viral RNA detection of SARS-CoV-2 in nasopharyngeal specimens of infected individuals. The viral kinetic dynamic of SARS-CoV-2 is adapted from (8-10).**



Once infected individuals can progress through an asymptomatic incubation period followed by increased viral load; symptomatic phase and propensity to spread the virus, followed by symptom recovery. Four key points are essential in the viral kinetics: (1) Point of infection by SARS-CoV-2; (2) Virus detection starts to emerge and is dependent on patient factors and variant viral strain; (3) Symptom onset and period around symptom onset marks a highly infectious state; although asymptomatic spread is also evidenced; and (4) Late infection is marked by low transmission potential and high Ct values. This period is variable and dependent on patient factors, vaccination status and analytical considerations. The final temporal ranges and Ct values in infection are dependent on patient factors, specimen type and NAAT assay utilised in viral detection.

Host factors may also affect the persistence of the virus, and pre-existing immune status via vaccination or prior infection or disease severity may also affect the duration of infectivity (9). In general, patients with severe disease or immunocompromised status remain infectious for longer, in contrast to non-severe patients with viral infectiousness shown to be present for up to ten days from symptom onset.

Many studies have shown that viraemia achieves peak levels around the symptomatic phase of COVID-19 and then gradually tapers over weeks. Asymptomatic infection at laboratory analysis is documented in various settings, and the potential for transmission is high in communities (11, 12). Patient factors can influence absolute viraemia, and the degree of viral load is higher (lower Ct values) in severe disease and age greater than 60 years. The persistence of viraemia may affect patient outcomes and possibly the degree of infectiousness. Also, variants of concern have been reported to have shorter incubation periods than other variants to potentially affect transmission (4, 9, 10). Viral transmission is affected by viral load and viral shedding factors, amongst others. Viral load is very important in transmission, and higher viral loads are associated with increased secondary attack rates and also symptomatic disease progression (4, 13). The life-cycle and transmission potential highlight the importance of considering Ct values more holistically, considering clinical factors, symptomatology and time point post-infection. In addition, it is important to note that the detection of positive Ct values does not indicate definitive infectious potential.

The specimen type used for detecting SARS-CoV-2 can affect the Ct value. In a systematic review and metanalysis of viral shedding behaviour, the mean duration of SARS-CoV-2 RNA shedding was 17.0 days (95% CI 15.5-18.6; 43 studies, 3229 individuals) in the upper respiratory tract, 14.6 days (9.3-20.0; seven studies, 260 individuals) in the lower respiratory tract, 17.2 days (14.4-20.1; 13 studies, 586 individuals) in stool, and 16.6 days (3.6-29.7; two studies, 108 individuals) in serum samples. The maximum shedding duration was 83 days in the upper respiratory tract, 59 days in the lower respiratory tract, 126 days in stools, and 60 days in serum (14). Ct values thus are influenced by specimen matrix and this pre-analytical factor can determine spurious early diagnostic results, and persistent viral clearance in late infection or resolved infection.

3. GENERAL DESIGN OF THE SARS-COV-2 RT-PCR QUALITATIVE ASSAY

The SARS-CoV-2 virus is measured in the laboratory by NAAT, most commonly, the reverse transcription polymerase chain reaction (RT-PCR), which measures a cycle threshold (Ct) value that identifies viral infection. The Ct value quantifies the amount of viral cDNA present in the specimen, which is detected from the assay background. Ct represents a PCR cycle number point on the PCR amplification plot where viral cDNA is exponentially amplified under optimal assay conditions where reagent, temperatures and incubation times are non-limiting. This enables the viral cDNA to double with each cycle and increase by a factor of 10 for every 3.3 cycles (15).

The SARS-CoV-2 qPCR assay consists of three vital analytical steps: (1) viral RNA isolation, (2) cDNA synthesis and (3) amplification of target viral genes in the cDNA. The steps can be performed in a single tube (one-step reaction) or split into two steps where viral RNA is first transcribed to cDNA and then transferred to the amplification phase of the analysis (two-step reaction) (16).

Firstly, viral SARS-CoV-2 RNA is extracted from the sample. Viral RNA is detected in upper, lower and gastrointestinal specimens, with various viral RNA shedding patterns observed

(14). There is uncertainty regarding the optimal upper respiratory tract specimen type for RT-PCT testing. The Infectious Diseases Society of America Guidelines (IDSA) suggests a nasopharyngeal swab, a mid-turbinate swab, an anterior nasal swab, saliva, or a combined anterior nasal/oropharyngeal swab rather than an oropharyngeal swab because of limited data suggesting lower sensitivity with oropharyngeal specimens (17). Notably, the Ct value will be influenced by the sample type, where it may be challenging to identify the infectious potential and glean an estimate of high viral RNA expression in the sample.

Extraction of the RNA is then followed by the reverse transcription step which copies the RNA viral genome to form complementary DNA (cDNA) catalysed by reverse transcriptase. And, thirdly, specific viral genes for the SARS-CoV-2 are then amplified using the cDNA input template in the qPCR reaction to identify the presence or absence of viral genome expression in the sample. Higher amounts of viral cDNA in the sample produce lower Ct values.

The quantitative polymerase chain reaction is based on real-time monitoring of DNA or cDNA amplification from input viral nucleic RNA. The RT-PCR amplification is detected by intercalating double-strand dye or probe-based emission of fluorescence (which is released by digestion of the attached probe to the newly amplified DNA strand). The fluorescence signal is detected by detectors in the instrument on a cycle-by-cycle basis in real-time (usually across 40 cycles) - the fluorescence output signal increases in each cycle. The measurable fluorescent signal is proportional to the viral cDNA present in the sample. Thus quantification cycle (Cq) or the cycle threshold (Ct) value of an amplification reaction is defined as the fractional number of cycles required for the fluorescence to reach a quantification threshold (15, 18).

The generation of an interpretative signal from RT-PCR produces a fluoresce emitted signal, which is detected by the instrument and proportional to the number of viral genes in the specimen. Thus, individuals with high viraemia produce a signal that results in a rapid increase in fluorescence output than low viraemia, requiring more amplification cycles to emit a detectable quantifiable signal. The Ct value is inversely proportional to viral gene expression and thus may serve as a surrogate marker of viral load for SARS-CoV-2. Current qualitative assays do not enumerate a viral load as the assays do not run calibration curves using reference samples to derive a viral count in the specimen objectively. These tests are thus able to produce a qualitative result that laboratories report (positive, negative, indeterminate) guided by manufacturer or laboratory-based guidelines on interpreting the results and the Ct values.

In contrast, in quantitative RT-PCR, reference samples spanning a range of known genome copies are simultaneously run alongside patient samples for each RT-PCR batch of tests, and the Ct value measured for the patient is used to calculate viral load by comparing the Ct value of the patient to the reference sample curve. The raw Ct value thus is not reported, but the laboratory issues a quantitative genome copy number.

The targets for the viral genes include structural or non-structural genes and in different combinations. The assays demonstrate various detection limits and analytical sensitivity (Table 1).

The targets for different nucleic acid amplification testing methods can produce false-negative results for variant SARS-CoV-2 virus. For example, S gene target failure with the recent Omicron variant in some RT-PCR tests can occur. However, other targets will amplify, and the result can be reported as positive for infection, guided by the assay manufacturer's interpretative recommendations. Also, as not all RT-PCR

Table 1 Commonly utilised SARS-CoV-2 molecular tests target various viral genes and demonstrate variable limit of detection (LOD) and analytical performance (19-24)

SARS-CoV-2 test	Company	Genes targeted; Limit of detect (LOD)	Analytical performance
Alinity m	Abbott Laboratories, Abbott Park, IL, USA	N, RdRp; 100 copies/ml	100% Sensitivity 100% Specificity
Abbott RealTime	Abbott Laboratories, Abbott Park, IL, USA	N, RdRp; 100 copies/ml	100% Sensitivity 100% Specificity
Xpert® Xpress	Cepheid, Sunnyvale, CA, USA	E, N; 8.26 copies/mL	97.80% Sensitivity 95.60% Specificity
Cobas®	Roche Molecular Systems Inc, Branchburg, NJ, USA	ORF1, E; 25-32 copies/mL	96.10% Sensitivity 96.80% Specificity
TaqPath™	Thermofisher Scientific, Waltham, MA, USA	S, E, N; 10 GCE/reaction	93.50% Sensitivity 93.30% Specificity
Allplex™	Seegene Inc, Seoul, South Korea	E, N, RdRp 4; 167 copies/mL	100% Sensitivity 96.70% Specificity

Abbreviations of RNA viral targets: N, nucleocapsid; RdRp, RNA-dependent RNA polymerase; E, envelope; ORF1, open reading frame; S, spike protein.

assays will result in failed S gene target amplification in Omicron variant infection, interpretation of a positive Ct value for the S gene cannot rule out Omicron variant infection (25).

4. CLINICAL AND LABORATORY CONSIDERATIONS FOR REPORTING CT VALUES DERIVED FROM QUALITATIVE NUCLEIC ASSAY AMPLIFICATION TESTING

The potential use of Ct values in clinical applications to predict disease severity, assess individuals' infectious potential and determine re-infection is not clearly understood. Many studies have demonstrated higher expression of SARS-CoV-2 biomarkers of infection by RT- PCR with disease severity. For example, a systematic review of 18 studies concluded that lower SARS-CoV-2 Ct values were associated with worse clinical outcomes. In 57 % of studies (n=8), Ct values were correlated with disease severity. The authors concluded that Ct values might help predict patients' clinical course and mortality with COVID-19, pending further confirmatory studies (26). In another systemic review of RT-PCR analysis for SARS-CoV-2, 29 moderate quality studies were identified. Twelve studies identified a significant inverse relationship between Ct values

and positive viral culture. Also, symptom onset was related to Ct value and disease severity. Two studies also showed that viral culture positivity reduced by 33% for every increase in one Ct value unit (27). Clinical studies also support the quantitative evaluation of Ct values for the prognostication of adverse patient outcomes. SARS-CoV-2 viral load (as assessed by CT values) can predict patients' adverse clinical outcomes and more invasive management (28, 29).

In contrast, other clinical studies have not found an association between Ct values and patient outcomes and management. Shah et al. (30) observed that patients admitted with positive SARS-CoV-2 RT-PCR diagnosis failed to evidence a correlation between COVID-19 disease severity and mortality. Patients with mild disease showed lower Ct values than patients with severe disease. Furthermore, patients who died had significantly lower Ct values than patient survivors (30). Additionally, a systematic review and meta-analysis of seven clinical studies identified no significant association between hospitalisation and Ct value. This metanalysis showed an association between Ct value <25 and severe disease and mortality in comparison to Ct values >30; however, increased disease severity and mortality were less pronounced at Ct values of 25-30 compared with >30 (31).

Some studies have demonstrated that the utility of Ct values in patient prognosis is limited. Ct values have not been found to support the prognosis of COVID-19 disease in community patients and were insignificantly associated with worse outcomes (32). The administration of oxygen treatment to positive SARS-CoV-2 patients was not associated with Ct values. The investigators concluded that Ct values should not be used as an isolated indicator of patient prognosis (33). The utility of Ct values in solid organ transplant patients did not help predict COVID-19 disease severity (34). Repeated Ct value analysis at initial and nadir levels found no differences in prognosticating patient survival and disease severity and suggested that Ct values have limited use in managing COVID-19 disease (35).

The Food and Drug Administration (FDA) has acknowledged the use of serial testing of asymptomatic individuals to decrease false-negative results and has attempted to improve screening pathway tools (36, 37). The utility of sequential testing may afford some positive benefit in patient diagnosis, management and risk-stratification. Sequential testing of SARS-CoV-2 in a retrospective cohort analysis showed that a three-fold increase in Ct value correlated with a 0.15 improvement of the disease severity index score: Sequential Organ Failure Assessment (SOFA). This finding implicates the potential utility of sequential measurement of Ct values for prognosis in specific patient populations with COVID-19 (38). Serial testing has also been found to help diagnose individuals living in shared quarters who would be misdiagnosed if symptom screening or testing at only one timepoint were used. Therefore, serial testing can reduce transmission in congregated settings such as correctional facilities (39). Serial testing can also be valuable in identifying new infections and curbing SARS-CoV-2 spread in hospital settings (40). Interestingly, the utility of Ct values at a population level to identify changing trends in virus infectivity and the evolution of new viral strains by extracting Ct values from population surveillance data can inform the trajectory of the SARS-CoV-2 pandemic. For example, an increase in aggregated population Ct values indicates a decline in clinical SARS-CoV-2 cases (41).

Various clinical studies have identified that Ct values from RT-PCR can assist as a proxy for infectious virus detection. The probability of viral growth in cell culture declines to approximately 6% after ten days from symptom onset (Public Health England, 2020). A large study that analysed 754 upper respiratory samples from 425 symptomatic cases that tested positive for SARS-CoV-2 by

Rt-PCR targeting the RNA-dependent RNA polymerase (RdRp) gene showed that the estimated odds ratio of infectious viral recovery decreased by 0.67 for each unit increase in Ct value (95% CI: 0.58–0.77) with 8.3% (95% CI: 2.8%–18.4%) recovery of virus from samples with Ct > 35. Regression analysis also indicated that pre-symptomatic samples were at least as likely to be culture-positive as samples taken during symptomatic phases. (42). Other studies have tried to link Ct values with infectiousness; for example, in a small cross-sectional study, the viral infectivity by cell culture was significantly reduced for SARS-CoV-2 E-gene Ct > 24, with the odds ratio for infection decreasing by 32% for every increase of 1 Ct unit above 24. (43).

Although higher expression of viral biomarkers usually correlates with culture positivity, inter-assay Ct variability for SARS-CoV-2 is significant (6). Thus attribution of a single Ct cut-off point that predicts cell culture positivity is not available using the current qualitative RT-PCR assays. In addition, positive culture specimens have also been identified with high Ct values (44, 45). It would also be erroneous to tailor clinical management decisions based exclusively on low Ct value test results. Furthermore, it is important to re-iterate that the amplification of viral RNA by qualitative RT-PCR may not be consistent with live virus detection. The use of viral cell culture is thus an important adjunctive, although non-routine, tool to identify the infectious replicative potential of virus in samples. Viral culture, however, is itself limited by non-standardised methodology and interpretation of the cytopathic viral cell features. Therefore, the utility of viral cell culture as a gold standard to determine the infectious potential of samples requires standardisation and studies comparing SARS-CoV-2 Ct values should consider this limitation. Standardising cell culture procedures and interpretation of results and using internal and external quality control to improve overall quality assurance can reduce analytical and post-analytical test variation (46-49). Improved standardisation of cell culture can tie in with standardising Ct values and potentially identifying universal Ct threshold cut-off points that define viable virus and culture positivity.

Cevik *et al.* (14) also noted that many studies failed to identify positive viral cultures beyond day nine post-infection. Their review supports an association between viral load and virus viability. Therefore, the latter observation suggests that a particular threshold Ct value may support clinical practice points for the duration of infectiousness and isolation of index cases. Moreover, it does emphasise the importance of tying viral load dynamics to clinical presentation in SARS-CoV-2 infection. There are broad caveats that should be considered when Ct values are being utilised to inform the clinical management of patients. Assay-specific between-run variation and inter-assay variability hamper a single cut-off Ct threshold point derivation that demarcates disease severity and informs clinical risk stratification approaches and prognostication of infected individuals.

The development of fit-for-purpose quantitative qPCR assays may support clinical applications on the proviso that a definite clinical utility for Ct is demonstrated. In a joint consensus statement issued by the Infectious Diseases Society of America (IDSA) and the Association for Molecular Pathology (AMP), the use of Ct values in clinical decision-making is cautioned and not advised for the correlation with disease severity or in the prediction of active infection (and thence transmission of SARS-CoV-2) (6). The American Association of Clinical Chemistry also recommended against the reporting of SARS-CoV-2 Ct values. It supports the position by highlighting various points along the total testing process for SARS-CoV-2 RT-PCR testing, which considers pre-examination, examination and post-examination factors. Furthermore, it

impresses a standardised post-analytical comment for the release of Ct values that acknowledges limitations across the total testing process.

During the pre-examination (pre-analytical and pre-pre-analytical phases of viral testing), the lack of standardisation of patient preparation for obtaining specimens, such as removing mucous from respiratory passages or ingesting food and drinks, can cause inaccurate results. The efficiency of the specimen collection, specimen type and media utilised to collect the specimen also introduce potential variation (50). A stronger focus on clinical factors of time of onset and resolution of symptomatology also influences viral RNA, and integrating the Ct value with the evolving clinical history is essential (51). He *et al.* (51) studied temporal viral shedding patterns in 94 laboratory-confirmed patients of COVID-19 and further modelled infectiousness profiles from 77 infector-infectee transmission pairs. The investigators estimated that 44% (95% confidence interval, 30-57%) of secondary cases were infected at the pre-symptomatic level. The highest viral load was present in throat swabs at symptom onset.

Broader relevant clinical factors of vaccination status and immunisation also need consideration. Traditional pre-analytical sample stability factors of transport and age of specimen are essential to ensure intact viral RNA is preserved to avoid false-negative results. (52). The Centre for Disease Control (CDC) also does not support Ct values to determine the viral load to guide decisions on infectiousness and releasing patients from quarantine. They contend an imperfect relationship between the amount of virus present in samples and the Ct value. They note that factors which may affect the Ct value are improper collection and storage methods, processing of the specimen, and molecular assay sensitivity, which can cause the imperfect relationship (53). The pre-analytical phase of laboratory testing can affect the amount of detectable viral RNA present in the specimen and produce variable or false results. In summary, pre-examination processes that can affect COVID-19 viral RNA concentration include the specimen collection method, specimen matrix and collection methods, transport media volume, and type and time taken to arrive at the laboratory. Ultimately, the amount of RNA present in the specimen and the quality of whole RNA molecules will affect the assay's ability to amplify the RNA and provide high analytical sensitivity.

The limit of detection (LoD) for molecular tests indicates the lowest concentration of gene target that can be detected in ≥ 95% of repeat measurements and thus measures the analytical sensitivity of the molecular assay(54). This property is varied between RT-PCR assays; for example, LoD variation up to 10 000 fold was evidenced by ± 275 applications of new Emergency Use Authorisation in vitro diagnostic molecular assays to the United States Food and Drug Administration (37). This can affect the detection of low viral copy numbers and produce false-negative results and variation in Ct values between assays. The units of reporting LoD are also varied between assays, and comparisons of LoD are confusing. LoD unit of reports includes copies of genomic RNA per millilitre of transport media (copies/ml), copies/microliter, copies per reaction volume and molarity of assay target (54). In an extensive study of 27 500 patient test results by the Abbott RealTime SARS-CoV-2 assay (with a LoD of 100 copies viral RNA/ml of transport medium), each 10-fold increase in LoD increased the false-negative rate by 13%. The investigators showed that the highest LoDs could thus produce false-negative rates as high as 70% (54). The variability of LoD between assays (table 1) foregrounds low viral RNA copies may produce false-negative Ct values and potentially misclassify early disease where viral copies are low during the incubation (asymptomatic) period of SARS-CoV-2 infection.

Assay design features consisting of variable primer sets, probes and fluorescent labels also can potentially affect Ct values. These assay design features can affect the efficiency of the RT-PCR reaction, the specificity of the reaction to identify the true positive viral target and the optimal binding of primers and probes to target sequences in variants of SARS-CoV-2. In addition, the calculation of the threshold level by manual or automated selection modes can impact the Ct value and affect the accuracy of patient results. In addition, Ct range reliability should span values that permit the amplification of viral RNA in a clinically relevant range and considers the natural life cycle of the virus. As a general principle, values outside the assay's linear range should not be reported to avoid false results and misclassification of patients.

The Ct value variation has been demonstrated between assays in various studies. Cycle thresholds and diagnostic performance of clinical samples assessed by ten nucleic amplification techniques that included RT-PCR and loop-mediated isothermal amplification (LAMP) methodologies, utilising the LightMix E-gene test as the gold standard, showed excellent specificity of 100%. However, sensitivity ranged between 68.2% (95% CI 45.1% - 86.1%) to 95.5% (95% CI, 77.2% - 99.9%). Notably, all samples with viral loads >100 copies/µl showed positive results. Furthermore, Ct values that amplified the same gene targets for SARS-CoV-2 demonstrated significant variation (55). This study highlights the potential inter-assay variation of Ct values for SARS-CoV-2 RNA detection amongst current qualitative RT-PCR assays. Moreover, it suggests that analytical accuracy, the potential for misdiagnosis and assessment of infectious status may be adversely affected by Ct value reporting of qualitative nucleic acid-based testing for SARS-CoV-2 infection. Nalla *et al.* (56) assessed the diagnostic performance of seven RT-PCR assays by analysing clinical samples by different primer-probe RT-PCR designs for SARS-CoV-2. All assays were highly specific for SARS-CoV-2, with no cross-reactivity with other respiratory viruses tested. The assay's sensitivity to detect SARS-CoV-2 varied between assays, with Center for Disease Control (CDC) N2-gene and Corman E-gene primer-probe sets demonstrating the highest sensitivity (100%) with detection limit at six genomic equivalents of the the the viral RNA. Kasteren *et al.* (57) have also investigated PCR efficiency, LoD and diagnostic performance by seven commercial RT-PCR assays for SARS-CoV-2 detection using viral RNA isolated from cell culture. The efficiency varied for similar RNA targets between assays; for example, RdRp-gene efficiency varied between 104% - 118%. Also, where two targets were detected within an assay, the efficiencies were variable between the targets in the same assay; for example, the KH Medical assay demonstrated the efficiency of 118% by RdRp versus S-gene efficiency of 99%. The variation in test amplification efficiency invites rigorous validation of analytical test performance. Furthermore, even on the same molecular-based assay, longitudinal repeat testing may produce false results reflecting assay efficiency and multi-target variability within an assay.

External quality assurance schemes also evidence variation in Ct values for single and multiple viral RNA targets between laboratories, potentially impacting patient management. Although samples with lower Ct values correspond in general to higher levels of viral RNA, there is inconsistent data which demonstrates the quantitation and precision of the observed differences in Ct values. Therefore, the College of American Pathologists (CAP) cautions against the limitations of Ct values by scientists and healthcare providers (20). In an external quality assurance survey by CAP, same-batch quality assurance material was administered to 700 laboratories to analyse SARS-CoV-2 RNA. CAP identified that the median value for the analysed

samples showed variation by up to 14 cycles. In addition, a single sample analysis by the same instrument showed that the difference in median Ct values for different viral targets was three cycles. Furthermore, the survey also noted that for a single gene target analysed by the same instrument amongst all laboratory participants, a difference of up to 12 cycles was observed (20). Laboratory-specific practices of selecting multiple testing platforms or analysing specimens by multiplex nucleic acid testing assays with different viral targets can further promote variation in measured Ct values. Therefore, the reporting of instrument-specific identity for analysis of patients specimens potentially could be relevant for the interpretative analysis of patients' results. Qualitative reporting does not routinely distinguish Ct values for individual amplified viral targets. This may also be a valuable consideration as the variation between different targets observed by the CAP survey may erroneously suggest a higher viral load.

The Austrian EQA for SARS-CoV-2 analysed data for qualitative outcomes for nucleic acid extraction and detection of the virus by the 52 participant laboratories by utilising three positive (Ct values: S1, 28.4; S2, 33.6; S3, 38.5) and one negative sample. All laboratories scored a 100% for analytical specificity. However, 60% of the laboratories detected all positive samples correctly, 37% did not detect the weakest positive specimen and 3% of laboratories obtained false-negative results for S2 and S3 (58).

Furthermore, a national EQA programme in South Korea showed that 110 (93.2%) laboratories reported correct results for all qualitative molecular tests, and 29 (24.6%) laboratories had >1 outlier according to cycle threshold values. (59). Collectively, these EQA data show that Ct value sensitivity is variable and influenced by pre-analytical nucleic acid extraction procedures and the amplification step of the RT-PCR assay. Assays also positively demonstrate excellent analytical specificity.

Until recently, the lack of standardised reference material to quantify SARS-CoV-2 viral load has made comparability between assays challenging. The recent designation of the first WHO International Standard for SARS-CoV-2 RNA for nucleic acid amplification technique-based assays consists of acid-heat inactivated England/2020 isolate of SARS-CoV-2 (NIBSC code 20/146), was evaluated in a WHO international collaborative study (60, 61). The unit for the potency is 7.40 Log 10 IU/lyophilised ampoule which after reconstitution is 7.70Log10/ml. Using this material to develop quantifiable NAAT will improve harmonisation between assays and move to the development of consensus assay threshold Ct value points for the management of patients.

5. CONCLUSIONS

An examination of qualitative molecular-based testing for SARS-CoV-2 infection, especially by RT-PCR analysis, reveals variation in Ct values between assays that mitigate against reporting of Ct values for qualitative analysis by NAAT. The sources of Ct variation are a consequence of pre-assessment factors that affect the quality of viral specimen RNA and variation at the analytical level of the NAAT.

Furthermore, the interaction between patient factors, viral life-cycle and shedding kinetics make assigning Ct threshold cut-off points problematic in guiding patient management. Currently, Ct value reporting for SARS-CoV-2 is not supported by many international laboratory regulatory bodies. The derivation of a WHO preparation of standardised RNA reference material provides an avenue to move toward quantifiable viral load measurement, and harmonisation of NAAT with the potential of deriving cut-off points to guide clinical management decisions.

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In this issue: Current laboratory aspects of COVID-19

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Systematic review of diagnostic accuracy of DiaSorin Liaison SARS-CoV-2 antigen immunoassay

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ABSTRACT

Background

Quantification of SARS-CoV-2 antigens by means of rapid, high-throughput and fully-automated techniques has been proposed as a feasible alternative to overcome the current shortage of resources for routine molecular diagnostics. To this end, we provide here a systematic review of diagnostic accuracy of DiaSorin Liaison SARS-CoV-2 antigen immunoassay.

Methods

An electronic search was conduced in Medline and Scopus, with no language or date restrictions (up to January 20, 2022), for identifying all published studies articles in which the diagnostic performance of the DiaSorin Liaison SARS-CoV-2 antigen immunoassay was compared with molecular diagnostic techniques.

Results

The electronic search identified a final number of 11 studies, totalling 4449 oro- and naso-pharyngeal specimens. The pooled diagnostic sensitivity, specificity and area under the curve (AUC) of the Liaison SARS-CoV-2 antigen immunoassay in all samples were 0.51 (95%CI, 0.49-0.54), 1.00 (95%CI, 1.00-1.00) and 0.994 (95%CI, 0.990-0.998), respectively, whilst the overall concordance with molecular diagnostics was 82.1%. The pooled diagnostic sensitivity, specificity and AUC of the Liaison SARS-CoV-2 antigen immunoassay in specimens with high viral load (i.e., cycle threshold values <25-30) were 0.79 (95%CI, 0.75-0.82), 1.00 (95%CI, 0.99-1.00) and 0.911 (95%CI, 0.879-0.943), respectively, whilst the overall concordance with molecular diagnostics in such samples increased to 94.2%.

Conclusion

The results of this systematic literature review suggest that there is sufficient accuracy of the DiaSorin Liaison SARS-CoV-2 antigen immunoassay in samples with high viral loads that would enable its reliable usage for identifying superspreaders, who are responsible for the vast majority of transmission events.

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INTRODUCTION

Coronavirus disease 2019 (COVID-19), a life-threatening infectious disease that first appeared at the end of 2019, is caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and now responsible for the worst human pandemic since the Spanish flu, which emerged over one century ago [1]. High rates of community transmission around the world are driving an extremely high number of daily positive cases and large demand for testing, contact tracing and isolation procedures, which are now further compounded by the emergence of highly mutated and infective variants such as the Omicron (B.1.1.529) lineage [2]. This unprecedented demand for testing has disrupted the capacity of most clinical laboratories to provide an efficient response to these immense test volumes. According to the Coronavirus Resource Center maintained by the John Hopkins University, over 573 million new cases of SARS-CoV-2 infection have been diagnosed up to the end of July 2022 [3], which represents only the tip of the iceberg of the huge number of diagnostic tests that have been performed (between 5- to 10-fold higher). It is hence not surprising to read the results of an ongoing worldwide survey promoted by the American Association for Clinical Chemistry (AACC), which highlights that nearly one-third of all responding laboratories are having issues acquiring reagents and test kits for SARS-CoV-2 diagnostics, and around 30% of labs also have a >1 week turnaround time for processing all the specimens that have been delivered for testing [4]. Moreover, diagnostic labs are also not immune from labor shortages, also in part now driven by widespread transmission of the Omicron variant and need for quarantining. This generated backlog of unanalyzed samples not only delays the diagnosis of several COVID-19 cases who may need timely and early treatment, but also makes it impossible to promptly isolate or quarantine asymptomatic or mildly symptomatic cases, who may be responsible for spreading the outbreak further, especially those bearing high viral loads [5].

One of the major COVID-19 testing challenges is the fact that the reference method for diagnosing SARS-CoV-2 infection encompasses detection (and quantification) of viral RNA in nasopharyngeal specimens, which is unsustainable for clinical laboratories when faced with enormous volumes of diagnostic samples with need for short turnaround time [6]. To overcome this limitation, quantification of SARS-CoV-2 antigens

has been proposed as a possible alternative to viral RNA detection [7]. The use of the so-called antigen rapid detection tests (Ag-RDTs) for quick SARS-CoV-2 diagnostics is now widespread, though the often-insufficient analytical sensitivity, arbitrary interpretation, along with the possibility to obtain only qualitative results are well-recognized and still unresolved drawbacks [8], which may be potentially offset by developing robust, quantitative, accurate and reproducible laboratory-based immunoassays [9].

The DiaSorin Liaison SARS-CoV-2 Antigen test is a fully-automated chemiluminescence sandwich-immunoassay (CLIA) for detection of SARS-CoV-2 nucleocapsid (N) protein in nasal swab and nasopharyngeal swabs. According to manufacturer's specifications [10], the test can be adapted on DiaSorin LIAISON XL and LIAISON platforms, has a throughput of 136 tests per hour (results are available on average in 40 min), the analytical sensitivity (limit of detection [LOD]) is 22.0 Median Tissue Culture Infectious Dose ($TCID_{50}$)/mL, the cut-off is 200 $TCID_{50}$/mL, whilst the overall imprecision is 11-15%. Additional information on preanalytical issues, buffers and biosafety requirements can be retrieved from the package insert [10]. As the DiaSorin immunochemistry platforms are already widespread in many clinical laboratories worldwide, we provide here a systematic review of diagnostic accuracy of DiaSorin Liaison SARS-CoV-2 Antigen Immunoassay. Clearly, defining the diagnostic accuracy of this test will help informing and guiding its clinical use.

MATERIALS AND METHODS

We carried out an electronic search in Scopus and Medline (PubMed interface) using the keywords "Liaison" OR "DiaSorin" AND "antigen" AND "SARS-CoV-2" or "COVID-19" within all search fields and without language or date restrictions (i.e., up to January 20, 2022), aimed at identifying all studies in which the diagnostic performance of Liaison SARS-CoV-2 antigen immunoassay was compared with a reference molecular diagnostic technique. Two authors (G.L. and B.M.H.) screened articles by title, abstract and full text (when available) were identified based on the predefined search criteria, selecting studies in which the rates of true positive (TP), false positive (FP), true negative (TN) and false negative (FN) cases based on manufacturer's recommended cut-off (i.e., 200 $TCID_{50}$/mL) were provided or could be extrapolated from data reported in the study. The reference lists were also hand-searched to screen for further potentially eligible investigations. The data reported in each investigation was then included in a pooled analysis for estimation of diagnostic sensitivity, specificity, and accuracy (Summary Receiver Operating Characteristic Curve; SROC; Agreement; Kappa statistics) with 95% confidence interval (95%CI). A subgroup analysis was performed in samples with higher viral load (when available). A random effects model was used for pooling data, whilst the heterogeneity was calculated using χ^2 test and I^2 statistic. The statistical analysis was carried out using MetaDiSc 1.4 (Unit of Clinical Biostatistics team of the Ramón y Cajal Hospital, Madrid, Spain) [11]. The study was conducted in agreement with the Declaration of Helsinki and within the terms of local legislation.

RESULTS

The search of electronic databases using the predefined criteria allowed for the identification of 54 publications after removing duplicate studies between the two scientific platforms. Forty-three publications were excluded because they did not report specific data regarding the diagnostic performance of Liaison SARS-CoV-2 antigen immunoassay (n=33), did not perform a clinical evaluation (n=9) or were correspondence/letter to the editor (n=1). Thus, a final

number of 11 studies, totalling 4449 specimens, was included in our pooled analysis [12-22].

Table 1 summarizes the principal aspects of all selected studies. Briefly, three studies were conducted in Germany, two each in Italy and Kuwait, and one each in Belgium, France, Spain and the Netherlands. In all but two studies, the diagnostic performance of Liaison SARS-CoV-2 antigen immunoassay was tested in nasopharyngeal swabs, whilst in the two other studies oro-nasopharyngeal swabs [20,22] were employed. The range of viral load (when available) has been summarized in Table 1, together with the sample size which ranged between 119 and 897.

A sub-analysis of diagnostic performance in specimens with high viral load (i.e., Ct values <25-30) could be performed including 7/11 studies (n=2626 specimens), as summarized in Table 2.

Table 1 **Summary of studies that investigated the cumulative diagnostic performance of the fully automated DiaSorin Liaison SARS-CoV-2 Antigen Immunoassay**

Study	Country	Sample matrix	Sample size	Molecular assay (gene targets)	Range of viral load
Alghounaim et al., 2021 (12)	Kuwait	Nasopharyngeal swabs	897	Applied Biosystems TaqPath COVID-19 RT PCR kit (ORF, N and S)	Unspecified
Altawalah et al., 2021 (13)	Kuwait	Nasopharyngeal swabs	300	Thermo Fisher TaqPath COVID-19 multiplex real-time RT-PCR test (Orf1ab, N and S)	11-28 Ct
Baj et al., 2021 (14)	Italy	Nasopharyngeal swabs	119	Abbott real-time SARS-CoV-2 assay (N and RdRP)	3-30 Ct
Fernandez-Rivas et al., 2022 (15)	Spain	Nasopharyngeal swabs	861	Seegene Allplex SARS-CoV-2 Assay (E and N)	11-40 Ct
Fiedler et al., 2021 (16)	Germany	Nasopharyngeal swabs	182	Altona RealStar SARS-CoV-2 RT-PCR Kit (E, N, S and RdRP)	~1×102-~1.5×108 copies/mL
Hartard et al., 2021 (17)	France	Nasopharyngeal swabs	378	In-house - Pasteur Institut (RdRP)	19±5 Ct
Häuser et al., 2021 (18)	Germany	Nasopharyngeal swabs	223	NeuMoDx Molecular SARS-CoV-2 Test Strip (N and Nsp2) and Qiagen QIAamp Viral RNA Mini Kit (E and RdRP)	14-36 Ct

Lefever et al., 2021 (19)	Belgium	Nasopharyngeal swabs	410	Certest Viasure SARSCoV-2 real-time PCR detection kit (N1 and N2)	10-40 Ct
Osterman et al., 2021 (20)	Germany	Oro-nasopharyngeal swabs	410	Multiple assays - Seegene Allplex, Roche Cobas and Cepheid GeneXpert System (unspecified gene targets)	0.8×102-1.6×109 Geq/mL
Salvagno et al., 2021 (21)	Italy	Nasopharyngeal swabs	421	Altona RealStar SARS-CoV-2 RT-PCR Kit (E and S)	16-40 Ct
Van der Moeren et al., 2021 (22)	The Netherlands	Oro-nasopharyngeal swabs	248	Abbott Alinity M SARS-CoV-2 Assay (N and RdRP)	12-39 Ct

Ct, cycle threshold.

Table 2 **Summary of studies that investigated the diagnostic performance of the fully automated DiaSorin Liaison SARS-CoV-2 Antigen Immunoassay in nasopharyngeal samples with high viral load (i.e., cycle threshold values <25-30)**

Study	Country	Sample matrix	Sample size	Cut-off of viral load
Alghounaim et al., 2021	Kuwait	Nasopharyngeal swabs	881	<25 Ct
Altawalah et al., 2021	Kuwait	Oro-nasopharyngeal swabs	300	<29 Ct
Baj et al., 2021	Italy	Nasopharyngeal swabs	94	<26 Ct
Fernandez-Rivas et al., 2022	Spain	Nasopharyngeal swabs	732	<30 Ct
Häuser et al., 2021	Germany	Oro-nasopharyngeal swabs	131	<30 Ct
Salvagno et al., 2021	Italy	Nasopharyngeal & oropharyngeal swabs	421	<30 Ct
Van der Moeren et al., 2021	The Netherlands	Oro-nasopharyngeal swabs	74	<30 Ct

Ct, cycle threshold.

The pooled cumulative diagnostic performance of Liaison SARS-CoV-2 antigen immunoassay in all oro- and naso-pharyngeal samples is shown in Figure 1. The pooled diagnostic sensitivity, specificity and AUC in all samples were 0.51 (95%CI, 0.49-0.54; I^2, 96.4%), 1.00 (95%CI, 1.00-1.00; I^2, 0.0%) and 0.994 (95%CI, 0.990-0.998), respectively. The overall concordance of this immunoassay with a reference molecular technique was 82.1% (kappa statistics, 0.57 and 95%CI, 0.55 to 0.59), thus indicating moderate agreement [23]. The pooled cumulative diagnostic performance of Liaison SARS-CoV-2 antigen immunoassay in specimens with high viral load (i.e., Ct values <25-30) is reported in figure 2. The pooled diagnostic sensitivity, specificity and AUC in these subsets of samples were 0.79 (95%CI, 0.75-0.82; I^2, 68.5%), 1.00 (95%CI, 0.99-1.00; I^2, 74.4%) and 0.911 (95%CI, 0.879-0.943), respectively. The overall concordance of this immunoassay with a reference molecular technique was 94.2% (kappa statistics, 0.84 and 95%CI, 0.81 to 0.86), thus indicating almost perfect agreement in the presence of high viral load [23].

DISCUSSION

Several lines of evidence now attest that providing reliable and timely results of SARS-CoV-2 testing not only enables a more appropriate and rapid management of symptomatic cases, as well as prompt isolation of potentially contagious asymptomatic or mildly symptomatic cases, [24], but also allows to efficiently predict the pressure on healthcare systems in terms of overall hospitalizations, intensive care unit (ICU) admissions and even mortality [25]. The pursuit of these otherwise unquestionably essential outcomes is now becoming an insurmountable effort. In the context of the ongoing COVID-19 pandemic and the attendant challenge imposed by the nearly 3 million new daily infections to laboratory medicine and the healthcare system as a whole, the availability of rapid, high-throughput and accurate techniques continues to be pursued as a primary objective for scaling up diagnostic capacities across many different settings, including in hospital laboratories [26]. Among the various fully automated SARS-CoV-2 antigen techniques that have been recently developed, validated and commercialized, the Liaison SARS-CoV-2 two-step sandwich chemiluminescence immunoassay (CLIA) has the potentiality to provide fast and high-throughput COVID-19 diagnostics in many clinical laboratories equipped with Liaison immunochemistry platforms.

With respect to the clinical performance of this method, the results of our pooled analysis demonstrate an overall satisfactory diagnostic accuracy (AUC, 0.994), absolute diagnostic specificity (i.e., 100%) compared to reference molecular techniques, yet compounded by a limited diagnostic sensitivity - slightly above 50% - which would not allow to conclude that it may be an adequate replacement of nucleic acid amplification test (NAAT), and is probably dependent on the use of a suboptimal (i.e., too high) diagnostic cutoff. Nonetheless, our pooled analysis in samples with high viral load (i.e., Ct <25-30) has evidenced that the still optimal diagnostic accuracy (AUC, 0.911) and specificity (i.e., 100%) are now combined with a satisfactory diagnostic sensitivity (i.e., close to 80%). This is a foremost aspect in terms of epidemic control, since the likelihood of obtaining a positive SARS-CoV-2 culture is strictly dependent on the viral load, with such possibility approximating zero in respiratory samples with Ct ≥30 [27,28]. Accordingly, Hirschfeld et al. reported that the Ct values corresponding to SARS-CoV-2 infectiousness reported in clinical studies would more frequently lie between 29-31, with very low probability that patients with higher Ct values (and thereby lower viral load) would carry a relevant infective risk [29].

A crucial question can hence be finally asked; what could be the value and the most suitable placement of this method within the COVID-19

Figure 1 **Cumulative diagnostic sensitivity, specificity and accuracy (Summary Receiver Operating Characteristic Curve; SROC) with 95% confidence interval (95%CI) of the fully automated DiaSorin Liaison SARS-CoV-2 Antigen Immunoassay for diagnosing SARS-CoV-2 infection in nasopharyngeal samples**

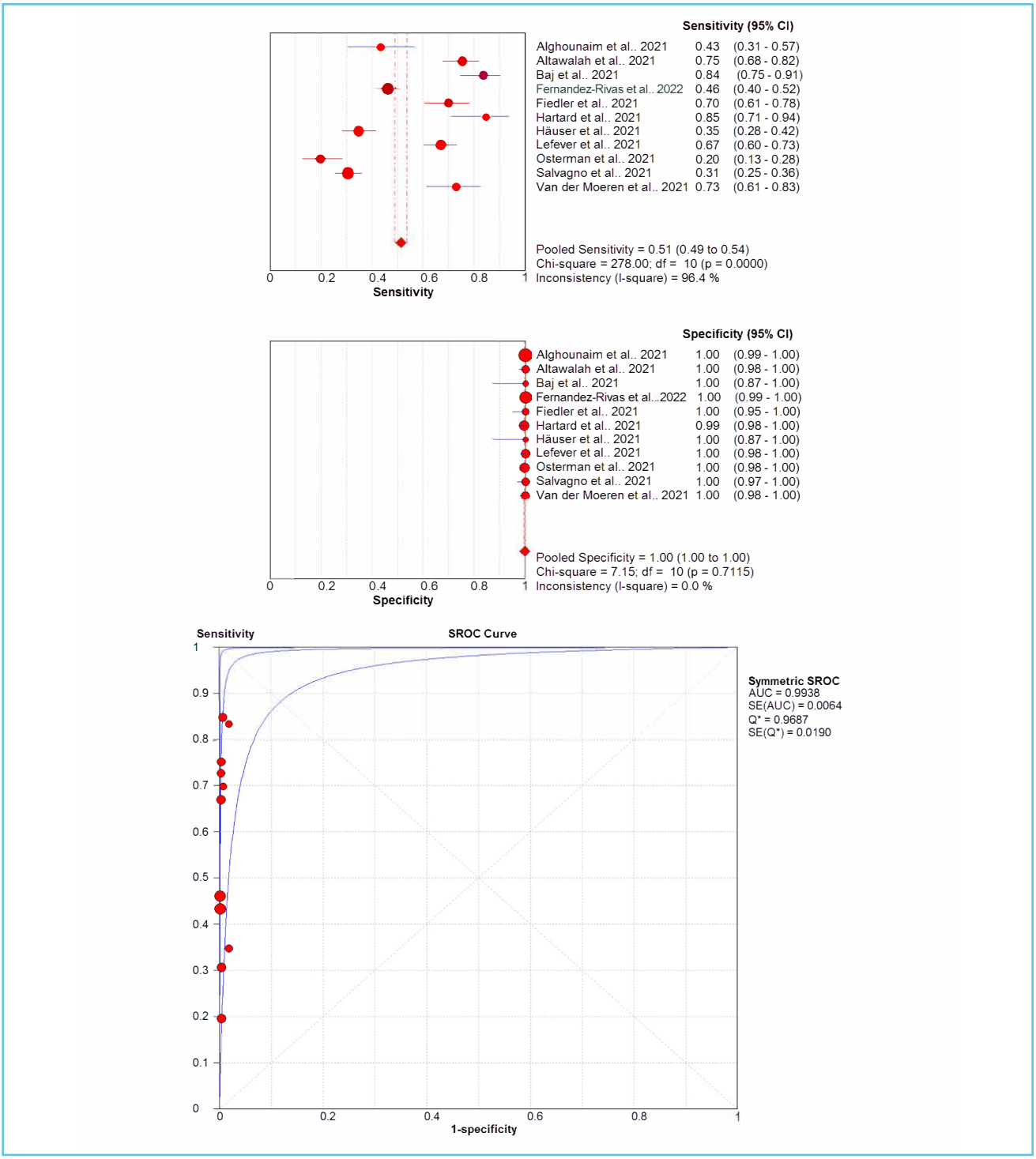
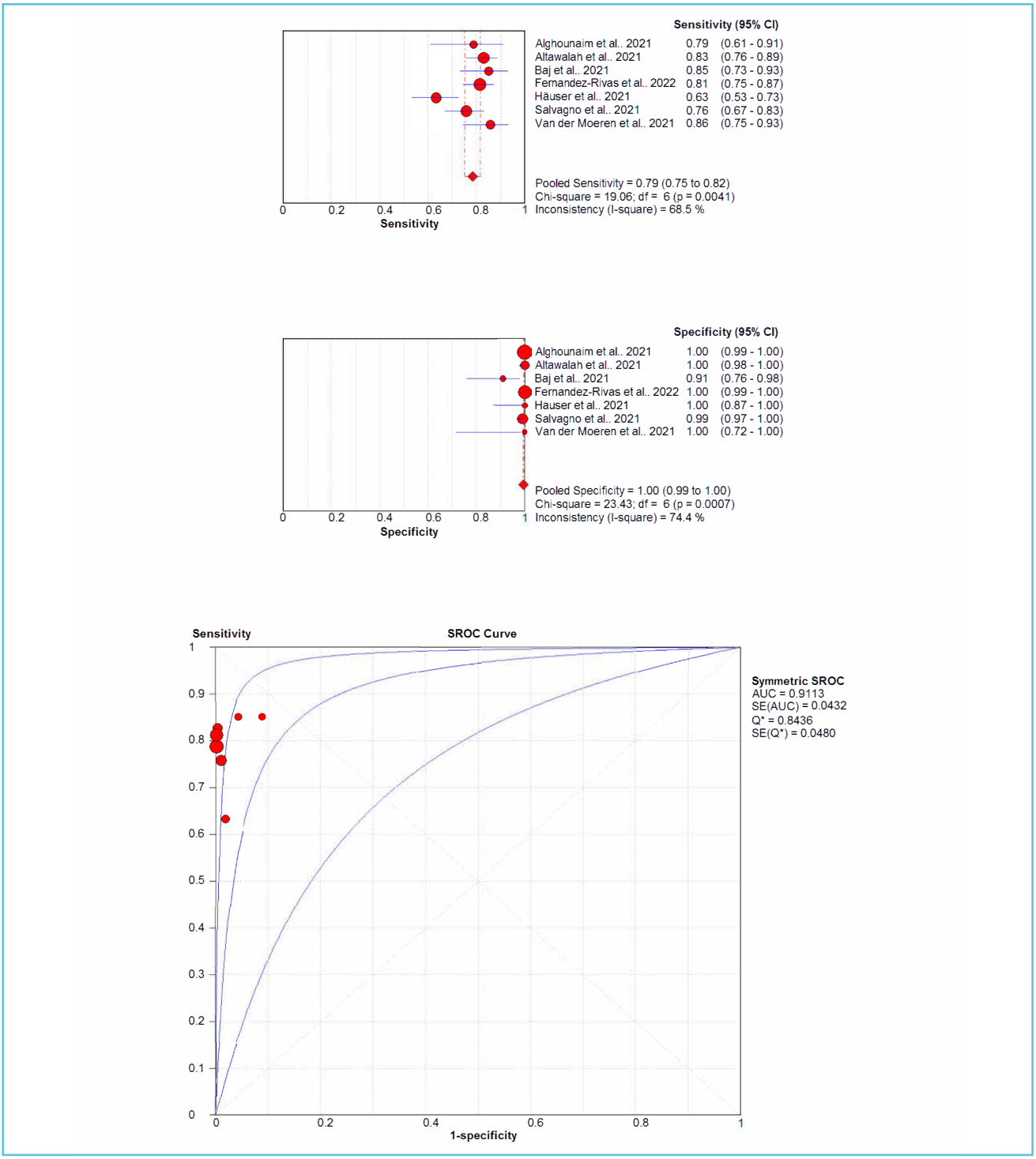


Figure 2 **Diagnostic sensitivity of the fully automated DiaSorin Liaison SARS-CoV-2 Antigen Immunoassay for diagnosing SARS-CoV-2 infection in nasopharyngeal samples with high viral load (i.e., cycle threshold values <25-30). The three lines represent the mean AUC and its 95% confidence interval.**



diagnostic strategy? As noted earlier, it is unlikely that this and other SARS-CoV-2 CLIAs will replace molecular techniques for diagnosing all SARS-CoV-2 infections as they do not have adequate sensitivity. Nonetheless, their higher accuracy at higher viral load thresholds would suggest that SARS-CoV-2 antigen immunoassays could be used for identifying the so called "super(viral)-carriers", who are responsible for the vast majority of transmission events (up to 80%), especially when asymptomatic or pre-symptomatic [5,30], up to the very unwarranted corollary that new infections caused by a "super-spreader" may be more likely to be highly contagious [31]. To this end, the use of these techniques for contact tracing and mass testing or population screening would enable to save precious personnel, technical and economic resources, thus prioritizing molecular testing in those cases where seems more urgently needed (i.e., for diagnosing acute infection in symptomatic or highly suggestive cases). Moreover, the high throughput of this technique can help enable multiple testing over the course of infection (when a first assay has been performed outside a diagnostic window) or several days following a high-risk exposure due to a variable incubation period, as well as be employed in strategies to test out of quarantine. However, it must be clearly noted that a negative results of a SARS-CoV-2 antigen test does not enable to definitely rule out an acute infection, thus the use of a more accurate NAAT would still be advisable in highly suspected cases with equivocal test results. It is also noteworthy that the diagnostic sensitivity of this SARS-CoV-2 antigen immunoassay using the recommended cut-off in samples with high viral load (i.e., 0.79) seems lower than that displayed by automated methods produced by other manufacturers such as Ortho VITROS (i.e., 0.98) [32], Fujirebio Lumipulse (i.e., ~1.00) [33], LumiraDX (i.e., ~1.00) [35], Roche Elecsys (i.e., 0.95) [35] and S-PLEX SARS-CoV-2 N (i.e., ~1.00) [36].

One important aspect that needs to be highlighted is that further studies shall be urgently planned to verify how the analytical and diagnostic performance of this and other immunoassays may be modified by emergence of new variants of concerns such as the former Delta (B.1.617.2) and the new Omicron (B.1.1.529) lineages.

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None

Ethics statements

This analysis was based on electronic searches in unrestricted, publicly available repositories, so that no informed consent or ethical committee approvals were needed.

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Laboratory features of hospitalised patients with COVID-19 in Jersey, UK

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COVID-19, severe acute respiratory syndrome, coronavirus 2 (SARS-CoV-2), laboratory features, mortality risk

ABSTRACT

COVID-19 is an acute respiratory infection caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). To date, more than 550 million cases and 6 million deaths have been reported worldwide. This study investigated the laboratory features in hospitalised patients with COVID-19 and determined risk factors for in-hospital mortality.

This retrospective observational study included laboratory results of confirmed cases of hospitalised patients with SARS-CoV-2 infection in Jersey (UK) between March–December 2020 (subject to inclusion criteria), and a control group. Furthermore, COVID-19 patients were split into two sub-groups, based on outcome (non-survivors vs. survivors). Logistic regression was used to determine risk factors for in-hospital mortality.

A total of 81 COVID-19 cases and 100 controls were included in this study. In the COVID-19 group, 59.3% of subjects were male, and the overall mortality was 33.3%. The main laboratory changes were

the following: 95.1% of patients presented with raised C-reactive protein (p<0.001), 85% showed increased fibrinogen (p<0.001), 70% had prolonged prothrombin time (p=0.014), 51.9% suffered from lymphopenia (p<0.001), 42% had elevated gamma glutamyl transferase (p=0.011) and 35.8% demonstrated raised creatinine concentration (p=0.002). Non-survivors were older than survivors (median age: 82 vs. 74 years, p=0.003) with substantial lymphopenia (p=0.018), high creatinine level (p=0.009), and leukocytosis (p=0.018). Increased in-hospital mortality risk was 6.7-fold in patients presenting with a lymphocyte count <0.85 x10^9/L, 5.3-fold with red blood cell distribution width >14%, 4.9-fold with white cell count >9.5 x10^9/L, and 3.3-fold for those presenting with creatinine >100 µmol/L. Age ≥82 years was significantly associated with death, and male gender a risk factor for hospital admission in COVID-19.

These results demonstrate that routine haematology and biochemistry tests may allow for risk-stratification of hospitalised patients with COVID-19.

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INTRODUCTION

COVID-19 is an acute respiratory infection caused by a new strain of coronavirus first identified in December 2019 in Wuhan - China, initially named 2019-nCoV, and now known as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (1,2). Initial epidemiological investigations suggested a seafood and wet animal wholesale market in Wuhan was associated with the outbreak (3). Current evidence indicates that SARS-CoV-2 has a zoonotic origin, which subsequently evolved resulting in human-to-human transmission (4).

Seven coronavirus species are known to cause human disease: four human coronavirus (HCoV) strains, known as HCoV-229E, HCoV-OC43, HCoV-NL63, and HCoV-HKU, are capable of infecting the upper respiratory tract, and are responsible for 15–30% of all common cold cases; and three highly pathogenic strains, capable of infecting the lower respiratory tract, causing severe pneumonia: severe acute respiratory syndrome coronavirus (SARS-CoV-1), Middle East respiratory syndrome coronavirus (MERS-CoV), and the newly identified SARS-CoV-2 (1,5,6). SARS-CoV-1 was responsible for outbreaks in Guangdong Province - China in 2002 and 2003 (about 8,000 cases worldwide with a case fatality rate of approximately 10%), whereas MERS-CoV caused outbreaks in the Middle East in 2012 (about 2,500 cases reported with an estimated case fatality rate of 36%) (7). SARS-CoV-2 which led to the current outbreak of COVID-19, rapidly spread to eighteen countries outside China between late December 2019 and the end of January 2020, leading the World Health Organisation (WHO) to declare COVID-19 a pandemic on the 11th March 2020 (1). At the time of writing, more than 180 million COVID-19 cases had been reported in 219 countries and territories around the world, with almost 4 million deaths (8). The United Kingdom is one of the worst affected countries, with 4.9 million cases and more than 128 000 deaths reported, whereas China (where the outbreak originated) reported 91 847 cases, and 4 636 deaths between December 2019 and July 2021. In contrast, Jersey (Channel Islands, UK) reported 3 674 cases and 69 deaths, over the same period (8,9). Direct comparisons between countries are challenging due to important variations in the testing and diagnosis criteria used, and the way COVID-19 deaths are recorded in different countries (10).

Studies have shown that up to 42.5% of all cases of SARS-CoV-2 infection may remain completely asymptomatic (11). However, up to 20% of infected individuals may develop severe disease, including acute respiratory distress syndrome (ARDS), pneumonia or pulmonary inflammation

(6). The latter has been associated with novel pulmonary-specific vasculopathy process classified as pulmonary intravascular coagulopathy (12,13). It is thought that SARS-CoV-2 may cause direct pulmonary infection of endothelial cells, via ACE-2 receptors, potentially triggering COVID-19 associated vasculopathy (14). Other mechanisms that may exacerbate endothelial cell damage and organ dysfunction in severe COVID-19 include pro-inflammatory cytokine generation, complement activation and severe hypoxia (15).

A number of recently published studies report potential changes linked to hospitalised patients with COVID-19, particularly lymphopenia, raised D-dimer, lactate dehydrogenase and C-reactive protein (CRP), and low albumin (16-19). In addition, older age has been systematically linked to higher mortality rates in COVID-19 patients (17,20–24). Published studies so far are very heterogeneous and important differences in reported findings exist between different cohorts. Most describe the clinical presentation of hospitalised patients with COVID-19 disease in China and the USA. However, European data is more limited. Analysis of the reported number of cases/deaths, and data from the first European studies revealed important differences in terms of the demographics, laboratory features, and mortality rates in hospitalised patients between countries (24,25), showing published findings cannot simply be extrapolated to individual countries.

The aim of this study is to investigate the main laboratory features of hospitalised patients with COVID-19 disease in Jersey – Channel Islands, UK, and to determine if certain changes on admission results may be associated with disease severity. Additionally, risk factors for in-hospital death in this COVID-19 group were also determined. This study also aims to contribute to the international data on this current topic, addressing the lack of published data on European cohorts of patients.

MATERIAL AND METHODS

Study design and participants

This retrospective observational study was performed at the General Hospital in Jersey (Channel Islands, UK), and approved by the local Research and Ethics Committee (Ref: 2020/HCSREC/03). All laboratory confirmed cases of SARS-Cov2 infection between March – December 2020, in patients admitted to hospital or already hospitalised at the time of testing were considered for inclusion in this study. Documented clinical information was reviewed to establish if COVID-19 was the primary reason for admission, and whether patients were symptomatic and/or required hospital treatment for COVID-19, either on admission or throughout hospitalisation (inclusion criteria). Individual signs and symptoms, and pre-existent comorbidities were excluded from the analysis due to this information not being available for all patients. Asymptomatic patients with laboratory confirmed SARS-Cov2 infection, who did not require COVID-19 treatment (either on admission or throughout their hospital stay), and had been admitted for other primary reasons, where deemed non-COVID-19 admissions, and excluded from this study. Hospital-acquired COVID-19 cases were assumed in light of prolonged hospitalisation with evidence of previous negative SARS-CoV-2 tests and flagged as known contact with other COVID-19 patients or healthcare workers in the hospital. The control group consisted of 100 patients admitted for other reasons, during the same period, had shown at least two negative SARS-CoV-2 tests on admission/during their hospital stay and remained negative until discharged from hospital.

Data collection and laboratory investigations

Patient demographics (age, gender) and laboratory results were extracted from the laboratory information management system (when available): haematology (haemoglobin (Hb), red

blood cell distribution width (RDW), platelets (PLT), white blood cells (WBC), and five-part differential), haemostasis (prothrombin time (PT), activated partial thromboplastin time (APTT), fibrinogen, and D-dimer), and biochemistry (renal profile (urea, creatinine), liver function tests (albumin, total protein (TP), bilirubin (BIL), gamma glutamyl transferase (GGT), alkaline phosphatase (ALP), and alanine aminotransferase (ALT)), and CRP). The electronic patient record system was used to inform the level of care received, length of hospital stay, and outcome.

Laboratory confirmation for SARS-CoV-2 was defined as a positive result of real time reverse transcriptase–polymerase chain reaction (RT-PCR) assay using a nasopharyngeal/oropharyngeal swab. Specimens were initially tested at Public Health England, Porton Down (UK), in-house testing commenced in April 2020 using qualitaive Gene Xpert SARS-CoV-2 RT-PCR test kits (Cepheid, California, USA).

Haematology tests were locally performed on venous blood samples collected into a 4-mL BD Vacutainer tube containing K_2 EDTA (0.184 mol/L; BD, Oxford, UK), and analysis performed on Sysmex XN-2000 analysers (Sysmex Corporation, Kobe, Japan), using flow cytometry technology, with the exception of Hb, which was measured by the sodium-lauryl-sulphate (SLS) method.

Haemostasis studies were performed on venous blood samples collected into 2.7 mL BD vacutainer tubes containing 0.109 mol/3.2% tri-sodium citrate (BD, Oxford, UK), spun at 4000 rpm for 4 min prior to analysis. Samples were analysed using the Werfen IL ACL TOP 550 coagulation analyser (Werfen, Bedford, MA, USA), by a photo-optical method for PT, APTT and fibrinogen assays, and a latex immunoassay method for the D-dimer assay.

Biochemistry tests were performed on venous blood sample collected into a 3.5-mL BD Vacutainer SST II gel tube (BD, Oxford, UK), centrifuged at 3500 rpm for 10 min prior to analysis. The tests were analysed using Ortho Vitros 5600 analysers (Ortho-Clinical Diagnostics, NY, USA) by various methods based on MicroSlide technology.

Statistical analysis

Statistical sample size calculation was not performed given that the sample size consisted of all COVID-19 cases admitted to the General Hospital during the study period, with proviso they met the inclusion criteria. All statistical analyses were performed in the IBM SPSS software (version 26). Differences between groups were calculated using the *t* test if data was normally distributed; otherwise, the Mann-Whitney test was used. Standard deviation (SD) and interquartile range (IQR) (IQR1 – 25th percentile; IQR3 – 75th percentile) were chosen to best describe the dispersion of the data for mean and median, respectively. Categorical variables were compared using the X^2 or Fisher exact test, as appropriate. Probability (p) <0.05 was considered significant for all tests. For consistency, a maximum of 3 decimal places were used for p values therefore, values under 0.001 were reported as p<0.001 (e.g., p=0.0004 was reported as p<0.001).

To ascertain if the statistically significant differences of mean/median values between groups/sub-groups were clinically significant, the percentage of patients with abnormal results were calculated for each parameter showing statistically significant changes, by setting the critical value of interest (e.g., PLT <150 x10^9/L) as a categorical variable; then, the X^2 or Fisher exact test was used (as appropriate) to determine if there was a statistically significant difference (p<0.05) in the percentage of patients showing abnormal results between groups. Normal ranges used to facilitate the interpretation of statistical analysis findings throughout the study are specific for the local adult population in Jersey.

Receiver operating characteristic (ROC) curves were calculated for continuous variables showing statistically significant differences between the survivor and non-survivor sub-groups. The area under curve (AUC) and the 95% confidence interval (CI) were determined to establish optimal cut-off points that maximised sensitivity and specificity to predict death by the Youden's index. These cut-offs were used to transform the continuous variables into binary variables, and univariate and multivariate logistic regression models were applied to calculate the estimated odds ratio and the 95% CI. Variables that were statistically associated with mortality in the univariate analysis were included in the multivariate model, using the forward stepwise likelihood ratio method.

RESULTS

A total of 113 COVID-19 hospitalised patients were identified as having had a positive SARS-CoV-2 RT-PCR test on admission or during hospitalisation: 81 patients met the inclusion criteria and were included in the test group (70 were new admissions, 11 were identified as part of the inpatient screening programme - likely hospital acquired cases); 32 patients were found not to meet the inclusion criteria because COVID-19 was not the primary reason for admission, and they remained completely asymptomatic/did not require any COVID-19 treatment on admission/throughout hospitalisation (13 were new admissions, 19 were identified as part of the inpatient screening programme – likely hospital acquired cases).

Patient group with hospitalised patients with COVID-19 vs. control group

There was no statistically significant difference in age and gender distribution between the COVID-19 group (median age: 75 years, overall range: 28-94 years; 59.3% males) and controls (median: 77 years old; overall range: 19-97 years, 54% males) (Table 1). An analysis of the haematology results revealed the test group showed statistically significant lower PLT, WBC, lymphocytes, monocytes, eosinophils, and basophils, compared with controls. Interestingly, platelet count could not be determined in 4 patients (out of 81) due to PLT clumping (5% of all patients). Haemostasis results showed significantly higher PT, fibrinogen, and D-dimer levels in the COVID-19 group. Biochemistry changes consisted of higher levels of creatinine, GGT, ALT, and C-reactive protein, and lower albumin. Analysis of the differences between categorical variables (Table 2) confirmed that the parameters showing abnormal mean/median values (based on the normal range) were associated with a higher percentage of abnormal results. Importantly, changes in WBC, albumin, and ALT were shown not to be clinically significant.

COVID-19 group were split into two sub-groups based on outcome

Non-survivors were found to be significantly older (median age: 82 years; overall range: 50-94 years) than survivors (median: 74 years; overall range: 28-92 years) and presented with higher median WBC, RDW and creatinine levels and lower lymphocyte count on admission (Table 3). The analysis of categorical variables (Table 4) confirmed the clinical significance of all these changes (except for RDW). No statistically significant differences in haemostasis results were found despite prolonged PT, and lower D-dimer levels were seen in non-survivors. In addition, survivors showed a slightly longer albeit non-significant hospital stay compared to non-survivors (median: 12 days, IQR: 6-23 in survivors vs. 11 days; IQR: 7-18 in non-survivors; p=0.343).

ROC analysis and logistic regression analysis

Table 5 shows the optimal cut-off points established using ROC curve analysis, based on modest but statistically significant AUC values that

Table 1 **Demographics & laboratory features – control and COVID-19 groups**

		COVID-19 group		Control group		
Parameter	**Normal range**	***n***	**Median (IQR) or Mean ± SD**	***n***	**Median (IQR) or Mean ± SD**	***p* value**
Age (years)	N/A	81	75 (61 – 83)	100	77 (56 – 86)	0.868*
Gender N (%)	N/A	81	♂ 48 (59.3%) ♀ 33 (40.7%)	100	♂ 54 (54%) ♀ 46 (46%)	0.478†
Hb (g/dL)	♂ 13.0 – 17.0 ♀ 11.0 – 15.0	81	13.03 ± 2.19	100	12.74 ± 2.10	0.370‡
RDW (%)	10.0 – 20.0	81	13.8 (12.9 – 14.7)	100	13.4 (12.6 – 14.6)	0.318*
PLT (10^9/L)	150 – 450	77	214 (156 – 291)	100	272 (212 – 338)	0.001*[a]
WBC (10^9/L)	3.5 – 11.0	81	8.00 (5.90 – 10.90)	100	9.65 (6.93 – 13.35)	0.004*[a]
Neutrophils (10^9/L)	1.8 – 8.0	81	6.14 (4.07 – 9.75)	100	7.29 (4.75 – 10.15)	0.061*
Lymphocytes (10^9/L)	0.8 – 4.0	81	0.74 (0.51 – 1.15)	100	1.40 (1.02 – 1.87)	<0.001*[a]
Monocytes (10^9/L)	0.2 – 1.0	81	0.52 (0.39 – 0.74)	100	0.67 (0.53 – 0.94)	0.001*[a]
Eosinophils (10^9/L)	0.01 – 0.50	81	0.02 (0.00 – 0.07)	100	0.10 (0.04 – 0.20)	<0.001*[a]
Basophils (10^9/L)	0.01 – 0.10	81	0.02 (0.01 – 0.03)	100	0.04 (0.03 – 0.06)	<0.001*[a]
PT (sec)	10 – 13.0	40	13.7 (12.6 – 16.4)	48	12.7 (11.7 – 14.4)	0.007*[a]
APTT (sec)	22.0 – 37.0	40	30.0 ± 3.6	48	31.0 ± 4.9	0.335‡
Fibrinogen (g/L)	1.7 – 4.8	40	6.41 (5.00 – 6.98)	48	4.48 (3.66 – 5.51)	<0.001*[a]
D-dimer (ng/mL)	0 – 250.0	24	336.5 (227.3 – 599.5)	5	170.0 (123.5 – 234.5)	0.008*[a]

Urea (mmol/L)	2.5 – 7.8	81	7.30 (5.55 – 11.35)	100	6.75 (4.50 – 9.25)	0.078*
Creatinine (µmol/L)	♂ 58 – 110 ♀ 46 – 92	81	80.0 (59.5 – 114.5)	100	69.5 (56.0 – 88.0)	0.023*[a]
Albumin (g/L)	35 – 50	81	37.4 ± 5.5	97	40.0 ± 6.1	0.004[‡a]
TP (g/L)	60 – 80	81	68.0 (64.0 – 73.0)	95	71.0 (65.0 – 76.0)	0.067*
BIL (µmol/L)	0 – 21	81	13.0 (10.0 – 18.0)	96	12.5 (8.3 – 19.5)	0.391*
GGT (U/L)	♂ 15 – 73 ♀ 12 – 43	81	44.0 (31.0 – 137.0)	96	32.5 (19.0 – 55.5)	<0.001*[a]
ALP (U/L)	30 – 130	81	79.0 (65.5 – 104.5)	96	81.0 (66.5 – 116.3)	0.517*
ALT (U/L)	♂ 0 – 50 ♀ 0 – 35	79	27.0 (18.0 – 38.0)	95	21.0 (16.0 – 29.0)	0.003*[a]
CRP (mg/L)	0 – 10	81	63.0 (34.0-168.0)	97	14.0 (5.0 – 35.5)	<0.001*[a]

Key: *♂ male; ♀ female;* Mann-Whitney U test; † X^2 test; ‡ t-test; [a] statistically significant (p<0.05).*
Abbreviations: n: total number of patients tested; IQR: Interquartile range (Q1, Q3); SD: Standard deviation; N/A: Not applicable; CRP: C-reactive protein.

Table 2 **Analysis of categorical variables for all parameters showing statistically significant differences – controls and COVID-19 group**

	COVID-19 group		Control group		
Categorical variable	***n***	**N (%)**	***n***	**N (%)**	***p* value**
PLT <150 x10^9/L	77	17 (22.1%)	100	6 (6.0%)	0.002*[a]
WBC >11.0 x10^9/L	81	20 (24.7%)	100	32 (32.0%)	0.280*
Lymphocytes <0.8 x10^9/L	81	42 (51.9%)	100	16 (16.0%)	<0.001*[a]
Monocytes <0.2 x10^9/L	81	4 (4.9%)	100	0 (0.0%)	0.038[†a]
Eosinophils <0.01 x10^9/L	81	25 (30.9%)	100	3 (3.0%)	<0.001*[a]

Basophils <0.01 x10^9/L	81	81 (8.6%)	100	0 (0.0%)	0.003†a
PT ≥13.0 sec	40	28 (70.0%)	48	21 (43.8%)	0.014*a
Fibrinogen >4.8 g/L	40	34 (85.0%)	48	18 (37.5%)	<0.001*a
D-dimer >250.0 ng/mL	24	16 (66.7%)	5	1 (20.0%)	0.130†
Creatinine ♂ >110 µmol/L ♀ >92 µmol/L	81	29 (35.8%)	100	16 (16.0%)	0.002*a
Albumin <35 g/L	81	23 (28.4%)	97	16 (16.5%)	0.056*
GGT ♂ >73 U/L ♀ >43 U/L	81	34 (42.0%)	96	23 (24.0%)	0.011*a
ALT ♂ ≥50 U/L ♀ ≥35 U/L	79	18 (22.8%)	95	16 (16.8%)	0.325*
CRP >10 mg/L	81	77 (95.1%)	97	60 (61.9%)	<0.001*a

Key: *♂ male; ♀ female;* X^2 test; † Fisher exact test; [a] statistically significant (p<0.05).*
Abbreviations: n: total number of patients tested; N: number of patients with abnormal results, based on categorical variable tested; CRP: C-reactive protein.

Table 3 **Demographics & laboratory features – hospitalised patients with COVID-19 based on outcome**

		COVID-19 non-survivors		COVID-19 survivors		
Parameter	**Normal range**	***n***	**Median (IQR) or Mean ± SD**	***n***	**Median (IQR) or Mean ± SD**	***p* value**
Age (years)	N/A	27	82 (74 - 87)	54	74 (57 - 81)	0.003*a
Gender N (%)	N/A	27	♂ 16 (59.3%) ♀ 11 (40.7%)	54	♂ 32 (59.3%) ♀ 22 (40.7%)	1.000†
Hb (g/dL)	♂ 13.0 - 17.0 ♀ 11.0 - 15.0	27	12.42 ± 2.49	54	13.33 ± 1.99	0.080‡
RDW (%)	10.0 - 20.0	27	14.1 (13.0 - 15.3)	54	13.4 (12.6 - 14.4)	0.028*a
PLT (10^9/L)	150 - 450	24	230 (167 - 330)	53	211 (153 - 281)	0.367*
WBC (10^9/L)	3.5 - 11.0	27	9.50 (6.10 - 13.60)	54	7.30 (5.48 - 9.40)	0.042*a

Neutrophils (10^9/L)	1.8 - 8.0	27	7.34 (4.20 - 11.83)	54	5.42 (3.89 - 7.51)	0.085*
Lymphocytes (10^9/L)	0.8 - 4.0	27	0.63 (0.47 - 0.81)	54	0.99 (0.54 - 1.35)	0.025*[a]
Monocytes (10^9/L)	0.2 - 1.0	27	0.58 (0.43 - 1.03)	54	0.52 (0.37 - 0.73)	0.300*
Eosinophils (10^9/L)	0.01 - 0.50	27	0.03 (0.01 - 0.08)	54	0.01 (0.00 - 0.06)	0.125*
Basophils (10^9/L)	0.01 - 0.10	27	0.02 (0.01 - 0.04)	54	0.02 (0.01 - 0.03)	0.058*
PT (sec)	10 - 13.0	12	15.4 (12.4 - 18.8)	28	13.6 (12.7 - 15.6)	0.400*
APTT (sec)	22.0 - 37.0	12	31.7 (27.8 - 33.0)	28	29.1 (27.1 - 30.8)	0.128*
Fibrinogen (g/L)	1.7 – 4.8	12	6.17 ± 2.08	28	6.33 ± 1.74	0.813‡
D-dimer (ng/mL)	0 - 250.0	5	262.0 (231.5 - 676.5)	19	358.0 (215.0 - 620.0)	0.915*
Urea (mmol/L)	2.5 - 7.8	27	7.70 (5.80 - 16.20)	54	6.95 (5.15 - 9.75)	0.092*
Creatinine (µmol/L)	♂ 58 – 110 ♀ 46 – 92	27	103.0 (63.0 - 123.00)	54	76.0 (55.8 - 96.5)	0.024*[a]
Albumin (g/L)	35 - 50	27	36.3 ± 5.1	54	38.0 ± 5.6	0.185‡
TP (g/L)	60 - 80	27	66.6 ± 6.3	54	69.6 ± 7.6	0.077‡
BIL (µmol/L)	0 - 21	27	12.0 (9.0 - 18.0)	54	13.5 (10.0 - 18.0)	0.488*
GGT (U/L)	♂ 15 – 73 ♀ 12 - 43	27	67.0 (31.0 - 160.0)	54	43.0 (30.5 - 93.0)	0.437*
ALP (U/L)	30 - 130	27	80.0 (68.0 - 102.0)	54	78.0 (57.5 - 110.8)	0.408*
ALT (U/L)	♂ 0 - 50 ♀ 0 - 35	26	26.5 (18.0 - 39.0)	53	29.0 (18.0 - 38.0)	0.830*
CRP (mg/L)	0 - 10	27	67.0 (37.0 - 176.0)	54	61.0 (33.8 - 158.3)	0.700*

Key: *♂ male; ♀ female;* Mann-Whitney U test; † X^2 test; ‡ t-test; [a] statistically significant ($p<0.05$).*
Abbreviations: n: total number of patients tested; IQR: Interquartile range (Q1, Q3); SD: Standard deviation; N/A: Not applicable; CRP: C-reactive protein.

Table 4 Analysis of categorical variables for all parameters showing statistically significant differences – hospitalised patients with COVID-19 based on outcome

	COVID-19 non-survivors		COVID-19 survivors		
Categorical variable	***n***	**N (%)**	***n***	**N (%)**	***p* value**
RDW >15 %	27	7 (25.9%)	54	11 (20.4%)	0.571*
WBC >11.0 x10^9/L	27	11 (40.7%)	54	9 (16.7%)	0.018*[a]
Lymphocytes <0.8 x10^9/L	27	19 (70.4%)	54	23 (42.6%)	0.018*[a]
Creatinine ♂ >110 µmol/L ♀ >92 µmol/L	27	15 (55.6%)	54	14 (25.9%)	0.009*[a]

Key: *♂ male; ♀ female;* X^2 test; † Fisher exact test; [a] statistically significant (p<0.05). Abbreviations: n: total number of patients tested; N: number of patients with abnormal results, based on categorical variable tested.*

maximised sensitivity and specificity to predict death. Univariate logistic regression analysis demonstrated that all selected parameters with determined cut-offs were significantly associated with death. Multivariate logistic analysis indicated that RDW >14% (OR = 5.335), WBC >9.5 x10^9/L (OR = 4.855), lymphocyte count <0.85 x10^9/L (OR = 6.694), and creatinine >100 µmol/L (OR = 3.280) (Table 6) were risk factors for death in hospitalised patients with COVID-19.

DISCUSSION

The median age of the hospitalised patients with COVID-19 included in this study was 75

Table 5 ROC curve analysis of selected parameters

Parameter	ROC curve analysis			
	AUC	**95% CI**	***p* value**	**Cut-off selected**
Age (years)	0.707	0.586-0.827	0.003[a]	≥ 82 years
RDW (%)	0.650	0.528-0.772	0.029[a]	> 14 %
WBC (10^9/L)	0.639	0.504-0.775	0.042[a]	> 9.5 x10^9/L
Lymphocytes (10^9/L)	0.653	0.530-0.777	0.025[a]	< 0.85 x10^9/L
Creatinine (µmol/L)	0.654	0.526-0.782	0.024[a]	> 100 µmol/L

Key: *[a] statistically significant (p<0.05 for the AUC = 0.500). Abbreviations: ROC: Receiver operating characteristic; AUC: Area under curve; CI: Confidence interval (CI of AUC).*

Table 6 ROC curve analysis of selected parameters

Variables	Univariate analysis			Multivariate analysis		
	OR	95% CI	*p* value	OR	95% CI	*p* value
Age ≥ 82 years	4.210	1.542-11.492	0.005[a]			
RDW >14%	4.156	1.560-11.069	0.004[a]	5.335	1.524-18.674	0.009[a]
WBC >9.5 x10^9/L	3.630	1.330-9.909	0.012[a]	4.855	1.358-17.364	0.015[a]
Lymphocytes <0.85 x10^9/L	4.717	1.642-13.555	0.004[a]	6.694	1.845-24.290	0.004[a]
Creatinine >100 µmol/L	5.091	1.872-13.845	0.001[a]	3.280	1.005-10.699	0.049[a]

Key: *[a] statistically significant (p<0.05). Abbreviations: OR: Odds ratio; CI: confidence interval.*

years, which is comparable to that reported in the UK (median age: 73 years) (21). An overall mortality rate of 33.3% was found, higher than the inpatient mortality reported in China (28%) (17) and Germany (24%) (24). Patients in this study had a higher median age than patients in China (median age between 48-62 years) (17,18,26–30), in the USA (median age between 58-63 years) (22,23,31), and in other European countries (median age between 63-69 years) (20,24,25). Like several other studies, an association was found between older age and increased mortality from COVID-19 (17,21–23), which might partially explain the higher mortality rate seen in this cohort. However, a direct comparison with overall mortality rates reported by other international studies is difficult given that the vast majority included patients who remained in hospital at the time of reporting; e.g., the UK study reported an overall mortality rate of 26%, with 41% survivors, and 34% still in hospital (21). If the number of hospitalised patients were considered the mortality rate would be between 26–38.8%. Factors to help explain the difference in mortality rates reported include important demographic and epidemiological differences between countries/regions, such as the percentage of elderly individuals, ethnicity, prevalence of co-morbidities/risk factors, such as hypertension, diabetes, obesity (21), and distinct healthcare models/resources available in each area.

Males accounted for most deaths in this cohort (16 deaths, 59.3%), although the mortality rate in males and females was undistinguishable (33.3% in both groups). Like other studies, no statistically significant difference was found in gender distribution between survivors and non-survivors (18,31). The cumulative number of COVID-19 cases reported in Jersey (9) showed more women tested positive (46% males vs. 54% females; p<0.001) however, most of the hospitalised patients were males (59.3% males, vs. 40.7% females; p=0.017) suggesting the male gender is a risk factor for hospital admission in COVID-19, which goes towards explaining the higher number of deaths seen in male patients.

This study found hospitalised patients with COVID-19 presented with a statistically significant lower median WBC, lymphocytes, monocytes, eosinophils, and basophils, compared with controls. Of these, the median lymphocyte

count (0.74 x10^9/L) was below the normal range, affecting 51.9% of patients, which was consistent with other studies (18,24). Lymphopenia was significantly more pronounced in non-survivors, affecting 70.4% of patients. Several studies have shown an association between lymphopenia and severe disease and/or death from COVID-19 (17,18,30). It is thought that SARS-CoV-2 may directly infect lymphocytes via ACE-2 receptors on their surface, contributing to their lysis. The cytokine storm seen in SARS-CoV-2 infection, which results in markedly increased levels of interleukins (IL), particularly IL-6, IL-2, IL-7, granulocyte colony stimulating factor (GCSF), and tumour necrosis factor alpha (TNF-α) may also promote lymphocyte apoptosis (33), having been described in three highly pathogenic coronavirus: SARS-CoV-1, MERS-CoV, and SARS-CoV-2 (30).

An analysis of categorical variables, based on clinically significant values, showed that 30.9% of COVID-19 patients presented with eosinopenia. This is consistent with several studies (26,29,30). Basopenia and monocytopenia were observed less frequently, affecting 8.6% and 4.9% of patients, respectively. Qin *et al.* also reported modest changes in these two parameters (30). Recent studies have shown that eosinophils play a key role against viruses and bacteria (not just in parasitic infections/allergic reactions) through synthesis, storage, and release of several cytokines. Eosinophils can act as antigen presenting cells, stimulating the immune capabilities of T lymphocytes, and are also capable of promoting humoral responses by interacting with B lymphocytes (34). This is thought to contribute to the destruction of these cells, together with the increased mobilisation of eosinophils onto the airway and other epithelial tissues affected by SARS-CoV-2 infection (26). Unlike other studies, we found no association between eosinophil levels and the severity of COVID-19 disease (26,29,30). However, WBC did appear to show prognostic potential given that 40.7% of non-survivors presented with leukocytosis, which was consistent with other studies (17,30) suggesting a more pronounced inflammatory response in severe cases.

Thrombocytopenia was identified in 22.1% of hospitalised patients with COVID-19, despite median values being within normal ranges. This is comparable with other studies, although other authors reported slightly lower median PLT values in their cohorts (17,18,25,35). Earlier studies suggested an association between low PLT count and increased risk of severe disease and mortality in COVID-19. however, no evidence-based cut-off has been defined (31,36). This study found no statically significant difference between survivors and non-survivors.

Haemostasis results revealed COVID-19 patients presented with deranged clotting: 85% of patients showed raised fibrinogen, 70% prolonged PT, and 66.7% elevated D-dimer, although the latter was not statistically significant (due to the low number of D-dimer tests performed). These findings are consistent with other studies (37). A comparison of haemostasis results between survivors and non-survivors suggests limited prognostic potential, although the low number of coagulation studies requested on admission (particularly in non-survivors) may have biased the data. Initial studies from China linked the coagulopathy seen in hospitalised patients with COVID-19 to disseminated intravascular coagulation (DIC) (27,37), however DIC was a rare finding in cohorts consisting of a majority of Caucasian patients (12,38), which is consistent with this study. These changes have been attributed to pulmonary intravascular coagulopathy, which is a distinct pathological process (12,13). D-dimer has been widely reported as a potential prognostic factor in COVID-19 (17,27) however, none of the non-survivors included in this study presented a D-dimer result on admission over potential prognostic cut-off values suggested by other studies (17,28). It would be inappropriate

to draw definite conclusions based on such low number of haemostasis tests, particularly for D-dimer.

The most evident biochemical change in hospitalised patients with COVID-19 was raised CRP (abnormal in 95.1% of patients), which is consistent with other studies (12,24,35). Unlike these studies, we found no significant differences between CRP levels in hospitalised patients with COVID-19, based on patient outcome. Raised CRP is an established finding in several types of pneumonia, and several studies have shown increased amounts of proinflammatory cytokines in serum (which will lead to an increase of several inflammatory markers) are associated with pulmonary inflammation and extensive lung damage in SARS-CoV-1, MERS-CoV and SARS-CoV-2 infection (30,39,40). High levels of CRP are suggestive of a developing cytokine storm in COVID-19 patients.

Other significant biochemical findings were higher creatinine levels in hospitalised patients with COVID-19, compared to controls. Despite median creatinine values being within normal ranges, 35.8% of COVID-19 patients presented with raised creatinine levels. This appeared to have prognostic potential, affecting 55.6% of non-survivors, being consistent with other studies (18,23). Wang *et al.* hypothesised that acute kidney injury could arise from direct effects of the virus, hypoxia, and shock (18) . Furthermore, 42% of hospitalised patients with COVID-19 presented with elevated GGT levels. Changes in albumin and ALT did not appear clinically significant despite statistical significance. This appears to suggest only a small proportion of COVID-19 patients in this cohort had clinically significant liver injury, which would be in keeping with findings from a meta-analysis performed by Li et al. (41). However, an association between low serum albumin and increased odds of in-hospital death has been documented by other studies (19). The relatively small number of non-survivors in our cohort make it impossible to draw definite conclusions.

Finally, this study also showed statistically significant differences in RDW between non-survivors and survivors. This was consistent with other studies (31,42,43). The fact that RDW results were largely within normal range, and no significant differences were found when looking at set categorical variables based on critical values, suggests this is not clinically significant. Despite this, RDW shows a clear prognostic potential, as recently demonstrated in a larger local cohort of COVID-19 patients (44). This test has been widely researched as an independent predictor of mortality in critically ill patients with sepsis (45). This suggests RDW may be a generic predictor of mortality, not directly linked to potential pathological changes directly associated with COVID-19, which might explain why we did not find differences between hospitalised patients with COVID-19 and the control group.

Overall, the wide range of changes in laboratory results seen in this study supports multi-organ involvement. Both SARS-CoV-2 direct invasion of different tissues/organs via ACE-2 leading to organ injury, and the hyperinflammatory response seen in severe cases of COVID-19 have been associated with disease progression, ARDS, heart failure, kidney injury, liver damage, and a wide range of neurological disorders (45).

This study found COVID-19 patients presenting with lymphocyte counts below 0.85 x10^9/L were 6.7 times more likely to die from the disease. Likewise, the mortality risk was 5.3 times higher in those presenting with an RDW above 14%, 4.9 times higher in patients presenting with WBC greater than 9.5 x10^9/L, and 3.3 times higher for those presenting with creatinine levels over 100 µmol/L. Age ≥82 years was significantly associated with death. This is partially in keeping with literature (17,31), even though suggested cut-off ranges vary considerably between studies. It is

important to note most of the studies published so far focused on investigating the association between laboratory results and the severity of COVID-19 disease. However, a few authors have determined the mortality risk associated with certain changes, making direct comparisons to the findings of this study difficult.

The limitations of this study include being undertaken on a single hospital site (findings may not be applicable to other locations); the retrospective study design meant not all laboratory tests were performed on all patients (potential bias due to a small number of test results, particularly D-dimer); patients may have presented to hospital at varying phases of the disease progression (admission results may not necessarily reflect the initial phase of the disease); earlier cases not offered the same treatments (e.g., steroids given pre-admission) which could have influenced the laboratory results on admission; potential inaccuracies when comparing laboratory data with other studies, given most authors did not include details of the laboratory methods used in their studies, whilst some authors clearly used different technology/assays. Additionally, the control group consisted of SARS-CoV-2 negative patients admitted for other reasons instead of healthy controls (potential bias), although the authors feel may make it more relevant to day-to-day practice in an acute hospital setting. The study design enabled the authors to capture all COVID-19 cases over a defined period, and with a definite outcome: discharged (survivor) or mortality, overcoming limitations seen in other studies, where data from patients still hospitalised at the time of reporting (unknown patient outcome) was included, leading to bias (e.g., lower mortality rates). The inclusion criteria in this study involved a careful review of the clinical data for each patient to exclude patients admitted for other reasons, who remained completely asymptomatic (not requiring COVID-19 treatment), which is important given that asymptomatic individuals might need to seek hospital treatment for a variety of medical reasons/emergencies (e.g., trauma) and consequently, present with underlying changes unrelated to COVID-19. The authors believe a different approach would have biased the results further. Some studies in the USA did demonstrate significant differences between COVID-19 patients seen only in the Emergency Department (mostly asymptomatic cases), and those requiring hospitalisation (22,23).

CONCLUSION

This study showed the highest in-hospital mortality risk was associated with a lymphocyte count <0.85 x10^9/L on admission, followed by RDW >14%, WBC >9.5 x10^9/L, and creatinine levels >100 µmol/L. Age ≥82 years was significantly associated with death, and male gender a risk factor for hospital admission in COVID-19. These results demonstrate that routine haematology and biochemistry tests, available in most laboratories, may allow for risk-stratification of hospitalised patients with COVID-19. Larger studies are necessary to confirm these findings.

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Conflict of interest

There are no competing interests to declare among the authors of this work.

Ethical approval

This study was approved by the Government of Jersey Research and Ethics Committee (reference: 2020/HCSREC/03).

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In this issue: Current laboratory aspects of COVID-19

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Prognostic value of routine blood parameters in intensive care unit COVID-19 patients

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ABSTRACT

Introduction

Laboratory medicine has an important role in the management of COVID-19. The aim of this study was to analyze routinely available blood parameters in intensive care unit COVID-19 patients and to evaluate their prognostic value.

Patients and methods

This is a retrospective, observational, single-center study including consecutive severe COVID-19 patients who were admitted into the intensive care unit of Ben Arous Regional Hospital in Tunisia from 28 September 2020 to 31 May 2021. The end point of the study was either hospital discharge or in-hospital death. We defined two groups based on the outcome: survivors (Group 1) and non-survivors (Group 2). Demographical, clinical, and laboratory data on admission were collected and compared between the two groups. Univariate

and multivariate logistic regression analysis were performed to determine the predictive factors for COVID-19 disease mortality.

Results

A total of 150 patients were enrolled. Eighty patients (53.3%) died and 70 (46.7%) survived during the study period. Based on statistical analysis, median age, Simplified Acute Physiology Score (SAPS II) with the serum levels of urea, creatinine, total lactate dehydrogenase (LDH), creatine kinase, procalcitonin and hs-troponin I were significantly higher in non-survivors compared to survivors. On multivariate analysis, LDH activity ≥ 484 U/L (OR=17.979; 95%CI [1.119-2.040]; p = 0.09) and hs-troponin I ≥ 6.55 ng/L (OR=12.492; 95%CI [1.691- 92.268]; p = 0.013) independently predicted COVID-19 related mortality.

Conclusion

Total LDH and hs-troponin I were independent predictors of death. However, further clinical investigations with even larger number of patients are needed for the evaluation of other laboratory biomarkers which could aid in assessing the prediction of mortality.

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INTRODUCTION

The outbreak of the SARS-CoV-2 infection began in Wuhan, Hubei, China and spread rapidly around the world (1). Since March 2020, Coronavirus Disease 2019 (COVID-19) has been declared as a pandemic (2). The clinical manifestations of COVID-19 vary highly, ranging from asymptomatic or mild infection to severe forms of pneumonia requiring hospitalization at intensive care unit (ICU). In severe forms, respiratory distress syndrome may be often accompanied by life-threatening multi-organ failure (3). Several recent studies have investigated serum biomarkers closely associated with COVID-19 severity (4). However, only a few studies have focused on the prognostic role of laboratory findings in ICU COVID-19 patients.

Therefore, the aim of this study was to analyze routine blood parameters of severe COVID-19 patients and to explore the mortality predicting factors in these ICU patients.

SUBJECTS AND METHODS

Study population

It is a retrospective, observational, single-center study including all patients hospitalized between 28 September 2020 to 31 May 2021 in a Tunisian ICU in Ben Arous Regional Hospital. COVID-19 infection was confirmed by using reverse-transcriptase polymerase-chain reaction (RT-PCR) assay, and/or a rapid antigen test, and/or a chest computed tomography scan (CT), and/or a positive serological test (positive for serum SARS-CoV-2 specific IgM or IgM and IgG antibodies). We excluded those patients who were still under treatment at the time of data collection. Two groups were defined: survivors (Group 1) and non-survivors (Group 2).

Data collection

Demographical, clinical, and laboratory data were collected and statistically analyzed. These data involved age, gender, comorbidities (hypertension, diabetes mellitus, dyslipidemia, coronary heart disease, kidney disease, respiratory disease, thyroid disorders, obesity) and the Simplified Acute Physiology Score (SAPS II) which is a severity clinical score and mortality estimation tool. It was designed to measure the severity of disease for patients admitted to ICU aged 15 years or above. The score is made of 12 physiological variables and 3 disease-related variables. Score point ranges between 0 and 163 and a predicted mortality between 0% and 100% (5).

The measurement of routinely available blood tests was performed on the date of ICU admission in the Central Laboratory of Ben Arous Regional Hospital. The laboratory tests included general parameters, such as C-reactive protein (CRP), procalcitonin (PCT), complete blood count and D-dimer. Hs-troponin I measurement was performed on admission since its prognostic value has been reported in several studies.

Evaluation criteria

Patients were followed up during their hospitalization. Our study's primary endpoint was COVID-19 related mortality. The clinical and laboratory data were compared between the two study groups.

Statistical analysis

Statistical analysis was performed with SPSS version 25.0 software. Continuous variables were presented as median values with interquartile range (IQR) and were compared by the Student's t-test or Mann-Whitney U-test according to the normality of the distribution. Qualitative variables were presented as counts and percentages and were compared by the Pearson χ^2 and Fisher's exact tests. Univariate and multivariate logistic regression analysis was used to determine the predictive factors for COVID-19 disease mortality. A p value < 0.05 was considered to be statistically significant.

RESULTS

Demographical and clinical characteristics of COVID-19 patients

In this study, 150 patients, 88 men and 62 women (gender-ratio M/F=1.41), were enrolled. The median age was 64.5 years. Among study participants, 121 patients (80.66%) had at least one comorbidity, while 47 patients (31.3%) were mechanically ventilated during ICU treatment.

The following medication was administered before ICU admission: antibiotic therapy (n=104, 69.3%), corticosteroid therapy (n=148, 98.6%),

Table 1 Drugs and other ICU treatment administrated in both study groups

	Group 1 (n=70)	Group 2 (n=80)	p value	RR [95% CI]
Prone position	10	59	0.000	2.636 [1.639; 4.240]
Dialysis	3	12	0.017	1.691 [1.214; 2.356]
Antibiotic therapy	48	56	0.193	1.06 [0.731 ; 2.157]
Corticosteroid therapy	73	75	0.515	1.66 [0.932 ; 1.873]
Curative anticoagulation	35	69	0.000	3.898 [2.043; 7.436]
Mechanical ventilation	16	73	0.000	12.713 [4.903; 32.966]
Tracheotomy	5	4	0.149	0.68 [0.521 ; 2.147]

curative anticoagulation (n=104, 69.3%), and mechanical ventilation (n=89, 59.3%) (Table 1).

Eighty patients (53.3%) in Group 2 died of COVID-19 and 70 individuals (46.7%) survived and were discharged from the hospital (Group 1) (Table 2). The median hospitalization duration in ICU was 10 days for non-survivors (IQR [6, 17.5] days). Mortality causes were the following: hypoxemia (n=99, 66%), septic shock (n=32, 21.3%), cardiogenic shock (n=1, 0.7%), and multi-organ failure (n=18, 12%). We noted one case of coronary syndrome in Group 2 during the hospitalization in ICU.

Comparison of clinical characteristics between the two groups is presented in Table 2. The median age and the SAPS II score were significantly

Table 2 Comparison of clinical characteristics between the two patient groups

Characteristics	Total (n=150)	Group 1 (n= 70)	Group 2 (n=80)	p value
Age (years) median (IQR)	64.5 [20-92]	61 [20-92]	65[31-68]	**0.004**
Gender, n (%) Male Female	 88 (58.7%) 62 (41.3%)	 38(54.3%) 32(45.7%)	 50 (62.5%) 30 (37.5%)	0.324
Median SAPS II score	32 [27-38]	29 [24-33]	34 [29-46]	**<0.001**
Comorbidities, n (%)	121 (80.6%)	58 (82.8%)	63 (78.7%)	0.52
Hypertension, n (%)	68 (45.3%)	27 (38.6%)	41 (51.2%)	0.14
Diabetes mellitus, n (%)	61(40.7%)	28 (40%)	41 (51.2%)	0.876
Dyslipidemia, n (%)	19 (12.7%)	9 (12.9%)	10 (12.5%)	0.948
Coronary disease, n (%)	24 (16%)	11 (15.7%)	13 (16.3%)	0.929
Renal disease, n (%)	10 (6.7%)	4 (5.7%)	6 (7.5%)	0.752
Respiratory disease, n (%)	23 (15.3%)	11 (15.7%)	12 (15%)	0.904
Thyroid disorders, n (%)	8 (5.3%)	5 (7.1%)	3 (3.8%)	0.474
Obesity, n (%)	13 (8.7%)	7 (10%)	6 (7.5%)	0.772
Mechanical ventilation, n (%)	97 (64%)	20(28%)	77 (96%)	**<0.001**

*IQR: interquartile range; SAPSII: Simplified Acute Physiology Score. Bold **p values** mean statistically significant difference.*

higher in Group 2 vs Group 1. There was no significant difference in terms of gender and comorbidities (hypertension, diabetes mellitus, dyslipidemia, coronary heart disease, kidney disease, respiratory disease, thyroid diseases and obesity) between the two groups. Mortality ratio was significantly higher in invasive ventilated patients.

Laboratory parameters of COVID-19 survivors and non-survivors

Blood routine parameter results studied on admission are presented and compared between the two groups in Table 3. The levels of blood glucose, alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyltranspeptidase (GGT), alkaline phosphatases (ALP), total bilirubin, sodium, potassium, chloride, calcium, magnesium, phosphorus, total protein, N-terminal prohormone of brain natriuretic peptide (NT-proBNP), D-dimer, CRP, hemoglobin, white blood cells (WBC), neutrophils, lymphocytes and platelets were not significantly different between the two groups (Table 3). In contrast, blood urea, creatinine, Lactate dehydrogenase (LDH), Creatine kinase (CK), PCT and hs-troponin I levels were significantly higher in Group 2 (non-survivors) than in Group 1 (survivors).

Multivariate analysis

Variables with statistically significant differences between the two groups (median age, SAPS II score, BUN, creatinine, LDH, CK, PCT and hs-troponin I) were included in logistic regression analysis. Accordingly, LDH ≥ 484 U/L (OR=17.979; 95%CI [1.119-2.040]; p = 0.09) and hs-troponin I ≥ 6.55 ng/L (OR=12.492; 95%CI [1.691- 92.268]; p = 0.013) were independent predictors for mortality.

Table 3 Comparison of blood routine parameters between the two groups

Laboratory tests, median (IQR)	Total (n=150)	Group 1 (n=70)	Group 2 (n=80)	p value
Glucose, mmol/L	9.72 (6.64-15.41)	8.87 (6.04-15.64)	10.35 (7.28-15.2)	0.36
ALT, U/L	30 (18.5-44)	33 (20-48.5)	28(18-42)	0.115
AST, U/L	41(27-58)	36 (25-55)	42(28-60)	0.245
GGT, U/L	50.5 (27.7-77.7)	49 (27-78.5)	52(28-80)	0.904
ALP, U/L	65 (52-86.2)	60.5 (49.7-84.2)	69(57-89)	0.055
Total bilirubin, µmol/L	9.05 (6.9-12.7)	8.75 (6.6-12.10)	10 (7.7-13.1)	0.225
Urea, mmol/L	6.85 (5.2-10.95)	5.65(4.45-8.22)	8.85(6.37-13.47)	**<0.01**
Creatinine, µmol/L	71.9 (61.1-104.4)	66.5(55.1-86-1)	77.85(66.5-123.2)	**<0.01**
LDH, U/L	528.5 (411.25-660.75)	480(361-587)	608 (472-740)	**<0.01**

CK, U/L	69.5 (42-209.25)	55.5 (39.2-146.2)	91 (48.5-346.2)	**0.011**
Sodium, mmol/L	137(134-140)	136.5(133-139)	138(136-141)	0.13
Potassium, mmol/L	4.2 (3.9-4.62)	4.15 (3.8-4.6)	4.25 (3.9-4.7)	0.291
Chlorides, mmol/L	102 (99.75-105)	101 (99.75-104)	102 (99.25-105)	0.47
Calcium, mmol/L	2.08 (1.95-2.21)	2.09 (2.00-2.22)	2.04 (1.93-2.2)	0.56
Magnesium, mmol/L	0.9 (0.8-1.0)	0.9 (0.8-1.0)	1.0 (0.9-1.1)	0.50
Phosphorus, mmol/L	0.95 (0.81-1.21)	0.91 (0.79-1.14)	0.96 (0.82-1.28)	0.137
Total protein,g/L	66 (59-70.75)	67 (61-71)	65 (57-70)	0.079
CRP, mg/L	108 (52.8-120)	96(43-112)	118 (68-120)	0.732
hs-Troponin I,ng/L	12.1 (4.6-57.3)	5.5 (3.1-19.4)	21.6 (7.4-125.5)	**<0.001**
NT-proBNP, pg/mL	376 (137-1277)	245 (90.5-738.7)	565 (177-1608)	0.59
PCT, ng/mL	0.22 (0.0057-0.597)	0.1 (0-0.26)	0.32 (0.14-1.35)	**0.002**
D-dimer, ng/mL	1305.47 (738.8-2784.2)	1305 (730-2225)	1371 (732-4142)	0.527
Hemoglobin, g/dL	12.2 (10.87-13.4)	12.2 (10.8-13.4)	12.15 (10.9-13.6)	0.792
WBC, $*10^3/\mu L$	10.58 (7.43-13.5)	9.87 (6.77-12.85)	10.89 (8.02-14.5)	0.131
Neutrophils, $*10^3/\mu L$	9.41 (6.21-12.04)	8.83 (5.40-11.17)	9.75 (7.30-13.56)	0.076
Lymphocytes, $*10^3/\mu L$	0.77 (0.53-1.09)	0.84 (0.60-1.07)	0.69 (0.48-1.13)	0.086
Platelets, $*10^3/\mu L$	251 (197-313)	277 (217-327)	232 (184-306)	0.093

ALP: Alkaline phosphatases; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; CK: Creatine kinase; CRP: C-reactive Protein; GGT : Gamma-glutamyl-transpeptidase; LDH: Lactate dehydrogenase; NT-proBNP: N-terminal prohormone of brain natriuretic peptide; PCT: Procalcitonin; WBC: White blood cells. Bold p values mean statistically significant difference.

DISCUSSION

COVID-19 is now recognized as a multisystem disease that can cause a complex disorder affecting many organs, which may require ICU hospitalization (6). Our study investigated the demographical profile, pre-existing comorbidities and routine blood parameters of 150 COVID-19 patients hospitalized in ICU comparing survivors and non-survivors. The clinical features in our study were comparable with other studies (7). Mortality reported in the literature ranges from 30 to 80% (8–13). These differences can be explained by the wide variety and population heterogeneity in different clinical studies. Economic and organizational obstacles in some countries may also partly explain the worse outcomes. For instance, the reduced number of ICU beds in developing countries may delay the hospitalization of severe COVID-19 patients in ICU wards.

As in other cohorts, the demographical and clinical risk factors for mortality were the age, SAPS II score and need for mechanical ventilation. Comorbidities did not significantly influence the COVID-19 related mortality in our study. Many authors showed that comorbidities were associated with a higher risk for death in patients with COVID-19. Estenssoro *et al.* identified cardiovascular disease, chronic kidney disease and diabetes as important mortality risk factors in mechanically-ventilated COVID-19 patients (14). The gender role in mortality was observed in different series; male gender was associated with worse outcomes and death (7). In our study, non-survivors were predominantly males (50 vs. 30). These non-significant differences can be explained by the retrospective nature of the study and the relatively low number of recruited patients.

Consistent with previous findings, univariate analysis showed that urea, creatinine, PCT, total LDH, CK and hs-troponin I were significantly different between the two groups. Renal injury was frequently reported in patients with COVID-19, even in those who had no underlying kidney disease (15). The systemic immune response to the SARS-COV-2 leading to so-called a cytokine storm can be an explanation for the high prevalence of kidney injury in patients with COVID-19 (16,17). Therefore, kidneys may be a susceptible target of the SARS-COV-2 infection. Elevated level of urea at admission maybe an indicator for early kidney injury. Consequently, early detection of acute kidney injury may facilitate appropriate treatment, including avoiding nephrotoxic drugs and adequate fluid therapy (2).

We also found that PCT was significantly associated with death without being an independent factor of mortality. Similarly, a study investigating this marker as a COVID-19 mortality predictor, showed an upward trend of acute-phase proteins, including PCT in non-survivors, and a stable or downward trend in survivors (18). PCT levels appeared to be disease-severity-dependent and may be associated with bacterial co-infection (19). In addition, a recent study hypothesized that a progressive increase in PCT levels may predict a worse prognosis (20). Consistently to other studies, CK, a marker of muscle tissue damage, was associated with an increased mortality in patients with COVID-19 (21,22).

It is relatively common that COVID-19 patients have clinical signs of dehydration and hypovolemia. This may contribute to renal impairment and consequently to a mild increase in CK levels. In addition, muscle damage and CK elevation, even without respiratory symptoms, should be considered as a potential COVID-19 manifestation. Consequently, it is important to monitor CK levels in COVID-19 patients, especially when they complain of muscle pain and weakness (23).

We used logistic regression analysis to screen independent significant factors associated with in hospital-mortality in ICU. LDH ≥ 484 U/L (OR=17.979; [95% CI: 1.119-2.04]; p = 0.09) was an independent predictor for mortality. LDH, an ubiquitous enzyme, is well recognized as a prognostic marker related to the severity of several pathologies. LDH elevation in COVID-19 occurs in cell lysis syndrome and may reflect the extent of lung and other tissue damage (24-26). Additionally, LDH levels are elevated in thrombotic microangiopathy, which is associated with renal failure and myocardial injury (25). In the latter, the elevation of LDH can be associated to the elevation of troponin. In our study, hs-troponin I ≥ 6.55 ng/L (OR=12.492; 95% CI [1.691-92.268]; p = 0.013) was an independent predictor of mortality. Interestingly, a meta-analysis concluded that cardiac injury biomarkers mainly increased in COVID-19 non-survivors (26). Data on acute myocardial injury associated with COVID-19 shows a very strong independent association between increased troponin concentrations and disease severity, including mortality. It has been hypothesized that the acute inflammatory response in COVID-19 disease can cause rupture of atherosclerotic plaques leading to ischemia. Inflammation also causes endothelial dysfunction and increases the procoagulant activity of the blood, which can contribute to the formation of an occlusive thrombus over a ruptured coronary plaque (27,28).

In contrast to other studies, we did not find any prognostic value of CRP. However, Zhang et al. did not find any significant difference in CRP levels between survivors and non-survivors on ICU admission. However, at 1-3 days after admission CRP levels were significantly altered between the two groups (29). Interestingly, D-dimer did not differ between our two groups. The same results were reported by a multicentric study including 1260 patients (30). However, this marker has been considered as a prognostic marker in COVID-19 (31). Survival analysis by Zhang *et al*. find an association between 14-day mortality and an increase in D-dimer with no difference in 7-day mortality rate. Monitoring CRP and D-dimer levels during hospitalization would be interesting to evaluate the prognostic role of these markers.

Our study has several limitations. First, this was a retrospective single center investigation with a relatively low number of patients. Therefore, the only evaluated event was mortality. Second, laboratory parameters were analyzed only at admission. The evaluation of the kinetics of some biological markers would be interesting, thus further studies are needed to overcome these limitations.

In conclusion, our study showed that the levels of LDH and troponin on admission, were independent predictors of mortality. This can help clinicians to predict disease prognosis and perform early therapeutic interventions.

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Ethical approval

The study was approved by the Institutional Ethics Committee and was in accordance with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Conflict of interest

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

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Utility of biochemical markers in predicting severe COVID-19: experience from a tertiary hospital in South India

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COVID-19, SARS-CoV-2, interleukin-6, D-dimer, procalcitonin, ferritin

ABSTRACT

Background

Coronavirus Disease 2019 (COVID-19) patients can present with a wide array of symptoms. For laboratory investigation of these patients several biochemical tests are routinely requested. Here we wanted to evaluate the utility of procalcitonin (PCT), ferritin, D-dimer, interleukin 6 (IL-6) and total lactate dehydrogenase (LDH) activity in predicting severe COVID-19 infection.

Patients and methods

This study was undertaken at a tertiary care medical hospital in Tamil Nadu, India representing 183 COVID-19 RT-PCR positive patients, who were grouped based on their disease severity as mild (n=21), moderate (n=115) and severe (n=47) cohorts. All routine

clinical chemistry analysis was performed as part of routine baseline assessment. Biomarkers of inflammation and infection were tested via the measurement of IL-6, PCT, ferritin, and D-dimer. Serum IL-6 concentration was estimated by ELISA, while total LDH activity was analyzed by kinetic colorimetric assay. Serum ferritin, PCT and D-dimer were measured by fluorescent immunoassay by sandwich immuno-detection method.

Results

Biomarkers were significantly different among subgroups, and the highest concentrations were found in those with intensive care unit (ICU) admission. Serum PCT showed the best power to predict the need for ICU treatment followed by D-dimer, IL-6 and total LDH. Based on the AUC-ROC analysis, mortality was most effectively indicated by D-dimer followed by PCT, LDH, IL-6 and ferritin.

Conclusion

Our study highlights the utility of some routinely available biochemical tests in the management of severe COVID-19. The higher baseline values of these biomarkers hint towards the probability of severe infection and a larger risk of death.

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INTRODUCTION

Severe Acute Respiratory Syndrome Corona Virus 2 (SARS-CoV-2) has gripped the world after being first reported in Wuhan, China in December 2019. An enveloped single stranded RNA virus belonging to the family Coronaviridae and subfamily of orthocoronavirinae was isolated as the cause of the pandemic [1,2]. Since millions of people across the globe have been prey to this infection and have succumbed due to it. A highly populous country like India with low sanitation levels has been an easy target. The Coronavirus Disease 2019 (COVID-19) patients can present with a wide array of symptoms, which include mild fever, cough, fatigue, upper respiratory symptoms and gastrointestinal symptoms. Anosmia and dysgeusia have been reported to be frequently found in these patients. Some cases can develop severe complications, such as Acute Respiratory Distress Syndrome (ARDS), respiratory and cardiac failure leading to multiorgan dysfunction and death [3]. Early therapeutic intervention and continuous monitoring during therapy play a critical role in reducing mortality.

Evidence accumulated in recent past has suggested the critical role of cytokines and chemokines released due to cellular destruction caused by rapid viral proliferation [4]. The molecular testing forms the basis for diagnosis, but the requirement of sophisticated instruments and unavailability of trained personnel for performing Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) has been challenging. Several biomarkers are being utilized to predict severity of the disease. Inflammatory markers like Procalcitonin (PCT), C-reactive Protein (CRP) and interleukin-6 (IL-6) are being reported to be associated with the severity of COVID-19 infection [5]. Liver enzymes and renal functions are also monitored in patients suffering from COVID-19 [6,7].

Several biochemical tests are being performed in COVID-19 subjects. Risk stratification of COVID-19 cases can be done using the array of biochemical tests available. Hence, it is desirable to find early and effective predictors of clinical outcomes in these patients. Patients with severe COVID-19 presented with an immunochemical profile like in cytokine storm. The intensified production of pro-inflammatory cytokines may be involved in pathophysiology causing severe pulmonary oedema, respiratory failure and damage to organs, such as liver heart

and kidney [8]. Increase in pro-inflammatory cytokines e.g., IL-6 and tumour necrosis factor-α (TNF-α), have been observed in patients with severe disease and found to be significantly associated with mortality [9]. PCT is a routinely used inflammatory marker in the daily routine. Any microbial infection can cause a significant raise in PCT, as endotoxins and pro-inflammatory cytokines induce its release from parenchymal tissues. Various studies have supported the theory that considerable increase in PCT levels from its baseline value denotes the beginning of critical phase of COVID-19 infection [10]. Formation and lysis of cross-linked fibrin gives rise to D-dimer. This reflects the activation of coagulation and fibrinolysis. Severity of COVID-19 symptoms are found to be associated with hemostatic abnormalities and elevated levels of plasma D-dimer values [11]. Ferritin, being an acute phase reactant, is linked to the underlying systemic vasculitis that cause lesions in major organ systems [12]. Lactate dehydrogenase enzyme (LDH) is present in numerous tissues throughout the body; thus, tissue damage easily leads to its serum elevation. LDH in COVID-19 cases is seen as a marker of lung injury in the initial stage of the disease [13].

The plethora of pathological processes in COVID-19 include hyperinflammation, cytokine storm, dysregulation of coagulation pathway, thereby producing a picture of systemic vasculitis leading to varied fatal complications. Our study was to assess the utility of widely used biochemical parameters in predicting the severity and mortality in COVID-19 infection. We aimed to define the relative cut-off values for various biomarkers to foretell disease morbidity in COVID-19 infected individuals.

MATERIALS AND METHODS

This clinical study was carried out by the Department of Biochemistry, in a tertiary care medical college hospital located in Madurai, India. Consecutive adult patients with positive RT-PCR results were enrolled at this hospital from August 2020 to October 2020. The study was approved by the Institutional ethics committee. This study is in compliance with the ethical principles for medical research involving human subjects, in accordance with the Declaration of Helsinki. The patients were grouped according to their clinical symptoms into mild cases as group I, moderate cases were grouped as group II and severe cases were grouped as group III, based on National Clinical Management Protocol COVID-19, Revised version 3, dated June 13, 2020, by the Ministry of Health and Family Welfare, Government of India. According to the guideline, patients with uncomplicated upper respiratory tract infection, and mild symptoms, such as fever, cough, sore throat, nasal congestion, malaise and headache were categorized as mild cases, who could be managed at home. Pneumonia with no signs of severe disease with presence of clinical features of dyspnoea and or hypoxia, fever, cough, including SpO_2 <94% (range 90-94%) on room air, respiratory rate more or equal to 24 per minute were categorized as moderate cases. Severe cases were those patients who developed severe pneumonia or ARDS with severe hypoxia. Patients who presented with sepsis or acute life-threatening organ dysfunction caused by an unregulated host response to suspected or proven infection were considered as severe cases. Patients presenting with persisting hypotension despite volume correction or even after correction with vasopressors were also grouped as severe cases [14].

Exclusion criteria

Subjects showing negative RT-PCR results for COVID-19, or having a history of any hepatic and renal diseases prior to being infected with viral pneumonia were excluded. Pregnancy and

the presence of malignancy were exclusion criteria as well.

Inclusion criteria

All adults, who were tested for COVID-19 infection and had positive result by RT-PCR during the defined study period were included into the study.

Assignment of study group

Patients in the mild group I were treated with home quarantine. The moderate cases (group II) were admitted to the hospital and were treated in isolation wards. The severe cases assigned to group III required admission to intensive care unit (ICU). The patients were sub-grouped as survivors and non-survivors based on the mortality at the time of discharge from the health care facility for further analysis.

SARS-CoV-2 RT-PCR testing was done by a closed system, Truenat from Molbio diagnostics private limited, India on Truelab workstation. Qualitative detection of SARS-CoV-2 was done from upper respiratory specimens (nasopharyngeal swabs and oropharyngeal swabs) in our hospital. Results were calculated based on graphical analysis and cycle threshold (Ct) values. The Envelope (E) gene and Open Reading Frame-1 (ORF1) gene were targeted for detection of infection by commercially available kit, as per manufacturer's instruction [15].

Data collection

Clinical data included gender, age, time of admission and time of discharge. Routine biochemical and hematological tests were conducted to assess their baseline values. All routine clinical chemistry analysis like renal and liver functions, serum electrolytes, complete blood count were performed as part of the routine baseline assessment. The routine clinical chemistry tests were performed using Toshiba 120FR fully automated system for baseline assessment of the patients. Biomarkers of inflammation and infection were tested, which consisted of IL-6, PCT, ferritin, and D-dimer. Serum IL-6 was estimated using a commercially available human IL-6 ELISA kit (Biotech Diaclone, Besançon, France) with a sensitivity of 2 pg/mL as per the manufacturer's instructions [16]. Serum ferritin, PCT and D-dimer were measured by fluorescent immunoassay by sandwich immuno-detection method using i-Chroma analyzer [17]. Total LDH activity was analyzed by kinetic colorimetric assay.

Statistical analysis

Data was analyzed using IBM SPSS v.16.0 statistical software. The non-normal distribution was confirmed by subjecting data for Kolmogorov-Smirnov test. Continuous variables with non-parametric distribution were expressed as the median (25th percentile, 75th percentile). Mean values with standard deviation were used to express data that was continuous and equally distributed. The categorical variables were summarized as frequencies and percentages. The data were compared between the groups based on severity of COVID-19 infection by using ANOVA and K independent sample test for parametric and non-parametric distribution, respectively.

Students unpaired t-test and Mann-Whitney U test were used for two-group comparisons of continuous variables in different groups based on the mortality. Statistical significance was assumed if $p < 0.05$. Receiver Operating Characteristic (ROC) curve analysis was performed to determine the diagnostic utility of various biomarkers of COVID-19 for determining ICU admission and for predicting mortality. The measures of diagnostic accuracy including the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), positive likelihood ratio and negative likelihood ratio were calculated using MedCalc's diagnostic test evaluation calculator [18].

RESULTS

A total of 183 patients were included into the analysis, 69% (n=126) were males and 31% (n=57) were females. The patients were divided based on the severity of their disease as group I (n=21), group II (n=115) and group III (n=47). The mean age of the study population was 57.89 ± 14.3 years. Amongst our study population, 19.6% (n=36) died of the disease. There was no casualty in group I. Group II with moderate cases had a mortality rate of 10%, i.e., eleven cases. 53% of all cases (25 cases) from group III constituting severe cases, died. All statistical analyses and conclusions drawn are based on baseline values of the parameters studied. Table 1 shows the distribution of age, gender and biochemical markers amongst the three groups. All parameters showed difference across the 3 groups. P values for baseline characteristics and biochemical markers between the three groups are depicted in Table 1. All biomarkers were distributed in a statistically significant ($p<0.05$) manner amongst the groups. There was a statistical

Table 1 Distribution of baseline characteristics and biochemical markers of COVID-19 infected patients based on the severity of the infection

	Biological reference interval	Group I n=21	Group II n=115	Group III n=47	p value
Age (years)		47 ± 15	57 ± 14	62 ± 12	**<0.001***
Males [N (%)]		10 (48%)	88 (77%)	28 (60%)	
Total Protein (g/dl)	6-7.8	6.8 ± 0.6	6.4 ± 0.7	6.1± 0.8	**0.003***
Albumin (g/dl)	3.5-5.5	4 ± 0.3	3.7 ±0.4	3.6 ± 0.6	**0.002***
Sodium (mEq/L)	136-145	137 ± 4	135 ± 4	133 ± 6	**0.009***
Potassium (mEq/L)	3.5-5.0	3.9 ± 0.4	4.1 ± 0.6	4.3 ± 0.8	0.203
Chloride (mEq/L)	98-106	84 ± 42	100 ± 10	100 ± 6	0.857
Aspartate Transaminase (U/L)	Less than 35	30 (24, 58)	40 (34, 60)	51(35,69)	0.175
Alanine Transaminase (U/L)	Less than 35	24 (21, 36)	33(23, 53)	33 (25,56)	0.717
Alkaline Phosphatase (U/L)	36-92	80(69, 95)	73(59,101)	83 (64,106)	0.357
Urea (mg/dl)	17-43	22 (17, 27)	30(23, 42)	44 (29, 68)	**<0.001***
Creatinine (mg/dl)	0.7-1.3	0.8 (0.6, 0.85)	0.8 (0.6, 1)	1.0 (0.8,1.4)	**0.007***

Procalcitonin (ng/ml)	<0.1	0.1 (0.1, 0.1)	0.1 (0.1, 0.2)	0.3 (0.1, 0.6)	**< 0.001***
D-dimer (ng/ml)	<500	212 (165, 251)	382 (203, 743)	818 (368, 4490)	**< 0.001***
Interleukin 6 (pg/ml)	5.3 - 7.5	6.9 (5.1, 10.6)	50.8 (11.4,172.7)	144 (63.5, 32605)	**< 0.001***
Ferritin (ng/ml)	M: 20-250 F: 10-120	43 (18, 130)	328 (136, 536)	442 (188, 686)	**< 0.001***
Total lactate dehydrogenase (U/L)	60-100	514 ± 170	837 ± 378	1055 ± 539	**0.001***

*Notes: Data are mean ± SD and median (25th Percentile, 75th Percentile). *p <0.05 is significant, M: Males, F: Females.*

significance in the age distribution across the 3 groups, with older individuals having a higher disease severity.

The AUC-ROC curves were used for comparing the potential of different biomarkers such as PCT, D-dimer, IL-6, Ferritin and LDH to predict severity and mortality due to COVID-19, respectively (Figures 1 and 2). Accordingly, serum PCT had the best power to predict ICU admission followed by D-dimer, IL-6 and LDH.

Figure 1 **Receiver operator characteristic curves comparing the potential of biochemical markers to predict severity of COVID-19**

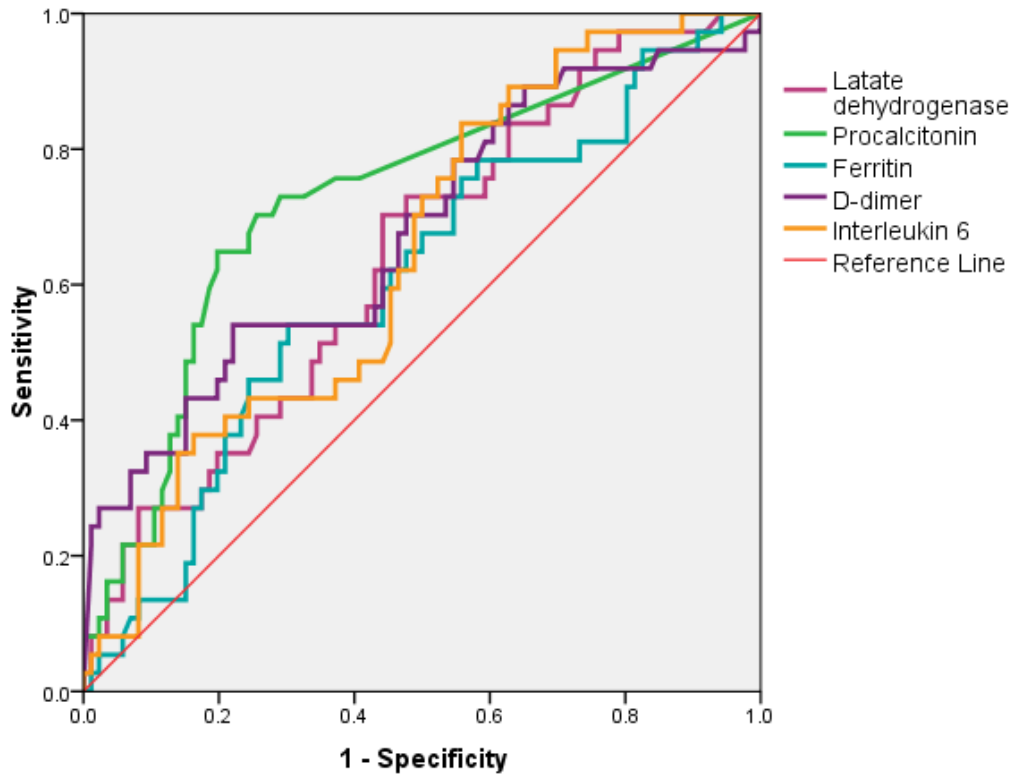
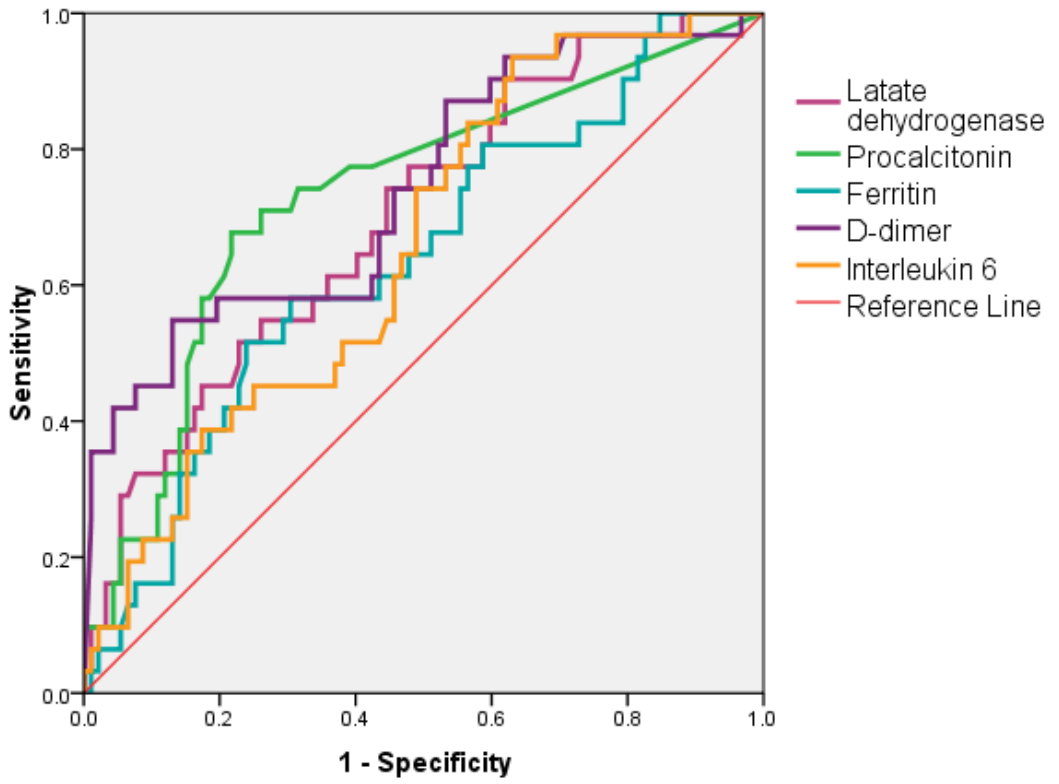


Figure 2 Receiver operator characteristic curves comparing the potential of biochemical markers to predict the mortality in cases infected with COVID-19



Based on ROC curves of biomarkers, comparison to predict mortality was done by analyzing the measures of diagnostic accuracy as displayed in Table 2. The ROC curve was used to obtain a specific cut-off for each biomarker. PCT had a sensitivity of 71% and a specificity of 70.7% at 0.15 ng/ml. Ferritin had a sensitivity of 58.1% and a specificity of 56.5% at 448 ng/ml. IL-6 showed a sensitivity of 74.2% and a specificity of 44.6% at 60 ng/ml, while D-dimer had a sensitivity of 58.1% and a specificity of 70.7% at 684 ng/ml. Finally, total LDH had a sensitivity of 77.4% and a specificity of 52.2% at 794 U/L (Figure 1).

PCT and D-dimer are seen to have better performance in comparison to IL-6, LDH and ferritin with respect to their AUC-ROC (Figure 1 and Table 2). D-dimer is seen to have best NPV followed by PCT to predict mortality. IL-6 was seen to have the highest PPV to predict mortality. The positive likelihood ratio for mortality prediction was seen to be best with IL-6 followed by PCT.

The distribution of biochemical markers among COVID-19 patients grouped based on their outcome are presented in Table 3. We found non-survivors to be significantly older than survivors.

DISCUSSION

This study is a retrospective study which was conducted to analyze the usefulness of some routinely available biochemical markers in the management of COVID-19 infection. Patients infected with SARS-CoV-2 infection tend to develop ARDS which requires early detection and monitoring from initial stages to prevent poor outcomes.

Table 2 Diagnostic performance of different biomarkers based on their cut-off value from the ROC curve analysis for the prediction of mortality in COVID-19

Biomarker (Cut-off)	AUC (95% CI)	p value	PPV (95% CI)	NPV (95% CI)	Positive Likelihood Ratio (95% CI)	Negative Likelihood Ratio (95% CI)
PCT (0.15 ng/ml)	0.731 (0.625, 0.838)	**<0.001***	71.43% (56.39-82.86)	70.25% (65.45-74.64)	3.89 (2.01-7.53)	0.66 (0.53-0.82)
Ferritin (448 ng/ml)	0.637 (0.525, 0.749)	**0.022***	55.56% (41.36 - 68.9)	62.61% (57.94-67.05)	1.75 (0.99 –3.09)	0.83 (0.69 -1.01)
IL-6 (60 pg/ml)	0.656 (0.551, 0.760)	**0.010***	80.56% (65.67-89.97)	58.22% (54.43-61.91)	4.23 (1.96-9.17)	0.73 (0.63-0.86)
LDH (794 U/L)	0.699 (0.594, 0.803)	**0.001***	72.22% (57.54-83.3)	56.07% (51.21-60.82)	2.49 (1.3-4.78)	0.75 (0.62-0.91)
D-dimer (684 ng/ml)	0.741 (0.636, 0.847)	**<0.001***	58.33% (44.7-71.24)	72.57% (67.55-77.07)	2.61 (1.48-4.62)	0.71 (0.56-0.90)

Abbreviations: AUC, area under the curve; ROC, receiver operator characteristic; positive predictive value (PPV), negative predictive value (NPV), PCT, Procalcitonin; LDH, Lactate dehydrogenase; IL-6, Interleukin 6.
**p <0.05 is significant.*

Table 3 Distribution of biochemical markers of COVID-19 infected patients grouped based on the outcome

	Survivors (N=147)	Non-Survivors (N=36)	t/z value	p value
Age (years)	56 ± 14	63 ± 10	-2.502	**0.013***
Total Protein (g/dl)	6.4 ± 0.7	6.2 ± 0.2	1.578	0.117
Albumin (g/dl)	3.7 ± 0.4	3.6 ± 0.4	1.337	0.183
Sodium (mEq/L)	135 ± 4	133 ± 7	2.270	**0.025***

Potassium (mEq/L)	4.1 ± 0.5	4.4 ± 0.8	-2.376	**0.019***
Aspartate Transaminase (U/L)	40 (31, 57)	54(40, 78)	-2.357	**0.018***
Alanine Transaminase (U/L)	31 (23, 49)	39(27, 64)	-1.565	0.118
Alkaline Phosphatase (U/L)	74(59, 102)	86(64, 101)	-0.986	0.324
Urea (mg/dl)	29(22, 41)	47(30, 71)	-4.552	**< 0.001***
Creatinine (mg/dl)	0.8 (0.6,1)	0.9(0.8, 1.3)	-2.184	**0.029***
Procalcitonin (ng/ml)	0.1(0.1, 0.2)	0.3(0.1, 0.7)	-4.486	**< 0.001***
D-dimer (ng/ml)	327(195,671)	1638(439, 9477)	-4.985	**< 0.001***
Interleukin 6 (pg/ml)	31.1(9.79, 164.4)	145(76.6, 312.3)	-4.371	**< 0.001***
Ferritin (ng/ml)	241(94,519)	619(313, 768)	-3.334	**0.001***
Lactate dehydrogenase (U/L)	820 ± 375	1133 ± 576	-3.714	**< 0.001***

*Note: Data are mean ± SD and median (25th Percentile, 75th Percentile). *p <0.05 is significant.*

Our study aimed at finding the utility of biomarkers for detecting severity of disease and predict disease outcome. We compared various biochemical tests among sub-groups based on disease severity. Five candidate biomarkers (Ferritin, PCT, IL-6, LDH and D-dimer) were chosen for comparison of their ability to predict severity and morality due to COVID-19 infection. We found that D-dimer and IL-6 had a vast difference between mild and severe cases. Similar finding was seen between survivors and non-survivors. PCT and D-dimer had a higher AUC-ROC curve for predicting severity and mortality as compared with other biomarkers.

Several studies have established that COVID-19 infected patients presented with pneumonia like symptoms [19,20,21]. The blood studies in COVID-19 infected cases at our tertiary care hospital revealed elevation of various inflammatory markers, such as IL-6, ferritin, D-dimer and PCT, which were comparable to previous reports [5,21,22]. ARDS associated with vast production of inflammatory cytokines, resulting in multi-organ dysfunction in viral infections resembles the features of secondary hemophagocytic lymphohistiocytosis (HLH) [23]. Such proinflammatory response due to exuberant elevation of cytokines has been previously documented in COVID-19 infections [24].

IL-6 is a pleiotropic cytokine, secreted by cells of innate and adaptive immune system as a response to microbial antigens. It causes enhanced activity of T and B cells, neutrophils and monocytes by triggering JAK2-STAT pathway. It induces the secretion of CRP, which helps in activation of classical complement pathway, thereby facilitating mediation of phagocytosis. IL-6 has been proposed to be a good marker of

prognosis in COVID-19 [5,25]. IL-6 contributes to the effective host defense against SARS-CoV-2 infection. However, extensive production of IL-6 can lead to cytokine storm which encompasses severe systemic inflammatory response [26]. IL-6 blockade therapy, using humanized anti-IL-6 receptor antibody, tocilizumab has been found to be beneficial in treating COVID-19 infections [25]. In our study, IL-6 levels were found to be elevated significantly in group III (severe COVID-19 infection). This was similar to the findings in a meta-analysis by Henry et al. [27] and Parsons et al. [28] suggesting the use of IL-6 as a biomarker for prognostic monitoring.

Bacterial infections stimulate amplified production of PCT from extrathyroidal tissue. In viral infections increased interferon-γ inhibits PCT production to remain it in normal limits in non-complicated cases of COVID-19 [28,29]. PCT is more likely to make a distinction between bacterial infection and other inflammatory processes than total leucocytes count or CRP levels [30]. We found PCT to be elevated in severe cases of COVID-19 infection as proposed by previous studies [4,30]. PCT is a crucial biomarker which if elevated at the time of hospitalization may be suggestive of severe COVID-19 infection.

Although lungs are the main target organ for COVID-19, kidneys and liver have been frequently affected due to the hyperimmune response caused by the virus [29]. Angiotensin converting enzyme-2 (ACE-2) receptors are known to ease binding of the virus and help in its entry into the cells [31]. ACE-2 receptors are present abundantly in small intestine, heart muscle, kidney, testis and thyroid [5]. The expression of ACE2 receptors on the renal tubules makes them a target organ for the virus [30]. Renal functions were seen to deteriorate with severe infection. The cholangiocytes have a higher expression of ACE2 receptors, thereby making them a suitable target for SARS-CoV-2 resulting in hepatic dysfunction. The mechanism proposed for transitory elevation in transaminases in COVID-19 infection is secondary liver damage due to hyperinflammatory response to infection. This can also be due to hepatotoxic drugs being used in the management of these patients [22]. Previous studies by Ferrari et al. [32] and Kumar et al. [6] claimed significant levels of elevation of transaminases in severe COVID-19 infections, whereas our findings did not show a statistical significance in the levels of transaminases amongst COVID-19 cases.

Serum ferritin, a marker of iron storage in the body, is seen to increase in cases of inflammation, hepatic disorder and malignancy [4]. It has been increased in patients with severe infection due to COVID-19 as a result of associated secondary HLH and cytokine storm [33]. Controlling of availability of iron to pathogens by ferritin plays a significant role in protecting the body against active infection [31]. Increase in ferritin levels is typically in the range of 500-3000 ng/mL. The increase in ferritin levels leads to activation of endothelial cells in the pulmonary vessels. This can cause imbalance in the normal hemostasis, regulation of fibrinolysis and maintenance of permeability of the vasculature. Such imbalance has a function in the development of COVID-19 vasculopathy resulted by inflammation [34]. The lower respiratory tract injury in COVID-19 patients explains elevated LDH levels. LDH being an indicator of lung injury, increases proportional to the severity of infection [26].

Hyper inflammation leading to elevated D-dimer and fibrinogen levels were seen to cause hypercoagulation and various complications such as Disseminated Intravascular Coagulopathy (DIC) [29]. D-dimer levels were seen to be higher in patients with severe infection as compared with milder infection of COVID-19. Such findings have been described earlier by Ponti et al., who suggested the activation of coagulation and secondary hyperfibrinolysis in mortality due to

COVID-19 infection [30]. D-dimer levels indicated thrombosis and elevated Fibrin Degradation Products (FDP) that occur due to thrombolysis [5]. Administration of anticoagulant therapy with low molecular weight heparin has been reported to be associated with a better prognosis due to decreased venous thromboembolism and DIC.

In our study, IL-6 levels were found to be elevated significantly in in non-survivors. Tjendra et al. studied various biomarkers to predict severity and outcomes in COVID-19, stated that patients with IL-6 >10 pg/ml had a concurrent elevation of various other biomarkers. Such candidates were more likely to develop sepsis and eventually die within 3 days of hospital admission [35].

Non-survivors showed higher values of PCT in our study which was similar to the findings of Gao et al. [20] and a meta-analysis by Malik et al. [22]. Haywood and colleagues studied hospitalization and mortality among COVID-19 patients and found that in-hospital mortality was related to abnormal level of biomarkers, such as lactate, creatinine, procalcitonin and platelet count [36]. Regarding laboratory changes in patients with fatal COVID-19, Henry et al. reported that elevation in the levels of certain biomarkers, such as IL-6, ferritin, PCT, LDH and D-dimer were often seen in cases with fatal COVID-19. PCT can serve as a marker of secondary bacterial infection, which could increase the probabilities of fatal outcome [27].

The non-survivors also exhibited increased serum creatinine and urea concentrations as compared with the survivors (Table 3). Non-survivors had significantly higher AST levels than other liver enzymes. AST with dominated increase was stated to reflect real liver injury [35]. Increased cytokine secretion, ACE2 receptor binding affinity of spike protein of the virus could be predominant cause of multiorgan injury in COVID-19 [37].

Elevation of serum ferritin could be either due to leakage from damaged cells or by active secretion from HepG2 cells and macrophages. Ferritin is seen to possess both immunosuppressive and pro-inflammatory effects [38]. The activation of monocyte-macrophage system causing inflammation is a primary cause of elevated serum ferritin. This supports the theory that diabetics are more prone to developing inflammatory storm which indirectly causes rapid worsening and a poor prognosis in COVID-19 patients [39]. Our patients exhibited high levels of ferritin in the severe and non-survivors of COVID-19 infection which was also observed in previous studies by Keddie et al. [29] and Aloisio et al. [40].

Li et al. evaluated the effect of serum LDH at admission and found it to be an independent risk factor for severity and mortality in COVID 19 cases. Under the influence of acute hypoxia or inflammation, due to lung infection, thrombogenesis and organ injury can occur, thereby making LDH an important marker in COVID-19 cases [41]. LDH is released from numerous tissues during death [29]. Bao et al. suggests LDH as a marker related to the risk of death in COVID-19 cases [42]. In our study, we found highest LDH levels in severe cases and among non-survivors.

Severe inflammation and hypoxia due to pneumonia cause activation of coagulation and fibrinolysis resulting in hypercoagulation state leading to DIC and multi-organ dysfunction. Zhang et al. have studied D-dimer in COVID-19 patients and concluded that D-dimer > 2µg/ml at baseline could predict in hospital mortality [11]. In addition, these patients were at a higher risk of developing pulmonary embolism. Malik et al. opined that elevated D-dimer was related to poor outcomes in COVID-19 patients [13]. Presence of prothrombotic milieu in non-survivors of COVID-19 infection could be the cause of elevated D-dimer levels. Patients with

severe infection and non-survivors exhibited higher levels of D-dimer. Thus, D-dimer is a reliable indicator of severity and can indicate outcome of the infection. This finding is supported by Ye et al. who suggested dynamic monitoring of D-dimer in hospitalized COVID-19 cases to monitor the risk of death [21].

Our observations reflect the efficacy of various biochemical markers. Biomarkers were significantly different amongst all groups and those with ICU admission had the highest concentrations. Serum PCT had the best power to predict ICU admissions followed by D-dimer, IL-6 and LDH (Figure 1). The areas under ROC curve was highest for D-dimer to predict the mortality followed by PCT, LDH, IL-6 and ferritin (Table 2). Finally, D-dimer is a better candidate amongst the chosen biomarkers based on its AUC-ROC curve for predicting mortality.

Our study being retrospective in nature is associated with few limitations. The lack of serial monitoring of various biomarkers is a drawback of our study. This was mainly due to the protocol followed at our institute that comprised of baseline laboratory assessment and continuous clinical monitoring. The inadequate knowledge about SARS-CoV-2 during the initial days were reasons behind such practice. Larger prospective studies with clinical correlation will help us to obtain valuable insights in the disease management and patient outcomes. One other limitation was that the study population included patients with comorbidities such as diabetes, hypertension, overweight, etc., which could also influence the severity and mortality of COVID-19. The sample population being heterogeneous in nature adds weightage to the study. We opine periodic monitoring of biomarkers among COVID-19 patients may aid the early detection of worsening of disease status. This can assist in timely escalation of the treatment protocol, which could be potentially lifesaving.

In conclusion, the higher baseline values of these biomarkers hints towards the probability of severe infection and increased mortality. Baseline biochemical markers help in segregation of high-risk cases and improve the management of patients resulting in an overall improvement. Stratification of cases helps in better management of hospital resources, manpower and aids early identification of requirement of ICU care. Our study highlights the utility of biochemical tests in management of COVID-19. The ease of testing makes them suitable for both triaging as well as monitoring of therapy.

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In this issue: Current laboratory aspects of COVID-19

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Verification and comparison of qualitative serological assays for Anti-SARS-COV-2 IgM and IgG antibodies detection

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SARS-CoV-2, COVID-19, serological assays, verification, antibodies

ABSTRACT

Background

Due to their wide application in the SARS-CoV-2 pandemic, we verified and compared three qualitative serological methods in order to select the most optimal that will best serve its purpose under laboratory conditions.

Methods

We assessed the diagnostic characteristics of two automated serological methods (Roche Elecsys® Anti-SARS-CoV-2 and Abbott SARS-CoV-2 IgG) and a POCT test (Colloidal Gold Method SARS-CoV-2 IgM/IgG Antibody Assay Kit). In the process of verification, analytical precision was also assessed for the automated assays.

Results

Diagnostic characteristics were determined by measuring antibodies against SARS-CoV-2 in 91 RT-PCR-negative and 60 RT-PCR-positive samples. The POCT test gave the highest number of false positive cases (8.61%). Roche Elecsys® Anti-SARS-CoV-2 gave only 2.65% false positivity and showed the highest diagnostic sensitivity of 98.33% (95% CI: 91.06–99.96), while Abbott SARS-CoV-2 IgG method showed 100.00% (95% CI: 96.03–100.00) diagnostic specificity and an almost perfect agreement with Roche Elecsys® Anti-SARS-CoV-2. When assessing the precision of the automated methods, we observed some variability in the positive control samples, but the values did not affect clinical interpretation.

Conclusion

Both automated methods demonstrate superior diagnostic characteristics compared to the Colloidal Gold Method, and this POCT test is not considered as an appropriate choice for routine testing. The two automated methods showed low variability without altering the results and their interpretation.

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INTRODUCTION

In December 2019, a new type of β-coronavirus began to emerge, which has been named Severe Acute Respiratory Syndrome Coronavirus, better known as SARS-CoV-2. Due to its severe pathogenicity and ability to spread in March 2020 the World Health Organization (WHO) declared it a global epidemic (1). Symptoms of COVID-19 infection are often nonspecific and heterogeneous, and depend on sex, age, immune status, viral load, associated diseases or possible history of other coronavirus infections (2–5). Despite the variation of responses from patient to patient, dry cough, fever, dyspnoea, loss of smell and taste are the most common symptoms and, in severe cases the infection may lead to death. In the same way, the kinetics of the immune response is also highly variable depending on the same factors as seen in symptoms (5,6). The severity and the magnitude of the epidemic with the variability of SARS-CoV-2 infection, which can affect the response of each person differently makes rapid, reliable and effective diagnosis essential. Diagnostic methods in an epidemic situation are playing an important role, particularly in controlling the epidemic and limiting the spread of the SARS-CoV-2 virus (7).

Diagnostics in SARS-CoV-2 epidemics is divided into indirect, serological assays that are measuring the humoral immune response, and direct diagnostic methods, which are detecting the presence of the virus by detecting viral RNA with RT-PCR or by detecting viral antigen, where POCT methods are most commonly used (7–9). POCT methods are particularly important for rapid diagnosis, whereas RT-PCR methods are more time-consuming and complex. Diagnostic sensitivity is crucial for both methods, as we want to minimise false negative results. To achieve this goal, it is important to be aware of the variability in viral load that is highest at the onset of symptoms, and sampling at the appropriate time point is also crucial for POCT methods, as they have inherently lower diagnostic sensitivity compared with RT-PCR (7,8,10–12).

Most common assays for serology are based on chemiluminescence or enzyme-linked immunosorbent principle. Immunochromatographic methods have also been developed for the purpose of point-of-care testing (6,13). Serological methods, which most often involve qualitative or quantitative detection of IgG and IgM antibodies, usually are directed against the nucleocapsid or spike protein of SARS-CoV-2. The combination of antibodies has been shown to be a more sensitive technique as part of the diagnostic approach

to infection identification and epidemic control (11). Seroconversion of IgM antibodies starts soon after the appearance of the symptoms but declines rapidly, whereas IgG antibodies appear in detectable concentrations around day 5 after the appearance of the symptoms and can remain detectable for several months (6,13,14). In relation to antibody seroconversion, serological methods are therefore not suitable to diagnose active infection, yet they are in an important adjunct to molecular methods, especially when the clinical picture is not consistent with the results (6,9,15). The combination of the two diagnostic approaches strongly increases the sensitivity of detecting the presence of infection in the acute phase (11,13).

Despite the limitations in detecting active infection, serological methods are of great importance for surveillance of the epidemiological situation, identification of patients who have been infected in the past and assessing the prevalence (8,9,12). Serological methods can also be a good approach to prognosis, since a correlation between the level of IgG antibodies against the nucleocapsid protein and the severity of the infection has been proven (12,16). In monitoring the immune response, serology may serve to identify those individuals who have developed a strong immune response and many of them can consequently be potential plasma donors for therapy of those, who have developed a more severe form of COVID-19 infection (8). The wide spectrum of use and importance of serological and other diagnostic methods, makes it essential to implement them as soon as possible especially during an epidemic. Despite the strong need for immediate implementation, a verification process is required before their use, mainly because of limitations, such as the impact of prevalence or disease stage, which may affect the sensitivity of the methods and consequently the quality of the results (8,10). Verification must cover the basic diagnostic and analytical properties of the method, as these are the characteristics that ensure the reliability of the results, and are crucial for the correct interpretation and comparison of the method with other methods, and also with other laboratories (17,18).

The aim of this paper is to verify three qualitative serological methods for the determination of specific antibodies against SARS-CoV-2 in order to determine which method gives the best results, best serves its purpose and consequently is the most optimal for early use in the laboratory. Repeatability, intermediate and intra-laboratory precision (intra- and inter-daily) were assessed to automated methods according to the CLSI EP15-A3 protocol to determine whether variability affects the results and final clinical interpretation. Coefficients of variation (CVs) were also compared with the manufacturer's claims.

METHODS & MATERIALS

Study design

Serological analyses were performed on all three methods in the Hormone and Tumour Marker Laboratory and the Body Fluid Laboratory during the onset of the epidemic. RT-PCR analysis was performed at the Institute of Microbiology and Immunology. Serum samples were obtained from the staff at the University Medical Centre Ljubljana, the Clinic for Infectious Diseases and Febrile Conditions and the Clinical Institute of Clinical Chemistry and Biochemistry. A proportion of the samples also belonged to hospitalised patients infected with COVID-19. The samples were anonymised residues of routine diagnostic samples.

Defining diagnostical properties

As part of the verification of serological methods used, diagnostic specificity, diagnostic sensitivity and predictive values were determined. The results obtained by the serological methods were compared with the RT-PCR results considering

the cut-off values of the manufacturer. For ease of overview, a 2x2 contingency table was drawn to calculate the diagnostic characteristics for each method. The methods were compared according to the number of false results and Cohen's kappa coefficient (κ).

Assesing analytical precision of automated assays

The precision of the automated methods was assessed according to the CLSI EP15-A3 protocol. We performed a 5x5 experimental model and assessed the repeatability, intermediate precision and intra-laboratory precision (intra and inter-daily) (18). We used a laboratory-prepared negative and positive control samples. The negative control was a 'pool' of two samples that were negative for SARS-CoV-2 antibodies and negative based on the RT-PCR test. The positive control was prepared from a 'pool' of two other samples reactive to SARS-CoV-2 antibodies and positive on the RT-PCR test. The precision was calculated by using one-way ANOVA. By monitoring variability, we observed the possible impact on the results and data interpretation. CVs were also compared with manufacturer's precision results. In case of deviation, statistical comparisons were performed to demonstrate that there is no statistically significant difference between the values.

Samples

In order to determine diagnostic properties and assess precision, we collected a total of 151 serum samples from subjects for whom we had information that a previous RT-PCR test had been performed. Out of the 151 samples, we used samples from non-hospitalised random subjects who were RT-PCR positive (n = 41), hospitalised patients with COVID-19 who were also RT-PCR positive (n = 19) and random subjects who were RT-PCR negative (n = 91) to determine the diagnostic properties of the methods. Age, sex, other possible infections, immune status, symptoms, the time since possible infection and time since RT-PCR result were not considered when collecting the samples. Serum samples were appropriately aliquoted and prepared for individual analyses, which were performed consecutively on all three methods within one day, avoiding repeated freeze-thawing.

Serological methods

We used three qualitative serological methods to determine specific antibodies against SARS-CoV-2. The general characteristics of the methods are listed in Table 1. The Roche Elecsys® Anti-SARS-CoV-2 method performed on a Cobas e411 analyser detects total Ig (IgG and IgM) by electro-chemiluminescence (ECLIA), whereas the Abbott SARS-CoV-2 IgG method performed on an ARCHITECT i1000SR analyser detects only IgG antibodies by chemiluminescence paramagnetic immunochemical immunoassay (CMIA). Both automated assays detect antibodies directed against the nucleocapsid (N) protein of the virus. The last method manufactured by Maccura Biotechnology is the SARS-CoV-2 IgM/IgG Antibody Assay Kit by Colloidal Gold Method POCT, which detects separately IgG and IgM antibodies against the SARS-CoV-2 antigen using the principle of colloidal gold immunochromatography.

Statistical analysis

Descriptive statistics were run in Microsoft Office Excel 2016 (Microsoft Corporation, Washington, USA). We calculated the diagnostic parameters and Cohen's kappa coefficient (κ) in GraphPad Prism 9 (GraphPad Software Inc., San Diego, CA, USA). Diagnostic parameters were presented with 95% confidence interval (CI) determined by the Clopper-Pearson method.

Precision of automated methods was calculated using one-way ANOVA in Microsoft Excel 2016 version Analyse-it Software Method Validation edition (Ltd. The Tannery, 91 Kirkstall Rd., Leeds,

Table 1 General characteristics of the three serological methods. Diagnostic properties of automated methods are presented with 95% confidence interval (CI)

Characteristics	Roche Elecsys® Anti-SARS-CoV-2	Abbott SARS-CoV-2 IgG	Maccura Biotechnology (Colloidal Gold Method)
Method	ECLIA*	CLIA**	Immunochromatography
Target	Nucleocapsid protein	Nucleocapsid protein	Antigen
Detection	Total antibodies IgG/IgM	IgG antibodies	Separate IgM and IgG antibodies
Way of interpretation	Automated	Automated	Manually
Unit	Cut-off index COI (S/C)***	Index (S/C)***	Not Applicable
Result interpretation	Positive: COI ≥ 1.0 Negative: COI < 1.0	Positive: Index ≥ 1.4 Negative: Index < 1.4	Positive: Colour reaction on the control line and test line Negative: Colour reaction on the control line
Diagnostic properties (manufacturer)	Sensitivity 100% (95% CI: 88.1–100) Specificity: 99.81% (95% CI: 99.65–99.91)	Sensitivity 100% (95% CI: 95.89–100) Specificity: 99.60% (95% CI: 98.98–99.98)	True positive: 3 out of 5 for IgG and 2 out of 5 for IgM False positive: none for IgG and 2 out of 20 for IgM

*Keys: *electro-chemiluminescence immunoassay, **chemiluminescent magnetic microparticle immunoassay, ***Signal (Sample/Calibrator).*

UK) and IBM SPSS Statistics 28.0 for Windows (Armonk, New York: IBM Corp.). We presented the results of the 5x5 experimental model using the average value, standard deviation (SD) and coefficient of variation (CV). Precision values that differed from the manufacturer's results were evaluated by F-test to assess whether the difference was statistically significant. The limit of statistical significance was $\alpha<0.05$. Graphical representations were produced in Microsoft Excel 2016 and GraphPad Prism 9.

RESULTS

Diagnostical properties of the serological assays

A total of 151 serum samples were analysed by all three methods to determine diagnostic sensitivity, diagnostic specificity, positive predictive value (PPV) and negative predictive value (NPV). Out of 151 samples, the Colloidal Gold Method detected the presence of at least one antibody class in 61 samples and no antibodies were

detected in 90 samples. A control line was visible in all test plates and therefore it can be claimed that no invalid results were observed. The automated Abbott SARS-CoV-2 IgG method detected the presence of IgG antibodies in 55 samples, the remaining 96 were negative. On the Roche Elecsys® anti-SARS-CoV-2 method, which automates the detection of total IgG and IgM antibodies, 62 out of 151 samples were positive and 89 samples were negative for SARS-CoV-2 antibodies. For each method, a 2x2 contingency table was plotted based on the RT-PCR result that previously confirmed or rejected the suspicion of COVID-19 infection. This is how we defined false and true results and presented them in absolute value and as a proportion of all samples analysed. The POCT SARS-CoV-2 IgM/IgG Antibody Assay Kit by Colloidal Gold Method correctly detected the presence or absence of at least one of the SARS-CoV-2 antibody classes in 138 (91.39%) samples. Out of all false results, 7 (4.64%) were false positive and 6 (3.97%) were false negative. The Abbott SARS-CoV-2 IgG automated method produced slightly fewer false results, 5 (3.31%), all of which were false negative. The second automated method, the Roche Elecsys® anti-SARS-CoV-2 method, gave the highest number of true results. Out of all false results, 3 (1.99%) were false positive and 1 (0.66%) was a false negative, according to the previous RT-PCR results. For all three methods, diagnostic parameters were calculated from the results and given with 95% confidence intervals. The results with 95% confidence intervals (CI) for all three methods are presented in Table 2. The lowest diagnostic characteristics were estimated for the POCT SARS-CoV-2 IgM/IgG IgM/IgG Antibody Assay Kit by Colloidal Gold Method. The highest diagnostic specificity was exhibited by Abbott SARS-CoV-2 IgG method and the highest diagnostic sensitivity by the automated Roche Elecsys® anti-SARS-CoV-2 method.

Comparison of serological assays

The results were initially compared in terms of the number of true results (TN + TP) and false results (FN + FP), which is graphically shown in Figure 1. We found that the POCT Colloidal Gold

Table 2 Diagnostic characteristics for all three serological methods applied in our study

Method	Diagnostic specificity		Diagnostic sensitivity		*NPV		**PPV	
	Value (%)	95% CI (%)	Value (%)	95% CI (%)	Value (%)	95% CI (%)	Value (%)	95% CI (%)
Maccura Biotechnology (Colloidal Gold Method)	92.31	84.79–96.85	90.00	79.49–96.24	93.33	86.05–97.51	88.52	77.78–95.26
Abbott SARS-CoV-2 IgG	100.00	96.03–100.00	91.67	81.61–97.24	94.79	88.26–98.29	100.00	93.51–100.00
Roche Elecsys® anti-SARS-CoV-2	96.70	90.67–99.31	98.33	91.06–99.96	98.88	93.90–99.97	95.16	86.50–98.99

**Negative predictive value, **Positive predictive value.*

Method by Maccura Biotechnology had the highest number of false results and the Roche Elecsys® anti-SARS-CoV-2 automated method had the lowest, which is also reflected in the better diagnostic performance shown in Table 2.

The methods were also compared with each other in terms of the level of agreement, which was determined by Cohen's kappa coefficient (κ). When comparing the POCT SARS-CoV-2 IgM/IgG Antibody Assay Kit by Colloidal Gold Method and the Abbott SARS-CoV-2 IgG method, we only observed an agreement between the presence or absence of IgG antibodies, as the automated method does not identify IgM antibodies. The level of agreement with the given Cohen's kappa coefficient (κ) and 95% CI are shown in Table 3. We found that the automated Roche Elecsys® anti-SARS-CoV-2 and Abbott SARS-CoV-2 IgG methods differed only in 7 results (7 results were negative by Abbott SARS-CoV-2 IgG but were positive with Roche Elecsys® anti-SARS-CoV-2), that is why these methods had the highest level of agreement. The weakest agreement was observed between POCT Colloidal Gold Method and the automated Roche Elecsys® anti-SARS-CoV-2 method where the methods differed in 17 results (9 results were positive with Roche Elecsys® anti-SARS-CoV-2 but were negative with Colloidal Gold Method, while 8 results were negative with the automated method but were positive by Colloidal Gold Method).

Figure 1 **Graphical presentation of the number of false and true results among all three serological methods where FN means false negative, FP false positive, TN true negative and TP means true positive data**

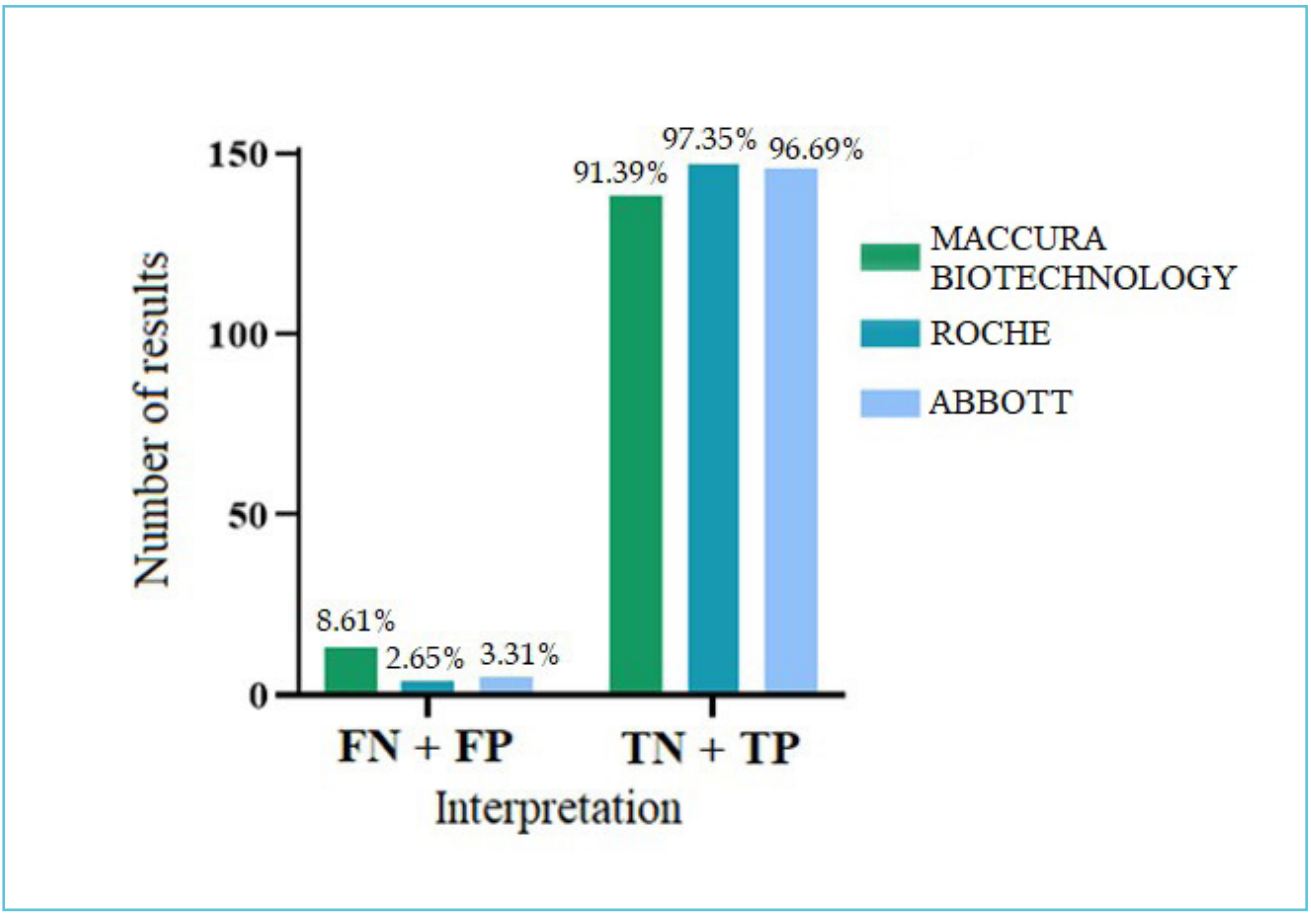


Table 3 Statistical agreement between serological methods demonstrated by Cohen's kappa coefficient (κ). The value of κ showed whether there was any agreement between two methods (19): none (κ = 0-0.20), minimal (κ = 0.21-0.39), weak (κ = 0.40-0.59), moderate (κ = 0.60-0.79), strong (κ = 0.80-0.90) or almost perfect (κ > 0.90)

Maccura Biotechnology vs Abbott		Abbott SARS-CoV-2 IgG		
		Positive	Negative	Total
Maccura Biotechnology (Colloidal Gold Method)	Positive	**43**	**0**	43
	Negative	**12**	**96**	108
	Total	55	96	151
κ = 0.82 (0.72–0.92)				
Maccura Biotechnology vs Roche		**Roche Elecsys® anti-SARS-CoV-2**		
		Positive	Negative	Total
Maccura Biotechnology (Colloidal Gold Method)	Positive	**52**	**8**	60
	Negative	**9**	**82**	91
	Total	61	90	151
κ = 0.76 (0.66–0.87)				
Roche vs Abbott		**Abbott SARS-CoV-2 IgG**		
		Positive	Negative	Total
Roche Elecsys® anti-SARS-CoV-2	Positive	**55**	**7**	62
	Negative	**0**	**89**	89
	Total	55	96	151
κ = 0.90 (0.83–0.97)				

Analytical precision of automated assays

The results for repeatability, intermediate precision and intra-laboratory precision (intra- and inter-daily) for the automated Roche Elecsys® anti-SARS-CoV-2 and Abbott SARS-CoV-2 IgG methods are presented in Table 4, together with the manufacturer's values given. The within-run and between-run signal variation is shown in Figures 2A and B for Abbott SARS-CoV-2 and Figures 2C and D for Roche Elecsys® anti-SARS-CoV-2. Based on the values listed in Table 4 and graphical representation, we can estimate that on both methods the variability of the between-run signal is slightly higher in positive control samples. Repeatability is slightly poorer for negative control samples on both methods. Despite the smaller variability, we can conclude that the precision of both automated methods was satisfactory, and the variability was too small to affect the results given by the method based on the values of coefficients of variation. Variability of the Roche Elecsys® anti-SARS-CoV-2 method did not exceed the manufacturer's values, so it can be concluded that both automated methods meet the manufacturer's criteria in terms of precision.

DISCUSSION

Despite some limitations of the serological methods, especially in identifying infection at an early stage, they are a very important complement to molecular methods and an important tool for epidemic surveillance, determining seroprevalence in the general population, understanding the

Table 4 Precision results for automated methods. The manufacturer's values are coloured in blue

		Abbott SARS-CoV-2 IgG				Roche Elecsys® anti-SARS-CoV-2			
		Negative control		Positive control		Negative control		Positive control	
Average		**0.07 Index**	0.04 Index	**3.51 Index**	3.53 Index	**0.08 COI**	0.059 COI	**66.03 COI**	2.97 COI
Repeatability	SD	**0.004**		**0.08**		**0.02**		**0.798**	
	CV (%)	**5.9**	5.9	**2.3***	1.1	**2.6**	2.6	**1.2**	1.3
Intermediate precision	SD	**0.002**		**0.109**		**0.000**		**0.971**	
	CV (%)	**2.8**		**3.1**		**0.0**	5.0	**1.5**	2.2
Intra-laboratory precision	SD	**0.004**		**0.136**		**0.002**		**1.257**	
	CV (%)	**6.5***	5.9	**3.9***	1.2	**2.6**		**1.9**	

** Imprecision value is higher than that declared by manufacturer. After further statistical analysis we concluded that there is no statistical difference between the values.*

immune response of individuals to infection, understanding the virus and the development, and monitoring the response to vaccines (8,9,12).

Several serological methods have been developed in recent years, which, like other diagnostic methods during an epidemic, need to provide rapid and, above all, high-quality and reliable results. In order to meet these requirements, irrespective of the urgency for a particular method, the laboratory should ensure that an appropriate verification step is performed before implementing the method, in which the user is informed about the properties and limitations of the method and an assessment is made as to whether the method serves its purpose under laboratory conditions (8,17,18).

In order to implement the most appropriate method in the laboratory, three qualitative serological methods were verified and compared - the automated Roche Elecsys® Anti-SARS-CoV-2 and Abbott SARS-CoV-2 IgG methods and the POCT SARS-CoV-2 IgM/IgG Antibody Assay Kit by Colloidal Gold Method. The most obvious difference between the methods was the class of antibodies detected, with the Roche Elecsys® Anti-SARS-CoV-2 measuring total IgG and IgM, the POCT Colloidal Gold Method analysing IgG and IgM separately, while the automated Abbott SARS-CoV-2 IgG method detected only IgG antibodies. The property of the POCT Colloidal Gold Method, which therefore detects the two types of antibodies separately, may be an advantage over the automated methods in terms of predicting the stage of disease. It is known that the separate identification of IgM and IgG antibodies together with molecular methods can predict whether an infection is acute or in a late-phase or convalescent, considering the kinetics of the immune response, the patient's status and the method's ability (11).

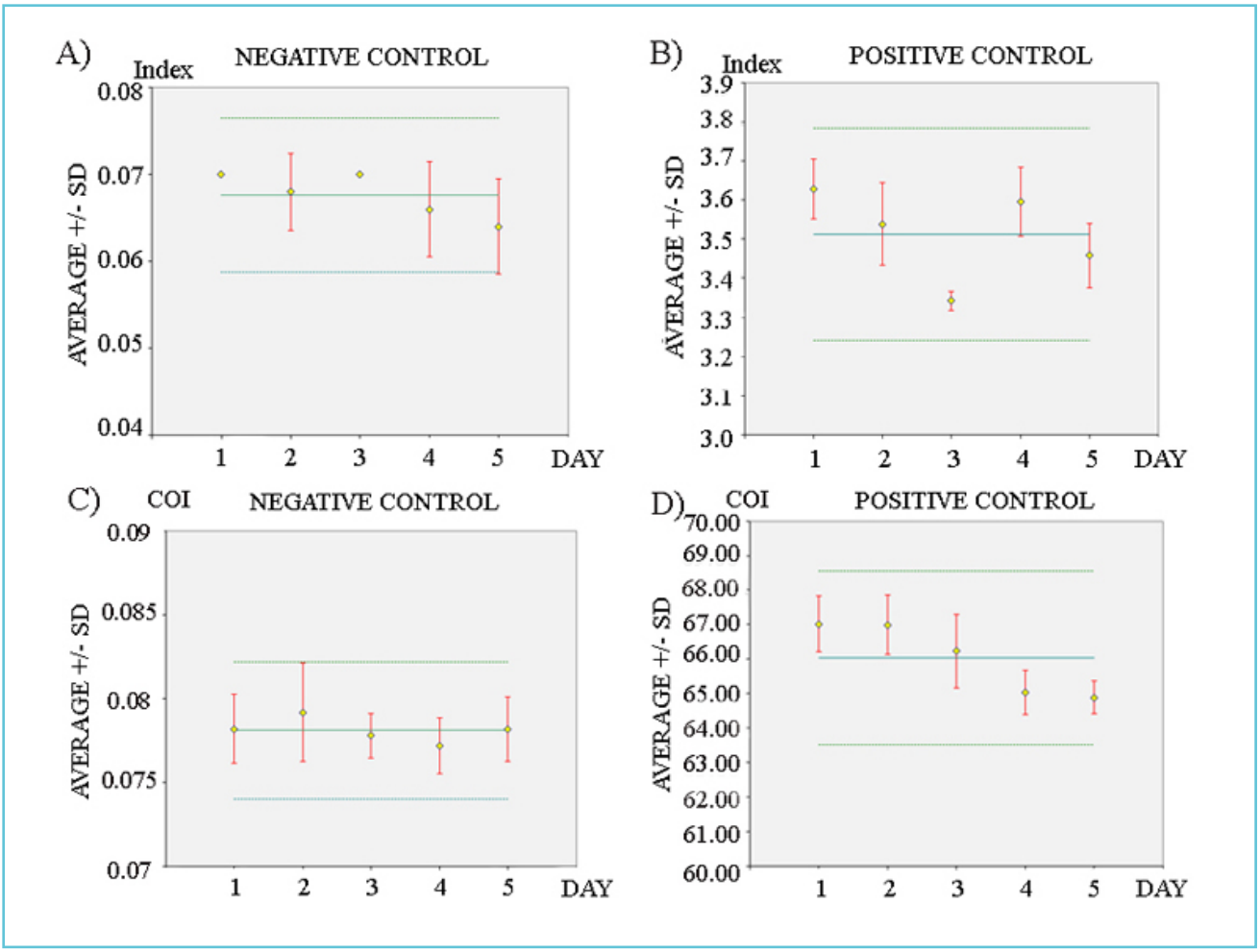
For this reason, the Abbott SARS-CoV-2 IgG method cannot be used in addition to molecular methods to detect early-phase disease, as this requires information on IgM antibodies as well. Nevertheless, the result obtained with the latter method is useful for demonstrating the presence of a history of COVID-19 infection (6,10). The limitations of IgG antibody detection in the early stages of infection were confirmed by Chew et al. who showed that the method had the highest clinical sensitivity after 14 days from the onset of symptoms (20). The known general properties of the selected qualitative serological methods already suggest that they are optimal in their use and performance. In order to implement the optimal method in the routine laboratory, we performed a verification study to determine the diagnostic characteristics of all three methods and to assess the precision of the two automated methods, in addition to the known properties.

The results of diagnostic sensitivity, diagnostic specificity and predictive values were obtained by measuring a total of 151 serum samples for which we had information on the result of a previously performed RT-PCR test (Table 2). We found that the POCT Colloidal Gold Method gave the highest number of false results (Figure 1), which was expected, as the sample preparation can influence the accuracy of the result and the visual reading makes the interpretation non-objective. We found that the most common cause for false-positive results was a reaction in the IgM antibody detection test line. The cause for false detection of IgM antibodies in POCT methods was investigated by Wang and his co-workers, who found that the presence of rheumatoid factor significantly increased the chance of false-positive results for IgM antibodies (21). In our case, this finding cannot be rejected or confirmed, as rheumatoid factor was not measured in these samples. In the case of false-negative results given by the POCT Colloidal Gold Method, we assumed that the reason was the low concentration, which was not detected

by the method because of its limited sensitivity compared to automated methods.

Since the diagnostic sensitivity is crucial, especially when using serological methods in the early phase of infection, it can be concluded that the automated Roche Elecsys® Anti-SARS-CoV-2 method is the most useful method with the lowest false-negative results according to the estimated sensitivity (11). The method is most likely to give the best results due to the identification of both classes of antibodies, which reduces the impact of the time elapsed since the onset of symptoms or a positive RT-PCR result. It can be assumed that this result could be further improved if this time were known and limited to a maximum of 14 days when seroconversion is usually definitely detectable (11,14).

Figure 2 **Graphical representation of in series and between series variability of each control sample where yellow dots represents average value in one day, dotted red line represents total average ± 2SD and continuous line represents total average of a signal.**
A) Abbott SARS-CoV-2 IgG variability of negative control sample
B) Abbott SARS-CoV-2 IgG variability of positive control sample
C) Roche Elecsys® anti-SARS-CoV-2 variability of negative control sample
D) Roche Elecsys® anti-SARS-CoV-2 variability of positive control sample



Despite the highest diagnostic sensitivity of the Roche Elecsys® Anti-SARS-CoV-2 method, it is important to be aware of that the method is still not suitable for the detection of acute infection. This was confirmed by Brochot and his coworkers, who investigated the issue of diagnostic sensitivity in their study, where, in particular for the detection of IgG class antibodies, false-negative results were detected at an early stage and also in asymptomatic patients. Because of these limitations, the study suggested that negative results of serological methods should be interpreted together with the patient's status and the method's capabilities (22).

Compared to diagnostic sensitivity, the Abbott SARS-CoV-2 IgG method had the highest diagnostic specificity, suggesting that there was no cross-reactivity with other respiratory viruses, which is the most common cause of false-positive results. Slightly lower diagnostic specificity was observed with the POCT Colloidal Gold Method and Roche Elecsys® Anti-SARS-CoV-2 methods, which could be explained by interferences that may cause false-positive data. The results could also be explained by the actual presence of antibodies in the presence of an otherwise negative RT-PCR result as is in the case of copresence of IgM antibodies in POCT Colloidal Gold Method and Roche Elecsys® Anti-SARS-CoV-2 methods. A situation can occur in the case of a false-negative RT-PCR result due to a low viral load at the time of collection (8). In this case, if the RT-PCR test was repeated and the serological results with true positive IgM were confirmed, an acute phase of infection could be inferred, as IgG antibody seroconversion has not yet occurred (13,14).

As part of the method verification and comparison, the level of agreement was assessed using Cohen's kappa coefficient (κ) and almost perfect agreement was found between the automated Roche Elecsys® anti-SARS-CoV-2 and Abbott SARS-CoV-2 methods (Table 3). This was the result we expected, based on the diagnostic properties found. In contrast to our assessment of agreement between the automated methods, Parai and colleagues found much poorer agreement between the Roche Elecsys® anti-SARS-CoV-2 and Abbott SARS-CoV-2 IgG methods (κ = 0.694; 0.641–0.746) in their study where they compared three chemiluminescent methods (23). According to our criteria, such level of agreement is considered to be moderate (19). Despite the lower level of agreement in some other studies, our results and those of other studies on the diagnostic performance of serological methods confirm that automated methods, in particular the Roche Elecsys® anti-SARS-CoV-2 method, have very good diagnostic characteristics.

In addition to the diagnostic properties, the precision of the two automated methods was assessed in the verification process according to the CLSI EP15-A3 protocol using a 5x5 experimental model. We assessed the repeatability, intermediate precision and intra-laboratory precision (intra and inter-daily) and estimated the possible impact on the results. In addition, we also compared the precision results with the manufacturer's data (Table 4). Despite the slightly higher variability in positive controls observed with both automated methods (Figure 2), we did not detect any major deviations that would affect the interpretation of the result. Based on the precision results, we can conclude that both methods also meet the manufacturer's criteria. Conflicting results were obtained by Padoan et al., who observed the highest variability in negative controls and concluded that the Abbott SARS-CoV-2 IgG method did not meet the manufacturer's criteria (24). The difference between the results compared with ours could be explained by the use of the 5×4 experimental model used in Padoan's study, as this model may give poorer results and may not capture all variability factors (18). Due to the difference in signals, we could not compare the precision of

the two methods. The problem of comparing qualitative methods due to signal differences was highlighted by Lee in her study. She also studied the importance of the signal and found a correlation between the CMIA-based method Index value and the severity of infection (25). Despite the satisfactory results, our assessment underestimated the variability between series, as we did not change reagents during the experimental work. The reagent replacement with different lot numbers or repeated calibrations could have been affected by random error.

Despite encouraging results, the paper has some limitations. First, the biggest limitation of our study is the relatively small number of samples, with which we verified all three serological methods. This reduced the statistical power of the results we obtained. Second, in the absence of information on the prevalence of SARS-CoV-2 virus in the studied population, we did not compare the predictive values with the manufacturer's data and with the data from other studies using the same serological methods. Third, when assessing analytical precision, we also compared the coefficients of variation with the manufacturer's results, which can often underestimate or overestimate the variability of the signal as shown by Martinello and colleagues (26). Also, even more accurate results on variability in the laboratory would be obtained, if the experimental model was extended over several days or several repetitions, as this would capture more of the potential causes of variation, such as changing reagents and performing calibrations (18).

Despite some limitations of our work, we can conclude that the automated methods have better diagnostic properties than POCT methods and we can also state that their precision is satisfactory, as the variability does not affect the results and CVs meet the manufacturer's criteria. Due to the better diagnostic sensitivity and performance, it can be concluded that the Roche Elecsys® Anti-SARS-CoV-2 automated method is better than the other methods for the identification of infected and recovered persons, as it gives fewer false results. We can conclude that, in terms of diagnostic properties and precision, the automated methods produce high-quality results that can be trusted and interpreted correctly. Despite the satisfactory results, further investigations could improve our work by including more patients in order to increase statistical power and via obtaining more data on the tested subjects to evaluate diagnostic methods more accurately. At the same time expanding the experimental model and comparing the results with other laboratories are also necessary (27).

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Ethical approval

We obeyed the ethical rules while obtaining and testing the samples.

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Performance of Elecsys Anti-SARS CoV-2 (Roche) and VIDAS Anti-SARS CoV-2 (Biomérieux) for SARS-CoV-2 nucleocapsid and spike protein antibody detection

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ABSTRACT

Introduction

Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), the novel viral pathogen that causes coronavirus disease 2019 (COVID-19) in humans, has spread worldwide since its identification in late 2019. The pandemic produced an accelerated development of new serological techniques for diagnosis.

Methods

We evaluated two commercial assays for serological diagnosis of SARS-CoV-2 infection, approved by the Administración Nacional de Medicamentos, Alimen-

tos y Tecnología Médica (ANMAT) in Argentina: Elecsys Anti-SARS-CoV-2; Roche for nucleocapsid total antibody detection, and VIDAS Anti-SARS-CoV-2 bioMérieux for spike protein IgG antibody detection. Sensitivity was assessed using a panel of 92 plasma samples from recovered COVID-19 patients who were positive for RT-PCR and positive for neutralizing antibodies by plaque reduction neutralization test (PRNT) and/or positive for IgG antibodies by indirect immunofluorescence assay (IFA). Specificity was determined studying 71 plasma samples collected during year 2018 prior to the COVID-19 pandemic. Assays were evaluated as stand-alone tests.

Results

Sensitivity was 97.8% and 98.9% for the Roche and bioMérieux assays, respectively, specificity: 98.5% (Roche) and 97.1% (bioMérieux), positive predictive value (PPV): 98.9% (Roche) and 97.8% (bioMérieux), and negative predictive value: (NPV) 97.2% (Roche) and 98.5% (bioMérieux). Additionally, Cohen's kappa coefficient demonstrated high concordance (k=0.950) between Roche and bioMérieux.

Discussion

In conclusion, our results evidenced a very good performance for the nucleocapsid antibody assay (Roche) and the spike protein antibody assay (bioMérieux), thus both platforms are equally adequate for indirect diagnosis of SARS-CoV-2 infection through total antibodies and IgG antibody detection, respectively.

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INTRODUCTION

During the year of 2020, different trademarks have developed assays with diverse antigenic configurations for clinical use in serological diagnosis of infection by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Some of these commercial assays received emergency authorization from the United States Food and Drug Administration (FDA) (1) and the Health and Safety Authority of Argentina: Administración Nacional de Medicamentos, Alimentos y Tecnología Médica (ANMAT).

Despite molecular assays are the gold standard for diagnosis of infection caused by SARS-CoV-2, serology is useful as diagnostic tool to complement viral RNA detection. Thus, RNA detection by RT-Polymerase Chain Reaction (RT-PCR) is most sensitive within the first 7 days after onset of symptoms and after that point, it diminishes below 50% (2). In contrast, many reports describe that antibodies against SARS-CoV-2 are detectable in only 50% of patients one week after onset of symptoms and sensitivity for their detection is enhanced up to 90% after two weeks (3). Likewise, it has been shown that a certain ratio of close contacts of patients with confirmed coronavirus disease 2019 (COVID-19) yield negative results or they are not tested at all with molecular techniques (4). In these cases, diagnosis of infection can be achieved with serological assays. Thus, serology arises as a very important complementary resource for diagnosis and control of this viral infection.

On the other hand, evaluation of the humoral immune response against SARS-CoV-2 by serological tests is very important for epidemiological surveillance to control the COVID-19 pandemic. In this sense, serological assays are economical, fast, easy to implement, and allow effective identification of people exposed to the virus (5, 6). In addition, serology is useful to determine immune status in workers, which facilitates return-to-work decisions and other relevant public health measures in the context of the COVID-19 pandemic (7, 8).

Previous studies regarding coronavirus SARS-CoV and MERS-CoV have revealed that the most

immunogenic antigens are the spike (S) and nucleocapsid (N) proteins; therefore, most serological techniques developed for detection of SARS-CoV-2 antibodies have focused on these viral proteins (9). In this sense, several commercial kits have been developed and evaluated (10, 11). Two of the most widely used commercial platforms to detect specific antibodies against SARS-CoV-2 are: Elecsys Anti-SARS-CoV-2 by Roche, which detects total antibodies against the viral nucleocapsid (anti-N) and VIDAS SARS-COV-2 IgG (9-COG) by bioMérieux, which contains spike protein of the virus as antigenic conformation, allowing detection of antibodies against the S protein (anti-S) (1,12,13). These assays are available in Argentina, and they have been approved by ANMAT for diagnosis of SARS-CoV-2 infection. Hence, we evaluated their performance for detection of specific total and IgG antibodies against the virus using a panel of plasma samples from subjects recovered from infection by the SARS-CoV-2 pandemic strain (B.1 lineage).

MATERIALS AND METHODS

Sample selection

One panel of positive and one of negative plasma samples for SARS-CoV-2 antibodies were used for this study. The panel of positive samples was obtained from the sample bank of the Virology Institute "Dr. J. M. Vanella", Facultad de Medicina, Universidad Nacional de Córdoba, Argentina, and was composed by 92 plasma samples with ethylenediaminetetraacetic acid (EDTA) as anticoagulant. They were collected during the year of 2020 from patients recovered from COVID-19 infection, 40-85 days after onset of symptoms. These patients were: i) positive by RT-PCR in nasopharyngeal swab samples and positive for both neutralizing antibodies (NTAbs) by plaque reduction neutralization test (PRNT) and IgG antibodies against SARS-CoV-2 by *in house* Indirect Immunofluorescence assay (IFA) (n=78), and ii) positive by RT-PCR in nasopharyngeal swab samples, positive for IgG antibodies against SARS-CoV-2 by IFA, but negative for NATbs by PRNT (n=14).

The characterization of plasma samples (positive panel) for antibodies against SARS-CoV-2 by PRNT and IFA was performed at the Virology Institute within the framework of the agreement with the Cordoba Ministry of Health for characterization of convalescent plasma for therapeutic use. Assays were carried out as previously described (14) and SARS-CoV-2 strain B.1 lineage (hCoV-19/Argentina/PAIS-G0001/2020, GISAID, ID: EPI_ISL_499083) was used for both tests.

The negative panel included plasma samples (with EDTA) collected from blood donors in 2018 prior to the COVID-19 pandemic (n=71).

Methods

The Elecsys Anti-SARS CoV-2 assay was performed on a Cobas e411 analyzer (Roche Diagnostics, Mannheim, Germany) and conducted according to the manufacturer's instructions. This sandwich assay uses a SARS-CoV-2 specific recombinant antigen representing the nucleocapsid protein. The electrochemiluminescent signal produced is compared to the cut-off signal value previously obtained with two calibrators. Results are expressed as (cut-off index, negative COI <1.0 or positive COI ≥1.0) for anti-SARS CoV-2 total antibodies.

The VIDAS SARS CoV-2 is a two-step sandwich enzyme-linked fluorescent assay (ELFA) performed on a VIDAS analyzer (bioMérieux, Marcy-l'Étoile, France). The VIDAS SARS-CoV-2 IgG assay was conducted according to the manufacturer's instructions. Briefly, the IgGs present in the sample are captured by a recombinant SARS-CoV-2 subdomain spike antigen coated on a solid phase, and then an anti-human IgG labelled with alkaline phosphatase is added. The intensity of the fluorescence produced by the substrate hydrolysis is

measured at 450 nm and is proportional to the antibody level. An index is calculated as the ratio between the relative fluorescence value (RFV) measured in the sample and the RFV obtained for the calibrator (humanized recombinant anti-SARS CoV-2 IgG) and interpreted as negative (index <1.0) or positive (index ≥1.0).

Table 1 shows manufacturer names, assays, methods, principles of antibody detection, recombinant antigens and types of immunoglobulins recognized by the two commercial immunoassays.

Samples from the negative panel that yielded false-positive results were also analyzed for potentially unspecific cross-reactions: HIV antigen/antibody, hepatitis B virus (HBV) surface antigen, hepatitis C virus (HCV) total antibody, rheumatoid factor (RHF) and antinuclear antibody (ANA). Viral serology was performed by Cobas e411 analyzer (Roche Diagnostics) and RHF was performed by immunoturbidimetry with a Cobas 6000 analyzer (Roche Diagnostics). ANA was performed by indirect immunofluorescence assay and imprints with Hep-2 cell line (human laryngeal carcinoma, Biosystem) were used. Briefly, samples were diluted 1/80 with phosphate buffered saline (PBS, pH=7) and incubated for 30 minutes at room temperature. Then, two washes with PBS were performed and anti-human IgG Abs conjugated with fluorescein isothiocyanate (Biocientífica S.A) was added to all wells, which were subsequently incubated for 30 minutes at room temperature. After two washes with PBS, Evans Blue was added to enhance the fluorescent signal. The samples were then dried, and a mounting solution was added for observation under Fluorescence microscope (Nikon Optiphot-2). The results were reported as negative or positive according to their fluorescence pattern. To guarantee the quality of the methodology internal and external controls were used and the results were interpreted and reported according to the criteria published by the Regional Committee for Laboratory Standardization based on international consensus (15).

Statistical analyses

Statistical analyses were conducted using Graph Pad Prism software version 6.0. Categorical variables were compared using Fisher's exact test. Sensitivity, specificity, PPV and NPV values were calculated. A p-value lower than 0.05 was

Table 1 **Characteristics of the commercial anti-SARS-CoV-2 serological assays from Roche and bioMérieux**

Manufacturer (platform)	Assay	Method	Principle	Capture antigen	Isotype detected	Cut-off values	
						Neg.	Pos.
ROCHE	Elecsys Anti SARS-CoV-2	ECLIA	Sandwich immunoassay	Nucleocapsid	Total antibodies	<1.0	≥1.0
bioMérieux	SARS-CoV-2 IgG (9-COG)	ELFA	Sandwich immunoassay (two-step)	RBD	IgG	<1.0	≥ 1.0

Abbreviations: ECLIA: electro-chemiluminescence immunoassay; ELFA: enzyme-linked fluorescent assay; RBD: Receptor Binding Domain; IgG: immunoglobulin G. RBD is a domain within the S1 subunit of the spike protein; Neg: negative, Pos: positive.

considered statistically significant. Additionally, concordance between the two commercial assays was analyzed using Cohen's kappa coefficient (κ). The κ value was classified as slight (0.00 to 0.20), fair (0.21 to 0.40), moderate (0.41 to 0.60), substantial (0.61 to 0.80) and almost perfect (0.81 to 1.00) according to Landis and Koch criteria (16).

RESULTS

Table 2 shows the overall performance of each automated analyzer (sensitivity, specificity, PPV and NPV values). Results from the positive panel were 90 positive plasma samples for total antibodies by Elecsys Anti-SARS CoV-2 and 91 positive plasma samples for VIDAS anti SARS-CoV-2 IgG. In addition, we evaluated specificity in the negative panel and found three false positive results. We analyzed these samples containing potentially cross-reactive factors and observed that HIV, HCV and HBV were negative in all cases, while three samples were positive for antinuclear antibodies; in addition, one of these samples was also positive for RHF. The Elecsys anti-SARS-CoV-2 assay yielded one false positive result containing autoantibodies for both RHF and ANA, while VIDAS Anti-SARS CoV-2 IgG produced two false-positive results containing only ANA.

When the results obtained by Roche and bioMérieux were compared to each other, a Cohen's kappa coefficient (κ) of 0.95 (95%CI, 0.90 to 0.99) was obtained, demonstrating high concordance between Elecsys Anti-SARS CoV-2 and VIDAS Anti-SARS CoV-2 IgG.

DISCUSSION

In the current study, we compared two commercial serology platforms for detection of antibodies against SARS-CoV-2 using panels of positive and negative plasma samples. We tested total antibodies against nucleocapsid protein with the assay from Roche and IgG-specific antibodies against spike protein with the bioMérieux assay; performance of the assays as stand-alone tests was also assessed. We found overall comparable sensitivity of 97.8% and 98.9% for Elecsys Anti-SARS CoV-2 and VIDAS Anti-SARS CoV-2 IgG, respectively. Results are in accordance with previous reports showing that Elecsys and VIDAS assays have better performance than other automated assays (12), reporting high rates of sensitivity, similar to what is described herein (13).

Moreover, other studies describe good levels of sensitivity for Elecsys anti-SARS-CoV-2 assay, supporting its use for detection of SARS-CoV-2 infection in areas of low prevalence (10) and evidencing a good performance as stand-alone test (1). Additionally, The National SARS-CoV-2 Serology Assay Evaluation Group from Oxford recommended the Elecsys anti-SARS-CoV-2 assay

Table 2 Clinical sensitivity, specificity, PPV and NPV of serological assays from Roche and bioMérieux in patients recovered from COVID-19

Platform	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	PPV (%) (95% CI)	NPV (%) (95% CI)
ROCHE	97.8 (92.3-99.7)	98.5 (92.4-99.9)	98.9 (94.0-99.9)	97.2 (90.3-99.6)
bioMérieux	98.9 (94.0-99.9)	97.1 (90.1-99.6)	97.8 (92.4-99.7)	98.5 (92.3-99.9)

Abbreviations: CI: confidence interval; PPV: positive predictive values; NPV: negative predictive values.

for serological testing due to its high sensitivity (17). In this sense, VIDAS anti-SARS-CoV-2 IgG assay probed to be a sensitive serological test, suitable for detecting specific antibody subtypes (11).

To assess specificity of the two automated assays, we analyzed a panel of pre-pandemic samples obtained two years before the first report of SARS-CoV-2 infection in the world. As a result, we found specificity rates of 98.5% and 97.1% for Elecsys Anti-SARS CoV-2 and VIDAS anti-SARS-CoV-2 IgG assays, respectively. These rates are concordant with values previously reported, when high-throughput assays for detection of antibodies against SARS-CoV-2 were analyzed (18). Similarly high rates of specificity have been described for Elecsys Anti-SARS CoV-2 (1, 10, 17) in studies of different populations, Moreover, the high rate of specificity found for VIDAS Anti-SARS CoV-2 IgG by bioMérieux was also concordant with the findings of other researchers (12, 13) and this is the reason why this assay has been previously used as a useful tool for antibody detection and epidemiological surveillance (11).

A low cross-reactivity rate due to non-specific factors when using both automated assays was observed. In this study, only 1/92 and 2/92 plasma samples containing potential cross-reacting analytes showed reactivity with Elecsys Anti-SARS CoV-2 and VIDAS Anti-SARS CoV-2 IgG, respectively. Previous reports have described similar results for these platforms (1, 10, 11, 13). Together with the evaluation of sensitivity and specificity, both assays showed similarly high rates of PPV and NPV. This finding, along with the high concordance between Roche and bioMérieux assays determined by Cohen's kappa index (0.95), proved that these two immunoassays are equally suitable for diagnosis of SARS-CoV-2 infection through antibody detection, being also adequate for sero-epidemiological surveillance in Argentina.

In conclusion, the relevance of this study was to determine the clinical usefulness of two commercial platforms with regional samples reporting these results, which show that both platforms are highly recommended for detection of specific antibodies against SARS-CoV-2 in medium and high-complexity laboratories at Argentina. Furthermore, these results demonstrate that reliable decisions can be made based on serological results obtained with these commercial assays, whether for health policies, return-to-work decisions and/or epidemiological studies to control viral spread.

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Personal and financial conflicts of interest

The authors declare that there are not competing interest.

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In this issue: Current laboratory aspects of COVID-19

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Variation of total anti-SARS-CoV-2 antibodies after primary BNT162b2 vaccination and homologous booster

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ABSTRACT

Background

In this serosurveillance study, we investigated the variation of total anti-SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2) antibodies in healthcare workers receiving primary BNT162b2 vaccination and homologous booster.

Methods

A total number of 524 subjects (median age, 46 years; 65.3% females), were studied. All received primary BNT162b2 vaccination (two doses) and homologous booster (one dose) >8 months after completing the primary cycle. Blood samples were collected before the first and second vaccine doses, at 1, 3 and 6 months after the second dose, as well as before and 1 month after booster. Total anti-SARS-CoV-2 neutralizing anti-

bodies were assayed with Roche Elecsys Anti-SARS-CoV-2 S chemiluminescent immunoassay.

Results

Overall, 65.1% subjects were baseline (i.e., pre-vaccination) SARS-CoV-2 seronegative and always tested SARS-CoV-2 negative ("N/N"), 16.2% were baseline SARS-CoV-2 seronegative but tested SARS-CoV-2 positive after receiving the vaccine booster dose ("N/P"), whilst 18.7% were baseline SARS-CoV-2 seropositive and always tested SARS-CoV-2 negative afterwards ("P/N"). All groups displayed a similar trend of total anti-SARS-CoV-2 S antibodies throughout the study period, though the P/N cohort exhibited higher values compared to the other two groups until receiving the booster, after which the levels become similar in all cohorts. Significant differences in total anti-SARS-CoV-2 S antibodies values were not found between N/N and N/P groups, neither 1 month after booster. The rate of subjects with protective antibodies values become 100% in all groups after booster.

Conclusions

Although baseline seropositivity is associated with more pronounced humoral immune response following primary vaccination compared to never infected subjects, SARS-CoV-2 infection after booster does not significantly foster antibody titers.

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INTRODUCTION

Several lines of evidence now attest that no existing vaccine would be completely effective against an infectious diseases, thus including those that have been developed against coronavirus disease 2019 (COVID-19) [1]. For viruses like SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2), global herd immunity is unlikely to be achieved for a variety of reasons that include lower than advisable compliance to vaccination [2], progressive accumulation of non-synonymous genomic mutations that promote escape from vaccine-elicited immunity [3], as well as waning vaccine efficacy and protection over time, which is mostly attributable to a decline in the titer of anti-SARS-CoV-2 neutralizing antibodies [4,5]. To this end, epidemiological and laboratory investigations are needed to precisely recognize and measure the impact of the many determinants that may contribute to impair vaccine efficacy over time, thus allowing for the establishment of timely and appropriate measures that could be effective to limit SARS-CoV-2 circulation, prevent or reduce the risk of developing severe COVID-19 illness, and limit emergence of novel variants [6].

In a world with limited resources, where vaccine coverage remains extremely heterogeneous across different countries and populations mostly for insufficient supply [7], prioritization of primary vaccination and booster doses administration to those parts of the population that may be more exposed to complications and adverse consequences of SARS-CoV-2 infection is crucial [8]. Among the potentially more vulnerable subjects, those with blunted immunogenic response and sharper and/or faster decay of anti-SARS-CoV-2 neutralizing antibodies were found to have magnified risk of breakthrough infections and unfavorable progression of COVID-19, including increased rates of hospital admission, need of mechanical ventilation or intensive care, and a greater risk of death [9,10]. Personalized vaccine administration would also be effective to concomitantly avert the risk of rare side effects in those who could safely delay primary cycle or boosters [11].

Since the extent of vaccine-elicited protection varies considerably when combined with SARS-CoV-2 infections [12], this serosurveillance study was aimed to explore the variation of total anti-

SARS-CoV-2 antibodies in healthcare workers receiving primary vaccination with BNT162b2 and homologous booster, with or without SARS-CoV-2 infection before primary vaccination or after vaccine booster.

MATERIALS AND METHODS

The initial study population consisted of 925 ostensibly healthy individuals recruited from the healthcare staff of the Pederzoli Hospital in Peschiera del Garda (Italy), who received a primary vaccination cycle with BNT162b2 COVID-19 vaccine (Pfizer Inc., New York, NY; two doses of 30 µg, separated by 3 weeks), and an additional homologous booster (a single dose of 30 µg) more than 8 months after completing the primary vaccination. BNT162b2 is a lipid nanoparticle-formulated, nucleoside-modified RNA vaccine encoding a pre-fusion, stabilized, membrane-anchored SARS-CoV-2 full-length spike protein. A nucleic acid amplification test (NAAT) for diagnosing incident SARS-CoV-2 infection was performed every 2-4 weeks throughout the study period, using Altona Diagnostics RealStar SARS-CoV-2 RT-PCR Kit (Altona Diagnostics GmbH, Hamburg, Germany) or Seegene Allplex SARS-CoV-2 Assay (Seegene Inc., South Korea). Venous blood was collected before administration of the first (baseline) and second BNT162b2 doses, then 1, 3 and 6 months after the second BNT162b2 dose, and finally immediately before and 1 month after the homologous BNT162b2 booster dose. Subjects who became SARS-CoV-2 positive between the first dose of vaccine and the booster were actually excluded from our analysis. This is due to the fact that the time passed between these vaccine doses was so long that the influence of a SARS-CoV-2 infection on humoral immunity throughout nearly 8 months could not be standardized.

Total anti-SARS-CoV-2 neutralizing antibodies were assayed using the Roche Elecsys Anti-SARS-CoV-2 S chemiluminescent immunoassay, on a Roche Cobas 6000 immunochemistry platform (Roche Diagnostics, Basel, Switzerland). This double-antigen sandwich method encompasses a recombinant form of the receptor binding domain (RBD) of the SARS-CoV-2 spike (S) protein, displays total imprecision <4% [13], as well as optimal agreement with plaque reduction neutralization test (PRNT) in vaccinated subjects (area under the curve, 0.990; sensitivity, 0.98; specificity, 0.95) [14]. According to manufacturer's specifications, results are positive when the serum total anti-SARS-CoV-2 antibodies concentration is ≥0.8 kBAU/L (kilo binding antibody units/L). A total anti-SARS-CoV-2 antibodies titer of 656 kBAU/L was considered as predictive of ≥80% protection against modest or severe COVID-19 illness, in keeping with previously published data [15,16].

Results of testing were finally reported as median and interquartile range (IQR), and the statistical analysis was performed using Analyse-it (Analyse-it Software Ltd, Leeds, UK). Between-group comparisons were carried out with Mann-Whitney test. All participants gave informed consents for vaccination and undergoing serial anti-SARS-CoV-2 antibodies testing. This observational study was reviewed and cleared by the Ethics Committee of Verona and Rovigo provinces (3246CESC), and was conducted in accordance with the Declaration of Helsinki, under the terms of relevant local legislation.

RESULTS

The final study population consisted of 524 subjects (median age, 46 years and IQR, 34-53 years; 65.3% females; 56.6% of the original sample), as 401 subjects were lost on follow-up (for either not completing vaccination, failing to provide blood samples at one or more time point throughout the study). Eleven additional subjects tested positive for SARS-CoV-2 mRNA

after the first and before the third vaccine dose, but were also excluded due to the insufficient sample size to enable analysis of this subgroup. Of all subjects finally included, 341/524 (65.1%) were baseline (i.e. pre-vaccination) SARS-CoV-2 seronegative (i.e., <0.8 kBAU/L) and then always tested SARS-CoV-2 negative ("N/N"; median age, 47 years and IQR, 36-54 years; 63.9% females); 85/524 (16.2%) were baseline SARS-CoV-2 seronegative (i.e., <0.8 kBAU/L) and then tested SARS-CoV-2 positive after receiving the vaccine booster dose ("N/P"; median age, 43 years and IQR 31-50 years; 68.2% females); whilst 98/524 (18.7%) were baseline SARS-CoV-2 seropositive (i.e., >0.8 kBAU/L) and then always tested SARS-CoV-2 negative ("P/N"; median age, 44 years and IQR, 33-52 years; 67.3% females), respectively. We excluded the cohort of subjects who were baseline SARS-CoV-2 seropositive (i.e., >0.8 kBAU/L) and then tested SARS-CoV-2 positive after receiving the vaccine booster dose always tested (i.e., P/P) because the final sample size of this cohort was considerably low (n=9), so that inclusion in the statistical analysis may be misleading. Subjects in the N/N cohort were slightly younger than those in the two other groups (i.e., p=0.035 vs. N/P and p=0.039 vs. P/N, respectively), whilst the sex distribution was similar across the three groups (all p>0.05).

The main variations of total anti-SARS-CoV-2 S antibodies in the three groups are summarized in table 1 and figure 1. As predicted, although all groups displayed a rather similar trend throughout the study period, the P/N cohort exhibited significantly higher values compared to the other two groups until these subjects received the vaccine booster dose, after which the serum antibodies levels become comparable to those of the other two cohorts. No significant differences were observed between the N/N and N/P groups, including at the 1 month time point after receiving the vaccine booster dose (Table 1).

The rate of subjects with protective total serum anti-SARS-CoV-2 S antibodies values (i.e., >656 kBAU/L) displayed rather consistent trend across groups. Specifically, in N/N and N/P the rate of positive subjects increased from 0% to 82-84% 1 months after the second vaccine dose, but then gradually declined to 32-35% before receiving the vaccine booster, after which such rate increased in both groups to 100% (Figure 2). Unlike these subjects though, the rate of P/N subjects with protective total anti-SARS-CoV-2 S antibodies values was 7% at baseline assessment, then remained always >88% throughout the subsequent time points, and increased further to 100% after receiving the vaccine booster dose.

DISCUSSION

Some important conclusions can be made from the results of this serosurvillance study in a group of healthcare workers who received a primary BNT162b2 vaccination followed by a homologous booster.

The first important aspect, which supports previously published evidence [17-19], is that a prior SARS-CoV-2 infection before primary vaccination provides a rather efficient priming to COVID-19 vaccination, in that the total anti-SARS-CoV-2 S antibodies values appear to be consistently higher in baseline seropositive individuals (P/N) compared to seronegative ones (both N/N and N/P groups in our study). The protection against the risk of developing moderate/severe COVID-19 illness (i.e., the subjects with predictably protective antibodies values) seems also to persist for longer after primary vaccination in baseline seropositive compared to baseline seronegative subjects (i.e., 88% in P/N vs. 32-35% in N/N and N/P, respectively), which would allow to safely prioritize the administration of vaccine boosters to seronegative individuals.

The second important aspect that emerged from this serosurvillance study, is that the vaccine

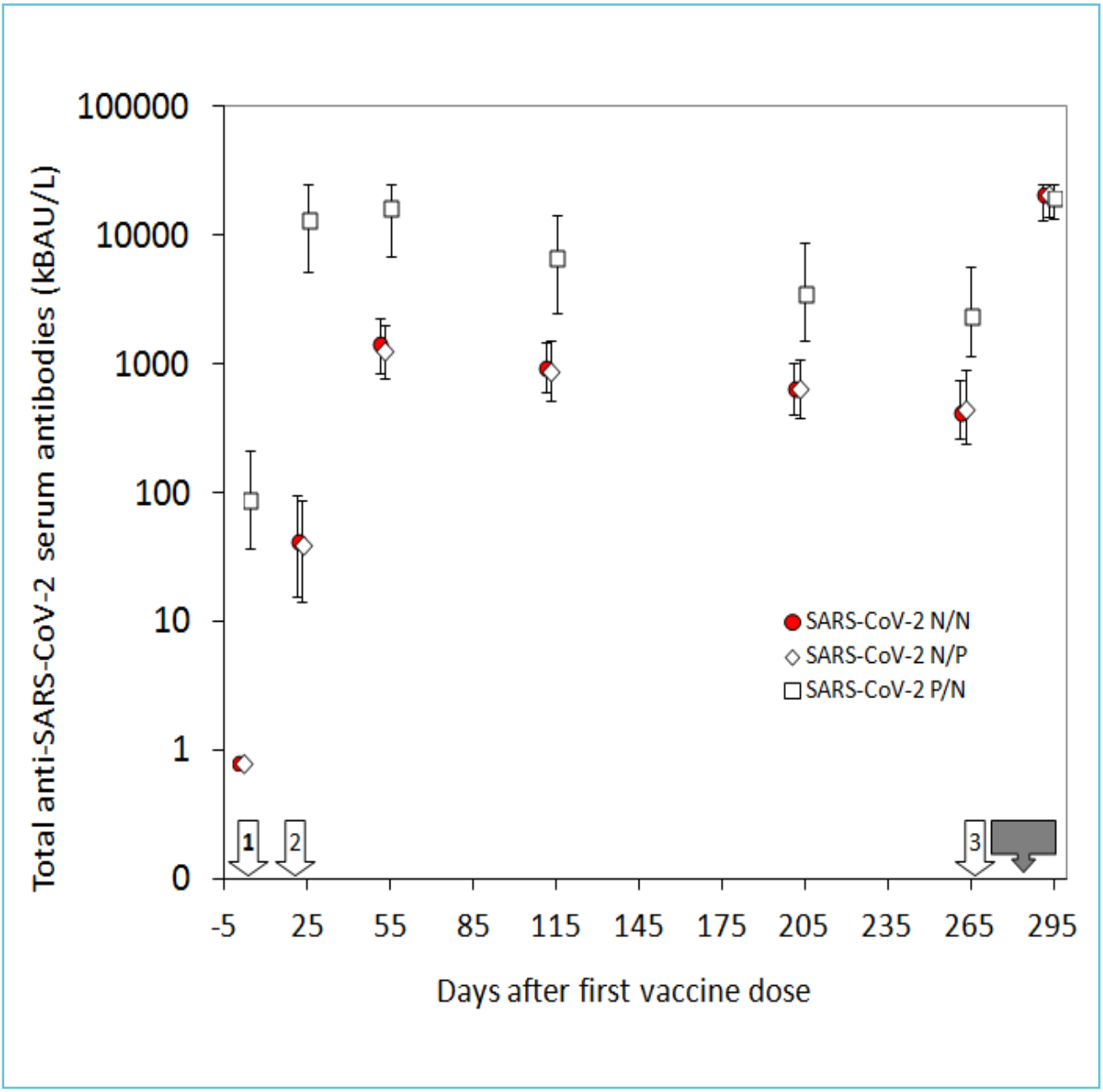
booster dose generates a considerable impact on total anti-SARS-CoV-2 S antibodies, increasing their concentration by around 50- and 8-fold in seronegative and seropositive subjects, respectively, though the final values reached do not significantly differ among all groups (Table 1). This is basically consistent with two, non-mutually exclusive, hypotheses. It can be first conjectured that the priming effect of a pre-vaccination SARS-CoV-2 infection has progressively declined over time, being almost completely lost before receiving the vaccine booster dose, such that baseline SARS-CoV-2 seronegative and seropositive subjects would become a more homogenous population. This is not really surprising, since total anti-SARS-CoV-2 antibodies display a half-life between 50-110 days [20], with a seropositive rate decreasing to less than 36% after 12 months [21].

Table 1 Serum concentration (median and interquartile range) of total anti-SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2) antibodies in a cohort of healthcare workers receiving primary BNT162b2 vaccination and homologous booster

Population	Baseline	Pre-2nd dose	1 M after 2nd dose	3 M after 2nd dose	6 M after 2nd dose	Pre-booster	1 M post-booster
SARS-CoV-2 N/N							
Values (kBAU/L)	<0.8	42.8 (15.4-96.1)	1440.0 (854.5-2269.0)	936.4 (601.2-1464.0)	661.6 (407.7-1023.0)	429.8 (265.8-744.0)	20848.0 (13218.0-25000.0)
SARS-CoV-2 N/P							
Values (kBAU/L)	<0.8	39.6 (14.1-87.4)	1289.0 (770.8-2011.0)	902.9 (517.4-1538.0)	656.0 (380.5-1088.0)	452.5 (244.0-911.8)	20891.0 (14028.0-25000.0)
p vs. N/N	1.000	0.258	0.510	0.321	0.180	0.110	0.392
SARS-CoV-2 P/N							
Values (kBAU/L)	80.8 (31.1-209.8)	13312.0 (5198.3-25000.0)	16358.0 (6898.8-25000.0)	6673.5 (2525.8-14395.0)	3529.5 (1523.0-8664.3)	2366.0 (1150.5-5777.8)	19290.0 (13720.5-25000.0)
p vs. N/N	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.364
p vs. N/P	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.306

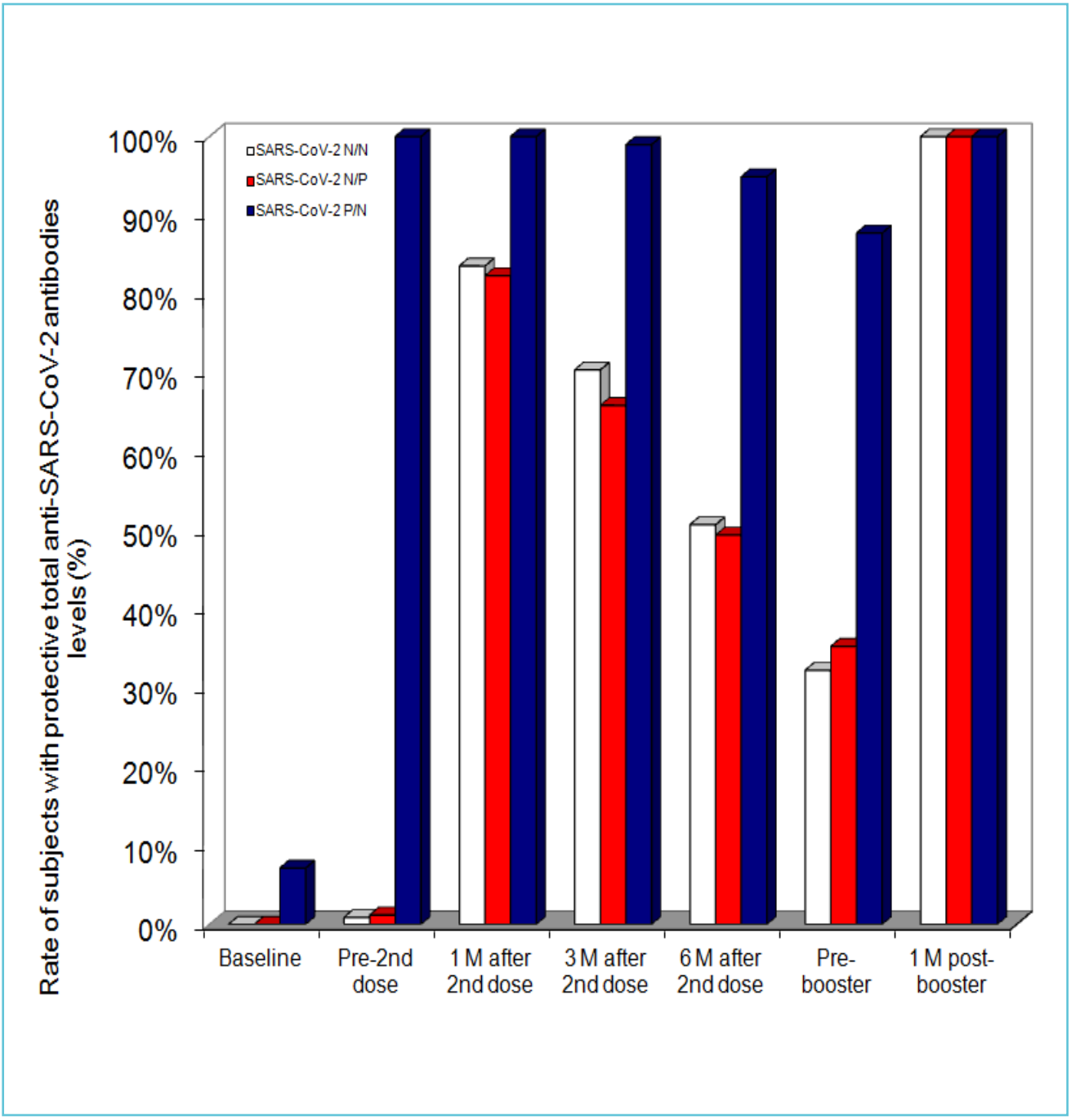
N/N, baseline SARS-CoV-2 seronegative and then always testing SARS-CoV-2 negative; N/P, baseline SARS-CoV-2 seronegative and testing SARS-CoV-2 positive after booster; P/N; baseline SARS-CoV-2 seropositive and then always testing SARS-CoV-2 negative; M, months; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

Figure 1 **Serum concentration (median and interquartile range) of total anti-SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2) antibodies in a cohort of healthcare workers receiving primary BNT162b2 vaccination and homologous booster. The white arrows indicate the timing of BNT162b2 vaccine doses, whilst the gray arrow indicates the SARS-CoV infection in the N/P group.**



N/N, baseline SARS-CoV-2 seronegative and then always testing SARS-CoV-2 negative; N/P, baseline SARS-CoV-2 seronegative and testing SARS-CoV-2 positive after booster; P/N; baseline SARS-CoV-2 seropositive and then always testing SARS-CoV-2 negative; M, months; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

Figure 2 **Rate of subjects with protective levels of total anti-SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2) antibodies (i.e., >656 kBAU/L) in a cohort of healthcare workers receiving primary BNT162b2 vaccination and homologous booster.**



N/N, baseline SARS-CoV-2 seronegative and then always testing SARS-CoV-2 negative; N/P, baseline SARS-CoV-2 seronegative and testing SARS-CoV-2 positive after booster; P/N; baseline SARS-CoV-2 seropositive and then always testing SARS-CoV-2 negative; M, months; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

On the other hand, it is also conceivable that the administration of a BNT162b2 booster dose has provided such a strong stimulus to an already primed immunological memory (i.e., memory B cells) [22], such that another potent immunogenic trigger like an incident SARS-CoV-2 infection occurred after vaccine booster would be incapable to produce further significant increases of total anti-SARS-CoV-2 S antibodies levels over the threshold achieved with vaccine boosters.

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Author contributions

All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

Informed consent

Informed consent was obtained from all individuals included in this study.

Ethical approval

This observational study was reviewed and cleared by the Ethics Committee of Verona and Rovigo provinces (3246CESC).

Conflict of interest statement

All the authors declare that they have no conflict of interest in this work.

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In this issue: Current laboratory aspects of COVID-19

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Kinetics of antibody response to repeated vaccination with Sputnik V: a pilot study with a series of five cases

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COVID-19, SARS-COV-2, antibody, immunoassay, Sputnik V vaccine

ABSTRACT

Objectives

Widespread vaccination is considered as one of the best methods in combating any pandemic including COVID-19. Gam-COVID-Vac also known as Sputnik V, is one of the first vaccines that was registered in 74 countries and received an emergency approval for immunization. Monitoring anti-SARS-CoV-2 antibodies over time is essential for evaluation of post-vaccination humoral immune response.

To date, there are only a limited number of clinical studies regarding the analysis of immune response after Sputnik V administration. It is of crucial importance to report independently on safety and efficiency of this vaccine with the aim to speed up the process of its final approval by the WHO.

Methods

Humoral immune response was monitored by seven immunoassays to analyze different classes of anti-SARS-CoV-2 Ig in five health workers after receiving the combined vector vaccination. This vaccine is based on two replication-deficient rAd26 and rAd5 viral vectors that carry the gene SARS-CoV-2 full-length glycoprotein S(rAd26-S and rAd5-S). Sputnik V was administered with a 21-day interval between the first and second dose. Venous blood was collected two hours before vaccination as a baseline, and then followed by 18 series up to 170-day post-vaccination.

Results

The participants in this study used a self-report form in which they noted their observations on safety at 72 h post-immunization. One participant reported mild side effects, such as muscle pain and fever, while the other four individuals had no noticeable complications. Seroconversion was detected in all individuals at 28 days of post-vaccination. Plateau of seropositivity has been achieved by 50th day of vaccination, while titer values decreased after 6 months.

Conclusion

This study provides some clinical data regarding the kinetics of antibody levels elicited after administration of heterologous rAd26-S and rAd5-S vaccine. Based on the preliminary data from this pilot study, it appears that Sputnik V vaccine generates a solid humoral immune response lasting at least 6 months after immunization.

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INTRODUCTION

The new coronavirus disease 2019 (COVID-19) disease is caused by severe acute respiratory syndrome coronavirus 2 (SARS-COV-2) infection, which has resulted in a global pandemic. The vast spread of this disease became a serious threat to global public health [1]. In essence, SARS-COV-2 is a single RNA virus and belongs to the beta-coronavirus of the family Coronaviridae, which most commonly induces respiratory symptoms, such as fever, unproductive cough, myalgia, and fatigue [2, 3]. Although several preventive actions, e.g., social distancing, hand hygiene, extensive use of face masks and contact monitoring have been implemented worldwide with the aim to limit the impact and spread of SARS-CoV-2 infection, the vast and enormous transmission of the SARS-CoV-2 coronavirus was unmanageable [4].

Currently, with the new variants emerging out of the Wuhan species, humanity is facing more than 6 million registered deaths as well as many unreported deaths with the possibility of the overall balance to account to the third leading cause of death, and the second largest cause of death due to viral infection within one century [5, 6, 7]. Furthermore, a new challenge in 2021 was to cope with the emergence of new, unknown and potentially more destructive variants of the virus [8].

According to the World Health Organization (WHO), vaccination is a safe and effective way of reducing the risk of developing serious disease and lowering the risk of generation of new, more powerful strains of the virus [9]. The reduction of severe COVID-19 disease after vaccination is associated with the synthesis of circulating neutralizing monoclonal antibodies, primarily IgG class, which specifically targets the SARS-CoV-2 spike protein, its S1 unit of receptor binding domain (RBD), to restrict or completely prevent the binding with host receptor (i.e., Angiotensin-Converting Enzyme 2, ACE2) [10].

Referring to WHO, as of September 17, 2020, 117 COVID-19 vaccine candidates were under clinical evaluation and 194 candidate vaccines were processed in the preclinical evaluation [11,12].

From those approved for clinical use, the vaccines based on mRNA technology were the first permitted and administered in many developed countries due to some technical advantages. Unfortunately, countries with smaller incomes lagged in procuring the vaccines and immunization began not only a few months later, but also with insufficient quantities for rapid vaccination of a large part of their populations. Additionally, the specific manner of proper storage of the new mRNA vaccines also made them less accessible. North Macedonia procured several different types of vaccines. One of the available vaccines was adeno-based vector vaccine Gam-COVID-Vac (Sputnik V), developed by Gamaleya National Research Centre, Russia. This vaccine still has not received the authorization for general use by the WHO yet, however it has been administered to millions around the globe, typically in low-income countries due to its low cost.

The use of viral vector-based vaccines allows the signaling pathways to produce both humoral immunities through antibody expansion and cellular immunity by stimulating a robust cytotoxic T lymphocyte (CTL) response to eliminate virus-infected cells [13]. Phase III trials have shown that these vaccines are effective in alleviating the severity of COVID-19 as a result of the development of effective humoral and cellular immunity, in particular the development of neutralizing antibodies against SARS-COV-2 [14, 15]. However, to the best of our knowledge, there is still a lack of published data describing the early and comprehensive humoral immune response after Sputnik V in subjects not included in clinical trials.

Since the immunological response varies between different types of COVID-19 specific vaccines, here our aim was to evaluate the humoral immune response after heterologous recombinant adenovirus (rAd26-S + rAd5-S) vaccine from the Gamaleya Research Institute-Sputnik V in samples of healthcare workers who were seronegative before vaccination via the assessment of antibodies of IgG and IgM classes targeting the entire SARS-CoV-2 Spike protein trimer (anti-spike trimeric IgG), the RBD (anti-spike RBD IgG) or the S1 subunit. We have used different assays for the analysis of antibodies for reliably reflecting the immunological response developed after vaccination with Sputnik V.

MATERIALS AND METHODS

Study design and participants

This five-case series was based on four female participants (at the age of 45, 50, 52 and 58 years) and one male participant who was 39 years old. All participants in this study were healthcare workers at the University Clinic for Gynecology and Obstetrics, Skopje, Macedonia who underwent vaccination with two doses of vector-based vaccine Sputnik V. The vector vaccine consists of adenovirus DNA, in which the SARS-CoV-2 coronavirus gene was integrated. Adenovirus is used as a “flask” to deliver the coronavirus gene to the cells for synthesizing the envelope proteins of SARS-CoV-2 virus.

The first received dose of replication-deficient human adenovirus-26 expressing full-length S protein (1011 viral particles) was administered between 7-10 April 2021. The second dose of human adenovirus- 5 expressing full-length S protein (1011 viral particles) was administered after 21 days following the protocol given by the manufacturer [15]. The participants in this study were healthy volunteers and none of them were taking immunomodulatory drugs. In addition, these volunteers did not have any infectious diseases at the time of vaccination or 14 days before vaccination and did not receive any other vaccination within the whole period of study. On the day of the administration of the first dose, all participants had a negative SARS-COV-2 specific RT-PCR test as well as negative result for the titers of anti-SARS-CoV-2 IgG and IgM antibodies.

Baseline venous blood sample was collected two hours before the vaccination by venipuncture using 6 ml serum tubes containing gel and clot activator (Becton Dickinson, Plymouth, UK), and then on the 4, 8, 12, 14, 18, 21, 23, 29, 32, 35, 38, 42, 50, 55, 63, 73, 80, 170 days. Blood samples were centrifuged at 1500 x g for 15 min, aliquoted and frozen at -70 °C.

Immunoassays

After the time of collection all samples were thawed, centrifuged and sera were tested with seven different anti-SARS-CoV-2 Ig immunoassays for detection of SARS-CoV2 antibodies to measure total IgG or IgM and IgA SARS-CoV-2 antibody according to the manufacturer protocol instructions (Table 1).

During this study, each participant was asked to report on any self-perceived post-vaccination adverse reactions that included local reactions (e.g., injection site pain, redness, and swelling) and/or systemic reactions (such as fatigue, headache, myalgia, arthralgia, chills and fever). All volunteers signed two written consents: one according to the national standard for receiving vaccination, and another one for the participation in the serological monitoring study.

Statistical analysis

All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS version 17.0 for Windows, SPSS Inc., Chicago, IL, USA). Results are expressed as the mean ± standard deviation (S.D). Cumulative results of antibodies testing were presented as mean ± standard deviation (SD), as ratio with baseline antibodies level (i.e., [time point value]/[baseline value and/or limit of detection]). Spearman test was used to test the correlation of levels of different antibodies over time. The clinical and laboratory characteristics in the groups were compared by analysis of variance (ANOVA test). For all analysis, a P-value less than 0.05 were considered statistically significant.

RESULTS

None of the participants experienced any clinical sign or symptom related to SARS-CoV-2 infection, and molecular nasopharyngeal PCR testing remained negative during the whole period of the study in all five tested subjects. One of the participants recorded moderate systemic side effect after the first and second dose, i.e., body temperature of more than 38,9 °C, headache, fatigue, chills, shivers and arthralgia. Three subjects suffered from mild pain at the injection site lasting no more than 48 hours after receiving the first and the second dose.

A total number of 100 samples were collected at the end of the study during a period of 20 weeks. The kinetics of antibodies against SARS-CoV-2 was determined all together at 19 time points for the five study volunteers. The cumulative data and kinetics of antibody development after the combined adenovirus-based Gam-COVID-Vac vaccine are shown in Figure 1.

Serological testing of the participants in the study revealed that all participants were seronegative for the virus specific immunoglobulins of IgM, IgA and IgG classes and initially began to produce anti-SARS-2 antibodies between days 12 and 14 of the first dose of the vaccine, with slight differences in kinetics of seroconversion (Table 2).

Anti-S1-RBD total IgG seroconversion began to elevate and increased gradually between 182.7 (IQR, 137.3 – 228.2) to 899.8 (IQR, 587.5 – 1212.1) folds from day 21 after the first dose, when the first pick was reached. Seroconversion for SARS-COV-2 IgG antibodies on the 21st day after the first dose increased from 335.7 (IQR, 276.3-434.7) to 1,624.7 (IQR, 1,154.2-4,424.8) folds. The first vaccine dose initiated a median increase of 38.8 (IQR, 20.8- 56.7) folds for IgG anti-S1-RBD, and 769.2 (IQR, 305.8-834.4) folds

for S1-RBD, and 30.4 (IQR, 16.26- 44.58) for total IgG anti-N and S. Further slow increase was noted after the second dose, and on the 38th day the second peak was reached. The second vaccine dose prompted a median increase from baseline of 898.0 (IQR, 578.5-1.212.0) folds for RBD total Ig and 1625.1 (IQR, 1.154-2.096) folds for SARS-COV-2 IgG, 177.0 (IQR,112.0-242.1) for SARS-COV-2 IgG anti-S1-RBD, 5361.1 (IQR, 2132.0-8591.0) for anti-S1-RBD and 159.0 (IQR, 83.64-234.3) for total IgG anti-N and S. After this exact time period and up to the 50th day, a plateau was achieved and the level of antibodies then started to slowly decline, however, 16 weeks after the first dose, the values were still higher than the first peak (Figure 2).

Table 1 **Technical and analytical characteristics of anti-SARS-CoV-2 immunoassays used in this study**

Test	Company	Analyzer	Principle	Detection	Ig class	Target	Cut-off
Elecsys Anti-SARS-CoV-2	Roche	COBAS ELECSYS	Sandwich	ECLIA	SARS-COV-2 total Ig	N	1.0 U/ml
Elecsys Anti-SARS-CoV-2 S	Roche	COBAS ELECSYS	DAGS	ECLIA	SARS-COV-2 total Ig	S RBD	0.8 U/ml (1.03 BAU/ml)
Vidas Sars-CoV-2 IgG II	bioMerieux	VIDAS	Sandwich	ELFA	SARS-COV-2 IgG	S1-RBD	1.00 index (20.33 BAU/ml)
SARS-CoV-2 IgG II Quant	Abbott	ARCHITECT	Indirect	CMIA	SARS-COV-2 IgG	S1-RBD	50.0 AU/ml (7.1 BAU/ml)
SARS-CoV-2 IgG (SCOVG)	Siemens	CENTAUR XPT	DAGS-2 steps	DCLIA	SARS-COV-2 IgG	S1-RBD	1.00 index (21.8 BAU/ml)
COVID-19 VIRCLIA IgG MONOTEST	Vircell	VIRCLIA	indirect	CLIA	SARS-COV-2 IgG	N, S	0.7 (index)
COVID-19 VIRCLIA IgM+IgA MONOTEST	Vircell	VIRCLIA	indirect	CLIA	SARS-COV-2 IgM+IgA	N, S	0.6 (index)

Abbreviations: DAGS, double-antigen sandwich assay; ECLIA, Electro-chemiluminescent Immunoassay; ELFA, Enzyme Linked Fluorescent Assay; CMIA, Chemiluminescent Microparticle Immunoassay; DCLIA, Direct Chemiluminescent Immunoassay CLIA, Chemiluminescent Immunoassay; Ig, Immunoglobulin; N, nucleocapsid; RBD, Receptor Binding Domain; BAU, binding antibody unit.

Table 2 Kinetics of anti-SARS-CoV-2 antibodies development after Sputnik V vaccination

Antibodies	Baseline	21 days	38 days	77 days	170 days
COBAS ELECSYS ASARS-COV-2, anti-S-RBD total Ig					
Serum values **(WHO BAU/mL)**	0.412	75.3 (56.6-94.04)	370.72 (242.05-499.39)	175.1 (129.39-221.71)	142.35 (53.35-231.17)
VIDAS SARS-COV-2 anti-S1-RBD IgG					
Serum values **(WHO BAU/mL)**	0.19	67.621 (52.57-82.67)	308.77 (219.3-398.24)	164.87 (67.28-262.47)	59.98 (54.12-65.86)
ARCHITECT SARS-COV-2 anti-S1-RBD IgG					
Serum values **(WHO BAU/mL)**	1.47	57.08 (30.58-83.44)	260.3 (164.7-355.9)	66.9 (41.04-92.76)	37.56 (19.35-55.77)
SIEMENS CENTAUR XPT SCOVG anti-S1-RBD IgG					
Serum values **(WHO BAU/mL)**	0.1	76.92 (55.67-98.19)	536.11 (213.2-859.1)	173.37 (37.47-309.29)	45.86 (34.79-56.93)
VIRCLIA SARS-COV-2 anti-N and S IgG					
Serum values **(index)**	0.05	1.52 (0.81-2.22)	7.95 (4.18-11.71)	5.9 (2.05-9.74)	2.3 (1.37-3.25)
COBAS ELECSYS SARS-COV-2 anti-N total IgG					
Serum values **(index)**	0.08	0.08	0.082 (0.077-0.086)	0.082 (0.077-0.086)	0.082 (0.077-0.086)
VIRCLIA SARS-COV-2 S, anti-N and S IgM + IgA					
Serum values **(index)**	0.01	0.04 (0.001-0.08)	0.091 (0.04-0.14)	0.18 (0.04-0.33)	0.048 (0.096-0.087)

Abbreviations: BAU, binding antibody units; Ig, Immunoglobulin; RBD, Receptor Binding Domain; S1, Spike protein S1 subunit.

Anti S1-RBD IgA+IgM antibody levels did not display any kinetic during the full period of study and stayed on the primary low levels as before vaccination. In a similar manner, the kinetics of total IgG anti-N antibodies response with the immunoassay used in this study, did not provide any response after vaccination. The levels of total IgG anti-N antibodies in all patients, and in all tested points have remained on the basal, undetectable levels and therefore are not presented in this study.

The Spearman's correlations between the levels of different antibodies over time were performed and these results are shown in Table 3.

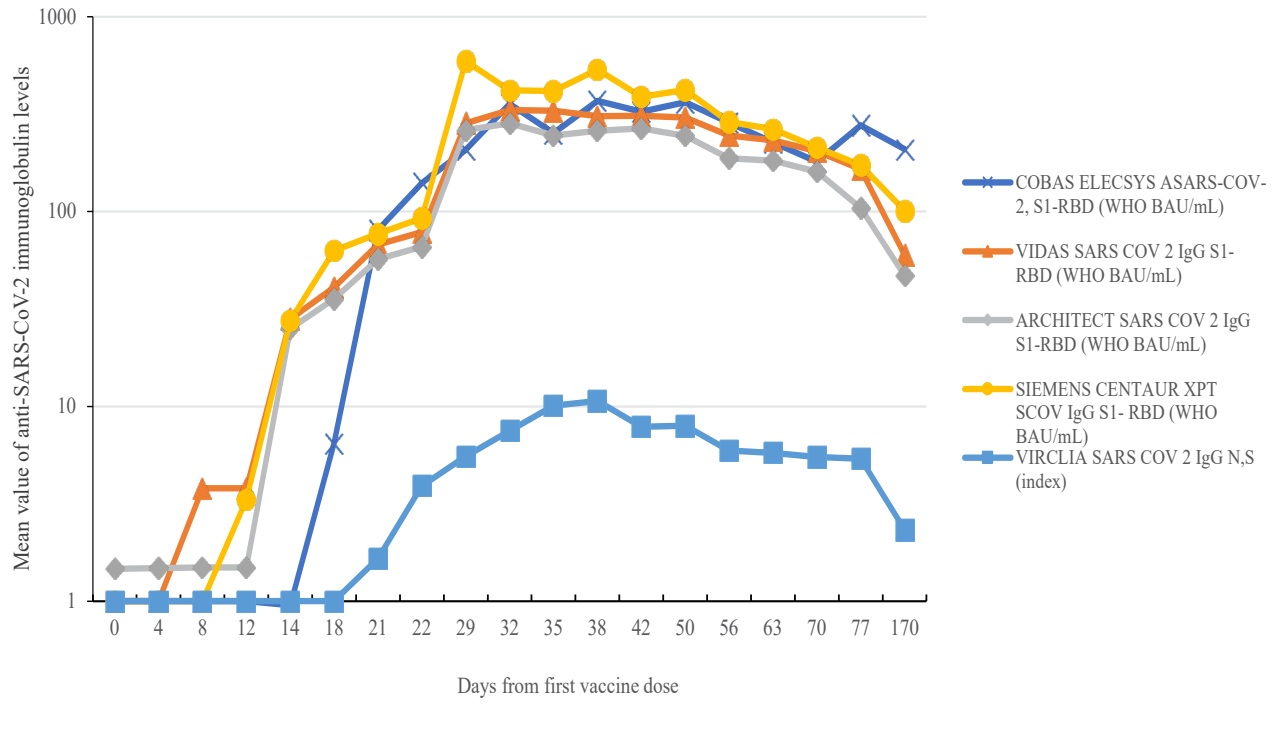
Significant correlation was found between VidasSARS-CoV-2 IgG S1-RBD and Architect SARS-CoV-2 IgG-S1-RBD (r=0.98; p<0.001), Vidas SARS-COV-2 IgG S1-RBD and Virclia IgG anti N/S (r=0.966; p<0.001), whereas a smaller but statistically significant correlation was found among the other anti-SARS-CoV-2 antibody classes.

As a reference for comparison among laboratories, IgG levels were expressed in international units (IUs) after normalization with the WHO International Standard for anti-SARS-CoV-2 antibody (Table 1).

DISCUSSION

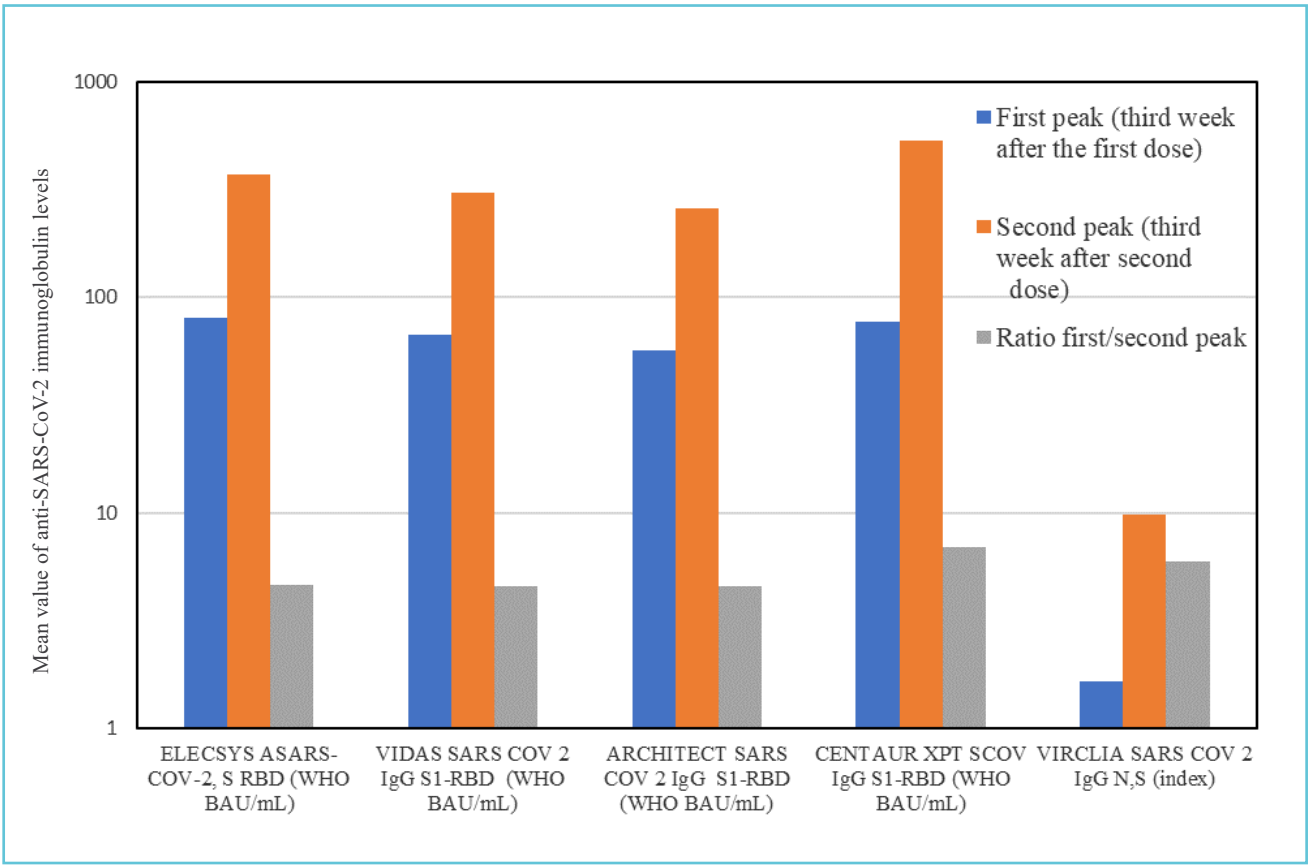
In less than a year, the entire world population has experienced a change in psychological, economic, medical as well as sociological and mental status as a result of the high rates of infection and mortality rate due to the novel SARS-CoV-2 virus infection. Universal and

Figure 1 **Kinetics of anti-SARS-2 antibodies following Gam-COVID-Vac-Adeno-based recombinant vaccine. Values are shown as mean ± SD**



Abbreviations: Ig,Immunoglobulin; N,nucleocapsid; RBD,receptor binding domain; S, spike protein.

Figure 2 **Median increase-peak values of different classes of anti-SARS-CoV-2 antibodies elicited by two doses of adeno-vector based "Sputnik V" -Gamaleya vaccine. The day of the peak is reported in the text.**



Abbreviations: Ig, immunoglobulin; N, nucleocapsid; RBD, receptor binding domain; S, spike protein.

comprehensive vaccination against COVID-19 is most likely the key to all strategies for stopping or reducing the circulation and reducing the contagiousness of SARS-COV-2. Several published studies have provided evidence that there is a direct correlation between the distribution of vaccines and the reduction of the number of SARS-COV-2 positive cases, hospitalization and mortality due to COVID-19 [18]. Although the efficacy of most currently licensed vaccines appears to be considerably high, especially in reducing the risk of clinical exacerbation among patients in different clinical risk groups [19], little is known about the immunogenicity of adenoviral vector-based vaccine Sputnik V. Although this vaccine has not been licensed by the WHO yet, it has been accepted to use by many countries in the relentless race for vaccines which have failed to provide doses of those vaccines approved by the WHO. Here we present our preliminary data on the safety, tolerability and immunogenicity of Sputnik V. In this study, only candidates who had no previous contact with the SARS-CoV-2 virus and have developed antibodies to the SARS-CoV-2 before the first dose of vaccine were eligible to participate. In terms of vaccine safety, we found that Sputnik V vaccine was well tolerated among participants and the most common reported systemic side effect were influenza-like symptoms. Only one participant had moderate side effects. All observed minor side effects during the study were transient, lasting no more than

48 hours and no serious adverse reactions have been reported. Tukhvatulin et al. have reported about the safety and immunogenicity of single-dose vaccine "Sputnik Light" vaccine, where only 5.2% of participants without immunity to SARS-CoV-2 complained of muscle and joint pain after vaccination, and only 5.5% participants had moderate grade adverse effects who persisted no more than 24 hours. A group of seropositive participants after vaccination demonstrated less and milder adverse effects when compared with seronegative participants [20].

The high seroconversion rates found in the participants of this study are in an agreement with what has been previously published for Sputnik V and other COVID-19 vaccines [14,20, 21]. Rossi et al. monitored antibody response in 62 seronegative participants and 227 participants with prior SARS-COV-2 infection receiving two doses of Sputnik V vaccine [22]. They reported that 94% of seronegative participants showed positive SARS-CoV-2 IgG response with geometric mean titer (GMT) of 244 [95% CI 180-328], and after second dose 100% of seroconversion with GMT of 2.148 [95% CI 1.742-2.649]. Antibody response was stronger in seropositive people receiving Sputnik V adeno-based vaccine compared with those negative at baseline, interestingly with no significant differences after one or two doses. In our study, 4 of 5 participants had a seroconversion before receiving the second dose, and after the second dose, all participants have developed specific antibodies against SARS-CoV-2. Salvagno et al. have demonstrated that two doses of Pfizer

Table 3 Spearman correlations and CI (95%) between overall serum increase of anti-S-RBD total Ig, anti-S1-RBD IgG, IgG anti-N and S elicited after administration of two doses of Sputnik V

	VIDAS (SARS-COV-2 anti-S1-RBD IgG)	ARCHITECT (SARS-COV-2 anti-S1-RBD IgG)	CENTAUR XPT (SCOVG anti-S1-RBD IgG)	VIRCLIA (SARS-COV-2 anti-N and S IgG)
ELECSYS (SARS-COV-2S-RBD total Ig)	0.903 (CI 95% 0.761-0.962) P<0.001	0.883 (CI 95% 0.716-0.954) P<0.001	0.890 (CI 95% 0.731-0.957) P<0.001	0.937 (CI 95% 0.841-0.976) P<0.001
VIDAS (SARS-COV-2 anti-S1-RBD IgG)		0.982 (CI 95% 0.953-0.993) P<0.001	0.942 (CI 95% 0.853-0.978) P<0.001	0.966 (CI 95% 0.912-0.987) P<0.001
ARCHITECT (SARS-COV-2 anti-S1-RBD IgG)			0.957 (CI 95% 0.889-0.984) P<0.001	0.933 (CI 95% 0.831-0.974) P<0.001
CENTAUR XPT (SCOVG anti-S1-RBD IgG)				0.940 (CI 95% 0.848-0.977) P<0.001

Abbreviations: IgG - Immunoglobulin G, RBD - Receptor binding domain, S1 - spike protein S1 subunit, N - Nucleocapsid.

and Moderna mRNA vaccines have elicited secretion of different classes of anti-SARS-CoV-2 where the first vaccine dose triggered an increase from baseline to the median of 103.3 folds for anti-spike trimeric IgG, 210.9 folds for anti-spike RBD IgG, and 13.3 folds for anti-spike S1 IgA, but the second boost triggered additional modes median increase of antibodies of 6.3 folds for anti-spike trimeric IgG, 7.2 folds for anti-spike RBD IgG, and 1.5 folds for anti-spike S1 IgA, respectively [23].

Even though our report is limited to 5 cases, it has many strengths in assessing post-vaccination immune response. Namely, we have presented an extensive sequence of blood sampling, which has given us an opportunity to present an early identification of SARS-CoV-2 antibodies as well as monitoring their progression over period of 6 months. In this study, we have also presented measurement of different antibodies responses (total IgG anti-S-RBD, IgG anti-S1-RBD, IgM+IgA anti-S and N) after vaccination with "Sputnik V" vaccine giving the opportunity for monitoring the elicited humoral response. In view of all above, it is possible for us to show that in total IgG anti-S-RBD and IgG anti-S1-RBD a constant linear increase was observed after the first vaccine, showing additional growth induced by the second vaccine dose.

As known for many viral infections, humoral immune kinetics show that antibody levels peak after natural contact with the viruses or after vaccination begin to drop after a certain number of weeks [24]. Similar as with other viruses, antibodies following the infection with SARS-CoV-2 decline after 8 months [25]. Few studies have evaluated the half-life of the antibodies elicited by mRNA-vaccines, suggesting that they may last for 6-8 months [26, 27]. We have demonstrated that after the adeno-based vaccine Sputnik V, the plateau of seropositivity was achieved at the 50th day and remained in the same range until it started to decline slowly after the 80th postvaccination day, thus 16 weeks after the first dose values were still higher than the first pick and dropped after 180 days, providing sustainable immunity in the same manner as other available vaccines against SARS-CoV-2.

Although the year of 2021 was supposed to be a year of equality and solidarity, in which the approved vaccines for SARS-CoV-2 would be equally distributed, in the race for timely vaccination, small and poor countries managed to procure and vaccinate their population with adeno-based vector "Sputnik V" vaccine. One of the currently available vaccines is Sputnik V, developed and manufactured by the Gamaleya, National Research Center for Epidemiology and Microbiology in Moscow, Russia. Although more than 80 countries, mainly in Eastern Europe such as Serbia, North Macedonia, but also in many countries in South America and Africa etc., have approved the vaccine, not many studies have been published on the effects and efficacy of this vaccine [28]. The few studies that have been officially published have confirmed the safety and efficacy of Sputnik V, as originally reported in phase 1/2/3 [29]. In contrast to this lack of peer-reviewed and published studies there is a growing number of published studies and abundance of data and information on the mRNA-1273 (Moderna/NIAID), BNT162b2 (Pfizer/BioNTech) and AZD1222 (AstraZeneca/University) vaccines [26,27,30].

The results of our study provide further knowledge about "Sputnik V", since in this study we aim to provide evaluation of the synthesis and viability of antibodies up to 6 months after immunization with this vaccine. Our data provide evidence that seronegative individuals with a negative titer prior to vaccination achieve an adequate and long-lasting humoral immune response that is maintained for more than 180 days after immunization with this vaccine. The disadvantage of our study is the limited number of individuals and that we were not able to examine

cellular immunity, as is the case for most Sputnik V research reports. We believe that Sputnik V should be approved for immediate use by the WHO and the European Medicines Agency, the first of which is crucial for the vaccine to be deployed in low-income countries through the COVID Global Access 19 (COVAX) initiative [28].

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In this issue: Current laboratory aspects of COVID-19

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Newly diagnosed chronic lymphocytic leukemia during symptomatic COVID-19: two cases

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ABSTRACT

Patients suffering from malignant diseases have a high risk of developing severe or critical forms of COVID-19 (Coronavirus Disease 2019). Chronic lymphocytic leukaemia (CLL) is characterized by dysregulated adaptive and innate immune responses, because both T and B cells, the function of phagocytes and the activity of the complement system may be affected. Severe SARS-CoV-2 infection also influences the immunological functions mainly via causing the depletion of CD4+ and CD8+ T cells. We present the cases of two patients, whose *de novo* CLL were observed during severe COVID-19 pneumonia. A 43-year-old man with IDDM (Insulin dependent diabetes mellitus) was sent to hospital in February 2021. He had a bilateral severe COVID-19 pneumonia. There was a suspected sign of malignancy on a thoracic vertebra in his chest CT, and haematological consultation was

requested. In parallel, a 53-year-old man was hospitalized in March of 2021 because of severe COVID-19 pneumonia. CLL was suspected based on his haematology test results (WBC: 123 G/L, lymphocytes: 91%, haemoglobin: 107 g/L). Flow cytometric analysis revealed CLL in both cases. Based on the result of the molecular genetic tests, the first patient had a good prognosis in Rai 0 stage, while the other patient suffered from Rai I stage with a worse prognosis. Both patients recovered from bilateral COVID-19 pneumonia without the need for intensive care unit treatment. The follow-up of these CLL patients that manifested during symptomatic COVID-19 disease further enriched our knowledge on such clinical conditions where the immune system is dysfunctional due to different simultaneous causes.

❖❖❖❖❖

INTRODUCTION

CLL is the most common type of leukaemia among adults in developed countries with an annual incidence of 3/100 000 people in Central Europe in 2019 [1]. It is characterized by the monoclonal accumulation of mature B lymphocytes of which immunophenotype and immunomodulating functions are changed resulting in the dysregulation of both the adaptive and innate immune responses. These changes affect both T and B cells, phagocytosis and the complement system leading to an immunosuppressive condition [2,3,4], thus the general risk of severe infections critically rises the morbidity and mortality [5,6]. Although 'watch-and-wait' strategy is recommended for low-risk patients (*i.e.,* Rai 0 stage), patients in higher risk category (e.g., Rai III-IV stages) require chemotherapy which includes not only conventional agents, but also new regimens, such as Bruton's tyrosine kinase inhibitors (BTKi) or B-cell lymphoma 2 (BCL-2) inhibitors. Treating patients with Rai I-II stages is feasible and highly indicated if the disease starts to progress [6,7]. CLL therapy also contributes to immunosuppression which further increases the risk of infections [5,6].

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection is a serious risk factor for cancer patients [8]. It causes the depletion of CD4+ and CD8+ T cells, B cells and natural killer cells causing an impairment of the immune system [9]. These complications and the increased level of cytokines producing CD14+CD16+ monocytes contribute to the development of cytokine storm and related fatal outcome [10]. Moreover, initiation of treatment can induce additional immune modulation that further increases the risk for severe infections [11].

Here we present two cases where CLL was confirmed during the clinical phase of COVID-19 pneumonia. SARS-CoV-2 infection causes lymphopenia in contrast to lymphocytosis that is typical in CLL. Our aim was to investigate the effects of these comorbidities on laboratory results and to accomplish the follow-up of acute and chronic clinical conditions when the immune system is under attack from two directions simultaneously.

TWO CASES

The first patient was a 43-year-old male patient with insulin dependent diabetes mellitus and transient ischaemic attack in his medical history. He was admitted to hospital with severe respiratory symptoms in February 2021 when his COVID-19 pneumonia was treated by the current protocol including remdesivir, steroids and antibiotic therapy. His chest CT scan for COVID-19 pneumonia suggested signs of malignancy on a thoracic vertebra and he was sent to a haematology consultation. In April, his laboratory parameters were as follows: white blood cell count (WBC): 17.2 G/L with 62.9% relative lymphocyte

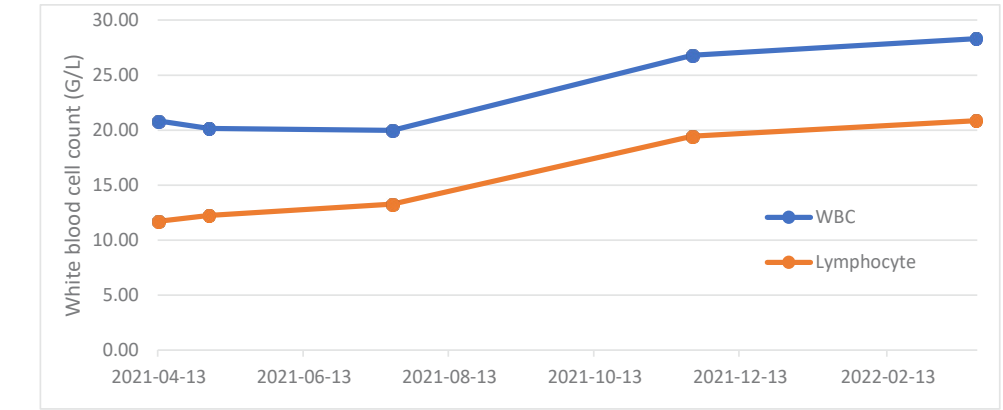
ratio, haemoglobin was 144 g/L, thrombocyte count was 214 G/L. In the peripheral blood smear, there were lymphocytes in 45% and their atypical forms in 6%. The result of the flow cytometric analysis in the peripheral blood found CD19 positive pathological B cells in 33% which were divided into two subclones (CD38+ and CD38-). FISH (fluorescence in situ hybridization) analysis proved the presence of del(13)(q14) deletion. The final diagnosis was CLL. The bone scintigraphy did not prove any solid tumour. Three months after the onset of SARS-CoV-2 infection, WBC count was elevated (20-21 G/L) with higher absolute lymphocyte count (11-12 G/L), but there was no anaemia or thrombocytopenia. The patient had neither hypogammaglobulinaemia nor paraproteinaemia, and the level of β2 microglobulin was 1.73 µg/ml. No lymph nodes or the spleen were palpable, however, the liver could be reached. After three months of these analyses, anti-SARS-CoV-2 IgG antibody test was positive (Table 1). Further genetic tests were performed as IgH gene rearrangement could not be detected, and IgHV somatic hypermutation status was uninterpretable. In November, WBC and absolute lymphocyte count began to rise to 20 G/L, but other laboratory parameters remained stable (Figure 1). CLL in this patient was determined in Rai 0 stage and 'watch and wait' strategy was suggested under his follow-up.

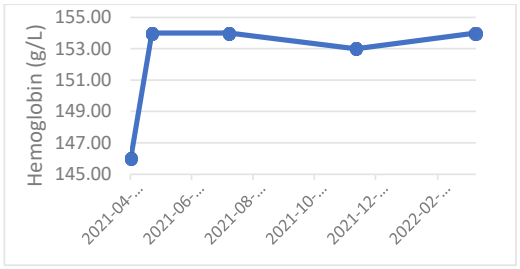
The other patient at the age of 53-years was treated in hospital with bilateral SARS-CoV-2 pneumonia in the end of March in 2021. He did not receive remdesivir or steroid therapy. The suspicion of CLL arose this time due to his haematology parameters (WBC: 123 G/L, lymphocytes: 91%, haemoglobin: 107 g/L), with enlargement of mediastinal and axillar lymph nodes. His peripheric blood smear showed lymphocytes in 93% and several smudge cells. The result of his flow cytometric analysis showed 82% pathologic

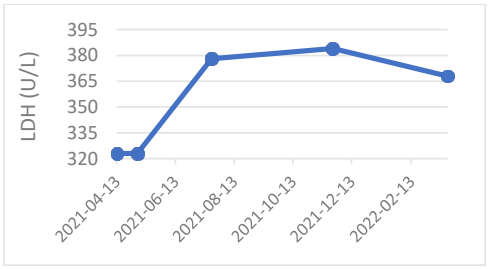
Table 1 Three months after SARS-CoV-2 infection, the test for anti-SARS-CoV-2 antibodies (IgM and IgG) was positive and they were neutralizing in both patients. First patient received Moderna vaccinations without any complications in May and June 2021. The other patient was vaccinated with Pfizer/BioNTech in December 2021 after his steroid therapy was ended.

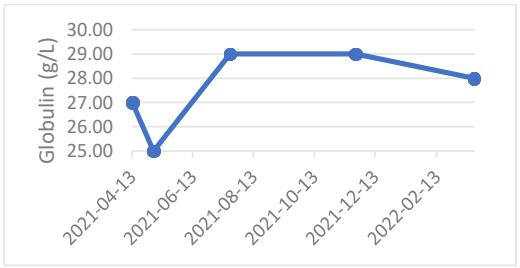
Antibody (AB) test	Results of 1st patient	Evaluation	Results of 2nd patient	Evaluation
SARS-CoV-2 IgM Architect (S/C)	33.56	Positive	6.61	Positive
SARS-CoV-2 IgG (AU/mL)	113	Positive	108	Positive
SARS-CoV-2 IgG Architect (S/C)	5.18	Positive	4.52	Positive
SARS-CoV-2 AB Neutralizing (%)	96.0	Positive	59.4	Positive

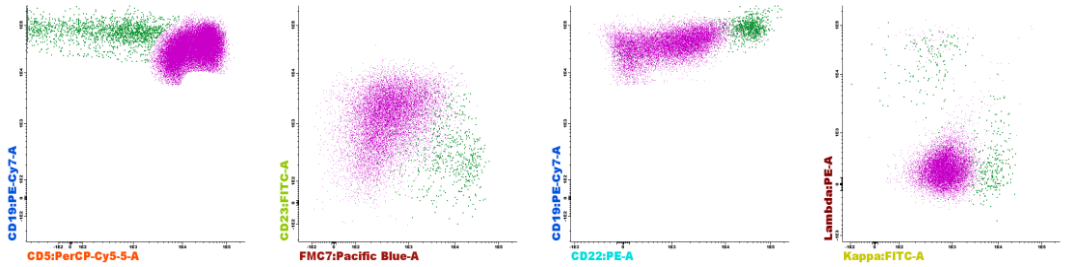
Figure 1 **Kinetics of different routine laboratory parameters during the follow-up and the main results of flow cytometric analysis in patient (No 1) with CLL (Rai 0 stage) depicted with dot plots***





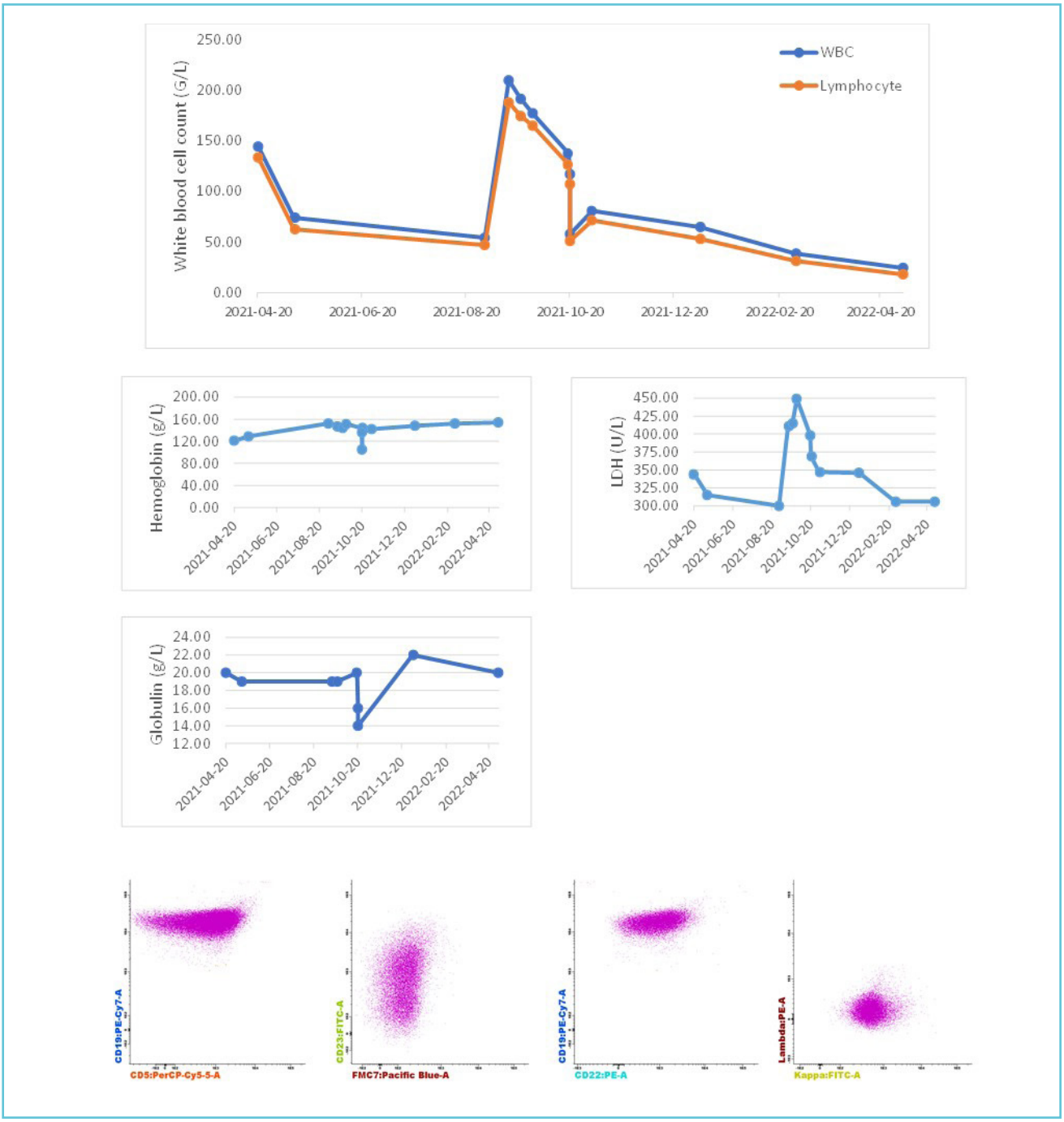






**Laboratory findings in accordance with the clinical conditions did not display a significant difference in the observed period (13 April 2021 – 05 May 2022). Cell counts were moderately elevated (WBC 20.8-28.36 G/L, lymphocyte 11.7-20.31 G/L, globulin 27-29 g/L, IgG 11.5 g/L, IgA 2.7 g/L, IgM 1.2g/L, with no sign of paraproteins). The infection was eliminated and the CLL did not show a progression.*

Figure 2 **Changes in different routine laboratory parameters during the study period and the characterization of pathological B-cell population by flow cytometry in COVID-19 positive patient (No 2) diagnosed with CLL (Rai I stage)***



**The extremely high WBC and lymphocyte counts were halved in the first four months (WBC 144→74 G/L, lymphocyte 133 → 62 G/L). Mild hypogammaglobulinaemia (IgG 6.1 g/L, IgA 2.6 g/L, IgM 1.3 g/L) with the presence of monoclonal IgG (1.2 g/L) was observed. Severe thrombocytopenia occurred at the end of August 2021. This state proved to be a secondary immune thrombocytopenia that responded well to the treatment. In September, WBC was increased to an extremely high level again, therefore urgent leukapheresis and ibrutinib therapy (280 mg/day) was required for a year.*

and CD38- B cells supporting the diagnosis of $CD38^-$ CLL. FISH analysis proved the presence of del(13)(q14) and *ATM* gene deletion. Molecular genetic test detected the monoclonal *IGH* gene rearrangement, while *TP53* gene mutation and IgHV somatic hypermutation status were negative (UM-CLL status). The patient had anti-CMV IgG and anti-EBV IgG titers in association with a mild hypogammaglobulinaemia and slightly elevated β2 microglobulin level (2.77 μg/ml). The immunofixation showed the presence of 1.2 g/L monoclonal IgG κ paraprotein in the gamma fraction. In May, WBC was 144 G/L, lymphocyte count was 133.4 G/L, and haemoglobin was 121 g/L. He did not have palpable lymph nodes, and the spleen was not enlarged either. The test for anti-SARS-CoV-2 antibodies were positive (Table 1). This patient had Rai I stage CLL, and he had no post-COVID-19 symptoms. In August, severe autoimmune thrombocytopenia developed with a platelet count of 35 G/L, which was treated successfully by steroid administration. One month later WBC and lymphocyte count were increased permanently, his disease showed a rapid progression with an extremely short (one-week long) lymphocyte doubling time. These results indicated the initiation of CLL-related treatment. In October, leukapheresis was required and BTKi (ibrutinib) was administrated to the patient with UM-CLL. In a couple of days, his clinical status and laboratory parameters gradually improved (Figure 2).

DISCUSSION

Both COVID-19 patients recovered from bilateral COVID-pneumonia uneventfully. They had a sufficient level of anti-SARS-CoV-2 antibody in the observed period. Their chronic lymphocytic leukaemia was diagnosed during SARS-CoV-2 infection. The stage of CLL and the clinical symptoms did not change in the case of the first patient. The second patient had CLL with a poor prognosis. The progress of their diseases was probably independent from the subsequent infection. The long-term follow-up of patients with CLL that manifested during symptomatic COVID-19 could further enrich our knowledge on such conditions where the immune system is attacked from multiple sides.

Our data potentially suggests a protective role of the complex immune dysfunction caused by CLL; this effect needs to be further investigated in case of severe SARS-CoV-2 infection that might cause an excessive inflammatory response.

CONCLUSION

The observation of these CLL patients with different case history implies that simultaneous manifestation of COVID-19 with a newly emerging CLL does not automatically cause difficulties in laboratory data interpretation neither during the diagnostic procedures nor under the follow-up period.

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In this issue: Current laboratory aspects of COVID-19

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Impact of COVID-19 on pediatric laboratory medicine: an IFCC C-ETPLM, SSIEM, ISNS global survey

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ABSTRACT

Objective

Pediatric laboratory medicine is a unique practice serving a vulnerable group of patients including highly specialized testing aiming to detect and treat inherited conditions early to avoid adverse outcomes. Data on the actual impact of COVID-19 pandemic on this speciality is lacking.

Methods

A survey was conducted by the IFCC Committee on Emerging Technologies in Pediatric Laboratory Medicine in partnership with the Society for the Study of Inborn Errors of Metabolism and International Society for Neonatal Screening, to assess the impact

on the clinical service provision during the initial wave (January to July 2020) of the COVID-19 pandemic and to gather experiences learned in order to improve laboratory preparedness for future outbreaks.

Results

217 survey responses were received from 69 regions. Sixty-three laboratories (29%) reported a restriction or suspension of service for a median period of four months. The common tests/services suspended were new-born screening program, body fluids and sweat testing. The reasons for the suspension were related to biosafety risks of COVID-19 transmission, manpower constraints and supplies disruption. A minority (9-10%) of laboratories did observe delayed/missed diagnoses or a more severe presentation of a clinical disorder. The critical operational decisions that helped manage the initial wave of COVID-19 included modifying work shift patterns, split-teams arrangement, use of personal protection equipment and social distancing.

Conclusion

The provision and delivery of pediatric laboratories services were affected during the initial wave of the COVID-19 pandemic. Manpower preparedness for future potential disruptions to pediatric laboratory services is a key finding and recommendation from this survey.

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INTRODUCTION

The coronavirus disease-2019 (COVID-19) caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a global pandemic first detected in December 2019. Global healthcare priorities have needed to primarily focus on the adult population who were clinically compromised or exhibited symptoms. During this pandemic, laboratory medicine has also been under considerable pressure and needed to change its operations to manage its services, including reorganizing laboratory operations, change management, diverting resources, deferring services, and overall ensuring business continuity of services defined as essential (1, 2). Reviewing the success or otherwise of these changes is important for future planning.

The global stress caused by the COVID-19 pandemic on healthcare services is unprecedented. Whilst there have been restrictions, there has also been a reluctance to seek pediatric care during the pandemic (3). With limited or reluctant access to normal healthcare brings the potential for later presentations of common childhood conditions (such as diabetes and appendicitis), delayed or stopped vaccination or screening programs, resulting in potentially worse prognosis (4-6). In addition, unique presentations of acutely sick children post-viral infection, mimicking the previously described Kawasaki disease (*e.g.,* pediatric multisystem inflammatory syndrome temporally associated with COVID-19), also presented a new challenge for pediatric laboratories.

Pediatric laboratory medicine is a unique practice serving a vulnerable group of patients (7). It includes highly specialized testing that aims to detect and treat inherited conditions early to avoid adverse outcomes. Anecdotes of diversion or limitation of pediatric laboratory testing, particularly those related to inborn errors, have been discussed in various online laboratory medicine professional fora. Whilst a reduction in pediatric health care access has been reported, the direct influence of the initial wave of the COVID-19 pandemic on pediatric laboratory medicine services is less clear (8-10).

It is important to understand the impact globally of COVID-19 on pediatric laboratory service delivery, especially through the initial wave when the greatest changes and hesitancy were

seen. Understanding the changes enforced or initiated due to COVID-19 during the initial impact and response will allow pediatric laboratories and relevant authorities to learn from their past experiences and implement appropriate mitigation measures. Additionally, it can serve to provide important comparisons between laboratories to support future planning.

This survey by the IFCC Committee on Emerging Technologies in Pediatric Laboratory Medicine (C-ETPLM), in partnership with the Society for the Study of Inborn Errors of Metabolism (SSIEM) and International Society for Neonatal Screening (ISNS), was conducted to:

1. understand how laboratories serving the pediatric population changed their clinical service delivery in response to the initial wave (January to July 2020) of the COVID-19 pandemic;
2. gather experiences learned from managing the initial wave(s) of the pandemic to improve laboratory preparedness for future outbreaks.

METHODS

Questionnaire

A descriptive electronic survey comprising 17 questions in English was constructed using SurveyMonkey software (Momentive Inc, San Mateo, California, United States). The survey questions were developed by four co-authors (TPL, TL, RG, CMM) from Singapore, the United Kingdom, Australia and Hong Kong, respectively on behalf of the IFCC Committee for Emerging Technologies in Pediatric Laboratory Medicine (C-ETPLM; https://www.ifcc.org/ifcc-emerging-technologies-division/etd-committees/c-et-plm/). The survey comprised a variety of question formats including multiple-choice and open-ended questions. Where relevant, the participants were invited to elaborate on their responses using free text. The survey was organized into the following areas:

a. general information (4 questions);
b. change of practice - delivery of laboratory services (6 questions);
c. effect - impact on clinical care or pathology of pediatric diseases during the initial wave of COVID-19 (3 questions);
d. retrospective - lessons learned from the initial wave of COVID-19 (4 questions).

For this survey, the "initial wave" was defined as the period between January 2020 and July 2020. The draft of the survey was piloted and reviewed by laboratories in Singapore, the United Kingdom and Australia before further refinements. No individually identifiable data was collected for this survey. This survey was exempted from Ethics Board review. A copy of the complete questionnaire is provided in Supplement 1.

DISTRIBUTION

This survey was distributed between February and April 2021. Participation was entirely voluntary, and participants were assured that complete anonymity would be preserved. The survey was publicized through multiple channels including email alerts, electronic news alerts, electronic newsletter and social media of the IFCC C-ETLM, SSIEM, ISNS and national societies. This network distribution approach provided a mechanism for the broadest reach. However, it did not allow for the calculation of the response rate as the distribution channels overlapped significantly.

DATA ANALYSIS

The electronic survey responses were reviewed in Survey Monkey and then exported into an Excel (Microsoft Excel, Microsoft, Seattle, WA, USA) spreadsheet for additional analysis. Question 2 of the survey was designed as an exclusion

criterion if the participants only answered to the option: "We do not serve pediatric patients" or only "other" for Question 2 (laboratory testing type).

The survey responses contained both quantitative and qualitative data, together with some free-text comments. To quantify the numerical data, results are presented as the percentage (%) of participants providing a particular response (numerator), compared to the total number of participants who responded to the question (denominator). As some survey questions allowed for multiple responses, the denominator reflects the addition of all responses made in these instances. That is, where multiple responses were solicited, the denominator may show a total response greater than the actual number of survey participants. Qualitative responses were reviewed and summarized categorically.

RESULTS

In total, the survey generated 238 responses. Responses from 21 laboratories were excluded as they did not indicate that they were involved in pediatric laboratory medicine in Question 2. These excluded laboratories either indicated that they serviced only adult patients or other settings. This left 217 valid responses with 96 (44%) of these deemed to be "complete" by Survey Monkey. The most frequent question avoided was number 15 relating to "operational decisions you wish your laboratory had avoided when managing the initial wave of COVID-19". Following examination of the complete versus incomplete response group, it was decided to include all responses obtained for the quantitative data. These responses were received from 69 geographical regions (Figure 1).

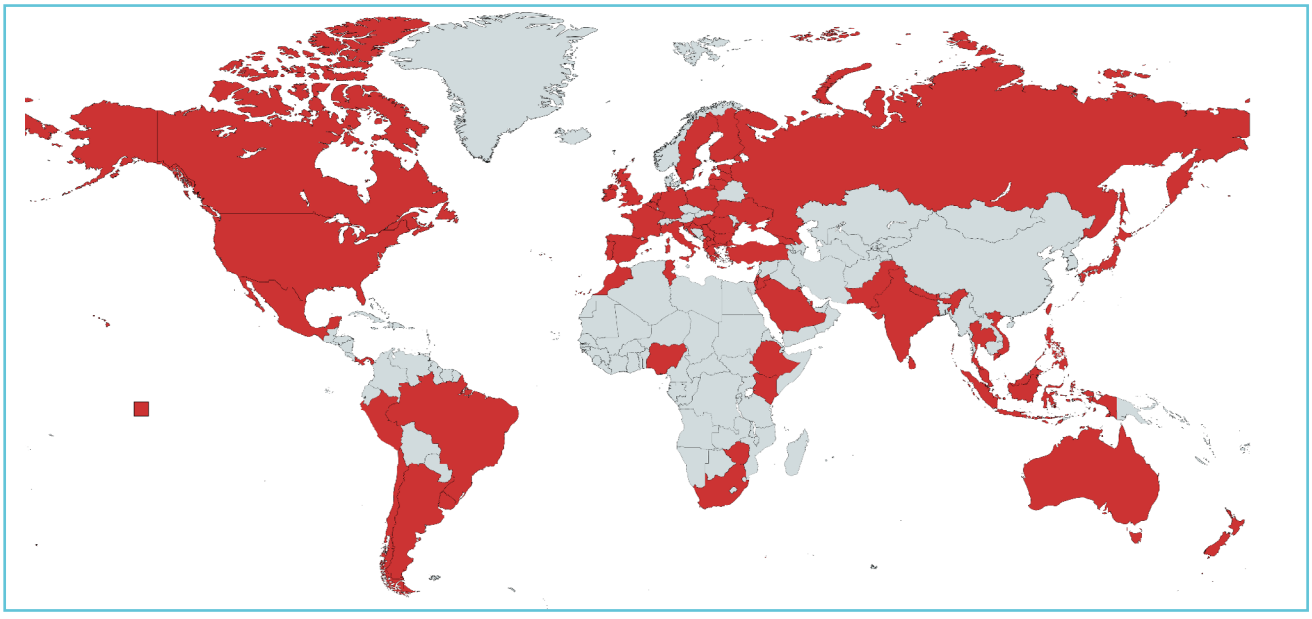
GENERAL INFORMATION (QUESTIONS 1-4)

The characteristics of the participating laboratories are summarized in Table 1.

DELIVERY OF LABORATORY SERVICES (QUESTIONS 5-10)

The ten clinical laboratory tests and services that were most restricted or suspended during the initial wave of the COVID-19 pandemic

Figure 1 World map showing valid responses from 69 region for the global survey



This figure was created with mapchart.net.

are summarized in Table 2. The restriction or suspension was in force for a median of four months. The most common reasons for suspension were the following: concern over the risk of COVID-19 transmission (41%, n = 37/90), manpower diversion to other areas (20%, n = 18), insufficient manpower (e.g., due to split team arrangement; 20%, n = 18), and disruption of reagent/consumable delivery due to COVID-19 (19%, n = 17). A variety of additional responses were recorded by 35% of participants. One laboratory reported diversion of funding to COVID-19 testing as the reason for service restriction/suspension. At the same time, the participating laboratories reported a significant decrease in workload for 15 clinical tests which are summarized in Table 3.

Quality standards related to national, or accreditation standards were reported by 15 % (n = 19/124) of laboratories. The laboratories reporting difficulty meeting national standards for newborn/pediatric screening programmes in their local setting with delayed turnaround time (n=3), suspension of confirmatory testing for newborn screening owing to maternal COVID-19 concerns (n=2), delay in sample delivery (n=2), insufficient reagent (n=1) and manpower constraints (n=1). This was consistent with the cited difficulty of meeting laboratory accreditation standards due to delayed turnaround time (n=2), delayed laboratory audits (n=2), non-availability of instrument technical support (n=1), manpower constraints in following quality issues (n=1), and delay in laboratory testing or diagnosis of pediatric conditions (n=1).

IMPACT ON CLINICAL CARE OR PATHOLOGY OF PEDIATRIC DISEASES (QUESTIONS 11-13)

Most participants reported no impact from COVID-19 on clinical care. However, an increase in missed detection of pediatric diseases related to the clinical laboratory tests/services stopped or restricted due to COVID-19 was noted by 10% (n = 12/121) of participants. Similarly, 9% (10/114) of participants observed an increase in later (more severe) presentation of pediatric diseases related to the tests/services stopped, restricted due to COVID-19. Clinical conditions associated with increased missed detections were neonatal jaundice, congenital hypothyroidism, urosepsis, non-COVID-19 viral infections, phenylketonuria, anaemia, endocrine disorders, leukaemia and lysosomal disease; each was reported by one participant. Clinical conditions associated with delayed or more severe presentation were Crohn's disease, phenylketonuria, neonatal jaundice, and inborn errors of metabolism with one participant reporting each.

By contrast, almost a quarter (23%, n = 27/116) of participants observed positive effects of COVID-19 on their laboratory services or clinical conditions that can be detected via laboratory tests.

LESSONS LEARNED FROM THE INITIAL WAVE (QUESTIONS 14-17)

The most important operational decision made that helped the management of the initial wave of COVID-19 was changing the normal working patterns of their staff either by modifying the shift patterns or splitting the staff into teams to prevent further infections (34%, n = 30/91 participants).

The next ranked change (18%) was implementing measures to protect their staff from potential infection including provision of personal protection equipment, social distancing where possible, restricting access and reviewing risk assessments.

Fifty-one participants described at least one operational decision that they would wish to avoid when managing the initial wave of COVID-19. The most important operational decisions were

Table 1 Characteristics of the laboratories participating in the survey

	Frequency	Percentage
Laboratory services provided		
Newborn bloodspot screening	59	27.2
Inherited metabolic diseases testing	63	29.0
Sweat testing	41	18.9
Genetic / molecular testing	54	24.9
Specialist pediatric endocrinology testing	36	16.6
Pediatric blood sciences - general biochemistry / hematology / endocrinology / immunology	92	42.4
General adult laboratory serving neonatal/ pediatric population	116	53.5
Number of patient samples processed per day		
<200	91	41.9
200-500	48	22.1
>500	78	35.9
Pediatric specialty supported by laboratory		
General pediatric medicine	164	75.6
Community pediatrics	73	33.6
Neonatal unit	133	61.3
Specialist children's hospital serving multiple subspecialties	96	44.2
Maternity	102	47.0

Table 2 **Laboratory tests and services that were restricted or suspended during the initial wave of COVID-19 pandemic (Jan to July 2020)**

	Frequency
Clinical test / clinical laboratory service restricted/ suspended	
Newborn screening program (including congenital hypothyroidism, inborn errors of metabolism)	12
Body fluids	6
Sweat testing	5
Fecal elastase, calprotectin, copro-parasitology	5
Saliva, sputum tests	4
Endocrine tests	4
Procalcitonin	3
Outpatient department services	3
Urine tests	2
Therapeutic drug monitoring	2
Autoimmune, allergy markers	2
D-dimer	2
IL-6	2
Vitamin testing	2
Duration of service restriction or suspension (months)	
Median (interquartile range)	4 (5.5)
Min	1
Max	12

Table 3 Laboratory tests with a decrease in workload compared to pre-COVID-19 in your laboratory during the initial wave of COVID-19 (Jan to July 2020)

Laboratory tests	Response frequency	Workload reduction (%)		
		Median	Min	Max
General chemistry	19%	50	5	90
Diabetes testing (glucose, HbA1c)	5%	50	25	90
Endocrinology and lipids	6%	50	15	80
Vitamin testing (folate, B12, others)	3%	65	15	80
Inflammatory markers (CRP, procalcitonin)	2%	25	15	40
Tumour markers	1%	17	5	30
Special chemistry (markers, metals, drugs)	6%	55	5	100
Sweat test	2%	100	90	100
Inherited metabolic testing	18%	60	20	100
Newborn screening	3%	30	5	50
Haematology	10%	50	5	100
Histopathology and cytology	3%	80	50	90
Allergy and immunology testing	10%	55	15	95
Genetic and genomic testing	2%	50	40	100
Clinical microbiology, virology and parasitology	13%	60	10	100

problems in routine service interruption (49%, n = 25/51), inadequate safety measures and education to the staff (43%, n = 22), reagent shortages (20%, n = 10), and lack of alternating teams, long working hours and staff deployment (20%, n = 10).

Only 8% of laboratories introduced SARS-CoV-2 PCR testing to support the active management of suspected patients. Workload strategies were also implemented to ensure essential services such as neonatal and maternal screening were maintained, and COVID-19 patients were

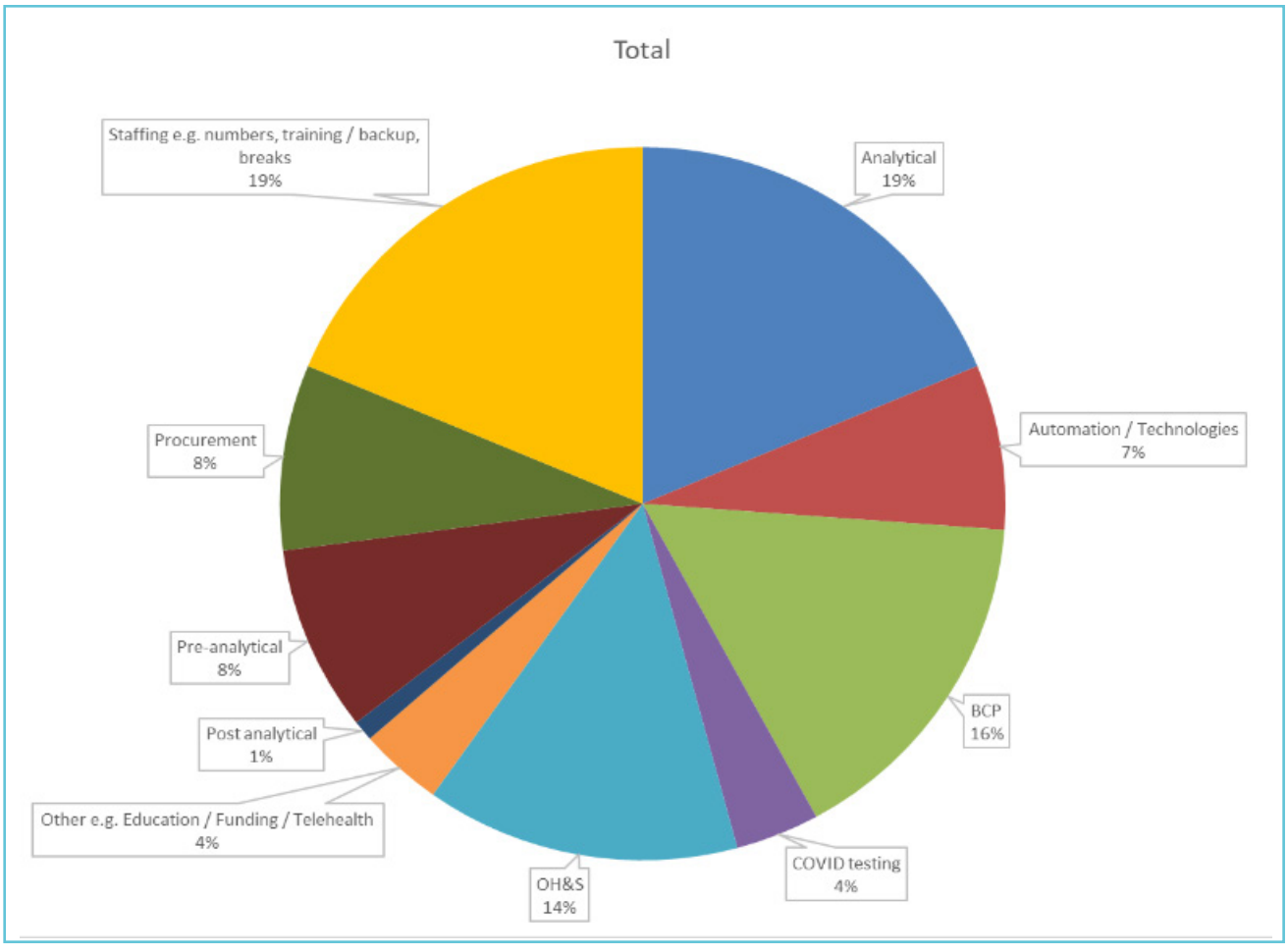
processed quickly. Some laboratories changed their inventory practices to reduce the number of deliveries by holding or ordering extra stock of essential consumables. A range of technologies (e.g., new laboratory platforms, automation) were employed by 44% (n = 41/93) of participants to help manage business continuity.

Similarly, there were 65 participants who responded the significant operational areas to prepare the laboratory for subsequent COVID-19 waves were staffing, analytical testing phase, business continuity planning, and occupational health and safety were given the highest priority by participants (Figure 2).

DISCUSSION

The result of this global survey showed that COVID-19 has significantly impacted the clinical laboratories serving the pediatric population during the initial wave of the pandemic. It was necessary to restrict or suspend certain clinical laboratory services. Among the most frequent restricted laboratory tests were newborn screening and sweat test programs that are designed to detect early hereditary abnormalities to avoid long-term adverse consequences. While the median duration of reduced access was relatively short at four months, a longer restriction (e.g., up to 12 months) may lead to

Figure 2 **Significant operational areas were identified to prepare the laboratory for subsequent COVID-19 waves***



BCP = business continuity planning; OH&S = occupational health and safety.

suboptimal patient care. Restriction or suspension of certain laboratory tests such as newborn screening is highly undesirable as it may be associated with severe consequences to infants in case of late diagnosis. If such a decision is unavoidable, such as the case of the unprecedented biosafety and resource challenges brought on by the pandemic, its duration should be kept to a minimum.

Importantly, the concerns over risk of COVID-19 transmission through laboratory procedures calls for early availability of risk-based biosafety/biohazard risk assessment (1). Nonetheless, the lack of sufficient personal protective equipment and biosafety facility impeded the ability of the laboratory to provide a safe environment for its staff. At the same time, the lack of manpower required improved and more consistent funding to build pandemic-preparedness and cross-training of staff to allow more dynamic deployment of resources. These approaches were thought to reduce the impact of a pandemic supporting the continuity of clinical services.

Split teams and triaging of activities have been employed in new born screening laboratories to mitigate the risk to continuity of service delivery during the initial wave of COVID-19 (2). In this survey, 12 (20%) of NBS laboratories had to restrict their services. This was similar to those reported by the COVID-19-NBS ISNS global network, which approximated the number of laboratories reporting moderate effects from COVID-19 on NBS laboratories (11). The magnitude of decrease in newborn screening activities reported by the survey participants corroborated with a recent study that examined 16 specialized medical centers treating inborn error of metabolism patients in Europe, Asia and Africa and reported a decrease of 60-80% in service activity compared to the pre-pandemic period (12).

At the same time, many clinical laboratory services saw a significant decrease in workload compared to the pre-pandemic period, with some services experiencing a near-complete reduction. Whilst not mentioning a specific reduction in workload, the ISNS global network quoted that up to 83.7% of NBS laboratories were affected by COVID-19 (11). Another study summarizing two surveys performed by the European Reference Network for Hereditary Metabolic Diseases have similarly found major disruption to clinical care delivery for patients with metabolic disorders (10). Besides the reduced clinical service delivery, the laboratory survey participants also reported difficulty meeting local national standards for newborn screening programmes as well as relevant accreditation standards, which may lead to suboptimal service quality.

Testing for other hereditary conditions, such as cystic fibrosis and hereditary angioedema, were also reduced. The reduction in the test volume for the other general laboratory tests may be related to the general reduction in patient visits to healthcare facilities. Moreover, the reduced laboratory testing for chronic conditions such as allergy, diabetes, dyslipidaemia, chronic renal conditions and tumour markers may lead to suboptimal care of these groups of patients. The reduced laboratory testing activity may be due to a combination of restricted laboratory services as well as reduced healthcare attendance during the initial wave of the pandemic.

The reduced laboratory testing, reduced clinical access and a deterioration in laboratory standards risked missed opportunity for early diagnosis and intervention, leading to delayed and potentially more severe clinical presentation. Such adverse consequences were reported by a dozen or fewer laboratories with the clinical conditions closely associated with laboratory tests that had reduced testing activity. Nevertheless, these survey responses should be considered anecdotal evidence as the information provided was not accompanied by curated objective

data. A national study in Scotland on general pediatric healthcare use found that while the rate of clinical consultations in primary care and secondary care fell during the lockdown, they were not associated with increased clinical severity scores or mortality (9). More studies are required that examine the journey of pediatric patients during the pandemic to provide more definitive evidence.

This study has some limitations. The number of laboratories participating in this study is relatively small, although pediatric laboratory medicine is a specialized area within the discipline. Many laboratories served mixed adult-pediatric populations, which may somewhat dilute the focus of the survey response. Additionally, this survey was designed with many open-ended, qualitative questions to best capture the response of the participants. Consequently, the summarization of the data involved subjective categorization and some fields were left unanswered, leading to suboptimal response rates.

There were various lessons learned from the first wave of COVID-19. Technology solutions are important to support business continuity but needs to be tailored according to local needs. With hindsight, several operational decisions made around manpower would not be repeated and this also was the focus for prioritization of future business continuity planning.

Manpower considerations ranged from staff wellbeing, minimizing the spread of COVID-19 between team members, the agility of team members, redeployment of team members to other areas and the maintaining a minimum number of staff available to run services. Some guidance on manpower arrangement in the laboratory setting has since been made available (12). Hence, manpower preparedness for future potential disruptions to pediatric laboratory services is a key finding and recommendation from this survey.

CONCLUSIONS

This survey supports our understanding of how laboratories serving the pediatric population changed their clinical service delivery in response to the initial wave of the COVID-19 pandemic. Staffing decisions were highlighted as both retrospective decisions that would have been done differently and as the priority for future business continuity planning. Overall, these gathered experiences learned from managing the initial wave(s) of the pandemic should improve laboratory preparedness for future outbreaks.

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SUPPLEMENTAL MATERIAL (SUPPLEMENT 1)

IFCC C-ETPLM, SSIEM, ISNS SURVEY ON IMPACT OF COVID-19 ON PEDIATRIC LABORATORY MEDICINE

Background

The coronavirus disease-2019 (COVID-19) pandemic has spread globally since its first detection in December 2019. In many regions, the COVID-19 is now entering a second and subsequent waves of outbreak. A recent survey by The International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) Task Force on COVID-19 has revealed that laboratories have had to change their operations to manage the pandemic, including diverting resources and deferring services.

Pediatric laboratory medicine is a unique practice serving a vulnerable group of patients. It includes highly specialized testing that aims to detect and treat inherited conditions early to avoid adverse outcomes. This survey by the IFCC Committee on Emerging Technologies in Pediatric Laboratory Medicine (C-ETLM), in partnership with the Society for the Study of Inborn Errors of Metabolism (SSIEM) and International Society for Neonatal Screening (ISNS), is conducted to:

1. Understand how laboratories serving the pediatric population have changed their clinical service delivery in response to the **initial wave (January to July 2020)** of the COVID-19 pandemic
2. Evaluate how these changes are affecting clinical care
3. Gather experiences learned from managing the initial wave(s) of the pandemic to improve laboratory preparedness for future outbreaks

This 17-item survey is completely voluntary and should take approximately 15 minutes to complete. The results of this survey will be analyzed in an aggregated manner and published to inform the larger clinical community. No individually identifiable information will be made public. By proceeding with this survey, you have provided an implied consent to the above. Thank you for your participation.

For queries about the survey, please contact Dr. Tim Lang (tim.lang@nhs.net).

General Information

1. Please indicate your country/ region of practice
2. Which of the following best describes your laboratory? Please select all that apply.
    a. Newborn Bloodspot Screening
    b. Inherited Metabolic Diseases Testing
    c. Sweat Testing
    d. Genetic / Molecular Testing
    e. Specialist Pediatric Endocrinology Testing

f. Pediatric Blood Sciences - General Biochemistry / Haematology / Endocrinology / Immunology
g. General adult laboratory serving neonatal/ pediatric population
h. Others, please specify: _____
i. We do not serve pediatric patients (this is an exclusion question)

3. Please indicate the number of patient samples your laboratory processes per day.
    a. <200 samples
    b. 200-500 samples
    c. >500 samples
4. Which of the following pediatric specialities are supported by your laboratory? Please select all that apply.
    a. General Pediatric Medicine
    b. Community Pediatrics
    c. Neonatal Unit
    d. Specialist Children's Hospital serving multiple subspecialties
    e. Maternity
    f. Others, please specify: _____

Delivery of Laboratory Services (to be mirrored for all laboratory areas)

5. What clinical test / clinical laboratory service did your laboratory stop OR restrict during the initial wave of COVID-19 pandemic (Jan to July 2020)? Please specify. Free text
6. How long was the laboratory test / laboratory service suspended for? Months
7. What were the two main reasons for the suspension?
    a. Manpower diversion to other areas
    b. Insufficient manpower (e.g. due to split team arrangement)
    c. Concern over risk of COVID-19 transmission
    d. Disruption of reagent/ consumable delivery due to COVID-19
    e. Others, please specify: _____
8. What are the top 5 tests with a decrease in workload compared to pre-COVID-19 in your laboratory during the initial wave of COVID-19 (Jan to July 2020)? Please indicate the estimated percentage decrease during the worst month.
9. Please indicate if the COVID-19 pandemic affected your laboratory's ability to meet any National Standards for newborn/ pediatric screening programmes available in your country?

    Yes/ No. If yes, please elaborate: _____

10. Please indicate if the COVID-19 pandemic affected your laboratory's ability to meet any Accreditation Standards (e.g. ISO 15189) available in your country?

    Yes/ No. If yes, please elaborate: _____

Impact on Clinical Care or Pathology of Pediatric Diseases During the Initial Wave of COVID-19 (Jan to July 2020)

11. Did your laboratory observe any increase in missed detection of pediatric diseases related to the clinical laboratory tests / services stopped or restricted due to COVID-19? Please specify the condition and related laboratory tests.
12. Did your laboratory observe any increase in later (more severe) presentation of pediatric diseases related to the tests / services stopped, restricted due to COVID-19? Please specify the condition and related laboratory tests.
13. Did your laboratory observe any positive effect of COVID-19 on laboratory services (or those that can be detected via laboratory tests)?
14. Lessons learned from the Initial Wave of COVID-19 (Jan to July 2020)
15. What are the three most important operational decisions your laboratory has made that positively helped the management of the initial wave of COVID-19? Please specify.
16. What are the three operational decisions you wish your laboratory has avoided when managing the initial wave of COVID-19? Please specify.
17. Was there any technology that your laboratory employed that were helpful in managing the COVID-19? Please specify.
18. What are the two operational areas you consider most important for preparing your laboratory for subsequent waves of COVID-19? Please specify.

This is the end of the survey. Thank you very much for your participation.

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