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Humorale Immunantwort in COVID-19 Patienten

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1 Einleitung

1.1 Humorale Immunantwort

Die humorale Immunantwort wird durch die nichtzellulären Bestandteile von Körperflüssigkeiten vermittelt. Sie umfasst Substanzen, die in den Körperflüssigkeiten, lateinisch „*humor*“, zu finden sind, und ist darauf ausgerichtet, Viren und Bakterien im extrazellulären Raum zu bekämpfen. Gemeinsam mit der zellulären Immunantwort bildet die humorale Immunantwort das Immunsystem von höheren Lebewesen [1]. Sie ist Teil der adaptiven Immunantwort, bei der die Erkennung hochspezifischer Oberflächenmerkmale von Fremdkörpern (beispielsweise Membranproteine von Bakterien oder Viren) durch die Lymphozyten erfolgt. Das adaptive Immunsystem wird durch die B- und T-Lymphozyten gebildet, wobei die B-Lymphozyten Antikörper produzieren und somit zur Generierung des humoralen Teils der Immunantwort beitragen und die T-Lymphozyten den zellvermittelten Teil der adaptiven Immunantwort darstellen [2]. B- und T-Lymphozyten entstehen aus einer gemeinsamen lymphoiden Vorläuferzelle im Knochenmark. Die Vorläuferzellen der T-Zellen gelangen von dort aus in den Thymus, wo sie sich weiter zu ausgereiften CD4-positiven T-Helferzellen oder CD8-positiven zytotoxischen T-Zellen differenzieren und die zelluläre Immunität bilden.

Die Vorläuferzellen der B-Lymphozyten entwickeln sich im Knochenmark zu ausgereiften B-Lymphozyten. Werden diese dann durch das Vorhandensein spezifischer Antigene aktiviert, differenzieren sie sich zu Plasmazellen, welche große Mengen von Immunglobulinen, den Antikörpern, synthetisieren und in löslicher Form sezernieren. Antikörper lösen die Aktivierung des Immunsystems aus, um die entsprechenden Fremdkörper zu eliminieren. Sie sind aus vier komplex miteinander verbundenen Proteinketten aufgebaut und besitzen eine Y-förmige Grundstruktur, welche der Erkennung von Antigenen und der Bindung an sie dient [3]. Alle Proteine, die eine solche Grundstruktur enthalten, gehören der "Immunoglobulin-Superfamilie" an [4]. Diese Grundstruktur ist aus zwei leichten und zwei schweren Ketten aufgebaut, welche durch Disulfidbrücken miteinander verbunden sind. Sowohl die leichten als auch die schweren Ketten enthalten variable und konstante Regionen. Jede leichte Kette besteht hierbei aus einer variablen und einer konstanten Domäne, während jede schwere Kette eine variable und drei konstante Domänen besitzt [5]. Die jeweilige leichte Kette und die ersten beiden Domänen der schweren Kette bilden zusammen das antigenbindende Fragment (Fab). Darunter liegt das kristallisierbare Fragment (Fc), welches die C-terminale konstante Domäne der beiden schweren Ketten enthält. Die Fabs sind durch die flexible Hinge-Region mit dem Fc verbunden, die in den verschiedenen Antikörperklassen und Isotypen in Länge und Flexibilität variiert [6] (Abbildung 1).

Die Immunglobuline jeder Spezies lassen sich aufgrund der Struktur ihres Grundgerüstes in verschiedene Klassen unterteilen. Beim Menschen können 5 verschiedene Grundgerüste oder Klassen

unterschieden werden, welche zu unterschiedlichen Zeiten gebildet werden: Immunglobulin G (IgG), Immunglobulin M (IgM), Immunglobulin A (IgA), Immunglobulin D (IgD) und Immunglobulin E (IgE) [7]. Frühe B-Gedächtniszellen treten innerhalb der ersten vier bis fünf Tage einer Immunantwort auf. IgM ist hier das erste Immunglobulin, das während der B-Zell-Entwicklung exprimiert wird und wird deshalb häufig verwendet, um eine akute Exposition gegenüber einem Immunogen oder einem Pathogen zu diagnostizieren. Es liegt als Pentamer (selten als Hexamer) vor, bei dem fünf einzelne IgM-Moleküle durch Disulfidbindungen in der CH4-Region miteinander verknüpft sind. Monomere IgM-Moleküle haben aufgrund ihrer noch nicht erfolgten Reifung eine geringe Affinität. Sie können aber durch die multimerische Interaktionen zwischen dem pentameren Antikörper und dem Antigen eine hohe Avidität erreichen. Sie beschichten hierbei durch Opsonisierung das Antigen und fördern dadurch die Zerstörung des Antigens. Zudem können sie das Komplementsystem aktivieren, was zu einer weiteren Aktivierung des Immunsystems führt und die Immunantwort gegen das Antigen verstärkt [8]. IgG ist mit einem Anteil von 75% die vorherrschende Immunglobulinklasse im Serum und hat die längste Serumhalbwertszeit aller Immunglobulin-Isotypen. Es kann als einziger Ig-Isotyp von der Mutter an den Fetus übertragen werden und sorgt so für eine zeitlich begrenzte Leihimmunität. IgG ist ein achsensymmetrischer Heterotetramer (Abbildung 1) und tritt als Monomer auf. Es kann die Bindung des Antigens an seine Zielstrukturen blockieren und so seine Wirkung neutralisieren. Basierend auf strukturellen, antigenen und funktionellen Unterschieden in der konstanten Region der schweren Kette können vier IgG-Unterklassen (IgG1, IgG2, IgG3 und IgG4) unterschieden werden, die unterschiedliche funktionelle Aktivitäten besitzen [3]. Zirkulierendes IgD ist dagegen im Serum nur in sehr geringen Mengen vorhanden und hat eine kurze Halbwertszeit. Seine Funktion ist noch unklar [9]. Die niedrigste Konzentration aller Immunglobuline im Serum weist IgE auf. Es hat nur eine Halbwertszeit von 2 Tagen und ist mit Überempfindlichkeits- und allergischen Reaktionen sowie der Reaktion auf parasitäre Infektionen verbunden [10]. Humanes IgA besteht aus zwei Untergruppen, IgA1 und IgA2. Monomeres IgA1 dominiert im Serum, während dimerisches und polymeres IgA1 und IgA2 – verbunden durch eine J-Kette – auf der Schleimhautoberfläche vorhanden ist, wo es eine wichtige Rolle zum Schutz vor Toxinen, Viren und Bakterien durch Neutralisierung oder Verhinderung der Anhaftung an das Schleimhautepithel spielt [11].

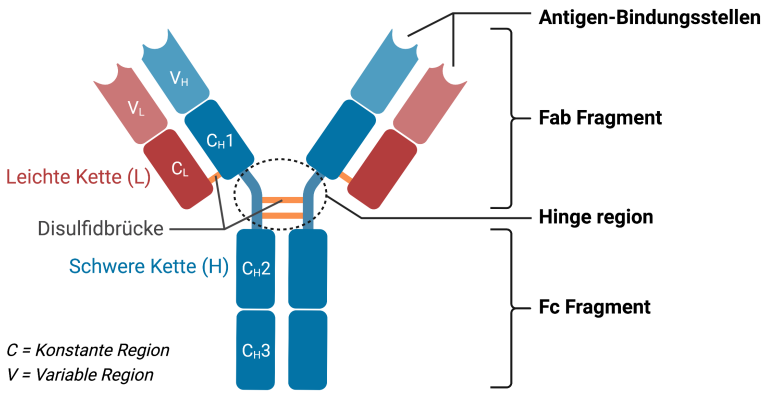

Abbildung 1: Aufbau eines Antikörpers am Beispiel von Immunglobulin G (erstellt mit BioRender.com)

Die initiale Synthese der Antigenbindungsstellen der Antikörper erfolgt in B-Zellen durch die Produktion von IgM und IgD wobei stets IgM als der erste Antikörper während einer Immunantwort gebildet wird [7]. In einer späteren Phase der Immunantwort kann ein Wechsel des Isotyps von IgM zu IgG, IgA oder IgE stattfinden, die effektiver und spezifischer als IgM sind. Dieser Prozess wird als Klassenwechsel-Rekombination, oder Isotypenwechsel von Immunoglobulinen, bezeichnet – ein genetischer Vorgang, bei dem B-Lymphozyten ihre konstante Region der schweren Kette des Immunoglobulins ändern. [12].

Die humorale Immunantwort wird durch den Kontakt von B- oder T-Lymphozyten mit spezifischen Antigenen ausgelöst. Die Aktivierung erfolgt, wenn Makrophagen, dendritische Zellen sowie B-Lymphozyten als antigenpräsentierende Zellen (APCs) Pathogene phagozytieren und prozessieren. Ein Haupthistokompatibilitätskomplex-Protein der Klasse II (MHC-II-Protein) wird mit dem prozessierten Fremdantigen beladen und den CD4+ T-Helferzellen präsentiert, welche es mittels ihres T-Zellrezeptors (TCR) erkennen. Das Cytokin Interleukin-1 (IL-1) sorgt hierbei für die Aktivierung der T-Helferzellen, welche daraufhin Interleukin-2 (IL-2) ausschütten [13]. IL-2 sorgt hier für eine klonale Expansion, indem es die Vermehrung von T-Helferzellen fördert. Diese werden aktiviert, wodurch sie wiederum IL-2 freisetzen. Dies führt zu einer erhöhten Anzahl von T-Helferzellen, die eine Immunantwort gegen das erkannte Antigen koordinieren und verstärken können. Gleichzeitig sorgt IL-2 auch für die Differenzierung von B-Zellen zu Plasmazellen und regt somit die Antikörperproduktion an [14]. Die produzierten Antikörper durchlaufen einen Reifungs- und Selektionsprozess, der ihre Bindungsstärke an das Zielantigen erhöht. Mutationen in den Genen, die für die variablen antigenbindenden Komponenten des Antikörpermoleküls kodieren, führen hierbei zu einer Vielzahl von B-Zell-Rezeptor-Varianten, von denen die meisten die Bindungsfähigkeit beeinträchtigen. Einige Mutationen verbessern jedoch die Bindungsaffinität und sorgen für spezifischere B-Zellen. Durch wiederholtes Selektieren entsteht eine hohe Affinität der hergestellten Antikörper. Diese somatische Hypermutation bewirkt, dass im Laufe einer Immunantwort die gegen ein bestimmtes Antigen gebildeten Antikörper durch die Affinitätsreifung mit zunehmend höherer Bindungsaffinität an das Antigen binden können [4, 15]. B-Lymphozyten entwickeln sich entweder zu B-Plasmazellen oder zu B-Gedächtniszellen. Die B-Gedächtniszellen sind langfristig im Körper vorhanden und ermöglichen bei erneutem Kontakt mit dem Antigen eine schnellere und effektivere Immunantwort. Es entstehen also zwei Arten von B-Zellen: die zirkulierenden Gedächtnis-B-Zellen, die im Körper zirkulieren und schnell aktiviert werden können, sowie die Plasmazellen mit hoher Affinität, die Antikörper produzieren. Diese Plasmazellen befinden sich in spezialisierten Bereichen der Milz und des Knochenmarks, wo sie jahrelang oder sogar jahrzehntelang überleben und kontinuierlich Antikörper absondern, unabhängig davon, ob das ursprüngliche Antigen noch vorhanden ist. Dieser Prozess trägt wesentlich zur langfristigen Immunität gegenüber dem betreffenden Antigen bei [16].

Die gebildeten Antikörper zerstören extrazelluläre Mikroorganismen und deren Produkte und verhindern mittels Neutralisierung, Opsonisierung und Komplementaktivierung, dass sich intrazelluläre Infektionen ausbreiten können [2]. Sie binden an die Oberflächenstruktur der Pathogene und verhindern so die Adsorption dieser an bestimmte Zelltypen durch die Neutralisation der Pathogene und verhindern somit die Infektion selbst [17]. Durch die Opsonisierung der Krankheitserreger bewirken sie deren Aufnahme durch Phagocytose, indem Fc-Rezeptoren an die konstanten Regionen der Antikörper binden. Dies führt zu einer chemotaktischen Anziehung für die Phagozyten und schließlich zur Phagozytose des Erregers [18]. Zusätzlich können an Pathogene gebundene Antikörper die Proteine des klassischen Signalwegs des Komplementsystems aktivieren und die Komplementkaskade durch die Bindung der ersten Komponente, des Komplementfaktors C1, an den Fc-Anteil des Antikörpers auslösen. Dies verstärkt die Opsonisierung, indem weitere Komplementproteine an die Oberfläche des Pathogens binden. Diese Aktivierung kann nur erfolgen, wenn C1 an IgM oder IgG bindet, wobei jedes C1-Molekül mindestens 2 benachbarte Fc-Anteile binden muss. Somit kann ein einzelnes IgM-Pentamer den klassischen Signalweg auslösen, wogegen die monomeren IgG-Moleküle zur Aktivierung in aggregierter Form vorliegen müssen. IgM ist in Hinsicht auf die Komplementbindung somit der wirksamere Antikörper [19].

1.2 SARS-CoV-2

Das Severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2) ist ein über die Atemwege übertragbarer Erreger, welcher für die Coronavirus disease 2019 (COVID-19) verantwortlich ist [20]. Es trat das erste Mal 2019 in der chinesischen Provinz Hubei auf und wurde von der WHO am 11. März 2020 zur Pandemie erklärt [21]. Bis zum Dezember 2023 gab es über 770 Millionen bestätigte Fälle mit über 7 Millionen Toten [22]. SARS-CoV-2 führte damit zur ersten Pandemie in der Geschichte, die durch ein Coronavirus verursacht wurde [23]. Es gehört der Familie der Beta-Coronaviren an, zu welcher auch das Severe acute respiratory syndrome coronavirus (SARS-CoV), das Middle East respiratory syndrome coronavirus (MERS-CoV) sowie die als „Erkältungsviren“ zirkulierenden saisonalen humanen Coronaviren (HCoV) HKU1, NL63, 229E und OC43 gehören. Letztere sind unter Säugetieren und Vögeln weit verbreitet sind und können beim Menschen vorwiegend milde Erkältungskrankheiten, aber auch schwere Lungenentzündungen hervorrufen können [24, 25]. Seroprävalenzstudien deuten darauf hin, dass über 90 % der Erwachsenen im Alter von 50 Jahren und älter Antikörper gegen alle vier häufig zirkulierenden HCoVs besitzen [25].

SARS-CoV-2 besitzt vier Hauptstrukturproteine, das Spikeprotein (S), das Membranprotein (M), das Hüllprotein (E) und das Nukleokapsidprotein (N), wobei S, E und M auf der Oberfläche der Virionmembran vorkommen und das N-Protein an der Bindung und Verpackung des RNA-Genoms beteiligt ist [26]. Das Nukleokapsidprotein von SARS-CoV-2 ist das Antigen mit der stärksten

Immundominanz unter den Coronaviridae und das vorherrschende Antigen, welches in Coronavirus-infizierten Zellen produziert wird [27, 28]. Die Infektion des Menschen mit SARS-CoV-2 erfolgt hauptsächlich durch die Interaktion des viralen S-Proteins mit den Rezeptoren des Angiotensin-konvertierenden Enzyms 2 (ACE2) auf der Oberfläche der Wirtszelle. Das S-Protein besteht aus den zwei Untereinheiten S1 und S2. Die S1-Untereinheit trägt hierbei die rezeptorbindende Domäne (RBD), welche für die Bindung an den Wirtszellrezeptor ACE2 verantwortlich ist, die S2 Untereinheit ist an der Fusion der Virusmembran mit der Wirtszellmembran beteiligt [29]. Das S-Protein wird nach der Bindung an ACE2 erst zwischen den Domänen S1 und S2 durch die wirtszelluläre Protease Furin gespalten. Diese einzigartige Furin-ähnliche Spaltstelle im Spike-Protein von SARS-CoV-2 scheint zur Übertragbarkeit und Infektiosität beizutragen [23]. Die Spaltung führt zu einer Konformationsänderung der S2-Untereinheit und dadurch zur Freilegung einer weiteren Spaltstelle, welche dann im weiteren Verlauf von der Transmembran-Serin-Protease vom Typ II (TMPRSS2) gespalten wird [30]. Dies aktiviert die S2-Untereinheit durch die Freilegung hydrophober Aminosäurereste, welche sich in die Wirtszellmembran einbetten und die Fusion von Virushülle und Wirtszellmembran erleichtern [31] (Abbildung 2).

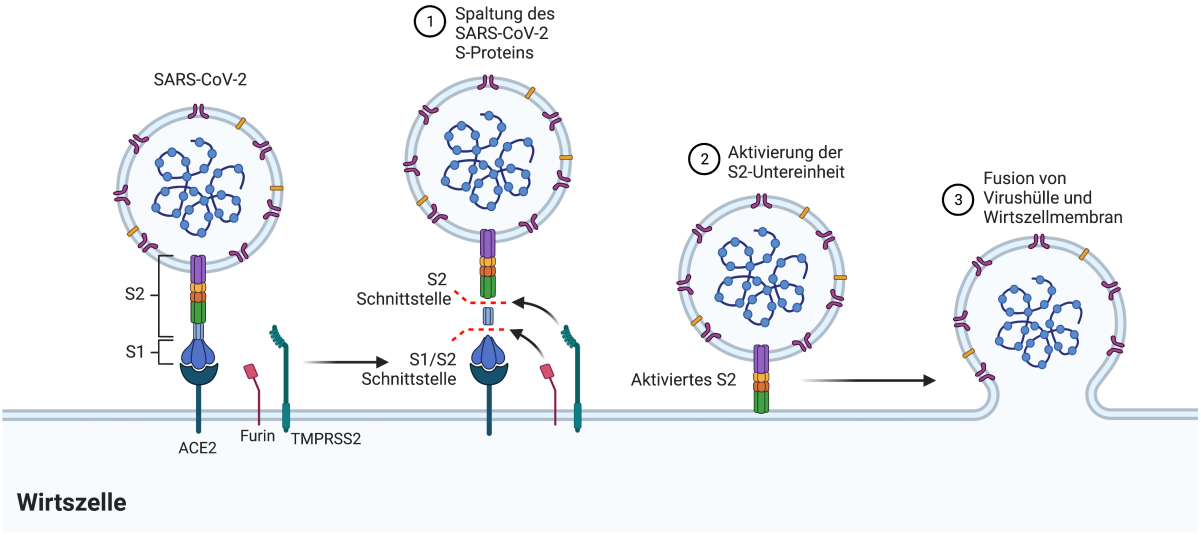

Abbildung 2: ACE2 vermittelter Eintritt von SARS-CoV-2 in die Wirtszelle (erstellt mit BioRender.com)

Die am häufigsten verwendete Methode zum direkten Nachweis von SARS-CoV-2 ist die Reverse Transkriptase-Polymerase-Kettenreaktion (RT-PCR) [32]. Im Gegensatz dazu ist der Nachweis einer Immunreaktion auf das Virus auf serologischer Basis ein indirekter Marker für die Infektion und kann als Ergänzung zur PCR eingesetzt werden, um die Identifizierung von SARS-CoV-2 bei Patienten zu unterstützen, die im Verdacht stehen, mit COVID-19 infiziert zu sein [33], da der Infektionsnachweis mittels PCR nur in einem begrenzten Zeitfenster möglich ist (Abbildung 3).

Serologische SARS-CoV-2-Tests, insbesondere IgG-basierte Tests können dazu für das Screening von genesenen COVID-19-Patienten, für die Therapie mit Rekonvaleszenzplasma, für SARS-CoV-2-Seroprävalenzstudien und für die Überwachung von Immunreaktionen auf COVID-19-Impfstoffkandidaten nützlich sein [34]. Auch sekretorisches IgA spielt eine wichtige Rolle beim Schutz

der Schleimhaut vor Krankheitserregern, indem es Atemwegsviren, einschließlich SARS-CoV-2, neutralisiert [35].

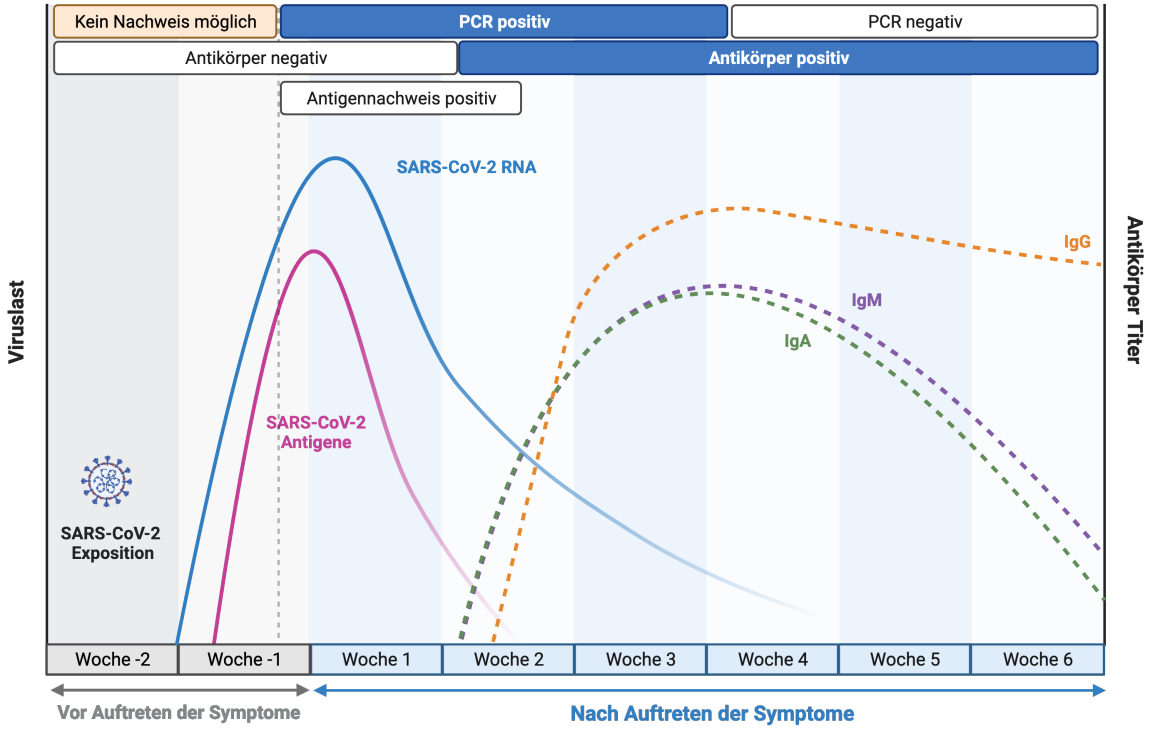

Abbildung 3: Direkter Nachweis und indirekter Nachweis von SARS-CoV-2 (erstellt mit BioRender.com)

2 Fragestellung

Ziel der vorliegenden kumulativen Dissertation ist es, die humorale Immunantwort gegen SARS-CoV-2 zu untersuchen und darzustellen. Untersucht wurden die Titer von IgG, IgM und IgA, sowie die Kapazität zur Virusneutralisation von Patientenseren im Laufe der Infektion (Serokonversion) und die Persistenz über die Zeit. Es wurden dazu Kollektive (Panels) von erstmalig mit SARS-CoV-2-infizierten Patienten untersucht, die stationär oder ambulant behandelt wurden. Serumproben wurden über den Zeitraum von der Infektion bis zur Rekonvaleszenzphase gesammelt und charakterisiert. Zusätzlich wurden Proben gesunder Blutspender, die vor dem Ausbruch der Pandemie gewonnen worden waren, Proben von gesunden Personen, die nicht aus Europa stammten, sowie Proben von mit anderen humanpathogenen Coronaviren (nicht SARS-CoV-2) infizierten Patienten untersucht. Dies diente dazu, potentielle serologische Kreuzreaktionen zu charakterisieren sowie die Sensitivität und Spezifität der verwendeten Testsysteme zu bewerten.

Zum Zeitpunkt der Veröffentlichung der Studien gab es noch keine oder nur wenige Erkenntnisse über die jeweilig veröffentlichten Inhalte. Die Ergebnisse sollten dazu dienen, die humorale Immunantwort zu beschreiben, besser zu verstehen und geeignete serologische Testsysteme aufzuzeigen, die in der Lage sind, diese darzustellen.

Originalarbeit 3.1:

Wie stellt sich die humorale Immunantwort bei einer SARS-COV-2 Infektion mit Augenmerk auf der Kinetik der verschiedenen Immunglobulinklassen sowie der Antigenspezifität dar?

Originalarbeit 3.2:

Sind Reinfektionen nach einer bereits durchgemachten SARS-CoV-2 Infektion möglich? Können reinfizierte Patienten infektiöses Virus ausscheiden, andere Personen anstecken und somit zur Infektionsdynamik beitragen?

Originalarbeit 3.3:

Wie verhalten sich Proben von Personen nichteuropäischer geografischer Herkunft, insbesondere solche aus dem afrikanischen Raum, in kommerziell erhältlichen ELISA Testen und erzeugen diese Probleme bei Ermittlung der humoralen Immunantwort?

Originalarbeit 3.4:

Wie nimmt die Antikörperantwort im Verlauf der Zeit ab? Können die verschiedenen Testsysteme dies darstellen? Wie verändert eine Impfung die Antwort? Kann man zwischen natürlicher Infektion und impfbasierter Antikörperreaktion unterscheiden? Entwicklung eines serologischen ELISA Tests auf Nukloekapsidprotein (NCP)-Basis zur Darstellung der Immunantwort für IgG- und IgM-Antikörper.

Originalarbeit 3.5:

Wie stellt sich die Kinetik der neutralisierenden Antikörperantwort dar? Ist die Darstellung mittels Surrogat-Testsystemen möglich und wie ist die Leistungsfähigkeit dieser Tests? Sind sie eine Alternative zu zellbasierten Assays und können sie die Entscheidungsfindung für das diagnostische Management von COVID-19 Patienten unterstützen?

3 Originalarbeiten

3.1 Emmerich P, von Possel R, Hemmer CJ, et al. Longitudinal detection of SARS-CoV-2 specific antibody responses with different serological methods. J Med Virol. 2021; 93(10):5816-5824. doi:10.1002/jmv.27113

3.1.1 Fragestellung

Die Diagnose einer SARS-COV-2 Infektion erfolgt durch den direkten Erregernachweis mittels RT-PCR. Zur Bestätigung akuter oder zurückliegender Infektionen werden serologische Tests zum Nachweis von Antikörpern gegen SARS-COV-2 genutzt. Eine Serokonversion kann zu verschiedenen Zeitpunkten auftreten und sich gegen verschiedene Strukturproteine von SARS-CoV-2 richten. Die humorale Immunantwort variiert erheblich zwischen verschiedenen Personen und in ihrer Kinetik gegenüber Immunglobulinklassen und Antigenspezifität.
Ziel dieser Arbeit war es, die Kinetik die Kinetik der humoralen Immunreaktion gegen SARS-CoV-2 darzustellen und Anhaltspunkte zu liefern, wann serologische Tests wirksam für das Screening oder die Überwachung der Infektion eingesetzt werden können.

3.1.2 Material und Methoden

Es wurden vier Serum Panels zur Darstellung der humoralen Immunantwort verwendet.
Panel A umfasste 82 sequenzielle und einzelne Serumproben nach Ende der Quarantäne von 25 deutschen Patienten mit PCR-bestätigter SARS-CoV-2 Infektion, welche leichte bis mittelschwere COVID-19-Symptome aufwiesen. Panel B umfasste 47 akute, sequenzielle und einzelne Serumproben von 17 PCR-bestätigten deutschen COVID-19 Patienten, welche eine schwere Erkrankung hatten und stationär behandelt werden mussten. Panel C umfasste Serumproben, die vor August 2019 von 42 gesunden deutschen Blutspendern genommen worden waren, und Panel D umfasste Proben, welche in der indirekter Immunfluoreszenz (IIF) positiv für IgG gegen saisonale Coronaviren waren.

Die Proben wurden auf das Vorhandendsein von IgA-, IgG- und IgM-Antikörpern gegen SARS-CoV-2 mittels eines indirekten Immunfluoreszenztests (IIFT) vorcharakterisiert und anschließend mit fünf verschiedenen Enzyme-linked Immunosorbent Assays (ELISA) (Euroimmun: Anti-SARS-CoV-2 ELISA (IgG); Anti-SARS-CoV-2 ELISA (IgA); Anti-SARS-CoV-2 QuantiVac ELISA (IgG); Anti-SARS-CoV-2 NCP ELISA (IgG) und Anti-SARS-CoV-2 NCP ELISA (IgM)) auf Antikörper gegen SARS-CoV-2 untersucht. Alle ELISAs verwenden rekombinante, virale Antigene, die auf der S1-Domäne des Spike-Proteins einschließlich der immunologisch relevanten RBD oder auf einem modifizierten NCP als Antigen basieren. Der Nachweis von SARS-CoV-2-spezifischen Antikörperreaktionen wurde auch im Hinblick auf die Infektionsphase untersucht. Die Proben wurden dazu in 11-20 Tage nach Symptombeginn (days post syndrom onset -

dpso) = frühe Phase der Infektion, 21-60 dpso = mittlere Phase der Infektion und >60 dpso = abgelaufene Infektion unterteilt.

3.1.3 Ergebnisse

Die Sensitivitäten zwischen den Assays variierten stark. Die Sensitivitäten der IgA- und IgM-Tests war in der frühen Phase der Infektion am höchsten, während positive Ergebnisse für IgG-Antikörper am häufigsten in der mittleren Phase auftraten. Die Titer der Anti-SARS-CoV-2-IgG-Antikörper gegen S1 erreichten in der intermediären Phase der Infektion ihr Maximum. Im Gegensatz hatten die Titer der Anti-SARS-CoV-2-IgA-Antikörper, die mit dem IIFT gemessen wurden, ihren Höhepunkt in der frühen Phase der Infektion, gefolgt von einem deutlichen Rückgang nach 60 dpso. Im Verlauf der Infektion sank die Anzahl der positiven Ergebnisse für Anti-SARS-CoV-2-IgM-Antikörper, die sowohl mit ELISA als auch mit dem IIFT gemessen wurden. Vergleicht man die Anzahl der positiven IgG-Testergebnisse des IIFT mit denen des S1- und NCP-basierten ELISAs, zeigt sich in der frühen Phase der Erkrankung eine höhere Sensitivität des NCP ELISAs, welche im weiteren Krankheitsverlauf im Vergleich zum S1-basierten ELISA jedoch wieder abnimmt.

3.1.4 Schlussfolgerungen

Ziel dieser Publikation war die Untersuchung der Langzeitkinetik der Antikörper gegen SARS-CoV-2 sowie die Bewertung charakteristischer Merkmale verschiedener serologischer Methoden.
Die Sensitivitäten der einzelnen Tests variierten je nach Test und Infektionsphase. Die in den verschiedenen Tests verwendeten Substrate dienen allerdings auch unterschiedlichen Zielsetzungen.
Der IIFT besitzt eine höhere Sensitivität für IgG-Antikörper als jeder der antigenspezifischen ELISAs. Die heterogenen IgG-Reaktionen gegen S1 und NCP spiegeln sich in den vorliegenden Panels in einer geringeren Empfindlichkeit der S1-spezifischen ELISAs gegenüber den NCP-ELISAs in der frühen Phase der Infektion wider, da nicht alle infizierten Personen Antikörper gegen die S1-Domäne von SARS-CoV-2 bilden. Die Verwendung von mit dem gesamten SARS-CoV-2 infizierten Zellen als Substrat in der IIFT bewirkt eine hohe Sensitivität, da das gesamte Antigenspektrum vorhanden ist. Rekombinante Antigensubstrate, die in der ELISA-Technik verwendet werden, sind für den Nachweis und die genaue Identifizierung von Antikörpern gegen ausgewählte Proteine von SARS-CoV-2, wie S1/RBD und NCP, geeignet und können damit einen Einblick in die Prävalenz spezifischer Antikörper gegen ausgewählte Antigene von SARS-CoV-2 geben. Aufgrund der Heterogenität der individuellen Antikörperreaktionen liefert ein ELISA möglicherweise nicht für alle Patienten positive Ergebnisse, eine Kombination von ELISAs mit verschiedenen Antigenen kann diese diagnostische Lücke jedoch verringern.

3.2 Brehm TT, Pfefferle S, von Possel R, et al. SARS-CoV-2 Reinfection in a Healthcare Worker Despite the Presence of Detectable Neutralizing Antibodies. Viruses. 2021;13(4):661. Published 2021 Apr 12. doi:10.3390/v13040661

3.2.1 Fragestellung

Sind Reinfektionen nach einer Infektion mit SARS-CoV-2 möglich und können reinfizierte Personen infektiöses Virus ausscheiden und somit am Infektionsgeschehen teilnehmen?

3.2.2 Material und Methoden

SARS-CoV-2 RNA wurde unter Verwendung eines kommerziellen, quantitativen Referenzstandards mittels PCR bestimmt. Es wurde eine Virusisolierung durchgeführt und aus dem Rachenabstrich eine Vollgenomanalyse durchgeführt. Zur Darstellung der humoralen Immunantwort wurde ein automatisierter Anti-SARS-CoV-2-IgG Test, welcher auf die S1/S2-Spike Domäne abzielt, und ein IIFT für IgA-, IgG- und IgM-Antikörper verwendet. Neutralisierende Antikörper wurden mittels einem für diese Arbeit etablierten *in vitro* Virusneutralisationstest (VNT) bestimmt.

3.2.3 Ergebnisse

Eine 27-jährigen Krankenschwester, welche auf einer COVID-19-Station arbeitete und sich im März 2020 mit SARS-CoV-2 infizierte, wurde im Dezember 2020 bei einer routinemäßigen PCR-Überwachung erneut positiv auf SARS-CoV-2 getestet. Nach der Erstinfektion entwickelte sie moderate IgG-, niedrige IgM-und keine messbaren IgA-Antikörpertiter. Nach der Reinfektion konnte ein deutlicher Anstieg der IgG-Titer beobachtet werden. IgA- und IgM-Antikörper konnten nicht nachgewiesen werden. Der Titer der neutralisierenden Antikörper war nach der Reinfektion wesentlich höher als nach der Erstinfektion. Die Vollgenomanalyse ergab, dass die Reinfektion mit einem anderen Virusstamm als die Erstinfektion erfolgte, welcher jedoch im Neutralisationstest keine Immunevasion zeigte.

3.2.4 Schlussfolgerungen

Reinfektionen mit HCoVs wurden zuvor berichtet und verliefen in der Regel milder als die Erstinfektion. Bei SARS-CoV-2-Reinfektionen wurden asymptomatische, weniger schwere oder auch schwerere Fälle im Vergleich zur Erstinfektion beobachtet, eine postinfektiöse Immunität scheint aber vor schweren Erkrankungen zu schützen. Die Mechanismen für Reinfektionen sind noch unklar, aber Faktoren wie hohe Viruslast, virale Variabilität und Immunantwort könnten eine Rolle spielen. Beschrieben wird hier den Fall von einer Person, welche sich 288 Tage nach Erstinfektion mit einer Virusvariante reinfizierte, die sich von der Variante der Erstinfektion unterschied. Die Viruslast bei der Reinfektion war gegenüber der Erstinfektion nicht verringert, das Virus wurde jedoch schneller eliminiert. Virusneutralisationstests mit der bei der Reinfektion isolierten Virusvariante zeigten im Vergleich zu der Variante der Erstinfektion keine veränderte Neutralisationsfähigkeit.

3.3 Emmerich P, Murawski C, Ehmen C, von Possel R, et al. Limited specificity of commercially available SARS-CoV-2 IgG ELISAs in serum samples of African origin. Trop Med Int Health. 2021;26(6):621-631. doi:10.1111/tmi.13569

3.3.1 Fragestellung

Sind kommerziell erhältliche, zertifizierte serologische SARS-CoV-2-IgG-ELISAs in Serum-/Plasmaproben aus Afrika, Südamerika und Europa spezifisch genug?

3.3.2 Material und Methoden

Es wurden IgG-Antikörper in 882 Serum-/Plasmaproben von symptomfreien Spendern, die vor der COVID-19-Pandemie in drei afrikanischen Ländern (Ghana, Madagaskar, Nigeria), Kolumbien und Deutschland gesammelt wurden, mit drei NCP-basierten und einem Spike/S1-basierten, CE-IVD zertifizierten, ELISAs bestimmt und mit dem IIFT als Goldstandard verglichen. Zusätzlich wurden alle Proben mittels einem In-house ELISA auf das Vorkommen von saisonalen HCoVs untersucht.

3.3.3 Ergebnisse

Für alle SARS-CoV-2-IgG-ELISAs wurde eine hohe Spezifität für Proben aus Madagaskar, Kolumbien und Deutschland bestätigt. Im Gegensatz dazu war die Spezifität für die ghanaischen und nigerianischen Serumproben deutlich niedriger, wobei die Spezifität im Spike/S1-basierten ELISA höher als in den verwendeten NCP-basierten war. IgG-Antikörper, die durch vorherige Infektionen mit saisonalen HCoVs gebildet wurden, konnten in allen Proben nachgewiesen werden, beeinflussten die Assayspezifität jedoch nicht zwangsläufig.

3.3.4 Schlussfolgerungen

Abhängig vom gewählten Testantigen und Assay-Protokoll kann die Spezifität von SARS-CoV-2-IgG-ELISAs in bestimmten Populationen signifikant reduziert sein. Gründe hierfür könnten eventuell Immunantworten gegen andere, endemische Viren oder Parasiten sein. Die Ergebnisse SARS-CoV-2-Seroprävalenzstudien (nicht nur) in Afrika sollten kritisch betrachtet werden. Falls erforderlich, sollte der Assay-Cutoff, unter Verwendung von vor der Pandemie erhaltenen SARS-CoV-2 IgG-negativen Serumproben, angepasst werden. Ergebnisse sollten sorgfältig interpretiert und wenn möglich, durch die Kombination von zwei unabhängigen serologischen Tests unter Verwendung verschiedener Antigene bestätigt werden.

3.4 Deschermeier C, Ehmen C, von Possel R, et al. Fcγ-Receptor-Based Enzyme-Linked Immunosorbent Assays for Sensitive, Specific, and Persistent Detection of Anti-SARS-CoV-2 Nucleocapsid Protein IgG Antibodies in Human Sera. J Clin Microbiol. 2022; e0007522. doi:10.1128/jcm.00075-22

3.4.1 Fragestellung

Für die Überwachung der SARS-CoV-2 Pandemie, insbesondere in Regionen der Welt, in denen der Zugang zu medizinischer Versorgung und molekularen Testkapazitäten begrenzt ist, sind zuverlässige serologische Tests unerlässlich. Benötigt werden SARS-CoV-2-IgG-Assays mit hoher Sensitivität, die auch dann noch ansprechen, wenn die Antikörper-Titer mehrere Monate nach der Infektion bereits wieder abgenommen haben. NCP-basierte Testsysteme können bei Personen, die mit Impfstoffen auf Spike-Basis geimpft worden sind, zwischen einer natürlichen SARS-CoV-2-Infektion und einer Impfantwort unterschieden. Dabei sind hochsensitive Tests erforderlich, um eine reduzierte humorale Anti-NCP-Antwort nachzuweisen, welche bei Impfstoff-Durchbruchsinfektionen beobachtet wurde.

3.4.2 Material und Methoden

Zur Herstellung von rekombinanten SARS-CoV-2-Antigenen wurden prokaryotische Expressionsvektoren in E. coli transformiert, die Bakterien lysiert, die Fusionsproteine aus der löslichen Fraktion gereinigt und die erhaltenen rekombinanten Antigene biotinyliert. Die Validierung des ELISAs erfolgte mit einem Serumpanel, welches 213 Longitudinalproben von 35 COVID-19-Patienten, einem Negativ-Kontrollpanel, das aus 790 Prä-COVID-19-Proben aus verschiedenen Regionen der Welt (Deutschland, Ghana, Madagaskar, Nigeria, Kolumbien, Demokratische Volksrepublik Laos), und das internationale Standardplasma 20/136 der WHO umfasste. IgG-Antikörper gegen SARS-CoV-2 wurden mit dem IIFT nachgewiesen. Es wurden zwei SARS-CoV-2-IgG-ELISAs mit unterschiedlichen Serum- und Antigenverdünnungen validiert und mit einem kommerziellen NCP ELISA verglichen. Zusätzlich wurden alle Seren auf Antikörper gegen humanpathogen Nicht-SARS-Coronaviren (HCoV-OC43, HCoV-HKU1, HCoV-NL63, HCoV-229E) mit entsprechend angepassten In-house IgG-ELISAs untersucht.

3.4.3 Ergebnisse

Ein verkürztes N-terminales SARS-CoV-2 NCP wurde rekombinant in E. coli exprimiert, aus der löslichen Fraktion des bakteriellen Lysats gereinigt und als Testantigen verwendet. Es wurden zwei ELISA-Protokolle auf Grundlage der im Bernhard-Nocht-Institut für Tropenmedizin (BNITM) patentierten FcγR basierten Plattformtechnologie entwickelt und mit unterschiedlichen Serum- und Antigenverdünnungen validiert. Beide ELISAs lieferten sehr gut reproduzierbare Ergebnisse. Die

diagnostische Sensitivität von ELISA 1 nahm bei rekonvaleszenten Patienten im Laufe der Zeit schnell ab. Der ELISA 2 ermöglichte den Nachweis von Anti-SARS-CoV-2-NCP-IgG-Antikörpern über einen wesentlich längeren Zeitraum, zeigte aber eine deutlich niedrigere Nachweisgrenze und wies bei hohen Antikörperkonzentrationen einen starken Prozone-Effekt (falsch niedrige oder falsch negative Messergebnisse bei extrem hohen Antikörpermengen in der Probe) auf. Der IIFT als hochsensitiver Goldstandard-Antikörperbindungstest ergab die höchsten IgG-Antikörpertiter zwischen einem und zwei Monaten nach Auftreten der Symptome, die mittleren Titer fielen in der Rekonvaleszenzphase stark ab, blieben aber bei den meisten Patienten mindestens zwölf Monate lang nachweisbar. Impfstoffe auf Spike-Basis erhöhten die SARS-CoV-2-IgG-IIFT-Titer stark. Während Proben mit einem hohen IgG-IIFT-Titer von beiden ELISAs als positiv erkannt wurden, war die analytische Sensitivität für ELISA 2 bei Proben mit einem niedrigeren Titer deutlich höher. Jedoch wurde bei Proben mit einem sehr hohen IgG-IIFT-Titer ein Prozone-Effekt für den ELISA 2 beobachtet. Der Vergleich zwischen ELISA 1 und einem kommerziell erhältlichen, NCP-basierten indirekten ELISA ergab eine gute Übereinstimmung des kommerziellen ELISAs mit dem ELISA 1.

3.4.4 Schlussfolgerungen

Es wurden zwei ELISA-Tests zur Messung von IgG-Antikörpern gegen SARS-CoV-2 entwickelt und validiert. Als Antigen wurde ein Fragment des NCP von SARS-CoV-2 verwendet. Ähnliche Fragmente des NPC von SARS-CoV-1 haben bereits gezeigt, dass sie den sensitiven Nachweis von Anti-SARS-CoV-1-IgG-Antikörpern in Patientenseren ermöglichen und dabei weniger falsch-positive Reaktionen hervorrufen als das Protein in voller Länge. ELISA 1 wies eine ähnliche diagnostische und analytische Sensitivität auf wie ein handelsüblicher ELISA, der bei COVID-19-Patienten eine deutliche Abnahme der Signalintensität ab etwa 6 Monate nach der Infektion zeigte. Im Gegensatz dazu ermöglichte ELISA 2 den Nachweis von Anti-NCP-IgG-Antikörpern für mehr als 12 Monate nach der Infektion. Wie vor kurzem gezeigt wurde, zeigen mehrere handelsübliche indirekte SARS-CoV-2-NCP-IgG-ELISAs in afrikanischen Probenpanels, die vor der COVID-19-Pandemie gesammelt wurden, inakzeptabel hohe falsch-positive Raten, obwohl dieselben Assays in europäischen Proben eine ausgezeichnete Spezifität aufweisen. Daher wurde die Spezifität der neu entwickelten ELISA-Tests mit einem umfangreichen Panel von 790 Negativ-Kontrollproben vor COVID-19 aus Europa, Südamerika, Asien und Afrika überprüft und erwies sich als hochspezifisch. Das Vorhandensein von IgG-Antikörpern, die durch frühere Infektionen mit HCoVs ausgelöst wurden, führte zu keinerlei Kreuzreaktionen.

Da das SARS-CoV-2-NCP als Testantigen verwendet wurde, werden Immunreaktionen, die durch Impfstoffe auf Spike-Basis (z. B. BioNTech/Pfizer und AstraZeneca) ausgelöst werden, mit diesen Tests nicht erfasst. Dadurch wird nicht nur bei ungeimpften Personen, sondern auch bei mit Spike-basierten Vakzinen geimpften Personen der serologische Nachweis einer natürlichen SARS-CoV-2-Infektion möglich.

3.5 von Possel R, Menge B, Deschermeier C, et al. Performance Analysis of Serodiagnostic Tests to Characterize the Incline and Decline of the Individual Humoral Immune Response in COVID-19 Patients: Impact on Diagnostic Management. Viruses 2024, 16, 91. https://doi.org/10.3390/v16010091

3.5.1 Fragestellung

Für die Frage nach der Impfantwort und der Gabe einer Boosterimpfung ist ein Verständnis der neutralisierenden Antikörperantwort unerlässlich. Ziel der Arbeit war die Darstellung der Kinetik der neutralisierenden Antikörper in humanen Proben im VNT im Vergleich zu zwei kommerziell erhältlichen Surrogat Virus-Neutralisationstests (sVNT) auf ELISA Basis. Da der VNT sehr zeit- und arbeitsintensiv ist und ein Labor der Biosicherheitsstufe 3 (BSL-3) erfordert, sind Tests, welche selbst bei großen Probenmengen auch in einem S1-Bereich durchgeführt werden können, die bessere Wahl.

3.5.2 Material und Methoden

Der Nachweis von SARS-CoV-2-spezifischem IgG erfolgte mittels IIFT und einem kommerziell erhältlichen Lineblot (EUROIMMUN, EUROLINE Anti-SARS-CoV-2-Profil (IgG)). Neutralisierende Antikörper wurden mittels einem VNT bestimmt. Es wurden vier verschiedenen Serumpanels mit dem VNT vorcharakterisiert und mit zwei kommerziellen sVNTs (cPass und NeutraLISA) verglichen. Panel A umfasste 268 Longitudinalproben von 115, teils hospitalisierten, COVID-19 Patienten mit leichten bis schweren Symptomen über einen Zeitraum von insgesamt 0-154 Tagen nach Symptombeginn. Das Negativkontrollpanel B enthielt Seren aus der Zeit vor der Pandemie von 95 Blutspendern, die vor August 2019 gewonnen worden waren. Panel C umfasste 237 Longitudinalproben von 36 leichtkranken COVID-19-Patienten mit natürlicher, PCR-bestätigter, SARS-CoV-2-Infektion sowie nachfolgender Impfung bei 21 dieser Patienten in einem Zeitraum von bis zu 474 Tagen nach der Infektion. Panel D enthielt Seren von Patienten mit saisonalen HCoV- (n = 12) und Rhinovirus-Infektionen (n = 1), welche zwischen Januar 2020 und April 2020 gewonnen und per IIFT bestätigt worden waren.

3.5.3 Ergebnisse

In der akuten Krankheitsphase der Patienten des Panels A konnte mit dem IIFT ein Anstieg des Anteils der IgG-Seropositivität um 21,0 %, und mit dem Lineblot um 8,1 % im ersten Zeitintervall, und um bis zu 55,6 % bzw. 45,9% im letzten Zeitintervall beobachtet werden. Auch der Anteil der Proben mit neutralisierenden Antikörpern nahm im Verlauf zu. Im letzten Zeitintervall waren bei allen Tests 85,0 % der Proben positiv für neutralisierende Antikörper. In den präpandemischen Proben aus Panel B wurden mit keinem der Tests ELISA-Antikörper oder neutralisierende Antikörper nachgewiesen, was auf eine

diagnostische Spezifität von 100 % hinweist. In den Longitudinalproben der rekonvaleszenten COVID-19-Patientenkohorte aus Panel C konnten anti-SARS-CoV-2-IgG Antikörper mit dem IIFT bei 100 % aller Proben nachgewiesen werden. Die IgG-Antikörperspiegel sanken oder stagnierten im Laufe der Zeit bis zu einer weiteren Antigenexposition durch eine erneute Infektion oder eine Impfung. Dabei führte die Impfung zu einem stärkeren Anstieg der IgG-Titer als eine Infektion ohne Impfung. Dieser Anstieg wurde sowohl im IIFT, als auch im Lineblot nachgewiesen. Durch eine Spike-mRNA-basierte Impfung wurde im Profil IgG nur eine starke Anti-S1/2-IgG-, aber keine Anti-NCP-IgG-Antwort ausgelöst – auf diese Weise kann zwischen einer natürlichen Infektion und einer Impfantwort unterschieden werden.
Der mittlere VNT- und sVNT- Titer sank im Laufe der Zeit. In den späten Zeitintervallen wurde ein Rückgang der Maximalwerte für den VNT und den NeutraLISA, aber nicht für den cPass festgestellt. Die mit beiden sVNTs ermittelten Inhibitionswerte korrelierten mit den Titern, die mit dem VNT bestimmt wurden. Ein Unterschied ergab sich jedoch in der Anzahl der neutralisierenden Antikörper-positiven und negativen Proben. Der cPass wies zu allen Zeitpunkten in mehr Proben neutralisierende Antikörper nach als der VNT und der NeutraLISA.
In den Patientenproben, die nach einer Infektion mit saisonalen HCoVs oder Rhinoviren gewonnen worden waren (Panel D) konnte kein SARS-CoV-2-IgG durch den IIFT oder den Lineblot nachgewiesen werden. SARS-CoV-2-spezifische neutralisierende Antikörper wurden hier in 8 von 14 Seren mit dem cPass, aber in 0 von 14 Seren mit dem VNT oder dem NeutraLISA nachgewiesen.

3.5.4 Schlussfolgerungen

Die Übereinstimmung der Ergebnisse des Lineblots mit denen des IIFT ist hoch und steigt in den Tagen nach der Infektion noch weiter an. Da der IIFT das gesamte Spektrum der viralen Antigene enthält, der Lineblot aber nur immobilisierte S1, S2 und NCP als Antigene nutzt, dürfte hier die Sensitivität im IIFT höher sein. Eine Serokonversion von IgG wird hauptsächlich zwischen einer und drei Wochen nach Auftreten der Symptome beobachtet. Die neutralisierenden Antikörper zeigen analog der Kinetik der IgG-Antikörper einen deutlichen Titerabfall nach 3 bis 6 Monaten. Eine Impfung sorgt für einen starken Titeranstieg, welcher im weiteren Verlauf persistiert. Der Nachweis von neutralisierenden Antikörpern lässt sich am besten mit einem zellkulturbasierten VNT ermitteln. Da dieser aber sehr zeitaufwendig ist und das Vorhandensein eines BSL-3 Labors voraussetzt, stellen die beiden untersuchten sVNTs eine gute Alternative dar. Die beiden sVNTs sollten aufgrund ihrer unterschiedlichen positiven und negativen Übereinstimmung mit dem VNT für unterschiedliche Zwecke verwendet werden. Der cPass ist gut geeignet um eine frühe und geringe Immunantwort festzustellen, während der NeutraLISA sich besser für die Überwachung eines abnehmenden Titers neutralisierender Antikörper eignet.

4 Diskussion

Die COVID-19-Pandemie bestimmte über mehr als 2 Jahre hinweg einen großen Anteil des Lebens weltweit. Umso wichtiger war die rasche Aufklärung der Übertragungs- und Schutz-Mechanismen für diese Erkrankung. Hierzu gehört besonders auch die Untersuchung der humoralen Immunantwort bei an COVID-19 erkrankten Patienten, um Wissenschaftlern und Experten für öffentliche Gesundheit zu helfen, SARS-CoV-2 besser zu verstehen und um Bekämpfungsmaßnahmen besser planen zu können [28]. Durch die Untersuchung unterschiedlicher Kollektive konnte ein breiter Querschnitt von COVID-19-Patienten erfasst werden. Mit den Seren von hospitalisierten Patienten aus der Universitätsmedizin Rostock, welche seit Beginn der Pandemie gesammelt wurden, konnte die Antikörperentwicklung von Tag 1 der Infektion an dargestellt werden. Dieses Kollektiv umfasste, zumal sich zu Beginn der Pandemie Patienten auch mit leichten Symptomen im Krankenhaus vorstellten, alle Schweregrade, bis hin zu Patienten, die beatmet werden mussten oder auch verstarben. Bei einem im Rahmen dieser Doktorarbeit untersuchten Kollektiv von Patienten, die ab Ende ihrer gesetzlich vorgeschriebenen Quarantäne, in Zeitintervallen von 2 bis 6 Wochen und im weiteren Verlauf von 3 bis 6 Monaten untersucht wurden, konnte der Verlauf der humoralen Immunantwort nach erfolgter SARS-CoV-2 Infektion über einen sehr langen Zeitraum dargestellt werden. Diese Seren wurden von Beginn der Pandemie Anfang 2020 bis in den Dezember 2022 gesammelt. Ein Großteil dieser Patienten wurde im weiteren Verlauf gegen COVID-19 geimpft und infizierte sich ein zweites oder drittes Mal, zum Teil auch mit bis dahin unbekannten SARS-CoV-2-Varianten.

Im Rahmen der vorliegenden Dissertation wurde die humorale Immunantwort gegen SARS-COV-2 auf Grundlage der Serokonversion von IgA, IgM und IgG (Originalarbeit 3.1) sowie der Möglichkeit einer Reinfektion (Originalarbeit 3.2) untersucht. Zusätzlich wurden neutralisierende Antikörper und deren Titerverlauf im Laufe der Zeit analysiert (Originalarbeit 3.5). Als Nachweismethode für die IgA-, IgM und IgG-Antikörper wurde ein IIFT mit virusinfizierten Zellen [36], und für die neutralisierenden Antikörper ein *in vitro* Neutralisationstest (Originalarbeit 3.2) – jeweils unter BSL-3 Bedingungen – etabliert. Im Vergleich mit diesen beiden Nachweis-Methoden für Antikörper wurden kommerzielle ELISA Teste und Immunoblots auf ihre Sensitivität und auf ihre Spezifität untersucht. Besonderes Augenmerk wurde hier auf eventuelle Kreuzreaktionen mit saisonalen HCoVs oder Seren aus verschiedener geografischer Herkunft (Originalarbeit 3.3) gelegt. Zusätzlich wurde mit dem für die vorliegende Dissertation gesammelten und charakterisierten Panel an humanen Serumproben im BNITM ein hochsensitiver NCP-basierter ELISA für den Nachweis von IgG-Antikörpern gegen SARS-CoV-2 entwickelt (Originalarbeit 3.4).

Der hier verwendete IIFT, welcher das Vollvirus als Antigen nutzt, ist sensitiver als die rekombinanten Spike- und NCP-basierten ELISAs. Dies erklärt sich aus der Tatsache, dass im IIFT das vollständige Virus in seiner natürlichen Faltung mit allen möglichen Bindungsstellen verwendet wird, die rekombinanten Nachweissysteme aber lediglich einen definierten, kleinen Bereich der Bindungsstellen präsentieren

können. Der IIFT ist daher der Goldstandard zur Darstellung der humoralen Immunantwort. Da für diesen Test jedoch BSL-3 Sicherheitslabore benötigt werden, der zeitliche Aufwand zur Herstellung von IIFT-Präparaten hoch ist, die Interpretation der Fluoreszenz eine langjährige Expertise voraussetzt, und ein hoher Proben-Durchsatz, wie bei einer Pandemie erforderlich, nicht möglich ist, sind rekombinante, kommerziell verfügbare ELISAs, welche auch unter S1-Bedingungen durchgeführt werden können, die bessere Wahl. Ob diese die humorale Immunantwort ausreichend darstellen, wurde in dieser Arbeit untersucht.

Mit dem IIFT konnten bereits in der frühen Phase der Infektion (Tag 10 bis Tag 22) in fast allen Proben Antikörper gegen SARS-CoV-2 nachgewiesen werden. Dies ist im Einklang mit publizierten Daten, die IgM- und IgA-Antikörper im Serum zwischen Tag 5 und 7 sowie IgG-Antikörper zwischen Tag 7 und 10 nach der Infektion nachweisen [37]. Der hochsensitive IIFT wird daher als Referenz verwendet, um kommerzielle Teste zu bewerten. Betrachtet man zu Beginn der Infektion die IgG-Antikörper separat, zeigen die NCP-basierten ELISAs eine höhere Sensitivität als die Spike-basierten ELISAs. Dies deckt sich mit Daten, die in der Frühphase der Infektion dem NCP-basierten ELISA eine höhere Sensitivität aufgrund der Immundominanz des N-Proteins zuschreiben [27, 28]. Dieser Test hat bei der Detektion eines akuten Infektionsgeschehens, wenn kein IIFT verwendet werden kann, einen Sensitivitätsvorteil im Vergleich zu einem Spike-basierten ELISA, da letzterer in der Frühphase der Infektion falsch negative Ergebnisse liefern kann.

Zwischen Tag 21 und Tag 60 dpso sind alle Proben von Infizierten im IIFT und fast alle Proben in IgA- und IgG-spezifischen ELISAs positiv. Der rekombinante IgM-ELISA ist dagegen deutlich weniger sensitiv als die Immunfluoreszenz. Aufgrund des Klassenwechsels virusspezifischer B-Zellen von IgM- zur IgG-Antikörperproduktion [12] ist allerdings bereits in dieser Phase ein Rückgang der IgM-Antikörper-Titer zu beobachten. In diesem Zusammenhang kann verstärkt ins Gewicht fallen, dass rekombinante ELISAs, die lediglich Antikörper gegen einen Teil aller möglichen Bindungsstellen erfassen, zu einer erniedrigten Sensitivität im Vergleich zum IIFT führen können.

In der Spätphase der Infektion, länger als 60 Tage dpso, zeigte sich im IIFT kein Rückgang der IgG-Antikörpertiter, wohl nimmt jedoch im S1 ELISA die Quote der IgG-positiven Proben ab, besonders dann, wenn das Testantigen das NCP ist. Dies deckt sich mit Beobachtungen, dass IgG-Antikörper gegen das N-Protein schneller abnehmen als IgG-Antikörper gegen das S-Protein [38]. Abhängig vom verwendeten Testsystem und auch dem verwendeten Antigen erhält man so unterschiedliche Aussagen zur Persistenz der IgG-Antikörper und damit auch zu einem Schutz vor einer Reinfektion. Daher sollten in der Spätphase der Infektion sowie bei Seroprävalenzstudien zur Vermeidung falsch negativer Ergebnisse (verglichen mit dem IIFT als Goldstandard), Spike-Proteine gegenüber Nukleokapsid-Proteinen als Test-Antigene für ELISAs vorgezogen werden. Zudem können rekombinante ELISAs auch eine Unterscheidung zwischen einer Infektion oder eine Impfung treffen, sofern Impfstoffe auf Spike-Basis

verwendet wurden. Für Infektions-Prävalenzstudien bei einem teilweise geimpften Kollektiv sollte jedoch ein NCP-basierter ELISA verwendet werden.

Zusammenfassend zeigt der IIFT in allen Phasen der Infektion für alle Immunglobulinklassen eine höhere Seropositivitätsrate als die Spike- und NCP-basierten ELISAs. Daher sollte man je nach Fragestellung und Zeitdauer nach Infektion das zu verwendende Testsystem auswählen.

Nicht nur der Zeitpunkt der Infektion, sondern auch die geografische Herkunft der Seren, welche mit den jeweiligen Tests untersucht werden, sollte bei der Auswahl des Testsystems und der Interpretation der Ergebnisse berücksichtigt werden (Originalarbeit 3.3). Serumproben aus Ländern in Subsahara-Afrika wiesen für die hier untersuchten kommerziell erhältlichen ELISAs eine deutlich reduzierte Spezifität auf, was im Einklang mit anderen Studien steht [39]. Auch eine kürzlich vorangegangene Malaria- [39-41] oder Dengue- [42] Infektion könnte hier zu falsch positiven Antikörper-Ergebnissen führen. Eine mögliche Erklärung hierfür ist die Tatsache, dass insbesondere die Malaria mit einer ausgeprägten polyklonalen B-Zell-Aktivierung einhergeht. NCP-basierte ELISAs zeigten in dieser Arbeit eine deutliche höhere falsch-positiv Rate als Spike-basierte ELISAs. Dies ist auch für andere, nicht-identische, NCP basierte ELISAs [43] und in einer erst kürzlich erschienenen Publikation mit identischen ELISAs in anderen Populationen [44] beobachtet worden. Die hohen falsch-positiven Antikörperbefunde in präpandemischen Seren könnten zum Beispiel durch vorherige Infektionen mit noch unbekannten Coronaviren entstehen. Auch die Tatsache, dass N-Proteine innerhalb der Coronaviren – im Gegensatz zu den S-Proteinen – weitgehend konserviert sind, könnte hierfür eine Erklärung sein [45].

Hieraus ergibt sich die Empfehlung, Proben aus den geografischen Regionen, die vor dem Ausbruch der SARS-CoV-2-Epidemie gesammelt wurden, zum Festlegen eines eigenen, für diese Region spezifischen Cut-offs zu nutzen, um falsch positive Antikörper-Befunde zu vermeiden. Auch sollte nach Möglichkeit eine Kombination aus verschiedenen Testen verwendet werden, um die Vorteile der einzelnen Teste zu kombinieren und um Nachteile auszugleichen.

Die Mehrheit der Personen entwickelt sieben bis 15 Tage nach der Infektion neutralisierende Antikörper gegen SARS-CoV-2. Die Antikörpertiter steigen zwischen 14 und 22 Tagen weiter an, erreichen dann ein Plateau, bevor sie wieder abnehmen [46]. Die mit zellbasierten VNTs und mit sVNTs hier erhobenen Daten für neutralisierende Antikörper (Originalarbeit 3.5) bestätigen dies.

Das Spike-Protein und hier besonders die RBD ist der Hauptbindungsort für neutralisierende Antikörper [47]. Die verwendeten sVNTs interagieren beide mit dieser Domäne, woraus sich die gute Korrelation mit den Testergebnissen des VNT erklärt. Zur Darstellung der neutralisierenden Antikörper sind zellbasierte VNTs zwar die beste Wahl, da sie hochsensitiv sind und wie auch der IIFT das Virus in seiner natürlichen Form präsentieren. Kommerzielle sVNTs liefern dagegen schneller Ergebnisse, ohne dass ein BSL-3 Labor nötig wäre. Es sollten hier aber immer die Leistungsdaten der einzelnen sVNTs beachtet

werden, da sie für unterschiedliche Anwendungsszenarien unterschiedlich gut geeignet sind, wie für die Untersuchung früh auftretender niedriger Antikörpertiter im Vergleich zur Überwachung von Impftitern. Der Nachweis dieser neutralisierenden Antikörper kann einen Rückschluss auf eine vorhandene Immunität gegen eine Infektion oder Reinfektion mit SARS-CoV-2 geben. Leider ist bei allen Virusneutralisationstests letztlich unklar, welcher minimale neutralisierende Antikörpertiter mit einer schützenden Immunität korreliert, und ob die Ergebnisse der kommerziell erhältlichen serologischen SARS-CoV-2-Tests eine solche Immunität zuverlässig vorhersagen [34]. Gedächtnis-B-Zellen können jedoch eine dauerhafte humorale Immunität vermitteln, selbst wenn die Titerhöhen der bereits im Serum vorhandenen neutralisierenden Antikörper abnehmen [48]. In früheren Studien wurde zudem eine höhere Prävalenz der Serokonversion bei schwer erkrankten Personen im Vergleich zu Personen mit asymptomatischer oder leichter Krankheit festgestellt [49] und SARS-CoV2-Antikörperspiegel korrelieren nachweislich positiv mit dem Schweregrad von COVID-19 [50]. Dies gilt auch für die Titer von neutralisierenden Antikörpern. Somit sind Patienten mit einer schwereren Erkrankung möglicherweise besser gegen eine Reinfektion geschützt als Patienten mit einer asymptomatischen oder leichten Erkrankung [51]. Reinfektionen nach durchgemachter SARS-CoV-2-Infektion und die Übertragung von SARS-CoV-2 durch das Ausscheiden von infektiösem Virus sind auch in Gegenwart neutralisierender Antikörper möglich (Originalarbeit 3.2). Trotz durchgemachter Krankheit und nachgewiesenen, moderaten neutralisierenden Antikörper Titern im VNT infizierte sich eine Person nach 288 Tagen erneut mit SARS-CoV-2. Nach der Erstinfektion waren IgG- und IgM-Titer mit dem IIFT nachweisbar. Die IgG-Titer stiegen nach der Reinfektion ebenso an wie die Titer im Neutralisationstest stark an. IgA- und IgM-Antikörper konnten dagegen nach der Reinfektion nicht nachgewiesen werden. Eine Vollgenomanalyse ergab, dass für die Erstinfektion und für die Reinfektion unterschiedliche SARS-CoV-2 Varianten verantwortlich waren. Die Untersuchung der neutralisierenden Aktivität der Antikörper aus Proben vor und nach der Reinfektion mit den jeweils zu dieser Zeit zirkulierenden Virusvarianten ergab keine signifikanten Unterschiede im VNT-Titer. Die neutralisierenden Antikörper erwiesen sich als wirksam gegen beide Virusvarianten, die Virusvariante der Reinfektion zeigte demnach keine Immunevasion.

Im Einzelfall kann die Kenntnis von Antikörper-Titern gegen SARS-CoV-2 oder über das Vorhandensein von neutralisierenden Antikörpern eine Entscheidungshilfe bei der Frage nach einer Auffrischungsimpfung oder der Gabe von Antikörpern sein. Hier ist jedoch zu beachten, dass therapeutische monoklonale Antikörper in Abhängigkeit der zirkulierenden Virusvarianten in ihrer neutralisierenden Wirkung vermindert oder ganz unwirksam werden können. So konnten wir in einer retrospektiven Studie zeigen, dass therapeutische, monoklonale Antikörper (Casivirimab/Imdevimab), welche gegen die bis dahin vorherrschenden Virusvarianten eine hohe klinische Wirksamkeit hatten, gegen die später auftretende Omikron Variante nur noch in maximaler Dosis eine neutralisierende Wirkung hatten, während andere therapeutische monoklonale Antikörper (Sotrovimab) aber auch in

niedrigeren Dosen neutralisierend wirkten [52]. Das Wissen über aktuell vorherrschende Virusvarianten und die Wirksamkeit therapeutischer monoklonaler Antikörper informiert somit Therapieentscheidungen.

Wünschenswert wäre, dass quantitative Antikörpertests – einschließlich Neutralisationstests – standardisiert werden, so dass sie z.B. anhand des internationale Standardplasmas 20/136 der WHO [53] untereinander vergleichbar werden, da sich hieraus möglicherweise auch Aussagen zu einer klinischen Schutzwirkung treffen lassen [54]. Da das Virus sich im Rahmen seiner Evolution ständig an neue Bedingungen anpasst und da laufend neue Varianten entstehen, sollte dieser Standard, wie von der WHO im Juli 2022 vorgeschlagen [55], regelmäßig an neue zirkulierende Varianten angepasst werden.

5 Zusammenfassung

In der vorliegenden Arbeit wurde die humorale Immunantwort gegen SARS-CoV-2 in COVID-19-Patienten untersucht. Es wurden Seren aus verschiedenen Kollektiven verwendet, um die Immunantwort zu bestimmten Zeitpunkten, aber auch eventuell auftretende Kreuzreaktionen zu analysieren. Dazu wurden unterschiedliche Testmethoden, wie ein für diese Arbeit etablierter IIFT, welcher das natürliche Virus verwendet, aber auch kommerziell erhältliche ELISAs, miteinander verglichen. Der IIFT zeigte hierbei eine höhere Sensitivität als die verwendeten S- und N-Protein basierten ELISAs. Dagegen sind die ELISAs schneller und einfacher durchführbar, erfordern kein Sicherheitslabor der Stufe 3 und können das Antigen benennen, gegen welches Antikörper nachgewiesen wurden. Sie sind somit u.a. für Seroprävalenzstudien im Hintergrund einer Spike-basierten Impfung geeignet. Je nach Anwendungs-Situation und Fragestellung sollte der passende Test ausgewählt werden, um eine optimale Sensitivität und Spezifität zu erreichen. Gerade in Proben aus afrikanischen Ländern variiert die Spezifität der einzelnen ELISAs, u.a. in Abhängigkeit vom verwendeten Testantigen. Hier sollten *a priori* Seren aus der zu untersuchenden geografischen Region, welche vor der Pandemie gesammelt wurden, zur Festlegung eines spezifischen Cut Offs verwendet werden, und die Ergebnisse sorgfältig im Kontext bewertet werden, da so eine Überschätzung der Seroprävalenz vermieden werden kann. Wenn möglich, sollten verschiedene serologische Teste kombiniert werden um die Ergebnisse zu validieren.

Die meisten Patienten entwickeln nach einer SARS-CoV-2-Infektion neutralisierende Antikörper, deren Titerhöhe jedoch im Laufe der Zeit abnimmt. Auch sind trotz des Vorhandenseins neutralisierender Antikörper Reinfektionen möglich. Die Kenntnis der Antikörpertiter, insbesondere der Titer der neutralisierenden Antikörper, kann für Auffrischungsimpfungen und Antikörpertherapien relevante Informationen bieten. Bei letzterem kann die Wirkung therapeutischer, monoklonaler Antikörper je nach vorherrschender Virusvariante verringert sein. Die Standardisierung quantitativer Antikörpertests ist wichtig, um eine aussagekräftige Bewertung der Schutzwirkung zu ermöglichen.

Die vorliegende Arbeit liefert somit Erkenntnisse zur humoralen Immunantwort gegen SARS-CoV-2 und zur Wertigkeit verschiedener Nachweismethoden. Auf diese Weise kann sie dazu beitragen, das Verständnis der Epidemiologie und Immunität zu verbessern und möglicherweise Entscheidungshilfen für Auffrischungsimpfungen und Antikörpertherapien bieten.

6 Abkürzungsverzeichnis

APC	antigenpräsentierende Zelle
BNITM	Bernhard-Nocht-Institut für Tropenmedizin
BSL-3.	Biosicherheitsstufe 3
dpso	days post symptom onset
ELISA	Enzyme-linked Immunosorbent Assay
E-Protein	Hüllprotein
Fab	antigenbindendes Fragment
Fc.	kristallisierbares Fragment
HCoV	humane Coronaviren
IgA	Immunglobulin A
IgD	Immunglobulin D
IgE	Immunglobulin E
IgG	Immunglobulin G
IgM	Immunglobulin M
IIFT	indirekter Immunfluoreszenztest
Il-1	Interleukin-1
Il-2	Interleukin-2
MERS-CoV	Middle East respiratory syndrome coronavirus
MHC-II-Protein	Haupthistokompatibilitätskomplex-Protein Klasse II
M-Protein	Membranprotein
NCP	Nukloekapsidprotein
N-Protein / NCP-Protein	Nukleokapsidprotein
RBD	rezeptorbindende Domäne
RT-PCR	Reverse Transkriptase-Polymerase-Kettenreaktion
SARS-CoV	Severe acute respiratory syndrome coronavirus
S-Protein	Spikeprotein
sVNT	surrogat Virusneutralisationstest
TMPRSS2	Transmembran-Serin-Protease vom Typ II
T-Zellrezeptors	TCR
VNT	*in vitro* Virusneutralisationstest

7 Abbildungsverzeichnis

8 Literaturverzeichnis

8.1 Der Dissertation zugrunde liegende Arbeiten mit Darstellung des Eigenanteils

Originalarbeit 3.1

Emmerich P, von Possel R, Hemmer CJ, et al. Longitudinal detection of SARS-CoV-2-specific antibody responses with different serological methods. J Med Virol. 2021;93(10):5816-5824. doi:10.1002/jmv.27113

Ronald von Possel: Datenerhebung, Revision und Überarbeitung des Manuskripts

Originalarbeit 3.2

Brehm TT [*], Pfefferle S[*], von Possel R[*], et al. SARS-CoV-2 Reinfection in a Healthcare Worker Despite the Presence of Detectable Neutralizing Antibodies. Viruses. 2021;13(4):661. Published 2021 Apr 12. doi:10.3390/v13040661

[*]*Diese Autoren haben zu gleichen Teilen zu dieser Arbeit beigetragen*

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Originalarbeit 3.5

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9 Originalarbeiten

9.1 Originalarbeit 3.1

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RESEARCH ARTICLE

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Longitudinal detection of SARS-CoV-2-specific antibody responses with different serological methods

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Abstract

Serological testing for anti-severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) antibodies is used to detect ongoing or past SARS-CoV-2 infections. To study the kinetics of anti-SARS-CoV-2 antibodies and to assess the diagnostic performances of eight serological assays, we used 129 serum samples collected on known days post symptom onset (dpso) from 42 patients with polymerase chain reaction-confirmed coronavirus disease 2019 (COVID-19) and 54 serum samples from healthy blood donors, and children infected with seasonal coronaviruses. The sera were analyzed for the presence of immunoglobulin G (IgG), immunoglobulin M (IgM), and immunoglobulin A (IgA) antibodies using indirect immunofluorescence testing (IIFT) based on SARS-CoV-2-infected cells. They were further tested for antibodies against the S1 domain of the SARS-CoV-2 spike protein (IgG, IgA) and against the viral nucleocapsid protein (IgG, IgM) using enzyme-linked immunosorbent assays. The assay specificities were 94.4%–100%. The sensitivities varied largely between assays, reflecting their respective purposes. The sensitivities of IgA and IgM assays were the highest between 11 and 20 dpso, whereas the sensitivities of IgG assays peaked between 20 and 60 dpso. IIFT showed the highest sensitivities due to the use of the whole SARS-CoV-2 as substrate and provided information on whether or not the individual has been infected with SARS-CoV-2. Enzyme-linked immunosorbent assays provided further information about both the prevalence and concentration of specific antibodies against selected antigens of SARS-CoV-2.

KEYWORDS
antibodies, COVID-19, ELISA, immunofluorescence, SARS-CoV-2, serology

Christina Deschermeier and Katja Steinhagen contributed equally to this study.

1 | INTRODUCTION

In the current pandemic, direct pathogen detection via reverse transcription and polymerase chain reaction amplification as well as real-time detection (real-time RT-PCR) is the gold standard for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) detection and enables early identification of acute SARS-CoV-2 infections. Serological testing for anti-SARS-CoV-2 antibodies is used to confirm ongoing or past infections with SARS-CoV-2. The detection of antibodies enables confirmation of SARS-CoV-2 infection in patients with typical symptoms and in suspected (asymptomatic) cases. Analysis of anti-SARS-CoV-2 antibodies is typically performed at an advanced stage of infection and thus expands the time frame for coronavirus disease 2019 (COVID-19) diagnostics.

Seroconversion of anti-SARS-CoV-2 antibodies can occur at different points in time after virus contact.[1,2] The features of immune responses to SARS-CoV-2 infections vary significantly between individuals,[3] especially regarding the kinetics, immunoglobulin classes, and antigen specificity. In the majority of COVID-19 patients, anti-SARS-CoV-2 antibodies are detectable within two weeks after infection.[4–6] Usually, specific immunoglobulin M (IgM) and immunoglobulin A (IgA) antibodies are detectable earlier than specific immunoglobulin G (IgG) antibodies.[5,7,8] In individual cases, anti-SARS-CoV-2 antibodies are either only detectable more than four weeks after onset of symptoms or not at all due to generally absent antibody secretion.[8–10]

Anti-SARS-CoV-2 antibodies target different structural proteins of SARS-CoV-2. The main immunogens are the spike and nucleocapsid proteins. The highly immunogenic S1 domain of the spike protein of SARS-CoV-2 is a major target for neutralizing antibodies and is being used as the antigen in many serological assays.[11] The immunologically relevant receptor-binding domain (RBD) represents another important target antigen for virus-neutralizing antibodies.[12] The nucleocapsid protein (NCP) of SARS-CoV-2 is the antigen with the strongest immune dominance among *Coronaviridae*[13] and contains diagnostically relevant epitopes of SARS-CoV-2. Previous studies suggested heterogeneous binding antibody responses to S1/RBD and NCP viral antigens,[14] and hence the presence of antibodies against one protein of SARS-CoV-2 does not necessarily coincide with the presence of antibodies against another.

The current research is determined to illuminate the kinetics of the humoral immune response against SARS-CoV-2, potentially providing guidance on when to use serological tests effectively for screening or monitoring of the infection. Results of serological tests can provide answers to important epidemiological, clinical and virological questions concerning SARS-CoV-2, for instance, on the traceability of infection chains and the role of asymptomatic or presymptomatic transmission. Moreover, the exact determination of the course of concentration of IgG antibodies against SARS-CoV-2 before and after vaccination can provide valuable information on the effectiveness of vaccination.

Currently, knowledge about SARS-CoV-2 antibody persistence is scarce, although it would help to understand the possible role of humoral immunity in the protection against reinfection. The aim of this study was to study the kinetics of antibodies against SARS-CoV-2 and to explore the characteristic features of eight serological assays.

2 | METHODS

2.1 | Human serum samples

Panel A comprised 82 sequential and single serum samples from 25 German patients (Table 1). Infection with SARS-CoV-2 was confirmed by PCR[15] by regional health authorities. These patients had mild to moderate COVID-19 symptoms.

Panel B comprised 47 sequential and single serum samples from 17 German patients (Table 1). Infection with SARS-CoV-2 was confirmed by PCR using the Allplex 2019-nCoV Assay (Seegene Inc.). These patients required hospitalization.

All patient samples were also serologically precharacterized by indirect immunofluorescence testing (IIFT).

Panel C comprised serum samples taken before August 2019 from 42 healthy German blood donors (Table 1).

Panel D comprised serum samples taken between January and March 2020 from twelve German children (Table 1) positive for IgG against seasonal coronaviruses (e.g., HCoV 229-E) by indirect immunofluorescence testing (IIFT, for research use only).

2.2 | Detection of anti-SARS-CoV-2 antibodies

The detection of antibodies against SARS-CoV-2 (genus: *Betacoronavirus*, family: *Coronaviridae*) using IIFT was performed with anti-IgG-, anti-IgA-, and anti-IgM-fluorescein isothiocyanate-labeled secondary antibodies on infected Vero E6 cells fixed in acetone-methanol.[16,17] Sample dilutions from 1:20 to 1:80 were screened. Samples with positive results were titrated to the final titer. An immunofluorescence signal at titers ≥1:20 was rated as positive and otherwise as negative.

Samples were further tested for the presence of antibodies against SARS-CoV-2 with five enzyme-linked immunosorbent assays (ELISA, from EUROIMMUN Medizinische Labordiagnostika AG). All ELISAs apply viral antigens recombinantly expressed in human cells. The anti-SARS-CoV-2 ELISA (IgG) and anti-SARS-CoV-2 ELISA (IgA) are based on the S1 domain of the spike protein of SARS-CoV-2 as antigen, including the immunologically relevant RBD, to detect anti-SARS-CoV-2 IgG and IgA antibodies, respectively. The anti-SARS-CoV-2 QuantiVac ELISA (IgG) was used for quantitative detection of anti-SARS-CoV-2 IgG antibodies by means of a six-point calibration curve. The anti-SARS-CoV-2 NCP ELISA (IgG) and Anti-SARS-CoV-2 NCP ELISA (IgM) are based on a modified NCP as antigen to detect anti-SARS-CoV-2 IgG and IgM antibodies, respectively. ELISA results were evaluated as recommended by the manufacturer. Borderline results were reported but excluded from the subsequent analyses

TABLE 1 Descriptive information of all panels

Panel	Age (mean ± *SD*)	Sex (*n* females, *n* males, *n* unknown)	*N* samples in panel	*N* samples per patient (mean ± *SD*, range)	Dpso (mean ± *SD*, range)	*N* sample ≤10 dpso	*N* samples 11–20 dpso	*N* samples 21–60 dpso	*N* sample >60 dpso
A 25 Patients	43.2 ± 13.7	17, 8, 0	82	3.3 ± 1 (1, 5)	78.4 ± 49.7, [10, 178]	1	10	28	43
B 17 Patients	68.0 ± 16.1	9, 8, 0	47	2.8 ± 1.4, (1, 6)	26.1 ± 30.8, (1, 109)[a]	13	19	3	6
C 42 Blood donors	>18 42 unknown	0, 0, 42	42	1 ± 0	-	-	-	-	-
D 12 Children	7.8 ± 4.2 0 unknown	5, 7, 0	12	1 ± 0	-	-	-	-	-

Abbreviation: Dpso, days after symptom onset.

[a]In Panel B, information on dpso was available for only 41 out of 47 samples.

as they do not allow secure evaluation and are subjected to retesting by means of other diagnostic methods and/or serological investigation of a follow-up sample in the laboratory practice.

The detection of SARS-CoV-2-specific antibody responses was also investigated with respect to the infection phase. As the diagnostic window for serological testing opens several days after pathogen contact, only samples taken later than ten dpso were considered. The phases were split into 11–20 dpso (early phase of infection; *n* samples in Panel A: 10, Panel B: 19), 21–60 dpso (intermediate phase of infection; n samples in Panel A: 28, Panel B: 3) and >60 dpso (past infection; n samples in Panel A: 43, Panel B: 6, Table 1).

The overall agreement between the qualitative results obtained with the anti-SARS-CoV-2 ELISA (IgG) and Anti-SARS-CoV-2 QuantiVac ELISA (IgG) was calculated, their degree of agreement was quantified using Cohen's κ including borderline results,[18] and the statistical association between results was described using Pearson correlation and 95% confidence intervals as determined by Clopper-Pearson interval.

3 | RESULTS

The sensitivities varied largely between assays (Table 2). The IIFT revealed positive results for anti-SARS-CoV-2 IgG, IgA, and IgM antibodies in 94.6%, 72.9%, and 65.9% of the patient samples, respectively. As a major part of samples in the panels, A + B was taken in the late phase of infection, the overall prevalence of IgM antibodies (representing the acute phase response) is lower than that of IgG. The ELISAs detected specific antibodies against S1 IgG and IgA in 75.8% and 80.3% of the patient samples, respectively. Anti-SARS-CoV-2 IgG and IgM antibodies against NCP were detected in 82.0% and 19.8% of the patient samples, respectively. The specificity was 100% by IIFT, Anti-SARS-CoV-2 ELISA (IgG, IgA), and Anti-SARS-CoV-2 NCP ELISA (IgM), while the four remaining assays reached specificities between 92.9% and 97.6%. Cross-reactivities were not observed.

Qualitative results (positive/borderline/negative) for individual serum samples have been visualized in Figure 1. The sensitivities of IgA and IgM assays were the highest in the early phase of infection, while positive results for IgG antibodies occurred most often in the intermediate phase (Table 3).

Positive results for anti-SARS-CoV-2 IgG antibodies against S1 reached a peak during the intermediate phase of infection. In contrast, positive results for anti-SARS-CoV-2 IgA antibodies as measured by IIFT showed an initial peak followed by a pronounced decrease after 60 dpso. During the course of infection, the number of positive results for anti-SARS-CoV-2 IgM antibodies dropped as measured both by ELISA and IIFT.

In the early phase of infection (11–20 dpso), IgG and IgA antibodies against S1 of SARS-CoV-2 were detected in 70.4% and 88.9% of the samples ($n = 29$), respectively, while IgG and IgM antibodies against NCP were detected in 86.2% and 50%, respectively. The IIFT

TABLE 2 Diagnostic performance of the assays

		IIFT			ELISA				
Panel		IgG	IgA	IgM	S1 IgG	QuantiVac S1 IgG	S1 IgA	NCP IgG	NCP IgM
A	*n* Positive	82	59	45	66	66	60	65	4
	n Borderline	-	-	-	4	5	10	6	3
	n Negative	0	23	37	12	11	12	11	75
	Sensitivity	100%	72%	54.9%	84.6%	85.7%	83.3%	85.5%	5%
	CI (%)	(95.6, 100)	(60.9, 81.3)	(43.5, 65.9)	(74.7, 91.8)	(75.9, 92.7)	(72.7, 91.1)	(75.6, 92.6)	(1.4, 12.5)
B	*n* Positive	40	35	40	28	30	34	35	21
	n Borderline	-	-	-	1	0	2	1	0
	n Negative	7	12	7	18	17	11	11	26
	Sensitivity	85.1%	74.5%	85.11%	60.9%	63.8%	75.6%	76.1%	44.7%
	CI (%)	(71.7, 93.8)	(59.7, 86.1)	(71.7, 93.8)	(45.3, 74.9)	(48.5, 77.3)	(60.5, 87.1)	(61.2, 87.4)	(30.2, 59.9)
A + B	*n* Positive	122	94	85	94	96	94	100	25
	n Borderline	-	-	-	5	5	12	7	3
	n Negative	7	35	44	30	28	23	22	101
	Sensitivity	94.6%	72.9%	65.9%	75.8%	77.4.%	80.34%	82.0%	19.8%
	CI (%)	(89.1, 97.8)	(64.3, 80.3)	(57.0, 74.0)	(67.3, 83.0)	(69.0, 8.4)	(72.0, 87.1)	(74.0, 88.3)	(13.3, 27.9)
C	*n* Positive	0	0	0	0	1	0	1	0
	n Borderline	-	-	-	1	0	1	1	0
	n Negative	42	42	42	41	41	41	40	42
	Specificity	100%	100%	100%	100%	97.6%	100%	97.6%	100%
	CI (%)	(91.6, 100)	(91.6, 100)	(91.6, 100)	(91.4, 100)	(87.4, 99.9)	(91.4, 100)	(87.4, 99.9)	(91.6, 100)
D	*n* Positive	0	0	0	0	0	0	0	0
	n Borderline	-	-	-	0	0	0	0	0
	n Negative	12	12	12	12	12	12	12	12
	Cross-reactivity	None	None	None	None	None	None	None	None

Note: Sensitivities were determined based on Panel A (*n* samples = 82), Panel B (*n* samples = 47) and Panels A + B (*n* samples = 129). Specificities were determined based on Panel C (n samples = 42). Cross-reactivities were determined based on panel D (*n* samples = 12). CI: 95% confidence interval. Borderline ELISA results were excluded for calculation of the sensitivity and specificity.

Abbreviations: ELISA, enzyme-linked immunosorbent assay; IgA, immunoglobulin A; IgG, immunoglobulin G; IgM, immunoglobulin M; IIFT, indirect immunofluorescence testing; NCP, nucleocapsid protein; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

detected SARS-CoV-2 specific IgG, IgA, and IgM antibodies in 96.6%, 93.1%, and 96.6% of the samples, respectively.

In the intermediate phase of infection (21–60 dpso), IgG and IgA antibodies against S1 of SARS-CoV-2 were detected in 93.3% and 82.8% of the samples (*n* = 31), respectively, while IgG and IgM antibodies against NCP were detected in 96.8% and 12.9%, respectively. The IIFT detected specific IgG, IgM, and IgA antibodies in 100%, 87.1%, and 100%, respectively.

In the late phase of infection (>60 dpso), IgG and IgA antibodies against S1 of SARS-CoV-2 were detected in 85.1% and 80.5% of the samples (*n* = 49), respectively, while IgG and IgM antibodies against NCP were detected in 81.4% and 0%, respectively. The IIFT detected specific IgG, IgA, and IgM antibodies in 98%, 44.9%, and 30.6%, respectively.

Overall, in samples taken later than 10 dpso, IgG and IgA antibodies against S1 of SARS-CoV-2 were detected in 83.7% and 83.5% of the samples (*n* = 109), respectively, while IgG and IgM antibodies against NCP were detected in 87.4% and 17%, respectively. The IIFT detected specific IgG, IgA, and IgM antibodies in 98.2%, 73.4%, and 64.2%, respectively.

Comparison of the qualitative results obtained using the Anti-SARS-CoV-2 QuantiVac ELISA (IgG) and the anti-SARS-CoV-2 ELISA (IgG) showed a high total agreement (98.9%, Table 4) and, corrected for the probability of random coincidence, an almost perfect degree

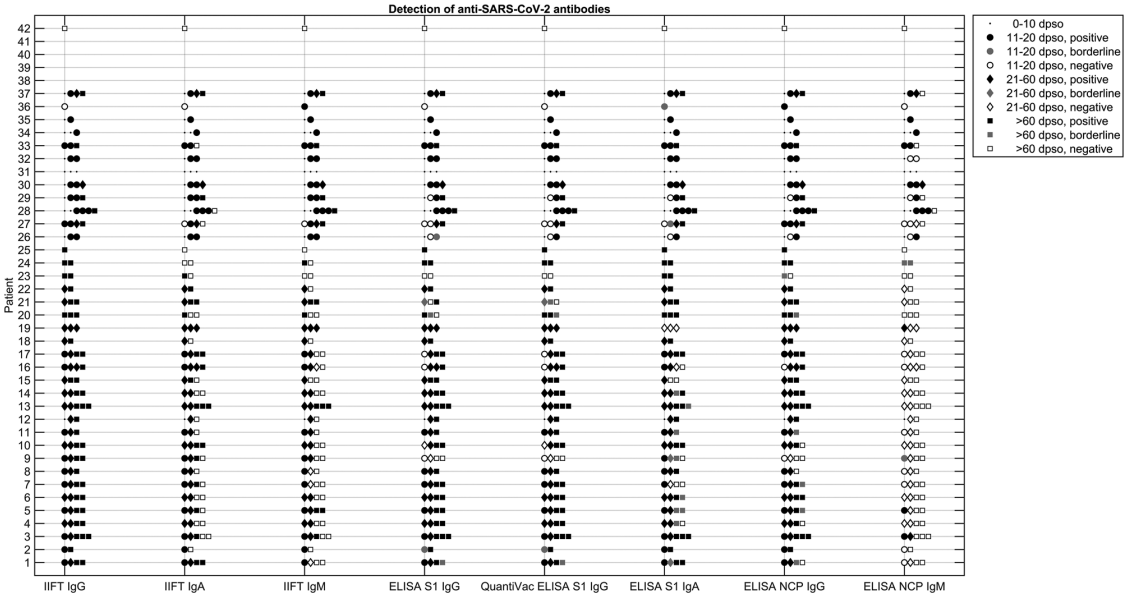

FIGURE 1 Longitudinal detection of SARS-CoV-2-specific antibody responses in serum samples from panel A (25 patients, 82 samples) and B (17 patients, 47 samples, results of six samples with unknown dpso are not displayed) with respect to the phase of infection using different serological methods (IIFT and ELISA). Dpso: days after onset of symptoms. SARS-CoV-2, severe acute respiratory syndrome coronavirus 2

TABLE 3 Number of positive results and sensitivity (%) per infection phase based on 109 serum samples from panels A + B taken later than ten days after onset of symptoms (dpso)

		IIFT						ELISA									
		IgG		IgA		IgM		S1 IgG		QuantiVac S1 IgG		S1 IgA		NCP IgG		NCP IgM	
Phase [dpso]	*N* samples	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
11–20	29	28	96.6	27	93.1	28	96.6	19 (2)	70.4	20 (1)	71.4	24 (2)	88.9	25 (0)	86.2	14 (1)	50.0
21–60	31	31	100	31	100	27	87.1	28 (1)	93.3	28 (1)	93.3	24 (2)	82.8	30 (0)	96.8	4 (0)	12.9
>60	49	48	98.0	22	44.9	15	30.6	40 (2)	85.1	40 (3)	87.0	33 (8)	80.5	35 (6)	81.4	0 (2)	0.0
≥11	109	107	98.2	80	73.4	70	64.2	87 (5)	83.7	88 (5)	84.6	81 (12)	83.5	90 (6)	87.4	18 (3)	17.0

Note: For the ELISAs, the number of borderline results are reported in brackets but were excluded for calculation of the sensitivity.

Abbreviations: ELISA, enzyme-linked immunosorbent assay; IgA, immunoglobulin A; IgG, immunoglobulin G; IgM, immunoglobulin M; IIFT, indirect immunofluorescence testing; NCP, nucleocapsid protein; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

of agreement ($\kappa = 0.93$, 95% confidence interval: [0.87, 0.98]). In addition, there was a strong positive, statistically significant correlation ($r = 0.98$, $p < 0.001$) between (semi)quantitative results of the two ELISAs (Figure 2).

4 | DISCUSSION

The aim of this study was to study the long-term kinetics of antibodies against SARS-CoV-2 and to assess the characteristic features of different serological methods. We present findings of the temporal profiles of IgG, IgA, and IgM antibody responses against SARS-CoV-2 determined in sera from patients with mild to severe COVID-19 by means of eight serological assays.

The sensitivities varied between assays and phases of infection but corroborated that the different substrates used in the assays serve different purposes. Due to the use of the whole SARS-CoV-2 as substrate instead of isolated recombinant antigens, the IIFT showed a higher sensitivity for IgG antibodies (94.6%) than each of the antigen-specific ELISAs (77.4% to 82.0%), when referring to all patient samples independent of the infection phase (Table 2). Similarly, the overall sensitivity of the IgM IIFT exceeded that of the anti-NCP

IgM ELISA by 46.1%, again reflecting the wider antigenic spectrum in IIFT.

The ELISAs, in contrast, provide information about the prevalence of specific antibodies against selected antigens of SARS-CoV-2. Hence, lower sensitivities of the S1-specific ELISAs compared to the NCP-specific ELISA probably reflect the known fact that not all infected individuals produce antibodies against the S1 domain of SARS-CoV-2.[10] Importantly, previous research showed that responses of specific IgG against S1 and NCP may be heterogeneous between individuals, time-delayed and do not always coincide with each other.[8,12,14] In the present panels, the prevalence of specific IgG antibodies against NCP in the early phase of infection was higher than that against S1 (Table 3). However, the findings of the current study do not support previous research by Herroelen et al., who undertook a comparative evaluation of commercial SARS-CoV-2 serological assays and observed no clear differences in the seroconversion kinetics of antibodies targeting SARS-CoV-2 S and N protein epitopes between severe and milder SARS-CoV-2 infections.

Exclusively in the early phase of infection, the prevalence of specific IgA antibodies against S1 was higher than that of specific IgG antibodies against NCP as well as S1. This observation reflects that of Okba et al.[1] However, it is in contrast to a previous study that showed a higher sensitivity of the Anti-SARS-CoV-2 IgG compared to the Anti-SARS-CoV-2 IgA ELISA in patient samples taken later than fourteen dpso,[19] whereby the discrepancy might be due to heterogeneous definitions regarding the early phase of infection.

The IgA IIFT showed a pronounced decrease in the antibody detection rate after 60 dpso, which was not observed for the IgA ELISA (Table 3). A possible explanation for this might be that the IgA antibody response against the S1 protein largely remains constant, while the production of IgA antibodies against other antigens of SARS-CoV-2 decreases.

More patients were seropositive for IgM by IIFT than by ELISA (Table 2, Table 3), which could be accounted for by the low sensitivity of the NCP IgM ELISA, warranting further investigations. However, the continuously low sensitivity of the NCP-specific IgM ELISA (Table 3) is in accordance with previous results indicating a

TABLE 4 Agreement between qualitative results of Anti-SARS-CoV-2 ELISA (IgG) and anti-SARS-CoV-2 QuantiVac ELISA (IgG) based on 183 serum samples (Panels A–D)

		Anti-SARS-CoV-2 ELISA (IgG)		
***n* Samples = 183**		**Positive**	**Borderline**	**Negative**
Anti-SARS-CoV-2 QuantiVac ELISA (IgG)	Positive	93	3	1
	Borderline	0	3	2
	Negative	1	0	80
Positive agreement		98.9%		
Negative agreement		98.8%		
Total agreement		98.9%		

Abbreviations: ELISA, enzyme-linked immunosorbent assay; IgG, immunoglobulin G; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

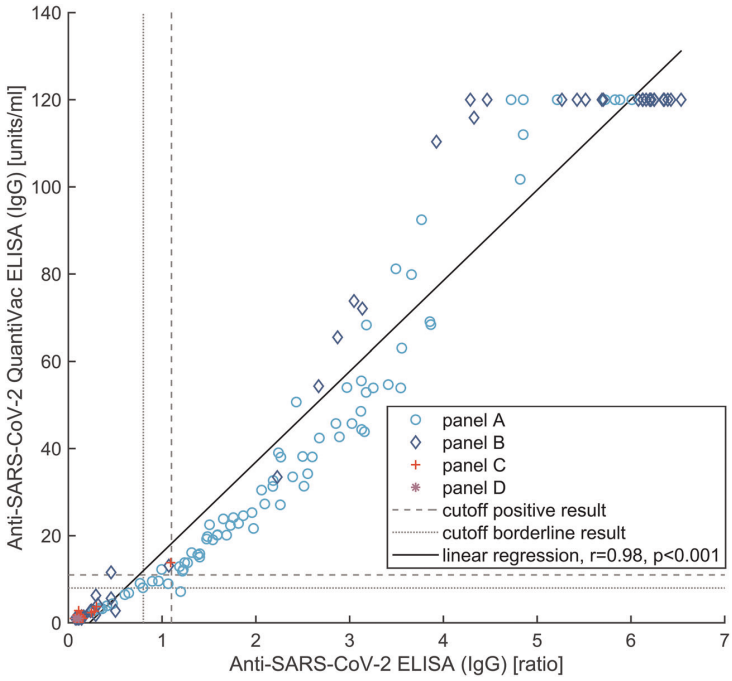

FIGURE 2 Correlation between semiquantitative results of anti-SARS-CoV-2 ELISA (IgG) and anti-SARS-CoV-2 QuantiVac ELISA (IgG). Detailed information on the serum panels is given in Table 1. ELISA, enzyme-linked immunosorbent assay; IgG, immunoglobulin G; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2

sensitivity of 55% at week 3–4 after disease onset.[20] Liu et al also observed a higher sensitivity of an ELISA based on the spike protein compared to an NCP-based ELISA for detection of IgM antibodies.[8] Two months after symptom onset, we observed a decline in the sensitivity of both IgM-specific assays (Table 3). Independent of the serological method, the two IgM-specific assays reached maximal sensitivities between 11 and 20 dpso (Table 3) and could therefore especially be applied to detect antibodies in samples taken during the early phase of infection. If patients develop specific IgM against NCP, these antibodies seem to be present for only a short time during the early phase of infection. A sharp decline in the IgM prevalence is to be expected because isotype switching of virus-specific B-cells from IgM to IgG antibody production causes a decline in circulating IgM.[21] The fact that SARS-CoV-2-specific IgM is detected mostly in the early infection phase but only in rare cases[22] invites the question of whether all isotypes should be measured during serodiagnostics.

The agreement analysis revealed a very high correlation between results obtained with the anti-SARS-CoV-2 ELISA (IgG) and the QuantiVac ELISA (IgG). The two samples that showed inconsistent qualitative results between these assays (Table 4, Figure 2) were taken relatively early and late (7 and 116 dpso) in the course of the disease. An explanation for these inconsistencies might be that the assays were incubated using the same aliquot but on different days, hence the experimental conditions might have differed slightly. Another reason might be that the artificial division between positive and negative results does not match the natural range of activity of some samples.

In general, the use of cells infected with the whole SARS-CoV-2 as a substrate has the great advantage of obtaining a high sensitivity due to the presence of the complete antigenic spectrum, as evident in the present IIFT results (Table 2). This is, however, linked to the disadvantage that a positive IIFT result does not allow for a conclusion on the molecular identity of the antigen(s) binding the antibody. In contrast, recombinant cell substrates used in the ELISA technique are ideally suited for the detection and precise identification of antibodies against selected proteins of SARS-CoV-2, such as S1/RBD and NCP. During the purification required for Anti-SARS-CoV-2 NCP ELISA production, tertiary or quaternary structured epitopes are often destroyed or weakened. Nevertheless, a selective loss of reactivity does have advantages, since undesired antibody binding aside from the recombinant target protein can be suppressed. Thereby, the specificity of the ELISA can be improved, which was evident in the present results. Moreover, the ELISA technique has the advantage of yielding results in numeral form, which allows an objective evaluation of results. The use of SARS-CoV-2 IIFT is (currently) reserved for specialized research laboratories with high biosafety restrictions due to the handling of the full virus. Compared to other serological techniques, IIFT is less implemented in standard diagnostic laboratories.

The presence of anti-SARS-CoV-2 S1/RBD IgG antibodies seems to correlate with the development of both virus neutralization and immunity.[1,3,23] Previous research found that titers of neutralizing antibodies were significantly correlated with the levels of anti-RBD IgG,[12] and RBD-specific IgG titers were suggested as a surrogate of neutralization potency against SARS-CoV-2 infection.[24] Nevertheless, it is possible that a patient does not develop antibodies against S1 of SARS-CoV-2, but only against NCP. However, this would suggest that neutralizing antibodies might not be present since binding antibodies against NCP seem to correlate to a lesser degree with immunity than binding antibodies against S1/RBD.[25] The development of immunity to SARS-CoV-2 is induced both by the humoral and the cellular immune response, whereby especially IgG directed against the S1 subunit of the SARS-CoV-2 spike protein and specific long-lived T cells are of great interest, as they are suspected to play the most relevant roles in virus neutralization and sustained immunity. A combination of serological tests to quantify both the interferon-gamma release by SARS-CoV-2-specific T cells, stimulated by SARS-CoV-2 specific antigens and the presence of anti-S1/RBD IgG antibodies will enable differentiated investigation of the immune response in the progression of infection and vaccination. In particular, the determination of relevant antibody concentrations will probably be one of the most important instruments for determining the vaccination success, although it is yet unknown how many antibodies against S1/RBD an individual must produce after vaccination to be protected from COVID-19. Surrogate neutralization assays detect circulating neutralizing antibodies against SARS-CoV-2 that block the interaction between the RBD of SARS-CoV-2 with the ACE2-cell surface receptor of the human host cell, thus supporting a quick diagnostic statement about the degree of immunity. In contrast to plaque-reduction neutralization tests, which require handling of the virus, surrogate neutralization assays can easily be integrated into the laboratory routine and do not require biosafety level 3 laboratories.

A detailed analysis of potential associations between antibody kinetics and disease severity was not performed because symptoms were not systematically recorded and the disease severity could therefore not be rated other than that patients in Panel A had no or mild symptoms and patients in Panel B required hospitalization. Nevertheless, the assay sensitivities were also reported for each panel separately (Table 2). Analysis of temporal profiles was performed on samples from both patient panels because the distribution of samples in the three infection phases was unbalanced between Panels A and B (Table 2).

ELISA or immunoblot techniques might be used in the future to differentiate between reactivities against distinctive SARS-CoV-2 antigens, which might be useful for the determination of biomarkers indicative of early or late infection phases.

In summary, the evidence of this study emphasizes that the assays have different advantages as well as intended purposes. ELISAs provide an insight into the prevalences of specific antibodies against selected antigens of SARS-CoV-2. Due to the heterogeneity of individual antibody responses, an ELISA may not yield positive results for all patients but a combination of ELISAs with different antigens can reduce this diagnostic gap. The three Anti-SARS-CoV-2 ELISAs that detect IgG antibodies can be used to confirm pathogen contact,

starting from week two of the infection, to monitor the humoral response following an acute infection confirmed by direct detection and to detect past infections. The highly immunogenic S1 domain of the spike protein of SARS-CoV-2 is a major target for neutralizing antibodies and showed a good correlation with different test systems for the detection of neutralizing antibodies.[19,26,27] IgA-specific ELISAs might further be used to monitor the immune response in COVID-19 patients. IIFT showed the highest sensitivities due to the use of the whole SARS-CoV-2 as substrate and provide information on whether or not an individual has been infected with SARS-CoV-2.

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CONFLICT OF INTERESTS

Babett Menge, Claudia Messing, Viola Borchardt-Lohölter, and Katja Steinhagen are employees of EUROIMMUN Medizinische Labordiagnostika AG, a company that commercializes serological assays and co-owns a patent application related to immunoassays for the diagnosis of a SARS-CoV-2 infection.

AUTHOR CONTRIBUTIONS

Petra Emmerich completed conceptualization, investigation, funding acquisition, project administration, writing, review, and editing. Ronald von Possel completed investigation, review, and editing. Christoph Hemmer completed the investigation, data curation, review, and editing. Hilte Geerdes-Fenge completed investigation, data curation, review, and editing. Carlos Fritzsche, completed investigation, data curation, review, and editing. Babett Menge completed investigation, data curation, writing, review and editing. Claudia Messing completed the resources, writing, review, and editing. Viola Borchardt-Lohölter completed the formal analysis, visualization, writing, and original draft preparation. Christina Deschermeier completed funding acquisition, project administration, review, and editing. Katja Steinhagen provided resources, completed project administration, writing, review, and editing.

ETHICS STATEMENT

The observational study has been performed in agreement with the Declaration of Helsinki. It has been approved by the Ethics Review Board of the Faculty of Medicine of the University of Rostock (registration number: A2020-0086) and the Ethics Review Board of the Medical Association Hamburg (registration number: 2020-10162-BO-ff). The samples from healthy adults (Panel C, Table 1) were collected via blood donation. Diagnostic leftover samples after completion of all diagnostic measures from children (Panel D, Table 1) were collected by a routine clinical laboratory (Lübeck, Germany). All samples were processed anonymously.

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Communication

SARS-CoV-2 Reinfection in a Healthcare Worker Despite the Presence of Detectable Neutralizing Antibodies

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Abstract: So far, only a few reports about reinfections with SARS-CoV-2 have been published, and they often lack detailed immunological and virological data. We report about a SARS-CoV-2 reinfection with a genetically distinct SARS-CoV-2 variant in an immunocompetent female healthcare worker that has led to a mild disease course. No obvious viral escape mutations were observed in the second virus variant. The infectious virus was shed from the patient during the second infection episode despite the presence of neutralizing antibodies in her blood. Our data indicate that a moderate immune response after the first infection, but not a viral escape, did allow for reinfection and live virus shedding.

Keywords: SARS-CoV-2; reinfection; COVID-19; healthcare worker; immunity; neutralizing antibodies

1. Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has infected more than 135 million people and caused more than 3 million deaths worldwide until now [1]. Infection of the immunocompetent host normally leads to the development of neutralizing antibodies, yet antibody levels may wane over time [2]. Reports of reinfections have been anecdotally published with increasing frequency [3–10]. A more profound understanding of the virological and immunological characteristics of SARS-CoV-2 reinfections may help to define reliable correlates of immunity. Here, we present detailed clinical, virological, and immunological data of the first well-documented case of a SARS-CoV-2 reinfection in a highly exposed immunocompetent female healthcare worker in Germany, which occurred seven months after her initial infection. Importantly, all criteria suggested by the recently published Centers for Disease Control and Prevention (CDC) protocol for investigating

2.5. Virus Neutralization Assay (NT)

Patient sera drawn at different timepoints before and after the reinfection were available for virus neutralization assays. The sera used were decomplemented at 56 °C for 30 min prior to serial dilution in triplicate starting at 1:20. Triplicates of the dilutions were mixed with an equal volume of SARS-CoV-2 (isolate HH-1/isolate HH-24.2), equivalent to 20 TCID50 isolate HH-1/4 TCID50 isolate HH-24.2 per sample. After incubation at 37 °C for one hour, the serum/virus mixtures were transferred to 96-well plates containing 5.0×10^6 cells/plate of Vero cells (ATCC CRL-1008) seeded the previous day. Following incubation for 96 h at 37 °C, supernatants were discarded, and the plates were fixed in 4% formaldehyde and stained with crystal violet. The highest serum dilution protecting 2 of 3 wells from cytopathic effect (CPE) was taken as the neutralizing antibody titer.

3. Results

A 27-year-old female nurse working in a COVID-19 ward of the University Medical Center Hamburg-Eppendorf (UKE), Germany, developed fever, chills, and exertional dyspnea on 18 March 2020. She had no history of any underlying medical conditions and no indication of a compromised immunity. The patient immediately placed herself into self-quarantine and was tested positive for SARS-CoV-2 on March 20 by qRT-PCR of a naso- and oropharyngeal swab with 1×10^6 copies/mL (Figure 1) [21]. While the fever and chills resolved during domestic isolation on March 25, she reported exertional dyspnea for another four weeks. Notably, mild arterial hypertension was first diagnosed a few weeks after the infection, and treatment with bisoprolol was initiated. She returned to work at her ward after 17 days of quarantine, after testing negative for SARS-CoV-2 by qRT-PCR in two subsequent samples. Anti-SARS-CoV-2 spike (S1/S2) IgG levels in July 2020 were 40 AU/mL in July 2020 and thus clearly above the 15 AU/mL detection limit suggested by the manufacturer. At later time points, anti-spike (S1/S2) IgG levels remained stable (60 AU/mL in September 2020) (Figure 1 and Supplementary Materials Table S1). During the night shift of 26 to 27 December 2020, she developed a dry cough and mild rhinorrhea. Routine hospital surveillance by qRT-PCR (twice weekly using gargling solution) returned a positive result for SARS-CoV-2 on 27 December (<5000 copies/mL), 282 days after the first positive test. Consecutive qRT-PCR tests from naso- and oropharyngeal swabs on 28 and 29 December showed viral loads of 9×10^5 and 2×10^7 copies/mL, respectively.

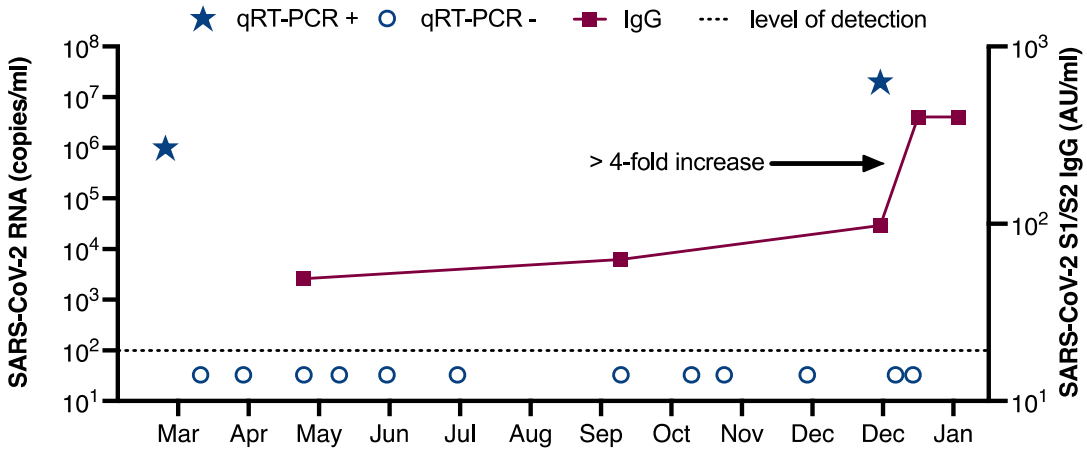

Figure 1. Time course with quantitative detection of SARS CoV-2 RNA [log copies/mL] (blue) and quantitative anti SARS CoV-2 S1/S2 antibody levels [log AU/mL] (red). RNA level was 1×10^6 copies/mL and 2×10^7 copies/mL at first infection and reinfection respectively. Anti-SARS-CoV-2 spike (S1/S2) IgG was 40 IU/mL after first infection and a > 4-fold booster during reinfection was observed (97 AU/mL on 29 December 2020, and >400 AU/mL on 13 January 2021).

suspected SARS-CoV-2 reinfection were met (duration since previous test > 90 days, CT value < 33, symptoms typical of coronavirus disease 2019 (COVID-19), observation of different clades between the first and second infection) [10]. Successful SARS-CoV-2 isolation in cell culture at the time of reinfection proves that shedding of the infectious virus was possible despite the presence of preformed neutralizing antibodies.

2. Materials and Methods

2.1. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

For detection and quantification of SARS-CoV-2 RNA, the fully automated qRT-PCR system Cobas6800 (Roche Molecular Solutions, Pleasanton, CA, USA) was used. The viral load was calculated using the E-gene target (T2) and a standard curve to convert from Ct to viral loads using a commercial quantitative reference standard (from Instand, Düsseldorf, Germany). The linear range and matrix validation have previously been published by our group [11].

2.2. Cell Culture and Virus Isolation

For virus isolation, 500 µL of the swab specimen taken at the time of reinfection (29 December 2020) was used to infect Vero E6 cells (ATCC CRL-1008) [12]. Virus growth was confirmed by qRT-PCR at 72 h post-infection. The supernatant was filtered and transferred to fresh Vero cells. After two days, cells showed a strong cytopathogenic effect (CPE) and the supernatant was harvested and frozen. The median tissue culture infectious dose (TCID50) was calculated based on the infection of Vero cells with serial ten-fold dilutions of the stock and was 1.57×10^7/mL.

2.3. Viral Whole Genome Sequence Analysis

The viral genomes from the first and second episode of infection were sequenced from the pharyngeal swab material and were named #HH-24.I and HH-24.II, respectively. Amplicon sequencing and a bioinformatic analysis were performed, as recently published [13,14]. Library generation was performed using the CleanPlex SARS-CoV-2 Panel (Paragon Genomics, CA, USA). Merged reads were aligned to NC_045512.2 using minimap2 [15] with default settings for short read alignment. Major variants ($\geq$50% of reads) were called using freebayes Bayesian haplotype caller v1.3.1 [16] with ploidy and haplotype independent detection parameters to generate frequency-based calls for all variants passing input thresholds (-K -F 0.5). Input thresholds were set to a minimum coverage of 10 and minimum base quality of 30 (min-coverage 10, -q30). Resulting variants were annotated using ANNOVAR [17]. Pangolin lineage and nextstrain clade assignment of consensus sequences were performed using the pangolin (https://github.com/cov-lineages/pangolin, accessed on 7 March 2021) and nextclade (https://github.com/nextstrain/nextclade, accessed on 7 March 2021) packages. Phylogenetic analysis and tree visualization were performed using nextstrain [18]. To visualize the investigated samples in the context of European SARS-CoV-2 strains, 100 European sequences were randomly sub-sampled from the data available in the GISAID database [19]. For more detailed methods of phylogenetic analyses see [13,14].

2.4. Analysis of Humoral Immune Response

An automated quantitative anti-SARS-CoV-2 IgG assay targeting the S1/S2 spike domain (DiaSorin, Saluggia, Italy) was used according to the manufacturer's recommendations [20]. For the immunofluorescence assay, Vero E6 cells (ATCC CRL-1008) infected with SARS-CoV-2 isolate HH-1 were spotted on glass slides, air-dried, and fixed in ice-cold acetone. Serial dilutions of patient sera were incubated on slides for 1 h at 37 °C. The slides were washed twice with PBS. SARS-CoV-2 antibodies (IgM, IgG, IgA) were detected by indirect immunofluorescence using anti-human IgG (Medac, Cat. No 5230–0288), anti-human IgM (Medac, Cat. No 02-10-03) and anti-human IgA (ThermoFisher, Waltham, MA, USA, Cat. No. A18782) (FITC labeled secondary antibodies and incubation at 37 °C for one hour).

At the time of reinfection, inflammatory parameters were not elevated and leukocyte subsets, as well as total IgM, IgG and IgA levels, were within normal range, without any indication of immunodeficiency (Supplementary Materials Table S2). Symptoms were resolved by 30 December 2020, and she was tested negative for SARS-CoV-2 on 11 January 2021. During the second infection, a rapid increase in the anti-SARS-CoV-2 spike (S1/S2) IgG was observed (97 AU/mL on 29 December 2020 and >400 AU/mL on 13 January 2021). Indirect immunofluorescence assays demonstrated a low IgM titer only at the first infection, and no measurable IgA titers, but significant IgG titer increases after the first and second infection (Supplementary Materials Table S1). The SARS-CoV-2 variant causing the reinfection was successfully isolated in cell culture (Figure 2a). No samples of the first infection were available for virus rescue attempts. Therefore, neutralizing antibody assays (NT IC50) were performed with both our local Hamburg reference isolate (HH-1) [12] and the virus isolated at the time of reinfection (HH-24.II) on patient sera drawn before and after the reinfection. We observed similar neutralizing titers with 1:80 and 1:160 after the first infection and strongly increased neutralizing titers of 1:1280 and 1:2560 after the second infection for both isolates HH-1 and HH-24.II, respectively (Figure 2b).

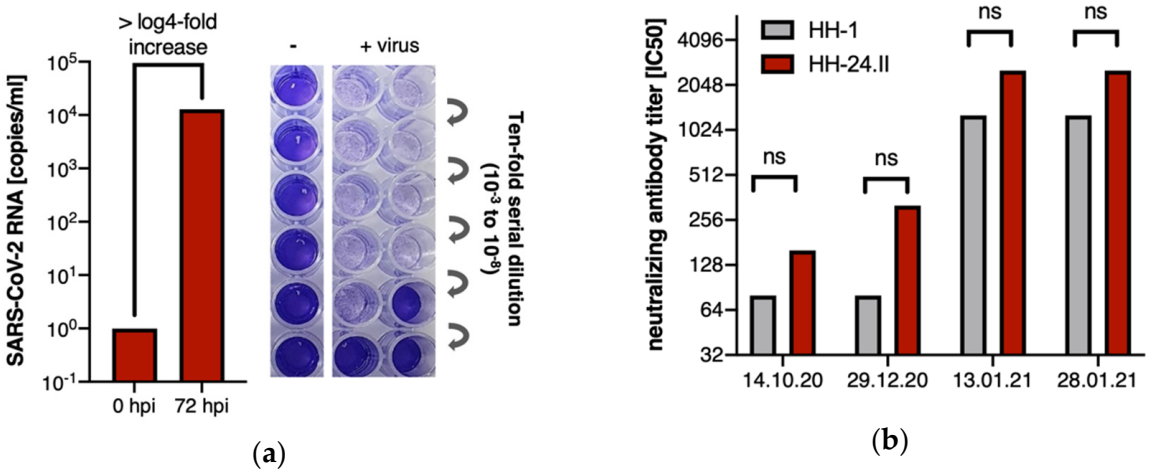

Figure 2. (**a**) Successful isolation of SARS-CoV-2 from swab sample (HH-24.II) reflected by > log4-fold in-crease of viral RNA in the supernatant of Vero cells at 72 h post infection (hpi) detected by qRT-PCR. Quantification of the virus stock produced of the rescued virus in cell culture revealed a TCID50 of 1.57×10^7; (**b**) Virus neutralization assay was performed with serial dilutions of patient sera of one time point before (14 October 2020) and three time points after the reinfection (29 December 2020, 13 January 2021, 28 January 2021) and both the isolated virus of the patient (HH-24.II, red bars) and the HH-1 isolate (gray bars). Neutralizing antibody titers (IC50) were detected at all time points. No significant differences in the neutralizing capacity of the two linages were observed. Between 29 December 2020 and 13 January 2021 a > 4-fold titer increase was observed which reflects a significant increase.

Next generation sequencing (NGS) revealed that the viral sequences from the initial virus variant in March (HH-24.I) and the variant in December (HH-24.II) belonged to pangoline lineages B.3 and B.1.177, respectively [22] (Figure 3b). In total, both sequences differ in 21 positions (Supplementary Materials Table S3 and Figure 3a), including two typical variations in spike proteins A222V and D614G.

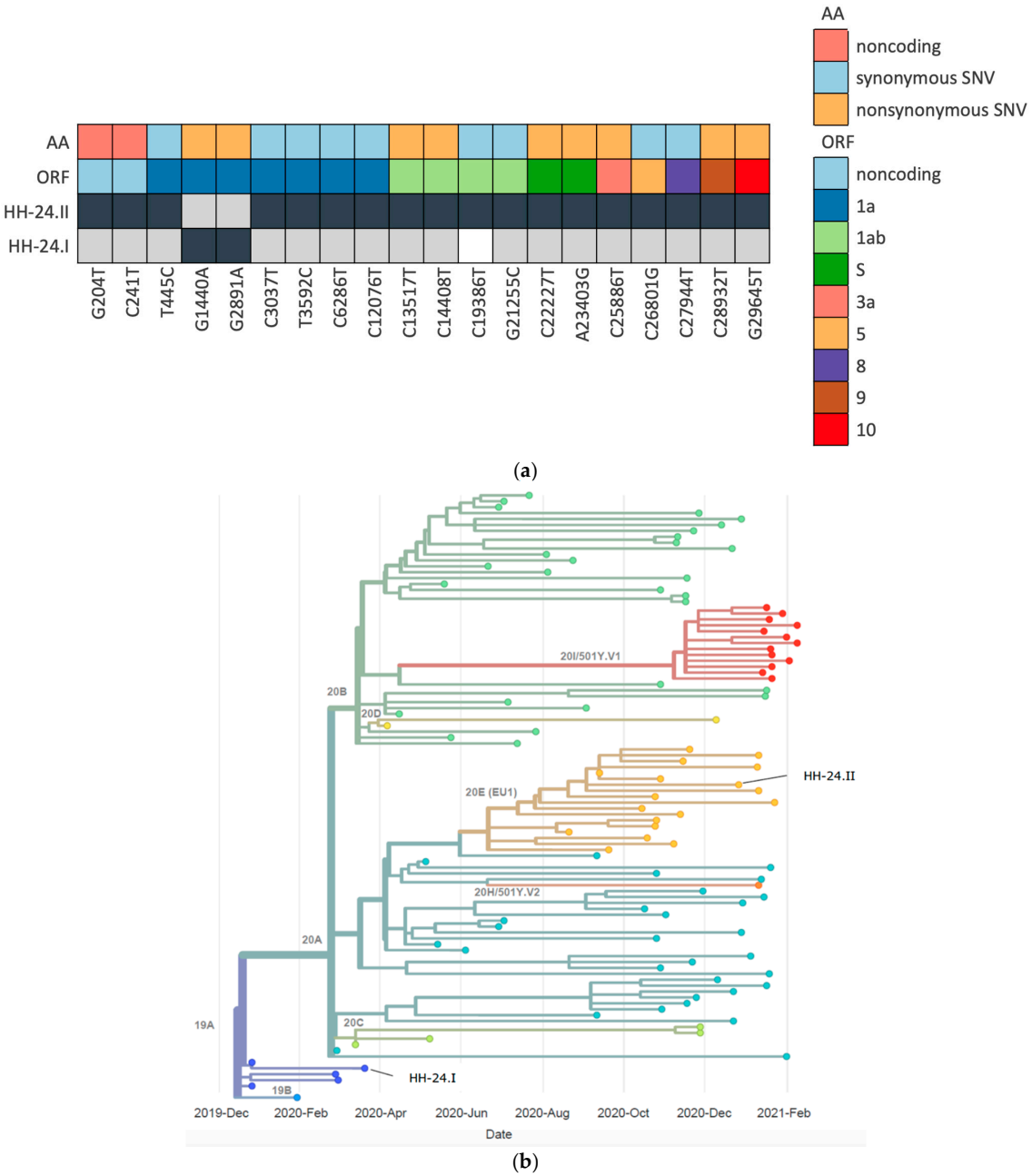

Figure 3. (**a**) Variant nucleotide positions of the sequences HH-24.I and HH-24.II with respect to the reference sequence NC_045512.2 are given in dark grey, whereas light grey boxes indicate reference bases. Uncovered positions by amplicon seq are depicted as white boxes. The color bars on top represents feature assignment (ORF, open reading frame; AA, amino acid changes); (**b**) nextstrain phylogenetic visualization of HH-24.I and HH-24.II in the context of 100 random samples present in Europe from onset of the pandemic until February 2021. Clades are indicated by different colors.

4. Discussion

Homologous reinfections with seasonal human betacoronaviruses within one year have previously been reported and are generally associated with milder symptoms in reinfected patients compared to primary infections [23,24]. The previously reported reinfections with SARS-CoV-2 have been either entirely asymptomatic [3,9], less severe [5], like in our patient, or more severe [4,6–8] in relation to the initial episode. Since reinfection cases are usually noticed because of clinical symptoms, there is likely a reporting bias towards symptomatic cases and post-infection immunity to SARS-CoV-2 may generally protect from severe illness. This is supported by a recent study which demonstrated that seropositive healthcare workers had a substantially reduced risk of SARS-CoV-2 reinfection in the six months following the initial infection [25]. However, correlates of protection remain to be established and it is currently unknown whether the mode of reinfection (e.g., overwhelmingly high titers of SARS-CoV-2, virus-intrinsic virulence), the magnitude, breadth, and quality of the humoral immune responses, waning T-cell immunity or other viral or host factors alone or in combination allow for reinfections [26]. According to recently published case definitions for confirmed SARS-CoV-2 reinfections, viral RNA sequencing is required to differentiate a true reinfection by distinct viral variants from prolonged viral shedding or a reactivation of a lingering virus infection [10,27]. While it has been shown that in most patients, SARS-CoV-2 RNA is undetectable four weeks after the onset of symptoms, prolonged PCR-positivity of up to 104 days after the initial infection has been reported [28,29]. We demonstrate that the same person was infected with SARS-CoV-2 in March 2020 and was reinfected 282 days after the first positive qRT-PCR with a different viral lineage (19A and 20 EU1 respectively). The SARS-CoV-2 sequences retrieved from both infections matched the epidemiology of the typical clades circulating in Germany during the respective time without any additional changes in the essential parts of the spike gene associated with immune escape. Moderate levels of SARS-CoV-2 spike IgG and an NT (IC50) antibody assay were observed after the initial infection. Those were comparable to levels detectable after a mild clinical course of COVID-19 or two weeks after a single injection of an mRNA vaccine [30]. The current reinfection episode was associated with only a few symptoms and was detected only after a routine screening of exposed healthcare workers at our hospital [31]. It resulted in a strong (approximate factor of 10) boost of antibody levels in both the quantitative anti-spike (S1/S2) IgG and in a 4-fold titer increase in the NT (IC50) antibody assay. Notably, the peak virus titer at the time of reinfection was not reduced compared to the initial infection, but the virus was cleared rapidly and was below the limit of detection of diagnostic qRT-PCR after nine days. There is currently little knowledge about the level and quality of humoral immune responses that can render protection from clinical disease, and a better understanding about reinfection events may help to identify serological correlates of immunity. Further studies are needed to investigate whether vaccination after COVID-19 or reinfection reliably boosts the SARS-CoV-2-specific immune responses to levels where sterilizing immunity and longer lasting protection from clinical disease and transmission are achieved.

5. Conclusions

The presented case of a SARS-CoV-2 reinfection of an immunocompetent patient in a high-risk healthcare setting indicates that a moderate immune response after the first infection rather than viral escape did allow for the reinfection. The shedding of the infectious virus in the presence of neutralizing antibodies indicates that during reinfection, further transmission of SARS-CoV-2 is conceivable.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/v13040661/s1, Table S1: Serological results, Table S2: Laboratory parameters at the time of reinfection, Table S3: Variant description.

Author Contributions: Conceptualization, T.T.B., S.P., J.S.z.W. and M.L.; methodology, S.P., R.v.P., D.N., A.G., F.O., P.E., A.R., T.G., M.A., N.F. and M.L.; software, T.T.B., S.P., M.L.; formal analysis,

T.T.B., S.P., R.v.P., J.S.z.W., M.L.; investigation, T.T.B., S.P., R.v.P.; resources, P.E., M.M.A., A.W.L. and M.A.; writing—original draft preparation, T.T.B., S.P., R.v.P., J.S.z.W., M.L.; writing—review and editing, R.K., D.N., S.S., A.G., F.O., P.E., A.R., T.G., P.B., G.A., J.K.K., M.M.A., A.W.L., M.A., N.F.; visualization, T.T.B., S.P., R.v.P., M.L.; supervision, J.S.z.W., M.L. All authors have read and agreed to the published version of the manuscript.

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Limited specificity of commercially available SARS-CoV-2 IgG ELISAs in serum samples of African origin

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Abstract

OBJECTIVES Specific serological tests are mandatory for reliable SARS-CoV-2 diagnostics and seroprevalence studies. Here, we assess the specificities of four commercially available SARS-CoV-2 IgG ELISAs in serum/plasma panels originating from Africa, South America, and Europe.

METHODS 882 serum/plasma samples collected from symptom-free donors before the COVID-19 pandemic in three African countries (Ghana, Madagascar, Nigeria), Colombia, and Germany were analysed with three nucleocapsid-based ELISAs (Euroimmun Anti-SARS-CoV-2-NCP IgG, EDI™ Novel Coronavirus COVID-19 IgG, Mikrogen *recom*Well SARS-CoV-2 IgG), one spike/S1-based ELISA (Euroimmun Anti-SARS-CoV-2 IgG), and in-house common cold CoV ELISAs.

RESULTS High specificity was confirmed for all SARS-CoV-2 IgG ELISAs for Madagascan (93.4–99.4%), Colombian (97.8–100.0%), and German (95.9–100.0%) samples. In contrast, specificity was much lower for the Ghanaian and Nigerian serum panels (Ghana: NCP-based assays 77.7–89.7%, spike/S1-based assay 94.3%; Nigeria: NCP-based assays 39.3–82.7%, spike/S1-based assay 90.7%). 15 of 600 African sera were concordantly classified as positive in both the NCP-based and the spike/S1-based Euroimmun ELISA, but did not inhibit spike/ACE2 binding in a surrogate virus neutralisation test. IgG antibodies elicited by previous infections with common cold CoVs were found in all sample panels, including those from Madagascar, Colombia, and Germany and thus do not inevitably hamper assay specificity. Nevertheless, high levels of IgG antibodies interacting with OC43 NCP were found in all 15 SARS-CoV-2 NCP/spike/S1 ELISA positive sera.

CONCLUSIONS Depending on the chosen antigen and assay protocol, SARS-CoV-2 IgG ELISA specificity may be significantly reduced in certain populations probably due to interference of immune responses to endemic pathogens like other viruses or parasites.

keywords SARS-CoV-2, seroepidemiologic studies, immunoglobulin G, Enzyme-Linked Immunosorbent Assay, specificity, Africa

Sustainable Development Goals (SDGs): Good Health and Well-Being

Introduction

In February and early March 2020, the first COVID-19 cases were reported from African countries with a high level of international contacts (Egypt, Algeria, Nigeria, Morocco, South Africa) [1]. Following these primary importation events, case numbers rapidly increased due to intra-continental and intra-national transmission as well as further importation mainly from Europe and Asia [1, 2]. As of February 14th 2021, 2,723,431 laboratory-confirmed SARS-CoV-2 infections and 68,294 deaths caused by COVID-19 have been reported from the WHO Africa region [3].

Nevertheless, the observed incidence rates are still well below the estimated numbers that were expected based on the socioeconomic challenges many African countries are facing [4] and the numbers observed in Europe and the Americas [3]. Although testing capacities vary widely between African countries, this observation is not solely based on underreporting, but probably reflects both the lower median age of the African population and a different immune status induced by contact with endemic pathogens [4]. Besides modulating COVID-19 morbidity and mortality rates, the latter may also influence performance data of SARS-CoV-2 serological tests; in particular assay specificity may be challenged by previous or current infections with other endemic pathogens [5–7]. Therefore, analyses of this parameter in different populations are an important prerequisite to ensure reliable diagnostic procedures and seroprevalence studies.

Up to now, a plethora of ELISA tests for detection of anti-SARS-Cov-2 antibodies has been developed and commercialised [8]. Usually, these assays employ one of the major immunogenic coronavirus proteins [9] as antigen, that is the nucleocapsid protein (NCP) or defined subdomains of the spike protein (e.g. S1 or the isolated receptor binding domain (RBD)). In this study, we investigate the specificity of three NCP-based SARS-CoV-2 IgG ELISA kits and one spike/S1-based kit in pre-COVID-19 serum/plasma panels from three African countries (Ghana, Madagascar, Nigeria) as well as from South America (Colombia) and Europe (Germany).

Methods

Human serum/plasma samples

The study was performed using stored human serum/-plasma samples collected before the COVID-19 pandemic (Table 1). Collection of samples was approved by

Table 1 Sample panels used in the study

Serum panel	Ghana 1	Ghana 2	Madagascar	Nigeria	Colombia	Germany
Number	150	133	167	150	134	148
Sampling year	2014–2015	1999	2010	2018	2014	2004–2015
Sample type	Serum	Serum	Plasma	Serum	Serum	Serum
Donors	Children	Teens, adults	Pregnant women	Adults	Adults	Adults
Age (median, IQR)	6 (3–7)	22 (16–45)*	23 (20–30)	41 (30–58)†	27 (23–36)	39 (28–48)‡
Sex m/f (*n* (%))	72/78 (48/52)	61/72 (46/54)	0/167 (0/100)	71/79 (47/53)	55/79 (41/59)	79/35 (69/31)‡
Sampling site	Agogo	Villages in the central region of Ghana	Coastal (n = 99) and highland (n = 68) regions	Irrua	Valledupar	Hamburg
Plasmodium spp. Positive (*n* (%))	55 (37.7)§	Not tested	4 (2.4)§	Not tested	Not tested; no previous infections reported	Not tested
SD Bioline Dengue Duo Rapid Test IgG pos (*n* (%))	Not tested	Not tested	Not tested	Not tested	29 (21.6)	Not tested

f, female; IQR, interquartile range; M, male.
*n = 89 (exact age information not available for 43 donors (all 43: age ≥ 18 years))
†n = 149 (age information not available for one donor)
‡n = 114 (sex and exact age information not available for 34 donors (all 34: age ≥ 18 years))
§Parasitaemic samples were identified by microscopy (Ghana) and *Plasmodium*-specific RT-PCR (Madagascar), respectively.

the Ethics Committees of the Kwame Nkrumah University of Science and Technology (Kumasi/Ghana; CHRPE/AP/427/13, 2013), the Comité d'ethique de la Vice Primature Chargée de la Santé Publique (Antananarivo/Madagaskar; no. 051-CE/MINSAN, 2009), the Irrua Specialist Teaching Hospital (Irrua/Nigeria; ISTH/HREC/20171019/28, 2017), the Hospital Rosario Pumarejo de Lopez (Valledupar/Colombia, 2013), and the Medical Association Hamburg/Germany (no. PV4608, 2013). All studies complied with the Declaration of Helsinki. Written informed consent was obtained from all individuals or, in case of minors, from parents or legal guardians before enrolment. Data privacy protection was guaranteed by anonymisation of samples.

Commercially available assays

SARS-CoV-2 IgG ELISAs (Euroimmun Anti-SARS-CoV-2-NCP IgG ELISA (Euroimmun, Germany), Euroimmun Anti-SARS-CoV-2 IgG ELISA (Euroimmun, Germany), EDI™ Novel Coronavirus COVID-19 IgG ELISA (Epitope Diagnostics, US), Mikrogen *recom*Well SARS-CoV-2 IgG ELISA (Mikrogen, Germany), Table 2), line blotting (Euroline Anti-SARS-CoV-2 Profile IgG line blot (Euroimmun)), and surrogate neutralisation assays (sVNT, Genscript, US) were performed and evaluated according to the manufacturers' instructions.

SARS-CoV-2 IgG immunofluorescence testing (IIFT)

Testing was performed as described previously [10] using SARS-CoV-2 infected Vero cells fixed with acetone/methanol.

In-house common cold CoVs ELISA

IgG antibodies were detected using a patented platform technology [11, 12]. Briefly, diluted patient serum/plasma samples were co-incubated together with a biotinylated recombinant antigen for 24 h at 4 °C in a microwell plate coated with a recombinant IgG immune complex specific capture molecule. Following a washing step, the bound IgG/antigen immune complexes were visualised by subsequent application of horseradish peroxidase (HRP)-labelled streptavidin and the colorimetric HRP substrate tetramethylbenzidine (TMB). After stopping the enzymatic reaction, the assay result was generated by measuring the optical density of the solution in the well at 450/620 nm.

As antigens, bacterially expressed, N-terminally truncated common cold CoV nucleoproteins comprising the protein's dimerisation domain were used (OC43 (AIL49389.1): NCPΔ258, HKU1 (AGT17773.1): NCPΔ256/S290F, NL63 (AFO70495.1): NCPΔ222, 229E (NP_073556.1): NCPΔ235). An assay cut-off of OD450-OD620 = 0.3 was determined by comparison of the ELISA results with the results obtained with a commercially available lineblot (Euroline Anti-SARS-CoV-2 Profile IgG, Euroimmun) for 32 German healthy blood donors. Index values (iv) were calculated by dividing the measured OD450-OD620 values by 0.3; samples were classified as negative (iv < 0.7), borderline (0.7 ≤ iv < 1.3), or positive (iv ≥ 1.3).

Table 2 Commercially available ELISA kits used in the study

	Euroimmun Anti-SARS-CoV-2-NCP-ELISA IgG	Euroimmun Anti-SARS-CoV-2-ELISA IgG	*EDI*™ Novel Coronavirus COVID-19 IgG ELISA kit	Mikrogen *recom*Well SARS-CoV-2 IgG
Manufacturer	Euroimmun AG Lübeck, Germany	Euroimmun AG Lübeck, Germany	Epitope Diagnostics, Inc. San Diego, US	Mikrogen GmbH Neuried, Germany
Status	CE-IVD	CE-IVD, FDA EUA	CE-IVD	CE-IVD
Antibody isotype	IgG	IgG	IgG	IgG
Test format	96 well microplate	96 well microplate	96 well microplate	96 well microplate
Test principle	indirect ELISA	indirect ELISA	indirect ELISA	indirect ELISA
Antigen	NCP (modified)	spike (S1 domain)	NCP	NCP
Sample dilution	1:101	1:101	1:101	1:101
Interpretation index value (iv)	negative: iv < 0.8 borderline; 0.8 ≤ iv < 1.1 positive: iv ≥ 1.1	negative: iv < 0.8 borderline; 0.8 ≤ iv < 1.1 positive: iv ≥ 1.1	negative: iv ≤ 0.9 borderline; 0.9 < iv < 1.1 positive: iv ≥ 1.1	negative: iv < 1.0 borderline; 1.0 ≤ iv < 1.2 positive: iv ≥ 1.2

CE, Conformité Européenne; EUA, Emergency Use Authorisation; FDA, Food and Drug Administration; IFU, Instructions for Use; IVD, *in vitro* diagnostics; NCP, nucleocapsid protein.

Statistical analysis

Statistical analyses (calculation of 95% confidence intervals, Fisher's exact test) were performed using GraphPad Prism.

Results

High percentage of false positive SARS-CoV-2 IgG ELISA results in pre-COVID-19 serum samples from Ghana and Nigeria

To assess the specificities of commercially available SARS-CoV-2 IgG ELISA tests in sample panels of different origin, *a priori* SARS-CoV-2 IgG negative samples (Table 1) collected from symptom-free donors before 2019 in Africa (Ghana, Madagascar, Nigeria), South America (Colombia) and Europe (Germany) were analysed with the Euroimmun Anti-SARS-CoV-2-NCP IgG ELISA (Euroimmun, Germany), the Euroimmun Anti-SARS-CoV-2 IgG ELISA (Euroimmun, Germany), the EDI™ Novel Coronavirus COVID-19 IgG ELISA (Epitope Diagnostics, US), and the Mikrogen *recom*Well SARS-CoV-2 IgG ELISA (Mikrogen, Germany) (Table 2). While IgG ELISA specificities where good to excellent for pre-COVID-19 samples originating from Colombia, Madagascar, and Germany, increased false positive rates were observed in *a priori* SARS-CoV-2 IgG negative sera from Ghana and Nigeria (Figure 1, Table 3).

Correlation of NCP- and spike/S1-based ELISA results

The index values obtained with the three assays employing the same antigen (recombinant NCP) showed a clear correlation (Figure 2). In contrast, only a small number of 15 out of 600 African samples (Ghana 1: 4/150, Ghana 2: 3/133, Madagascar: 0/167, Nigeria: 8/150) were concordantly classified as positive by both the NCP-based and the spike/S1-based Euroimmun IgG ELISA (Figure 3a–c), a criterion that is fulfilled by the vast majority of sera from PCR-confirmed COVID-19 patients in the convalescent phase [13]. Positive or borderline serum reactivity with recombinant SARS-CoV-2 NCP (respectively spike/S1) was confirmed for 5/7 (7/7) Ghanaian and 6/8 (7/8) Nigerian samples using a commercially available line blot (Euroline Anti-SARS-CoV-2 Profile IgG line blot (Euroimmun)) (Figure 3d). In addition, antibodies binding to SARS-CoV-2 spike S2 domain were found in 7/7 Ghanaian and 4/8 Nigerian samples (Figure 3a–d). However, all 15 samples tested negative in IgG IIFT using SARS-CoV-2-infected Vero cells [10] and showed no or only very weak activity in a SARS-CoV-2 surrogate virus neutralising test (sVNT, Genscript, US) (Figure 3d).

Assessment of antibodies directed against common cold CoVs

To investigate the influence of previous infections with common cold CoVs on SARS-CoV-2 ELISA specificity, the complete sample panel (n = 882) was assayed for IgG antibodies interacting with the C-terminal dimerisation domains of the OC43, HKU1, NL63, and 229E NCPs using an in-house ELISA. In concordance with the worldwide occurrence of these viruses, considerable fractions of all serum panels, including the ones from Germany and Colombia, reacted positive in these tests (Figure 4).

In addition, the 15 African sera concordantly classified as positive by both the NCP-based and the spike/S1-based Euroimmun ELISA (Figure 3a–c) were also analysed with the Euroline Anti-SARS-CoV-2 Profile IgG line blot (Euroimmun) (Figure 3d). All of them showed a strong signal with OC43 NCP (mean index value 5.6) that by far exceeded the signals detected for the NCPs of HKU1 (mean index value 0.68), NL63 (mean index value 1.2), 229E (mean index value 1.0), and also SARS-CoV-2 (mean index value 1.3). Strikingly, only 2/7 Ghanaian samples but 7/8 Nigerian samples reacted positive in the in-house OC43 ELISA employing an N-terminally truncated OC43 NCP as antigen (Figure 3d).

Influence of Plasmodium parasitaemia on ELISA specificity

Information about *Plasmodium* parasitaemia was only accessible for one of the Ghanaian panels (Ghana 1, 55/150 samples from symptom-free children with microscopically detectable parasitaemia) and the Madagascan (4/167 *Plasmodium*-PCR positive samples) panel. Here, a reduced specificity in Ghanaian parasitaemic *vs.* non-parasitaemic samples was observed for the Euroimmun Anti-SARS-CoV-2-NCP IgG ELISA but not for the other SARS-CoV-2 IgG ELISAs (Table 4).

Discussion

In our study, we report a markedly reduced specificity of four SARS-CoV-2 IgG serological assays in serum samples originating from countries of sub-Saharan Africa, that is Ghana and Nigeria. This observation is in concordance with recent reports from Benin [7], Malawi [14], Tanzania [6], and Zambia [6] describing performance data of commercially available ELISAs [7, 14] and IIFT using eukaryotic cells overexpressing SARS-CoV-2

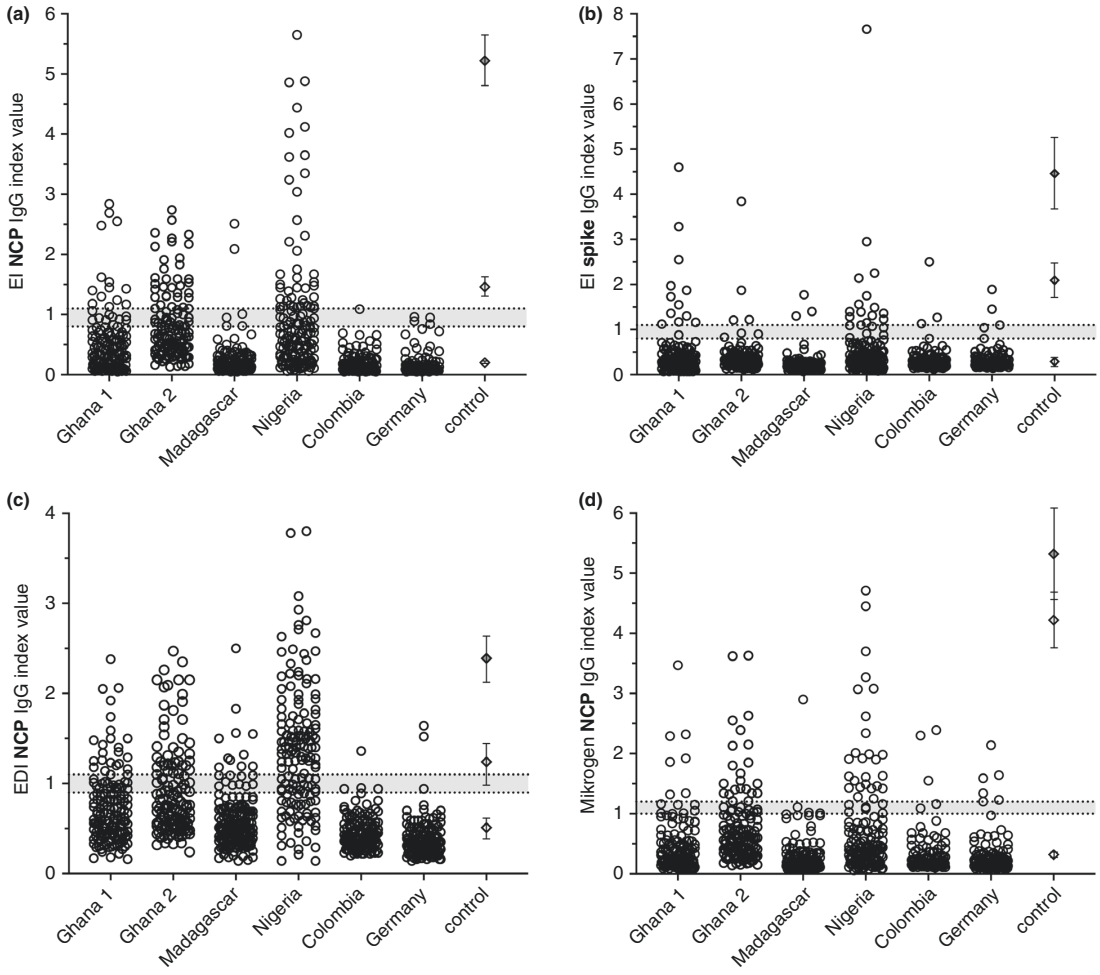


Figure 1 SARS-CoV-2 IgG ELISA results. (a–d) Index values obtained for serum/plasma samples collected before 2019 in three different African countries (Ghana panel 1 (n = 150), Ghana panel 2 (n = 133), Madagascar (n = 167), Nigeria (n = 150)), Colombia (n = 134), and Germany (n = 148) with the Euroimmun (EI) Anti-SARS-CoV-2-NCP IgG ELISA (a), the Euroimmun (EI) Anti-SARS-CoV-2 IgG ELISA (b), the EDI Novel Coronavirus COVID-19 IgG ELISA (c), and the Mikrogen *recom*Well SARS-CoV-2 IgG ELISA (d). Diamonds: index values obtained for two IgG positive COVID-19 patient sera sampled on day 19 post onset of symptoms (dark/-light grey: SARS-CoV-2 IgG IIFT titre 1:640/1:160) and one negative control serum; error bars: standard deviation of 13 (a, c) and 14 (b, d) independent measurements. Dotted lines: negative and positive cut-off values; grey shading indicates index values rated as 'borderline' according to the manufacturers' instructions.

proteins [6], respectively. Positive reactivity with both SARS-CoV-2 NCP and spike/S1 detected by ELISA could be confirmed by line blotting for 11/15 and 14/15 African sera, respectively. Thus, the line blot is slightly less sensitive in picking up the apparently false positive signals in the pre-COVID-19 African sera. As has previously been recognised for serological tests aiming at detection of anti-SARS-CoV-1 antibodies [9], our study revealed a higher rate of false positive assay results in the NCP-based IgG ELISAs than in the spike/S1-based IgG ELISA. This finding may reflect the higher degree of sequence conservation between the immunodominant regions of

Table 3 SARS-CoV-2 IgG ELISA specificities

				Euroimmun Anti-SARS-CoV-2-NCP-ELISA IgG			Euroimmun Anti-SARS-CoV-2-ELISA IgG			*EDI*™ Novel Coronavirus COVID-19 IgG ELISA kit			Mikrogen *recom*Well SARS-CoV-2 IgG		
Antigen				NCP (modified)			Spike S1 domain			NCP			NCP		
Ghana 1	pos	bl	neg	14	11	125	12	1	137	24	17	109	7	7	136
(*n* = 150)	specificity (95% CI)			90.7 (84.8–94.5)			92.0 (86.4–95.5)			84.0 (77.2–89.1)			95.3 (90.5–97.9)		
Ghana 2	pos	bl	neg	34	21	78	4	3	126	39	15	79	22	10	101
(*n* = 133)	specificity (95% CI)			74.4 (66.4–81.1)			97.0 (92.3–99.1)			70.7 (62.4–77.8)			83.5 (76.2–88.9)		
Ghana all	pos	bl	neg	48	32	203	16	4	263	63	32	188	29	17	237
(*n* = 283)	specificity (95% CI)			83.0 (78.2–87.0)			94.3 (90.9–96.6)			77.7 (72.5–82.2)			89.7 (85.6–92.8)		
Madagascar	pos	bl	neg	2	4	161	3	0	164	11	10	146	1	3	163
(*n* = 167)	specificity (95% CI)			98.8 (95.5–99.9)			98.2 (94.6–99.6)			93.4 (88.5–96.4)			99.4 (96.3–100.0)		
Nigeria	pos	bl	neg	42	18	90	14	7	129	91	19	40	26	6	118
(*n* = 150)	specificity (95% CI)			72.0 (64.3–78.6)			90.7 (84.8–94.5)			39.3 (31.9–47.3)			82.7 (75.8–87.9)		
Colombia	pos	bl	neg	0	1	133	3	1	130	1	3	130	3	2	129
(*n* = 134)	specificity (95% CI)			100.0 (96.6–100.0)			97.8 (93.3–99.5)			99.2 (95.5–100.0)			97.8 (93.3–99.5)		
Germany	pos	bl	neg	0	4	144	2	3	143	2	1	145	6	0	142
(*n* = 148)	specificity (95% CI)			100.0 (97.0–100.0)			98.6 (94.9–99.9)			98.6 (94.9–99.9)			95.9 (91.2–98.3)		

CI, confidence interval; pos/bl/neg, number of samples rated as positive (pos), borderline (bl), and negative (neg) by the respective test. For calculation of specificities, both negative and borderline results were classified as 'not positive'.
Cells indicating the number of samples tested positive, borderline, and negative with the respective assay were shaded dark grey, medium grey, and light grey, respectively.

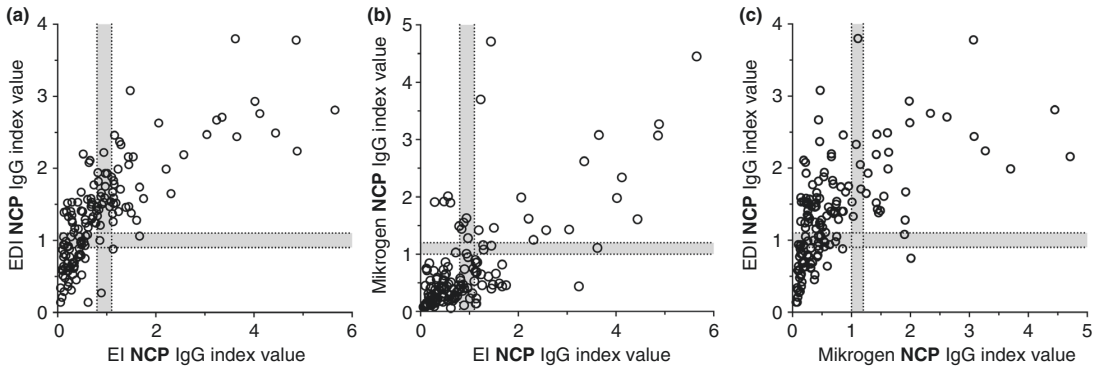
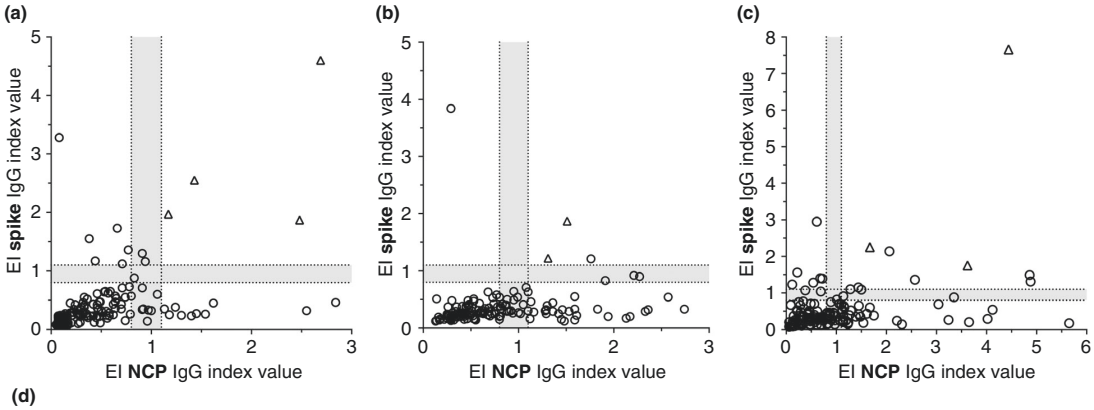


Figure 2 Correlation of SARS-CoV-2 IgG ELISA test results (NCP-based tests), exemplified for the Nigerian sample panel (*n* = 150). (a) Euroimmun (EI) Anti-SARS-CoV-2-NCP IgG ELISA vs. EDI Novel Coronavirus COVID-19 IgG ELISA, (b) Euroimmun (EI) Anti-SARS-CoV-2-NCP IgG ELISA *vs*. Mikrogen *recom*Well SARS-CoV-2 IgG ELISA, (c) Mikrogen *recom*Well SARS-CoV-2 IgG ELISA *vs*. EDI Novel Coronavirus COVID-19 IgG ELISA. Dotted lines: negative and positive cut-off values; grey shading indicates index values rated as 'borderline' according to the manufacturers' instructions.

the coronavirus NCPs [9], rendering assays based on this antigen more prone to cross-reactivity than tests employing the less conserved spike protein.

Indeed, one possible cause of the observed limited specificity of SARS-CoV-2 IgG ELISAs may be cross-reactivity with antibodies elicited by previous infections with other CoVs [6]. We detected such antibodies in considerable fractions of those sample panels for which excellent SARS-CoV-2 IgG ELISA specificity has been observed. Thus, their presence does not inevitably lead to



(d)

ID	sVNT % inh	Euroimmun ELISA		Euroimmun Lineblot							in-house ELISA			
		SARS-CoV-2		SARS-CoV-2			NCP				NCP-CTD			
		NCP	S1	NCP	S1	S2	OC43	HKU1	NL63	229E	OC43	HKU1	NL63	229E
G1-1	1.9	1.2	2.0	0.4	3.1	3.5	5.0	0.7	0.1	0.1	1.5	0.5	0.1	0.1
G1-2	9.4	2.7	4.6	0.9	5.3	5.2	6.2	0.5	0.4	0.2	0.4	0.3	0.2	0.2
G1-3	10.0	1.4	2.5	1.1	3.6	1.9	5.1	0.2	0.6	0.5	0.2	0.2	0.3	0.2
G1-4	4.1	2.5	1.9	0.5	1.6	2.4	4.1	0.9	2.8	1.2	2.9	0.3	10.1	0.2
G2-1	25.6	1.8	1.2	0.9	1.0	0.6	1.6	0.2	0.3	0.4	0.6	0.2	0.2	0.1
G2-2	24.6	1.5	1.9	0.9	1.7	2.2	7.1	0.4	2.1	0.9	0.2	0.3	2.0	0.1
G2-3	21.2	1.3	1.2	1.4	2.4	4.3	7.3	0.2	0.8	0.2	0.2	0.3	0.2	0.1
N-1	24.4	3.6	1.8	3.7	1.3	1.0	5.3	0.3	1.0	2.8	5.3	0.2	6.0	0.2
N-2	10.7	4.9	1.3	2.3	0.4	0.3	6.2	0.4	1.4	0.5	1.5	0.2	0.2	0.2
N-3	23.7	4.4	7.7	1.1	6.8	5.6	7.2	2.7	1.4	0.8	7.5	2.8	0.6	0.3
N-4	28.4	4.9	1.5	3.3	1.8	0.8	4.1	1.0	3.0	2.2	1.4	0.6	7.4	0.3
N-5	19.4	2.6	1.4	0.3	0.7	0.2	5.8	0.5	1.1	0.7	1.5	0.4	0.3	0.2
N-6	21.5	2.1	2.1	1.2	1.6	0.2	4.2	0.3	0.7	3.5	4.3	0.9	1.1	2.6
N-7	27.7	1.7	2.2	1.3	2.2	2.3	5.1	0.9	1.5	0.7	7.4	2.9	9.8	2.0
N-8	28.3	1.4	1.2	0.4	1.0	0.3	5.0	1.0	1.2	0.7	1.0	0.7	0.9	0.3

Figure 3 (a–c) Correlation of Euroimmun Anti-SARS-CoV-2-NCP IgG ELISA and Euroimmun Anti-SARS-CoV-2 IgG ELISA test results (NCP-based vs. spike/S1-based test). Shown are index values for sample panels Ghana 1 (a), Ghana 2 (b), and Nigeria (c). Triangles indicate spike/S1/NCP IgG positive samples containing IgG antibodies reacting positive with SARS-CoV-2 spike/S2 in the Euroimmun line blot. (D) ELISA, sVNT and line blot results for sera testing positive in both the Euroimmun NCP- and spike/S1-based IgG ELISA. Serum samples (Ghana 1 (G1): $n = 4$, Ghana 2 (G2): $n = 3$, Nigeria (N): $n = 8$)) were tested using the SARS-CoV-2 sVNT (Genscript) and the Euroline Anti-SARS-CoV-2 Profile IgG (Euroimmun) according to the manufacturer's instructions. Rating of index values (iv): Euroimmun ELISA: negative: iv < 0.8, borderline; 0.8 ≤ iv < 1.1, positive: iv ≥ 1.1; Line blot: negative: iv < 0.6, borderline: 0.6 ≤ iv < 1.0, positive: iv ≥ 1.0; in-house ELISA: negative: iv < 0.7, borderline: 0.7 ≤ iv < 1.3, positive: iv ≥ 1.3; sVNT: negative: % inhibition < 20.0, borderline; 20.0 ≤ % inh < 30.0, positive: % inh ≥ 30.0; CTD: C-terminal domain; dark grey fields: positive; light grey fields: borderline.

false positive signals. Nevertheless, the most challenging sample panel originating from Nigerian donors displayed the by far highest percentage of OC43 in-house-ELISA reactive samples (43.3%) and all 15 African samples (8 from Nigeria, 7 from Ghana) showing up false positive in both NCP- and spike/S1-based assays show extraordinarily high signals in an OC43 NCP line blot. Strikingly, all 8 Nigerian samples, but only 2/7 Ghanaian samples reacted with an N-terminally truncated, recombinant OC43 NCP in the in-house ELISA. This discrepancy between the line blot and the in-house ELISA results may be caused by (i) a lower sensitivity of the ELISA employing a truncated NCP displaying fewer epitopes as antigen (although an ELISA based on similar fragment of SARS-CoV-1 has been shown to detect anti-SARS-CoV-1 antibodies with excellent sensitivity [15]), (ii) a different conformational status of the used antigens (line blot: at least partially denatured, ELISA:

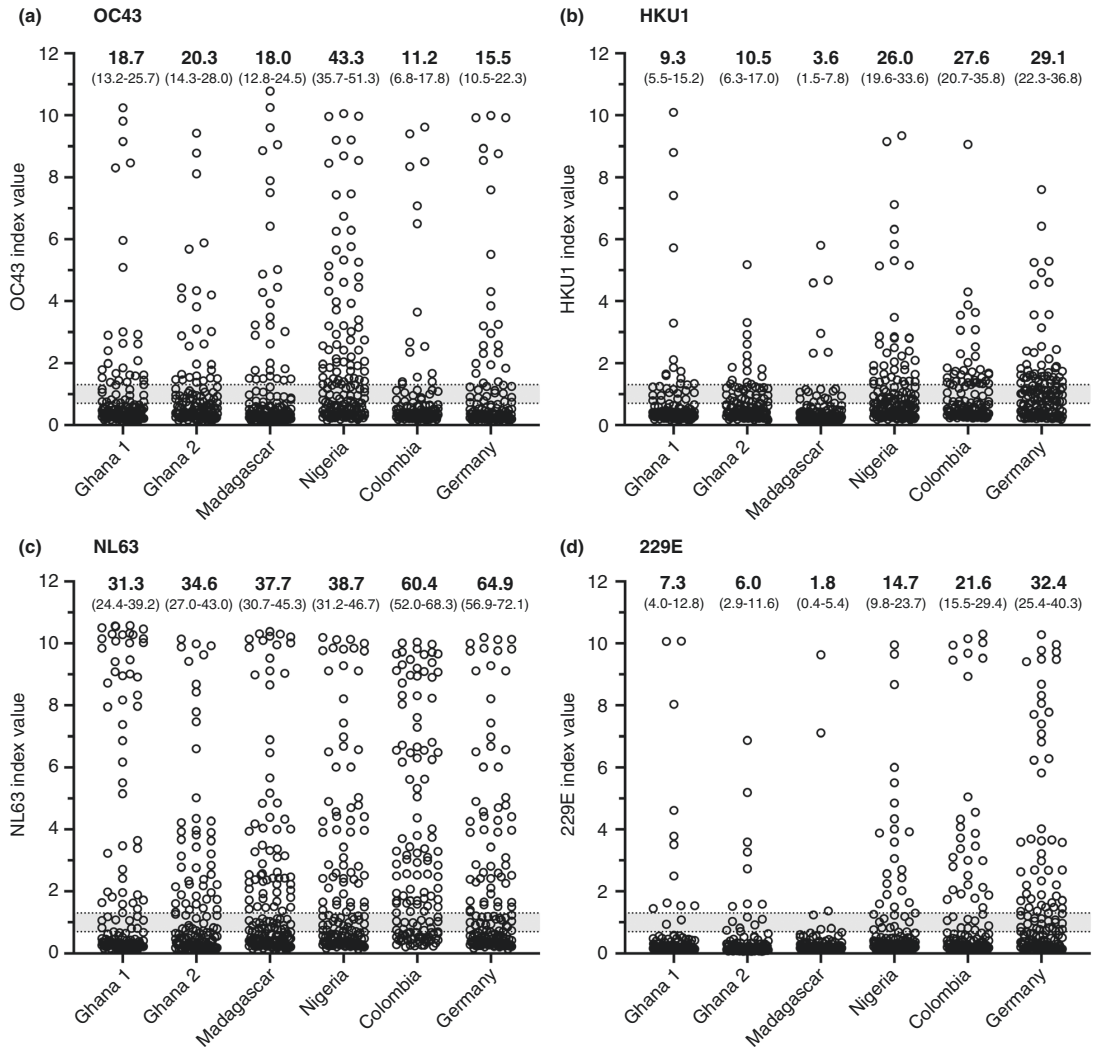


Figure 4 Common cold CoVs ELISA results. Index values obtained for the sample panels from Ghana (1: $n = 150$, 2: $n = 133$), Madagascar ($n = 167$), Nigeria ($n = 150$), Colombia ($n = 134$), and Germany ($n = 148$) using an in-house IgG ELISA protocol employing the C-terminal dimerisation domain of (a) OC43 NCP, (b) HKU1 NCP, (c) NL63 NCP, and (d) 229E NCP as antigen. Bold numbers: % of samples for which an iv ≥ 1.3 was obtained, numbers in brackets: 95% confidence interval. Grey shading indicates ivs rated as 'borderline' ($0.7 \leq$ iv < 1.3).

natively folded, soluble protein), (iii) blockage of epitopes by biotinylation of the ELISA antigen, (iv) different OC43 genotypes or closely related CoVs eliciting cross-reactive immune responses in the Nigerian and Ghanaian samples, respectively (this assumption is supported by the finding that 6/7 Ghanaian samples but only 4/8 Nigerian samples react borderline or positive with SARS-CoV-2 spike/S2 in the line blot). Taken together, at least some of the observed false positive signals could be due to previous infections with other coronaviruses sharing B cell epitopes with both OC43 and SARS-CoV-2.

Table 4 SARS-CoV-2 IgG ELISA specificities for donor subgroups from Ghana with and without *Plasmodium* parasitaemia (Ghana panel 1, symptom-free children)

				Euroimmun Anti-SARS-CoV-2-NCP-ELISA IgG			Euroimmun Anti-SARS-CoV-2-ELISA IgG			*EDI*™ Novel Coronavirus COVID-19 IgG ELISA kit			Mikrogen *recom*Well SARS-CoV-2 IgsG		
Parasitaemic (n = 55)	pos	bl	neg	9	6	40	6	1	48	12	9	34	1	4	50
	specificity (95% CI)			83.6 (71.5–91.4)			89.1 (77.8–95.3)			78.2 (65.5–87.2)			98.2 (89.5–100.0)		
Not parasitaemic (n = 95)	pos	bl	neg	5	5	85	6	0	89	12	8	75	6	3	86
	specificity (95% CI)			94.7 (88.0–98.0)			93.7 (86.6–97.3)			87.4 (79.1–92.8)			93.7 (86.6–97.3)		
P value				0.0387			0.3578			0.1675			0.4235		

pos/bl/neg: number of samples rated as positive (pos), borderline (bl), and negative (neg) by the respective test. CI: confidence interval. For calculation of specificities, both negative and borderline results were classified as 'not positive'. P values were calculated using Fisher's exact test (2 x 2 contingency table, pos versus 'not positive').
Cells indicating the number of samples tested positive, borderline, and negative with the respective assay were shaded dark grey, medium grey, and light grey, respectively.

As has already been reported for ZIKV [16] and SARS-CoV-2 IgG ELISAs [7], hypergammaglobulinaemia resulting from polyclonal B-cell activation induced by pathogens like *Plasmodia* can challenge assay specificity. Indeed, we observed a slightly reduced specificity of the Euroimmun Anti-SARS-CoV-2-NCP IgG ELISA in Ghanaian parasitaemic *vs*. non-parasitaemic samples (83.6% (95% CI: 71.5%-91.4%) *vs*. 94.7% (95% CI: 88.0%-98.0%), $P = 0.0387$)). While *Plasmodium falciparum* malaria is holoendemic in many regions in Ghana and Nigeria [17], length and intensity of malaria transmission varies significantly between different areas in Madagascar [17, 18]. In Colombia, 78% of the population lives in malaria free areas, and *Plasmodium falciparum* accounts only for 58% of malaria cases (42%: *Plasmodium vivax*)[19]. None of the 134 Colombian donors included in our study reported a previous malaria episode.

Recently, Lustig *et al.* [5] observed false positive results of spike-based IgA and IgG ELISAs in sera from donors with acute or past dengue virus infection. In the context of the study for which the samples were originally collected, 29 (21.6%) of the 134 Colombian donors had been tested positive in the SD Bioline Dengue Duo IgG Rapid Test (Alere/Abbott, USA), indicating a high titre of anti-DENV IgG antibodies [20]. Indeed, two of the three Colombian sera tested positive in the spike/S1-based Euroimmun Anti-SARS-CoV-2 ELISA belonged to this subgroup; a robust statistical evaluation of this finding would require significantly higher sample numbers.

Although antibodies binding to all three recombinant SARS-CoV-2 antigens presented on the Euroline Anti-SARS-CoV-2 Profile IgG line blot (NCP, spike/S1, spike/S2) were detected in some pre-COVID-19 African sera generating false positive ELISA results (Figure 3d), none of these samples showed up false positive in the in-house IgG IIFT using SARS-CoV-2 infected Vero cells. A possible explanation for this surprising observation might be the use of full virus displaying native proteins in their natural context vs. highly concentrated, purified, recombinant antigens. Indeed, increased numbers of false positive SARS-CoV-2 IgG IIFT results have been observed in African sera in a study using transfected HEK cells overexpressing SARS-CoV-2 antigens [6]. Furthermore, the specific SARS-CoV-2 staining pattern might be only generated when antibodies recognising different viral proteins with sufficiently high affinity are present in a tested serum. Interestingly, line blot intensity patterns for the different SARS-CoV-2 proteins (NCP, spike/S1, spike/S2) vary strongly between the 15 sera generating false positive signals in both the NCP and Spike IgG ELISA (Figure 3d).

Limitations of the study

Our study has some limitations that have to be acknowledged when interpreting the presented data. First of all, the sample panels (originating from previous studies with different scientific objectives) do not reflect representative cross-sections of the respective countries' populations and also comparability between sample panels is limited. Although in total 600 African samples have been analysed, the number of samples per country giving rise to false positive SARS-CoV-2 IgG assay results is still

relatively small, impeding statistical analyses. Furthermore, limited accessible sample volumes prevented us from performing material-intensive assays as the SD Bioline Dengue Duo IgG Rapid Test or *Plasmodium*-specific RT-PCR for the complete panel.

Conclusions

Our work shows that several commercially available SARS-CoV-2 IgG ELISAs, especially those employing recombinant NCP as antigen, are prone to generate a high number of false positive results when testing serum/-plasma samples originating from Sub-Saharan Africa. Beside other factors, high antibody titres resulting from previous infections with other coronaviruses and/or acute or previous malaria episodes may cause this phenomenon.

Based on these findings, the following recommendations should be considered when testing sera from African individuals or performing SARS-CoV-2 seroprevalence studies (not only) in Africa: (i) Carefully assess false positive signals obtained with the chosen serological test(s) in the target population (using *a priori* SARS-CoV-2 IgG negative serum samples which were stocked before 2019); if necessary adjust assay cut-off [14]. (ii) Be aware of potentially interfering/cross-reacting endemic pathogens and carefully interpret SARS-CoV-2 IgG test results in this context. (iii) If possible, combine information from two independent serological tests employing different antigens. In the context of the upcoming implementation of SARS-CoV-2 vaccine programmes, even other antigens than NCP and spike, for example ORF8 and ORF3b [21], might become relevant for differentiation of natural infections from vaccine responses although their use is limited due to high genetic variability [22, 23]. (iv) Re-evaluate samples generating a positive ELISA result by SARS-CoV-2 IgG IIFT and SARS-CoV-2 neutralisation testing.

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Fcγ-Receptor-Based Enzyme-Linked Immunosorbent Assays for Sensitive, Specific, and Persistent Detection of Anti-SARS-CoV-2 Nucleocapsid Protein IgG Antibodies in Human Sera

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ABSTRACT Sensitive and specific serological tests are mandatory for epidemiological studies evaluating severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) prevalence as well as coronavirus disease 2019 (COVID-19) morbidity and mortality rates. The accuracy of results is challenged by antibody waning after convalescence and by cross-reactivity induced by previous infections with other pathogens. By employing a patented platform technology based on capturing antigen-antibody complexes with a solid-phase-bound Fcγ receptor (FcγR) and truncated nucleocapsid protein as the antigen, two SARS-CoV-2 IgG enzyme-linked immunosorbent assays (ELISAs), featuring different serum and antigen dilutions, were developed. Validation was performed using a serum panel comprising 213 longitudinal samples from 35 COVID-19 patients and a negative-control panel consisting of 790 pre-COVID-19 samples from different regions of the world. While both assays show similar diagnostic sensitivities in the early convalescent phase, ELISA 2 (featuring a higher serum concentration) enables SARS-CoV-2 IgG antibody detection for a significantly longer time postinfection (≥15 months). Correspondingly, analytical sensitivity referenced to indirect immunofluorescence testing (IIFT) is significantly higher for ELISA 2 in samples with a titer of ≤1:640; for high-titer samples, a prozone effect is observed for ELISA 2. The specificities of both ELISAs were excellent not only for pre-COVID-19 serum samples from Europe, Asia, and South America but also for several challenging African sample panels. The SARS-CoV-2 IgG FcγR ELISAs, methodically combining antigen-antibody binding in solution and isotype-specific detection of immune complexes, are valuable tools for seroprevalence studies requiring the (long-term) detection of anti-SARS-CoV-2 IgG antibodies in populations with a challenging immunological background and/or in which spike-protein-based vaccine programs have been rolled out.

KEYWORDS immunoassay, infectious disease, immunoglobulins, laboratory methods and tools, viral diseases

Reliable serological assays are mandatory for monitoring the progression of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic, particularly in regions of the world where access to medical care and molecular testing capacities are limited. To enable a conclusive estimation of SARS-CoV-2 seroprevalence, a SARS-CoV-2 IgG assay has

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to fulfill several crucial requirements. First, due to postconvalescence antibody waning (1), high assay sensitivity in coronavirus disease 2019 (COVID-19) patient samples collected more than 6 months after infection is needed. Second, although SARS-CoV-2 IgG assay specificity in general is good to excellent in sample panels from Europe, Asia, and the United States (2), several African serum panels were recently shown to strongly challenge assay specificity due to a different immunological background caused by endemic infectious diseases such as *Plasmodium falciparum* malaria and others (3–6). Therefore, if use in these settings is intended, critical assessment and (where necessary) optimization of assay specificity are required. Third, with the progressive rollout of country-specific vaccine programs, humoral immune responses induced by natural SARS-CoV-2 infection must be differentiated from vaccine responses. If spike-based vaccines like, e.g., Comirnaty (BioNTech/Pfizer) or Vaxzevria (AstraZeneca) are administered, assays employing the SARS-CoV-2 nucleocapsid protein (NCP) (7) as the antigen still allow the unequivocal detection of antibodies induced by natural SARS-CoV-2 infection. Notably, highly sensitive assays will be required to detect the apparently reduced humoral anti-NCP response that has been observed in vaccine breakthrough infections (8). In contrast, vaccines based on inactivated SARS-CoV-2 (e.g., the Sinopharm vaccine) potentially elicit antibodies against all structural virus proteins. In principle, natural infection in individuals immunized with those vaccines could be proven by the detection of antibodies targeting nonstructural proteins like open reading frame 3 (ORF3) or ORF8 (9). Nevertheless, several SARS-CoV-2 lineages lacking the functional expression of these proteins have been described (10, 11).

During the last few years, we have employed a patented platform technology (12, 13) methodically combining antigen-antibody binding in solution with isotype-specific detection of *in vitro*-formed immune complexes to develop several highly sensitive and specific plate-based enzyme-linked immunosorbent assays (ELISAs) for the detection of both IgM and IgG antibodies directed against viral pathogens such as dengue virus (DENV) (14), Lassa virus (LASV) (15), Crimean-Congo hemorrhagic fever virus (CCHFV) (16), and Zika virus (ZIKV) (17, 18). Briefly, for the detection of IgG, patient sera are coincubated with soluble, labeled recombinant antigen (and, if necessary, unlabeled competitor molecules suppressing cross-reactive antibody binding) on a 96-well plate coated with the recombinantly produced immunoglobulin-like (Ig) domain of *Homo sapiens* Fcg receptor IIA_{H131} (*Hs*FcγRIIA_{H131}) (mediating the isotype-specific binding of IgG-antigen immune complexes). Thereby, the use of natively folded antigen in the liquid phase guarantees the preservation of conformational epitopes and prevents nonspecific interactions with hydrophobic amino acid stretches that may be exposed in partially unfolded or denatured proteins. If necessary, even high serum concentrations can be applied without increasing the assay background (19). Captured immune complexes are then detected by a colorimetric reaction that can be quantified using a conventional ELISA reader.

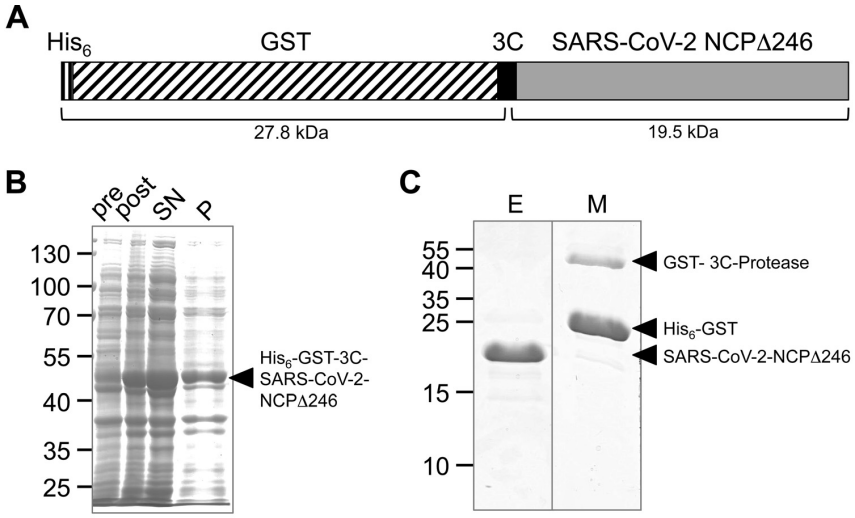
Here, we utilize this technology to develop ELISA protocols for the detection of antibodies directed against the SARS-CoV-2 NCP and present detailed validation data on reproducibility, diagnostic and analytical sensitivities, specificity, and interference.

MATERIALS AND METHODS

Generation of prokaryotic expression vectors (pOPIN-J-CoV-N2b/SB/N3). Amplicons encoding the N2b/SB/N3 domain (7) of coronavirus (CoV) NCPs were generated by PCR (see Table S1 in the supplemental material) using either random-primed cDNA transcribed from virus RNA or synthesized cDNA (codon optimized for expression in *Escherichia coli*; GenScript) as a template and inserted by in-fusion cloning (TaKaRa) into pOPIN-J (20) cut with HindIII/KpnI. Insert sequences were verified using Sanger sequencing (Seqlab).

Recombinant expression and purification of recombinant antigens. Expression vectors encoding tandem-affinity-tagged, N-terminally truncated CoV NCPs (Fig. 1A) were transformed into *E. coli* BL21 RIG cells. Upon induction with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), recombinant fusion proteins were expressed for 3 h at 30°C or 37°C. Harvested bacteria were lysed, and fusion proteins were purified from the soluble fraction of the bacterial lysate (Fig. 1B) by Ni-nitrilotriacetic acid (NTA) chromatography under native conditions followed by on-column cleavage of the tandem His_6–glutathione *S*-transferase (GST) tag (Fig. 1C) as described previously (16).

Biotinylation of recombinant antigens. Purified, recombinant antigens were covalently coupled to biotin using EZ-Link sulfo-NHS (*N*-hydroxysuccinimide)-biotin reagent (Thermo Fisher) at a 30-fold molar excess according to the manufacturer's instructions. Biotinylated proteins were stored in 0.5× phosphate-buffered saline (PBS)–50% glycerol at −20°C.



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	SARS-CoV-2 NCPΔ246	SARS-CoV-1 NCPΔ247	MERS NCPΔ238	HKU1 NCPΔ256	OC43 NCPΔ258	NL63 NCPΔ222	229E NCPΔ235
SARS-CoV-2 NCPΔ246	100.0	88.6	40.8	26.4	25.3	20.5	19.9
SARS-CoV-1 NCPΔ247	94.3	100.0	39.8	26.4	25.3	19.1	19.7
MERS NCPΔ238	53.6	51.9	100.0	27.8	29.9	19.4	20.0
HKU1 NCPΔ256	43.5	43.0	44.8	100.0	59.6	18.6	18.6
OC43 NCPΔ258	43.3	42.3	46.4	71.5	100.0	20.2	17.6
NL63 NCPΔ222	37.5	35.4	35.0	34.6	37.3	100.0	38.2
229E NCPΔ235	34.7	33.7	37.2	31.4	30.6	62.4	100.0

FIG 1 Prokaryotic expression and purification of N-terminally truncated SARS-CoV-2 NCP. (A) Schematic representation of the fusion protein His_6–GST–3C–SARS-CoV-2 NCPΔ246 (calculated molecular weight, 47.3 kDa). (B) Total lysates preinduction (pre) and postinduction (post) and soluble (supernatant [SN]) and insoluble (pellet [P]) lysate fractions. (C) Eluate (E) and matrix (M) after on-column cleavage. (D) Amino acid sequence comparison of truncated CoV NCPs. Above the diagonal/light gray shading are identity scores (percent), and below the diagonal/dark gray shading are similarity scores (percent). MERS, Middle East respiratory syndrome.

Human sera. This study complies with the Declaration of Helsinki. Written informed consent was obtained from all individuals or, in the case of minors, from parents or legal guardians before enrollment. Data privacy protection was guaranteed by the anonymization of samples.

(i) COVID-19 patient sera. Longitudinal serum samples (range of days after the onset of symptoms [days postonset {dpo}], 10 to 446; number of serum samples per patient, 1 to 13) from 35 patients (median age, 45 years; age range, 19 to 64 years; 23 females and 12 males) with PCR-confirmed SARS-CoV-2 infection collected in Germany between March 2020 and May 2021 were analyzed. All patients displayed ambulatory mild disease (21). Fifteen patients received a COVID-19 vaccination after more than 6 months of convalescence (10 BioNTech/Pfizer, 2 Moderna, and 3 AstraZeneca). The collection of samples was approved by the Medical Association, Hamburg, Germany (no. 2020-10162-BO-ff). World Health Organization (WHO) international standard plasma 20/136 (1,000 binding antibody units [BAU]/mL) was obtained from the National Institute for Biological Standards and Control (NIBSC).

(ii) *A priori* SARS-CoV-2 IgG-negative control sera. The study was performed using stored human serum/plasma samples from symptom-free donors collected before the COVID-19 pandemic in Germany (2004 to 2015), Ghana (1999 and 2014 to 2015), Madagascar (2010), Nigeria (2018), Colombia (2014), and Lao People's Democratic Republic (PDR) (2014) (Table S2) (3). The collection of samples was approved by the Ethics Committees of the Kwame Nkrumah University of Science and Technology (Kumasi, Ghana) (CHRPE/AP/427/13, 2013), the Comité d'Ethique de la Vice Primature Chargée de la Santé Publique (Antananarivo, Madagascar) (no. 051-CE/MINSAN, 2009), the Irrua Specialist Teaching Hospital (Irrua, Nigeria) (ISTH/HREC/20171019/28, 2017), the Hospital Rosario Pumarejo de Lopez (Valledupar, Colombia) (2013), Lao People's Democratic Republic (no. 030/NECHR), and the Medical Association, Hamburg, Germany (no. PV4608, 2013).

SARS-CoV-2 IgG indirect immunofluorescence testing (IIFT). Immunofluorescence analysis detecting IgG antibodies targeting SARS-CoV-2 full-virus antigens was performed as described previously (22). Briefly, SARS-CoV-2-infected Vero-E6 cells (ATCC CRL-1586) were fixed with acetone-methanol and incubated with serial dilutions of patient sera. After washing, the detection of bound IgG was performed using a fluorescein isothiocyanate (FITC)-labeled anti-IgG antibody (Sifin).

SARS-CoV-2 IgG ELISAs 1 and 2. For the SARS-CoV-2 IgG FcγR ELISA, Nunc MaxiSorp ELISA plates were coated with 5 μg/mL *Hs*FcγR-Igl (CD32 [$FcγRIIA_{H131}$]) (14) in PBS (pH 7.4). After blocking for 2 h at room

temperature with 1× PBS (pH 7.4)–0.25% bovine serum albumin (BSA)–0.05% Tween 20, plates were stabilized with a liquid plate sealer (Candor) and stored at 4°C until use. Human sera (ELISA 1, 1:50 in 1× PBS [pH 7.4]–0.05% ProClin 300–0.01% phenol red; ELISA 2, pure sera) and biotinylated recombinant antigen (ELISA 1, 1:10,000; ELISA 2, 1:25,000) (in conjugate dilution buffer [CDB] [1× PBS {pH 7.4}–1% BSA–0.5% fetal bovine serum–1% Nonidet P-40–0.1% ProClin 300]) were mixed 1:1 on the *Hs*FcγR-Igl-coated plates and coincubated overnight at 4°C. After washing with 100 mmol/L Tris-HCl (pH 7.4)–150 mmol/L NaCl–0.05% Tween 20–0.005% ProClin 300, horseradish peroxidase (HRP)-labeled streptavidin (0.1 μg/mL in CDB) was applied to the wells for 1 h at 4°C. After washing again, tetramethylbenzidine (TMB; KPL) was added for 20 min at room temperature, and the reaction was stopped by the addition of 1 N sulfuric acid (Merck). The TMB reaction product was quantified by measuring the absorbances at 450 nm (*A*450) and 620 nm (*A*620) on a SPECTROstar Nano ELISA reader (BMG Labtech) and calculating the difference *A*450-*A*620. The Euroimmun anti-SARS-CoV-2 NCP ELISA (IgG) was performed and evaluated according to the manufacturer's instructions.

Common cold CoV IgG ELISAs. IgG antibodies interacting with the N-terminally truncated NCPs of OC43, HKU1, NL63, and 229E were detected in human sera as described previously (3). Briefly, IgG FcγR ELISA 1 described above was performed using biotinylated OC43 NCPΔ258, HKU NCPΔ256, NL63 NCPΔ222, and 229E NCPΔ258 (Table S1), respectively, as the antigens.

Interference testing. To evaluate interference by hemolytic, icteric, and lipemic serum conditions as well as high biotin and rheumatoid factor (RF) concentrations, assay mixtures were spiked with hemoglobin (Sigma), bilirubin (Sigma), triglycerides (Sigma), biotin (Sigma), and RF (Lee Biosolutions), respectively. Simulated serum concentrations were 5 mg/mL and 10 mg/mL for hemoglobin, 0.5 mg/mL for bilirubin, and 5 mg/mL for triglycerides. Biotin interference was tested at simulated serum concentrations of between 0.1 ng/mL and 100,000 ng/mL; simulated RF serum concentrations were 50 IU/mL, 100 IU/mL, and 1,000 IU/mL. Assays were performed by employing three COVID-19 patient serum samples covering a range of A_{450}-A_{620} readings ("low," "medium," and "high") and at least three prepandemic negative-control serum samples.

Sequence alignments and protein structure visualization. Multiple-amino-acid sequence alignments and identity/similarity scores were generated using the MUSCLE algorithm in MacVector (version 12.7.5). The three-dimensional (3D) structure of truncated SARS-CoV-2 NCP (comprising the dimerization domain and the C-terminal tail) was predicted using AlphaFold2 (23). Predicted structures were visualized with UCSF Chimera (version 1.13.1) (24) and aligned to the experimentally determined structure of the dimerization domain (25) using the MatchMaker utility.

Data analysis. Receiver operating characteristic (ROC) analyses were performed with MedCalc (version 19.2.1); statistical analyses (calculation of 95% confidence intervals [CIs] according to the modified Wald method and Fisher's exact test) were done using GraphPad Prism QuickCalcs. To avoid statistical bias, a maximum of 1 sample per patient per category was included for calculations of diagnostic and analytical sensitivities. If several samples from an individual patient belonged to the same category, the sample taken on the latest day after the onset of symptoms was included in the analysis.

RESULTS

Production of N-terminally truncated SARS-CoV-2 NCP for use as an ELISA antigen. SARS-CoV-2 NCPΔ246 was recombinantly expressed in *E. coli* and purified from the soluble fraction of the bacterial lysate (Fig. 1A to C). The amino acid sequence identities of SARS-CoV-2 NCPΔ246 with the respective NCP fragments of other human-pathogenic betacoronaviruses ranged between 25.3% for OC43 and 88.6% for SARS-CoV-1 (Fig. 1D; see also Fig. S1 in the supplemental material).

Development of FcγR-based SARS-CoV-2 IgG ELISA protocols. Assay protocols were developed based on the FcγR-based platform technology patented by the Bernhard Nocht Institute for Tropical Medicine (BNITM) (12). Thereby, human sera are coincubated with a biotinylated antigen in a 96-well plate coated with the recombinantly produced immunoglobulin-like extracellular domain of human FcγRIIA (CD32 H131) (14). Subsequently, bound IgG-antigen complexes are detected by the application of HRP-labeled streptavidin and the colorimetric HRP substrate TMB. Two assay versions with different serum and antigen dilutions were validated. In assay version 1 ("ELISA 1"), a final in-well serum dilution of 1:100 (as utilized in the previously developed CCHFV and ZIKV IgG FcγR ELISAs [16, 17]) was employed, while in assay version 2 ("ELISA 2"), a high serum concentration (1:2, final in well) was applied. Biotinylated antigen was titrated at final dilutions in well of 1:20,000, 1:50,000, and 1:75,000 (data not shown); based on the obtained signal-to-noise ratios, final in-well antigen dilutions of 1:20,000 and 1:50,000 for ELISAs 1 and 2, respectively, were chosen.

Reproducibility. Intra- and inter-assay variations were assessed using four SARS-CoV-2 IgG-positive serum samples and four *a priori* SARS-CoV-2 IgG-negative serum samples (Table S3). Both ELISAs generated highly reproducible results, with mean intra-assay coefficients of variation (CVs) of <5% and mean inter-assay CVs of <10% for positive signals.

Detection range and linearity. The detection ranges of the two ELISAs were determined using serial dilutions of WHO international standard plasma 20/136 simulating

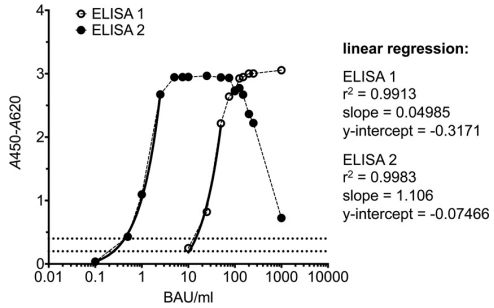

FIG 2 Detection range. WHO international standard plasma 20/136 (1,000 BAU/mL) was serially diluted with serum dilution buffer (SDB) (1× PBS [pH 7.4], 0.05% ProClin 300, 0.01% phenol red) to simulate samples with antibody concentrations ranging between 0.1 BAU/mL and 1,000 BAU/mL. Simulated samples were tested with ELISA 1 (open circles) (final in-well dilutions of 1:100 for samples and 1:20,000 for the conjugate) and ELISA 2 (filled circles) (final in-well dilutions of 1:2 for samples and 1:50,000 for the conjugate). Dotted lines indicate A_{450}-A_{620} values of 0.2 and 0.4, respectively. Solid lines indicate the linear regression fit.

samples with antibody concentrations of between 0.1 BAU/mL and 1,000 BAU/mL (Fig. 2). Assuming an assay cutoff of an A_{450}-A_{620} of 0.2 (see below), ELISA 2 showed a significantly lower detection limit (0.25 U/mL) than ELISA 1 (10.4 U/mL) but exhibited a strong prozone effect at high antibody concentrations (Fig. 2). In particular, signal saturation was observed for ELISA 2 for antibody concentrations of between 7.5 BAU/mL and 75 U/mL; higher antibody concentrations induced a continuous signal drop to an A_{450}-A_{620} of 0.725 for 1,000 BAU/mL.

Diagnostic sensitivity. To determine the diagnostic sensitivities of the two SARS-CoV-2 IgG FcγR ELISAs, 213 longitudinal serum samples obtained from 35 German patients with PCR-confirmed SARS-CoV-2 infection and 139 serum samples from healthy German blood donors (HDs), collected before the COVID-19 pandemic, were analyzed (Fig. 3). ROC analyses were performed separately for sample sets collected at different time points after the onset of symptoms (Fig. S2). By applying the lowest possible cutoff value generating 100% specificity in the negative HD panel (A_{450}-A_{620} cutoff = 0.2), diagnostic sensitivities were 90.5% (95% CI, 69.9 to 98.5%) and 100.0% (95% CI, 81.8 to 100.0%) in samples collected 1 to 2 months after the onset of symptoms for ELISAs 1 and 2, respectively (Table 1). While the diagnostic sensitivity of ELISA 1 (sera, 1:100; conjugate, 1:20,000) was found to drop quickly over time in convalescent patients, ELISA 2 (sera, 1:2; conjugate, 1:50,000) enabled the detection of anti-SARS-CoV-2 NCP IgG antibodies for a much longer time period after acute infection (Fig. 3 and Table 1). With the assays being based on NCP antigen, the time course of the humoral response to SARS-CoV-2 infection could still be monitored in individuals having received spike-protein-based vaccines (filled dots in Fig. 3).

Analytical sensitivity. Immunofluorescence analysis of COVID-19 patient samples using SARS-CoV-2-infected Vero cells revealed the highest IgG antibody titers between 1 and 2 months after the onset of symptoms (Fig. 4A and B). Later in the convalescent phase, the median IgG antibody titers dropped considerably but persisted at detectable levels in most patients for at least 12 months; spike-based vaccines strongly boosted SARS-CoV-2 IgG IIFT titers (filled dots in Fig. 4A and B). To determine the analytical sensitivities of the two SARS-CoV-2 IgG FcγR ELISAs in relation to IIFT (serving as a highly sensitive gold-standard antibody binding assay), 109 samples from 35 patients with PCR-confirmed SARS-CoV-2 infection taken prior to an eventual vaccination with a spike-based vaccine were stratified according to their respective IgG IIFT titers (≤1 sample/patient/category; median/range, 123/13 to 443 dpo). While samples with a high IIFT titer of SARS-CoV-2 IgG antibodies (≥1,280) were readily detected as positive by both ELISAs, the analytical sensitivity was significantly higher for ELISA 2 when analyzing samples with an IgG IIFT titer of between 160 and 640 (Fig. 4C and D and Table 2). In samples with a high SARS-CoV-2 IgG IIFT titer (≥5,120), a prozone effect was observed for SARS-CoV-2 IgG FcγR ELISA 2 (Fig. 4D).

Comparative testing of a subset of 82 IIFT positive samples from 26 COVID-19 patients (dpo range: 10 to 178) with a commercially available, NCP-based indirect ELISA (Euroimmun SARS-CoV-2 NCP IgG) revealed a high concordance with SARS-CoV-2 FcγR IgG ELISA 1 (Fig. S3).

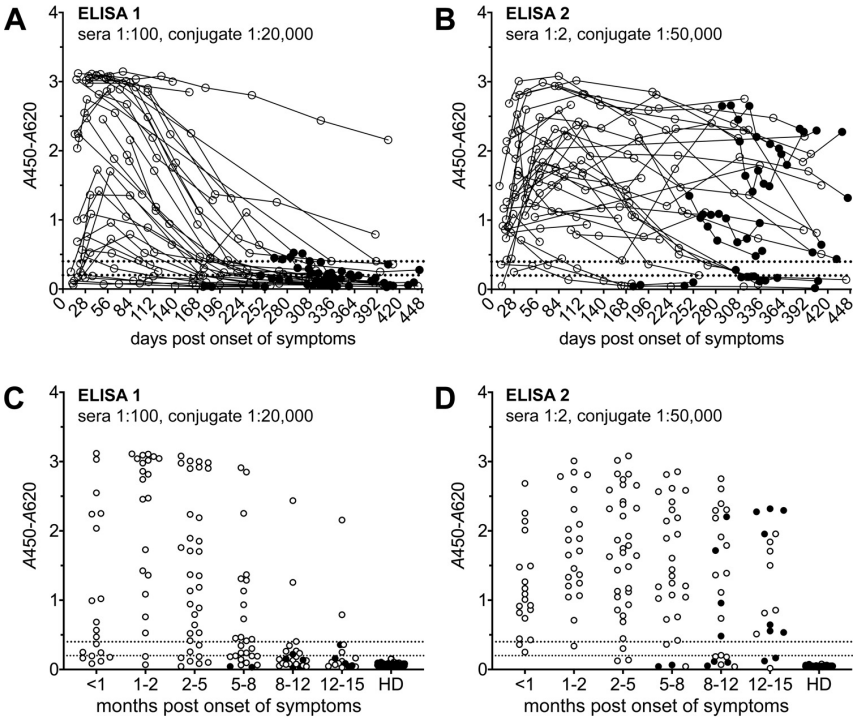

FIG 3 Diagnostic sensitivity. Longitudinal serum samples from 35 COVID-19 patients were analyzed using ELISA 1 (A and C) and ELISA 2 (B and D). (A and B) Trajectories (individual patients; 1 to 13 samples/patient; $n = 213$). (C and D) Stratification according to months after the onset of symptoms (≤1 sample/patient/category; $n = 146$). Dotted lines indicate A_{450}-A_{620} values of 0.2 and 0.4. HD, healthy donors (Germany) ($n = 139$). Filled dots indicate postvaccination samples (spike-based vaccine).

Specificity. The specificities of the two SARS-CoV-2 IgG FcγR ELISAs in comparison with the Euroimmun SARS-CoV-2 IgG NCP ELISA (Table 3; Fig. S4) were evaluated using *a priori* SARS-CoV-2 IgG-negative serum panels acquired from symptom-free donors before the COVID-19 pandemic in Europe (Germany), Africa (Ghana, Madagascar, and Nigeria), Asia

TABLE 1 Diagnostic sensitivity[a]

mpo	dpo	No. of samples	A_{450}-A_{620} cutoff	No. of positive samples, % sensitivity (95% CI) for ELISA 1 (sera, 1:100)	No. of positive samples, % sensitivity (95% CI) for ELISA 2 (sera, 1:2)	*P*
<1	10–28	19	0.200	14, 73.7 (50.9–88.5)	19, 100.0 (80.2–100.0)	0.0463
			0.400	11, 57.9 (36.2–76.9)	17, 89.5 (67.4–98.3)	0.0625
1–2	29–56	21	0.200	19, 90.5 (69.9–98.5)	21, 100.0 (81.8–100.0)	0.4878
			0.400	19, 90.5 (69.9–98.5)	20, 95.2 (75.6–100.0)	1.0000
2–5	57–140	34	0.200	28, 82.3 (66.1–92.0)	32, 94.1 (79.9–99.3)	0.2585
			0.400	26, 76.5 (59.8–87.8)	31, 91.2 (76.3–97.7)	0.1863
5–8	141–224	27	0.200	17, 63.0 (44.2–78.5)	24, 88.9 (71.1–96.8)	0.0537
			0.400	13, 48.1 (30.7–66.0)	23, 85.2 (66.9–94.7)	0.0084
8–12	225–336	24	0.200	8, 33.3 (17.8–53.4)	17, 70.8 (50.6–85.3)	0.0199
			0.400	3, 12.5 (3.5–31.8)	16, 66.7 (46.6–82.2)	0.0003
12–15	337–396	17	0.200	5, 29.4 (13.0–53.4)	14, 82.3 (58.2–94.6)	0.0049
			0.400	2, 11.8 (2.0–35.6)	14, 82.3 (58.2–94.6)	0.0001

[a]Longitudinal serum samples obtained from 35 COVID-19 patients were stratified according to months postonset (mpo) (≤1 sample/patient/category). Sensitivities as well as 95% confidence intervals (CIs) were calculated for two alternative cutoff values (A_{450}-A_{620} = 0.2 and 0.4). dpo, days postonset. *P* values were determined using Fisher's exact test.

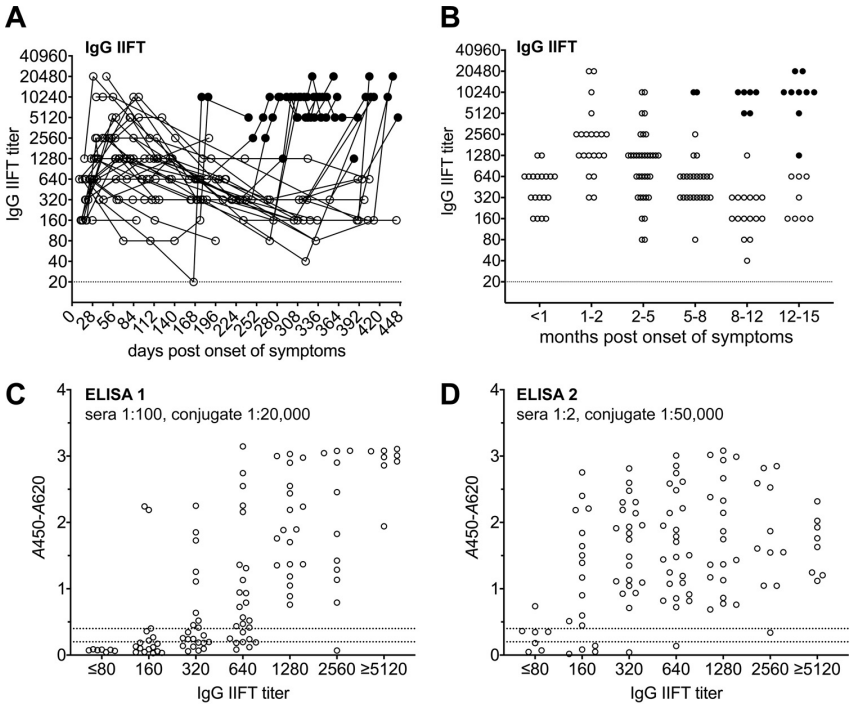

FIG 4 Analytical sensitivity. (A and B) Longitudinal serum samples from 35 COVID-19 patients were analyzed by IgG IIFT. Filled dots indicate postvaccination samples (spike-based vaccine). (A) Trajectories (individual patients; 1 to 13 samples/patient; $n = 213$); (B) stratification according to months after the onset of symptoms (≤1 sample/patient/category; $n = 146$). (C and D) Results of ELISA 1 (C) and ELISA 2 (D) for prevaccination samples stratified according to IIFT titers (≤1 sample/patient/category; $n = 109$). Dotted lines indicate A_{450}-A_{620} values of 0.2 and 0.4.

(Lao PDR), and South America (Colombia) (Table S2) (3). While false-positive rates were low for both the commercially available ELISA and the newly developed tests with the European serum panel, both SARS-CoV-2 IgG FcγR ELISAs showed superior specificity in application to African serum panels (Table 3; Fig. S4).

Although IgG antibodies interacting with the corresponding NCP fragments of the betacoronaviruses OC43 and/or HKU1 (Table S1) were detected in 244 (31%) of 790 tested serum samples (Table 3; Fig. S5) (3), no obvious cross-reactivity of these antibodies with the SARS-CoV-2 antigen has been observed. Indeed, false-positive signals obtained for a small number of Nigerian serum samples with SARS-CoV-2 IgG FcγR ELISA 1 could not be suppressed with an excess of unlabeled OC43 NCP, although high levels of antibodies interacting with this protein were present in these samples (data not shown).

Interference. For SARS-CoV-2 IgG FcγR ELISA 1 (sera, 1:100, final in well; antigen, 1:20,000, final in well), no significant influence on the obtained A_{450}-A_{620} values was observed for final serum concentrations of 10 mg/mL hemoglobin, 0.5 mg/mL bilirubin, and 5 mg/mL triglycerides and biotin concentrations of up to 100,000 ng/mL. While a rheumatoid factor serum concentration of 50 IU/mL did not influence the signal height, concentrations of 100 IU/mL and 1,000 IU/mL suppressed positive signals by ca. 30% and 50%, respectively, without increasing the assay background.

For SARS-CoV-2 IgG FcγR ELISA 2 (sera, 1:2, final in well; antigen, 1:50,000, final in well), although a final biotin serum concentration of 100,000 ng/mL induced a significant signal loss for positive sera, a concentration of 3,500 ng/mL did not significantly influence A_{450}-A_{620} values. Hemoglobin serum concentrations of up to 5 mg/mL did not influence assay performance, but signals were impacted at 10 mg/mL. Rheumatoid factor serum concentrations of 50 IU/mL and 1,000 IU/mL reduced the intensities of positive signals by ca. 40%, and 50%, respectively, without increasing the assay background. The influence of bilirubin and

TABLE 2 Analytical sensitivity[a]

IgG IIFT titer	No. of samples	A_{450}-A_{620} cutoff	No. of positive samples, % sensitivity (95% CI), for ELISA 1 (sera, 1:100)	No. of positive samples, % sensitivity (95% CI), for ELISA 2 (sera, 1:2)	*P*
≤80	7	0.200	0, 0.0 (0.0–40.4)	4, 57.1 (25.0–84.2)	0.0699
		0.400	0, 0.0 (0.0–40.4)	1, 14.3 (0.5–53.3)	1.0000
160	17	0.200	6, 35.3 (17.2–58.8)	13, 76.5 (52.2–90.9)	0.0366
		0.400	3, 17.6 (5.4–41.8)	13, 76.5 (52.2–90.9)	0.0016
320	22	0.200	14, 63.6 (42.9–80.4)	21, 95.4 (76.5–100.0)	0.0212
		0.400	9, 40.9 (23.2–61.3)	21, 95.4 (76.5–100.0)	0.0002
640	25	0.200	20, 80.0 (60.4–91.6)	24, 96.0 (78.9–100.0)	0.1895
		0.400	17, 68.0 (48.3–82.9)	24, 96.0 (78.9–100.0)	0.0232
1,280	19	0.200	19, 100.0 (80.2–100.0)	19, 100.0 (80.2–100.0)	1.0000
		0.400	19, 100.0 (80.2–100.0)	19, 100.0 (80.2–100.0)	1.0000
2,560	11	0.200	10, 90.9 (60.1–100.0)	11, 100.0 (70.0–100.0)	1.0000
		0.400	10, 90.9 (60.1–100.0)	10, 90.9 (60.1–100.0)	1.0000
≥5,120	8	0.200	8, 100.0 (62.8–100.0)	8, 100.0 (62.8–100.0)	1.0000
		0.400	8, 100.0 (62.8–100.0)	8, 100.0 (62.8–100.0)	1.0000

[a]Longitudinal serum samples obtained from 35 COVID-19 patients were stratified according to SARS-CoV-2 IgG IIFT titers (≤1 sample/patient/category). Sensitivities as well as 95% confidence intervals (CIs) were calculated for two alternative cutoff values (A_{450}-A_{620} = 0.2 and 0.4). *P* values were determined using Fisher's exact test.

triglycerides could not be studied for ELISA 2 due to the strong impact of the solvent used (chloroform) on antigen integrity.

DISCUSSION

Assay protocol. The SARS-CoV-2 IgG ELISAs developed and validated in this work employ a patented platform technology (12, 14–17) in which immune complexes between a labeled, recombinant antigen and human serum IgG antibodies are formed in solution and subsequently captured by an isotype-specific FcγR immunoglobulin-like domain. Thereby, the use of a soluble, natively folded antigen has a direct positive impact on sensitivity and specificity because conformational epitopes are preserved and nonspecific interactions with hydrophobic amino acid stretches that may be exposed in incorrectly folded proteins cannot occur. With the main intended use of our assays being the monitoring of seroconversion and seroprevalence in archived samples, development was focused on the optimization of sensitivity and specificity rather than a minimized time to result. Nevertheless, preliminary results indicate that upon adjustments of conjugate and serum concentrations, shortening the antigen-sample incubation step from overnight to 2 h might be possible, with only minor losses in sensitivity and no increase in the assay background (data not shown). Importantly, the assays, which are procurable via the European Virus Archive Goes Global (EVAg) webpage (26), can be performed manually, and results are generated using a standard absorbance ELISA reader, facilitating their use also in settings where no highly specialized technical equipment is available.

Choice of antigen. As an antigen, a SARS-CoV-2 NCP fragment comprising the dimerization and C-terminal tail domains of SARS-CoV-2 NCP (see Fig. S1 in the supplemental material) was used. Similar fragments of SARS-CoV-1 NCP have previously been shown to allow the sensitive detection of anti-SARS-CoV-1 IgG antibodies in patient sera (27, 28) while generating fewer false-positive responses than the full-length protein in negative-control sera (28). As for other viral nucleocapsid proteins (16, 28), the cost-efficient production of large quantities of natively folded SARS-CoV-2 full-length NCP and subdomains is possible in *E. coli* (7).

Assay sensitivity. Complete validation was performed for two SARS-CoV-2 IgG FcγR ELISA protocols employing different final serum and conjugate dilutions (ELISA 1, sera at 1:100 and conjugate at 1:20,000; ELISA 2, sera at 1:2 and conjugate at 1:50,000). ELISA 1 displayed diagnostic and analytical sensitivities similar to those of a commercially available, manually performed, plate-based indirect ELISA (Euroimmun SARS-CoV-2 NCP IgG ELISA) (29), thus showing a significant waning of signal intensity in COVID-19 patients at approximately 6

TABLE 3 Specificity of SARS-CoV-2 IgG ELISAs[a]

Panel	No. of samples	No. (%) of betacoronavirus IgG-positive samples	A_{450}-A_{620} cutoff	No. of negative samples, % specificity (95% CI), for ELISA 1 (sera, 1:100)	No. of negative samples, % specificity (95% CI), for ELISA 2 (sera, 1:2)	No. of negative/bl samples, % specificity (95% CI), for Euroimmun NCP IgG
Germany	139	51 (36.7)	0.200	139, 100.0 (96.8–100.0)	139, 100.0 (96.8–100.0)	139, 100.0 (96.8–100.0)
			0.400	139, 100.0 (96.8–100.0)	139, 100.0 (96.8–100.0)	
Ghana A	131	33 (25.2)	0.200	128, 97.7 (93.2–99.5)	129, 98.5 (94.3–99.9)	97, 74.0 (65.9–80.8)
			0.400	129, 98.5 (94.3–99.9)	131, 100.0 (96.6–100.0)	
Ghana B	145	34 (23.4)	0.200	142, 97.9 (93.8–99.6)	145, 100.0 (96.9–100.0)	132, 91.0 (85.1–94.8)
			0.400	145, 100.0 (96.9–100.0)	145, 100.0 (96.9–100.0)	
Madagascar	166	30 (18.1)	0.200	165, 99.4 (96.3–100.0)	166, 100.0 (97.3–100.0)	164, 98.8 (95.4–100.0)
			0.400	166, 100.0 (97.3–100.0)	166, 100.0 (97.3–100.0)	
Nigeria	149	79 (53.0)	0.200	143, 96.0 (91.3–98.3)	149, 100.0 (97.0–100.0)	107, 71.8 (64.1–78.4)
			0.400	146, 98.0 (94.0–99.6)	149, 100.0 (97.0–100.0)	
Colombia	40	12 (30.0)	0.200	40, 100.0 (89.6–100.0)	40, 100.0 (89.6–100.0)	40, 100.0 (89.6–100.0)
			0.400	40, 100.0 (89.6–100.0)	40, 100.0 (89.6–100.0)	
Lao PDR	20	5 (25.0)	0.200	20, 100.0 (81.0–100.0)	20, 100.0 (81.0–100.0)	20, 100.0 (81.0–100.0)
			0.400	20, 100.0 (81.0–100.0)	20, 100.0 (81.0–100.0)	

[a]Serum/plasma samples collected from symptom-free donors before 2019 in Europe, Africa, South America, and Asia were analyzed with SARS-CoV-2 IgG FcγR ELISAs 1 and 2 and the Euroimmun anti-SARS-CoV-2 NCP ELISA (IgG). Specificities and 95% confidence intervals (CIs) were calculated for two alternative cutoff values (A_{450}-A_{620} = 0.2 and 0.4). bl, borderline.

months postinfection. In contrast, ELISA 2 allowed the persistent detection of anti-NCP IgG antibodies for more than 12 months postinfection for most patients. Thus, SARS-CoV-2 IgG FcγR ELISA 2 displays a sensitivity comparable to that of Elecsys anti-SARS-CoV-2 (Roche), a commercially available NCP-based automated electrochemiluminescence immunoassay system detecting total Ig through double-antigen binding (30, 31).

Assay specificity. As we have shown recently, unacceptably high false-positive rates of up to 61% are obtained with several commercially available indirect SARS-CoV-2 NCP IgG ELISAs in African sample panels collected before the COVID-19 pandemic, although the very same assays displayed excellent specificity in European samples (3). Therefore, the specificity of the newly developed SARS-CoV-2 IgG FcγR ELISAs was challenged with an extensive panel of 790 pre-COVID-19 negative-control samples originating from Europe (n = 139), South America (n = 40), Asia (n = 20), and Africa (n = 591). Both ELISAs were found to be highly specific (>96%), even in the most challenging sample panel, originating from Nigeria. For applications requiring maximum specificity in African samples, we recommend using a slightly elevated cutoff value of an A_{450}-A_{620} of 0.4 (instead of 0.2 for samples from European residents). As for the commercially available assays evaluated in our previous study (3), the presence of IgG antibodies elicited by previous infections with the common cold CoVs OC43, HKU1, NL63, and 229E did not *per se* compromise SARS-CoV-2 IgG FcγR ELISA specificity. Indeed, in concordance with data from previous studies (1, 32), those antibodies were found in a significant proportion of donor sera of each origin (Table 3; Fig. S5). Three Nigerian samples generated false-positive signals in ELISA 1 but not ELISA 2. Although high levels of anti-OC43 IgG antibodies were detected in all three serum samples, signals in the SARS-CoV-2 IgG FcγR ELISA could not be suppressed by the addition of excess OC43 NCP, ruling out OC43 cross-reactivity as a cause. A possible explanation for these false-positive signals occurring in ELISA 1 but not ELISA 2 could be the presence of high levels of IgG antibodies elicited by another, as-yet-unidentified member of the *Coronaviridae* family binding with low affinity to SARS-CoV-2 NCP.

Caveats and limitations. When applying the SARS-CoV-2 IgG FcγR ELISAs presented in this study in seroepidemiological research projects, several caveats and limitations have to be considered. First of all, due to the use of SARS-CoV-2 NCP as the antigen, humoral immune responses elicited by spike-based vaccines (e.g., BioNTech/Pfizer and AstraZeneca) are not detected by these assays. Correspondingly, detection of natural

SARS-CoV-2 infection is possible in naive individuals and probands vaccinated with spike-based vaccines but not with vaccines based on inactivated full virus (e.g., Sinopharm and Sinovac). The assay version should be chosen according to the respective research questions: while ELISA 1 is most suitable to monitor the development of the humoral immune response during the acute and early convalescent phases of the disease, ELISA 2 allows the detection of SARS-CoV-2-specific antibodies for a much longer time period postinfection and therefore is the assay version of choice for large-scale seroprevalence studies (30). Nevertheless, this assay shows a strong prozone effect in serum samples with very high anti-NCP IgG titers. Therefore, misleadingly low readings could occur in certain situations, such as during early convalescent stages of severe infections (1) or upon reinfections/vaccinations strongly boosting the anti-NCP response. If these scenarios are likely, the parallel performance of ELISAs 1 and 2 is strongly recommended. Furthermore, ELISA 2 is (due to the direct use of pure, undiluted serum samples) more susceptible to interfering substances than ELISA 1. Therefore, serum quality should be monitored and critically assessed, particularly when using this assay version. Further potential limitations, relevant for both assay versions, are the differential affinities of the recombinant capture molecule CD32 H131 for the various IgG subclasses (33) and the potential masking of relevant epitopes by the biotin label. Nevertheless, the dominant IgG subclasses induced by SARS-CoV-2 infection, IgG1 and IgG3 (34, 35), have been shown to strongly interact with CD32 H131 in a previous study (33).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 7.4 MB.

SUPPLEMENTAL FILE 2, PDF file, 0.1 MB.

SUPPLEMENTAL FILE 3, PDF file, 0.1 MB.

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C.D. and P.E. are inventors on patent EP3207375, and P.E. is an inventor on patent EP2492689. C.D. and B.R. are cofounders and shareholders of Panadea Diagnostics GmbH. We declare no other potential conflicts of interest.

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Performance Analysis of Serodiagnostic Tests to Characterize the Incline and Decline of the Individual Humoral Immune Response in COVID-19 Patients: Impact on Diagnostic Management

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Abstract: Serodiagnostic tests for antibody detection to estimate the immunoprotective status regarding SARS-CoV-2 support diagnostic management. This study aimed to investigate the performance of serological assays for COVID-19 and elaborate on test-specific characteristics. Sequential samples (n = 636) of four panels (acute COVID-19, convalescent COVID-19 (partly vaccinated post-infection), pre-pandemic, and cross-reactive) were tested for IgG by indirect immunofluorescence test (IIFT) and EUROIMMUN EUROLINE Anti-SARS-CoV-2 Profile (IgG). Neutralizing antibodies were determined by a virus neutralization test (VNT) and two surrogate neutralization tests (sVNT, GenScript cPass, and EUROIMMUN SARS-CoV-2 NeutraLISA). Analysis of the acute and convalescent panels revealed high positive (78.3% and 91.6%) and negative (91.6%) agreement between IIFT and Profile IgG. The sVNTs revealed differences in their positive (cPass: 89.4% and 97.0%, NeutraLISA: 71.5% and 72.1%) and negative agreement with VNT (cPass: 92.3% and 50.0%, NeutraLISA: 95.1% and 92.5%) at a diagnostic specificity of 100% for all tests. The cPass showed higher inhibition rates than NeutraLISA at VNT titers below 1:640. Cross-reactivities were only found by cPass (57.1%). Serodiagnostic tests, which showed substantial agreement and fast runtime, could provide alternatives for cell-based assays. The findings of this study suggest that careful interpretation of serodiagnostic results obtained at different times after SARS-CoV-2 antigen exposure is crucial to support decision-making in diagnostic management.

Keywords: COVID-19; individual humoral immune response; serodiagnostic tests

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1. Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) causes coronavirus disease-19 (COVID-19) [1–3] with mild, severe, or fatal courses. Due to the rapid global spread of SARS-CoV-2, the World Health Organization declared a pandemic in March 2020 [4,5]. Along with the human coronaviruses (hCoV) HKU1 and OC43, SARS-CoV-2, like SARS-CoV and MERS-CoV, belongs to the beta-CoVs. Additionally, alpha-CoVs 229E and NL63 are found worldwide. CoVs typically cause mild upper respiratory disease [4].

The entry of SARS-CoV-2 into cells is mainly mediated by the interaction of the homotrimeric viral spike (S) glycoprotein with the human angiotensin-converting enzyme 2 (hACE2) on host cells [6]. The S protein is composed of two subunits, S1 and S2 [7],

with the receptor binding domain (RBD) located on S1. Following the binding of S1/RBD to hACE2, S1 and S2 are cleaved by host proteases, which induce the activation of S2. Activated S2 mediates the fusion of the viral particle with the cellular membrane [6]. This fusion results in the release of viral RNA, which is encapsulated by the nucleocapsid (N) protein, for virus replication [8]. N is an abundantly produced viral protein [9] and serves multiple functions, such as the regulation of viral replication and RNA assembly [8].

Nucleic acid amplification tests, such as Real-Time Reverse Transcription Polymerase Chain Reaction (rRT-PCR), are used to detect acute SARS-CoV-2 infections [10,11]. Analysis of the host immune response can indirectly identify individuals with a current or past infection and monitor vaccine efficacy [12,13]. Indirect immunofluorescence tests (IIFT) can detect seroconversion by determining immunoglobulin (Ig) classes IgG, IgM, and IgA antibodies (Ab) in response to the full antigenic spectrum of SARS-CoV-2-infected cells [14]. Virus neutralization tests (VNT) detect the inhibition of viral infection by neutralizing antibodies (NAb) present in patient serum [15]. These cell culture-based assays serve as the gold standard even though they are time-consuming, labor-intensive, and require a biosafety level 3 (BSL3) laboratory [16]. Another disadvantage is their unsuitability for large-scale use and the lack of inter-laboratory standardization [15]. Commercially available technologies such as enzyme-linked immunosorbent assays (ELISA) and immunoblots that specifically detect Abs targeting the main SARS-CoV-2 immunogens S1/RBD, S2, and N [17,18] are potential alternatives for cell-based serological assays. They are less time-consuming, do not require a BSL3 environment, and improve comparability between laboratories. Surrogate virus neutralization assays (sVNT) detect the binding between recombinant hACE2 and recombinant viral RBD in an ELISA format with a colorimetric readout [19]. The color intensity is inversely proportional to the NAb concentration in the sample.

Several studies have investigated commercially available S1/RBD-based assays, mainly analyzing a single sample per patient [16,20–23]. In this study, the performance of a line blot was compared with that of an IIFT for the detection of SARS-CoV-2-specific IgG and of two sVNTs with a VNT for the detection of NAbs, thereby focusing primarily on sequential sera at different phases of COVID-19 without and with vaccination. By doing so, this study aimed to achieve a better understanding of the performance of the different assays in different stages of COVID-19 and after vaccination and how this affects their interpretation.

2. Materials and Methods

Patient data are summarized in Table 1. Panel A consists of 268 sequential and single samples obtained from 115 patients with mild to severe COVID-19 symptoms over a total period of 0–154 days post-symptom onset (dpso) between March 2020 and April 2021. SARS-CoV-2 was confirmed by real-time RT-PCR (Allplex 2019-nCoV Assay, Seegene Inc., Seoul, Republic of Korea). The panel comprises 15 patient samples with the following comorbidities: cancer (n = 10), immunosuppression after organ transplantation (n = 3), and ongoing Ab therapy (n = 4), including 2 cancer patients. Panel B contains pre-pandemic sera from 95 blood donors obtained before August 2019. Panel C is composed of 237 sequential and single samples from 36 convalescent COVID-19 patients (PCR [24] confirmed) with mild symptoms. The samples were obtained between March 2020 and March 2021 and cover a period of combined 10 to 474 dpso. Of those patients, 21 were vaccinated within the time of this study using the vaccines Ad26.COV2.S, BNT162b2, mRNA-1273, AZD122, or combination schedules of these vaccines. Panel D contains sera from patients with seasonal hCoV infection (n = 12) and rhinovirus infection (n = 1) obtained between January 2020 and April 2020. The infection was confirmed by IIFT [13].

Table 1. The representation of cohorts used in this study.

	Panel A (Acute)	Panel B (Pre-Pandemic)	Panel C (Convalescent)	Panel D (Seasonal hCoV, Non-SARS-CoV-2)
Total number of patients	115	95	36	13
Age (mean ± SD, range) [years]	61.6 ± 18.6, 21–89	unknown	43.1 ± 13.4, 19–64	7.8 ± 4.2, 4–15 75.7 ± 0.5, 75–89
Sex (*n* females, *n* males, *n* unknown)	58, 57, 0	unknown	24, 12, 0	3, 7, 0 1, 2, 0
N samples in the panel	268	95	237	14
Number of sequential samples per patient	1–6	-	1–14	1–2
dpso/dpPCR (mean ± SD, range, unknown)	14.3 ± 20.6, 0–154, 31	-	199.1 ± 134.6, 10–474, 0	unknown 68.7 ± 46.2, -, -
Total number of vaccinated patients in the cohort	-	-	21	-
N samples post-vaccination	-	-	63	-
Number of sequential samples post-vaccination	-	-	1–9	-
dpVac (mean ± SD, range)	-	-	52.1 ± 40.7, 6–181	-

Abbreviations: dpso: days post-symptom onset; dpPCR: days post-PCR; dpVac: days post-vaccination; SD: standard deviation.

The detection of SARS-CoV-2-specific IgG was performed by means of IIFT as previously described [25] using cells infected with the SARS-CoV-2 patient isolate HH-1 [26]. Briefly, SARS-CoV-2-infected VeroE6 cells (ATCC CRL-1008) were fixed with acetone-methanol and incubated with diluted (1:10 to 1:80) patient sera. Human IgG was detected using an anti-human IgG fluorescein isothiocyanate conjugate. Specimens were analyzed with a fluorescence microscope. Titers below 1:20 were considered negative.

Samples were additionally analyzed using the EUROLINE Anti-SARS-CoV-2 Profile (IgG) (EUROIMMUN, Medizinische Labordiagnostik AG, Lübeck, Germany). The Profile IgG is a line blot coated with purified S1, S2, and N of SARS-CoV-2 (Wuhan-Hu-1), as well as N of seasonal hCoVs, for the determination of the corresponding IgG in serum or plasma. The assay was performed according to the manufacturer's instructions with diluted (1:51) serum. Samples were classified as positive (≥18), equivocal (12–18), or negative (≤11) based on line intensities for each antigen measured using the EUROLineScan software 3.4.32 (EUROIMMUN). A sample was anti-SARS-CoV-2 positive when at least two of the three line intensities (S1, S2, and N of SARS-CoV-2) were above the cutoff. It was considered equivocal if S1 or N were positive, and at least one further band was equivocal. If none of this applied, the sample was negative.

The VNT for the detection of NAbs was performed according to Brehm et al. [26]. Heat-inactivated (56 °C, 30 min) serially diluted patient sera (1:20 to 1:5120, triplicates) were mixed with an equal volume of viral solution containing 20 tissue culture infectious dose 50 ($TCID_{50}$) of SARS-CoV-2 (patient isolate HH-1) and transferred to VeroE6 cells, seeded the previous day with a concentration of 5×10^6 per well of a 96-well plate. Cells were incubated at 37 °C with 5% CO_2 in a humidified environment for 96 h. The supernatant was discarded, and cells that were not detached by the cytopathic effect (CPE) were fixed with 4% formaldehyde and stained with crystal violet for CPE evaluation. The highest

dilution protecting cells from CPE in two of three wells was assessed as NAb titer. A titer of 1:40 was used as a cutoff.

In addition to the studied VNT, two different surrogate virus neutralization tests, the GenScript cPass (GenScript, Piscataway Township, NJ, USA) and the SARS-CoV-2 NeutraLISA (EUROIMMUN), were used to determine NAb. The tests were performed according to the instructions of the respective manufacturer. Briefly, the cPass was performed using diluted serum (1:10) preincubated with horseradish peroxidase (HRP)-labeled RBD (37 °C, 30 min) and transferred to hACE2-precoated wells (37 °C for 15 min). RBD and hACE2 binding were detected by HRP-catalyzed color reactions. A signal strength of ≥30% was considered positive, and <30% was negative.

The NeutraLISA was performed using serum samples diluted (1:5) with sample buffer containing biotinylated hACE2 and added to RBD-precoated wells (37 °C, 60 min). HRP-labeled streptavidin was added (30 min), followed by detection using a chromogen and absorbance measurement. Samples were evaluated as positive, equivocal, and negative if the inhibition rate was ≥35%, between 20% and 35%, and ≤20%, respectively.

Inter-assay concordances were calculated as percentages of agreement with Clopper-Pearson confidence intervals (CI). Equivocal results (EUROLINE Profile IgG and EUROIMMUN SARS-CoV-2 NeutraLISA) were counted as positive. Statistical analysis was performed using the Wilcoxon rank sum test (GraphPad Prism 9) with Bonferroni correction.

3. Results

3.1. Assessment of Humoral Responses in Acute COVID-19 Using Different Assays

The development of the humoral immune response during acute COVID-19 was analyzed in samples from panel A (Table 1), irrespective of the severity of symptoms and comorbidities. Panel B samples served as a negative control.

Samples were categorized according to their time of collection (dpso/dpPCR) into T1 (0–5), T2 (6–10), T3 (11–20), and T4 (>20) and tested for IgG seroconversion (Table 2). In T1, anti-SARS-CoV-2-specific IgG was detected in 21.0% (13/62) of the samples by IIFT and in 8.1% (5/62) by the Profile IgG. As the infection progressed, the seropositivity increased to 61.3% (49/80) and 48.8% (39/80) in T2, as well as 80.0% (60/75) and 72.0% (54/75) in T3 using the IIFT and Profile IgG, respectively. In T4, IIFT and Profile IgG found 80.0% (16/20) and 85.0% (17/20) of samples positive, respectively (Supplementary Figure S1). Overall, SARS-CoV-2-specific IgG antibodies were detected in 57.1% (153/268) of patient samples by IIFT and in 47.0% (126/268) by Profile IgG. Calculations revealed a positive agreement of 78.4% and a negative agreement of 94.8% for the Profile IgG in relation to the results obtained by IIFT. No Abs were detected in the pre-pandemic samples using both tests, indicating a diagnostic specificity of 100% for the IIFT and Profile IgG.

Neutralizing activity was determined in the same panels using an in-house VNT in comparison with the sVNTs (cPass and NeutraLISA). In T1, the VNT revealed NAbs in 11.3% (7/62) of sera, the cPass determined inhibition in 12.9% (8/62) and the NeutraLISA in 6.4% (2 positive plus 2 equivocal/62) (Table 2). With increasing dpso/dpPCR, the proportion of samples with NAb increased. In T2 as well as T3, the VNT, cPass, and NeutraLISA characterized 61.3% (49/80), 52.2% (42/80), and 35.1% (23 positive plus 5 equivocal/80) as well as 77.3% (58/75), 76.0% (57/75), and 65.3% (40 positive plus 9 equivocal/75) samples as positive, respectively. Remarkably, the cPass yielded positive results in a higher number of early samples compared to the NeutraLISA. In T4, all tests found 85.0% (17/20) of samples positive (15 positive plus 2 equivocal/20, NeutraLISA). Using the VNT as a reference, an overall positive agreement of 88.3% and a negative agreement of 92.7% for the cPass, as well as a positive agreement of 71.7% and a negative agreement of 95.9% for the NeutraLISA, were calculated. No tests detected NAbs in prepandemic sera, which indicates a diagnostic specificity of 100% for all assays.

Table 2. Comparative determination of SARS-CoV-2-specific IgG and NAb during acute COVID-19 infection (panel A) and in pre-pandemic samples (Panel B) by different diagnostic tests.

		Anti-SARS-CoV-2-IgG		**Anti-SARS-CoV-2 NAb**			
		In-House IIFT	**EUROLINE Profile IgG**	**In-House VNT**	**GenScript cPass**	**EUROIMMUN SARS-CoV-2 NeutraLISA**	
Period (dpso/dpPCR)	*N* total	*N* pos (%)	*N* pos (%)	*N* pos (%)	*N* pos (%)	*N* pos (%)	*N* equ (%)
T1 (0–5)	62	13 (21.0)	5 (8.1)	7 (11.3)	8 (12.9)	2 (3.2)	2 (3.2)
T2 (6–10)	80	49 (61.3)	39 (48.8)	49 (61.3)	42 (52.2)	23 (28.8)	5 (6.3)
T3 (11–20)	75	60 (80.0)	54 (72.0)	58 (77.3)	57 (76.0)	40 (53.3)	9 (12.0)
T4 (>20)	20	16 (80.0)	17 (85.0)	17 (85.0)	17 (85.0)	15 (75.0)	2 (10.0)
Unknown	31	15 (48.4)	11 (35.5)	14 (45.2)	13 (41.9)	11 (35.5)	2 (6.5)
All samples incl. unknown	268	153 (57.1)	126 (47.0)	145 (54.1)	137 (51.1)	91 (34.0)	20 (7.5)
Positive agreement % [a]		reference	78.4	reference	88.3	71.7	
95% CI			71.1–84.7		81.9–93.0	63.7–78.9	
Negative agreement %		reference	94.8	reference	92.7	95.9	
95% CI			86.1–98.1		83.6–96.6	88.2–98.7	
Pre-pandemic sera	95	0	0	0	0	0	
Specificity % [b]		100	100	100	100	100	
95% CI		96.2–100	96.2–100	96.2–100	96.2–100	96.2–100	

[a] Calculation of agreements was based on the respective in-house tests, with equivocal results from EUROIMMUN SARS-CoV-2 NeutraLISA counted as positive. [b] Calculation of specificities was based on a clinical diagnosis. Abbreviations: pos, positive; equ, equivocal; dpso/dpPCR, days post-symptom onset/days post-PCR.

The cohort of acute cases included patients diagnosed with cancer, treated with immunosuppressives after organ transplantation, or receiving SARS-CoV-2 antibody therapy (Supplementary Figure S2). One patient with follicular lymphoma, which affects the immune system, did not develop a humoral immune response within 30 dpso. On the other hand, a patient with Hodgkin's lymphoma established IgG seroconversion and produced NAbs at levels comparable to patients without comorbidities. In two of three patients treated with immunosuppressives, seroconversion was detectable at 7 dpso/dpPCR. Noteworthy are the results from patients on SARS-CoV-2 therapy with S1-based antibodies. These samples were positive for NAb in all assays and for IgG in the IIFT as well as for anti-S1 IgG, yet overall negative in the Profile IgG.

3.2. Dynamics of the Humoral Immune Response in a Convalescent COVID-19 Patient Cohort

Sequential convalescent samples (n = 237) from 36 PCR-confirmed patients over a period of up to 474 days (Table 1, Panel C) were analyzed. Anti-SARS-CoV-2 IgG antibodies were detected in 100% using the IIFT, whereas the Profile IgG showed a positive result for 91.6% (216 positive plus 1 equivocal/237) of samples, corresponding to a positive agreement of 91.6% (Table 3).

Table 3. Comparative determination of SARS-CoV-2-specific IgG and NAb in the convalescent COVID-19 cohort (panel C) by different diagnostic tests.

		Anti-SARS-CoV-2-IgG			**Anti-SARS-CoV-2 NAb**			
		In-House IIFT	**EUROLINE Profile IgG**		**In-House VNT**	**GenScript cPass**	**EUROIMMUN SARS-CoV-2 NeutraLISA**	
dpso/dpPCR	*N* total	*N* pos (%)	*N* pos (%)	*N* equ (%)	*N* pos (%)	*N* pos (%)	*N* pos (%)	*N* equ (%)
(T5 (0–150)	99	99 (100)	89 (89.9)	1 (1.0)	81 (81.8)	87 (87.9)	35 (35.4)	16 (16.2)
T6 (151–300)	45	45 (100)	39 (86.7)	0 (0)	33 (73.3)	38 (84.4)	11 (24.4)	7 (15.6)
T7 (301–500)	25	25 (100)	21 (84.0)	0 (0)	15 (60.0)	18 (72.0)	6 (24)	2 (8.0)
TVac (Vaccination)	68	68 (100)	68 (100)		68 (100)	68 (100)	68 (100)	0 (0)
All samples	237	237 (100)	216 (91.1)	1 (0.4)	197 (83.1)	211 (89.0)	120 (50.6)	25 (10.5)
Positive agreement % [a]		reference	91.6		reference	97.0	72.1	
95% CI			87.3–94.8			93.5–98.9	65.3–78.2	
Negative agreement %		reference			reference	50.0	92.5	
95% CI						33.8–66.2	79.6–98.4	

[a] Calculation of agreements was based on the respective in-house tests, with equivocal results from EUROLINE Profile IgG and EUROIMMUN SARS-CoV-2 NeutraLISA counted as positive. Abbreviations: pos, positive; equ, equivocal; dpso/dpPCR, days post-symptom onset/days post-PCR; TVac, days post-vaccination.

In all patients, IgG antibody levels decreased or stagnated over time until another antigen exposure by re-infection (sample CoV24) or vaccination (Supplementary Figure S3). This second stimulus led to a stronger increase in IgG detected in both the IIFT and Profile IgG than in most cases of infection alone. Noteworthy, the vaccination induced a strong anti-S1/2 IgG but no anti-N IgG response.

Additionally, the dynamics of the NAbs were investigated using the VNT as well as both sVNTs (Table 3). Samples were grouped based on their collection times (dpso/dpPCR), as follows: T5 (0–150), T6 (151–300), T7 (301–500), as well as TVac (days post-vaccination).

Over time, the median NAb titers decreased from 1:160 (T5) to 1:40 (T7). This decrease was also seen by cPass and NeutraLISA, as reflected by median inhibition rates declining from 57.5% to 42.8% and from 21.2% to 10.8%, respectively (Figure 1A). Similarly, a decline in maximum values from T5 to T7 for the VNT (titers of 1:2560 to 1:640) and the NeutraLISA (inhibition of 100% to 87%) was noticed. In contrast, no relevant titer change was observed for the cPass (96% for T5 and T7).

The inhibition values detected with both sVNTs correlated with the corresponding titers determined by the VNT, as indicated by Spearman's rank correlation coefficients (cPass: r = 0.83 [95% CI: 0.78–0.86], NeutraLISA: r = 0.79 [95% CI: 0.74–0.84]). However, the assays differed in the number of NAb-positive and negative samples. In T5, the VNT detected NAbs in 81.8% (81/99) of sera, the cPass in 87.9% (87/99), and the NeutraLISA in 51.5% (35 positive plus 16 equivocal/99). In T6, 73.3% (33/45), 84.4% (38/45) and 40.0% (11 positive plus 7 equivocal/45) and in T7, 60.0% (15/25), 72.0% (18/25) and 32.0% (6 positive plus 2 equivocal/25) of samples were found NAb positive by the VNT, cPass, and NeutraLISA, respectively. Noteworthy, in all intervals, the cPass detected more NAb-positive samples than the VNT and the NeutraLISA. The TVac subpanel consists of sequential samples from 21 patients taken after vaccination or re-infection. During this period, the highest NAb titers ($\geq$1:1280) were found in 94.1% (64/68) of sera using the VNT. Similarly, the highest inhibition values (75% to 100%) were detected in 100% (68/68)

and 98.4% (67/68) by cPass and NeutraLISA, respectively. The dynamics of SARS-CoV-2-specific IgG and NAb are shown in Supplementary Figure S4, and the course of NAbs in all single samples is shown in Supplementary Figure S5.

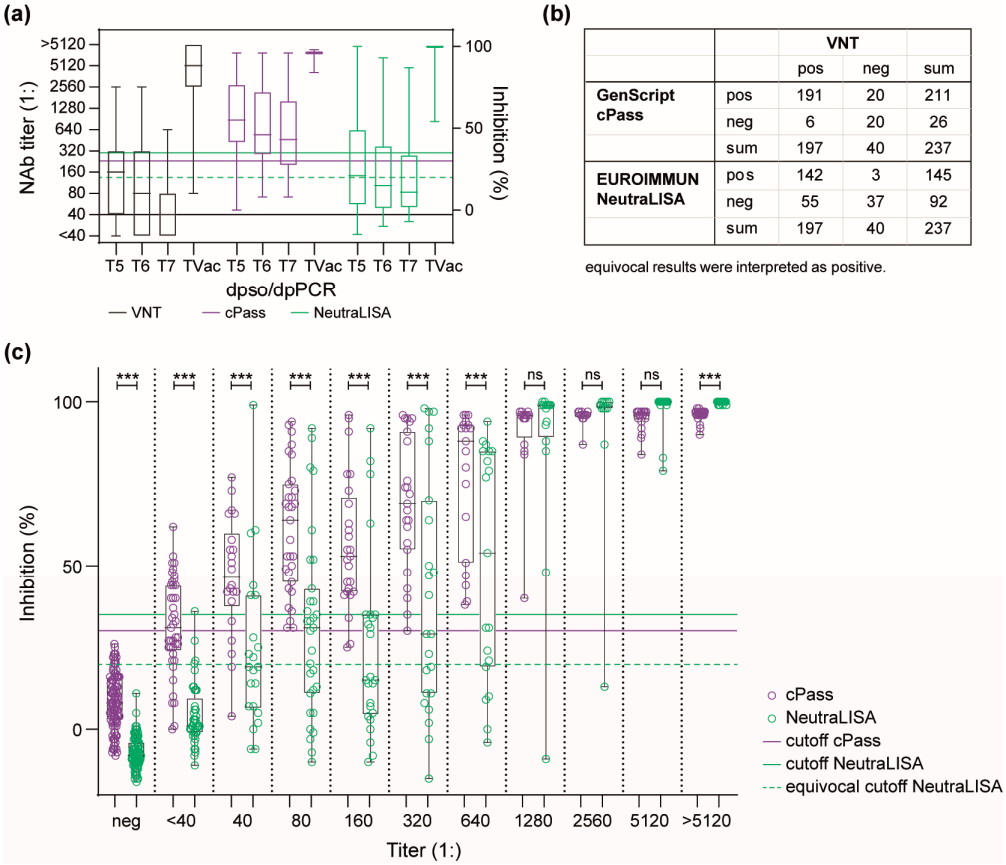

Figure 1. (**a**) Decrease of NAbs in sera from convalescent patients over time, as determined by three different NTs. Results obtained using VNT (black) are plotted on the left *Y*-axis; those obtained using cPass (purple) and NeutraLISA (green) are plotted on the right *Y*-axis. (**b**) Number of NAb positive and negative sera by cPass and NeutraLISA compared to VNT. (**c**) Comparison of inhibition detected by cPass and NeutraLISA at the corresponding VNT NAb titers: *** = $p \leq 0.001$, ns = not significant, and neg = pre-pandemic samples.

Overall, based on the samples from convalescent patients and related to the VNT, there was a positive agreement of 97.0% and a negative agreement of 50.0% for the cPass, as well as a positive agreement of 72.1% and a negative agreement of 92.5% for the NeutraLISA. Although both sVNTs are based on the inhibition of binding between S1/RBD and ACE2, there were numerous differences in measured inhibition values and hence NAb-positive or negative evaluation (Figure 1B). Therefore, the medians of inhibition rates determined by sVNTs at different NAb titers were compared. A significant difference ($p < 0.001$) was found at all titers ≤1:640. Here, the inhibition values determined by cPass were on average 2.4 times higher than those obtained by NeutraLISA, except for values at titers <1:40, where measured values were 7.2 times higher. In samples of the negative cohort, there was a median difference of 16% (Figure 1C). At titers ≥1:2560, inhibition rates reached saturation in both sVNTs. Only at titers > 1:5120, there was a marginal (1.04-fold) but significantly higher inhibition rate with NeutraLISA.

Panel C included one patient with SARS-CoV-2 reinfection (sample CoV24, Supplementary Figure S3) [26]. At day one pso of the reinfection, the detected NAb titer was 1:80 and increased to 1:1280 at 16 dpso. At the same time, the inhibition rate detected by NeutraLISA increased similarly, from −10% to 99%. However, the increase in inhibition seen by cPass was less dramatic (84% to 96%). Interestingly, the cPass detected an increase in inhibition from 74% (75 days before reinfection) to 84% (one day post-reinfection), whereas a decrease from 33% to −10% was seen by the NeutraLISA and a stagnation of NAb titer at 1:80 (VNT) during the same time.

3.3. Performance of Antibody Detection in Patient Samples after Infection with Seasonal hCoVs

As structural similarities between SARS-CoV-2 and other seasonal hCoV may affect the analytical specificity of a serodiagnostic test, cross-reactivities were studied in a cohort comprising pediatric (n = 10) and adult sera (n = 4) from patients with seasonal hCoV infections (n = 12) or rhinovirus infection (n = 1). No SARS-CoV-2-positive IgG was detected by the IIFT or Profile IgG. Anti-N IgG against the respective seasonal hCoV was found in 6 out of 14 sera, but not in the sample with rhinovirus infection. SARS-CoV-2-specific NAb were neither detected by VNT nor by NeutraLISA. However, the cPass detected SARS-CoV-2-specific NAbs in 8/10 pediatric and 0/4 adult sera (Figure 2).

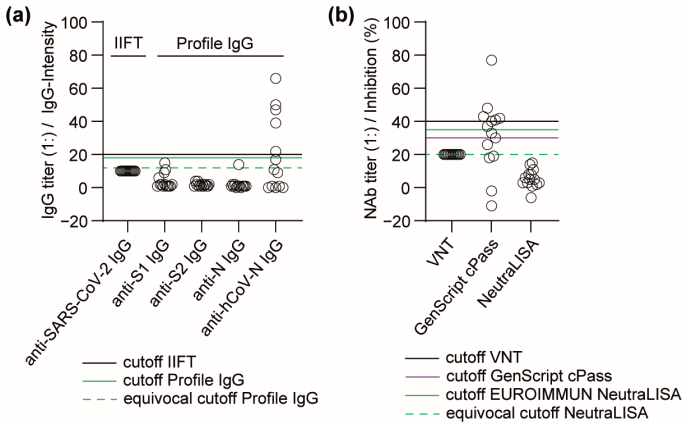

Figure 2. Analytical specificity of (**a**) Profile IgG and (**b**) cPass and NeutraLISA in sera from patients with seasonal hCoV infection.

4. Discussion

As COVID-19 is a crisis affecting public health and the global economy [27], it is important to know the immune status of individuals for planning vaccination campaigns [28] as well as for implementing healthcare and economic measures [27]. Here, the results obtained by different diagnostic tests were compared, and the tests' analytical performance in the determination of IgG and NAb during acute (panel A) and convalescent (panel C) COVID-19 infections as well as in pre-pandemic (panel B) and seasonal hCoV infection (non-SARS-CoV-2 infection, panel D) cohorts was assessed.

The positive agreement of the results of the Profile IgG with those of the IIFT is high. Nevertheless, it increases from panel A (78.3%) to panel C (91.6%). This could be due to different test principles and the manufacturer's instructions on how to interpret the results. In contrast to the IIFT, which contains the full spectrum of viral antigens in the correct conformation, the Profile IgG comprises immobilized S1, S2, and N as antigens, which are immunodominant yet limited [17,29]. As more epitopes are available in the IIFT, its sensitivity might be generally higher. Additionally, according to the instructions for the interpretation of Profile IgG results, a sample is positive if at least two of the three antigen bands show intensities above the cutoff. N of CoVs induces an antibody response earlier than S [17,30,31]. This potentially contributes to the lower positive agreement in Panel A.

Seroconversion of IgG is mainly observed between one and three weeks after symptom onset [32]. After this time, SARS-CoV-2-specific Abs were detected with both assays, as reflected by the high agreement in Panel C. However, the decrease of Abs against CoV antigens occurs at different times in different individuals.

Different results between the IIFT and Profile IgG were observed in patients receiving S1-based immunoglobulin therapy. Although the highest values for S1 were obtained with the Profile IgG, the samples were overall negative due to the instructions for result interpretation mentioned above but positive with the IIFT.

Immobilized N of seasonal hCoVs on the Profile IgG provides additional information on past CoV infections and could also be useful for cross-reactivity screenings [25,33] in vaccine studies, as novel N-based vaccines have been suggested [34,35].

Although IgG and NAb levels tend to correlate well [15], IgG-positive patients may be NAb-negative [22,36]. Thus, the determination of NAbs is probably a better predictor of the immune protection of a person [37] than simple IgG measurement. In the present study, the neutralizing activity of samples from the four different panels was tested with the cell culture-based VNT and two sVNTs, cPass and NeutraLISA. As NAbs are usually detected later than non-neutralizing IgG [38–40], the early detection of NAbs in T1 (0–5 dpso/dpPCR) by all three NTs was surprising. As dpso and dpPCR were equalized in this study and samples from panel A were analyzed irrespective of disease severity, the detectability of NAbs might shift to earlier times. By revising the data, two sera in T1 were found to originate from a patient receiving Ab therapy. However, in T1, the cPass yielded the same number of positive samples as the VNT and one false positive. Very early detection of SARS-CoV-2-specific Abs in acute COVID-19 and false-positive reactivity in pre-pandemic samples by the cPass have been previously described [23,41], and it has been speculated that non-neutralizing Abs were also recognized [22]. Here, no false-positive samples were found in panel B. Nevertheless, the inhibition rates seen in the cPass were closer to the cutoff levels than the inhibition rates seen in the NeutraLISA. However, the detection of non-specific Abs could explain the different course of NAbs in the CoV24 sample found by cPass compared to NeutraLISA and VNT.

The higher positive agreement (97.0%) with VNT at a lower negative agreement (50.0%) for the cPass and vice versa for the NeutraLISA (71.6% and 92.5%) in the convalescent sera are in line with previous studies [20,23,42]. Interestingly, the cPass had a lower negative agreement in panel C (50.0%) compared to panel A (92.3%), while the NeutraLISA showed similar agreements (92.5% and 95.2%). The difference in agreements is striking, as both tests are based on the inhibition of binding between RBD of SARS-CoV-2 and hACE2. In the cPass, the preincubation of serum with HRP-labeled RBD might enhance the assay's sensitivity but could also lead to overestimation of the neutralizing activity [43,44]. The competitive binding of serum antibodies and biotinylated hACE2 to coated RBD in the NeutraLISA could decrease the assay's sensitivity and thus lead to more false-negative samples [23,42], even though competitive binding might reflect the in vivo situation. Although most NAbs are directed against the S1/RBD domain (the detected antigen in sVNTs), NAbs against other epitopes were described [45]. Thus, the false-negative results of sVNTs could be attributed to the broader antigenic spectrum of cell culture-based NTs. Since both sVNTs correlate strongly with the VNT, they should also yield a negative result for these samples.

Despite the correlation, higher inhibition rates by cPass compared to those obtained by NeutraLISA in almost all sera, excluding post-vaccination samples, were observed, which was similarly shown by others without emphasizing this fact [16,23,46]. The higher inhibition rates in cPass may be caused by the different assay setup. However, they were also shown in a comparative study of assays with the same underlying principle [43].

When investigating the cross-reactivity of sVNTs in a small cohort, SARS-CoV-2-specific NAbs were observed in stored pediatric samples (8 of 10) from patients with seasonal hCoV infection but not in adult samples (0 of 4) using cPass. No cross-reactivity was found using NeutraLISA. Interestingly, patients with syphilis infections but not with seasonal hCoV infections were reported to be positive for cPass [47,48]. This false-positive

detection might be caused by preserving agents such as sodium azide in stored samples, as azide inhibits peroxidase activity [49]. Due to the different test setups, this inhibition impacts the cPass but not the NeutraLISA, although in both tests HRP catalyzes the color reaction. This makes NeutraLISA more robust for sample additives.

The data presented in this study are based on sera obtained between March 2020 and April 2021 from persons infected with the variants of SARS-CoV-2 circulating at that point. This is presumably the biggest drawback of this study. Many variants of concern (VOCs) have evolved since then. Since mutations mainly occur in S, they cause a substantial limitation in the detection ability of S1/RBD-based serological assays [23,50]. Assuming a similar immune response to S1 regardless of VOC, times of increase and decrease of NAbs after a COVID-19 infection should be similar and could be estimated, as well as conclusions regarding the immunoprotective status of an individuum drawn.

5. Conclusions

For serodiagnostics, the Profile IgG can be used as a fast alternative to cell culture-based IIFT to confirm the immune responses to COVID-19 infection and vaccination, starting approximately two weeks after the immunologic event. The immunoprotective function of Abs can be best determined by cell culture-based virus infection assays containing the full viral antigenic spectrum. However, as infection assays are restricted to BSL3 diagnostic labs and require a runtime of several days, depending on the procedure, sVNTs represent a less restricted alternative. The sVNTs studied here should be used for different purposes, as they differ in their positive and negative agreements with VNT. After adapting S1/RBD to the currently circulating VOCs, an early and low immune response can be determined by the cPass due to its high positive agreement, whereas the NeutraLISA is helpful to monitor waning NAbs, especially after vaccination, to support decision-making about booster shots as well as in patients receiving Ab therapy. All assays should be interpreted carefully, and the results should be complemented by the outcomes of other diagnostic tests. For instance, a combination of sVNT and a blot-based assay or IIFT could increase diagnostic accuracy, as more than one SARS-CoV-2-specific antigen would be used and test results would serve as internal controls.

Supplementary Materials: The following supporting information can be downloaded: https://www.mdpi.com/article/10.3390/v16010091/s1, Figure S1: Dynamics of SARS-CoV-2-specific IgG and NAbs during an acute infection. Tests used for Ab measurement are indicated on x-axes. The percentage of samples in which Ab was detected is depicted in black (positive) and in which Ab was not detected in grey (negative). Figure S2: Course of antibodies in patients (a) undergoing cancer treatment, (b) treated with immunosuppressives, or (c) receiving antibody therapy. The course of NAb is shown on the left plot, with NAbs determined by VNT plotted on the left *Y*-axis (black), by cPass plotted on the right *Y*-axis (purple), and by NeutraLISA plotted on the right *Y*-axis (green). Plots on the right side show IgG detected in the same samples with total IgG determined with IIFT (black), plotted on the left *Y*-axis, S1 IgG (green), S2 IgG (blue), and NP IgG (red), all determined with Profile IgG and plotted on the right *Y*-axis; Figure S3: Course of SARS-CoV-2-specific IgG antibodies determined with IIFT, or Profile IgG, in the sera of convalescent patients Total IgG (black) determined by IIFT plotted on the left *Y*-axis; IgG values for anti-S1 (green), anti-S2 (blue), and anti-NP (red) determined by Profile IgG plotted on the right *Y*-axis; Figure S4: Dynamics of SARS-CoV-2-specific IgG and NAbs in samples from convalescent patients Tests used for Ab measurement are indicated on x-axes. The percentage of samples with Ab presence is depicted in black (positive) and without Ab detection in gray (negative). Figure S5: Course of SARS-CoV-2-specific NAbs in sequential serum samples from convalescent patients. NAbs were determined with VNT (black) plotted on the left *Y*-axis, and inhibition rates were determined with cPass (purple) and NeutraLISA (green) plotted on the right *Y*-axis.

Author Contributions: P.E. and C.D. contributed to the design and conception of this study. R.v.P., B.M., C.D., C.F., C.H., H.G.-F., M.L., K.S., E.L., R.T., and R.A. were involved in sample collection, data acquisition, and initial data analysis. Data analysis and original draft writing were performed by A.S. and finalized with the help of R.v.P., P.E., and B.M. All authors have read and agreed to the published version of this manuscript.

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Institutional Review Board Statement: This study was performed in accordance with the Declaration of Helsinki and approved by the Ethics Review Board of the Faculty of Medicine of the University of Rostock (panel A, registration number: A2020-0086) and the Ethics Review Board of the Medical Association Hamburg (panel C, registration number: 2020-10162-BO-ff). Sera obtained by blood donation served as negative control samples (panel B). Leftover diagnostic samples collected by a routine diagnostic lab (Lübeck, Germany) were used to investigate cross-reactivities (panel D). All samples were processed anonymously.

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Conflicts of Interest: Babett Menge, Katja Steinhagen, Erik Lattwein, and Anette Schulz are employed by EUROIMMUN Medizinische Labordiagnostika AG, a manufacturer of diagnostic reagents and co-owner of patent applications related to immunoassays for diagnostics of SARS-CoV-2 infections. Ralf Tönnies and Reiner Ahrendt are employees of medac GmbH, Wedel, Germany, a company that distributes diagnostic tests. Christina Deschermeier is the founder and shareholder of Panadea Diagnostics GmbH. Petra Emmerich and Christina Deschermeier are inventors of patents EP2492689 (PE) and EP3207375 (PE, CD). The other authors declare no conflicts of interest.

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10 Selbstständigkeitserklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbstständig und ohne fremde Hilfe verfasst und keine anderen Hilfsmittel als die angegebenen verwendet habe.

Insbesondere versichere ich, dass ich alle wörtlichen und sinngemäßen Übernahmen aus anderen Werken als solche kenntlich gemacht habe.

Hamburg, 30.01.2024 _____ (Unterschrift)

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12 Lebenslauf

In elektronischer Veröffentlichung nicht verfügbar.