PEER REVIEW HISTORY

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ARTICLE DETAILS

TITLE (PROVISIONAL)	Evaluation of the IgG antibody response to SARS CoV-2 infection and performance of a lateral flow immunoassay: cross-sectional and longitudinal analysis over 11 months
AUTHORS	Robertson, Louise; Moore, Julie; Blighe, Kevin; Ng, Mark Kok Yew; Quinn, Nigel; Jennings, Fergal; Warnock, Gary; Sharpe, Peter; Clarke, Mark; Maguire, Kathryn; Rainey, Sharon; Price, Ruth; Burns, William; Kowalczyk, Amanda; Awuah, Agnes; McNamee, Sara; Wallace, Gayle; Hunter, David; Sager, Steve; Chao Shern, Connie; Nesbit, M. Andrew; McLaughlin, James; Moore, Tara

VERSION 1 – REVIEW

REVIEWER	Kadkhoda, Kamran Cleveland Clinic
REVIEW RETURNED	04-Feb-2021

GENERAL COMMENTS	1. Please mention how the sample size was calculated. 2. Only RT-PCR-positive samples should be used. 3. It is pivotal to confirm their positives (i.e., tested positive by all 4 tests) by PRNT similar to this great study: https://wwwnc.cdc.gov/eid/article/27/2/20-4088_article 4. It's key to mention if the samples were from asymptomatic, mild, moderate, or sever cases as it makes a huge difference re duration of Ab response.

REVIEWER	Gaebler, Christian The Rockefeller University
REVIEW RETURNED	02-Apr-2021

GENERAL COMMENTS	The main goal of this manuscript is the cross-validation of a point-of-care rapid lateral flow immunoassay for the detection of SARS-CoV-2 spike specific antibodies. Using a large set of patient plasma samples the authors also intend to cross-sectionally assess the longevity of SARS-CoV-2 specific antibody responses. The authors emphasize that they created a set of rules that allows for the clear identification of SARS-CoV-2 antibody positive reference samples without the need of further PCR-confirmation of prior viral infection. Strength: • Large cohort of participants and samples including pre-pandemic control samples

	• Extensive comparison of three large-scale serological assays with a point-of-care rapid lateral flow immunoassay for the detection of SARS-CoV-2 specific antibodies • Good correlation between visual score of lateral flow immunoassay and semiquantitative cut-off values of serological assays including different antigenic targets. • Assessment of cross-reactivity against patient samples with other respiratory viruses including seasonal coronavirus NL63 and 229E. Limitations: • The emphasized need for a non-PCR reliant reference sample is not entirely clear. This becomes especially important regarding the large fraction of individuals in the study cohort that did not seroconvert and lack RT-PCR confirmed COVID-19 diagnoses (see. Fig.2 Week 13-16 across all serological assays). Reference samples based on prior PCR-confirmed viral infection would lack this potential source of bias. • Cross-sectional analysis not optimal for analysis of longevity of humoral antibody responses. Findings are more important for evaluation of test performance. In addition, several follow-up studies have examined dynamics and durability of humoral immune responses longitudinally. • It would be helpful to visualize the concordant or discrepant results of the different serological assays in a Venn diagram. • The relevance of the age analysis is not clear besides showing statistical significance. The observed differences most likely reflect cohort characteristics rather than showing importance for the test performance. The authors should at least comment and/or expand their demographic analysis. • Discussion would benefit from a stronger focus on the strength of the manuscript (i.e. validation of serological and lateral flow immunoassay). Very broad talking points without further detailed discussion (“immune passport”, “Asymptomatic individuals may be unaware of infection and others may harbour pre-existing immunity or elucidate a T cell response” etc.) should be avoided. Minor points: Line 106: Coronavirus Disease 2019 (COVID-19) 120: SARS-CoV-2 (virus) 141: (antigenic) protein 156: All participants provided informed consent with no adverse events. What is meant by that? No adverse events were observed during the sample collection? 330: (COVID-19) SARS-CoV-2 infection 338: COVID-19 (disease)

VERSION 1 – AUTHOR RESPONSE

Reviewer: 1
Dr. Kamran Kadkhoda, Cleveland Clinic

Comments to the Author:
1. Please mention how the sample size was calculated.
We refer the reviewer to the Statistical Analysis sub-section of the methods (lines 215-224) where the minimum sample size of 240 individuals was determined for a prevalence of 10% and 506 individuals was required if the prevalence of SARS-CoV-2 was 5%. Our data represents assessment of a sample size of 657 participants.
The sample size for the assessment of performance metrics for the LFIA AbC-19 was stipulated by the UK Government Medicines and Healthcare products Regulatory Agency as a minimum sample size of 200 known positives and 200 known negatives for SARS-CoV-2 LFIA antibody immunoassays. Our data represents assessment of a sample size of 330 known positives and 488 known negatives.

2. Only RT-PCR-positive samples should be used.
It is increasingly recognised that the high false positive and false negative rate of RT-PCR makes it an imperfect reference standard and its use could greatly penalise the evaluation of diagnostic accuracy of new tests if RT-PCR is used as a stand-alone reference standard.

Information regarding SARS-CoV-2 RT-PCR-positivity was gathered wherever possible. We wish to highlight to Dr Kadkhoda that this study was conducted at the peak of the pandemic in Northern Ireland and very early in the first lock down. The availability of, and access to, RT-PCR testing was very limited during this early stage of the first wave of the COVID-19 pandemic in Northern Ireland. Many samples were collected from individuals many weeks after their self-reported date of onset of COVID-19 symptoms.

While SARS-CoV-2 RT-PCR positivity has been used as a reference standard for determination of prior infection, this has been brought into question by reports of significant numbers of both false negatives (up to 29%) and false positives (up to 4%). We refer Dr Kadkhoda to a recent diagnostic accuracy study of lateral flow immunoassays published in BMJ that uses a reference standard of antibody positivity by a composite of two laboratory immunoassays (Moshe et al., 2021).

We therefore determined the seropositivity of individuals based on the presence of both antibodies to Nucleocapsid (pan-Ig and IgG) by two assays (Roche and Abbott, respectively) and, the S1 subunit of the spike protein (IgG) by a Euroimmun ELISA assay, all of which have been widely accepted. We then measured the performance metrics of the AbC-19 Spike protein LFIA against these assays.

To address this comment, we have now added additional analysis of SARS-CoV-2 RT-PCR positive individuals, analysing AbC-19 test sensitivity against any samples from existing positive and negative cohorts with RT-PCR positive status (n=227). We determine and report within the resubmission a sensitivity of 92.07% (87.76%- 95.23%), however highlight that 12 out of 18 RT-PCR positive individuals negative for IgG antibodies by AbC-19 showed no detectable antibodies by all three laboratory assays (EuroImmun, Roche or Abbott); these could possibly be attributed to false positive RT-PCR results, failure to seroconvert, or a decrease in antibody levels below assay positivity thresholds by the time the sample was taken.

3. It is pivotal to confirm their positives (i.e., tested positive by all 4 tests) by PRNT similar to this great study:
https://eur03.safelinks.protection.outlook.com/?url=https%3A%2F%2Fwwwnc.cdc.gov%2Feid%2Farticle%2F27%2F2%2F20-4088_article&data=04%7C01%7Ca.nesbit%40ulster.ac.uk%7C414b119370fd4a78f3eb08d8fb3e0a17%7C6f0b94874fa842a8aeb4bf2e2c22d4e8%7C0%7C0%7C637535591162947171%7CUnknown%7CTWFpbGZsb3d8eyJWIjoiMC4wLjAwMDAiLCJQIjoiV2luMzIiLCJBTiI6Ik1haWwiLCJXVCI6Mn0%3D%7C1000&sdata=O1ucCzX9fYz9fciyQ5oPMcnTijYjbwgUsm64XA7NYbc%3D&reserved=0

PRNT assays, which measure the neutralisation of live SARS-CoV-2 virus, require access to a Biological Safety Level 3 laboratory and specialist staff which are not available to us at Ulster University.

Whilst we agree that these would be interesting results, we note that our use of a composite reference standard strengthens our data- a positive result by the S1 EuroImmun ELISA (when we know spike antigen antibodies correlate highly with neutralising antibodies, Dan et al., 2021) and one additional assay is sufficient. In addition, we show the degree of agreement, but also the discordance between the tests (Figure 1, Figure S3- newly added Venn diagram).

Interestingly, the paper to which the reviewer refers does not use PCR positivity as an indicator of infection but rather measures seroprevalence using two assays both of which measure antibodies to nucleocapsid protein alone, thus potentially underestimating total seroprevalence by not assessing spike protein antibody seroprevalence.

4. It's key to mention if the samples were from asymptomatic, mild, moderate, or sever cases as it makes a huge difference re duration of Ab response.

A growing number of manuscripts, published and submitted, report on the longevity of the persistence of antibodies to SARS-CoV-2 antigens. The minority of these present detailed information on the severity of infection and it is largely left to the reader to make this inference, depending on whether the participants were recruited from hospitalised, symptomatic or asymptomatic cohorts. In our experience, self-reported symptoms are a poor indicator of previous infection (note the number of self-reported individual samples below positive thresholds, represented by black dots in Figure 2). Furthermore, the reported longevity of antibody responses varies between studies, and while those with higher initial antibody titres generally maintain these higher titres, this is not always the case, and those with lower initial titres may maintain these over many months.
Unfortunately, samples in our cross-sectional cohort obtained from Southern Health and Social Care Trust (SHSCT) Healthcare workers (n=195), and Northern Ireland Blood Transfusion Service (NIBTS, n=184) were provided anonymised, with minimal demographic information (age and gender), meaning we do not have severity data for them.

By way of addressing this reviewer's comment we have now added additional longitudinal data demonstrating detectable antibodies to SARS-CoV-2 Nucleocapsid and Spike antigens up to 46 weeks following a positive RT-PCR result in samples obtained through the Northern Ireland convalescent plasma program (Figure S6).

Reviewer: 2
Dr. Christian Gaebler, The Rockefeller University

Comments to the Author:
The main goal of this manuscript is the cross-validation of a point-of-care rapid lateral flow immunoassay for the detection of SARS-CoV-2 spike specific antibodies. Using a large set of patient plasma samples the authors also intend to cross-sectionally assess the longevity of SARS-CoV-2 specific antibody responses.

The authors emphasize that they created a set of rules that allows for the clear identification of SARS-CoV-2 antibody positive reference samples without the need of further PCR-confirmation of prior viral infection.

Strength:
• Large cohort of participants and samples including pre-pandemic control samples
• Extensive comparison of three large-scale serological assays with a point-of-care rapid lateral flow immunoassay for the detection of SARS-CoV-2 specific antibodies
• Good correlation between visual score of lateral flow immunoassay and semiquantitative cut-off values of serological assays including different antigenic targets.
• Assessment of cross-reactivity against patient samples with other respiratory viruses including seasonal coronavirus NL63 and 229E.

Limitations:
• The emphasized need for a non-PCR reliant reference sample is not entirely clear.
We have reworded these sections of the manuscript to make this clearer.
At the time that the participants recruited to this study first became infected with SARS-CoV-2 and ill with COVID-19 (early 2020), PCR testing was not routinely available in Northern Ireland. Many of the samples were collected weeks or months after symptom onset. Thus, RT-PCR positivity was not available as an indicator of past infection. To assess the performance of the AbC-19 LFIA tests, we therefore needed to use samples that had been measured using well-accepted laboratory immunoassays.

This becomes especially important regarding the large fraction of individuals in the study cohort that did not seroconvert and lack RT-PCR confirmed COVID-19 diagnoses (see. Fig.2 Week 13-16 across all serological assays). Reference samples based on prior PCR-confirmed viral infection would lack this potential source of bias.
We acknowledge this reviewer comment and indeed this was a major challenge as we progressed this study at a time when no reference standard was available for serological assessment of SARS-CoV-2 antibodies and when lock down restrictions meant only hospitalised severely ill patients were able to obtain RT-PCR virus detection. There were ethical barriers with consenting hospitalised participants due to the high mortality rate in the initial months of the pandemic.

The participant blood samples included in Fig 2. are samples collected during 2020, for which we have either an RT-PCR result (red dots) or a self-reported symptom onset date (black dots). The number of RT-PCR positive individuals (red dots) that do not show any detectable antibodies (14 in total, Fig 2, Fig S2) emphasizes the drawbacks/bias of using RT-PCR as a reference sample; whether this is due to lack of seroconversion or false positive RT-PCR result. The large number of self-reported symptoms (black dots) that do not produce antibodies highlights the unreliability of using self-reported symptoms to indicate Covid-19 infection.

These samples cannot introduce bias into our LFIA evaluation presented within this study (case use for detection of SARS-CoV-2 antibodies), as they are not included in the positive cohort for AbC-19 performance assessment (some, in fact, fall into our negative cohort as they meet the criteria of negative by all three laboratory assays, see the newly included Figure S3 Venn diagram).

To address Dr. Gaeblers concern, we have now added additional analysis of SARS-CoV-2 RT-PCR positive individuals, analysing AbC-19 test sensitivity against any samples from existing positive and negative cohorts with RT-PCR positive status (n=227). We determine and report within the resubmission a sensitivity of 92.07% (87.76%- 95.23%), however highlight that 12 out of 18 RT-PCR positive individuals negative for IgG antibodies by AbC-19 showed no detectable antibodies by all three laboratory assays (EuroImmun, Roche or Abbott); these could possibly be attributed to false positive RT-PCR results, failure to seroconvert, or a decrease in antibody levels below assay positivity thresholds by the time the sample was taken.

• Cross-sectional analysis not optimal for analysis of longevity of humoral antibody responses. Findings are more important for evaluation of test performance. In addition, several follow-up studies have examined dynamics and durability of humoral immune responses longitudinally.
We have now added longitudinal data from the NIBTS convalescent plasma program, showing persistence of detectable IgG antibodies more than 10 months following positive RT-PCR result. Spike and Nucleocapsid antibody levels are now reported within a new Figure S6 representing sequential blood samples (2-9 samples per subject) from over 100 RT-PCR positive individuals. The data we now include are broadly in agreement as regards longevity of antibody presence to that of Dan et al., (2021) from their combined cross-sectional and longitudinal studies performed in USA.
• It would be helpful to visualize the concordant or discrepant results of the different serological assays in a Venn diagram.
Thank you for this suggestion. We agree with Dr. Gaebler and have added Venn diagrams to supplementary material (Figure S3), denoting concordant/discrepant results, as well as RT-PCR positive results within each cohort.
• The relevance of the age analysis is not clear besides showing statistical significance. The observed differences most likely reflect cohort characteristics rather than showing importance for the test performance. The authors should at least comment and/or expand their demographic analysis.
We have now added further demographic descriptions for each cohort (Page 10 Line 252- 256, Page 12 Line 302-303) and comment on the statistically significant result in the discussion. We agree with Dr. Gaebler that this is likely due to cohort characteristics and does not reflect the wider population or the performance of the immunoassays used.
• Discussion would benefit from a stronger focus on the strength of the manuscript (i.e. validation of serological and lateral flow immunoassay).
We thank reviewer Dr. Gaebler for this positive comment on the strength of the manuscript and we have made substantial changes to the discussion to better highlight the strengths of the study.
Very broad talking points without further detailed discussion ("immune passport", "Asymptomatic individuals may be unaware of infection and others may harbour pre-existing immunity or elucidate a T cell response" etc.) should be avoided.
We have now removed these sections from the manuscript.

Minor points:
Line

106: Coronavirus Disease 2019 (COVID-19) updated (now Line 111)
120: SARS-CoV-2 (virus) sentence removed from manuscript
141: (antigenic) protein updated (now Line 148)
156: All participants provided informed consent with no adverse events.
What is meant by that? No adverse events were observed during the sample collection? Yes, we have simplified this to remove confusion (now Line 164).
330: (COVID-19) SARS-CoV-2 infection updated (now Line 411)
338: COVID-19 (disease) updated (now Line 420)

We trust that this extensive and thorough response adequately address the reviewers' comments. We would like to thank the reviewers for their time and input into significantly improving this manuscript and we also thank you for considering our research study for publication in BMJ Open.

VERSION 2 – REVIEW

REVIEWER	Kadkhoda, Kamran Cleveland Clinic
REVIEW RETURNED	27-Apr-2021

GENERAL COMMENTS	Any such studies will require confirmation by PRNT even if it’s done at the peak on any outbreak. Here’s one example: https://wwwnc.cdc.gov/eid/article/27/2/20-4088_article

REVIEWER	Gaebler, Christian The Rockefeller University
REVIEW RETURNED	04-May-2021

GENERAL COMMENTS	In this revised manuscript, the authors responded very carefully to most of the comments of the reviewers and have improved their manuscript by adding a longitudinal analysis for the assessment of the durability of SARS-CoV-2 specific antibody responses. In addition, the now included Venn diagrams are helpful to clarify test results between different study participants. Minor comments: 381-384: See studies below Kanika Vanshylla, Veronica Di Cristanziano, Franziska Kleipass, Felix Dewald, Philipp Schommers, Lutz Gieselmann, Henning Gruell, Maike Schlotz, Meryem S. Ercanoglu, Ricarda Stumpf, Petra Mayer, Matthias Zehner, Eva Heger, Wibke Johannis, Carola Horn, Isabelle Suárez, Norma Jung, Susanne Salomon, Kirsten Alexandra Eberhardt, Birgit Gathof, Gerd Fätkenheuer, Nico Pfeifer, Ralf Eggeling, Max Augustin, Clara Lehmann, Florian Klein, Kinetics and correlates of the neutralizing antibody response to SARS-CoV-2 infection in humans,Cell Host & Microbe, https://doi.org/10.1016/j.chom.2021.04.015. Petersen, M. S. et al. SARS-CoV-2 natural antibody response persists up to 12 months in a nationwide study from the Faroe Islands. medRxiv, 2021.2004.2019.21255720, doi:10.1101/2021.04.19.21255720 (2021). Li, C. et al. Twelve-month specific IgG response to SARS-CoV-2 receptor-binding domain among COVID-19 convalescent plasma donors in Wuhan. bioRxiv, 2021.2004.2005.437224, doi:10.1101/2021.04.05.437224 (2021).

VERSION 2 – AUTHOR RESPONSE

Reviewer: 1
Dr. Kamran Kadkhoda, Cleveland Clinic

Comments to the Author:
Any such studies will require confirmation by PRNT even if it’s done at the peak on any outbreak. Here’s one example:
https://eur03.safelinks.protection.outlook.com/?url=https%3A%2F%2Fwwwnc.cdc.gov%2Feid%2Farticle%2F27%2F2%2F20-4088_article&data=04%7C01%7Ctara.moore%40ulster.ac.uk%7Ccc298ef2364647406b0808d9155aecd3%7C6f0b94874fa842a8aeb4bf2e2c22d4e8%7C0%7C0%7C637564302550695231%7CUnknown%7CTWFpbGZsb3d8eyJWIjoiMC4wLjAwMDAiLCJQIjoiV2luMzIiLCJBTiI6Ik1haWwiLCJXVCI6Mn0%3D%7C1000&sdata=MWn8nNLzPhY5zhqEZVz06JYmUrEZ5KbH8RJCOwmq1uI%3D&reserved=0

As per my email correspondence I would like to stress again on behalf of the team involved in this study, it was not and is not possible for us to conduct the neutralising antibody assays within our University setting, due to health and safety restrictions. In addition, we wish to bring to your attention that the paper referred to by Reviewer 1 confirmed neutralising antibody by PRNT in only 8 samples that were positive by both of their serological assays– a completely different order of magnitude of positive samples than we have in our study (296 positive by all three tests). The lack of ability to do this neutralising antibody assay ourselves would mean we would have to outsource COVID blood samples to an external laboratory, even if we could source one with such substantial assay capability. This, combined with the large number of samples assessed and taken alongside the obvious cost implications, leaves us in a situation that we have to declare we genuinely cannot address this comment any further. It is our belief that our study's methodology and validation processes are sound, and that the reliability of the results would not be significantly altered by further confirmation by the PRNT assay.

Reviewer: 2
Dr. Christian Gaebler, The Rockefeller University

Comments to the Author:
In this revised manuscript, the authors responded very carefully to most of the comments of the reviewers and have improved their manuscript by adding a longitudinal analysis for the assessment of the durability of SARS-CoV-2 specific antibody responses. In addition, the now included Venn diagrams are helpful to clarify test results between different study participants.

Minor comments:
381-384: See studies below
Kanika Vanshylla, Veronica Di Cristanziano, Franziska Kleipass, Felix Dewald, Philipp Schommers, Lutz Gieselmann, Henning Gruell, Maike Schlotz, Meryem S. Ercanoglu, Ricarda Stumpf, Petra Mayer, Matthias Zehner, Eva Heger, Wibke Johannis, Carola Horn, Isabelle Suárez, Norma Jung, Susanne Salomon, Kirsten Alexandra Eberhardt, Birgit Gathof, Gerd Fätkenheuer, Nico Pfeifer, Ralf Eggeling, Max Augustin, Clara Lehmann, Florian Klein, Kinetics and correlates of the neutralizing antibody response to SARS-CoV-2 infection in humans,Cell Host &
Microbe, https://eur03.safelinks.protection.outlook.com/?url=https%3A%2F%2Fdoi.org%2F10.1016%2Fj.chom.2021.04.015&data=04%7C01%7Ctara.moore%40ulster.ac.uk%7Ccc298ef2364647406b0808d9155aecd3%7C6f0b94874fa842a8aeb4bf2e2c22d4e8%7C0%7C0%7C637564302550695231%7CUnknown%7CTWFpbGZsb3d8eyJWIjoiMC4wLjAwMDAiLCJQIjoiV2luMzIiLCJBTiI6Ik1haWwiLCJXVCI6Mn0%3D%7C1000&sdata=wy2VamE5zwWAoZJCovj3hVMnstFwGWWeOssg6wXphts%3D&reserved=0.
Petersen, M. S. et al. SARS-CoV-2 natural antibody response persists up to 12 months in a nationwide study from the Faroe Islands. medRxiv, 2021.2004.2019.21255720, doi:10.1101/2021.04.19.21255720 (2021).
Li, C. et al. Twelve-month specific IgG response to SARS-CoV-2 receptor-binding domain among COVID-19 convalescent plasma donors in Wuhan. bioRxiv, 2021.2004.2005.437224, doi:10.1101/2021.04.05.437224 (2021).

Thank you for alerting us to these recent studies, we have now changed our wording and added these references.

These lines (now 428-431) read

“The longevity of IgG antibodies to both spike and nucleocapsid protein more than 10 months after RT PCR positive status (and beyond in a small number of samples, Figure 2, Figure S6) is consistent with that observed in other recent studies(19–21).”