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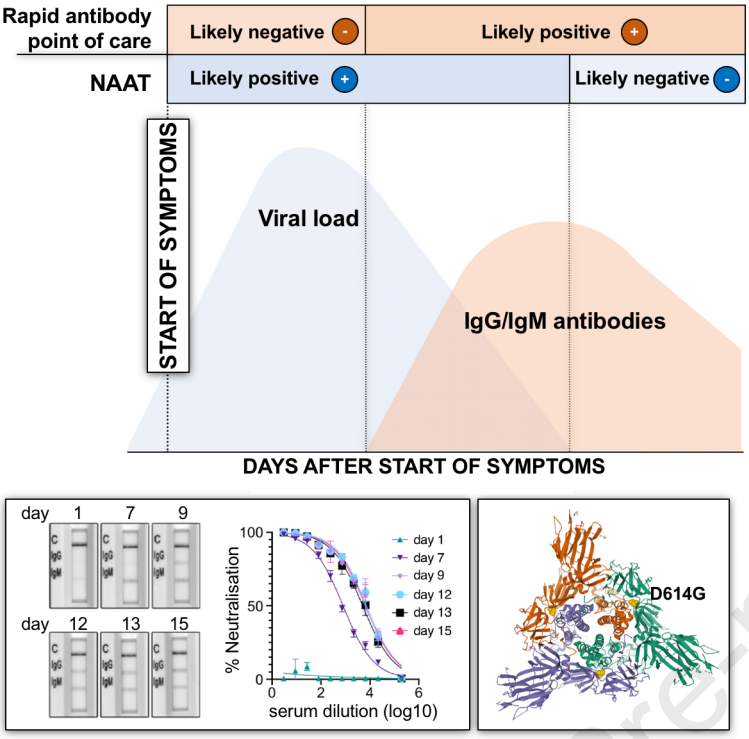

Combined point of care nucleic acid and antibody testing for SARS-CoV-2 following emergence of D614G Spike Variant

Petra Mlcochova[1,2]*, Dami Collier*[1,2,3], Allyson Ritchie[4], Sonny M. Assennato[4], Myra Hosmillo[5], Neha Goel[4], Bo Meng[1,2], Krishna Chatterjee[6], Vivien Mendoza[6], Nigel Temperton[7], Leo Kiss[8], Leo C. James[8], Katarzyna A. Ciazynska[8], Xiaoli Xiong[8], John AG Briggs[8], James A. Nathan[1,2], Federica Mescia[1,2], Laura Bergamaschi[1,2], Hongyi Zhang[9], Petros Barmpounakis[10], Nikos Demeris[10,11], Richard Skells[6, 11], Paul A. Lyons[1,2], John Bradley[3,12], Steven Baker[1,2] , Jean Pierre Allain[4], Kenneth GC Smith[1,2], Rachel Bousfield[13], Michael Wilson[13], Dominic Sparkes[13], Glenn Amoroso[14], Effrosyni Gkrania-Klotsas[13], Susie Hardwick[14], Adrian Boyle[14], Ian Goodfellow[5], Ravindra K. Gupta[1,2,13,15, 16]

The CITIID-NIHR COVID BioResource Collaboration: Stephen Baker, John Bradley, Gordon Dougan, Ian Goodfellow, Ravi Gupta, Paul J. Lehner, Paul Lyons, Nicholas J. Matheson, Kenneth G.C. Smith, Mark Toshner, Michael P. Weekes**,** Nick Brown, Martin Curran, Surendra Palmar, Hongyi Zhang, David Enoch, Daniel Chapman**,** Ashley Shaw, Sherly Jose, Areti Bermperi, Julie Ann Zerrudo, Evgenia Kourampa, Laura Watson, Jieniean Worsley, Caroline Saunders, Ranalie de Jesus, Jason Domingo, Ciro Pasquale, Bensi Vergese, Phoebe Vargas, Marivic Fabiculana, Marlyn Perales, Lee Mynott, Elizabeth Blake, Amy Bates, Anne-Laure Vallier, Alexandra Williams, David Phillips, Edmund Chiu, Alex Overhill, Nicola Ramenatte, Jamal Sipple, Steven Frost, Helena Knock, Richard Hardy, Emily Foster, Fiona Davidson,Viona Rundell, Purity Bundi, Richmond Abeseabe, Sarah Clark, Isabel Vicente, Laura Watson, Jieniean Worsley, Anne Elmer, Carla Ribeiro, Jenny Kourampa, Sherly Jose, Jane Kennet, Jane Rowlands, Anne Meadows, Criona O'Brien, Rebecca Rastall, Cherry Crucusio, Sarah Hewitt, Jane Price, Jo Calder, Laura Canna, Ashlea Bucke, Hugo Tordesillas, Julie Harris, Valentina Ruffolo, Jason Domingo, Barbara Graves, Helen Butcher, Daniela Caputo and Emma Le Gresley, Benjamin J Dunmore, Jennifer Martin, Ekaterina Legchenko, Carmen Treacy, Christopher Huang, Jennifer Wood, Rachel Sutcliffe, Josh Hodgson, Joy Shih, Stefan Graf, Zhen Tong, Federica Mescia, Tobias Tilly, Ciara O'Donnell, Kelvin Hunter, Linda Pointon, Nicole Pond, Marta Wylot, Emma Jones, Stuart Fawke and Ben Bullman, Lori Turner, Isobel Jarvis, Ommar Omarjee, Aloka De Sa, Joe Marsden, Ariana Betancourt, Marianne Perera, Maddie Epping, Nathan Richoz, Georgie Bower, Rahul Sharma, Francesca Nice, Oisin Huhn, Stuart Fawke, Natalia Savoinykh Yarkoni, Nika Romashova, Daniel Lewis, Andrew Hinch, Chiara Cossetti, Mateusz

Strezlecki, Richard Grenfell, Hannah Stark, Neil Walker, Kathy Stirrups, Nigel Ovington, Eleanor Dewhust, Emily Li and Sofia Papadia.

*Equal contribution

[1] Cambridge Institute of Therapeutic Immunology & Infectious Disease (CITIID), Cambridge, UK.

[2]Department of Medicine, University of Cambridge, Cambridge, UK.

[3]Division of Infection and Immunity, University College London, UK. WC1E 6BT

[4]Diagnostics for the Real World EU Ltd., Chesterford Research Park, UK.

[5]Department of Pathology, University of Cambridge, Cambridge

[6] NIHR Cambridge Clinical Research Facility, Cambridge, UK.

[7]Viral Pseudotype Unit, Medway School of Pharmacy, University of Kent, UK

[8]Medical Research Council Laboratory of Molecular Biology, Cambridge, UK

[9]Clinical Microbiology & Public Health Laboratory, Cambridge University NHS Hospitals Foundation Trust, Cambridge, UK.

[10] Department of Statistics, Athens University of Economics and Business, Athens, Greece

[11]Cambridge Clinical Trials Unit-Cancer Theme

[12] National Institutes for Health Research Cambridge Biomedical Research Centre, Cambridge, UK.

[13]Department of Infectious Diseases, Cambridge University NHS Hospitals Foundation Trust, Cambridge, UK.

[14]Department of Emergency Medicine, Cambridge University NHS Hospitals Foundation Trust, Cambridge, UK.

[15]Africa Health Research Institute, Durban, South Africa

[16]Twitter:@GuptaR_lab

[17]Lead contact

Address for correspondence:

Ravindra K. Gupta
Cambridge Institute for Therapeutic Immunology and Infectious Diseases
Jeffrey Cheah Biomedical Centre
Cambridge Biomedical Campus

Puddicombe Way
Cambridge CB2 0AW

Tel: +44 1223 331491
rkg20@cam.ac.uk

Summary

Rapid COVID-19 diagnosis in hospital is essential, though complicated by 30-50% of nose/throat swabs being negative by SARS-CoV-2 nucleic acid amplification testing (NAAT). Furthermore, the D614G spike mutant now dominates the pandemic and it is unclear how serological tests designed to detect anti-Spike antibodies perform against this variant. We assess the diagnostic accuracy of combined rapid antibody point of care (POC) and nucleic acid assays for suspected COVID-19 disease due to either wild type or the D614G spike mutant SARS-CoV-2. The overall detection rate for COVID-19 is 79.2% (95CI 57.8-92.9%) by rapid NAAT alone. Combined point of care antibody test and rapid NAAT is not impacted by D614G and results in very high sensitivity for COVID-19 diagnosis with very high specificity.

Introduction

As of the 2nd of August 2020, more than 18.0 million people have been infected with SARS-CoV-2 with over 690,000 deaths[1]. The unprecedented numbers requiring SARS-CoV-2 testing has strained healthcare systems globally. There is currently no gold standard for diagnosis of COVID-19. Detection of SARS-CoV-2 by nucleic acid amplification testing (NAAT) is largely done by real time RT-PCR on nose/throat swabs in centralised laboratories. RT-PCR specimens are often batch analysed and the turnaround time for this test can be as long as 2- 4 days in real world settings[2]. NAAT tests from a single nose/throat swab are negative in up to 50% in patients who have CT changes consistent with COVID-19 and/or positive antibodies to SARS-CoV-2 [3-5]. The lack of detectable virus in upper airway samples is not only a serious barrier to making timely and safe decisions in the emergency department, but also leads to multiple swab samples being sent, frequently from the same anatomical site, leading to additional strain on virology laboratories. Nonetheless, NAAT remains important in identifying infectious individuals. Additionally, in severely ill patients

tracheo-bronchial samples might be NAAT positive even when the nose/throat swab is negative[4,6].

Multiple factors might contribute to negative results by NAAT, including test sensitivity, sampling technique and timing of the sampling in the disease course[6]. The viral load in the upper respiratory tract is detectable from around 4 days before symptoms[7] and frequently wanes after a week post symptom onset[8 9]. Similarly, a case series from Germany found the detection rate by RT-PCR was <50% after 5 days since onset of illness[10]. A proportion of patients develop a secondary deterioration in clinical condition requiring hospitalisation and respiratory support, at a time when immune pathology is thought to be dominant rather than direct pathology related to viral replication [9,11].

An antibody response to SARS-CoV-2 is detectable 6 days from infection and is almost always neutralising [12,13]. Antibody based diagnosis of COVID-19 shows increasing sensitivity in the latter part of the infection course when NAAT testing on nose/throat samples is more likely to be negative[14-17]. As a result, diagnosis of infection as well as identification of infectivity would benefit from a combination of virologic and immunologic markers to inform patient initial triage and subsequent management. It is critical to determine whether a rapid point of care combined antibody and nucleic acid testing strategy could improve diagnosis.

We previously evaluated the diagnostic accuracy of the SAMBA II SARS-CoV-2 rapid test compared with the standard laboratory RT-PCR and found similar accuracy with a turnaround time of 2-3 hours even in real world settings [18]. Several studies have now reported head-to-head comparisons of immuno-chromatographic lateral flow immunoassays (LFAs)[15-17,19]. These assays are cheap to manufacture and give a binary positive/negative result, thereby lending themselves well to point of care (POC) testing. Even though they have variable performance and in general are negative in the early phase of infection, they become highly sensitive in the later stage of illness[15-17,19] and some are also highly specific.

In this study we evaluated the diagnostic performance of a POC combination comprising NAAT and antibody testing against a composite reference standard of laboratory RT-PCR and a serum neutralisation assay. Notably, SARS-CoV-2 viruses with a D to G mutation in Spike at position 614 have increased in prevalence globally[20]. Cryo EM studies suggest that

D614 may play a role in Spike inter-molecular stability [21], potentially contributing to increased infectivity [20]. Given POC antibody tests were designed to detect antibodies to wild type S protein, we also aimed to investigate whether SARS-CoV-2 infections with D614G Spike mutant virus could be diagnosed by POC antibody tests.

Results

In phase one, 45 prospectively recruited participants in the COVIDx study with suspected COVID-19 disease had nose/throat swabs specimens tested for nucleic acid as well as stored sera for antibody testing. Samples at hospital admission were collected at a median of 7 (IQR 7-13) days after illness onset. The sera from 42.2% (19/45) participants showed neutralising antibody response against SARS-CoV-2 Spike protein pseudotyped virus infection in a neutralisation assay using a cut-off of 50% inhibition at 1:4 dilution (Figure 1A). 26 participants' sera showed no neutralising response (Figure 1B). The neutralisation ability of participants' sera was compared with an in house ELISA IgG assay for Spike specific antibodies based on a recently reported method[22] (Supplementary Figure 1), and significant association between positive results in both assays was demonstrated (Figures 1C, $p<0.0001$). Figures 1D-G shows significant associations between the point of care antibody test result and both ELISA ($p<0.0001$) and neutralisation assays, $p<0.0025$. POC antibody testing showed no cross reactivity in sera obtained before the pandemic (Supplementary table 1). The neutralisation assay also demonstrated lack of cross-reactivity with SARS-CoV-1 on a limited subset of sera (Supplementary Figure 2).

Results from the four IgG antibody assays utilised in this study were confirmed (4 or 3 concordant) in 38/45 samples and, against this classification, neutralisation (Figure 1A-C), spike ELISA ([22] Figure C,D,F and Supplementary Figure 1), Surescreen and COVIDIX Healthcare assays gave a correct result in 100%, 97.4%, 92.1% and 86.8%, respectively, justifying the choice of the neutralisation assay as part of a composite reference standard.

53.3% (24/45) of participants had COVID-19 disease, as determined by the composite reference standard (lab RT-PCR and neutralisation assay). Median age was 73.5 (IQR 54.0-86.5) years in those with SARS-CoV-2 infection by our composite reference standard and 63.0 (IQR 41.0-72.0) years in those without disease (Table1). CRP and procalcitonin were significantly higher in confirmed COVID-19 patients and 'classical' chest radiograph appearances were more common in confirmed COVID-19 patients (Table1, $p<0.001$).

However, 6/24 (25%) had normal or indeterminate chest radiographs in the confirmed COVID-19 group.

As expected from the clinical study inclusion criteria, more than 80% of patients presented with influenza like illness (ILI) with documented fever and approximately one third had clinical or radiological evidence of pneumonia (Table 1). Highly experienced internal medicine physicians were caring for suspected COVID-19 cases at our institution, and this was partly due to the significant co-morbidities in the local population that mandated a broad differential diagnostic approach in hospitalised individuals (Table 1). Amongst patients with COVID-19 one suffered from rheumatoid arthritis and was currently immunosuppressed with Prednisolone. Amongst patients without COVID-19, five were immunosuppressed for the following conditions: psoriatic arthritis - Usekinumab (anti IL-12, IL-23); multiple myeloma - Lenalidomide and dexamethasone; Lymphoma – ciclosporin; hypersensitivity pneumonitis - mycofenalate and prednisolone; renal transplant - mycofenalate and tacrolimus. No patients in the study were under treatment with the anti-B cell monoclonal antibody rituximab.

During the peak of the first wave routine respiratory virus testing was halted at our institution due to the demands of SARS-CoV-2 testing and low seasonal prevalence of these pathogens. Multiplex PCR for other respiratory viral pathogens was performed in only 8 participants. Seven of these participants were negative and one participant tested positive for influenza A.

The overall COVID-19 diagnosis rate (positive predictive agreement) by rapid nucleic acid testing was 79.2% (95% CI 57.8-92.9), decreasing from 100% (95% CI 65.3-98.6%) for days 1-4 to 50.0% (95% CI 11.8-88.2) for days 9-28 post symptom onset (Table 2 and Supplementary Figure 3). When IgG/IgM rapid tests were combined with NAAT, the overall positive predictive agreement increased to 100% (95% CI 85.8-100) (Table 2). Additional cases of COVID-19 detected in NAAT negative patients were identified by POC tests under investigation (Figure 2). Among 21 COVID-19 negative individuals, there were three false positive results for one POC antibody test and one false positive result for the other, resulting in positive predictive values of 88.9% and 96.0% for the two POC antibody/ SAMBA II NAAT combinations.

Those with positive NAAT and sequence available were predominantly infected with strains containing the D614G mutation in Spike, downstream of the receptor binding domain and

located on the Spike surface (Figure 3A, B). 14/24 (58.3%) patients deemed to be COVID-19 positive by the reference composite standard were positive by both rapid NAAT and antibody testing and 14/14 were infected with strains bearing D614G, indicating that point of care serological tests were able to detect infections with this variant.

To understand the relationship between POC band intensity and neutralisation activity further, we identified three participants (all infected with D614G Spike mutant) with stored samples at multiple time points in their illness (Figure 4). Two individuals were sampled from early after symptom onset and the third presented three weeks into illness. In the first two cases (Figure 4A-F), we observed an increase in neutralisation activity over time that was mirrored by band intensities on rapid POC antibody testing. As expected IgM bands arose early on with IgG following closely. Of note in patient 1 there was a weakly detectable IgM band by rapid test with no serum neutralisation activity (Figure 4A, B). Over time the band intensity for IgM and IgG increased along with serum neutralisation activity. In the individual presenting 21 days into illness (Figure 4G-I), only IgG was detected with rapid POC antibody testing and as expected band intensity did not increase over the following days.

In phase 2, we performed a prospective evaluation of combined testing in 128 patients presenting with possible COVID-19 from July 13th to 27th 2020. Their clinical presentation was less severe and diagnoses broader than in phase 1 (Table 3), with cardiovascular and gastrointestinal disease significantly represented and respiratory disease representing just 60% of cases - likely as a result of the increased appreciation of diverse presentations of COVID-19 disease [23]. Patients did have significant comorbidities and around 10% were immune suppressed, though without B cell depleting agents (Table 3). By this time the POC NAAT test had been validated in a head to head study against the lab RT-PCR and entered routine use (Collier et al., 2020), replacing the RT-PCR. Given the need to further assess the specificity of the POC antibody tests in routine clinical practice and with fresh blood rather than serum, we compared the performance of POC antibody tests on finger prick blood against serum neutralisation (Figure 5A and B).

In this second phase there was only one NAAT positive case, who was also positive by both POC antibody tests and serum neutralisation. There were three NAAT negative individuals presenting with respiratory symptoms who had positive POC antibody tests by both

COVIDIX and SureScreen, along with serum neutralisation activity. The POC antibody tests showed 100% negative predictive agreement with serum neutralisation and the kappa correlation between POC antibody tests and serum neutralisation was extremely high at 0.97.

Discussion

Here we have shown that POC NAAT testing in combination with antibody detection can significantly improve diagnosis of COVID-19. Overall positive predictive agreement against the composite reference standard under clinical trial conditions was around 79% for rapid NAAT testing of nose/throat swab samples, reaching 100% with a combined approach of rapid NAAT testing and either of the two POC lateral flow-based antibody tests. The specificity of the combined approach was 85-95% on stored serum under clinical trial conditions and 100% on fingerprick blood in routine clinical care.

As expected, nucleic acid detection in nose/throat samples was highest in those presenting within the first few days (100% in samples taken in the first 4 days after symptom onset). Conversely antibody detection by LFA increased with time since symptom onset with 100% efficacy beyond 9th day post-symptoms. One study reported that combined lab based RT-PCR with lab based antibody testing could increase sensitivity for COVID-19 diagnosis from 67.1% to 99.4% in hospitalised patients[24]. However, in that study this assessment of sensitivity was made using clinical diagnosis. A major strength of this study is the use of an objective reference standard that included NAAT and serum neutralisation - a phenotypic test for functionality of antibodies. This assay was shown to be robust and accurate, using a recently described ELISA method for SARS-CoV-2 IgG detection that is now used globally[22].

The D614G Spike mutant has spread globally. Wild type Spike protein antigen is used in the development and validation of POC antibody tests, including those tested here. Of critical importance is the fact that both POC antibody tests (and ELISA) were able to detect antibody responses in patients infected with the D614G Spike mutant and that band intensity of POC testing increased with neutralisation activity in these individuals. Given that POC antibody tests are far cheaper and simpler to deploy, they will likely be used in low resource settings that do not have access to NAAT[25]. Demonstration that POC antibody LFA tests can detect the D614G spike mutant is therefore of importance.

Use of antibody tests for COVID-19 diagnosis in hospitals has been limited for a number of reasons. Firstly, we know from SARS-CoV-1 that previous humoral immunity to HCoV OC43 and 229E can elicit a cross-reactive antibody response to N of SARS-CoV-1 in up to 14% of people tested in cross-sectional studies[26], and previous exposure to HCoV can rarely elicit a cross-reactive antibody response to the N and S proteins of SARS- CoV-2 [16,27]. Secondly, antibody tests do not achieve the same detection rates as nucleic acid based tests early in infection, as humoral responses take time to develop following viral antigenic stimulation. However, by day 6 post symptom onset detection of IgG to Spike protein has been reported to reach 100% sensitivity [12] and this is useful in cases with immune mediated inflammatory disease where RT-PCR on respiratory samples is often negative, for example in the recently described Kawasaki-like syndrome named PIMS (paediatric inflammatory multi-system syndrome) [28].

In phase one (COVIDx trial) we tested stored sera rather than whole blood finger prick, though this was intentional given the caution needed in interpreting LFAs and concern regarding potential cross-reactivity of antibodies and poor specificity. Although SARS-CoV-2 ELISA testing of our pre-pandemic sera did reveal occasional N reactivity to SARS- CoV-2, likely due to cross reactivity with seasonal CoV, these samples were negative on POC antibody testing. However, the specificity of the COVIDIX test was estimated at only 85%, compared to a more acceptable 95% for SureScreen. We therefore carried out prospective evaluation of POC antibody testing on finger prick blood in 128 suspected cases of COVID-19 in order to further evaluate specificity of both tests in routine clinical practice. We found no false positives in patients whose sera were non-neutralising. This is consistent with an estimated specificity of above 99% with the SureScreen assay observed in an independent analysis using stored pre-pandemic sera[29]. The greater incidence of false positive POC antibody tests, predominantly with COVIDIX, on stored sera as compared to fresh finger prick blood may be due to processing and storage of sera, contamination of sera with other blood products, or other causes, including patient factors that differed between the two phases. Nevertheless, now that we are in a low incidence period it is advisable to perform confirmation testing using an alternative platform for either a single positive antibody or NAAT test, as is now the policy at our institution. One should note in particular that antibody tests may be negative in patients with immune suppression, highlighting that patient factors can influence interpretation of results and that alternative diagnoses should be considered.

We envisage a deployment approach whereby both test samples, finger prick whole blood and nose/throat swab, are taken at the same time on admission to hospital. The finger prick antibody test result is available within 15 minutes. Due to the possibility of false positive results from POC serology testing, a positive POC antibody test result as the only positive marker should ideally be confirmed with a second rapid POC test / laboratory IgG/IgM test before movement to a COVID-19 area, or recruitment into a clinical treatment study. At our institution further diagnostic data from chest imaging and blood indices such as lymphocyte count and C-reactive protein when assessing patients for COVID-19 and clinical decision making. Further swabs for NAAT testing are also taken where possible.

A confirmed positive NAAT result remains critical not only to identify early infection but, more importantly to triage infectious patients to be isolated from other patients and be handled with particular care by staff. NAAT is also valuable in milder and asymptomatic cases given severity appears to correlate with magnitude of antibody responses [16,30]. In conclusion rapid combined testing could be important in diagnosis and management of COVID-19, particularly given the pandemic is not well controlled in many parts of the world and as diverse manifestations of disease emerge.

Limitations of study

This study was limited by the fact that it was conducted at a single centre with relatively small numbers of individuals in the clinical study (phase 1), largely due to a lack of available stored serum. Phase 1 of the study used stored serum where there was a higher false positive rate than phase 2 where whole blood was used. The implementation study (phase 2) had greater numbers and was able to effectively demonstrate the high specificity of POC antibody tests and very low false positive rate for both POC antibody tests on whole blood, though itself was hampered by the low incidence of COVID-19 infection during the period it was undertaken. This low incidence rate in phase 2 limited further evaluation of the sensitivity of the combined approach. There was also a lack of data on repeated sampling and sampling from deeper respiratory sites in those suspected cases who were NAAT negative. Future larger studies are warranted.

Author contributions

Conceptualization, R.K.G., P.M., D.A.C., E.G.K., A.B., K.G.C.S.

Methodology, R.K.G., P.M., D.A.C., A.R., S.M.A., M.H., I.G., N.T., L.K., L.C.J., K.A.C., X.X., J.A.G.B., J.A.N., R.S., J.P.A.

Investigation and Data Collection, R.K.G., P.M., D.A.C., A.R., S.M.A., M.H., I.G., N.G., B.M., K.C., F.M., V.M., H.Z., L.B., P.B., N.D., P.A.L., J.B., S.B., J.P.A., R.B., M.W., D.S., G.A., S.H.

Writing – Original Draft, R.K.G., P.M., D.A.C.,

Writing – Reviewing and editing, A.R., S.M.A., E.G.K., A.B., H.Z.

Declaration of interests

The authors declare no competing interests

Table 1: Characteristics of participants in diagnostic accuracy study. COVID-19 status is based on composite reference standard test of nose/throat swab SARS-CoV-2 RT-PCR + serum neutralisation of pseudovirus bearing SARS-CoV-2 Spike. [§] Wilcoxon rank sum test used except where indicated. [a] Chi-square test.

	COVID-19 Pos N=24	COVID-19 Neg N=21	P value [§]
Male sex (%)	14 (58.3)	9 (42.9)	0.30[a]
Median age (IQR) years	73.5 (54.0-86.5)	63.0 (41.0-72.0)	0.03
Influenza-like illness with documented fever	20 (83.3)	17 (81.0)	0.84
Clinical or radiological evidence of pneumonia	10 (41.7)	7 (33.3)	0.57
Immunosuppressed Yes No	 1 (4.2) 23 (95.8)	 5 (23.8) 16 (76.2)	 0.053
Cardiovascular disease	6 (25.0)	2 (9.5)	0.25
Chronic Respiratory disease	5 (20.8)	6 (28.6)	0.73
Chronic Renal disease	4 (16.7)	2 (9.5)	0.67
Diabetes Mellitus	6 (25.0)	3 (14.3)	0.47
Median SpO2 (IQR) %	95.0 (92.5-96.0)	96.0 (94.0-98.0)	0.09
Median FiO2 (IQR)	0.21 (0.21-0.24)	0.21 (0.21-0.21)	0.40
Median PaO2 (IQR) Kpa	5.0 (3.0-9.1)	7.2 (3.8-9.0)	0.30
Median PaO2:FiO2 ratio (IQR)	20.5 (13.3-32.9)	30.9 (18.1-36.2)	0.09
Median Respiratory rate (IQR) breaths/min	22.0 (19.0-27.5)	20.0 (17.0-23.0)	0.06
Median heart rate (IQR) beats/min	86.0 (77.5-99.5)	88.0 (78.0- 107.0)	0.44
Median Systolic BP (IQR) mmHg	139.5 (117.5-149.0)	135.0 (119.0-152.0)	0.90
Median duration of illness (IQR) days	7 (1-8)	10 (3-14)	0.10

Median Hb (IQR) g/dL	12.9 (12.0-13.8)	13.1 (11.6-14.1)	0.46
Median WCC (IQR) $x10^9$/L	7.0 (5.0-8.0)	9.0 (7.0-14.0)	0.08
Median lymphocyte count (IQR) $x10^9$/L	0.8 (0.5-1.2)	1.2 (0.8-1.5)	0.12
Median platelet count (IQR) $x10^9$/L	213.5 (188.5-303.5)	271.0 (186.0-305.0)	0.59
Median Ferritin (IQR) µg/L	684.7 (206.2-1059.1)	112.3 (49.6-323.6)	0.02
Median CRP (IQR) mg/L	72.0 (28.5-214.5)	12 (4.0-53.0)	0.004
Median procalcitonin (IQR) ng/mL	0.2 (0.1-0.6)	0.0 (0.0-0.1)	0.03
Radiological findings			
Normal	2 (8.3)	9 (42.9)	<0.001 [a]
Indeterminate	4 (16.7)	3 (14.3)	
Classic	18 (75.0)	3 (14.3)	
Non-COVID	0 (0.0)	6 (28.5)	

Table 2. Individual and combined diagnostic accuracy of point of care rapid NAAT-based and antibody tests according to time from initial symptoms. Positivity predictive agreement is the percentage of positive test results in samples deemed positive by the composite reference standard. Negative predictive agreement is the percentage of negative test results in samples deemed negative by the composite reference standard. *43 out of 45 patients had SureScreen antibody results

% (95% CI)	Days 1-4 N=14	Days 5-8 N=14	Days 9-28 N=17	Overall N=45*
SAMBA II NAAT				
Positive predictive agreement	100 (65.3-98.6)	81.8 (48.2-97.8)	50.0 (11.8-88.2)	79.2 (57.8-92.9)
Negative predictive agreement	100 (69.2-100)	100 (29.2-100)	100 (71.5-100))	100 (83.9-100)
COVIDIX Ig M & IgG				
Positive predictive agreement	100 (59.0-100)	90.9 (58.7-99.8)	100 (54.1-100)	95.8 (78.9-99.9)
Negative predictive agreement	100 (59.0-100)	66.7 (9.4-99.2)	81.8 (48.2-97.7)	85.7 (63.7-97.0)
SAMBA II NAAT & COVIDIX IgM &IgG				
Positive predictive agreement	100 (59.0-100)	100 (71.5-100)	100 (54.1-100)	100 (85.8-100)
Negative predictive agreement	100 (59.0-100)	66.7 (9.4-99.2)	81.8 (48.2-97.7)	85.7(63.7-97.0)
SureScreen IgM & IgG*				
Positive predictive agreement	42.9 (9.9-81.6)	90.9 (58.7-99.8)	100 (54.1-100)	79.2 (57.8-92.9)
Negative predictive agreement	100 (54.1-100)	66.7 (9.4-99.2)	100 (69.2-100)	94.7 (74.0-99.9)
SAMBA II NAAT & SureScreen IgM & IgG*				
Positive predictive agreement	100 (59.0-100)	100 (71.5-100)	100 (54.1-100)	100 (85.8-100)
Negative predictive agreement	100 (54.1-100)	66.7 (9.4-99.2)	100 (69.2-100)	94.7(74.0-99.9)

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Table 3: Characteristics of 128 individuals hospitalised with suspected COVID-19 during implementation of combined POC testing. *testing done on stored serum due to fingerprick test failure. NIV- non invasive ventilation; LTOT–long term oxygen therapy; NEWS- national early warning score; NAAT - nucleic acid amplification testing

Male gender (%)	42.2
Median age (IQR) yrs	67 (50.8-80.0)
Median SpO2 (IQR) %	96 (95-97)
Median fiO2 (IQR)	0.21 (0.21-0.21)
Maximal Additional Ventilatory Support	
nasal cannulae	24
facemask	7
LTOT/NIV	4
Intubation	1
Median duration of illness (IQR) days	2.5 (1-7)
NAAT positive (%)	2 (1.6)
Neutralisation positive (%, n=101)	8(7.9)
COVIDIX Healthcare IgG/M positive (%)*	6 (3.9)
SureScreen IgG/M positive (%)*	6 (3.1)
Median lymphocyte count (IQR) $x10^9$/L	1.3(0.76-1.76)
Median CRP (IQR) mg/L	46 (15-129)
Comorbidities	
Cardiovascular disease	44 (34.3)
Chronic respiratory disease	62 (48.4)
Chronic kidney disease	11 (8.6)
Diabetes Mellitus	24 (18.8)
Immune suppression	13 (10.2)
Diagnosis	
1. Respiratory	61
2. Cardiovascular	16
3. Gastrointestinal	13
4. Genitourinary	7
5. Other	30
NEWS score	2 (1-5)
Chest radiograph findings (n=114)	
Normal	59
Indeterminate	31
Classic	0
Non-COVID-19	24
CT findings (n=24)	
Normal	3
Indeterminate	7
Classic	1
Non-COVID-19	13

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Figure titles and legends

Figure 1: Antibody detection for SARS-CoV-2: cross validation of lateral flow diagnostic tests (POC antibody tests) with ELISA and SARS-CoV-2 pseudotype virus neutralisation assays. A, B. Serum from COVID-19 suspected participants inhibited (n=19) (A) or did not inhibit (n=26) (B) SARS-CoV-2 pseudotype virus infection in a neutralisation assay. Serum from a healthy donor was used and a negative control. Error bars represent SEM. C. Comparison between ELISA and positive/negative results from neutralisation assay. n=37, $p<0.0001$. D. Comparison between ELISA Spike protein reactivity and positive/negative POC antibody test results (COVIDIX SARS-CoV-2 IgM/IgG Test). n=38, $p<0.0001$. E. Comparison between EC50 dilution titre from neutralizing assay and positive/negative POC antibody test results (COVIDIX SARS-CoV-2 IgM/IgG Test). n=44, $p=0.0025$. F. Comparison between ELISA IgG and positive/negative POC IgG band results for SureScreen SARS-CoV-2 IgM/IgG test. n=38, $p<0.0001$. G. Comparison between EC50 dilution titre from neutralisation assay and positive/negative SureScreen SARS-CoV-2 IgM/IgG antibody band test results. n=43, $p=0.005$. The assays were performed in duplicate.

Figure 2: Venn diagrams comparing positive and negative diagnostic test results in hospitalised patients by NAAT (SARS-CoV-2 nucleic acid amplification testing) and point of care (POC) antibody testing by A. COVIDIX Healthcare IgM/IgG kit (n=45) and B. SureScreen IgM/IgG kit (n=43).

Figure 3. A. **Spike D614G characterisation in clinical cohort.** Genome map of SARS-

CoV-2, with overall topography of Spike expanded. NTD- N-terminal domain; RBD- receptor binding domain; FP- fusion peptide; HR1- heptad repeat 1; HR2- heptad repeat 2; TM- transmembrane region; IC- intracellular domain. The aligned sequence of 10 amino acids on either side of D614 is shown for 16 participants for whom sequence data were available. A dot represents where the amino acid is unchanged from wildtype, the mutant glycine is represented by G. **B.** Top view of SARS-CoV-2 Spike glycoprotein trimeric structure in a closed state, with position 614 in yellow in each protomer. Structure determined by cryogenic electron microscopy. RCSB PDB 6VXX.

Figure 4: Longitudinal antibody responses in patients infected with D614G mutant SARS-CoV-2 detected by rapid lateral flow and neutralisation assays. A, D, G. An immune-chromatographic lateral flow rapid diagnostic test (POC antibody test - COVIDIX SARS-CoV-2 IgM IgG Test) on longitudinal samples in individual patients detecting SARS-CoV-2 IgM and IgG bands. Band intensities were acquired using ChemiDoc MP Imaging System and quantified using Image Lab software. B, E, H. SARS-CoV-2 pseudotyped virus neutralisation assay from longitudinal serum samples in individual patient examples. The assays were performed in duplicate. Error bars represent SEM. C, F, I. Comparison of IgG band intensities from lateral flow rapid diagnostic test with EC50 neutralisation titres from SARS-CoV-2 pseudotyped virus neutralisation assay in individual patients. Correlations were estimated by linear regression analysis.

Figure 5: Distribution of serum neutralisation activity against SARS-CoV-2 in hospitalised patients during implementation phase (A) Neutralisation EC50 dilution titre interpreted as positive or negative using a cut off for positive neutralisation of 1:4 dilution **(B)** Neutralisation data for individual patients stratified by POC antibody test result (both tests were fully concordant in phase 2). Data points represent reciprocal dilution of serum required to inhibit 50% of infection by lentivirus pseudotyped with SARS-CoV-2 Spike glycoprotein. The assays were performed in duplicate. Line represents mean and bar represents standard deviation (n=101 sera tested).

STAR Methods

RESOURCE AVAILABILITY

Lead Contact

Further information should be directed to and will be fulfilled by the Lead Contact, Ravindra K. Gupta rkg20@cam.ac.uk.

Materials Availability

This study did not generate new unique reagents.

Data and Code Availability

Raw anonymised data are available from the lead contact without restriction.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Clinical Study

The study was conducted in two phases; a clinical validation phase followed by an implementation phase. The study participants in phase one were part of the COVIDx trial[18], a prospective analytical study which compared SAMBA II SARS-CoV-2 point of care test to the standard laboratory RT-PCR test for the detection of SARS-CoV-2 in participants admitted to Cambridge University Hospitals NHS Foundation Trust (CUH) with a possible diagnosis of COVID-19. Consecutive participants were recruited during 12-hour day shifts over a duration of 4 weeks from the 6th of April 2020 to the 2nd of May 2020. We recruited adults (>16 years old) presenting to the emergency department or acute medical assessment unit as a possible case of COVID-19 infection. This included any adult requiring hospital admission and who was symptomatic of SARS-CoV-2 infection, demonstrated by clinical or radiological findings [18]. 45 participants who had available stored sera were included in this sub-study and underwent further antibody testing. Phase 2, from July 13th to 27th 2020, comprised a service evaluation of clinical practice whereby adults (>16 years old) presenting to the emergency department or acute medical assessment unit as a possible case of COVID-

19 infection were included. This included any adult requiring hospital admission and who was symptomatic of SARS-CoV-2 infection, demonstrated by clinical or radiological findings.

Cell lines

293T cells were cultured in DMEM complete (DMEM supplemented with 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 10% FCS) and maintained at 37^{O}C in %% CO_2.

Ethical approval

COVIDx (NCT04326387) was approved by the East of England - Essex Research Ethics Committee (REC ref: 20/EE/0109**)**. Serum samples were obtained from patients attending Addenbrooke's Hospital with a suspected or confirmed diagnosis of COVID19. Prospective combined point of care testing of suspected COVID-19 cases was done under CUH NHS Trust service evaluation 3163.

METHOD DETAILS

Test methods

NAAT tests

The standard laboratory RT- PCR test, developed by public health England (PHE), targeting the RdRp gene was performed on a combined nose/throat swab. This test has an estimated limit of detection of 320 copies/ml. In parallel, SAMBA II SARS-CoV-2 testing was performed on a combined nose/throat swab and inactivated in a proprietary buffer at the point of sampling. SAMBA II SARS-CoV-2 targets 2 genes- Orf1 and the N genes and uses nucleic acid sequence based amplification to detect SARS-CoV-2 RNA, with limit of detection of 250 copies/ml.[31]

Pseudotype virus preparation

Viral vectors were prepared by transfection of 293T cells by using Fugene HD transfection reagent (Promega) as follows. Confluent 293T cells were transfected with a mixture of 11ul

of Fugene HD, 1µg of pCAGGS_SARS-CoV-2_Spike or pCDNAΔ19Spike-HA, 1ug of p8.91 HIV-1 gag-pol expression vector[32,33], and 1.5µg of pCSFLW (expressing the firefly luciferase reporter gene with the HIV-1 packaging signal). Viral supernatant was collected at 48 and 72h after transfection, filtered through 0.45um filter and stored at -80˚C. The 50% tissue culture infectious dose ($TCID_{50}$) of SARS-CoV-2 pseudovirus was determined using Steady-Glo Luciferase assay system (Promega).

Pseudotype neutralisation assay

Spike pseudotype assays have been shown to have similar characteristics as neutralisation testing using fully infectious wild type SARS-CoV-2[34].Virus neutralisation assays were performed on 293T cell transiently transfected with ACE2 and TMPRSS2 using SARS-CoV-2 Spike pseudotyped virus expressing luciferase. Pseudovirus was incubated with serial dilution of heat inactivated human serum samples from COVID-19 suspected individuals in duplicates for 1h at 37˚C. Virus and cell only controls were also included. Then, freshly trypsinized 293T ACE2/TMPRSS2 expressing cells were added to each well. Following 48h incubation in a 5% CO_2 environment at 37°C, the luminescence was measured using Steady-Glo Luciferase assay system (Promega).

Enzyme-linked immunosorbent assay (ELISA)

We developed an ELISA targeting the SARS-CoV-2 Spike and N proteins. Trimeric spike protein antigen used in the ELISA assays consists of the complete S protein ectodomain with a C-terminal extension containing a TEV protease cleavage site, a T4 trimerization foldon and a hexa-histidine tag. The S1/S2 cleavage site with amino acid sequence PRRAR was replaced with a single Arginine residue and stabilizing Proline mutants were inserted at positions 986 and 987. Spike protein was expressed and purified from Expi293 cells (Thermo Fisher). N protein consisting of residues 45-365 was initially expressed as a His-TEV-SUMO-fusion. After Ni-NTA purification, the tag was removed by TEV proteolysis and the cleaved tagless protein further purified on Heparin and gel filtration columns.

The ELISAs were in a stepwise process; a positivity screen was followed by endpoint titre as previously described[22]. Briefly, 96-well EIA/RIA plates (Corning, Sigma) were coated with PBS or 0.1µg per well of antigen at 4°C overnight. Coating solution was removed, and wells were blocked with 3% skimmed milk prepared in PBS with 0.1% Tween 20 (PBST) at ambient temperature for 1 hour. Previously inactivated serum samples (56°C for 1 hour) were

diluted to 1:60 or serially diluted by 3-fold, six times in 1% skimmed milk in PBST. Blocking solution was aspirated and the diluted sera were added to the plates and incubated for 2 hours at ambient temperature. Diluted sera were removed, and plates were washed three times with PBST. Goat anti-human IgG secondary antibody-Peroxidase (Fc-specific, Sigma) prepared at 1:3,000 in PBST was added and plates were incubated for 1 hour at ambient temperature. Plates were washed three times with PBST. ELISAs were developed using 3,5,3′,5′- tetramethylbenzidine (TMB, ThermoScientific); reactions were stopped after 10 minutes using 0.16M Sulfuric acid.

COVIDIX 2019 SARS-CoV-2 IgG/IgM Test (COVIDIX Healthcare, Cambridge, UK). This colloidal-gold lateral flow immunoassay is designed to detect IgG and IgM to SARS-CoV-2. The test is CE marked. It was used according to the manufacturer's instructions. 10µl of serum was added to the test well followed by 2 drops of the manufacturer's proprietary buffer. In order to rule out cross reactivity of this test with seasonal coronavirus antibodies we tested 19 stored specimens from before 2020, some of which had N and S protein SARS-CoV-2 cross reactivity (Supplementary table 2).

SureScreen SARS-CoV-2 IgG/IgM Test (SureScreen Diagnostics Ltd, Derby, UK). This colloidal-gold lateral flow immunoassay is designed to detect IgG and IgM to SARS-CoV-2. It was used according to the manufacturer's instructions. The test has been CE marked and previously validated against a large panel of negative historical controls and in serum from confirmed PCR positive COVID-19 cases[16]. 10µl of serum was added to the test well followed by 2 drops of the manufacturer's proprietary buffer.

Next generation sequencing of SARS-CoV-2 isolates in nose/throat swabs

Samples with CT values above 33 were sequenced with a multiplex PCR approach according to the ARTIC version 2 protocol with version 3 primer set. Amplicons were sequenced using MinION flow cells version 9.4.1 (Oxford Nanopore Technologies, Oxford, UK). Genomes were assembled as previously described[35]. The sequences are freely available from GISAID EpiCoV^{TM} under accession IDs: EPI_ISL 433757, 433754, 433792, 433850, 433751, 433778, 433869, 433875, 433874, 433917, 433962, 433956, 434034, 438681, 438711 and 444331. The submitting laboratory is the COVID-19 Genomics UK (COG-UK) Consortium and the originating laboratory is Department of Pathology, University of Cambridge.

QUANTIFICATION AND STATISTICAL ANALYSIS

Enzyme-linked immunosorbent assay (ELISA) quantification

The optical density at 450 nm (OD450) was measured using a Spectramax i3 plate reader. The absorbance values for each sample were determined by subtracting OD values from uncoated wells. All data analyses were performed using Prism 8 version 8.4.2 (GraphPad). An OD cut off of 0.3 was used to define a positive IgG response to full length Spike protein.

COVIDIX 2019 nCoV IgG/IgM Test band density

For quantification of IgG and IgM band density in COVIDIX 2019 nCoV IgG/IgM Test, high resolution images of completed POC antibody test cassettes were acquired using ChemiDoc MP Imaging System (Bio-Rad) at 20min post-addition of the human serum. Band intensities were analysed using Image Lab software (Bio-Rad).

Quantification of neutralisation sensitivity

The 50% inhibitory dilution (EC_{50}) was defined as the serum dilution at which the relative light units (RLUs) were reduced by 50% compared with the virus control wells (virus + cells) after subtraction of the background RLUs in the control groups with cells only. The EC_{50} values were calculated with non-linear regression, log (inhibitor) vs. normalized response using GraphPad Prism 8 (GraphPad Software, Inc., San Diego, CA, USA). The neutralisation assay was positive if the serum achieved at least 50% inhibition at 1 in 3 dilution of the SARS-CoV-2 spike protein pseudotyped virus in the neutralisation assay. The neutralisation result was negative if it failed to achieve 50% inhibition at 1 in 3 dilution.

Assessment of neutralisation assay performance

Four assays detecting IgG to COVID-19 were utilised in this study. 38 of the 45 samples were identified as concordant with at least three of the four assays and considered confirmed either negative or positive. Against this group of samples validated for content of COVID-19 IgG, each individual assay was assessed. Neutralisation, ELISA, SureScreen and COVIDIX assays gave a correct result in 100%, 97.4%, 92.1% and 86.8%, respectively, justifying the choice of the neutralisation assay as standard.

Analyses

The performance of SAMBA II SARS-CoV-2 test and COVIDIX SARS-CoV-2 IgG/IgM Test or SureScreen SARS-CoV-2 IgG/IgM Test for diagnosing COVID-19 were calculated alone and then in combination along with binomial 95% confidence intervals (CI). A composite reference standard was used - standard lab RT-PCR and a neutralisation assay. Descriptive analyses of clinical and demographic data are presented as median and interquartile range (IQR) when continuous and as frequency and proportion (%) when categorical. The differences in continuous and categorical data were tested using Wilcoxon rank sum and Chi-square test respectively. Statistical analysis were conducted using Stata (version 13) and GraphPad Prism (version 8), with additional plots generated using GraphPad Prism. Venn diagrams were prepared using Venny[36]. Structural modelling of location of D614G was done using Mol*: D. Sehnal et al (doi:10.2312/molva.20181103).

ADDITIONAL RESOURCES

COVIDx was registered with the ClinicalTrials.gov Identifier NCT04326387.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Goat anti-human IgG antibody	Sigma	Cat# A0170
Bacterial and Virus Strains		
Biological Samples		
Participants combined nose and throat swab	This study	N/A
Participants serum	This study	N/A
Chemicals, Peptides, and Recombinant Proteins		
SARS-CoV-2 Spike protein	Laboratory of J. Briggs	[37]
SARS-CoV-2 N protein	Laboratory of J. Nathan	N/A
Critical Commercial Assays		
SAMBA II SARS-CoV-2 test	Diagnostics for the real World	Cat# 8500-12
SARS-CoV-2 RT-PCR in-house test on was performed on Qiagen Roto gene platform	Qiagen	N/A
COVIDIX 20019 SARS-CoV-2 IgG/IgM Test	COVIDIX Healthcare	Cat# ICOV-402
SureScreen SARS-CoV-2 IgG/IgM Test	SureScreen Diagnostics	Cat# COVID19
Deposited Data		
Mapping and structural mapping of D614G was done on S protein structure deposited in PDB	PDB	RCSB PDB 6VXX.
Sequences of SARS-CoV-2	GISAID EpiCoV™	www.gisaid.org
Experimental Models: Cell Lines		
Expi293 cells	Laboratory of J. Briggs	[37]
293T	Laboratory of Greg Towers	N/A
Experimental Models: Organisms/Strains		
Oligonucleotides		
Next generation sequencing 3 primer set	Laboratory of I. Goodfellow	[35]
Recombinant DNA		
pCAGGS_SARS-CoV-2_Spike	NIBSC	#100976
pCDNAΔ19Spike-HA	Laboratory of P. Lehner	N/A
pCSFLW	Laboratory of G. Towers	N/A
pCAGGS/ACE2	Laboratory of N. Temperton	N/A
pCAGGS/ TPMPSS2	Laboratory of N. Temperton	N/A
Software and Algorithms		
STATA version 13	STATA	https://www.stata.com/order/download-details/
R 2.6.3	The R project	https://www.r-project.org/
Image Lab	Bio-Rad	N/A
GraphPad Prism 8	GraphPad Software	N/A
Venny	Website	https://bioinfogp.cnb.csic.es/tools/venny/

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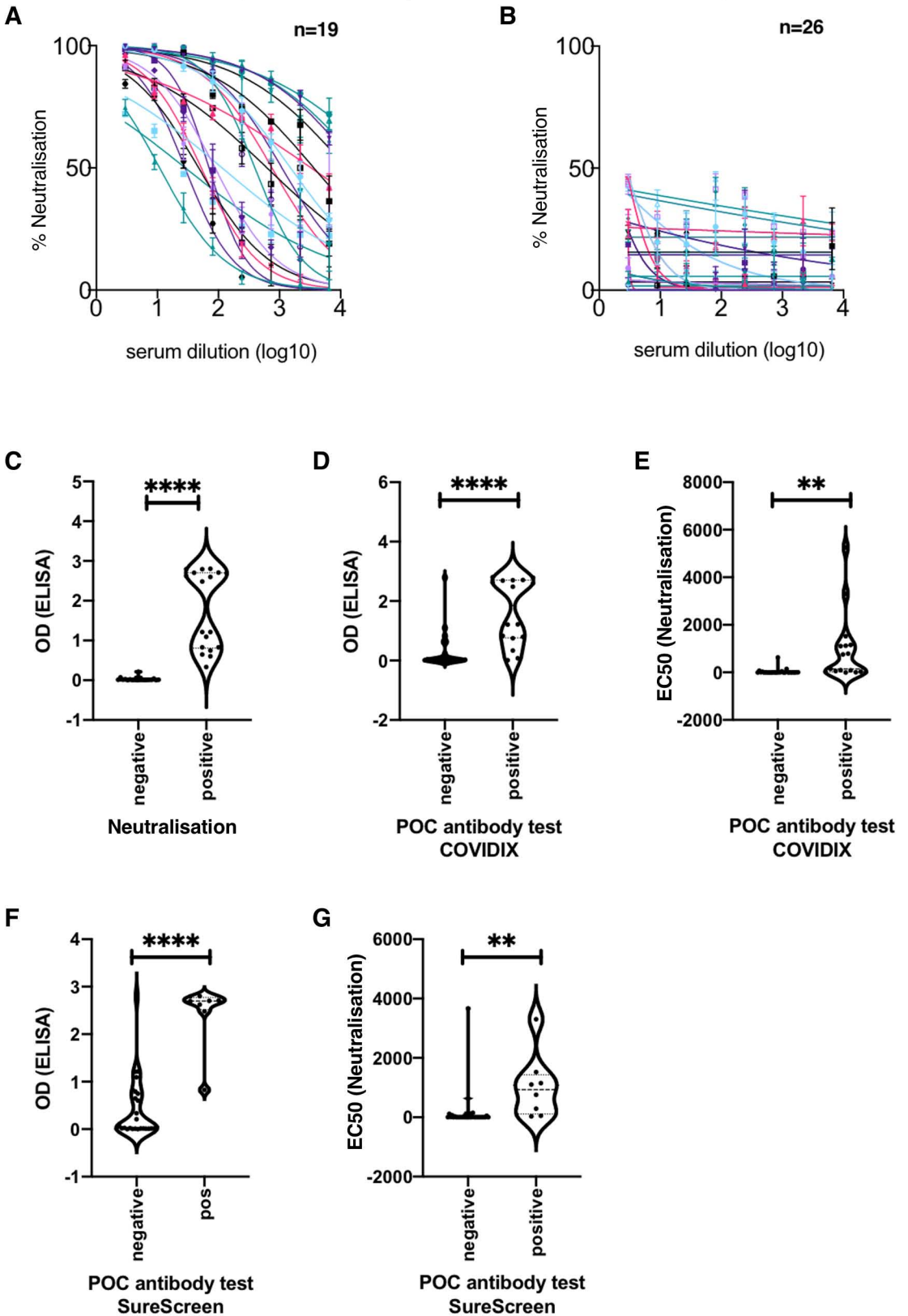
Highlights

- Combined rapid antibody + nucleic acid detection correctly diagnoses **SARS-CoV-2**
- Rapid antibody tests detect immune responses against **SARS-CoV-2** bearing D614G
- Rapid **SARS-CoV-2** antibody tests do not cross react with antibodies to seasonal CoV
- False positivity in **SARS-CoV-2** finger prick blood antibody tests can be very low.

ETOC blurb

Mlcochova et al. report that combined rapid nucleic acid amplification testing (NAAT) and finger prick blood antibody tests can substantially improve diagnosis of COVID-19 as compared to NAAT alone and are able to detect the SARS-CoV-2 Spike D614G variant that dominates the pandemic.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n=19
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EC50 (Neutralisation)
negative
positive
POC antibody test
COVIDIX
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OD (ELISA)
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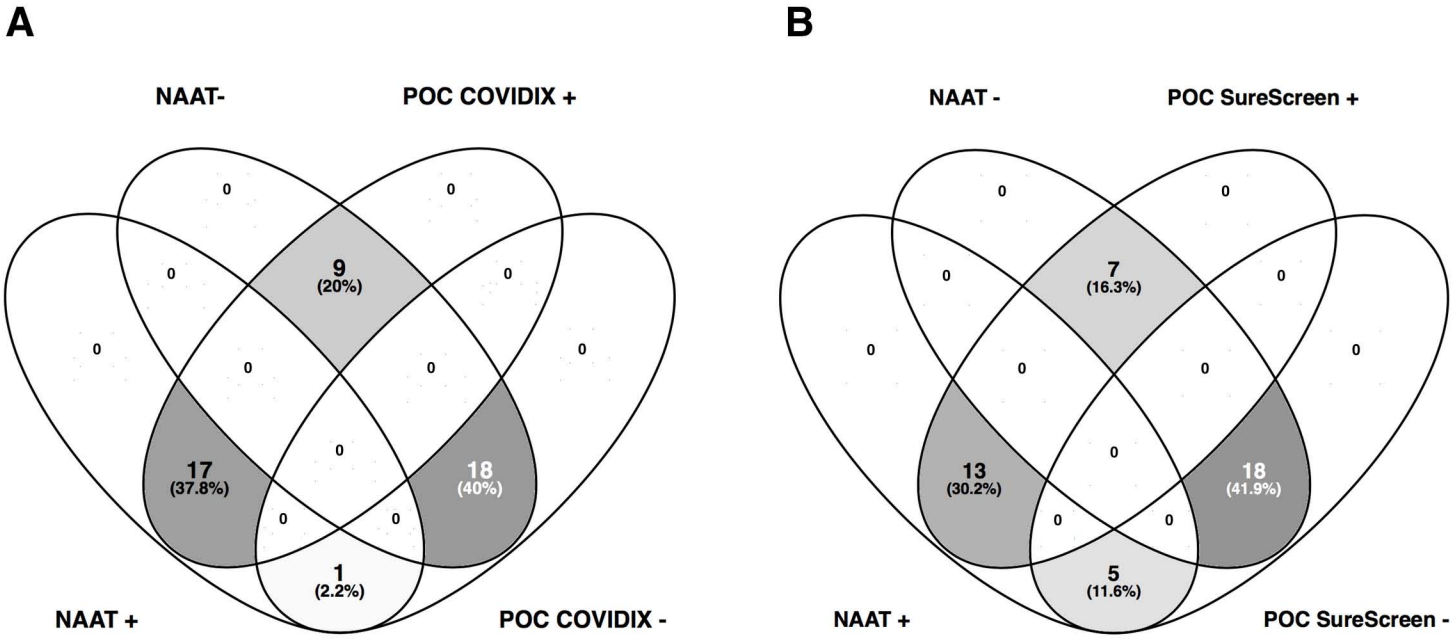
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POC COVIDIX -
B
NAAT -
POC SureScreen +
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0
0
0
7
(16.3%)
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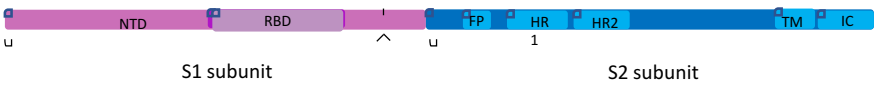
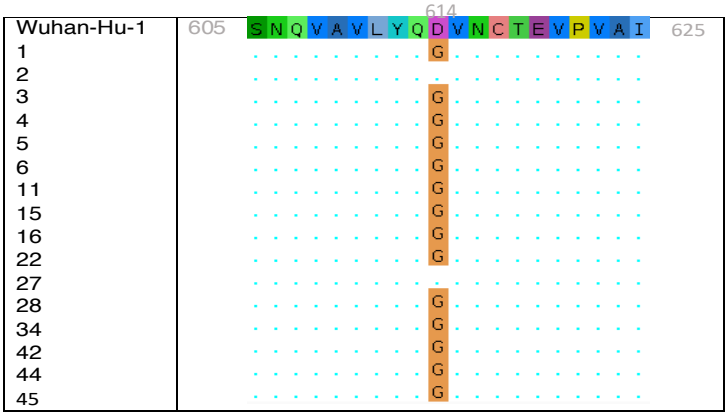
13
(30.2%)
18
(41.9%)
0
0
0
5
(11.6%)
NAAT +
POC SureScreen -

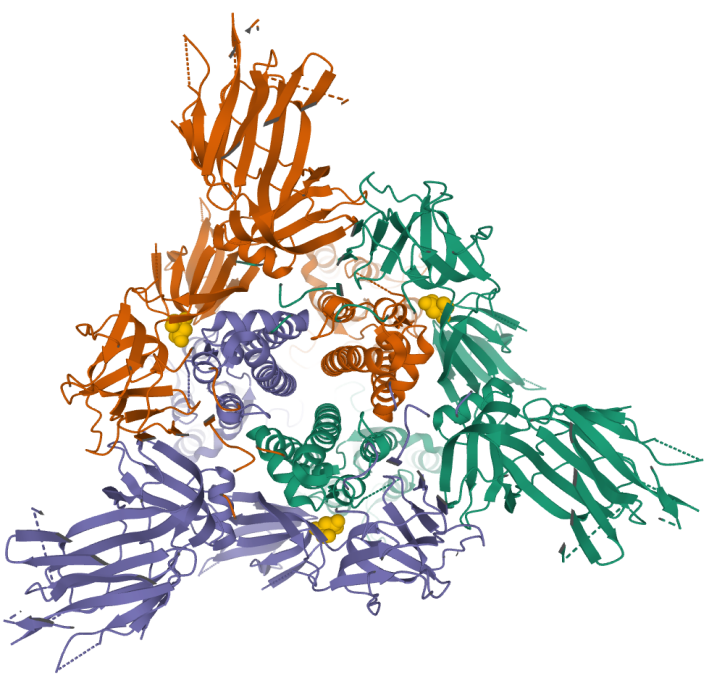
A

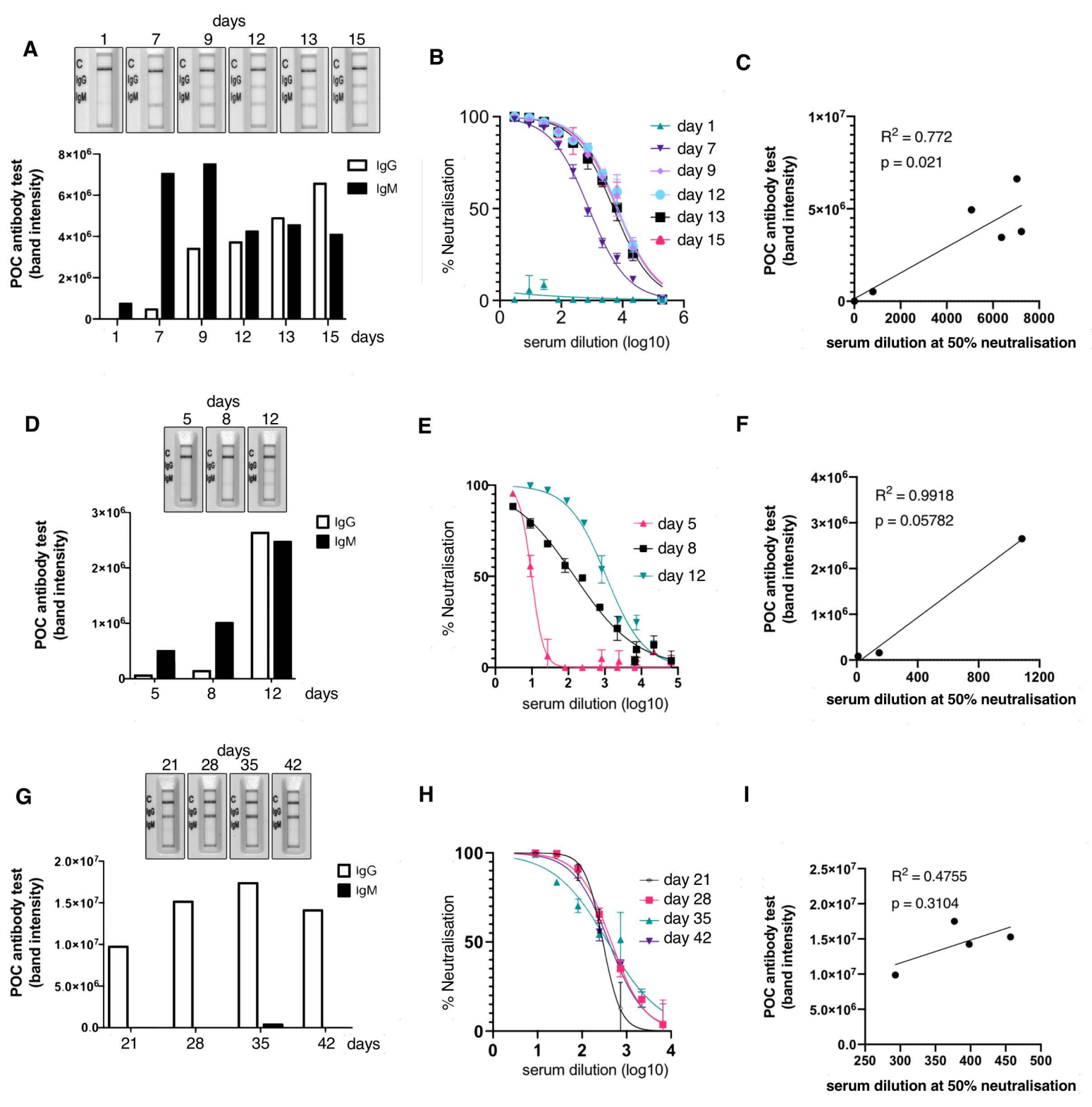

ORF1ab gene
S gene


NTD
RBD
FP
HR 1
HR2
TM
IC
S1 subunit
S2 subunit

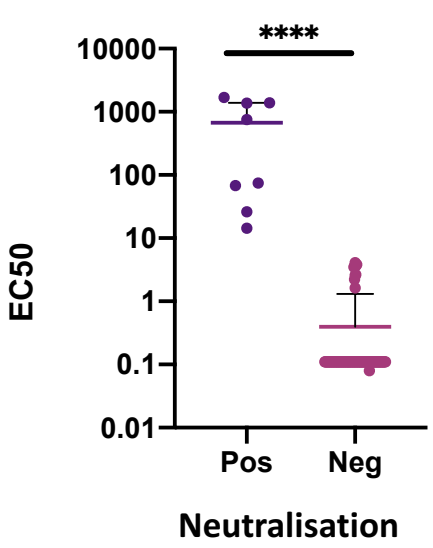

Wuhan-Hu-1
605
614
625
S N Q V A V L Y Q D V N C T E V P V A I
1
2
3
4
5
6
11
15
16
22
27
28
34
42
44
45

B

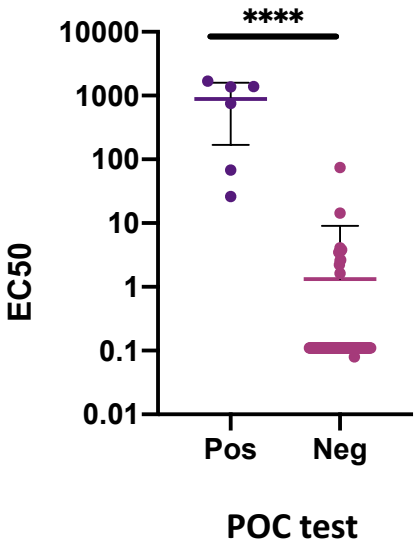



A
days
1
7
9
12
13
15
C
IgG
IgM
POC antibody test (band intensity)
8×10^6
6×10^6
4×10^6
2×10^6
0
IgG
IgM
1
7
9
12
13
15
days
B
% Neutralisation
100
50
0
day 1
day 7
day 9
day 12
day 13
day 15
0
2
4
6
serum dilution (log10)
C
POC antibody test (band intensity)
1×10^7
5×10^6
0
R^2 = 0.772
p = 0.021
0
2000
4000
6000
8000
serum dilution at 50% neutralisation
D
days
5
8
12
POC antibody test (band intensity)
3×10^6
2×10^6
1×10^6
0
IgG
IgM
5
8
12
days
E
% Neutralisation
100
50
0
day 5
day 8
day 12
0
1
2
3
4
5
serum dilution (log10)
F
POC antibody test (band intensity)
4×10^6
3×10^6
2×10^6
1×10^6
0
R^2 = 0.9918
p = 0.05782
0
400
800
1200
serum dilution at 50% neutralisation
G
days
21
28
35
42
POC antibody test (band intensity)
2.0×10^7
1.5×10^7
1.0×10^7
5.0×10^6
0.0
IgG
IgM
21
28
35
42
days
H
% Neutralisation
100
50
0
day 21
day 28
day 35
day 42
0
1
2
3
4
serum dilution (log10)
I
POC antibody test (band intensity)
2.5×10^7
2.0×10^7
1.5×10^7
1.0×10^7
5.0×10^6
0.0
R^2 = 0.4755
p = 0.3104
250
300
350
400
450
500
serum dilution at 50% neutralisation

A


****
10000
1000
100
10
1
0.1
0.01
EC50
Pos
Neg
Neutralisation

B


****
10000
1000
100
10
1
0.1
0.01
EC50
Pos
Neg
POC test