A SARS-CoV-2 nucleocapsid ELISA represents a low-cost alternative to lateral flow testing for community screening in LMI countries

Running Title: Low-cost ELISA testing for SARS-CoV-2

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Summary

Background: Controlling the spread of SARS-CoV-2 is problematic because of transmission driven by asymptomatic and pre-symptomatic individuals. Community screening can help identify these individuals but is often too expensive for countries with limited health care resources. Low-cost ELISA assays may address this problem, but their use has not yet been widely reported.

Methods: We developed a SARS-CoV-2 nucleocapsid ELISA and assessed its diagnostic performance on nose and throat swab samples from UK hospitalised patients and sputum samples from patients in Ghana.

Results: The ELISA had a limit of detection of 8.4 pg/ml antigen and 16 pfu/ml virus. When tested on UK samples (128 positive and 10 negative patients), sensitivity was 58.6% (49.6-67.2) rising to 78.3% (66.7-87.3) if real-time PCR Ct values >30 were excluded, while specificity was 100% (69.2-100). In a second trial using the Ghanaian samples (121 positive, 96 negative), sensitivity was 52% (42.8-61.2) rising to 72.6% (61.8-81.2) when a >30 Ct cut-off was applied, while specificity was 100% (96.2-100).

Conclusions: Our data show that nucleocapsid ELISAs can test a variety of patient sample types while achieving levels of sensitivity and specificity required for effective community screening. Further investigations into the opportunities that this provides are warranted.

Keywords: SARS-CoV-2, nucleocapsid, ELISA, diagnosis, test

Introduction

Coronavirus disease 2019 (COVID-19) is an acute life-threatening disease that can leave survivors with long-term sequelae [1]. COVID-19 deaths stand at over 3.7 million [2], and although there now are effective vaccines to prevent disease [3-5], it will likely take several years for them to be administered on a large scale in economically disadvantaged parts of the world. In the interim other measures will be required to control transmission, particularly in vulnerable settings such as hospitals.

Testing plays an important role in managing disease outbreaks, particularly in the case of SARS-CoV-2, where both pre-symptomatic and asymptomatic individuals are able to transmit virus to others [6]. Deciding which test to use in any particular setting depends on a trade-off between test sensitivity, turn-around times, logistic requirements and costs. The gold standard assay for detecting SARS-CoV-2 is the polymerase chain reaction (PCR); a nucleic acid-based test requiring expensive infrastructure and reagents. Loop-mediated isothermal amplification (LAMP) also detects nucleic acid and benefits from reduced infrastructure and reagent costs [7, 8]. However, reagent and infrastructure costs associated with LAMP are still significant, in particular those associated with RNA purification - a requirement for maximum sensitivity to be achieved [7].

In comparison to nucleic acid test modalities, tests detecting abundant viral protein antigens such as nucleocapsid (Nc) typically have lower infrastructure and reagent costs. Several commercial lateral flow device (LFD) tests detecting SARS-CoV-2 Nc already exist [9]. With short turnaround times, LFDs offer the advantage of point-of-care testing. They are also relatively cheap to run as there is no need for lab and/or additional equipment to process tests and the more basic ones do not require a reader. However, LFDs suffer from moderate to low sensitivity compared to PCR [10-14]. Also, while those LFD tests that do not require additional instrumentation are cheaper to perform than nucleic acid tests, there remains uncertainty as to their exact cost for low and middle-income countries (LMICs). Gates Foundation support has allowed commercial LFDs to be provided at no more than $5/test [15]. However, if this

upper limit cost is close to the actual cost the limited health care budgets in LMICs will still be stretched, particularly as pharma companies prefer to use third-party agents for distribution in LMICs, driving up costs as much as 5-10 fold.

Enzyme linked immunoassays (ELISAs) are another means by which Nc antigen can be detected. While ELISAs are slower to generate test results, they are typically able to detect lower levels of antigen compared to LFDs. Also while ELISAs do require lab processing, the laboratory infrastructure needed for this is minimal, with reading of the plates even being possible using mobile phone apps [16]. Importantly, ELISAs can be manufactured at a much lower unit cost/test than LFDs, making their use in certain LMIC settings attractive. In this study, we have developed an in-house ELISA and tested it using a variety of upper and lower respiratory tract samples from different population settings in both the UK and Ghana.

Materials and methods

Cloning and expression of nucleocapsid proteins

The gene sequences for SARS-CoV-2 nucleocapsid phosphoprotein (Nc, Gene ID: 43740575), HKU1 Nc (Gene ID: 3200423), OC43 Nc (Gene ID: 39105221), 229E Nc (Gene ID: 918763) and NL63 Nc (Gene ID: 2943504) were purchased as codon-optimized DNAs for expression in *E. coli* (GeneArt – ThermoFisher Scientific). For details of their cloning and expression see the methods section in Supplementary materials.

Polyclonal anti-SARS-CoV-2 sera

Recombinant SARS-CoV-2 Nc protein was used to immunize 2 rabbits and the polyclonal antibody (pAb) from each resultant sera was affinity-purified against the immunizing antigen (Eurogentec; Speedy Rabbit immunization programme). Pooled pAb was biotinylated with a 25 molar excess of N-hydroxysuccinimide (NHS)-4 x polyethylene glycol (PEG4)-Biotin (Sigma) for 2 hours at 4°C, dialysed overnight at 4°C against PBS and stored as aliquots at -20°C until used.

SARS-CoV-2 Nc-capture ELISA assay.

MAXISORP NUNC-IMMUNO plates were coated with 50 µl/well anti-SARS-CoV-2 pAb at 1.5 µg/ml overnight at 4°C. Plates were washed 5 x 5 min with Tris-buffered saline supplemented with 0.05% Tween-20 (TBS-T, pH 7.2), before blocking with 300 µl/well of 0.1 M NaH_2PO_4/Na_2HPO_4 (pH 7.2), 0.15 M NaCl, 0.05% (v/v) Tween-20 and 1% (w/v) bovine serum albumin (BSA) for 1 hr at room temp. The blocking buffer was removed and 50 µl/well sample added. Each plate also included a series of dilutions of recombinant SARS-CoV-2 Nc as an internal control. Antigen incubation was performed at RT for 1 hr. The plates were then washed 5 x 5 min with TBS-T before addition of 1.25 µg/ml (50 µl/well) of biotinylated, affinity-purified rabbit α-SARS-CoV-2 IgG, diluted in 0.1 M NaH_2PO_4/Na_2HPO_4 buffer (pH 7.2), 0.15 M NaCl,

0.05% (v/v) Tween-20 and 10% (v/v) rabbit serum (Sigma). Plates were incubated for 1 hr at room temp and washed again 5 x 5 min with TBS-T. Horseradish peroxidase (HRP) - Avidin conjugate (BioLegend) was diluted 1:2,500 in blocking buffer and added at 50 µl/well. After 1 hr incubation at RT, plates were washed 6 x 5 min with TBS-T before addition of 50 µl/well of 3,3',5,5'-tetramethylbenzidine substrate solution (SeraCare), which was left to develop for 1 hr at room temperature. Reactions were stopped with 50 µl/well of 1 M H_2SO_4 solution and a plate reader used to measure absorbance at 450 nm.

SARS-CoV-2 cell culture

A low passage SARS-CoV-2 isolate Australia/VIC01/2020 (GenBank ID: MT007544.1) was obtained from the Defence Science and Technology Laboratories at Porton Down and propagated a further 2 to 3 times on Vero E6 cells (European Collection of Authenticated Cell Culture) to obtain the working stocks used in all experiments. During virus propagation, cells were maintained in DMEM (Invitrogen) supplemented with 4% FCS, 50 u/ml penicillin, 50 ug/ml streptomycin, 25mM HEPES. Viral titre was determined by plaque assay on Vero E6 cells.

Clinical samples: Southampton

Nose and throat swab samples were collected from patients admitted to the Southampton General Hospital (University Hospital Southampton Foundation NHS Trust) within 24 hours of testing positive for COVID-19 by RT-PCR, using the QIAstat-Dx Respiratory SARS-CoV-2 panel [17]. Target sequences amplified encompass coding regions from both the viral polymerase and E gene. Ct values are derived from whichever is the most abundant of these two target sequences. Samples from PCR-negative healthy controls were also obtained. Patient information regarding date of sampling, age, gender, duration of symptoms prior to recruitment, Ct values and ELISA results are available in supplementary information. All swabs

were stored dried at -80℃ until processed for ELISA analysis, with both processing and subsequent testing being performed with operators blinded to infection status and Ct value.

Clinical samples: University of Ghana

The ELISA assay was evaluated in Ghana at the West African centre for Cell Biology of Infectious Pathogens(WACCBIP)- University of Ghana. Specimens used in this case were from a variety of individuals and collected by various methods; PCR-confirmed positive samples were from any of i) anonymised patient isolated obtained as part of WACCBIP's ongoing genomic surveillance study from Tamale Teaching Hospital, Cape Coast Teaching Hospital, Veterinary Services Directorate COVID-19 testing centres in Takoradi and Accra and ii) consented WACCBIP research participants who tested positive. Negative samples were obtained from WACCBIP research participants who tested PCR-negative during random screening activities. PCR detection used a World Health Organization (WHO)-approved commercial kit (Da An Gene Company). Target sequences for PCR amplification encompassed the coding region of ORF1a/b and the N gene. Ct values presented represent the average of these two reads. Where available, information regarding date of sampling, age, gender, the presence of symptomatic infection, Ct values and ELISA results for each individual in the cohort are presented in supplementary information. Samples obtained through the genomic surveillance project were a mixture of sputum, nasal lavage as well as swabs in transport medium, whereas samples collected by WACCBIP were primarily sputum. Specimen aliquots used for the ELISA were either taken from the sample used for PCR and sequencing, or collected at the same time as the sample used for PCR diagnosis. Samples obtained through genomic surveillance were stored upon collection (and after a positive-PCR result) at -80ºC or -20ºC depending on the facility. They were transported to WACCBIP in dry ice and either transferred to a -20ºC freezer or processed immediately for the ELISA. Samples collected by WACCBIP were either processed immediately or stored at -20ºC until processing.

Sample processing

Respiratory samples were processed into a viral lysis buffer (VLB) comprising 1% (v/v) Triton X-100 / 0.3% (v/v) tri butyl phosphate, 1X Complete EDTA-free protease inhibitor and 5 mM EDTA in Dulbecco Phosphate Buffered Saline (PBS) before use. Briefly, dry nasal swabs were immersed in VLB (0.4ml and 1.0ml for UK and Ghana samples respectively) for 15 minutes. Wet oropharyngeal swabs stored in viral transport medium (VTM) were supplemented with 0.1 volumes of 11X VLB. Sputum samples were initially mixed with 1mL of PBS to reduce the viscosity and 300 µL of this solution supplemented with 30µl volumes of 11X VLB. Sample processing of nasal lavage was similar to VTM from wet swabs. All samples processed for ELISA were either used immediately or stored frozen until needed.

Ethical approval

The Southampton clinical arm of the study was approved by the South Central - Hampshire A Research Ethics Committee: REC reference 20/SC/0138, on the 16th March 2020. The protocol is available at:
(https://eprints.soton.ac.uk/439309/2/CoV_19POC_Protocol_v2_0_eprints.pdf).
The Ghanaian clinical arm of the study conformed to the Ghanaian Public Health Act, 2012 (Act 851) and the Data Protection Act, 2012 (Act 843). Ethical approval for this study was obtained in Ghana from the Ethics Board of the College of Basic and Applied Sciences, University of Ghana and the Ethics Committee of the Ghana Health Service (ECBAS 063/19-20 and GHS-ERC 011/03/20).

Results

Developing a capture ELISA for SARS-CoV-2 Nc

Recombinant SARS-CoV-2 Nc expressed in *E.coli* was used to raise antisera in New Zealand rabbits. The resultant polyclonal antibody (pAb) displayed strong reactivity to SARS-CoV-2 Nc by indirect ELISA, producing a robust signal that reached saturation at concentrations ~1 µg/ml (Fig 1a). Western blot showed pAb recognised SARS-CoV-2 Nc but did not cross-react with recombinant Nc from the other 4 coronaviruses endemic within the human population (Fig 1b).

Using the pAb, a capture ELISA was developed that relied on a non-biotinylated form of the antibody to capture Nc from solution and a biotinylated form to detect captured antigen. After optimization the ELISA demonstrated a dynamic response signal up to concentrations of 1600 pg/ml using recombinant Nc and a linear dose-response below 400 pg/ml (R^2 = 0.999, SE = 0.005) (Fig 2a, b). Based on the regression analysis at this lower dose range the limit of detection (LoD) was 8.4 pg/ml (LoD = 3.3 x σ/S) and the limit of quantification was 25.5 pg/ml (LoQ = 10 x σ/S). Consistent with our previous Western blot data, the presence of recombinant Nc from other coronavirus species did not generate a signal in the ELISA except at very high concentrations ≥ 4 µg/ml (Fig 2c).

When expressed in an infected cell, Nc is phosphorylated. To ensure the capture ELISA recognised native viral Nc, we tested its ability to detect virus from uv-inactivated SARS-CoV-2 viral supernatants in the presence of detergent. Using a series of two-fold dilutions of three independently generated cell culture supernatants, the capture ELISA was found to have a LoD between 8-16 pfu/ml, depending on the viral supernatant used (Fig 3a). Further analysis established that the ELISA recognised Nc from both membrane-protected and

unprotected compartments within the viral supernatants, consistent with it recognising both virion-associated and non-virion-associated antigen (Fig 3b).

Analysis of clinical samples by nucleocapsid capture ELISA

To assess the capacity of the ELISA to diagnose SARS-CoV-2 infection, nose and throat swab samples were collected from patients admitted to the Southampton University Trust Hospital within 16 hours of testing positive for COVID-19 by quantitative syndromic PCR [17]. In total 138 samples were tested, 128 of which were from confirmed PCR positive cases (median Ct value = 29.8, interquartile range (IQR) = 23.0 to 32.2) and 10 of which came from PCR negative controls. The median (IQR) age of the infected patients was 64 years (60-74). The median (IQR) time from symptom onset to recruitment was 7 days (3-10). From the ELISA read values it was clear that the signal from the negative controls was universally low but the signal from the positive sample varied considerably (Fig 4a), with there being both negative signals as well as many strong signals exceeding the linear range of the Nc dose-response curve, present as an internal control for all assays. Specificity of the assay was excellent but given the low numbers of negative controls in the trial, confidence levels were wide (100% (95% CI, 69.2-100)). The sensitivity of the assay was lower but with tighter confidence intervals (58.6% (95% CI, 49.6-67.2). Stratifying the data against Ct values obtained from the earlier syndromic PCR values showed that the sensitivity of the assay increased if the analysis was restricted to patients with low Ct values (i.e. high viral loads) (Fig 4b). If samples with Ct values >30 were excluded sensitivity was 78.3% (95% CI, 66.7-87.3; 69 positive samples), and if samples with Ct values >26 were excluded this increased to 87.0% (95% CI, 73.7-95.1; 46 positive samples). Grouping the cohort into different Ct value ranges and examining the ELISA signals in each of these subdivisions suggested that there was a relationship between RNA concentration and Nc levels (Fig 4c). To analyse this relationship in more detail, concentrations of Nc were determined using the internal standard curves run within the assay. For those samples where the signal exceeded the linear range of the standard curve additional

ELISA analysis was done using serial dilutions of the samples. This revealed a significant inverse linear relationship between Ct values and log(10) Nc concentration ($p<0.0001$), albeit with a modest R^2 value of 0.54 (Fig 4d).

Analysing ELISA using diverse samples in Ghana

To examine the performance of the ELISA in an LMIC setting, the assay was evaluated in Ghana at the West African centre for Cell Biology of Infectious Pathogens (WACCBIP) at the University of Ghana. Specimens used in this case were from a variety of locations and individuals. Analysis of a small number of oropharyngeal swabs collected in VTM (n=24) and nasal lavage fluids (n=4) from COVID-19 patients suggested that such samples were very poor substrates for ELISA analysis (data not shown). For this reason, sampling predominately focussed on the collection of sputum which provided a more robust ELISA signal. In total 217 samples were tested, 121 of which were from confirmed positive cases (median Ct value = 25, IQR = 23.3 to 33.9). Consistent with results obtained with Southampton samples, all negative samples had low absorbance values as assessed by the Nc ELISA whereas the signal from the positive samples varied widely (Fig 5a). Again specificity was seen to be high, with much tighter confidence intervals because of the higher numbers in the negative control group (100% (95% CI, 96.2-100)). Sensitivity was broadly similar to that seen for the Southampton samples, either when all positive samples were included in the analysis (52% (95% CI, 42.8-61.2)) or when the Ct cut off threshold was reduced to 30 and below (72.6% (95% CI, 61.8-81.2; 84 positive samples)) (Fig 5b). Restricting the analysis to samples where there was information on clinical presentation (n=48), no obvious difference in sensitivity could be observed between the 21 symptomatic (81.0% (95% CI, 58.1-94.6)) versus 27 asymptomatic (74.1% (95% CI 53.7-88.9)) cases. These relatively high sensitivity values compared to the cohort overall likely reflect the high viral loads in this subsection of the cohort (mean Ct values of 20.9 and 22.7 respectively). Although no assessment of Nc concentration was undertaken, it was clear that ELISA signal strength increased with decreasing Ct values of the samples (Fig 5c). However, it was also apparent that there were small numbers of

244 samples with very low Ct values that that failed to generate a signal in the ELISA assay (i.e. 4
245 samples with a Ct ≤ 20 proved test negative).
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Discussion

There is currently an unmet need for low-cost SARS-CoV-2 diagnostic tests in LMICs. In this study, we investigated whether a standard ELISA-based platform offered a potential solution to this problem. Initial development work produced an Nc ELISA specific for SARS-CoV-2 that detected levels of recombinant Nc protein as low as 8.4 pg/ml and infectious virus from cell culture supernatants at levels between 8-16 pfu/ml. Assessment of ELISA performance using nose and throat swab samples from a UK cohort with severe COVID found the test to have a sensitivity of 58.6% (95% CI, 49.6-67.2) and specificity of 100% (95% CI, 69.2-100). A repeat assessment of performance using sputum samples from Ghana produced similar results, with a sensitivity of 52.0% (95% CI, 42.8-61.2) and specificity of 100% (95% CI, 96.2-100).

Accessibility, frequency and the time taken from sampling to obtaining a result are all key factors in ensuring effective population screening for SARS-CoV-2, with analytical limits of detection being of secondary importance [18]. The low cost and minimal lab equipment requirements needed to run ELISAs make them both accessible and cost-effective when performing frequent repeated sampling. Analysis of ELISA performance in large-scale SARS-CoV-2 community screening programmes has yet to be undertaken. However, there is reason to think that the findings reported here are of relevance and support the use of ELISAs in such settings. Firstly, ELISA performance was robust given it produced broadly similar results using samples from Ghana and the UK even though sample types were different and different lab personnel were running the two arms of the trial. Secondly, although the positive samples were predominately from symptomatic patients, looking at Ct values as an inverse proxy for the viral load it appears that these were either higher (UK median Ct 30, IQR 23.3-32) or equivalent (Ghana median Ct 26.2, IQR 19.7-31.1) to those that have been encountered in a national screening programme (median Ct 26.2, IQR 19.7-31.1) [19]. Ct values vary between studies due to variables such as the efficiency of the PCR reaction, target sequence, sample volume and sample handling so using them to make comparisons has to be approached with

caution. However, at least for the UK cohort, the high Ct values are also likely due to the time of sampling. Viral titres are considered to peak around the time individuals first become symptomatic [20]. Increasing the time between symptom onset and testing for the presence of Nc in patients still positive by PCR also reduces Nc-based test sensitivity [21]. As the UK cohort samples came from patients admitted to hospital with severe COVID-19, many had been symptomatic for a week or more (median time 7 days, IQR range of 3-10 days) and thus would be expected to have relatively low titres. Consequently, the overall sensitivity of the ELISA calculated from using our UK hospital cohort may be a slight underestimate of what might be seen if the ELISA was used in community screening.

A key aim of community screening is to identify individuals with high viral loads, as these individuals will be the most infectious [22]. While there is a correlation between Ct values and recovery of infectious virus, there remains some ambiguity as to what the Ct threshold might be above which individuals can be considered non-infectious, with different studies reporting widely varying results [23-27]. However, based on current WHO guidance those individuals most infectious have test Ct values lower than 25-30 [28]. When analysis of our ELISA test results was limited to Ct values of 30 or below, the sensitivity of the ELISA was 78.3% (95% CI, 66.7-87.3) and 72.6% (95% CI, 61.8-81.2) respectively for the UK and Ghana arms of the study. When the analysis was restricted to Ct values of 26 or below sensitivity increased to 87.0% (95% CI, 73.7-95.1) and 74.6% (95% CI, 62.5-84.5). These aforementioned sensitivities are broadly similar to that of a commercial direct LAMP saliva assay (83% and 94% with Ct cut-offs of <33 and <25) currently employed as a screening tool in the UK [29]. They also compare favourably to the sensitivities reported in a head-to-head comparison of 5 commercial LFDs looking at asymptomatic individuals with Ct values < 30 [30]. Nc ELISA performance also appears similar to that seen for the Innova LFD [13], a test system widely employed by the UK government for community surveillance; see Table S1 for comparison. An added advantage to the ELISA format is that it is compatible with sputum, the preferred sample type in LMICs due to difficulties and costs associated with swab collection. Community

screening requires robust test performance for both symptomatic and asymptomatic individuals with high viral loads. While we found no obvious difference in test sensitivity between symptomatic and asymptomatic cases in the Ghanaian arm of our study, a potential limitation of this study was the relatively few individuals for which clinical presentation data was available.

Ct values and Nc concentration have previously been reported to be tightly correlated [31, 32]. Therefore, one of the more puzzling observations from this study was the more limited correlation that we observed. Part of the explanation will be due to separate swabs being taken for PCR analysis and then ELISA analysis, with there being up to 24 hours between the two sampling events. Even if the two samples had been taken simultaneously, evidence suggests as much as a 100-fold variation can be observed in sampling when taking consecutive nasal swabs [33]. Nonetheless, it is harder to explain a similar phenomenon seen with the sputum samples, where occasional very low Ct value samples appeared to contain no detectable Nc. One explanation could be that protease inhibitors in sample processing buffer failed to completely suppress protease activity in some samples and Nc was subsequently destroyed. Antigen masking resulting from the presence of Nc antibodies in the sample is another possible explanation. However, antigen masking has been discounted as a significant problem by others [34] and use of pAb in our capture ELISA reduces the likelihood of it further. Failure to detect Nc in low Ct samples may of course reflect genuine absence of the antigen in the sample. However, given that virions contain a very high molecular ratio of Nc to viral RNA [35], the RNA for the PCR signal would have to then come from elsewhere. Replication associated vesicles released from infected cells could be this source [36], as although not established, it seems likely that the ratio of Nc to viral RNA in them would be lower that that of virions. However there would still need to be conditions in the lung, such as the presence of opsonizing antibodies directed against viral envelope proteins [37], for Nc levels to drop more precipitously than viral RNA levels. Given this later possible explanation and the implications it would have for antigen-based testing, it would be interesting to see whether there is a

downward shift in the ratio of Nc to viral RNA in both infected individuals that had been previously vaccinated as well as in re-infected individuals.

When obtaining 2 reads per sample and including Nc controls in each plate, the experimental cost of reagents and consumables for running a Nc sputum-based ELISA was 0.31 GBP/sample in the UK and 0.46 GBP/sample in Ghana (Table S2 and S3). Obviously other costs would be associated with use of the ELISA such as employment of a trained technician ($0.21 per sample in Ghana) and provision of basic lab infrastructure. However, even with these additional costs taken into account the ELISA is still significantly cheaper than the $50/individual commercial antigen test deployed for screening incoming travellers in Ghana. The cheap costs make large-scale screening both feasible and desirable and should also allow for repeated sampling from the same individual, increasing accuracy and reliability [18].

In summary, our results support further investigations into the use of Nc ELISAs for large-scale screening programmes in LMICs. Consumable costs associated with the Nc ELISA are approximately 10-fold lower than the current commercial prices charged for both lateral flow testing and direct LAMP testing. While large-scale production and internal validation of an ELISA system would require industry involvement, we anticipate that ELISA testing would still offer a much lower cost test platform than anything else currently employed, especially if developed in an LMIC such as Ghana since third party and transportation costs would be significantly diminished.

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Competing interests

TWC has received discounted equipment and consumables from QIAGEN to support the independent research study that collected the patient samples used in this study. He has received speaker fees, honoraria, travel reimbursement, equipment and consumables free of charge for research outside of this submitted study from BioFire Diagnostics and BioMerieux; consultancy fees from Synairgen Research, Randox Laboratories, and Cidara Therapeutics; is a member of an advisory board for Roche and a member of two independent data monitoring committees for trials sponsored by Roche; and has acted as the UK chief investigator for an investigational medicinal product study sponsored by Janssen. RWE, CS, IK and PH are co-founders of Highfield Diagnostics Ltd. All other authors declare no competing interests.

Data sharing

The data analysed and presented in this study are available from the corresponding author on reasonable request, providing the request meets local ethical and research governance criteria.

Contributions

Conceptualization: MVH, MC, TW, OQ, GAA, PKQ, CJM

Data Curation: MVH, PCO, TW, PKQ, CJM

Formal Analysis: MVH, CJM

Funding Acquisition: CS, MC, PKQ, CJM

Investigation: MVH, PCO, CJM

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Project Administration: MC, TWC, PKQ, CJM

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Validation: MVH, PCO

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Writing – Original Draft Preparation: CJM

Writing – Review & Editing: MVH, NJB, SP, PH, IK, PJD, RWE, MC, TWC, PKQ, CJM

Figure legends

Figure 1. Reactivity of rabbit polyclonal antibody against recombinant SARS-CoV-2 Nc. (a) Direct ELISA using using purified pAb against SARS-CoV-2 Nc to detect this same antigen. Each pAb was assessed in a separate ELISA that included negative control wells incubated with highest concentration of pAb tested and which resulted in a mean OD495 signal of less than 0.06 (data not shown). Data derives from a single experiment with technical replicates (mean +/- SD; n=3). (b) Specific detection of SARS-CoV-2 Nc by Western blot using pooled rabbit pAb (right panel) and where each well contains 100ng recombinant Nc. A Coomassie stained gel containing 1 mg/well of the same human endogeous coronavirus Nc protein used in the Western analysis is also shown (left panel).

Figure 2.Detection of Nc by capture ELISA. (a) Data from capture ELISA using a range of different concentrations of recombinant SARS-CoV-2 Nc. Values shown represent the data from 13 independent experiments after subtraction of the background signal (mean +/- 95% C.I.). (b) Subset of data from (a) examining the linear relationship between signal and Nc concentration at lower concentrations of Nc (<400 pg/ml). (c) Results from a single experiment looking at the response to different concentrations of recombinant Nc from different human coronaviruses (mean +/- S.D. from technical triplicates).

Figure 3. Detection of Nc from cell culture supernatant. (a) Detection of Nc from three separate viral supernatants of known titre that were first uv-inactivated and then treated with 1% Tx100 + 0.3% TBP. Data represents the mean +/- SD from 3 separate readings with values compared against the background control (* = $p<0.05$; paired t-test). (b) uv-inactivated viral supernatants were immunodepleted with anti-Nc pAb or mock immunodepleted, subsequently treated or not treated with 1% Tx100, 0.3% TBP and then assessed by ELISA. Data shown represents mean +/- SD (* p<0/05, # $p<0.01$; paired t-test) from one of 2 representative experiments.

Figure 4. An evaluation of the Nc capture ELISA to detect SARS-CoV-2 infection using samples from a hospitalised UK patient cohort. Dry nose and throat swabs were taken within 16 hours of patients receiving a positive syndromic PCR test. (a) ELISA signal from samples recovered from swabs after reconstitution in 0.4ml lysis buffer. Dotted line represents the cut-off threshold (mean+/- 3xS.D. of negative control signals). (b) Plot of assay sensitivity against maximum Ct cut-off thresholds generated from syndromic PCR test. (c) Plot of individual OD450 readings from all samples separated into different Ct range categories with the cut-off threshold represented as a dotted line. (d) Plot of Nc concentration versus Ct value; R^2 and SE values derived from linear regression analysis. All concentration values were derived from samples taken from PCR positive patients with those values falling below the LoQ (25 pg/ml) plotted as this threshold value.

Figure 5. An evaluation of the Nc capture ELISA to detect SARS-CoV-2 infection using sputum samples from a Ghanaian cohort. PCR was performed on the same sample used for testing by ELISA. (a) Plot of ELISA signal from all PCR +ve and PCR negative samples. Dotted line represents the cut-off threshold (mean +/- 3xS.D. of negative control signals). (b) Plot of assay sensitivity against maximum Ct cut-off thresholds. (c) Plot of individual OD450 readings from all samples separated into different Ct range categories with the cut-off threshold represented as a dotted line.

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572

Fig 1

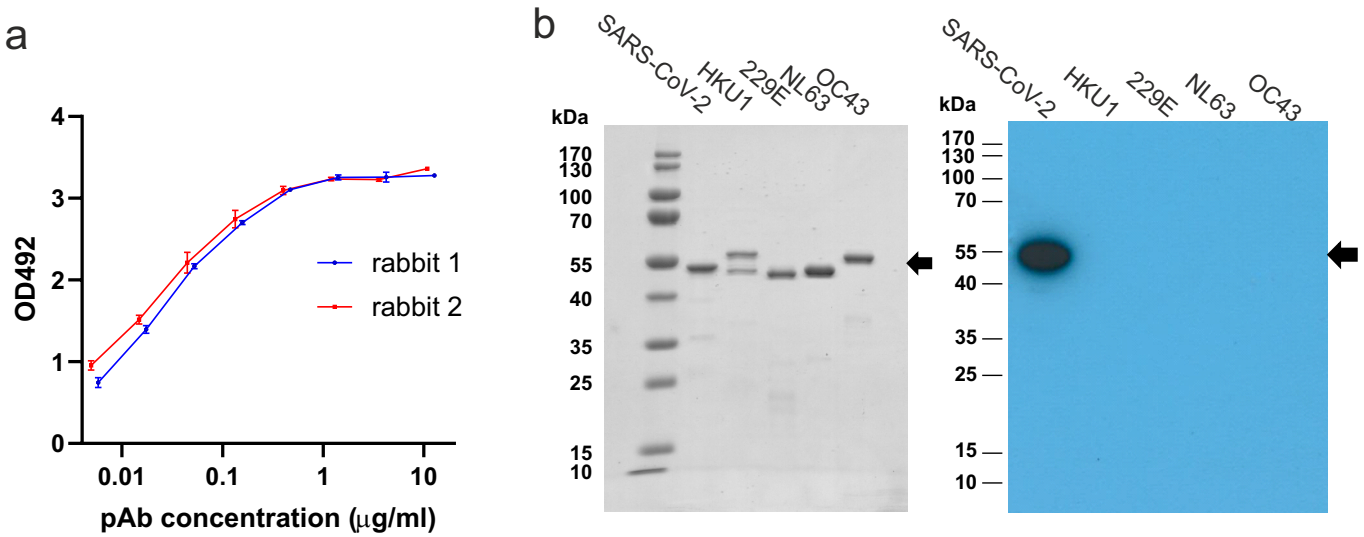

a
OD492
4
3
2
1
0
0.01
0.1
1
10
pAb concentration (μg/ml)
rabbit 1
rabbit 2
b
SARS-CoV-2
HKU1
229E
NL63
OC43
kDa
170
130
100
70
55
40
35
25
15
10
SARS-CoV-2
HKU1
229E
NL63
OC43
kDa
170
130
100
70
55
40
35
25
15
10

Fig 2

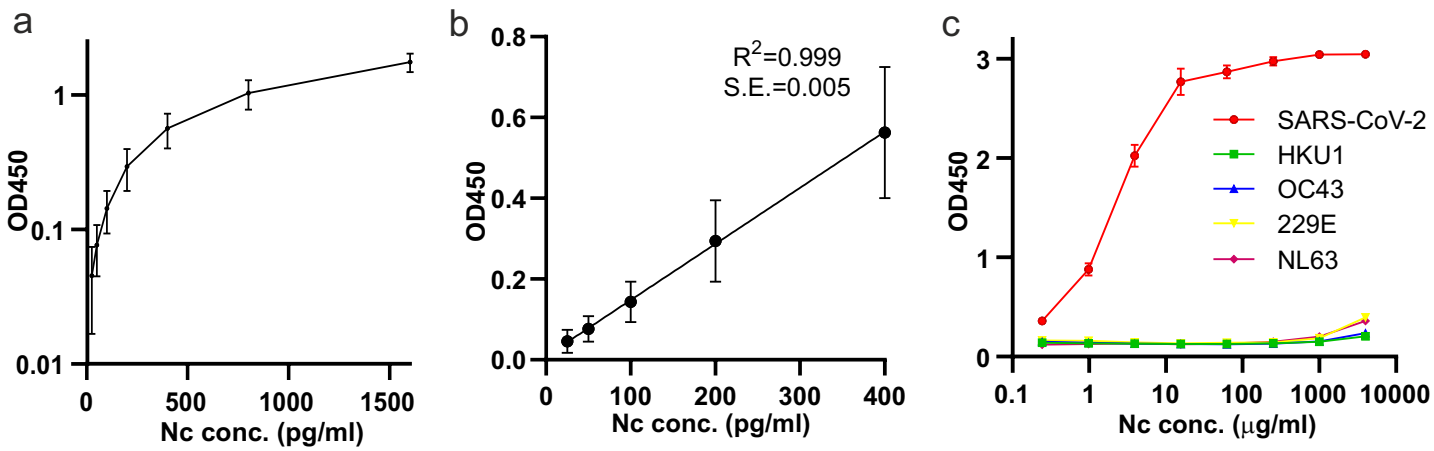
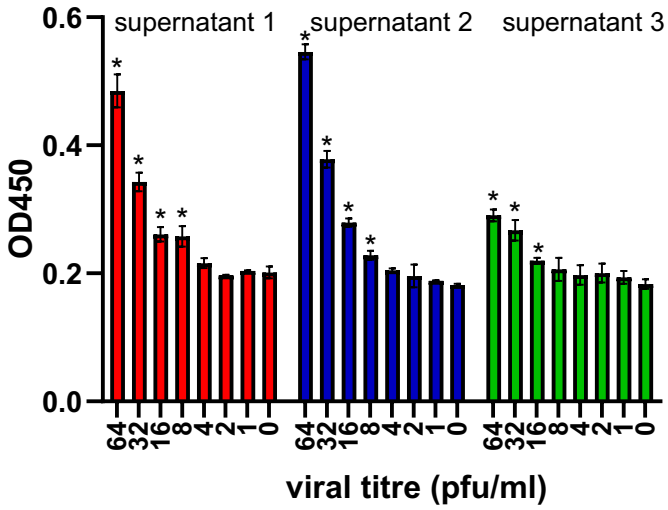

a
OD450
1
0.1
0.01
0
500
1000
1500
Nc conc. (pg/ml)
b
OD450
0.8
0.6
0.4
0.2
0.0
R2=0.999
S.E.=0.005
0
100
200
300
400
Nc conc. (pg/ml)
c
OD450
3
2
1
0
SARS-CoV-2
HKU1
OC43
229E
NL63
0.1
1
10
100
1000
10000
Nc conc. (μg/ml)

Fig 3

a


supernatant 1
supernatant 2
supernatant 3
OD450
0.6
0.4
0.2
0.0
64
32
16
8
4
2
1
0
viral titre (pfu/ml)

b

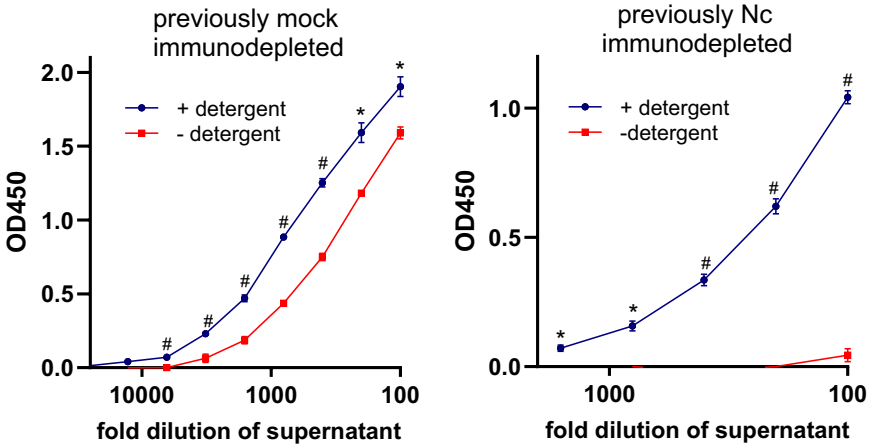

previously mock immunodepleted
+ detergent
- detergent
OD450
2.0
1.5
1.0
0.5
0.0
10000
1000
100
fold dilution of supernatant
previously Nc immunodepleted
+ detergent
-detergent
OD450
1.0
0.5
0.0
1000
100
fold dilution of supernatant

Fig 4

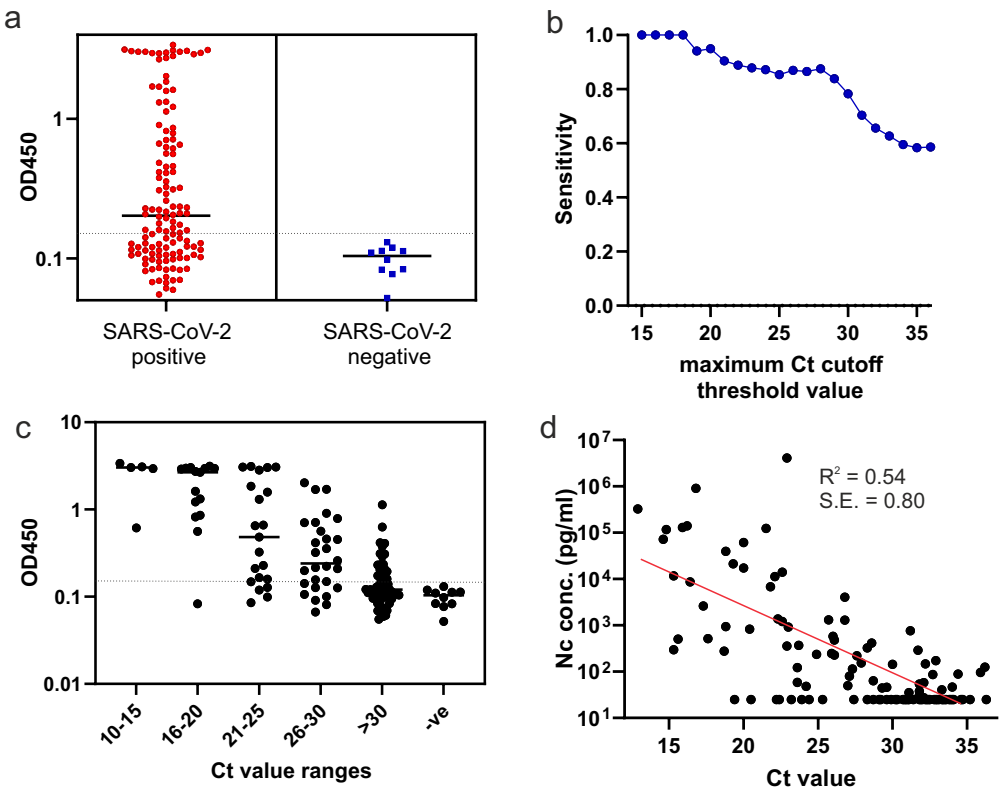
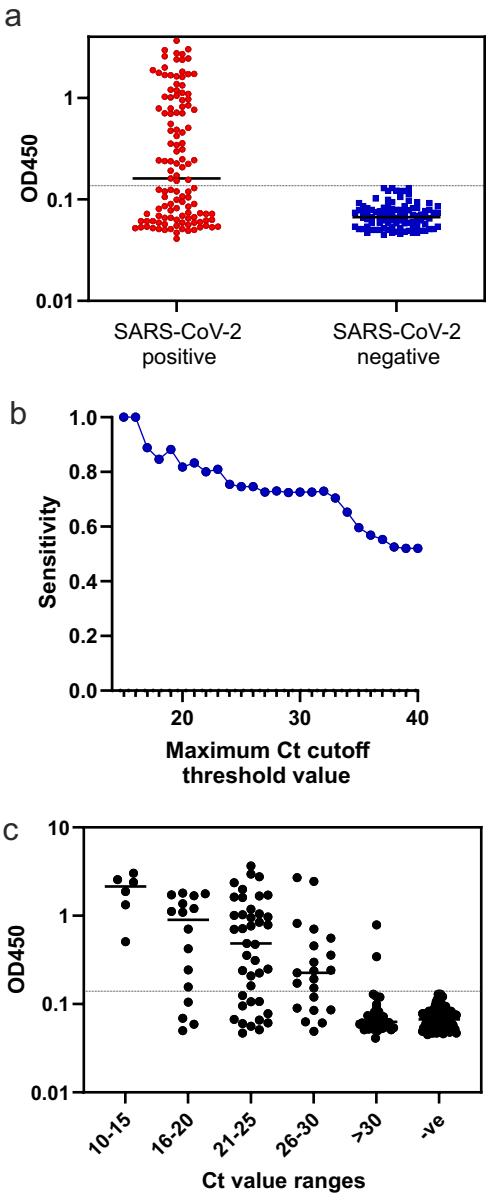

a
OD450
1
0.1
SARS-CoV-2 positive
SARS-CoV-2 negative
b
Sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
15
20
25
30
35
maximum Ct cutoff threshold value
c
OD450
10
1
0.1
0.01
10-15
16-20
21-25
26-30
>30
-ve
Ct value ranges
d
Nc conc. (pg/ml)
10^7
10^6
10^5
10^4
10^3
10^2
10^1
R^2 = 0.54
S.E. = 0.80
15
20
25
30
35
Ct value

Fig 5


a
OD450
1
0.1
0.01
SARS-CoV-2 positive
SARS-CoV-2 negative
b
Sensitivity
1.0
0.8
0.6
0.4
0.2
0.0
20
30
40
Maximum Ct cutoff threshold value
c
OD450
10
1
0.1
0.01
10-15
16-20
21-25
26-30
>30
-ve
Ct value ranges

Supplementary methods

Expression of nucleocapsid proteins

All Nc DNAs were cloned into pHYRSF53 (AddGene ref number #64696; doi: 10.1016/j.pep.2015.08.019) and the resulting plasmids transformed into Rosetta ™ (DE3) competent cells. For protein expression, bacterial cultures were grown in LB broth at 37°C until OD 0.4-0.5, induced with 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) for 4 hrs and harvested by centrifugation at 4°C. Bacterial pellets were resuspended in 20 mM HEPES (pH 8.0), 50 mM NaCl, 10% (v/v) glycerol, 1 mM β-mercaptoethanol and 1 mM PMSF, sonicated and supplemented with 0.1% Triton X-100 (v/v) before centrifugation at 15,000 rpm at 4°C for 20 min to pellet insoluble contents. Soluble fractions were passed through a 0.45 µm syringe filter, supplemented with 10 mM imidazole and incubated for 3 hours at 4℃ with Ni-NTA beads; a polishing step that facilitated binding of partially degraded 6xHIS-SUMO tagged protein to the resin while the full-length recombinant protein stayed in solution. After removal of the beads by centrifugation the supernatant was supplemented with NaCl, HEPES (pH 8.0) and imidazole solutions final concentrations of 1 M, 50 mM and 10 mM, respectively. It was then incubated with fresh Ni-NTA beads for 3 hrs incubation on a rotator at 4°C, transferred to a disposable column, and the Ni-NTA resin washed with ice-cold 50 mM HEPES (pH 8.0), 1 M NaCl, 10% (v/v) glycerol, 0.1% (v/v) Triton X-100 and 20 mM imidazole. Recombinant 6xHIS-SUMO-Nc protein was eluted with 50 mM HEPES (pH 8.0), 1 M NaCl, 10% (v/v) glycerol, 0.1% (v/v) Triton X-100 and 300 mM imidazole, dialysed overnight in 50 mM HEPES (pH 8.0), 1 M NaCl, 10% (v/v) glycerol and 0.1% (v/v) Triton X-100 at 4°C and re-purified as described above, but with 0.5 M NaCl in both the wash and elution buffers. Elution fractions containing protein were pooled and diluted with dialysis buffer (20 mM HEPES (pH 8.0), 0.35 M NaCl, 10% (v/v) glycerol and 1 mM β-mercaptoethanol) such that the final protein concentration was ≤ 2 mg/ml. After supplementation with 2 µg Ubiquitin-like protease (Ulp) per 1 mg of Nc protein, the recombinant protein was dialysed overnight at 4°C against a larger volume of the same dialysis buffer. Imidazole was then added to a final concentration of 10mM and the sample placed down a 0.5 ml Ni-NTA column pre-equilibrated with 20 mM HEPES

(pH 8.0), 0.35 M NaCl, 10% glycerol to remove both Ulp and the cleaved 6xHIS-SUMO tag. Recovered tag-free Nc was dialysed for 3 hrs at 4°C in 20 mM HEPES (pH 8.0), 0.35 M NaCl, 10% glycerol, quantified by BCA assay and purity checked by 12% (w/v) SDS-PAGE. All Nc proteins were stored at -80°C until use.

Western blot

One hundred nanograms per well of each of the recombinant nucleocapsid proteins were subject to SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membrane. After blocking PVDF with 1% BSA in TBS-T, the PVDF was immunoblotted with 1 μg/ml pAb. Bound pAb was detected using a HRP donkey anti-sheep secondary antibody (Sigma) in combination with Picowest chemilumiescence substrate (Pierce) and the image capture on film. One microgram per well of the same nucleocapid proteins were subject to SDS-PAGE on a parallel acrylamide gel and the proteins visualized with Coomassie Blue.

Indirect ELISA assessing pAb reactivity to immobilized SARS-CoV-2 Nc

Wells were coated with 100 ng/ml SARS-CoV-2 Nc in PBS for 16 hrs at 4℃, blocked with 1mg/ml BSA in PBS for 2 hrs at room temperature and incubated with different pAb concentrations for 2 hrs at room temperature in blocking buffer. Bound antibody was detected with anti-rabbit IgG HRP conjugate (Sigma) in combination with OPD substrate.

Immunodepletion of Nc and detergent treatment of viral supernatants

Forty microlitres of beads (50% protein A/G slurry – Thermo Scientific) were incubated with 100 ug of pAb in a final volume of 1.4 ml buffer (PBS supplemented with 0.1% (w/v) BSA) overnight at 4°C with rotation. Mock treated beads were incubated in buffer alone. All beads were subsequently washed with buffer and transferred to 1 ml of a uv-inactivated viral supernatant and incubated with rotation for 2 hr at 4°C before centrifugation and collection of supernatant. Detergent treatment involved the addition of Tx100 and TBP to a final concentration of 1% and 0.3% respectively.