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Development of a rapid diagnostic test for the detection of antibodies or antigens to Coronavirus (COVID-19)

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ABSTRACT

The global health crisis caused by COVID-19 has overwhelmed both healthcare settings and economies globally. While mass population testing has improved drastically, recent reviews of existing methods have highlighted various shortcomings with these methods.
The aim of this project was to investigate whether the LAA could be modified and utilised as a rapid detection test which either matched or exceeded the existing sensitivity and specificity values.
The latex agglutination assay (LAA) investigated whether the COVID-19 spike protein could be detected in samples. COVID-19-specific IgM and IgG were used in conjunction with a series of non-specific antigens. Control or A_G-containing samples were mixed with A_B-microsphere complexes on glass microscope slides.
Manual visualisation identified various levels of agglutination. Light microscopy and spectrophotometry at 405 nm determined that the LAA could detect at least 2.3 ng of spike protein.
The particle-counting tool of ImageJ was utilised to obtain a data set which was subjected to statistical analysis which indicated that there was a significant difference between control samples and live tests, $P = 0.000102$ for the spike protein assay and $P = 0.254$ for the non-specific assay respectively.
The results obtained fell in line with a similar study conducted by Buffin et al. in 2018.
The analytical methods used in this project twinned with data obtained in previous studies supports the significant difference between control values and live test values. The LAA is easier, quicker to use (results in ≤ 30 minutes) and cheaper, with potentially better sensitivity to existing methods. This could benefit high- and low-income countries alike upon further research and optimisation.

Introduction

What is COVID-19?

In December of 2019, cases of pneumonia with an unknown aetiology were first documented in the city of Wuhan, China. As these cases became more prevalent, research began to identify the causative agent of this outbreak. After extensive investigation, scientists in China identified this agent to be a novel, enveloped virus belonging to the *Coronaviridae* family (Zheng & Lai, 2020). Furthermore, due to this novel virus's similarity to a coronavirus outbreak in 2003–2004, which caused SARS, it was appropriately named SARS-CoV-2 (Patel & Jernigan, 2020). The disease caused by SARS-CoV-2 has been named as COVID-19 (Guan et al., 2020).

Clinical manifestation

The primary clinical manifestation of COVID-19 is a lower respiratory tract infection, causing a dry cough, fever and fatigue (Chen, J et al., 2020). However, as this disease became more prevalent throughout the population of various countries, COVID-19 has been documented as a multi-system afflicting disease, causing a conglomeration of symptoms of varying severity, ranging from the mild flu-like symptoms as mentioned earlier, to pneumonia, sputum production, headaches, sore throat, chills, chest pain, haemoptysis, nausea, vomiting and diarrhoea (Wang et al., 2020). Anosmia and ageusia are two of the most widely reported symptoms in COVID-19 patients (Gautier & Ravussin, 2020) and was officially included as a diagnostic criterion in the United Kingdom by the NHS ('Symptoms of coronavirus (COVID-19)', nhs.uk, 2020).

COVID-19 complications

Severe cases of COVID-19 require immediate hospitalisation and intensive care as disease progression has caused damage to one or multiple organ systems. Primarily affecting the lungs, intubation is required and if the prognosis does not improve, mechanical ventilation. This is to combat severe hypoxemia (Möhlenkamp & Thiele, 2020). Oxygen supplementation methods, particularly mechanical ventilation, have an associated poor mortality rate (35% in the Netherlands and 59.5% in the UK) (Mahase, 2020a).

The cause of increased hospitalisation and mortality is COVID-19's ability to provoke a whole-body immune response. Pro-inflammatory cytokines such as IL-6, IL-2, IL-7, IL-10, γ-interferon and TNF-α are uncontrollably released, causing secondary Haemophagocytic Lymphohistiocytosis (sHLH) which attacks multiorgan systems leading to specifically ARDS and poor patient prognosis (Luo et al., 2020). It was this immunological response which caused millions of deaths during the Spanish flu pandemic. (Liu et al., 2015).

Transmission

There are currently three universally accepted modes of transmission for COVID-19. Airborne transmission attributed to the inhalation of contaminated aerosols within a closed environment. Despite research being incomplete, multiple studies, two of which are yet to be peer reviewed, have successfully isolated genetic material from SARS-CoV-2 in air samples using various PCR methods (Chia et al., 2020; Jiang et al., 2020; Liu et al., 2020; Santarpia et al., 2020)).

The second mode focuses on the exposure to larger respiratory droplets that are produced (coughing, sneezing, talking) and spread in proximity from one person to another. The third and final mode, is the direct contact of an uninfected person with contaminated surfaces (Morawska et al., 2020).

The full extent of how this novel virus is transmitted is still open to debate. For example, researchers have successfully isolated genetic

material in stool samples from patients. This implies that the faecal/oral route is a possible mode of transmission (Xiao et al., 2020).

Testing progression

As the pandemic began to spread worldwide, governments across the globe began implementing mass testing and contact tracing programmes. The standard which has been implemented worldwide and is recommended by the Centres for Disease Control (CDC), is RT-PCR of naso/oropharyngeal samples obtained by swabbing. This molecular diagnostic test identifies and amplifies two regions (N1 and N2) in the viral genome. A positive result is confirmed when both regions are identified, a negative result is confirmed when neither region is identified and an inconclusive result is given when only one region is detected (Vinh et al., 2020).

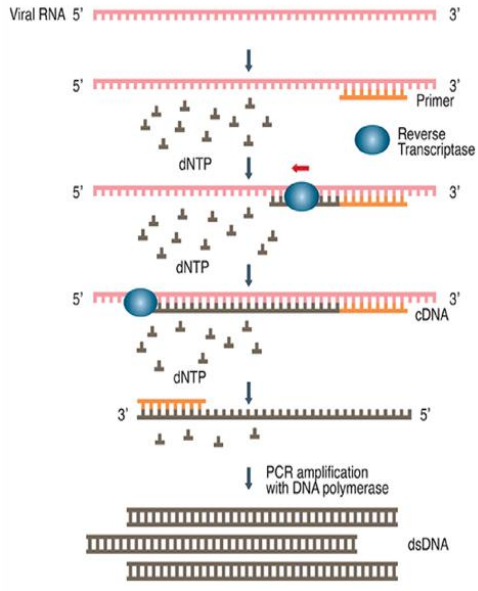

Figure 1: Molecular basis of RT-PCR to generate DNA (reproduced under the conditions of a creative commons license 3.0) (Carter et al., 2020, Figure 1)

Following on from the implementation of mass testing programmes came optimisation of these methods. The UK government has spent billions of pounds in research to ensure 'normal life' resumes as quickly as possible. One product of this spending has been the development of 'Nudgebox' machines, which can process 15 samples a day, with a specificity of 98% and sensitivity of 100%. These 'easy-to-use' machines implement the molecular technique of RT-PCR but in a setting that does not require laboratory assistance (Mahase, 2020c).

Another venture undertaken by the UK government is the development of high throughput testing capabilities using LAMP technology. Again, using the same premise as RT-PCR, but at a constant temperature and not requiring pure samples, this method can be implemented without the use of specialised laboratory equipment (Sharma et al., 2020).

This technology is promising as it possesses high throughput capabilities and other analytical techniques can be utilised, such as colorimetric analysis of samples (Park et al., 2020). Despite trials still being underway, the companies developing this technology are already boasting high sensitivity and specificity ranges (Mahase, 2020d).

Superseding the optimisation of the RT-PCR based assays came the development of lateral flow devices (LFDs). Lateral flow assays rely on the presence of immunological components such as the SARS-CoV-2 spike protein itself rather than genetic material. The LFD suffers from a lower general sensitivity,76.8% when compared to that of RT-PCR but boasts a sensitivity of >95% in patients with higher viral loads (Mahase, 2020e); in addition, it is a quick method that can be carried out at home or as part of a national screening programme, such as the one in place for university students.

The premise of LFDs is like that of a pregnancy test, with the samples in question being subjected to exposure of specific $A_{Bs.}$ The sample subsequently migrates along the device via capillary action. A positive result is confirmed via the presence of coloured lines on the device. Whereas results from RT-PCR assays can exceed 24 hours, LFDs can provide results within 10 to 30 minutes (Peto, 2021).

Current testing limitations

The gold standard RT-PCR method, while effective, is not without its drawbacks, requiring specialised operators/equipment and an efficient sample transport system. It was originally a lengthy and costly process, often exceeding 48 hours for results to be obtained. The process has now been optimised with nine out of 10 test results being returned the next day ('9 in 10 test results returned next day by NHS Test and Trace', GOV.UK., 2022). Original teething issues still remain, which limits the implementation in lower-income countries (Beeching et al., 2020; Dorlass et al., 2020). Despite this method reaching $\geqslant$ 96% efficiency (van Kasteren et al., 2020), it is not 100%, which means that false results can occur.

Testing of large populations would allow false results to go unnoticed, potentially exacerbating viral transmission (Surkova et al., 2020).

Operation Moonshot was a project undertaken by the UK government to implement mass population testing. Nudgebox and LamPORE technology were a creation of this operation and offer results within hours, with slightly elevated sensitivity and specificity ranges, without the use of specialised laboratory staff and equipment. This reduction in turnaround time will help identify and isolate cases of COVID-19 in a more efficient manner. Despite this vast improvement, the product data has been found to contain inaccuracies, which could hinder the use of these testing methods (Mahase, 2020d).

LFDs have been implemented across the United Kingdom to mass test specific populations, such as university students either returning or leaving their institution. Despite the initial positive results, studies conducted by Mahase (2020e) and Peto (2021), both of which have yet to be peer reviewed, have identified issues regarding this specific testing method. Both studies have identified that the highest test sensitivity is achieved by using highly trained laboratory scientists, with the sensitivity value decreasing dramatically to 58% when undertaken by self-trained members of the public. As mentioned previously, the >95% sensitivity value is only obtained from patients with a high viral load, meaning that the timing of testing is critical in order to catch them at their most infectious (Cevik et al., 2020), and using highly skilled operators is imperative for a more reliable result.

Project rationale

From reviewing the relevant literature (Mahase, 2020a; 2020b; 2020c; 2020d; 2020e; Peto, 2021), there is a distinct lack of rapid diagnostic and detection methods with adequate sensitivity/specificity. The current approaches are costly, time-consuming and produce a substantial number of false results.

The purpose of this research was to bridge the gap between current approaches and create a rapid diagnostic or detection method for COVID-19 which utilises IgG and IgM binding to the SARS-CoV-2 spike protein. This newly developed testing protocol could then, hypothetically, be scaled into a mass population testing system, subsequently assessing seroconversion levels after mass vaccination programmes. The method that was focussed on was the Latex Agglutination Assay.

Optimising. 4his technique involved identifying the key receptors in COVID-19 spike protein binding to host cells. A combination of Monoclonal and Polyclonal antibodies was used separately in this method. Antibodies are one of the key factors in the host immune response. Monoclonal antibodies only recognise one specific site on an invading pathogen, making them more specific compared to polyclonal antibodies which recognise multiple sites across the pathogen. Although this multiple site binding in polyclonal antibodies does generate higher sensitivity, it also runs the risk of nonspecific binding (Ascoli & Aggeler, 2018).

Developing less expensive detection methods without the need for laboratory assistance could also prove beneficial in lower-income countries, creating new testing regimens, aiding in the implementation of infectious disease control protocols and ultimately, saving lives. The

improved ease of testing has the potential to boost economic growth as faculty members will not need to be as highly trained as they are for the existing methods.

Methodology

Latex Agglutination Assay

The LAA is affordable, costing nearly three times less than RT-PCR and can provide results rapidly (Xiang et al., 2020), possibly without the need for specific analytical equipment. LAA methods provide a low-cost alternative that can be easily visualised but at no detriment to the sensitivity and specificity and could potentially be utilised in developing countries.

Visibly dyed microspheres (0.8 µm) (Bangs Laboratories) were coated with polyclonal A_{Bs} (Sino Biological) raised against the SARS-CoV-2 spike protein using Bangs Laboratories' B protocol (Laboratories, 2021b) and stored at 4°C until they were used. This protocol was undertaken by other members of the research team during a mandatory COVID-19 isolation period.

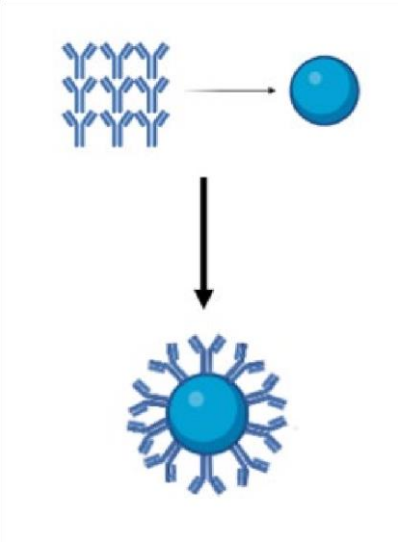

Figure 2: Visual representation of polyclonal antibodies binding to dyed microspheres. The blue circle represents the microsphere, and the 'Y'-shaped molecules represent A_{Bs} (Created using BioRender.com)

The product information sheet gave an A_G concentration of 1.18 mg/ml, and from these, various concentrations of A_G were created which are detailed in Table 1. A 1:1 (10 µl:10 µl) ratio of microspheres was added to either borate buffer (control) or to one of the A_G concentrations (test) on a standard glass microscope slide. This protocol was repeated in triplicate for every concentration.

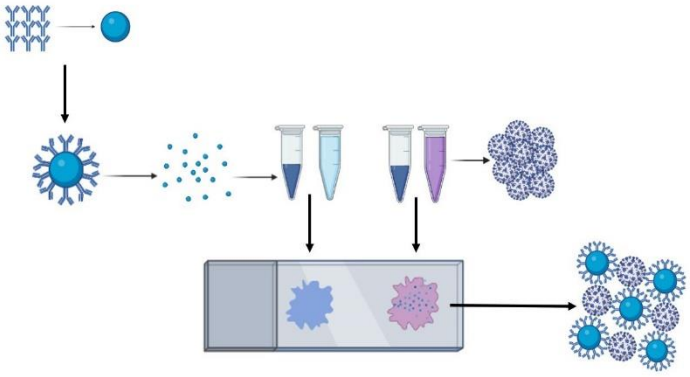

Figure 3: A flow diagram representing the LAA protocol (created using BioRender.com). On the left (blue) section of the slide is the control and, on the right, (pink) is the active test. Two distinct colours have been used to represent each test individually

After the initial round of LAA testing was completed, it was determined that the optimum testing concentration fell between the 10^{-2} and 10^{-3} concentrations. All other concentrations were removed.

Antigen Dilution	Antigen Volume (µl)	Buffer Volume (µl)	Antigen Concentration (ng/ml)
1 in 10 (10^{-1})	1	9	118
1 in 100 (10^{-2})	1	99	11.8
1 in 250	1	249	4.72
1 in 500	1	499	2.36
1 in 1000 (10^{-3})	1	999	1.18
1 in 10,000 (10^{-4})	1	9999	0.118

Table 1: The composition of each SARS-CoV-2 A_G dilution

In order to determine LAA specificity, three different A_{Gs} in the form of supernatants taken from different cell lines were recruited, as highlighted in Table 2. The LAA protocol was repeated in triplicate for each of these cell lines (using the same dilutions as detailed in Table 1).

Cell Line Name	Cell Line Origin
SF188	Cancer cell line
KNS42	Cancer cell line
U25159	Cancer cell line expressing Lentivirus particles

Table 2: Details of the origins of the cell lines used as non-specific A_{Gs}.

Latex Agglutination Assay modification

Disposable disc cards which were specialised for the LAA were used. The methodology remained the same, a 1:1 ratio of microspheres was added to the A_G concentrations outlined in Table 1 ($10^{-2} - 10^{-3}$). This process was repeated for the non-specific A_{Gs} detailed in Table 2.

Latex Agglutination Assay data acquisition

Initially, a visual examination was conducted by the operators. Following on from this, light microscopy at four times magnification (using a Leica CME Classic) was used to visually analyse the samples further. Images were acquired of these samples and subjected to ImageJ's particle counting tool. This data was subsequently analysed in SPSS using the Shapiro-Wilk test of normality and paired T-tests.
Finally, spectrophotometry (using a Novaspec ii, Pharmacia Biotech) was utilised to gain a real-time representation of agglutination levels.
For the spectrophotometric analysis, the same concentrations as mentioned previously were used but due to the low volume of reagents and time constraints, the volumes were reduced by a factor of 10. 50 µl of A_G was added to 50 µl of microsphere solution, still using the 1:1 ratio, to a cuvette.
The remainder of the sample was made up to 1ml with borate buffer.

The A_G concentrations were compared to a solution of 50 µl of microspheres mixed with 950 µl of borate buffer (Reference sample). The absorbance was measured at 405 nm at the time points 0, 0.5, 1, 2, 5, 10, 20 and 30 minutes. This was repeated for every A_G concentration. The cell lines had their absorbance measured at 30 minutes only.

Results

Latex Agglutination Assay

The LAA was performed on standard glass microscope slides and visually analysed in an almost exact manner to the AGID assays. The premise of this assay was based on the agglutination of polystyrene microspheres, thus the initial visualisation method focussed on assessing whether this process had occurred after the samples had been allowed to air-dry at room temperature. Upon manual visualisation by the operates, it was determined that the 10^{-1} and 10^{-4} A_G concentrations

fell outside of the optimum range and subsequently removed from testing. From this, the LAA was repeated in triplicate for each concentration.

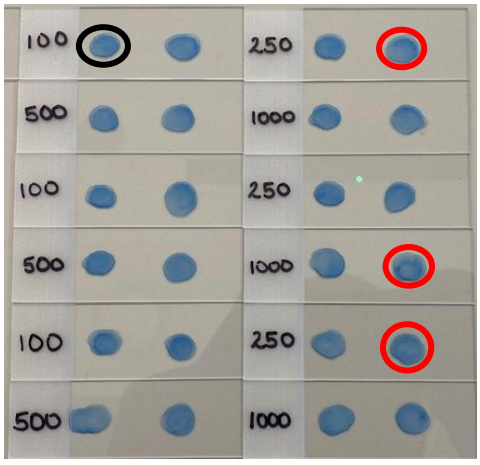

Figure 4: A photograph of completed LAAs on microscope slides using all the designated A_G concentrations (circled in black is the control test and circled in red is the live A_G test)

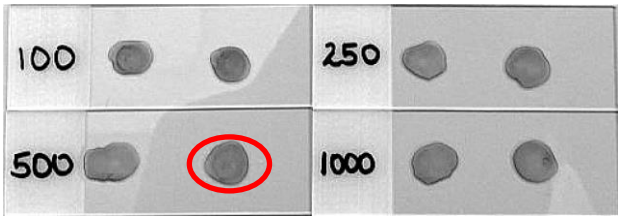

Figure 5: Image manipulation using ImageJ for one round of test A_G concentrations

For the LAAs, the control test was always placed on the left-hand side of the slide (circled in black in Figure 4) and the A_G test was always placed on the right (circled in red on Figure 4).

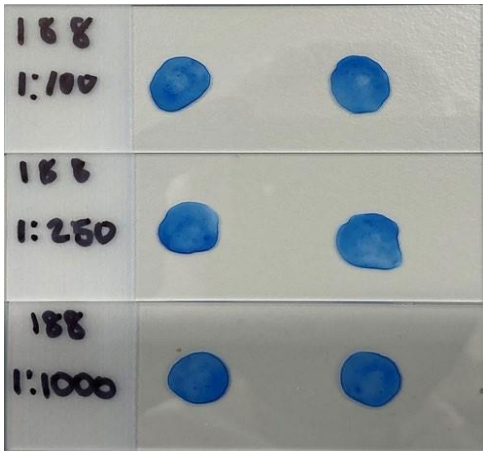

Figure 6: A photograph of completed LAAs on microscope slides of some of the designated concentrations of cell line SF188

Figure 7: Image manipulation using ImageJ for one set of LAAs using non-specific A_{Gs}

The LAAs for the non-specific cell lines were prepared and analysed using the exact same protocol. Control tests on the left, A_G test on the right and visually examined once the samples had air-dried.

Upon examination, the results highlighted in red on Figure 4 show a distinct band pattern, which is a deeper blue colour in comparison to the controls. After image manipulation using ImageJ, the band pattern can be more easily observed (circled in red on Figure 5).

The examination of the non-specific LAAs in Figure 6 did not reveal this banding in comparison with the controls. Further analysis using ImageJ (Figure 7)

also did not reveal any band patterns. However, there were similarities between some of the controls and A_G tests. Due to the uncertainty of the results, further analysis of all samples was undertaken.

Modified Latex Agglutination Assay

The modified LAA was carried out on specialised disposable disc cards and was visually analysed in the same manner as the LAAs carried out on microscope slides (data not included).

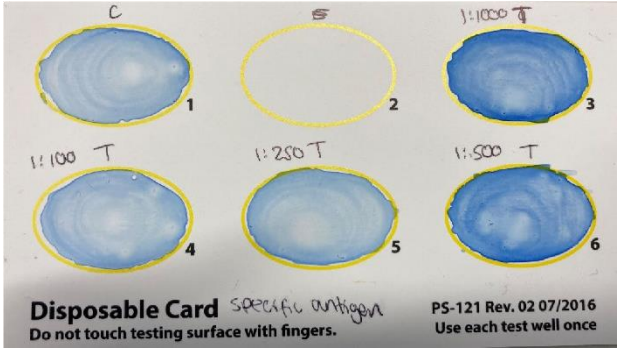

Figure 8: LAA completed on a specialised disc card for all A_G concentrations

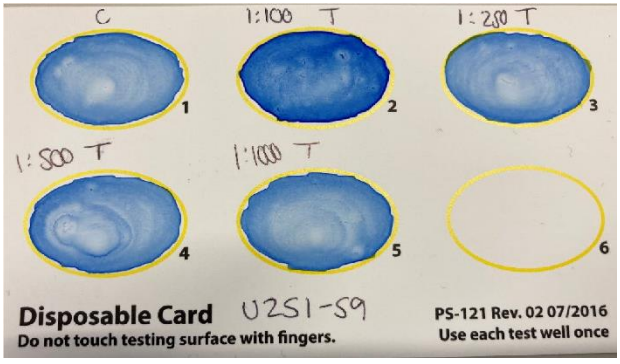

Figure 9: LAA completed on a specialised disc card for all concentrations of the non-specific A_G, U25159

The results obtained from this modified LAA fell in line with the method implemented using glass microscope slides. Agglutinates and concentric rings can be observed in Figure 8 but not in Figure 9.

Latex Agglutination Analysis using light microscopy

To gain a better understanding of what was occurring during the LAA, the method of choice for further visual examination was to use microscopy to analyse the morphology and contents of the band patterns. For this method, images were acquired using 4x magnification on a light microscope.

Figure 10: Analysis of a control sample using a 4x magnification (arrow highlighting agglutination)

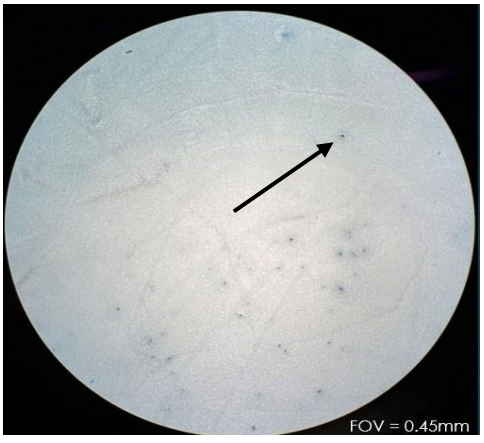

Figure 11: Analysis of a 1:100 test sample using 4x magnification (arrow highlighting agglutination)

As the samples were allowed to air-dry before being placed under the microscope, both Figures 10 and 11 show evidence that crystallisation has occurred. However, when comparing the two images, Figure 10 has a smaller number of circular blue agglutinates when compared to Figure 11. As Figure 10 was a control and Figure 11 was a live test, the appearance of these agglutinates was expected (this is shown via arrows in both Figures 10 and 11).

Particle counting using ImageJ

In addition to using ImageJ to manipulate the sample images to clarify the banding patterns, ImageJ's particle-counting tool was also utilised to quantify the number of agglutinates in each sample. This process involved removing any interference such as the crystallisation structures and background until only the agglutinates were left.

Test	Agglutinates Counted	Average Size
Live	54	31.444
Control	19	8.895

Table 3: Particle-counting results of Figures 10 and 11

Figure 10 (control) had a distinct lack of agglutinates at almost three times less than the live test and are harder to visualise compared to Figure 11, which has a substantially larger number of distinct agglutinates.

Table 3 shows the total number of agglutinates counted for the control and live test.

The overall trend between the specific A_G counts is that the test samples contained a larger number of agglutinates compared to the control.

However, when analysing the images obtained of the non-specific A_G test, there is a considerably higher level of agglutinates in both the control and test samples. Despite the higher level of microspheres incorporated, the trend shows a similar value of agglutinates between the control and test samples. Only the 1:100 and 1:1000 non-specific A_{Gs} were analysed using this method. It was predicted that as these are the optimum condition for binding to be observed; the remaining concentrations were excluded to save time.

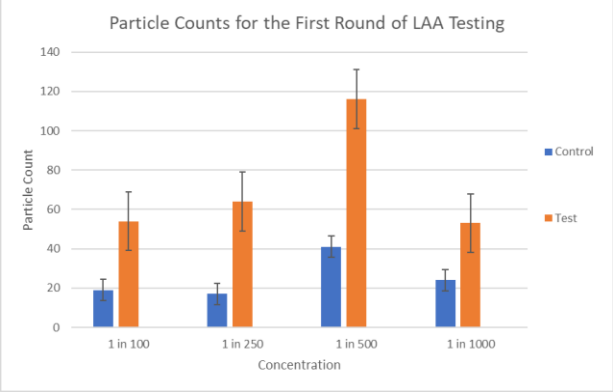

Figure 12: Particle-counting results of the first round of LAA testing

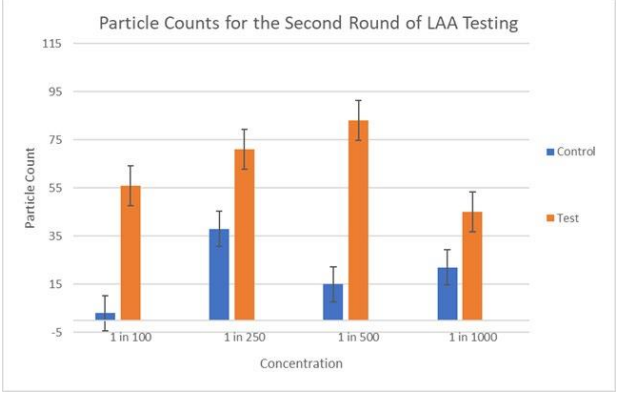

Figure 13: Particle-counting results of the second round of LAA testing

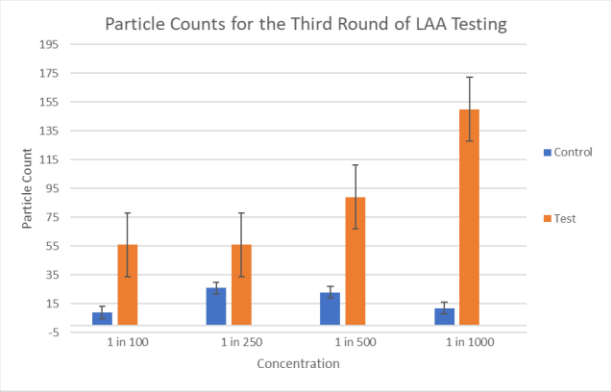

Figure 14: Particle-counting results of the third round of LAA testing

The error bars displayed in Figures 12 through to 14 display the range of values obtained from the particle-counting phase.

Real-time spectrophotometric analysis

To gain a better understanding of what is occurring in real time with respect to A_B-A_G binding, samples were analysed using a spectrophotometer at 405 nm over a half-hour time period. The specific and non-specific A_{G}s were compared to a control which was solely comprised of microspheres and buffer.

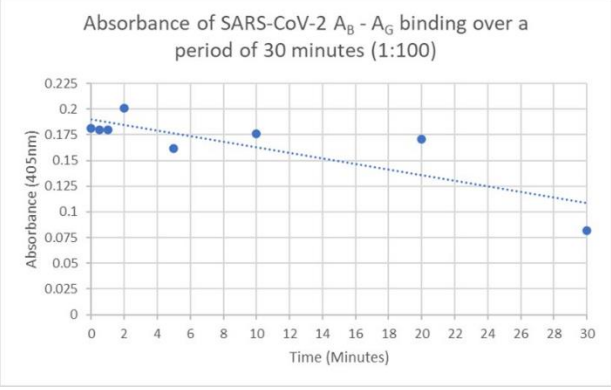

Figure 15: Absorbance values obtained for the specific A_G concentration 1:100 over a 30-minute time period

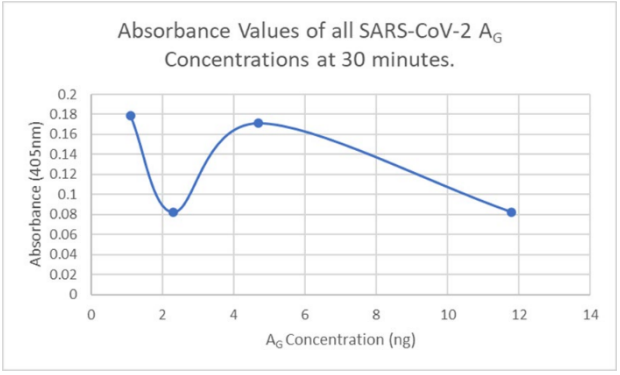

Figure 16: Absorbance values for all specific A_G concentrations at 30 minutes

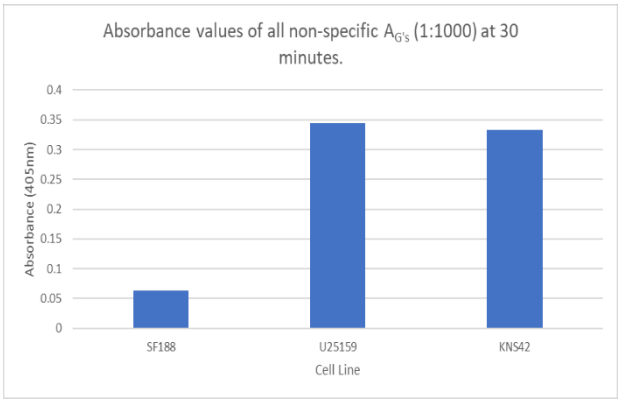

Figure 17: Absorbance values for all non-specific A_{G}s (concentration 1:1000) at 30 minutes

Figure 15 displays the absorbance values at 405 nm of the specific A_G concentration 1:100 measured at various time points over a total period of 30 minutes.

The overall trend displayed on this graph is that as time increases, absorbance decreases. The trend observed in Figure 16 which displays absorbance values at 405 nm for each specific A_G concentration at the 30-minute time point showcases that absorbance decreases as A_G increases. Due to the lack of reagents, each time point was measured once. These trends are in concordance with research conducted by Buffin et al. (2018).

After analysing all absorbance values, it was concluded that the optimum time point was at 30

minutes. Because of this, all concentrations for the non-specific A_{Gs} were only measured at this time point. Figure 17 details these absorbance values with cell line SF188 having the lowest absorbance and cell line U25159 having the highest (full data set not included).

Statistical analysis

Despite the noticeable differences between the specific and non-specific data sets, a general comparison is not sufficient to establish trends.

For this reason, statistical analysis of all the data was conducted using SPSS. The statistical analysis tests conducted included the Shapiro-Wilk test of normality and paired T-tests with a confidence interval of 95% for each respective test.

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
control	.281	6	.149	.873	6	.238
test	.267	6	.200*	.824	6	.096

Figure 18: Shapiro-Wilk test of normality results on the data acquired for the specific A_G

A Shapiro-Wilk test of normality was conducted on the data set (not included), as the population size was below 50. The results in Figure 18 show that the data is both parametric, P value above 0.05 (P=0.817) and nonparametric, P value below 0.05 (P=0.011). As there is only a minor deviation from normality, it would not affect the results of a parametric analysis (Ghasemi & Zahediasl, 2012) and thus, was deemed negligible, allowing further analysis using parametric methods.

	95% Confidence Interval of the Difference		t	df	Sig. (2tailed)
	Lower	Upper			
test - control	33.66727	73.66606	5.906	11	.000102

Figure 19: Paired T-test results on the data acquired for the specific A_G

Following on from the test of normality results, it was decided that a paired T-test was to be conducted on the data set. Figure 19 shows the T-test results as having a P value below 0.05 (P=0.000102). The results of this statistical analysis show that there is a statistical difference between the number of agglutinates counted on the control samples when compared to the test samples.

This process of statistical analysis was repeated for the data obtained for the non-specific A_G.

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Control	.150	12	.200*	.962	12	.817
Test	.223	12	.100	.808	12	.011

Figure 20: Shapiro-Wilk test of normality results on the data acquired for the non-specific A_{Gs}

Due to the population size being below 50, the Shapiro-Wilk test was conducted again, and both data sets had a P value above 0.05 (P=0.238 and P=0.096) (Figure 20), meaning that the data was parametric, and a paired T-test could be conducted.

	95% Confidence Interval of the Difference		t	df	Sig. (2-tailed)
	Lower	Upper			
Pair 1 control - test	-55.35284	166.68618	1.289	5	.254

Figure 21: Paired T-test results on the data acquired for the non-specific A_{Gs}

Figure 21 displays the results of the paired T-test, with a P value above 0.05 (P=0.254). This statistical analysis shows that there is no statistical difference between the number of agglutinates counted on the control samples in comparison to the test samples.

Discussion

Current global state

Development and deployment of mass population testing programmes has been critical throughout this pandemic and has improved dramatically, starting with the gold standard RT-PCR and progressing to rapid protocols utilising LAMP technology. Following on from this progression, mass testing programmes utilising LFDs have been undertaken across the country.

As mentioned previously, the implementation of LFDs to test large populations was incredibly beneficial, boasting a high sensitivity and specificity value, especially in patients with a high viral load and providing results within $\leqslant$ 30 minutes. In an article written by Peto (2020), the author described how mass testing and intensive contact tracing would have the potential to end the pandemic and ultimately, restore 'normal life'.

Latex Agglutination Assay

The current testing protocols in the UK, as mentioned previously, rely on two methods. The first of which is the gold standard RT-PCR which has an especially long turn-around time (>24 hours) and can ultimately take up to and often exceed 48 hours but boasts a sensitivity and specificity value of >95%. The second testing protocol is the use of LFDs, which have a variable sensitivity value (58% - >95%) but can generate results in up to 30 minutes (Mahase, 2020e). Despite vast improvements in testing protocols since the beginning of the pandemic, the current diagnostic methods still do not yield a 100% sensitive test, allowing false results to be generated which can either exacerbate viral transmission or require patients to undertake an unnecessary isolation period (dependent on the type of false result). Literature reviews conducted by Mahase (2020e) and Peto (2021), both identified that there is a distinct operator bias when performing diagnostic tests, potentially missing almost 50% of positive results.

This has led to the research of rapid diagnostic protocols that can be conducted without the need for highly trained individuals and laboratory equipment, thus removing the operator bias.

The LAA conducted in this research project could theoretically be considered a competitor to the already established ELISA methods. ELISA is available commercially and has also been implemented by the UK government to test for previous COVID-19 infections. RT-PCR and LFDs are the current methods of choice by the UK government ('Types and uses of coronavirus (COVID-19) tests', GOV.UK., 2021).

The premise of ELISA is similar to that of the LAA in that it focuses on the binding of A_{Bs} (IgM and IgG) and A_{Gs}. Thus, allowing both methods to be used as either a diagnostic tool or an indicator that a patient has been exposed to COVID-19. Despite the versatility of ELISA, it is a laborious protocol which requires trained operators and specialised equipment. Thus, making it fall into the same shortcomings as the RT-PCR.

The LAA conducted in this project, in its crude form, could potentially be conducted in a point of care setting without the need for specialised equipment or trained individuals, providing results

in the same ⩽ 30-minute time period as LFDs. Due to SARS-CoV-2 being a class 3 organism (Kaufer et al., 2020), the research team was unable to handle the live virus, making a sensitivity and specificity rating of the test impossible to generate. However, after testing the various A_G concentrations detailed in Table 1, it was calculated that, upon the visible development of agglutinates in the 1:500 concentration, the LAA could detect at least 2.3 ng of A_G.

The incorporation of ImageJ's particle-counting tool further supported these findings. A_G concentrations 1:100, 1:250 and 1:500 all showed a steady increase in agglutinates whereas the 1:1000 concentration had a reduced amount in comparison to the other samples.
The final value of 150 agglutinates counted does not fall in line with this trend. It can be theorised that the end of the microsphere solution was used, incorporating more microspheres into that one specific test.

ImageJ's particle-counting tool was implemented in the same way for the LAAs using non-specific A_{Gs}. The level of agglutinates in both control and test samples was considerably higher than the LAAs using the specific A_G. Again, this can be attributed to the use of the final volume of microspheres, incorporating larger amounts of the microspheres themselves. To determine whether this had an impact on the results, a statistical analysis of each data set was conducted. A smaller data set of non-specific A_{Gs}, consisting of only LAAs incorporating the 1:100 and 1:1000 concentrations were used. It was theorised that these were the optimum concentrations in which to view any non-specific binding. If this hypothesis was incorrect, the full data set would have been analysed.

Statistical analysis

Following the quantification of agglutinates was the implementation of statistical analysis methods, carried out using SPSS. The data was initially analysed using a Shapiro-Wilk test of normality, which deemed the data to be parametric, indicated by a P value of >0.05 in Figures 18 and 20. Figure 18, however, displayed a data set that had a P value of <0.05 (P=0.011). Upon further reading, this result was deemed unlikely to interfere with the results of further statistical analysis methods, thus the data set was considered parametric. Following this initial analysis, paired T-tests were conducted, all with a confidence interval of 95% on the data sets which can be viewed in Figures 19 and 21. For the paired T-test conducted on the specific A_G data set, a significant difference was observed between the data collected for the control and live tests. This was indicated by a P value < 0.05
(P = 0.000102). In comparison, the paired T-test conducted on the non-specific A_G data set, no statistical difference was observed between the control and test data. This was indicated by a P value > 0.05 (P = 0.254).
The acquisition of these results shows that the LAAs conducted had no cross-reactivity with the other non-specific A_{Gs} used.

Spectrophotometric analysis

As mentioned previously, spectrophotometric analysis of the LAA was implemented to gain a better understanding of the agglutination process in real time. An investigation conducted by Buffin et al. (2018) used the LAA in association with spectrophotometry. From this, an adapted protocol was created. The conclusion of the investigation undertaken by Buffin et al. (2018) discovered that sample absorbance is inversely proportional to the level of agglutination.

Figure 15 displays the absorbance values taken for specific A_G concentration 1:100 and that the optimum time point was 30 minutes. After this, the data collected for other concentrations at 30 minutes was compiled into a separate graph. Figure 16 shows these results. Despite an increase in absorbance at ~ 5 ng, the absorbance again decreased as the A_G concentration increased. The

results obtained do not specifically fit the trend observed in the investigation conducted by Buffin et al. (2018).
However, the overall trend that absorbance decreases with the increase of agglutination and ultimately, A_G concentration has been observed, further supporting the results obtained in this study.

Further research and applications

The nature of this project twinned with the current global climate has led to a very interesting investigation which has yielded a plethora of results. However, the restricted budget and time constraints associated with this project have meant that not every aspect has been investigated to its full potential.
Only the SARS-CoV-2 spike protein and associated A_{Bs} were used in this investigation. Ex vivo studies using a whole virus and unaltered immune components could theoretically produce different results. The inclusion of other viruses belonging to the *Coronaviridae* family could also help ascertain the true specificity value of this assay. This study has used IgM and IgG A_{Bs} to bind to the SARS-CoV-2 spike protein. This has proven to be efficient as there is a wide range of data supporting the use of these diagnostic tests on large populations. Other studies have detailed how SARS-CoV-2 gains entry to human cells via the binding of its spike protein to the ACE2 receptor (Chan et al., 2020). There is variable affinity between the spike protein and ACE2 which can be attributed to different polymorphisms within the gene (Calcagnile et al., 2021). This could potentially lead to the inclusion of alternative markers or an accumulation of different markers to enhance the sensitivity of COVID-19 testing across different populations.
If the LAA was able to be taken forward and used in a class 3 biohazard laboratory, a single blind study could be conducted in which researchers are provided with a mix of patient samples both positive and negative. Comparing the results of the LAA with the known results will produce sensitivity and specificity values which can be compared to the pre-existing LFDs and ELISA methods.
Finally, if there were no time or budgetary constraints, the LAA could have been incorporated into a mass testing study. The A_G concentrations used in this investigation ranged from 1:100 (11 ng) to 1:1000 (1.1 ng). If concentrations could be increased in small increments, such as 1:101 and then repeatedly tested, the statistical analysis methods used could be utilised to determine the exact A_G concentration in which the LAA is no longer effective.
The impact of COVID-19 on countries worldwide has been totally unprecedent in the 21st century. Many high-income areas with better quality of life and access to state-of-the-art healthcare have been overwhelmed during this pandemic. As more developed countries are beginning to ease their quarantine policies, those countries which do not have as strongly developed healthcare systems in place are at risk of being repeatedly decimated by the impact of this virus (Ali & Ali, 2020). Despite this, many third world countries, such as those on the African continent are welcoming assistance from governments across the world to help contain disease outbreaks. There is also an ethical obligation from these more developed countries to prevent the needless loss of life (Kavanagh et al., 2020). However, even if these resources are shared with other countries, it is always down to the public to engage and use testing facilities. This can be driven by
many civilians in low-income areas such as Nepal and Pakistan not having the option to not work, especially with the global economy suffering due to the inability of many people to go to work and as a result, many companies ground to a halt because of the global lockdown (Legese Feyisa, 2020).

There have been sporadic cases of COVID-19 in animals, with the first case appearing in a feline subject in late March 2020 (McNamara et al., 2020). Humans can and have successfully been subjected to strict social isolation policies, but animals pose a

unique struggle, especially in companion animals. This issue could possibly exacerbate viral transmission and cause further outbreaks. Agricultural practices keep large numbers of animals in close proximity, which poses a threat to not only employees but to their income.

There are already existing methods developed for detecting animal infection in an agricultural setting. Brüning et al. (1999) developed a chromatographic strip test for the detection of Rinderpest virus in cattle, which ultimately led to the eradication of the virus. This could theoretically be implemented in the human population. Rapid onsite testing twinned with adequate control measures plays a key role in isolating viral outbreaks and preventing further transmission, thereby preventing the need for further lockdown measures and allowing economies to progress as normal. The LAA is similar in the sense that it is cheap, easy to use and boasts high sensitivity and specificity. The existing experience with pen side testing highlights the attractiveness for the LAA to be utilised in more than healthcare settings.

The research into the LAA conducted in this project shows that the assay is rapid in comparison to existing methods, simple to use and is cost-effective. The LAA could be theoretically implemented into low-income countries as a means of infectious disease control and an analytical method (Kylilis et al., 2018) to detect the percentage of the population that have undergone seroconversion following exposure to SARS-CoV-2.

Conclusion

To conclude, the LAA was a prime candidate to be investigated for its potential to be involved with the rapid diagnosis or detection of COVID-19. The results obtained boast high specificity when used in conjunction with non-specific A_{Gs} and is sensitive enough to detect A_G concentrations into the nanogram range. The review of studies using similar testing protocols only further supports the results obtained.

The COVID-19 pandemic has been underway for over 14 months, and it is theorised that rapid and mass testing of populations twinned with the global vaccination programme is how 'normal life' will resume and with it, better economic stability and improved health, both physical and mental.

Subsequent, detailed research into this method is required to assess the true sensitivity and specificity values in relation to existing methods. However, I believe that based on the results obtained, the LAA could be modified and implemented for use in point of care settings and mass population testing programmes. The LAA can provide rapid and sensitive results while also removing the operator bias associated with other testing methods. The cost-effective nature of this assay makes it an integral part of paving a potential exit route from this pandemic in both high- and low-income countries.

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