Multiple indirect ELISAs for serological detection of SARS-CoV-2 antibodies

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Running title: Validated ELISAs for COVID-19 antibody response detection

Abstract

As the coronavirus disease 2019 (COVID-19), which is caused by the novel coronavirus SARS-CoV-2, continues to spread rapidly around the world, there is an urgent need for validated serological assays to evaluate viral specific antibody responses in COVID-19 patients or recovered individuals. In this study, we established and used indirect Enzyme Linked Immunosorbent Assay (ELISA)-based serological tests to study the antibody response in COVID-19 patients. In order to validate the assays, we determined the cut-off values, sensitivity and specificity of the developed assays using sera collected from COVID-19 patients in Saudi Arabia at different time points after disease onset, as well as sera that are seropositive to other human CoVs; namely MERS-CoV, hCoV-OC43, hCoV-NL63, hCoV-229E, and hCoV-HKU1. The SARS-CoV-2 S1 subunit of the spike glycoprotein and nucleocapsid (N) ELISAs that we developed here not only showed high specificity and sensitivity, but also did not show any cross-reactivity with other CoVs. We also showed that all RT-PCR confirmed COVID-19 patients included in our study developed both virus specific IgM and IgG as early as one week after the onset of disease. The availability of these validated assays will enable us to determine the nature and duration of the antibody response mounted in response to SARS-CoV-2 infection. It will also allow conducting large-scale epidemiological studies to determine evidence of previous exposure to the virus and assess the true extent of virus spread within communities.

Introduction

In December 2019, a cluster of atypical pneumonia was reported in Wuhan City, the capital of Hubei province in China. The etiological agent was quickly identified as a novel coronavirus, subsequently named as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), and identified as a cause of the Coronavirus Disease 2019 (COVID-19) [1]. Within weeks of its discovery, SARS-CoV-2 has rapidly spread to more than 200 countries around the world, causing large scale morbidity and mortality. Eventually, it was recognized as a pandemic by the World Health Organization (WHO) in early March of 2020. The rapid and continued spread of the virus has triggered the implementation of unprecedented public health measures by affected countries, including travel bans, borders closure, enforced curfew, lockdown of cities and shutdown of most businesses, public gatherings and other activities. Nevertheless, the spread of the virus was further complicated by the absence of vaccines and specific therapeutics to date.

Coronaviruses (CoVs) are a large group of viruses that can infect a wide range of hosts, including humans, animals and birds [2]. They are classified into four genera; alpha, beta, gamma and delta, in which only viruses from alphacoronaviruses (alpha-CoVs) and betacoronaviruses (beta-CoV) were recognized to infect humans so far [3]. SARS-CoV-2 belongs to the beta-CoV genus, which also contains two other highly pathogenic human CoVs; SARS-CoV and MERS-CoV as well as a number of animal CoVs [4]. Genome sequence analysis shows that SARS-CoV-2 shares nearly 79.5% identity with SARS-CoV and ~96% with a bat SARS-like CoV [1]. CoVs are enveloped viruses with a positive-sense, single-stranded, ~30 kb RNA genome, which contains at least 6 open reading frames (ORFs) [4]. The first two-thirds of the genome encodes for polyproteins: pp1a and pp1ab that are processed by viral and host proteases into 16 non-structural proteins (nsp1-16) [4,5].

The other third of the genome encodes the four main structural proteins (envelope (E), membrane (M), spike (S), and nucleocapsid (N) proteins) as well as other accessory proteins [4,5].

As SARS-CoV-2 continues to spread around the globe, it is important to understand the duration and nature of immunity mounted in response to infection, which is currently not fully understood and investigated. Furthermore, the actual extent of the current global COVID-19 pandemic is not well known; therefore, serological assays are critically needed to shed light on all these unanswered questions. Here, we report the development and validation of multiple indirect ELISA-based serological assays that can be adapted and used by laboratories to determine the immune status of individuals in surveillance and epidemiological studies, as we have previously described for MERS-CoV [6,7]. Using sera derived from either confirmed COVID-19 patients or known noninfected healthy controls, we validated our ELISAs and determined their cut off values, sensitivity and specificity. We also showed that our assays had no cross-reactivity using sera with known positivity to MERS-CoV and other common CoVs. Our study shows that SARS-CoV-2 IgM or IgG specific antibodies for either SARS-CoV-2 S1 or N antigens can be detected virtually in all real-time polymerase chain reaction (RT-PCR) confirmed COVID-19 patients included in our study as early as one week after disease-onset. Antibodies levels sharply increased by week two, with IgG persisting through week four compared to IgM, which peaked by week 2 or 3 before declining as previously shown [8].

Material and methods

Samples

A 100 serum samples from healthy controls collected before the COVID-19 pandemic with a positive control from a confirmed COVID-19 patient were used to determine the cut-off values for the developed indirect ELISAs. Another set of samples including 8 SARS-CoV-2 and MERS-CoV seronegative samples, two MERS-CoV seropositive samples and three SARS-CoV-2 seropositive samples were used to determine the cross-reactivity of the assays. A third cohort of pre-pandemic samples (n = 125) and RT-PCR confirmed COVID-19 patients (n = 52) including samples collected during the 1st week (n = 10), 2nd week (n = 23), 3rd week (n = 14) or 4th week (n = 5) of symptoms-onset were used to evaluate the developed ELISAs. All samples were obtained from multi ethnicity patients or donors residing in Saudi Arabia. All samples were anonymized and used based on ethical approvals obtained from the Unit of Biomedical Ethics in King Abdulaziz University Hospital (Reference No 245-20), the Institutional Review Board at the Ministry of Health, Saudi Arabia (IRB Numbers: H-02-K-076-0320-279 and H-02-K-076-0420-285), and the Global Center for Mass Gatherings Medicine (GCMGM) (No. 20/03A).

Expression and production of SARS-CoV-2 recombinant proteins.

Recombinant SARS-CoV-2 S1 submit of the S protein (amino acids 1–685), MERS-CoV S1 subunit (amino acids 1–725), and full-length S proteins from hCoV-OC43, hCoV-NL63, hCoV-229E and hCoV-HKU1 viruses tagged with histidine tag were purchased commercially (Sino Biological, China). Recombinant SARS-CoV-2 and MERS-CoV N proteins were expressed and purified from *Escherichia coli* BL21 (DE3) cells using a nickel-nitrilotriacetic acid (Ni-NTA)

column according to the manufacturer's protocol and as previously described [6]. Positive fractions of N proteins were pooled, aliquoted and stored at -80°C until used. SARS-CoV-2 proteins were confirmed by Western blot using anti-His tag antibodies as well as SARS-CoV-2 seropositive and seronegative human serum samples as previously described [6].

Indirect ELISA

Recombinant SARS-CoV-2 S1, MERS-CoV S1 and full-length S proteins from other human CoVs at a concentration of $1\text{ }\mu\text{g/mL}$ in phosphate-buffered saline (PBS) were used to coat 96-well high binding ELISA plates (Greiner Bio One, Monroe, NC) with $50\text{ }\mu\text{L}$ per well. Similarly, in-house produced SARS-CoV-2 and MERS-CoV N proteins were used to coat plates at a concentration of $4\text{ }\mu\text{g/mL}$. All plates were coated for overnight at 4°C , washed three times with PBS containing 0.05% tween-20 (PBS-T), and blocked with 5% skim milk in PBS-T buffer at 37°C for 1 h. After blocking, plates were washed three times and incubated with serum samples diluted at 1:100 in PBS-T with 5% milk for 1 h at 37°C . Plates were then washed three times again with PBS-T, incubated with HRP-conjugated goat anti-human IgG (H + L) or IgM antibodies (Jackson ImmunoResearch, West Grove, PA) for 1 h, washed again, and incubated with TMB (3,3',5,5' - tetramethylbenzidine) substrate (KPL, Gaithersburg, MD) at 37°C for 30 min. The reaction was terminated by adding $100\text{ }\mu\text{L}$ per well of the ELISA stop solution (0.16 M sulfuric acid). The absorbance was measured at 450 nm using the ELx808™ Absorbance Microplate Reader (BioTek, Winooski, VT).

Statistical analysis

The sensitivity of each ELISA was determined as (the number of samples that are true positives / the total number of samples that are true positives and false negatives $\times$ 100), and the specificity was determined as (the number of samples that are true negatives / the total number of samples that are true negatives and false positives) $\times$ 100. Receiver operating characteristic (ROC) analysis was calculated using GraphPad Prism V8 software (GraphPad Co.). Sensitivity, specificity and ROC analysis were calculated based on RT-PCR results. Each experiment was done twice with each serum sample run in duplicates.

Results

Expression and production of SARS-CoV-2 proteins

The S protein of SARS-CoV-2 is a major immunogen and is divided into two subunits; S1 which contains the receptor binding domain (RBD) and S2 that mediates the fusion with the host membrane [9]. The N protein is another target for most serological assays for CoVs because of its abundant expression [5,6,10]. We and others have shown that both proteins are suitable and comparable for the detection of virus-specific antibodies in MERS patients [6,10]. In this study, we have successfully expressed and purified a His-tagged SARS-CoV-2 N protein and subsequently used it for indirect ELISA development. Recombinant N protein was induced and expressed upon induction with IPTG, and purified on the Ni-NTA affinity chromatography column, while the recombinant S1-His tagged protein was purchased commercially. Western blot analysis showed that both S1 (~110 KDa, **Figure 1a**) and N (~46 KDa, **Figure 1b**) proteins were detected using anti-His antibodies and shown to bind specifically to sera derived from COVID-19 patients but not to COVID-19 seronegative sera from normal human donors collected prior to the pandemic. These data indicate that both S1 and N proteins are antigenically similar to native proteins and able to strongly and specifically detect SARS-CoV-2 antibodies in serum samples.

Development, optimization and determination of the cut-off values of the indirect ELISAs

We developed four different types of indirect ELISAs for the testing of IgM and IgG antibodies using purified SARS-CoV-2 S1 and N proteins as coating antigens. We initially optimized the coating conditions for the ELISA using known SARS-CoV-2 seronegative and seropositive sera and found that the optimal working concentrations of each antigen were 1 µg/mL and 4 µg/mL for

recombinant S1 and N proteins, respectively (data not shown). Furthermore, optimal serum dilution was determined using checkerboard titration where the highest OD ratio values of positive to negative samples (P/N) were obtained. After optimization, we tested sera from 100 normal human donors and one serum sample from an RT-PCR confirmed COVID-19 patient in the developed ELISAs at a dilution of 1:100 to determine the cut-off values (mean + 3 SD). As shown in **Figure 2**, the cut-off values were found to be 0.17 (mean = 0.09, SD = 0.3) for S1 IgG-ELISA, 0.30 (mean = 0.09, SD = 0.07) for S1 IgM-ELISA, 0.40 (mean = 0.17, SD = 0.08) for N IgG-ELISA, and 0.55 (mean = 0.24, SD = 0.10) for N IgM-ELISA. Almost all tested samples were below the determined cut-off values suggesting high specificity of the assays.

Determination of potential cross-reactivity with other CoVs

The ability of our developed assay to specifically detect and significantly differentiate SARS-CoV-2 antibodies in patients that might be co-infected with other CoVs was assessed. Here, ELISA plates were coated with different antigens representing MERS-CoV1 (S1 and N proteins) and the S protein of the other human CoVs, including hCoV-OC43, hCoV-NL63, hCoV-229E and hCoV-HKU1 at a concentration of 1 µg/mL. Using sera with known seropositivity to either MERS-CoV or to other known human CoVs, we found that our developed SARS-CoV-2 S1 and N-based ELISAs for IgG and IgM can only detect antibodies from COVID-19 seropositive sera but not from any of the other tested serum samples that are known to be IgG seropositive for MERS-CoV, hCoV-OC43, hCoV-NL63, hCoV-229E or hCoV-HKU1 (**Figure 3**). On the other hand, using S1 and N antigens of MERS-CoV only detected antibodies from MERS seropositive samples but not others. As expected, using S protein from other human CoVs showed presence of IgG antibodies only in all tested serum samples suggesting previous exposure to these common cold viruses.

Collectively, these data show that our assays can specifically detect and significantly differentiate SARS-CoV-2 specific IgG and IgM antibodies from those against other human CoVs in serum samples.

Validation of the developed ELISAs and testing of seroconversion

Testing of serum samples collected from another cohort of healthy donors (n = 125) or COVID-19 patients (n = 52) showed that our developed ELISAs could detect both IgG and IgM against both antigens as early as week one post symptoms-onset (**Figure 4**). Our data also show that IgG levels against both antigens increased over time, while IgM levels peaked by week 2 or 3 before starting to decline. Based on these data and on the assumption that all RT-PCR positive patients developed humoral response, we tried to determine the specificity and sensitivity of the developed ELISAs. As shown in **Table 1**, the specificity of the assays ranged between 91.2%-97.6%. The sensitivity, however, was dependent on the sampling time in relevance to disease onset. During the first week post symptoms onset, the sensitivity of IgM and IgG ELISAs ranged between 20%-30% and 40%-60%, respectively (**Table 1**). Nonetheless, the sensitivity of the assays increased to 88.5%, 84.6%, 100% and 88.5% for S1 IgG-ELISA, S1 IgM-ELISA, N IgG-ELISA and N IgM-ELISA, respectively by week two. Importantly, while these sensitivity values were maintained at 100% for N IgG-ELISA or increased to 100% for both S1 IgG-ELISA and S1 IgM-ELISA during week three and four post symptoms onset, N IgM-ELISA's sensitivity declined.

Next, we conducted ROC analysis to examine the diagnostic power of each developed assay as shown in **Figure 5**. Our analysis showed high accuracy of S1 IgG-ELISA, S1 IgM-ELISA and N IgG-ELISA with overall area under curve (AUC) of 0.938 ± 0.027 (95% CI: 0.886 - 0.990), 0.953

230 ± 0.021 (95% CI: 0.911 - 0.995) and 0.977 ± 0.015 (95% CI: 0.948 - 1.000), respectively, compared
231 to N IgM-ELISA which showed lower AUC of 0.886 ± 0.037 (95% CI: 0.812 - 0.959) (**Table 2**).
232 It was also clear that the accuracy of these assays was dependent on the sampling time as it was
233 low when testing samples collected during the first week after symptoms onset compared to those
234 collected during or after the second week of onset. Furthermore, high reproducibility was also
235 observed for all assays with very minimal variation (5%-10%) in obtained OD values including
236 inter-assay and intra-assay testing conducted on different days or by different individuals (data not
237 shown).

Discussion

In the current study, we report the development and validation of an ELISA-based serological assays for the detection of SARS-CoV-2 specific IgG and IgM antibodies in COVID-19 serum specimens. We showed that our ELISAs can specifically detect SARS-CoV-2 specific IgM and IgM antibodies in sera from COVID-19 patients, but not from sera derived from healthy human donors. Our data also show that our SARS-CoV-2 S1 and N ELISAs do not cross-react with sera that are seropositive to other human CoVs; including human CoVs that belong to the beta-CoV genus such as MERS-CoV, hCoV-OC43 and hCoV-HKU1, as well as those from alpha-CoVs such as hCoV-NL63 and the hCoV-229. Furthermore, using the developed ELISAs, we evaluated the production of SARS-CoV-2 specific IgG and IgM antibodies in a cohort of hospitalized COVID-19 patients (n = 52), including samples collected during the 1st week (n = 10), 2nd week (n = 23), 3rd week (n = 14) or 4th week (n = 5) of symptoms-onset. Our analysis showed that SARS-CoV-2 IgM or IgG specific antibodies for either SARS-CoV-2 S1 or N antigens can be virtually detected in all RT-PCR confirmed COVID-19 patients in this study. We showed that both virus specific IgG and IgM can be detected as early as one week after disease-onset but significantly increased by week two and three, with IgG persisting through week four (last time point in our study) compared to IgM which peaked by week 2 or 3 before declining. This increase in IgG over time and the decline in IgM antibodies by week 4 are consistent with some recent reports [11-14].

To be able to use the developed assays for large scale serosurveys, we determined the cut-off values, specificity and sensitivity of the different developed ELISAs. Our analysis showed that the cut-off values were found to be 0.17 (mean = 0.09, SD = 0.3) for S1 IgG-ELISA and 0.30 (mean = 0.09, SD = 0.07) for S1 IgM-ELISA. While for the N based ELISAs the cut-off values were

found to be 0.40 (mean = 0.17, SD = 0.08) and 0.55 (mean = 0.24, SD = 0.10) for IgG and IgM antibodies, respectively. Almost all seronegative samples were below the determined cut-off values, suggesting high specificity of the assays. Our ROC analysis also demonstrated powerful diagnostic performance of the developed assays.

The fact that all RT-PCR confirmed COVID-19 patients included in this study developed virus-specific antibody responses should be reassuring. The majority also showed detection of antibody response as early as week one. Although it has not been proven with SARS-CoV-2 in humans whether the mounted antibody response is protective and long-lasting, such responses are likely to be associated with protection from reinfection. Reinfection in humans has not been reported in SARS-CoV or MERS-CoV, and antibody responses against these two viruses were reported to last for up to three years [15,16]. Interestingly, a recent report examined the possibility of SARS-CoV-2 reinfection in non-human primates and showed that reinfection was unlikely after the induction of antibody responses [17]. Nevertheless, the possibility of reinfection in humans is a pressing question that warrants further investigations. The assays we presented here would be of a great utility not only to conduct such studies but also to examine the longevity of the mounted antibody responses against SARS-CoV-2 infection, which is critical for the vaccine development efforts. Serological assays like the ones we developed should be able to address these questions in the near future. The early detection of specific antibodies in COVID-19 patients also highlights the diagnostic importance of these assays especially in mild cases which usually present late to hospitals or go undetected.

Some seropositive COVID-19 sera were also found positive to other low pathogenic human CoVs, which may indicate that previous infections with other CoVs provide no immunity, at least in our cohort of COVID-19 patients. Interestingly, a recent study attempted to understand why SARS-CoV-2 infected children developed less severe symptoms compared to adults, suggested a possible cross-protection due to previous infections with circulating common cold CoVs, mostly through virus-specific T cell responses [18]. While we cannot confirm this suggestion here since the age range of the COVID-19 patients in our study was between 24 to 75 years and we only examined humoral immune responses, future studies clearly need to investigate this possibility further.

Another important finding in our study is that using both S1 and N in serology could lead to the detection of as many potential seropositive specimens as possible than using any of them alone. This is of great importance amid the current rapid and continuing spread of SARS-CoV-2 and the need for a quick and efficient method for contacts and cases tracing. It is now evident that asymptomatic infections occur and could play an important role in virus spread [19-21]. Thus, the ability to detect asymptomatic or mild cases is crucial for the epidemiological investigation [8,12].

Few serological assays have been reported thus far and most of them use the full S protein, S1 subunit or the RBD as capture antigens [8,11–13,22]. While these assays show high sensitivity and specificity rates, the use of the S1 or the RBD alone may result in missing cases or give less accurate estimation of the mounted antibody response since high levels of antibodies are generated to areas outside S1 or RBD [23]. Additionally, as it mediates binding and entry into cells, the S protein is under continuous selective pressure, which makes it more prone to acquire mutations that might affect the accuracy of S-based serological assays [24]. In our assays, we included N-

based ELISA in addition to S1 and found them complementary to each other with both showing high sensitivity and specificity. Another reason to include N-based ELISA in the serological testing algorithm is its relatively small size and lack of glycosylation sites, which makes it easy to clone and produce in prokaryotic expression systems, especially in resource-limited settings [3]. We believe that using both S1 and N ELISAs would capture as many potential SARS-CoV-2 positive cases as possible than using any of them alone.

The current standard method for the detection of SARS-CoV-2 relies on the detection of the viral RNA during the acute phase of the disease by RT-PCR. Although this highly sensitive method can effectively detect SARS-CoV-2 infection during acute infection phase, RT-PCR is time consuming and has limited detection rate of virus beyond week 3 after symptoms-onset [25,26]. Some of these issues could be addressed by the availability of well validated serological assays. Moreover, the development of serological assays is an essential step for the understanding of the epidemiology of SARS-CoV-2 infection. Of note, while our study reports a well validated ELISA assays, we have not assessed virus neutralization activities of detected antibodies. However, recent studies have shown positive correlation between high titers of IgG antibodies detected by ELISAs with neutralizing antibodies [22].

We believe that our assays are well validated, highly specific, sensitive and can be used for serosurveys to inform us about the extent of the current spread of COVID-19 pandemic in the population. Such studies are also important for a better understanding of the nature of the immune response to SARS-CoV-2, and the true estimate of the attack and infection fatality rates in different human populations.

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334

335 **Conflict of interest**

336 None

Figure 1. SARS-CoV-2 recombinant proteins. Recombinant SARS-CoV-2 (a) S1 or (b) N proteins were detected by Western blot using anti-His tag antibodies, known seropositive COVID-19 human samples or known seronegative COVID-19 human samples. All experiments showed protein bands with expected sizes (~110 KD and ~46 KD for S1 and N, respectively).

Figure 2. Cut-off values for the developed ELISAs. A 100 serum samples from healthy controls collected before the COVID-19 pandemic were used to determine the cut-off values for (a) S1 IgG-ELISA, (b) rS1 IgM-ELISA, (c) N IgG-ELISA and (d) N IgM ELISA. Values were calculated as mean + 3SD. The square is a serologically positive sample from COVID patient. The dotted lines represent the cut-off of each assay.

Figure 3. Specificity of the developed ELISAs. Developed ELISAs were tested for their specificity using sera known to be seronegative for SARS-CoV-2 and MERS-CoV (HC; n = 8), seropositive sera for MERS-CoV (MERS; n = 2) or seropositive sera for SARS-CoV-2 (COVID-19; n = 3). These serum samples were also tested for their reactivity in IgG and IgM ELISAs developed for MERS-CoV S1 and N proteins, as well as full S protein from hCoV-OC43, hCoV-NL63, hCoV-229E, and hCoV-HKU1 viruses. The dotted lines represent the cut-off of each assay.

Figure 4. Humoral immune response to COVID-19. Serum samples from healthy controls (n = 125) or COVID-19 patients collected during the first week (n = 10), second week (n = 23), third week (n = 14) or 4th week (n = 5) of symptoms onset were tested for IgG and IgM against SARS-CoV-2 S1 (a and b) and N (c and d) proteins using the developed ELISA. The dotted lines represent the cut-off of each assay.

360

361 **Fig. 5. Receiver operating characteristics (ROC) analysis.** ROC analysis was applied to positive
362 vs. negative SARS-CoV-2 samples as identified by RT-PCR assay for **(a)** S1 IgG-ELISA, **(b)** S1
363 IgM-ELISA, **(c)** N IgG-ELISA and **(d)** N IgM ELISA. Serum samples from healthy controls (n =
364 125) or COVID-19 patients collected during the first week (n = 10), second week (n = 23), third
365 week (n = 14) or 4th week (n = 5) of symptoms onset as well as all COVID-19 samples (n = 52).

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437 **Table 1. Specificity and sensitivity of the developed ELISAs based on sample time collection.**

ELISA	Specificity (%)	Sensitivity (%)			
		Week 1	Week 2	Week 3	Week 4
S1 IgG	97.6	40	88.5	100	100
S1 IgM	97.6	20	84.6	100	100
N IgG	91.2	60	100	100	100
N IgM	94.4	30	88.5	78.6	60

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Table 2. Area under the ROC curve (AUC) for the different developed ELISAs based on sample time collection.

ELISA	Samples	AUC ± SD	95% CI	P value
S1 IgG	All samples	0.940 ± 0.024	0.892 - 0.986	<0.0001
	Week 1 samples	0.746 ± 0.091	0.567 - 0.925	0.0099
	Week 2 samples	0.973 ± 0.020	0.935 - 1.000	<0.0001
	Week 3 samples	1.000 ± 0.000	1.000 - 1.000	<0.0001
	Week 4 samples	1.000 ± 0.000	1.000 - 1.000	0.0002
S1 IgM	All samples	0.963 ± 0.014	0.935 - 0.990	<0.0001
	Week 1 samples	0.829 ± 0.052	0.727 - 0.931	0.0006
	Week 2 samples	0.990 ± 0.007	0.977 - 1.000	<0.0001
	Week 3 samples	1.000 ± 0.000	1.000 - 1.000	<0.0001
	Week 4 samples	1.000 ± 0.000	1.000 - 1.000	0.0002
N IgG	All samples	0.971 ± 0.015	0.942 - 1.000	<0.0001
	Week 1 samples	0.863 ± 0.065	0.736 - 0.990	0.0001
	Week 2 samples	0.994 ± 0.005	0.985 - 1.000	<0.0001
	Week 3 samples	1.000 ± 0.000	1.000 - 1.000	<0.0001
	Week 4 samples	1.000 ± 0.000	1.000 - 1.000	0.0002
N IgM	All samples	0.871 ± 0.035	0.803 - 0.940	<0.0001
	Week 1 samples	0.528 ± 0.111	0.311 - 0.746	0.7655
	Week 2 samples	0.982 ± 0.009	0.965 - 1.000	<0.0001
	Week 3 samples	0.929 ± 0.038	0.854 - 1.000	<0.0001
	Week 4 samples	0.884 ± 0.067	0.753 - 1.000	0.0037