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Highlights

- Sciensano and its department Quality of Laboratories (QL) routinely organizes external quality assessment (EQA) as a part of the mandatory EQA program for SARS-CoV-2 serology in Belgium.
- Sample IS/17575 generated highly discordant results
- The importance of the choice of antigenic composition
- Antibody response against the N 184 protein appears to wane post-infection
- Difference in sensitivity of RBD 192 versus S1 versus S protein based immunoassays.

Journal Pre-proof

Importance of anti-SARS-CoV-2 assay antigenic composition as revealed by the results of the Belgian external quality assessment (EQA) scheme

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Keywords

SARS-CoV-2; serology; external quality assessment scheme; Nucleocapsid protein; Spike protein; receptor-binding domain

Abstract

Background: We report on sample IS/17575 since it generated highly divergent results in the Belgian SARS-CoV-2 serology EQA scheme.

Materials and methods: Sample IS/17575 was serum originating from a 30 years old male patient. 124 diagnostic laboratories analysed this sample.

Results: A total of 168 results was returned (including 5 doubles). Overall, 38 were positive. All tests against S1 were positive except the Euroimmun IgG ELISA and the Ortho clinical Diagnostics VITROS IgG CLIA. All tests against S1/S2 (Liaison, Diasorin) resulted in a signal above cutoff. Assays against RBD, mostly generate a negative result. An exception are the Wantai SARS-CoV-2 ELISA's. All tests targeting N protein were negative.

Conclusion: The survey shows, when > 6 months post-infection, assays targeting at least S1, and preferably S1 combined with S2, are the most sensitive. This finding accentuates the necessity of EQA schedules and antigenic composition of serologic SARS-CoV-2 assays.

Background

All Coronaviruses are enveloped, positive-stranded RNA viruses. Being an enveloped virus means that membrane fusion is essential for entrance in host cells and virulence. The fusion protein used is Spike (S) protein, which is present on the virion's surface. This is also the protein that gives rise to the neutralizing antibody response and is hence targeted by vaccines [1]. It initially occurs in the form of a trimer, that will be cleaved into receptor-binding unit S1 and fusion unit S2. S1 consists of 4 domains, the N-terminal domain (NTD), the receptor-binding domain (RBD), and two C-terminal domains (CTDs) [2].

Full commitment to diagnostic methods is especially important considering there are, at present, no curative medicines available. Serologic assays are the most important auxiliary tools to complement Nucleic Acid Amplification Tests (NAATs) [3]. The creation of these tests at an unprecedented speed consequently creates the need for a thorough assessment of their clinical performance.

An extra hurdle to overcome here is the fact that the commercially available serologic assays are anything but uniform, differing in the method of the immunoassay, the antibody class detected, the targeted viral components and the required specimen types [4]. At present there are tests on the market detecting total antibody (TAB) as well as IgA, IgM and IgG separately. Targeted antigens include Nucleocapsid (N) or S protein alone next to combined N and S proteins. Viral S protein targeted immunoassays can make use of the monomeric S protein (spike subunit 1 and/or 2) or the S protein in its native trimer form (spike receptor binding domain). Assay formats used comprise enzyme-linked immunosorbent assays

(ELISA), chemiluminescent immunoassays (CLIA), electrochemiluminescence immunoassay (ECLIA) and lateral flow immunoassays (LFIA) [5].

Almost all patients will develop detectable antibodies against SARS-CoV-2. It is generally assumed they appear 3 to 14 days post-onset [6]. The recommendation to test from day 14 after the alleged start of infection is the consequence of studies to reach the highest sensitivity [7].

Sciensano (formerly, the scientific public health institute for Belgium) and its department Quality of Laboratories (QL) routinely organizes external quality assessment (EQA) for a broad range of laboratory analyses under accreditation (ISO17043:2010). In order to ensure a scenically correct organization and evaluation of the results and to obtain useful and, if possible, commutable samples, QL is assisted by a panel of experts. The members of these panels are chosen in function of their expertise in a given domain and work in different types of laboratories (university, smaller hospitals, private laboratories) to ensure a link with the actual situation amongst Belgian patients and population in general.

EQA is an important tool for the assessment of a method's performance among the different participants. It aims to determine the possible differences in characteristics of the multiple available assays as a means to help harmonize the results generated by different methods and platforms [8]. Participating is mandatory for the licensed Belgian laboratories and contributes to ensuring and improving the quality of serological testing and providing the best patient care possible. The final goal is to ensure a reliable result, independent of the analyzing laboratory. EQA is the best way to compare the proficiency of the different assays for the same analysis. EQA also allows to put in evidence possible differences between different assays since all samples are identical.

The results were evaluated by comparison with a target value. This target value is the consensus of the panel of experts. Since it is a well-known fact that in infectious serology quantitative results between different methods and assays may differ even if the qualitative result (i.e. positive, negative or ambiguous) is the same, the target values were qualitative. Laboratories could however compare their quantitative results within their peer group (consisting of laboratories using the same method).

Each laboratory is indeed invited to compare its results with the expected result (target value) and with the results of its peer group. In case of a discordant result, a laboratory has the opportunity to demand a “repeat sample” to perform a second analysis in order to search for the reason for the discordant result. Each error in an EQA result should be considered a nonconformity in the laboratory's quality system.

We report here on the Sciensano SARS-CoV-2 serology. A particular sample (IS/17575), which draw attention during analysis of the results, will be commented. This sample was one of three that were sent out in survey 2020/2.

Methods

Samples and participants

The request to participate in the analysis was sent to all laboratories involved in the analysis of SARS-CoV-2 antibodies in Belgium and Luxembourg. This survey is part of the mandatory EQA program for SARS-CoV-2 serology in Belgium. In Luxembourg participating in the survey is voluntary. Three different serum samples, of whom two tested positive for SARS-CoV-2 through NAAT, were selected and delivered by CHU Tivoli (Centre Hospitalier Universitaire de Tivoli) and divided by Sciensano. The underlying clinical information was withheld so all laboratories performed the analysis without prior knowledge. The laboratories were asked

to analyze the samples on the platform(s) they routinely use for analysis of SARS-CoV-2 antibodies. 124 Belgian and Luxembourg laboratories participated. An overview of the distribution of the tests used in function of the technique for determining anti-Covid antibodies is provided in table 1.

Sample IS/17575 generated highly discordant results whilst the results for sample IS/17576 and IS/17577 were fully consistent across all participating labs. Background information (a summary is provided in table 2) was collected in order to get a better understanding of the discordant results. Sample IS/17575 was serum originating from a 30 years old male patient, developing minor Covid-19 symptoms on 12/04/2020. There was fatigue, headaches, muscle pains, a cough, a sore throat, nasal course, dyspnea, some abdominal complaints, ageusia and ansomia. There were no abnormalities on CT-scan neither was there pneumonia, fever, conjunctivitis, vomiting or skin lesions. The patient tested positive (low viral load/ high Cycle threshold (Ct) value) through SARS-CoV-2 NAAT testing on 16/04/2020. At 19/10/2020, serum was taken for serological analysis of SARS-CoV-2 IgG which was found to be positive with the Diasorin S1/S2 IgG kit, and negative with the Euroimmun IgG ELISA.

Assays

To gain a good insight in the results, it is important to understand the different immunoassay-methods, know which antibody class is detected and what is the targeted viral component. Therefore, we provided an overview of the different serological platforms used in the EQA in table 3 [9-27].

After performing the EQA and analyzing all results, it was decided to perform additional analyses in view of the inconsistency of the data. The first additional analysis was the SARS-CoV-2 ELISA by Vircell. Furthermore, a Luminex immunoassay targeting RBD, N Protein,

monomeric S1 protein and native S protein trimer was performed for analysis of IgG.

Luminex is a type of immunoassay that is able to precisely measure multiple analytes in one sample. This platform is currently used in research setting only [28]. Last, the sample was run on the Abbott SARS-CoV-2 IgG II Quant assay.

Evaluation of results

All laboratories sent the results of their analysis to Sciensano. Data were classified per antibody-type detected as well as stratified per platform and specific kit used, where rapid tests were listed separately. The viral target used in the kits was not taken into account. Sciensano had access to the quantitative data but interpreted these qualitatively according to the lab's used cutoff. Equivocal or borderline results were considered as such. The complete panel of results was sent to the participants in an anonymous manner.

Results

The 124 laboratories participating in the survey returned a total of 168 results for sample IS/17575. 96 lab's performed one analysis (77.42%), the others two or even multiple. Sciensano received 61 sets of TAB results (36.31%), 84 IgG determinations (50%), 20 IgM results (11.90%) and 3 IgA analysis (1.79%). Techniques used to screen the sera included ELISA (12.27%), CLIA (85.27%) and LFIA (2.45%). An overview of the results is provided in table 5.

For the TAB results, 10 out of 61 were positive (16.39%). 3 results were borderline or equivocal (4.92%) and 48 were negative (78.69%). Considering IgG CLIA and ELISA, 26 out of 80 results were positive (32.5%), 1 result was borderline/equivocal (1.25%) and 53 were negative (66.25%). All IgG results generated by rapid test analysis were negative. When looking at the results for IgM, only one out of 14 lab's (7.14%) or one out of 16 tests had a

positive result (6.25%). All rapid IgM tests were negative. For IgA, 2 tests were borderline and one was positive.

When stratifying the results according to viral target, 77 out of 78 analysis targeting N-antigen were negative, one was borderline/equivocal. 6 out of 8 tests targeting N- and S-antigen together were negative, one was borderline/equivocal and one was positive. 23 out of 30 analysis against RBD were negative, 2 were borderline/equivocal, 4 were positive.

Something that immediately catches the eye, and which made IS/17575 such an interesting sample, is the fact that 32 out of 47 results generated by testing against S1 were positive (68.09%). 2 results were borderline/equivocal (4.26%) and 13 were negative (27.66%).

In the light of these particular findings, the decision was made to perform some additional tests. The sample was reran with the Abbott Architect IgG and IgG II Quant assays. The IgG assay yielded a negative result, whilst the result with the IgG II Quant assay was positive. The bioMérieux Vidas IgG and IgM assay, which had already been performed during the survey, yielded again a negative result for both antibodies. The sample was also additionally analyzed with the SARS-CoV-2 ELISA by Vircell, which provided a positive result. The Luminex analysis yielded a positive IgG result for the N protein and the native S protein trimer.

Targeting RBD and monomeric S1 protein resulted in a signal below cutoff. An overview of the additional tests characteristics and results is provided in table 4 and 6.

Discussion

Taking a closer look at the results, it stands out that when stratifying the results according to viral target, almost all tests against the S1 moiety and all tests against S1/S2 resulted in a signal above cutoff. This emphasizes the importance of the choice of antigenic composition when performing a serological test in order to investigate the patient's immune status [29].

Choosing the most adequate assay is particularly important for pauci- or asymptomatic people, who are known to have a lower antibody response [30]. Remarkable is the fact that assays against RBD, which is after all part of the S1 moiety, mostly generate a negative result. An exception are the Wantai SARS-CoV-2 ELISA's. One could expect them to give a negative result when extrapolating from the other assays. On the other hand, the Euroimmun IgG ELISA gives negative results, while we would expect a signal above cutoff since this is an S1-based assay. A possible explanation may be found in the tested lower sensitivity of the Euroimmun ELISA, while the Wantai ELISA provides excellent sensitivity and specificity and was tested superior to other ELISA's [31,32,33]. Research on 30 NAAT positive patients showed that the Wantai assay detected antibodies in 28 cases, whilst the Euroimmun IgG assay picked up antibodies only 20 times. Remarkably, the Euroimmun IgA assay performed a lot better, with detecting 28 out of 30 positives [31]. This is also reflected in the results of the EQA, where the Euroimmun IgA assay did detect a positive or borderline signal.

The Ortho clinical Diagnostics VITROS TAB CLIA (just like the Diasorin Liaison S1/S2 IgG) performs excellent, with all laboratories using this test finding sample IS/17575 positive. The similar VITROS IgG assay then again appears to be less accurate. This difference could possibly be explained by the fact that TAB assays are in general more sensitive comparing to detecting only a single class of antibodies [33].

Another characteristic that can be deduced from this study is the fact that assays targeting the trimeric S protein combined with N protein, usually produce negative results. These assays seem to be noteworthy less sensible. The obvious explanation is that the vast majority of these assays are LFIA's [34]. This assumption is reinforced by the fact that the

Shenzen iFlash IgG assay, which is a CLIA, generates a positive result. None of the LFIA rapid tests was positive.

A possible explanation for this observed difference in N- and S protein based serological SARS-CoV-2 assays can be found in the recent insight that antibody response against the N protein appears to wane post-infection [29]. As a result, N protein assays could underestimate the true seroprevalence when tested on patients who were infected some time ago (in the order of magnitude of months rather than weeks). S protein directed antibody response tends to persist over time [29]. After all, in this particular case, the patient's antibody response was tested a little over 6 months after infection. In the acute phase of infection both antibody responses are equally sensitive, although IgG seroconversion for S protein would appear 2 days after this for N protein [15].

Then we still have to look for an explanation for the observed difference in sensitivity of RBD versus S1 versus S protein based immunoassays. Since these are all S protein based one could expect for all the assays to generate a positive result. However, there appears to be a difference. It has already been stated that the use of the S protein in its trimer form is more sensitive when compared to monomeric S proteins. This would be due to antibodies binding to the S2 subunit and the conservation of conformational epitopes within a higher order structure [35]. This theory seems to be confirmed by the Diasorin Liaison S1/S2 IgG tests. This higher sensitivity however comes at the expense of the specificity. S1 would be more specific compared to S since the spike S2 subunit is conserved among Coronaviruses. It is known that the specificity of the Diasorin Liaison S1/S2 IgG is lower when compared to its competitors [33]. If you reason that RBD is only one of the four subdomains of S1 [36] it seems plausible that this will be even less sensitive [37]. This seems confirmed by this EQA,

with almost all assays targeting RBD being negative, including the Diasorin Liaison IgM test. An exception to this “rule” is the Wantai ELISA, which seems exceptionally more sensitive in comparison to its competitors [31,32,33]. Finally, it is important to note that the data concern a single sample and however this EQA reveals some very interesting findings, more data and expansion of sample size are needed to be able to draw definitive conclusions.

Conclusion

In conclusion, the survey shows, when > 6 months post-infection, assays targeting S1 are the most sensitive. This can be explained by the recent insight that antibody response against the N protein appears to wane post-infection [29]. The highly divergent results highlight the importance of taking into account the antigenic composition in the light of intended use of the particular assay. Our findings also accentuate the necessity of EQA schedules for SARS-CoV-2 serology and use of sample drawn at different timepoints after Covid-19 episode.

Conflict of interest statement

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author statement

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Appendices

A. Figures

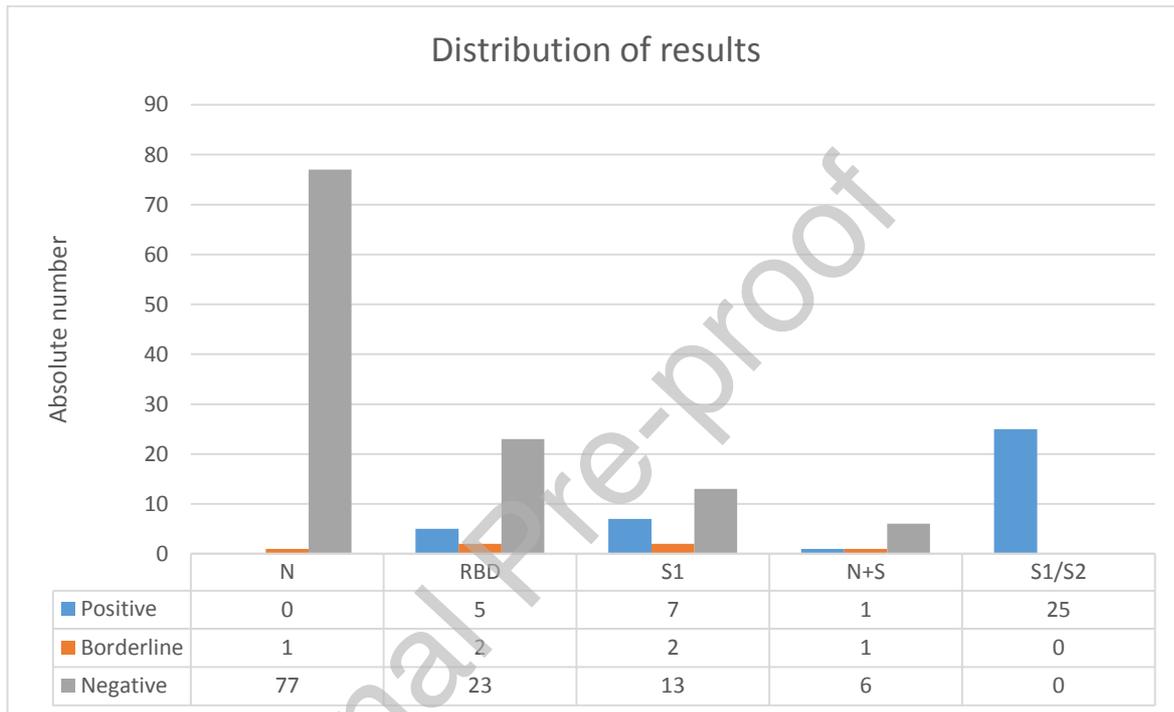


Figure 1. Graphic representation of the number of positive, borderline and negative results, recovered from the EQA (sample IS/17575), stratified per antigen.

B. Tables

Table 1. Overview of the number of participating lab's and their type of antibodies tested.

Tested antibodies	N laboratories reporting results
TAB	44
TAB and IgG	7
TAB and IgM	2
TAB and IgG and IgM	6
TAB and IgG and IgA	1
IgG	54
IgG and IgM	8
IgG and IgA	1
IgG and IgM and IgA	1

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Table 2. Classification of the results into positive, borderline and negative and further into antibody detected per antigen.

	N				RBD			S1			N+S			S1/S2
	IgM	IgG	IgM/IgA	TAB	IgM	IgG	TAB	IgA	IgG	TAB	IgM	IgG	IgM/IgG	IgG
+					1		4	1		6				25
+/-				1			2	2				1		
-	1	32	1	43	11	7	5		13		2		4	

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Table 3. Overview of the results obtained with the different assays used in the questionnaire, stratified per manufacturer [9-27].

Manufacturer	Kit	Assay Type	Target	N tests	Results (positive/borderline/negative)		
Abbott	SARS-CoV-2 IgG Assay (Architect)	CMIA	N	23			23-
	SARS-CoV-2 IgG Assay (Alinity)		N	9			9-
	SARS-CoV-2 IgM Assay (Architect)		RBD	2			2-
Beckman (Coulter)	Access SARS-CoV-2 IgG	CLIA	RBD	2			2-
Beijing Wantai Biological Pharmacy	Wantai SARS-CoV-2 Ab ELISA	ELISA	RBD	5	3+	2+/-	
	Wantai SARS-CoV-2 IgM ELISA		RBD	1	1+		
bioMérieux	VIDAS SARS-CoV-2 IgG	ELFA	RBD	4			4-
	VIDAS SARS-CoV-2 IgM		RBD	6			6-
Diasorin	LIAISON SARS-CoV-2 S1/S2 IgG tests	CLIA	S1/S2	25	25+		
	LIAISON SARS-CoV-2 IgM		RBD	3			3-
Epitope Diagnostics (EDI)	Novel Coronavirus COVID-19 IgM ELISA Kit	ELISA	N	1			1-
Euroimmun	Anti-SARS-CoV-2 ELISA IgG	ELISA	S1	8			8-
	Anti-SARS-CoV-2 NCP EI 2606-9601-2 G		S1	2			2-
	Anti-SARS-CoV-2 ELISA IgA		S1	3	1+	2+/-	
Healgen Diagnostics	COVID-19 IgG/IgM Rapid Test Cassette	LFIA	N+S	1			1-
Multi-G	Covid-19 IgM/IgA Ab test cassette	LFIA	N	1			1-
Ortho clinical Diagnostics	VITROS Immunodiagnostic Products Anti-SARS-CoV-2 Total	CLIA	S1	6	6+		
	VITROS Immunodiagnostic Products Anti-SARS-CoV-2 IgG		S1	3			3-
Roche	Elecsys Anti-SARS-CoV-2 Test (Cobas)	ECLIA	N	44		1+/-	43-
Shenzhen Yhlo Biotech	iFlash- SARS-CoV-2 IgG	CLIA	N+S	1	1+		
	iFlash- SARS-CoV-2 IgM		N+S	1			1-
Siemens	SARS-CoV-2 Total Antibody Test	CLIA	RBD	6	1+		5-
	SARS-CoV-2 IgG Assay		RBD	1			1-

Snibe	2019-nCoV IgG (CLIA)	CLIA	N+S	1		1+/-	
	MAGLUMI SARS-CoV-2 IgM/IgG Test		N+S	1			1-
	2019-nCoV IgM (CLIA)		N+S	1			1-
Xiamen Boson Biotech	Rapid 2019-nCoV IgG/IgM Combo Test Card	LFIA	N+S	2			2-

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Table 4. Overview of the results obtained with the additional assays performed after the questionnaire, stratified per manufacturer

Manufacturer	Kit	Assay type	Target	N tests	Results (positive/borderline/negative)		
Abbott	SARS-CoV-2 IgG II Quant	CMIA	N	1	1+		
	SARS-CoV-2 IgG Assay (Architect)		RBD	1			1-
bioMérieux	VIDAS SARS-CoV-2 IgG	ELFA	RBD	1			1-
	VIDAS SARS-CoV-2 IgM		RBD	1			1-
Luminex multiplex assay	In house assay	ELISA	RBD+ N+ S+ S1/S2	4	2+		2-
Vircell	SARS-CoV-2 ELISA	ELISA	N+RBD	1	1+		