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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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ABSTRACT

We report on sample IS/17575 since it generated highly divergent results in the Belgian SARS-CoV-2 serology external quality assessment scheme. Sample IS/17575 was serum originating from a 30 years old male patient. 124 diagnostic laboratories analysed this sample. 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. 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 external quality assessment schedules and importance of antigenic composition of serologic SARS-CoV-2 assays.

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1. 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 (Min and Sun, 2021). 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, the receptor-binding domain (RBD), and 2 C-terminal domains (Cai et al., 2020).

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) (Plebani et al., 2020). 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.

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(Theel et al., 2020). 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) (Lassaunière et al., 2020).

Almost all patients will develop detectable antibodies against SARS-CoV-2. It is generally assumed they appear 3 to 14 days post-onset (Lin et al., 2020). The recommendation to test from day 14 after the alleged start of infection is the consequence of studies to reach the highest sensitivity (Interim Guidelines 2021).

Sciensano (formerly, the scientific public health institute for Belgium) and its department Quality of Laboratories routinely organizes external quality assessment (EQA) for a broad range of laboratory analyses under accreditation (ISO17043:2010). In order to ensure a scientifically correct organization and evaluation of the results and to obtain useful and, if possible, commutable samples, Quality of Laboratories 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 (Haselmann et al., 2020). 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 1 of 3 that were sent out in survey 2020/2 Fig. 1.

2. Methods

2.1. 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 2 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 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 April 12, 2020. There was fatigue, headache, muscle pains, a cough, a sore throat, nasal course, dyspnea, some abdominal complaints, ageusia and anosmia. 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 April 16, 2020. At October 19, 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.

### 2.2. 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 provide an overview of the different serological platforms used in the EQA in Table 2 (Bryan et al., 2020; Egger et al., 2020; Garritsen et al., 2021; GeurtsvanKessel et al., 2020; Gutierrez-Cobos et al., 2021; Jaaskelainen et al., 2020; Lippi et al., 2020; Mahajan et al., 2020; Maine et al., 2020; Manthei et al., 2020; National SARS-CoV-2 Serology Assay Evaluation Group 2020; Padoan et al., 2020; Pieri et al., 2020; Plebani et al., 2020; Renard et al., 2021; Ruscio et al., 2021; San Tang et al., 2020; Steensels et al., 2020; Van Elslande et al., 2020).

| 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                               |

### 2.3. 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.

## 3. Results

The 124 laboratories participating in the survey returned a total of 168 results for sample IS/17575. 96 lab’s performed 1 analysis (77.42%), the others 2 or even multiple. Sciensano received 61 sets of TAB results (36.31%), 54 IgG determinations (30%), 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 Tables 2 and 3.

### 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                               |

### Table 2

Overview of the results obtained with the different assays used in the questionnaire, stratified per manufacturer (Bryan et al., 2020; Egger et al., 2020; Garritsen et al., 2021; GeurtsvanKessel et al., 2020; Gutierrez-Cobos et al., 2021; Jaaskelainen et al., 2020; Lippi et al., 2020; Mahajan et al., 2020; Maine et al., 2020; Manthei et al., 2020; National SARS-CoV-2 Serology Assay Evaluation Group 2020; Padoan et al., 2020; Pieri et al., 2020; Plebani et al., 2020; Renard et al., 2021; Ruscio et al., 2021; San Tang et al., 2020; Steensels et al., 2020; Van Elslande et al., 2020).

| 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)     | CLIA       | 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*                                  |
|                       | 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              | LIASON SARS-CoV-2 S1/S2 IgG tests | CLIA       | S1/S2  | 25      | 3*                                  |
|                       | LIASON SARS-CoV-2 IgM             | RBD        | 3      | 3-       |
| Epitope Diagnostics (EDI) | Novel Coronavirus COVID-19 IgM ELISA Kit | ELISA     | N      | 1       | 1-                                   |
|                       | Anti-SARS-CoV-2 ELISA IgG         | ELISA      | S1     | 8       | 8-                                   |
|                       | Anti-SARS-CoV-2 IgM               | S1         | 2      | 2-       |
|                       | Anti-SARS-CoV-2 ELISA IgA         | S1         | 3      | 3*       |
|                       | Anti-SARS-CoV-2 NCP EI 2606-9601-2G| S1         | 6      | 6*       |
|                       | Anti-SARS-CoV-2 ELISA IgA         | S1         | 3      | 3*       |
|                       | COVID-19 IgG/IgM Rapid Test Cassette | LFA      | N+S    | 1       | 1-                                   |
|                       | Covid-19 IgM/IgA Ab test cassette  | LFA        | N      | 1       | 1-                                   |
| European Pfennig      | VITROS Immunodiagnostic Products Anti-SARS-CoV-2 Total IgG | CLIA     | S1     | 3       | 3-                                   |
|                       | VITROS Immunodiagnostic Products Anti-SARS-CoV-2 IgG | CLIA     | S1     | 3       | 3-                                   |
| Roche                 | Elecsys Anti-SARS-CoV-2 Test (Cobas) | ECLIA     | N      | 44      | 1*/-                                 |
| Shenzhen Yhlo Biotech | fFlash SARS-CoV-2 IgG             | CLIA       | N+S    | 1       | 1*                                   |
| Siemens               | SARS-CoV-2 Total Antibody Test    | CLIA       | RBD    | 6       | 6*                                   |
|                       | SARS-CoV-2 IgG Assay              | RBD        | 1      | 1-       |
| Snibe                 | 2019-nCoV IgG (CLIA)              | CLIA       | N+S    | 1       | 1*                                   |
|                       | MAGUMS IgM SARS-CoV-2 IgM/IgG Test| CLIA       | N+S    | 1       | 1-                                   |
|                       | 2019-nCoV IgM (CLIA)              | CLIA       | N+S    | 1       | 1-                                   |
| Xiamen Boson Biotech  | Rapid 2019-nCoV IgG/IgM Combo Test Card | LFA  | N+S    | 2       | 2-                                   |
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 1 out of 14 lab’s (7.14%) or 1 out of 16 tests had a positive result (6.25%). All rapid IgM tests were negative. For IgA, 2 tests were borderline and 1 was positive.

When stratifying the results according to viral target, 77 out of 78 analysis targeting N-antigen were negative, 1 was borderline/equivocal. 6 out of 8 tests targeting N- and S-antigen together were negative, 1 was borderline/equivocal and 1 was positive. Twenty-three 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 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 (Lisboa Bastos et al., 2020). This assumption is reinforced by the fact that TAB assays are in general more sensitive comparing to detecting only a single class of antibodies (Harritshøj et al., 2021).

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 (Lisboa Bastos et al., 2020). This assumption is reinforced by the fact that TAB assays are in general more sensitive comparing to detecting only a single class of antibodies (Harritshøj et al., 2021).

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 (Fenwick et al., 2021). 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 (Fenwick et al., 2021). 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 (Van Elslande et al., 2020). Remarkably, the Euroimmun IgA assay performed a lot better, with detecting 28 out 30 positives (Acro biosystems 2021). 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 (Harritshøj et al., 2021).

To perform some additional tests. The sample was reran with the Abbott Architect 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.

### Table 3
Classification of the results into positive, borderline and negative and further into antibody detected per antigen.

| Antigen | TAB | ELISA | CMIA | Architect | VIDAS SARS-CoV-2 | In house assay | Luminex multiplex assay | Abbott SARS-CoV-2 | bioMérieux SARS-CoV-2 | bioMérieux SARS-CoV-2- IgG Assay | VIDAS SARS-CoV-2 | Luminex multiplex assay | Abbott SARS-CoV-2 | bioMérieux SARS-CoV-2- IgG Assay | VIDAS SARS-CoV-2 | Luminex multiplex assay | Abbott SARS-CoV-2 | bioMérieux SARS-CoV-2- IgG Assay | VIDAS SARS-CoV-2 | Luminex multiplex assay | Abbott SARS-CoV-2 | bioMérieux SARS-CoV-2- IgG Assay | VIDAS SARS-CoV-2 | Luminex multiplex assay |
|---------|-----|-------|------|-----------|------------------|----------------|------------------------|-------------------|------------------------|-------------------------------|------------------|------------------------|-------------------|-------------------------------|------------------|------------------------|-------------------|-------------------------------|------------------|------------------------|-------------------|-------------------------------|------------------|------------------------|
| N+      | IgM| IgG   | IgM/IgA| TAB       | CMIA             | N               | 1                      | 1*                 | 1                      | 1*                         | RBD              | 1                      | RBD                | RBD                         | RBD              | 1                      | 1*                 | RBD                         | RBD              | 1                      | 1*                 | RBD                         | RBD              | 1                      |
| N+/-    |    |       |       |           |                  |                 | 1                      | 1*                 | 1                      | 1*                         | RBD              | 1                      | RBD                | RBD                         | RBD              | 1                      | 1*                 | RBD                         | RBD              | 1                      | 1*                 | RBD                         | RBD              | 1                      |
| N-      | 1  | 32    | 1     | 43        | 11              | 7               | 5                      | 13                 | 2                      | 1                          |                  |                        |                    |                               |                   |                        |                    |                               |                   |                        |                    |                               |                   |                        |

### 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       |     | CMIA       | N      | 1       | 1*                                    |
| bioMérieux   | VIDAS SARS-CoV-2 | RBD     | 1       | 1*     |
| Lumines       | In house assay | ELISA | N+RBD  | 1       | 1*                                    |

### 4. 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 (Fenwick et al., 2021). Choosing the most adequate assay is particularly important for pauci- or asymptomatic people, who are known to have a lower antibody response (Milani et al., 2020). Remarkable is the fact that assays against RBD, which is after all part of the S1 moiety, mostly generate a negative result. An exception is 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 (Acro biosystems 2021; Harritshøj et al., 2021; Herroelen et al., 2020). 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 30 positives (Acro biosystems 2021). This is also reflected in the results of the EQA, where the Euroimmun IgA assay did detect a positive or borderline signal.
conservation of conformational epitopes within a higher order structure (Infantino et al., 2020). This theory seems to be confirmed by the Diaisorin 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 Corona-viruses. It is known that the specificity of the Diaisorin Liaison S1/S2 IgG is lower when compared to its competitors (Harritsjhej et al., 2021). If you reason that RBD is only 1 of the 4 subdomains of S1 (Yuan et al., 2021) it seems plausible that this will be even less sensitive (Tian et al., 2020). This seems confirmed by this EQA, with almost all assays targeting RBD being negative, including the Diaisorin Liaison IgM test. An exception to this “rule” is the Wantai ELISA, which seems exceptionally more sensitive in comparison to its competitors (Acro biosystems 2021; Harritsjhej et al., 2021; Herroelen et al., 2020). 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.

5. 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 (Fenwick et al., 2021). The highly divergent results highlight the importance of taking into account the antigenic composition in the light of intended use of the particular assays. Our findings also accentuate the necessity of EQA schedules for SARS-CoV-2 serology and use of sample drawn at different time-points after Covid-19 episode.

Authors’ contributions
All persons who meet authorship criteria are listed as authors, and all authors certify that they have participated sufficiently in the work to take public responsibility for the content, including participation in the design, concept, analysis, writing, or revision of the manuscript.

Declaration of competing interest
The author stated that there are no conflicts of interest regarding the publication of this article.

Appendices
A. Figure

Fig. 1

B. Tables

Table 1,2,3,4

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