FRIEDRICH-LOEFFLER-INSTITUT
FLI
Bundesforschungsinstitut für Tiergesundheit
Federal Research Institute for Animal Health

COVRIN

SARS-CoV2 Research Integration and Preparedness

One Health research integration on SARS-CoV2 emergence, risk assessment and preparedness.

WP1 - T1.1 Optimization and harmonization of immunological SARS-CoV2 antigen and antibody detection methods in domestic and wildlife animals.
ST1.2.1 Evaluation of antigen detection methods in clinical samples from animals

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INTERLABORATORY TESTING – SARS-CoV-2 IMMUNOLOGICAL ANTIGEN DETECTION Final report

1- Objective of the interlaboratory testing

Identification of samples containing SARS-CoV-2 antigens by immunological methods.

2- Introduction

For the detection of SARS-CoV-2 in samples of different origin, e.g. swabs, organs, environmental samples etc., methods for the detection of either viral genome (RT-PCR) of viral antigens can be used. For the latter, the immunological detection of specific viral antigens by rapid antigen tests (RAT), enzyme linked immunosorbant assays (ELISA) or immunofluorescence assays (iIFA) are common methods. However, due to the use of different protocols, antibodies for detection and type of assay, the comparability of different methods is not always given. Therefore, an interlaboratory comparative testing has been organized for a challenge of the test systems performed in the participating labs using a defined antigen sample panel.

Antigens widely used for the immunological antigen detection are the spike (S)- and the nucleocapsid (N)-protein of SARS-CoV-2. Due to easy quantification of isolated recombinant proteins, samples containing recombinant S- and N-proteins have been used besides heat-inactivated virus in this interlaboratory test.

Before we started the interlaboratory comparison, participants were asked to fill out a questionnaire asking about the methods used in the respective labs and available reference material for antigen as well as antibody detection.

3- General Information

A panel of nineteen samples were sent to the participating laboratories and the following information was given:

The samples sent may be recombinant antigens and heat-inactivated cell culture supernatant. No material capable of replication will be shipped.

The participating laboratories were asked to identify samples positive for SARS-CoV-2 antigens, identify the antigens which are included in the samples (spike (S) or nucleocapsid (N) protein). Furthermore, the method used for the detection should be specified. For a simple and clear comparison of the results, the participating laboratories were sent an Excel file in which the corresponding information and results were to be entered (supplement 1)

The participants were expected to perform either the assays they usually use in their labs or a in advance distributed common protocol for testing rapid antigen tests (RATs) provided as proposal for a harmonized protocol. Returned results were analyzed with regard to targeted antigen and the performance of the assay regarding sensitivity and specificity of the assays against the specified antigen.

4- Panel Composition and description

For the samples, recombinant S- and N-proteins of different coronaviruses and variants of SARS-CoV-2 were diluted to defined concentrations. The first concentration was deliberately chosen that is at the detection limit of RATs for certain variants according the standard protocol for the comparison of RATs at FLI. A tenfold higher concentration was chosen as a second concentration. The sample panel was completed with heat-inactivated cell culture supernatants and negative samples.

Table 1 shows the nineteen samples with the concentration of the recombinant antigens. The virus samples used were supernatants from cell cultures. Supernatants were heat-inactivated for shipment and a safe use under BSL2 conditions.

Immunological antigen detection in the samples by means of a RAT is shown in table 2 and figure 1. All samples including the heat inactivated cell culture supernatants containing N-protein have been detected by the selected RAT. Subsequent densitometric analysis of the line show clear concentration dependency. Variations between the samples of one concentration might be caused by dilution effects during preparation or different sensitivity of the RAT for different VOCs. Recombinant S-proteins and domains of the S-protein (RBD = receptor binding domain; NTD = N-termial domain) are not detected by the RAT.

Table 1: Sample description. Blue shaded: recombinant N-proteins, yellow shaded: recombinant S proteins or domains thereof, red shaded: inactivated viruses

Sample-No.	Sample	Concentration
1	N-Protein non-VOC	10 ng/µl
2	N-Protein SARS-CoV-2 VOC Alpha	10 ng/µl
3	N-Protein SARS-CoV-2 VOC Delta	10 ng/µl
4	N-Protein SARS-CoV-2 VOC Omicron	10 ng/µl
5	N-Protein Feline CoV	10 ng/µl
6	S-Protein SARS-CoV-2 VOC Delta	10 ng/µl
7	S-Protein NTD SARS-CoV-2 VOC Omicron	10 ng/µl
8	S-Protein RBD SARS-CoV-2 VOC Delta	10 ng/µl
9	N-Protein non-VOC	1 ng/µl
10	N-Protein SARS-CoV-2 VOC Alpha	1 ng/µl
11	N-Protein SARS-CoV-2 VOC Delta	1 ng/µl
12	N-Protein SARS-CoV-2 VOC Omicron	1 ng/µl
13	N-Protein Feline CoV	1 ng/µl
14	S-Protein SARS-CoV-2 VOC Delta	1 ng/µl
15	S-Protein NTD SARS-CoV-2 VOC Omicron	1 ng/µl
16	S-Protein RBD SARS-CoV-2 VOC Delta	1 ng/µl
17	negative	
18	Heat inactivated Delta	
19	Heat-inactivated Omicron	

Table 2: Expected results (exemplarily for rapid antigen test detecting N protein with subsequent densitometric analysis):

Sample-No.	Sample	RAT	Ratio SARS-CoV-2 antigen line / control line	SARS-CoV-2 detected?	Detected antigen
1	N-Protein non-VOC		1,35	y	N
2	N-Protein SARS-CoV-2 VOC Alpha		0,83	y	N
3	N-Protein SARS-CoV-2 VOC Delta		0,74	y	N
4	N-Protein SARS-CoV-2 VOC Omicron		1,17	y	N
5	N-Protein Feline CoV			n	----
6	S-Protein SARS-CoV-2 VOC Delta			n	----
7	S-Protein NTD SARS-CoV-2 VOC Omicron			n	----
8	S-Protein RBD SARS-CoV-2 VOC Delta			n	----
9	N-Protein non-VOC		0,29	y	N
10	N-Protein SARS-CoV-2 VOC Alpha		0,14	y	N
11	N-Protein SARS-CoV-2 VOC Delta		0,23	y	N
12	N-Protein SARS-CoV-2 VOC Omicron		0,26	y	N
13	N-Protein Feline CoV			n	----
14	S-Protein SARS-CoV-2 VOC Delta			n	----
15	S-Protein NTD SARS-CoV-2 VOC Omicron			n	----
16	S-Protein RBD SARS-CoV-2 VOC Delta			n	----
17	negative			n	----
18	Heat inactivated Delta		0,36	y	N
19	Heat-inactivated Omicron		1,56	y	N

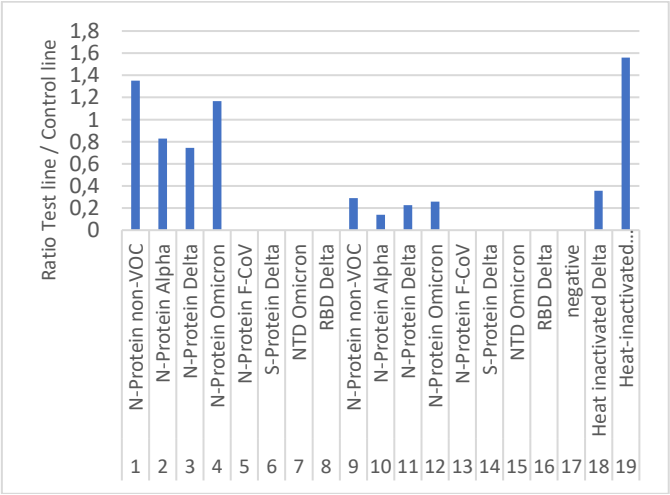

Figure 1: Densitometric analysis of samples. Densitometric volume ration of SARS-CoV-2 specific test line to control line is shown.

5- Participant Laboratories and contact persons

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6- Methods for antigen detection used by participants

The methods used by the participants for the detection of SARS-CoV-2 are listed in table 3. For the fast immunological detection, which can also made directly on-site, rapid antigen test have been used by several laboratories. Antigen-ELISAs have been used to detect either S- or N-antigen and a sandwich-ELISA has been established for the detection of N-antigen. One laboratory has used the E-Sarbeco RT-qPCR for the detection of viral genomic RNA and not an immunological method.

Table 3: Methods used for detection of viral antigen by participants. Green shaded assays use rapid antigen test for the detection of the antigens, in blue ELISA and in red RT-qPCR methods are listed.

Assay type	Name	Company	Targeted SARS-CoV-2 antigen	Protocol used
Rapid antigen test (RAT)	Rapid SARS-CoV-2 Antigen Test Card	Xiamen Boson Biotech Co., Ltd	N	Standard protocol for sample analysis by RATS: 25 µl of sample were diluted in 275 µl of RAT extraction buffer - 120 µl of extraction buffer containing diluted sample were applied to RAT and bands were analyzed by densitometric means

Rapid antigen test (RAT)	SARS-CoV-2 Ag Rapid Test	BioMaxima SA	N	One drop of each samples was applied to the test
Rapid antigen test (RAT)	Panbio™ COVID-19 Ag Rapid Test	Abbott	N	One drop of each samples was applied to the test
Rapid antigen test (RAT)	Coronavirus (2019-nCoV) Antigen Test	Beijing Hotgen Biotech Co.	N	Manufacturers instruction
Rapid antigen test (RAT)	NowCheck COVID-19 Ag Test	Bionote	N	One drop of each samples was applied to the test
Antigen-ELISA		in house	S	Antigen dilutions coated in Bicarbonate buffer pH 9.6 overnigth at 4°C. Rabbit antiserum (rabbit-anti-SARS-CoV-2-S1-2ST (619F)) was raised against the S1A subdomain of the SARS-CoV-2 spike protein (residues 1-294), fused to a triple Strep-Tag and produced in HEK293T cells (Davids Biotechnologie GmbH). Cut-off: A450 nm ≥ 0.100
Antigen-ELISA		in house	N	Antigen dilutions coated in Bicarbonate buffer pH 9.6 overnigth at 4°C. Rabbit serum (bleed) raised against insect-cell produced His-tagged NP of SARS-CoV-2. Detection of bound rabbit-Ig's with 1:4000 dilution of Goat-anti-rabbit Ig (H+L)-HRPO (Agilent-Dako) and TMB substrate system. Cut-off: A450 nm ≥ 0.100
Sandwich-ELISA	Mab sandwich ELISA for N-antigen	in house	N	Viruses 2022, 14(8), 1738
RT-qPCR	E-Sarbeco	in house	genomic RNA	200 µl sample extraction performed on Kingfisher Flex. E-gene and RdRp RT qPCR according to Corman et al 2020. Euro Surveill, 25(3) using AgPathID (Applied Biosystems)

7- Results

Depending on the kind of method used, the following tables show the results of the participants analyzing the samples. Table 4 depicted the results obtained by the different RATs. All used RATs should detect the nucleocapsid protein of SARS-COV-2. Positive reactions with samples containing SARS-CoV-2 N-protein as well as negative results with samples containing no SARS-CoV-2 N-protein are valued as correct (green shaded). No detection of N-proteins in samples 1-4, 9-12 and 18-19 is valued as wrong (red shaded). All RATs are able to identify heat inactivated viruses (samples 18 and 19) and there are no false positive

reactions with samples containing S-protein domains or N-protein from another virus (Feline Coronavirus). Unfortunately, samples with low concentration of N-protein are only detected by one RAT, two RATs detect only non-VOC and SARS-CoV-2 VOC Alpha but not VOC Delta or VOC Omicron (higher concentration). One RAT (Bionote) didn't detect any of the recombinant SARS-CoV-2 N-proteins.

Table 4: Results using RATs. Green shaded are the correct results, false negative results are shaded in red

Lab-Code:	1	2			4
Sample-No.	Rapid SARS-CoV-2 Antigen Test Card	SARS-CoV-2 Ag Rapid Test	Panbio™ COVID-19 Ag Rapid Test	NowCheck COVID-19 Ag Test	Coronavirus (2019-nCoV) Antigen Test
1	positive	weak positive	weak positive	negative	positive
2	positive	weak positive	weak positive	negative	positive
3	positive	negative	negative	negative	positive
4	positive	negative	negative	negative	positive
5	negative	negative	negative	negative	negative
6	negative	negative	negative	negative	negative
7	negative	negative	negative	negative	negative
8	negative	negative	negative	negative	negative
9	positive	negative	negative	negative	negative
10	positive	negative	negative	negative	negative
11	positive	negative	negative	negative	negative
12	positive	negative	negative	negative	negative
13	negative	negative	negative	negative	negative
14	negative	negative	negative	negative	negative
15	negative	negative	negative	negative	negative
16	negative	negative	negative	negative	negative
17	negative	negative	negative	negative	negative
18	positive	positive	positive	positive	positive
19	positive	positive	positive	positive	positive
Correctly identified	100%	68%	68%	58%	79%

Table 5 summarizes the results of the S-based and N-based ELISAs. The epitope of the S-based ELISA is located in the S1 subunit. Samples 6 and 14 also contain the S1 subunit and should therefore be recognized by the detection system. The negative results for these samples are therefore valued as false negative. In contrast to this, samples 7,8,15 and 16 contain only domains of the S1 protein. The (weak) positive result in sample 8 suggests the conclusion, that the epitope of the detecting antibody is located in this domain. Unfortunately, sample 16, the lower concentration of the protein has not been detected. Since it is not clear whether the antibody binds in the NTD, the two NTD samples (7, 15) are not evaluated as false negatives but are excluded from the analysis here. Unfortunately, the SARS-CoV-2 VOC Delta has not been detected properly-by this assay.

Only the N-based ELISA performed by laboratory 4 was able to detect the recombinant N-proteins of SARS-CoV-2 ancestral strain, VOC Alpha and VOC Delta, and the heat inactivated SARS-CoV-2 VOC Omicron. Like the double-antigen ELISA, the lower protein concentrations are detected. The double antigen ELISA did also not detect the higher concentrations of the recombinant N-proteins (samples 1-4)

Table 5: Results using ELISA. Green shaded are the correct results, false negative results are shaded in red. Orange shaded results are not valued, since real samples contain whole proteins and not only Subunits. It can't be excluded, that the assay would work with the whole protein (like with RBD):

Lab-Code	4		5
Sample-No.	S-ELISA	N-ELISA	Double antigen N-ELISA
1	negative	positive	negative
2	negative	positive	negative
3	negative	positive	negative
4	negative	negative	negative
5	negative	negative	negative
6	negative	negative	negative
7	negative	negative	negative
8	positive	negative	negative
9	negative	negative	negative
10	negative	negative	negative
11	negative	negative	negative
12	negative	negative	negative
13	negative	negative	negative
14	negative	negative	negative
15	negative	negative	negative
16	negative	negative	negative
17	negative	negative	negative
18	negative	negative	negative
19	positive	positive	positive
Correctly identified	68% (76% if samples 7 and 15 are excluded)	68%	52%

Since in this interlaboratory testing immunological methods should be used for antigen detection and mainly recombinant antigens were used, the detection by RT-qPCR couldn't be successful in most of the samples. However, the two heat-inactivated viruses were successfully detected by this method. Unfortunately, the E-gene based RT-qPCR gave positive results in samples 12 (N-Protein of SARS-CoV-2 VOC Omicron) and 13 (feline coronavirus), respectively. A RdRP-gene based RT-qPCR has been positive with sample 13 also. Since the Ct-values in these assays were between 33 and 38 it is difficult to say whether the reaction is based on a contamination or a wrong positive reaction.

8- Conclusion

For the immunological detection of SARS-CoV-2 antigens in samples with unknown content, the participating laboratories used two different principles: The detection of antigens by I) rapid antigen tests and II) ELISAs. Both methods have their advantages and disadvantages regarding their use for the fast and secure detection of SARS-CoV-2 antigens. RATs are easy to use, fast and don't need specialized laboratory equipment. However, depending on the RAT used for detection, differences in sensitivity and also specificity are observed. In contrast to this, validated ELISA systems are more sensitive and can easily adapted to special variants of concern. ON the other hand, ELISAs are time consuming and need trained personal and laboratory equipment for performance and analysis.

I) RATs

It was assumed that the higher antigen concentrations used for the interlaboratory tests would be high enough to be easily detected by RATs, while the lower concentrations would probably be at the detection limit of most RATs. To confirm this, a RAT routinely used in laboratory 1 was used. Specificity for SARS-CoV-2 N-protein was also demonstrated using the same RAT: The tests detected only N-protein from SARS-CoV-2, but not from FCoV, and no parts of S-proteins were detected. Surprisingly, none of the lower concentrations were detected by other RATs used in laboratories 2 and 4, and the four higher concentrations were only fully detected by the RAT of laboratory 4. The RATs used by laboratory 2 detected the higher concentration only with weak test lines or not at all. Also, differences between a weak detection of the ancestral strain and VOC Alpha on the one hand and no detection of VOC Delta and Omicron on the other hand could be observed.

II) ELISA

The participating laboratories used different ELISA systems for the detection of SARS-CoV-2 S- or N-protein. A direct antigen-ELISA based on the use of a S-protein specific polyclonal antisera should be able to detect at least the recombinant S1 subunit and the heat inactivated viruses. This ELISA could also be expected to be suitable to detect the NTD or RBD subunit. Nevertheless, only the heat-inactivated VOC Omicron and the high concentration of VOC Delta-NTD were scored as positive. The other S-protein positive samples were not detected. On the other hand, no N-protein has been detected by this assay.

The fail of detection might be due to the low concentration of antigens in the samples, meaning a low sensitivity of this assay. It cannot be excluded that the reduced sensitivity of ELISA is due to heat inactivation or denaturation of the recombinant proteins.

For the detection of N-antigen, also a direct antigen-ELISA has been used. This assay was able to detect the recombinant N-proteins of the ancestral strain as well as from VOC Alpha and VOC Delta and the heat-inactivated VOC Omicron. Since samples with low concentrations of N-protein were not detected,

the sensitivity of this assay could be improved. This is even necessary to a greater extent for double-antigen ELISA, too. Here, only the heat-inactivated VOC Omicron has been detected. Again, inactivation or denaturation might be responsible for a reduced detection by the specific antisera.

The results of this interlaboratory testing for immunological antigen detection methods show that the participating laboratories use different methods and protocols. The used protocols are all limited to the detection of a single antigen and show different sensitivity and specificity. For the future use, all methods must be adapted to the recently circulating variants of SARS-CoV-2 regarding these two properties. It is important that the methods are continuously checked to ensure that they are up to date and optimized according to requirements.

Since all participating laboratories used different methods which have been established and validated for diagnostic purposes, a harmonization of methods appears difficult. Comparison of the outcomes showed that the methods used, RATs and ELISA, can detect high viral loads well, but have difficulty detecting antigens reliably at low loads, such as in clinical samples at the beginning or end of an infection. Harmonization of methods is difficult to achieve under the current general setting, as the local conditions in the laboratories are often very different. Also, specific reagents such as individually produced antibodies or recombinant protein are not available to the same extent in all diagnostic laboratories. A coordinating body, responsible for establishing diagnostic methods, collecting and distributing resources, and continuously monitoring the quality of the tests performed, would be necessary to establish methods and protocols early in the occurrence of an epidemic and to ensure the possibility of performing them in all affected regions.

Supplement

Supplement 1: Results form to return the findings:

Contact		
	Institute	
	Name	
	E-Mail	

Protocol available for sharing or commercial assay?	

	Method(s) used for detection				
Sample:	Rapid antigen test (RAT)	ELISA	Other	SARS-CoV-2 detected?	Detected antigen
1					
2					
3					
4					
5					
6					
7					
8					
9					
10					
11					
12					
13					
14					
15					
16					
17					
18					
19					
20					

Comment:	