Design of an ELISA-based COVID-19 IgM, IgA and IgG Simultaneous Detection Test

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Riassunto

Una strategia efficace che testi i casi sospetti, tracci gli infetti e rintracci i loro contatti (*Test, Track, Trace* - TTT) è utile nel ridurre il dilagare della corrente pandemia di COVID-19, ma l'attuazione di uno screening sierologico di massa riguardante sia le IgM che le IgG (e eventualmente le IgA per valutare l'efficacia di diversi vaccini nell'elicitare l'immunità locale) rimane centrale per pianificare l'uscita dal *lockdown* in molti Paesi.

I test sierologici di massa potrebbero limitare i test molecolari sui tamponi nei casi sospetti che hanno sviluppato anticorpi durante una precedente infezione asintomatica di COVID-19; soprattutto essi sono essenziali per identificare i soggetti sieronegativi che devono essere vaccinati.

Il test progettato usa una nuova forma di fase solida per ELISA, una punta immunoadsorbente, e un nuovo metodo basato sulla doppia reazione eseguita utilizzando contemporaneamente due fasi solide: il micropozzetto standard che cattura le IgM (per poi rilevarne la specificità) impedendo interferenze con le IgG specifiche per il SARS-CoV-2, che vengono catturate dalla punta immersa nel medesimo micropozzetto. In questo modo si possono ottenere test sierologici per la ricerca simultanea di diversi isotipi anticorpali, limitando le reciproche interferenze.

Abstract

An effective strategy that tests suspected cases, tracks infected people and traces their contacts (TTT) will help reduce the spread of the current COVID-19 pandemic, but serological mass testing concerning both IgM and IgG (and IgA, in order to evaluate the local immunity stimulated by different vaccines) is central to lockdown exit plans in many countries.

Serological mass testing can avoid swab analyses in suspected cases which developed antibodies during a previous asymptomatic and not diagnosed COVID-19 infection; above all, it is essential to individuate seronegative persons to vaccinate.

The designed test uses a new shaped ELISA, the immunosorbent pin, and a new method based on the double reaction performed using simultaneously two different solid phases, able to separate, detect and differentiate immunoglobulin isotypes: the standard microwell (on which sample IgM are bound and tested about their specificity in the following steps; in this way IgM do not interfere with specific anti SARS-CV-2 IgG) and the pin, which binds specific anti SARS-CoV-2 IgG when immersed in the same microwell. The test has been conceived in order to run simultaneously different antibody detection tests and to avoid interferences generated by different antibody isotypes in serological tests.

Introduction

On 11 March 2020, the World Health Organization declared COVID-19 a pandemic [1]. "You cannot fight a fire blindfolded, and we cannot stop this pandemic if we don't know who is infected… Test, test, test", WHO Director-General Tedros Adhanom Ghebreyesus said on 16 March 2020.

The adopted RT-PCR based tests detect the recently emerged SARS-CoV-2 in nasopharyngeal and oropharyngeal swabs: nevertheless, even though they have high sensitivity and specificity, kits and equipment necessary to run them are limited in some geographical areas; moreover, they do not give information about past infections.
Serological tests are absolutely effective in estimating the real dimension of the COVID-19 pandemic, including current, recent and past infections.
Among them, rapid tests (lateral flow chromatographic immunoassay for the qualitative detection of IgG and IgM antibodies to SARS-CoV-2 in human fingerstick whole blood specimens) are useful in POCs, but they are unreliable to screen persons that need vaccination and those that do not, due to their sensitivity and specificity parameters.
Enzyme linked immuno-sorbent assay (ELISA) based tests are among the most sensitive and specific immunoassays and they are overall adopted to detect antibodies; running different antibody tests worldwide, however, could slow down sanitary interventions.
An effective strategy that tests suspected cases, tracks infected people and traces their contacts (TTT) will help reduce the spread of the current COVID-19 pandemic [2], but serological mass testing concerning both IgM, IgA and IgG is central to lockdown exit plans in many countries. They are absolutely effective in estimating the real dimension of the COVID-19 pandemic, including current, recent and past infections.

The new ELISA design and the innovative immunoreaction involving different solid phases

For the aforementioned, the development of a modular COVID-19 diagnostic kit - detecting IgM, IgA and IgG in blood serum or in saliva samples, even simultaneously, with the high sensitivity and specificity of the ELISA method - is of utmost importance in the course of the COVID-19 pandemic.
The test design can be achieved resorting to:

- the patent US7510687 [3], which describes the multi-immunosorbent device as an innovative shaped ELISA solid phase, consisting in a rod with multiple, protruding and immunosorbent ogival pins on which the immunocomplex is adsorbed, while in the meantime the microwells in which the pins are immersed act only as containers; the method has been successfully experimented in the frame of collaboration agreements with the EC - DG JRC concerning animal infectious diseases and food safety issues [4-7];
- the PCT/IB2012/052021 - WO 2012/143912, in which pins are immersed in oversized coated wells; different immunocomplexes are adsorbed on the pin surface and on the microwell surface [8].

Integrating both of the above said inventions, an innovative immunoreaction has been designed, involving immunosorbent solid phases having different formats: pins and microwells.
Each solid phase specifically adsorbs one of the three immunoglobulins to detect, in order to avoid reciprocal interferences in the detection of the immunocomplexes involving the SARS-CoV-2.
The proposed test design has been conceived as a modular tool, useful in the detection of a specific immunoglobulin isotype, up to the IgM, IgA and IgG simultaneous detection.
The differently shaped solid phases adopted in the test are differently coated:

- the microwell is coated with monoclonal antibody anti Human IgM (μ-chain specific) in order to bind sample IgM;
- ogival pins are coated with capturing antibody binding the SARS-CoV-2 selected antigen, in order to bind specific IgA or IgG potentially present in the sample;

some non-immunosorbent microwells serve as mere reagent containers (vessel), in which the coated pins are immersed.

IgM and IgG simultaneous detection

1. The sample is dispensed in the coated microwell containing a proper amount of diluting solution.

1a. Monoclonal antibody anti Human IgM (μ-chain specific) coating the microwell binds the sample IgM during the incubation step, as in the ELISA method (figure 1a).

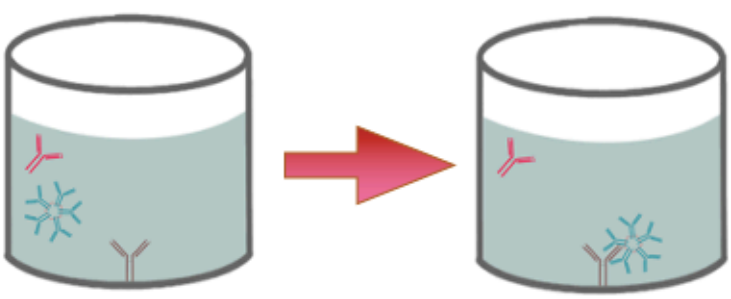
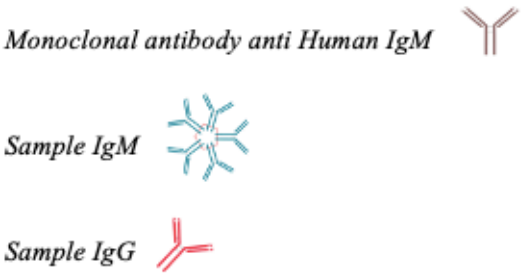


Figure 1a: the coated microwell containing the sample.
During the 1st incubation step, the monoclonal antibody anti Human IgM coating the microwell specifically binds the sample IgM.



1b. At the end of the previous incubation time, a pin coated with capturing antibody binding the SARS-CoV-2 selected antigen is immersed in the sample inside the microwell; the SARS-CoV-2 antigen binds the sample specific IgG which recognize its second epitope (figure 1b).

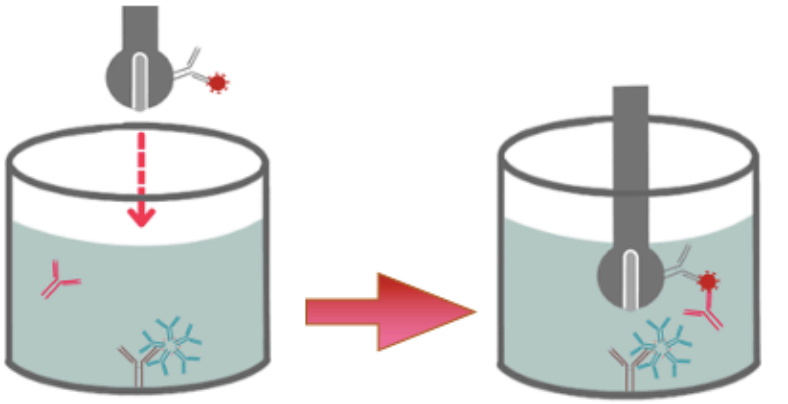
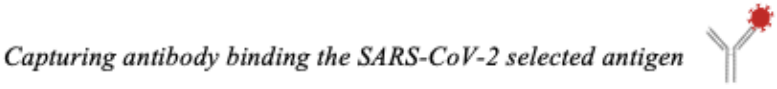


Figure 1b: the coated pin is immersed in the sample.
At the end of the 1st incubation step, the pin coated with the SARS-CoV-2 antigen is incubated in the coated microwell containing the sample, in order to bind the sample IgG recognizing the SARS-CoV-2 second epitope.



2. At the end of the incubation:

2a. the microwell is washed and filled with the HRP-conjugate SARS-CoV-2 antigen, which binds specific IgM adsorbed on the microwell surface (figure 2a);

2b. the pin is lifted and washed; the HRP-conjugate monoclonal antibody anti Human IgG (γ-chain specific) is dispensed in a vessel (a non-immunosorbent microwell), then the pin is immersed in it (figure 2b).

Figure 2a: HRP-conjugate SARS-CoV-2 antigen is dispensed in the microwell.

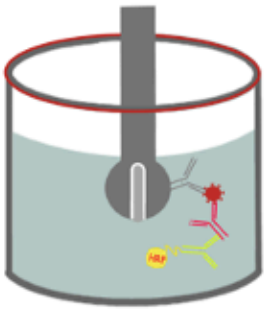


Figure 2b: HRP-conjugate monoclonal antibody anti Human IgG is dispensed in a vessel (not immunosorbent microwell, red marked) in which the pin is immersed and incubated.

HRP-conjugate SARS-CoV-2 antigen

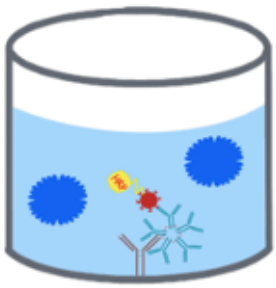
HRP-conjugate monoclonal antibody anti Human IgG

3. At the end of the incubation and the washing of the two immunosorbent solid phases:

3a. the microwell is filled with the chromogenic substrate (3,3',5,5'-tetramethylbenzidine - TMB) and the chromogenic reaction develops in it (figure 3a);

3b. the pin is immersed in the vessel containing the chromogenic substrate (TMB), in which the chromogenic reaction develops (figure 3b).

Figure 3a: TMB is dispensed in the microwell.
The chromogenic reaction develops.



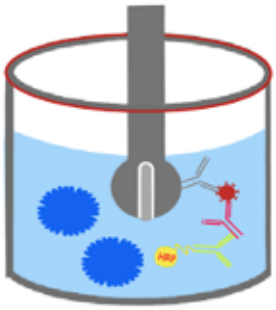


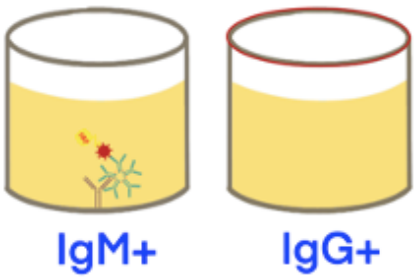
Figure 3b: TMB is dispensed in the vessel (red marked) and the pin is immersed in it.
The chromogenic reaction develops.

IgM+ IgG+

Blue coloured oxidised TMB

4. After incubation, the pin is lifted and thrown; the chromogenic reaction is stopped adding an acidic solution (sulphuric acid) both in the microwell and the vessel; the Absorbance / Optical Density (OD) is read in both of them through the ELISA-reader at λ = 450/620 nm (figure 4).

Figure 4: after incubation in TMB, the pin is lifted and thrown.
Adding an acidic solution both in the microwell and in the vessel, the chromogenic reaction is stopped.
The intense blue color turns in deep yellow color.
The OD is read in both the microwell and in the vessel at $\lambda = 450$ nm through the ELISA-reader.



Time by time, microstrips can be chosen (or arranged in microplates) in order to detect one, two or three immunoglobulin isotypes:

- by analysing one or two immunoglobulins isotypes, high through-put can be reached fitting mass screening purposes;
- by analysing IgM, IgG and IgA elicited by different viral antigens, accurate tests can deeply indagate the immunological response during the COVID-19 infection and its correlation with symptoms and viral excretion.

IgM and IgG simultaneous detection is performed diluting samples in the purple marked microwells, where IgM are detected and in which pins are immersed; IgG are then detected in the adjacent microwells (figure 5).

Figure 5: IgM and IgG simultaneous detection.
Microstrips can be arranged in the 96-well ELISA format in order to perform the IgM and IgG simultaneous detection for mass-screening purposes, preventing the IgM interference on the IgG test results.

	1	2	3	4	5	6	7	8	9	10	11	12
A	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG
B	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG
C	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG
D	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG
E	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG
F	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG
G	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG
H	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG

In the designed test:

- immobilizing IgM on the microwell surface, SARS-CoV-2 selected antigen adsorbed on the pin surface remains available to bind IgG even in case of low levels; therefore, IgG detection is scarcely affected by IgM;
- preventing the IgM interference in binding the viral antigens, that could hinder the low IgG adsorption, more reliable results can be obtained and especially when a quantitative test runs in diluted samples;
- since blood samples contain different IgG derived from past infections, the specific IgG are bound and adsorbed on the solid phases through the whole SARS-CoV-2 or its selected antigens;
- IgM anti SARS-CoV-2 are detectable only in case of COVID-19 current infections, when they are the predominant IgM in the sample; consequently, most IgM bound through the

monoclonal antibody anti Human IgM coating the microwell surface are potentially directed to the SARS-CoV-2 selected antigen and their specificity can be detected through the HRP-conjugate anti SARS-CoV-2 selected antigen in the following steps;

- in case the IgM high level could hinder the IgG detection, the positive IgM test highlights the positive cases anyway, also if the IgG detection tests give negative results due to the low level of IgG compared to the IgM level;
- controls or calibrators have to be included in the test (the negative and the positive control sera etc.).

IgM, IgA and IgG simultaneous detection

1. The sample is dispensed (in triplicate) and incubated in three microwells containing a proper amount of diluting solution:

- two microwells are coated with monoclonal antibody anti Human IgM;
- one non-coated microwell is dedicated to the specificity control in absence of antigen.

At the end of incubation:

- two pins coated with capturing antibody binding the SARS-CoV-2 selected antigen, alternatively dedicated to the IgA detection and to the IgG detection, are immersed in the coated microwells;
- the non-coated pin is immersed in the non-coated microwell.

During this incubation step, specific IgA and IgG adsorb on the pins.

In absence of non-specific binding, no adsorption occurs in the non-coated microwell and on the pin immersed in it.

2. After the incubation and the washing of microwells and pins:

- the pins are immersed in vessels (non-coated microwells) containing:
  - HRP-conjugate monoclonal antibody anti IgA (α-chain specific), which binds the specific IgA adsorbed on the pin surface;
  - HRP-conjugate monoclonal antibody anti IgG (γ-chain specific), which binds the specific IgG adsorbed on the pin surface;
  - HRP-conjugate polyclonal antibodies anti Human immunoglobulins useful in the specificity control;
- the HRP-conjugate SARS-CoV-2 antigen is dispensed in the microwells dedicated to the IgM detection and to the specificity control.

3. After the incubation and the washing of all the microwells, vessels and pins, the chromogenic substrate is dispensed; then, pins are immersed in it.

4. The pins are lifted and thrown, the chromogenic reaction is stopped adding acidic solution in all the microwells and vessels forming the ELISA microplate, then the OD is read through the ELISA reader at λ = 450/620 nm.

The ELISA-microplate can be arranged using 8-well microstrips:

- microstrips in columns 1, 2, 7 and 8 are coated with monoclonal antibody anti IgM; samples are dispensed in triplicate in columns 1-3 and 7-9;
- microstrips in columns from 3 up to 6 and from 9 up to 12 are non-coated; microwells in columns 4-6 and 10-12 act as mere containers (vessels).

The three pins used to test each sample are previously immersed in columns 1-3 (from which they are moved in columns 4-6) and in columns 7-9 (form which they are moved in columns 10-12), where proper reagents are sequentially dispensed (figures 6 and 7).
Each microplate row is dedicated to two samples.

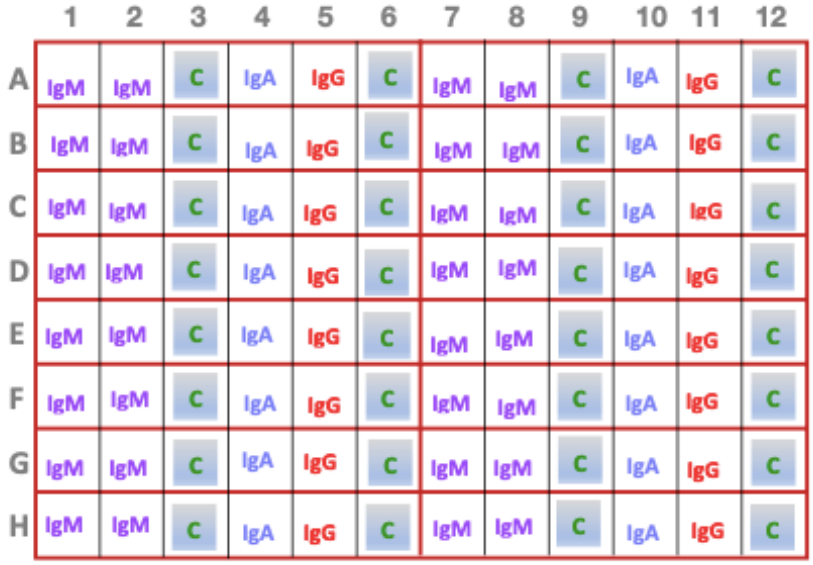


Figure 6: IgM, IgA and IgG simultaneous detection.
Microstrips can be arranged in the 96-well ELISA format in order to perform tests preventing the IgM interference on the test results.

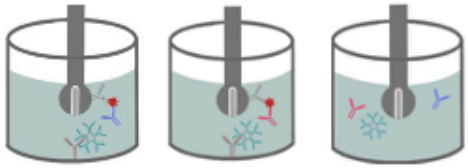
In the designed test aimed to accurate diagnosis:

- since it is of utmost importance to identify persons having current infections, which potentially excrete the SARS-CoV-2, the IgM detection runs in duplicate in order to obtain the most reliable results;
- the test includes, for each sample, two controls highlighting the false positive results / non-specific immunoreactions concerning the three analysed immunoglobulin isotypes (IgM, IgA and IgG);
- in case the IgM high level could hinder the IgG and the IgA detection, the positive IgM test highlights the positive cases anyway, also if the IgG and the IgA detection tests give negative results due to the low level of IgG or IgA compared to the IgM level;
- the immuno-response can be evaluated considering two different SARS-CoV-2 antigens: an "a" selected antigen can be HRP-conjugated and a "b" selected antigen having a second epitope can be immobilized on the pin surface; in that way, IgM detection concerns antibodies directed to the "a" antigen and IgG detection concerns antibodies directed to the "b" antigen, in order to evaluate simultaneously up to 4 epidemiological parameters in the current infected persons: IgM, IgG, immuno-response to SARS-CoV-2 "a" antigen and to SARS-CoV-2 "b" antigen (potentially, antigens can be selected in order to detect virus mutations) (figure 8); a third antigen can be used in the IgA detection in order to have information concerning up to six epidemiological parameters;
- the test can be arranged adsorbing the sample IgG on the microwell (replacing the IgM detection previously described) and the sample IgA on the pin or *vice versa*, in order to deeply investigate the IgA correlation with severe cases and to evaluate the local immunity stimulated by different vaccines;
- other controls have to be included in the test (negative and positive control sera or calibrators, etc.);
- quantitative results can be obtained carrying out tests in sample dilutions.

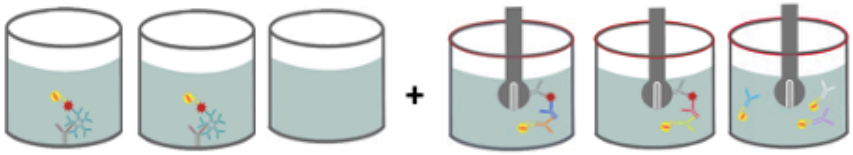
Figure 7: IgM, IgA and IgG detection.

1. Each sample is dispensed in three microwells containing the diluting solution; one microwell is no coated and it acts as negative control.

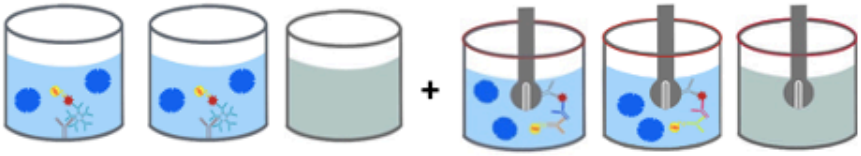
2. After incubation, pins are immersed and incubated in the diluted sample.



3. At the end of incubation, pins are washed and immersed in conjugates; microwells are washed and the conjugate is dispensed.



4. At the end of incubation, pins are washed and immersed in TMB; microwells are washed and TMB is dispensed in them.



5. After incubation, the acidic solution is dispensed; the chromogenic reaction is stopped and the intense bleu turns in deep yellow.

Monoclonal antibody anti Human IgM

Sample IgM

Sample IgA

Sample IgG

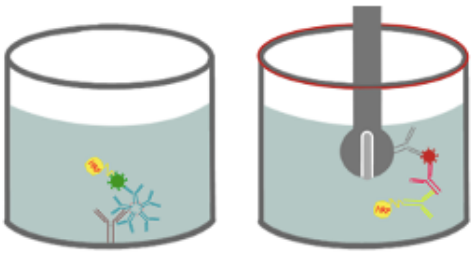
Capturing antibody binding the SARS-CoV-2 selected antigen

HRP-conjugate SARS-CoV-2 antigen

HRP-conjugate monoclonal antibody anti Human IgA

HRP-conjugate monoclonal antibody anti Human IgG

Figure 8: detection of IgM and IgG, immuno-response to SARS-Cov-2 "a" antigen and to SARS-CoV-2 "b" antigen.



SARS-CoV-2 "a" antigen HRP-conjugated

SARS-CoV-2 "b" antigen having a second epitope, immobilized on the pin surface

The two antigens can be selected among the main immunogenic SARS-CoV-2 proteins: the nucleoprotein (N) and - in the Spike glycoprotein trimer (S) - the S1 subunit, its N-terminal ($S1^A$) domain and its receptor-binding domain (RBD) [9].
S2 subunit is highly conserved and thus plays a role in the cross-reactivity of the whole S antigen.
S1 is a specific antigen for SARS-CoV-2 diagnostics, since no cross-reaction has been observed in patients seropositive for endemic HCoVs (HCoV-HKU1, HCoV-OC43, HCoV-NL63, and HCoV-229E) and MERS-CoV. The cross-reactivity resulting from the high degree of similarity between S1 and RBD of SARS-CoV and SARS-CoV-2 does not produce false-positives results due to the fact that SARS-CoV has not circulated in the human population since 2003.
N protein detects SARS-CoV-2 specific antibodies with high specificity and sensitivity.
RBD and N protein ELISAs are more sensitive than S1 ELISA in detecting antibodies in mildly infected patients.
Therefore, detecting antibodies against 2 different antigens might be needed to confirm the findings and avoid false-negative results in mass testing and surveillance studies [10].
Alternatively, the inactivated whole-virus can be used, in order to conduct a more comprehensive epidemiological survey.

Engineering

Since the protocol concerning the pins runs through UP and DOWN movements, the above described method could automatically run modifying and engineering the commercially available automated ELISA systems in order to add mechanisms able to grip and to move pins OUT and IN microwells and vessels filled at the moment by programmed dispensers.
Moreover:

- washing steps are easy to perform, thanks to the ogival shaped pins that can be squirted with washing solution and gently dried through air stream from the top, in order to allow the washing solution to flow away dragging the non-adsorbed antibodies or HRP-conjugates;
- the test can use miniaturized pins and ELISA 384-well microplates, in order to occupy the reduced space available in a compact instrument, maintaining the high through-put required for laboratories and POCs;
- pins can be used as single elements (figure 9) or they can be assembled as in the pin-device [5], in which one or more pins are dedicated to the sample analysis and one pin is dedicated to the negative control in absence of antigen, in order to evaluate the non-specific immuno-reactions / false positive test results;

- stop solution is unnecessary to stop the chromogenic reaction in the vessel, since it is stopped lifting the pin; in this case OD is read at $\lambda = 655$ nm.

Figure 9: the cut facets pin. The pin has the 90% of the surface extension than each microwell forming the ELISA 96-well microplate.

Test deliverables

Collecting data could efficiently contribute to estimate, worldwide, the following epidemiological parameters:

1. recent / current infections (if IgM detection occurs);
2. past infections, also the asymptomatic ones (if IgG detection occurs);
3. seroprevalence in large population;
4. a more affordable Transmission Rate (R_0) calculation;
5. a more affordable Case Fatality Rate (CFR) calculation;
6. correlation between IgA and IgG levels and case severity;
7. persons that do not need vaccination (since they have developed their own immuno-response);
8. vaccination durability concerning the humoral and the local immuno-response;
9. protecting effects of vaccines due to the elicited neutralising antibodies.

Moreover, the test could be adopted to individuate:

- persons not required to comply with restriction measures imposed in the countries and regions put under lockdown;
- persons who are already naturally immunized, which can resume productive, educational, artistic, commercial, professional, supporting and economic activities. These individuals will have to be identified until the launching of the vaccine campaign and monitored through repeated tests, in order to verify the permanence of antibodies over time and their correlation with the potential intermittent viral excretion, if serological and molecular tests are conducted.

Conflict of interest disclosure

The author declares no conflict of interests.

Acknowledgements

The author thanks the visual developer artist Beatrice Battista which created the explanatory pictures.

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