Direct Virus Capture assay for label-free detection of SARS-CoV-2 virions using laser microscopy

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ABSTRACT

SARS-CoV-2 is the causal agent of the COVID-19 pandemic, which from late 2019 endangered public health across the world. Since the start of the pandemic, it was immediately evident that early virus detection was crucial to managing the spread of the contagion. Then, many companies were prompted to investigate newer technologies for diagnosing viral diseases.

For this reason, the development of diagnostic tools quickly became a challenge taken on by many companies and research groups.

At present, real-time reverse transcription PCR (RT-PCR) is considered the gold standard assay for detection of SARS-CoV-2. However, detection of viral RNA in clinical samples does not discriminate between fully formed (infections) viral particles and fragmented or non-incapsidated (non-infectious) genomic material.

The present study focuses on the evaluation of an innovative biosensor, fast, label-free and variant-independent assay for capture and detection of SARS-CoV-2 whole virions named Direct Virus Capture (DVC). The proof-of-concept of this assay consists of a bioreceptor/antigen affinity interaction occurring between biological virus targets such ACE2 receptors printed on a glass coverslip substrate, and the SARS-CoV-2 S glycoproteins, located on the outer surface of virions.

The virions captured on the functionalized glass surface are detected using a digital laser microscope named Nano Eye Device - Virus Detector (NED-VD), specifically designed and calibrated to detect nanoparticles by light scattering.

The study was conducted on 191 human swab specimens collected from the upper respiratory tract (UTR; nasopharyngeal swabs and combined nose and throat swabs), 170 of these were tested and resulted positive by RT-PCR assay with cycle threshold (Ct) ranging from 14 to 25 and potentially containing intact SARS-CoV-2 virions.

The performance of this new assay for SARS-CoV-2 aimed at directly detecting intact virions was investigated and clinically evaluated achieving sensitivity of 40.5%, specificity of 90.4% and accuracy of 46% compared to the gold standard RT-PCR assay.

Similar DVC assays combined with a NED-VD instrument, are potentially applicable for detection of any other viruses characterized by a spherical structure of at least 40 nm diameter.

Keywords:
SARS-CoV-2
Laser Microscopy
Scattering
Label-free
Intact virions
Biosensing

1. Introduction

Since late 2019 an outbreak of a new severe respiratory disease in humans of unknown etiology emerged at first in Wuhan, China, and quickly spread all over the word (Zhu et al., 2020; Rotondo et al., 2021). Shortly after this outbreak, the chinese health authorities identified a novel coronavirus called SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2) as the causal agent of the new emerging clinical disease, which was subsequently named coronavirus disease 2019 (COVID-19). Due to the rapid spread and high mortality of the disease. the World Health Organization (WHO) declared it as a pandemic on 11 March 2020 (Cucinotta, D., Vanelli, M., 2020).

Consequently, from the moment of the identification of SARS-CoV-2, one of the critical objectives was to develop high-performance and quick diagnostic tests for the detection of the new coronavirus, in order to promptly isolate infected individuals to reduce the spread of contagion (Seyed et al., 2020). In addition, evaluation of infectivity as well as the viral shedding time frame in SARS-CoV-2 positive individuals became pressing issues.

Currently, the laboratory diagnostic tools and techniques for detection of SARS-CoV-2, and in general for all respiratory viruses, can be divided into molecular, antigen, and serological methods.

Molecular methods include detection of the viral genome using nucleic acid amplification tests (NAATs) and genome sequencing (Soler et al., 2020).

The availability on network databases of the genomic sequence of pathogens, including newly emerged viruses such as SARS-CoV-2, allows for the quick development and production of PCR kits with high specificity, sensitivity, and readiness for detection of emerging new viral variants (Soler et al., 2020).

Today, real-time reverse transcription PCR (RT-PCR) is the gold standard technique for diagnosing COVID-19, by detection of portions of the viral genome in clinical specimens quantifying the viral load in a sample by measuring the number of viral RNA copies per milliliter (Suo et al., 2020).

In addition, quantification or semiquantification of viral clinical samples proved to be useful for estimating the patients' infectivity (Piralla et al., 2021). This technique amplifies and detects specific RNA sequences of SARS-CoV-2 that may be present in different forms in the sample, including free RNA, RNA contained within intact virions, or RNA from damaged or degraded virions (Yu et al., 2020).

RT-PCR provides real-time quantification by first converting RNA into DNA (RT step), and then detecting the DNA using PCR. A sample is considered positive if the fluorescence reaches a specified threshold within a certain number of PCR cycles (Ct value). The Ct value

is inversely proportional to the viral load, with every ~3.3 increase in Ct value indicating a 10-fold reduction in the starting material (Tom, M. R., Mina, M. J., 2020).
However, even if viral nucleic acid detection by RT-PCR is considered the gold standard for the diagnosis of COVID-19, this assay cannot discriminate between viral RNA incapsidated into infectious particles and not-infectioviral nucleic acids.
Antigen detection techniques involve identifying specific viral proteins (known as viral antigens) usually by using specific antibodies. For example, the S glycoprotein (spike) in coronaviruses or the HA protein (hemagglutinin glycoprotein) in influenza virus can be detected through this method.
Enzyme-linked immunosorbent assays (ELISA) or immunofluorescence staining are among other conventional approaches used, which provide qualitative or semi-quantitative results with sufficient sensitivity (Soler et al., 2020; Safiabadi et al., 2021), although, those require skilled personnel and are time-consuming. Instead, rapid antigen diagnostic tests (RADTs) based on immunochromatography (IC), or lateral flow assays (LFA) have become popular due to their disposability, low cost, fast turnaround time, qualitative results (yes/no), and suitability for point-of-care testing.
Finally, serological assays indirectly identify microorganisms by detecting specific antibodies generated during the immune response. Typically, these tests are useful for retrospective diagnosis, surveillance, and epidemiological studies, providing estimates of prevalence and monitoring antibody levels over time to assess acquired protection or immunity (Leung et al., 2006, Soler et al., 2020).
Finally, conventional virus isolation still remains the only direct marker of virus replication and infectivity. However, this approach is cumbersome time consuming and requires specialized biosafety containment laboratories.
Before molecular, antigen or immunological methods as diagnostic tools for respiratory virus detection, viral culture has been considered the reference technique.
If on the one hand this technique had the great advantage of providing direct information about the virus pathogenicity, on the other hand its long turnaround time, usually days, and the requirement of skilled personnel for results interpretation make it useless for high-throughput tests (Soler et al., 2020, Seyed et al., 2020).
Nowadays, there are also several microscopy techniques able to visualize whole virions, providing direct morphology information and counts of viral particles, whether or not they are infectious. These include Transmission Electron Microscopy (TEM) (Roingeard et al., 2019), Scanning Electron Microscopy (SEM) (Blancett et al., 2017), Cryo-electron microscopy (Cryo-EM) (Cheng, 2015), Atomic Force Microscopy (AFM) (Moreno-Madrid et al., 2017) and fluorescence microscopy (FM) (Chen at al., 2017).
Overall, these techniques are widely used in research, although can be very expensive and require highly specialized expertise depending on the specific method and sample preparation required. In particular these techniques are sub-divided in label-free methods (all EM and AFM) or in labelling methods (FM).
Recently, we demonstrated as proof-of-concept an innovative, label-free, fast, variant-independent assay, named Direct Virus Capture (DVC), able to detect intact SARS-CoV-2 virions through direct imaging with Nano Eye Device - Virus Detector (NED-VD), a laser microscope working in evanescent wave illumination able to detect nanoparticles in scattering. We proved that the DVC assay has a minimum limit of detection (LOD) of ~ 10^7

viral particle/mL by using either HIV pseudotyped with SARS-CoV-2 spike proteins, and whole SARS-CoV-2, both cultured specimens (Lo Savio et al., 2022).
In particular, the DVC assay exploits intrinsic characteristics of the new coronavirus such as the biological high-affinity binding between spike glycoproteins (S) located on the outer surface, and angiotensin-converting enzyme 2 (ACE2) proteins printed on the glass substrate (Wang et al., 2020).
Here, we present and evaluate the performance of the DVC assay done in standard wells of 6 mm diameter in size (e.g., 96-well plate), using clinical samples.

2. Materials and methods

2.1. Specimen types

Between November 2021 and December 2022, a total of 152284 nasopharyngeal swab specimens were collected in 3ml Universal Transport Medium (UTM*TM*) at Microbiology and Virology Department, Fondazione IRCCS Policlinico San Matteo, Pavia and then tested for the presence of SARS-CoV-2 genome. Among these, 191 RT-PCR-positive swabs (Ct ranging from 14 and 25 and collected from different subjects) were included in this study tested with NED-VD within 48 hours from collection time or after being stored at -80°C.

2.2. SARS-CoV-2 nucleic acid detection

All the 191 human swabs specimens involved in the study were at first analyzed by RT-PCR assay. Total RNA was extracted on the MGISP-960 automated workstation using the MGI Easy Magnetic Beads Virus DNA/RNA Extraction Kit (MGI Technologies, Shenzhen, China). Detection of SARS-CoV-2 RNA was performed using the SARS-CoV-2 variants ELITe MGB® kit (ELITechGroup, Puteaux, France; cat. no. RTS170ING) targeting ORF8 and RdRp gene. Reactions were carried out on the CFX96 Touch Real-time PCR detection system (BioRad, Mississauga, ON, Canada).

2.3. NED-VD device

Nano Eye Device - Virus Detector (NED-VD) is an optical digital microscope, based on a patented technology (Carloni, 2016) developed for the optical detection of nanoparticles, consisting of a microscope module coupled with a line diode laser centered at 520 nm wavelength. The optical coupling between the laser and the coverslip causes total internal reflection of the beam in the substrate by Snell's law and is able to generate an evanescent wave upon the substrate itself. Nanomaterials down to 20 nm illuminated with this method scatter enough light be detected and observed by a camera located at the end of the microscope module. The optical device is equipped with 6 objectives (2×, 4×, 10×, 20×, 40×, 60×) and permit digital capture at these magnifications. This device has been described in detail in a previous publication (Lo Savio et al.,2022).

2.4. Bioconjugation of ACE2 proteins on the substrate

Glass coverslips (60 × 24 mm, 0.17 mm thickness) functionalized with aldehydic groups were purchased from Schott-Nexterion. The bioreceptors used to define the active regions on the substrate were angiotensin-converting enzyme 2 (ACE2) (Signal-Chem, concentration 0.5 µg/µL). The concentration of protein was chosen after investigating its effect on the assay performances (Lo Savio et al., 2022). Bioreceptors were dispensed on the coverslip surface by drop casting through manual deposition, with a volume of 0,5 µL. Drops were allowed to dry at 25 C° while shaking the coverslip at 1200 rpm in a thermo-shaker (Bio-San PST-100HL): after drying, a circular spot was formed, defining the active area on the coverslip. The drop volume determined the spot size, with a diameter in the range 1-2 mm. The estimated nominal surface concentration of bioreceptors in the active spot area varied slightly between 2.5 and 3.0 × 10^{11} / mm^2. This represents an upper limit estimation only based on the number of bioreceptors deposited on the substrate; due to the deposition method, a random orientation is expected, thus reducing the amount available for further interactions (Lo Savio et al., 2022).

2.5. DVC assay

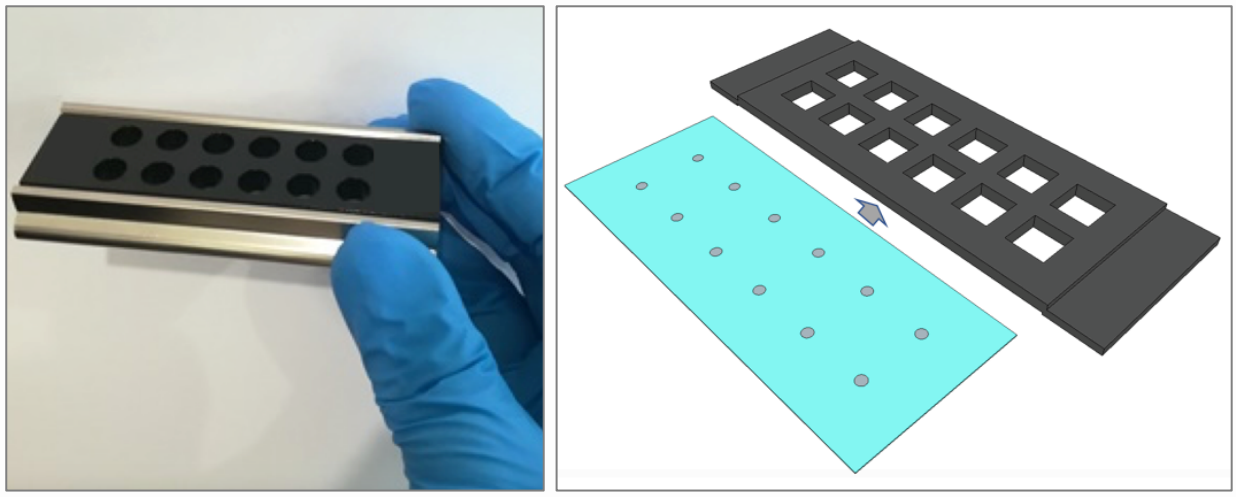
As substrate, glass coverslips divided in 12 separated cylindrical wells of 6 mm in diameter using a silicone isolator gasket and a reaction chamber (Fig. 1 a) with springs (Grace Biolabs). To capture SARS-CoV-2 intact virions, the coverslip regions outside the spot were blocked to prevent non-specific adhesion (blocking step). After removal of the blocking solution the nasopharyngeal swab extract was dispensed and incubated (reaction step). During the incubation phase, the reaction chamber was sealed, thus preventing evaporation and cross-contamination between adjacent wells. Finally, the coverslips were washed with distilled water (Milli-Q) to remove unbound or fragments of virions, or other nanomaterials potentially present in the starting sample, such as crystals or clusters of salts in the buffer solution, to avoid stray light or unwanted centers of light scattered.

In the blocking step, each well was covered with 50 µL of blocking solution (PBS, 1% BSA, 0.05% V/V Tween-20) and the entire reaction chamber was incubated in a thermo-shaker (Bio-San PST-100HL) for 15 minutes at 25°C with 250 rpm agitation. Each well was then washed at room temperature twice with 100 µL of a wash buffer (PBS, 0.05% Tween-20).

In the reaction step, 40 µL of swab extract was added to each well and the reaction chamber was incubated in the thermo-shaker for 30 min at 34° C with agitation at 1000 rpm. After the incubation time swab extract was removed from each well.

Finally, the reaction chamber was dismantled, the glass coverslip recovered and immersed for wash in a 50 mL conical tube with distilled water (Milli-Q) for 3 minutes at room temperature, then dried with air and inserted in a support (Fig. 1 b) before being analyzed with NED-VD, mainly at 4× magnification for a first view of the spot and 20× for the analysis of detected intensity values. In particular, the values were obtained by subtracting from the values obtained inside the spot those obtained in the area

surrounding the spot. To test intra-swab repeatability, the experiments were repeated in duplicate.
The DVC assay was entirely performed under a fume hood, in a biosecurity level 2 environment, whereas the reading of the slides using the NED-VD was performed outside of it, at room temperature, both in-situ, just beside the hood, both from a remote location by exploiting NED-VD's capabilities in IP sharing.



a) b)

Fig.1. a) Reaction chamber for the coverslip with 12 separated wells. b) Coverslip ready to be inserted in the support for NED-VD reading.

2.6. Evaluation of DVC assay

In order to determine sensitivity, specificity and the accuracy of the DVC assay, we compared data obtained from the RT-PCR tests with the results of the DVC assay. In particular, human swabs tested positive with corresponding Ct values were used to evaluate the sensitivity of DVC assay. The specimens tested negative by RT-PCR were analyzed to assess the specificity of the DVC assay. Finally, the totality of information collected from human swabs tested positive and negative by RT-PCR was used to evaluate the accuracy of the DVC assay. Cross reactivity of ACE2 towards other targets was beyond the scope of this study.

3. Results

A panel of 191 human swab specimens tested by RT-PCR were considered in this study, including 170 resulted positive with Ct values ranging from 14-25 (10^4-10^7 copies of RNA/mL) and 21 negative. Result of DVC assay showed concordant of positive in 40.5% and concordant of negative in 90.4% (Table 1) of the samples. Moreover, among the 170 RT-PCR positive swab with their relative Ct values, the correspondent DVC assay results are reported (Table 2). We observed no qualitative differences in results between fresh and

frozen swabs and the repeatability of DVC test in duplicate was experimentally performed for each patient to confirm the qualitative reproducibility of the assay.

Table 1

Confusion matrix for the DVC assay. The confusion matrix below summarizes the performance of DVC assay showing data relative to sensitivity (TP / TP + FN), specificity (TN / FP + TN) and accuracy (TP + TN / TP + FN + FP + TN). Sensitivity= 40,5%; Specificity= 90,4% and Accuracy= 46%. TP= True Positive; FP= False Positive; TN=True Negative; FN= False Negative.

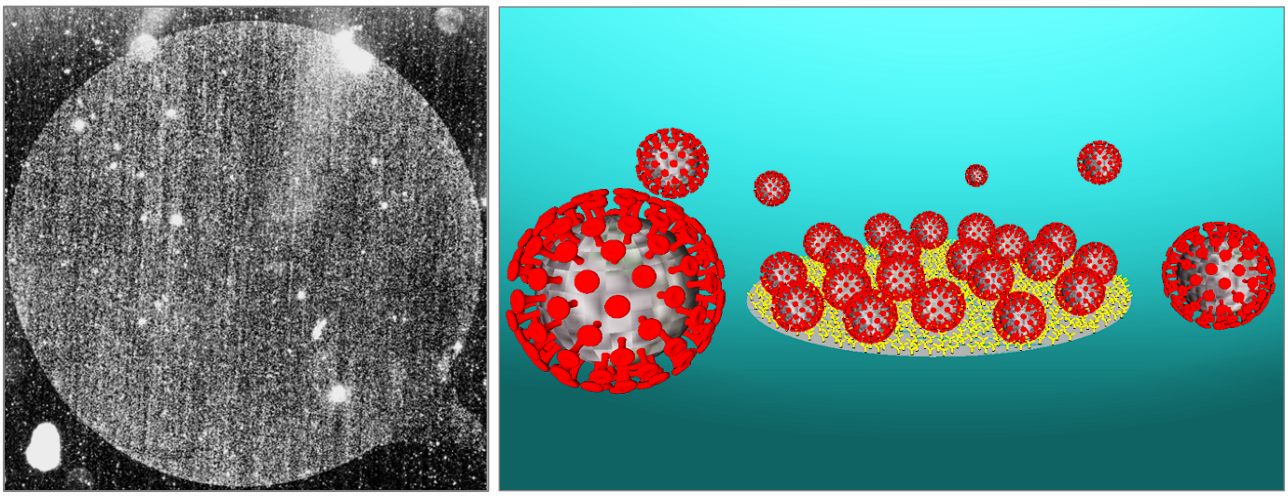
		Actual values	
		Positive	Negative
Predicted values	Positive	TP 69	FP 2
	Negative	FN 101	TN 19

Table 2

Panel of 170 swabs RT-PCR positive with related Ct values, and corresponding DVC assay results with positive/negative outcome.

RT-PCR positive (Ct values)	DVC	
No. of swabs	No. of Positive	No. of Negative
5 (14)	3	2
9 (16)	4	5
13 (17)	8	5
27 (18)	14	13
32 (19)	16	16
31(20)	12	19
13 (21)	5	8
20 (22)	3	17
9 (23)	2	7
7 (24)	1	6
4 (25)	1	3

In the DVC assay the signal intensity into the spot area relates to the number of analytes captured, that are represented by SARS-CoV-2 intact virions. Each data point is obtained by subtracting an average of the intensity value inside the spot, the signal, with one obtained in the area surrounding the spot, the background (Fig. 2). The intensity value of the signal in the images can depend on several criteria, such as laser power and camera acquisition parameters, in particular exposure time and digital gain. We discounted spurious signals due to impurities and to non-specific adhesion of SARS-CoV-2 whole virions outside the spot region.



a) b)

Fig. 2. a) Spot positive to the DVC assay at 4×. b) Representation of virions in solution docking on ACE2 spot.

As expected, testing the DVC a gradual trend of decrease (Fig. 3) in the light scattering signal of detected virions was noted, when Ct values increase. However, inhomogeneous values of signal intensity have been observed in positive samples having the same Ct value. A detailed discussion of these results is reported in the following section.

Hereafter, we show, at the spot edge, several representative images captured at 20× magnification by NED-VD of spots positive to DVC assay at various Ct values (Fig. 3 a-h), and of a negative spot, (Fig. 3 i).

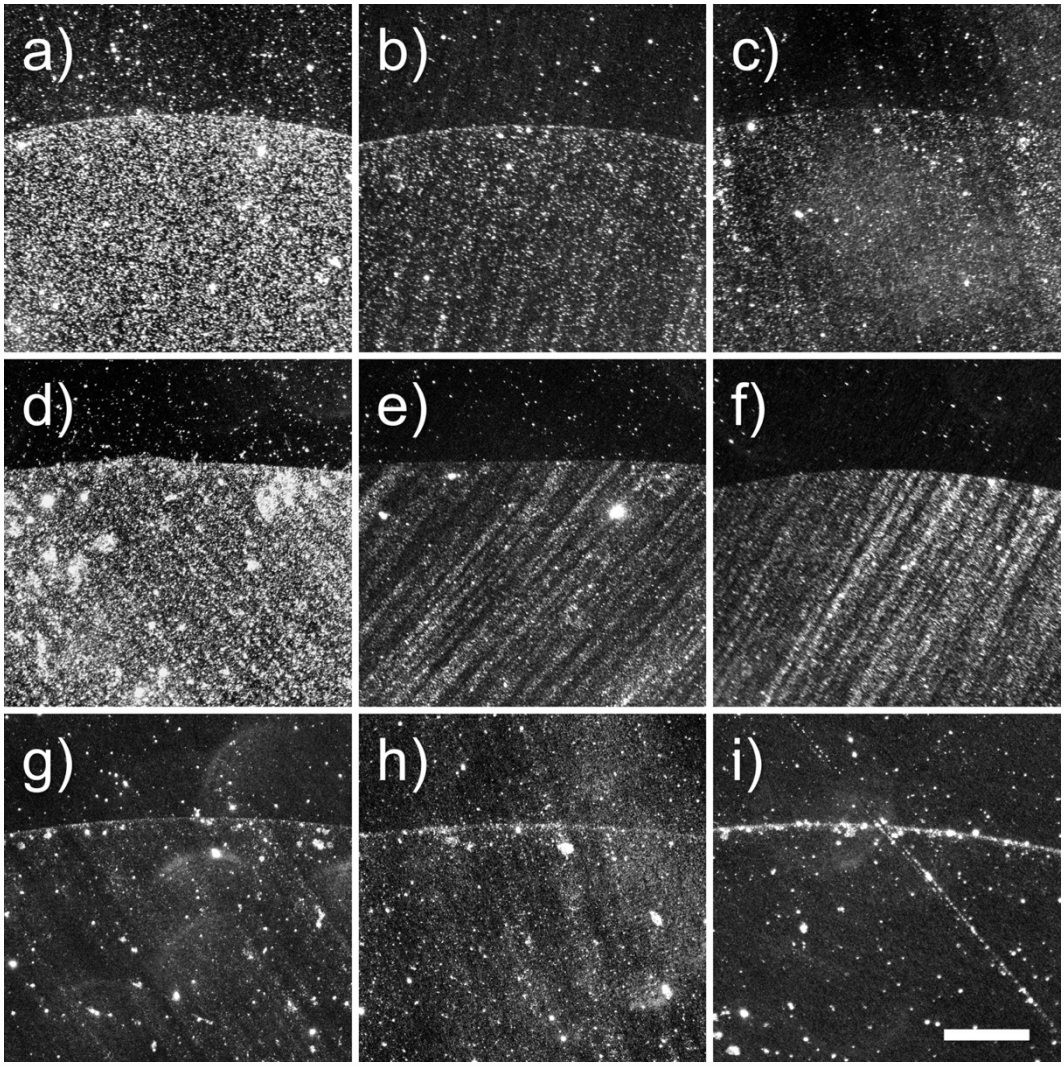


Fig. 3 a-h) NED-VD images of spots edge observed at 20× magnification after incubation with positive samples with corresponding Ct values of 14, 16, 17, 18, 19, 20, 21, 23, respectively; i) NED-VD image of a spot after incubation with negative sample. The scale bar is the same in all images and corresponds to 100 μm.

The DVC assay demonstrate better concordance with PCR results up to Ct values of 21 (corresponding approximately to 10^7 copies/ml), then this value decreases gradually (Fig. 4) mainly due to the biophysical constraints implicit in the methodology adopted. Over Ct values 22 the concordance decreases, presumably due to the lower probability of having of SARS CoV-2 whole virions (Singanayagam et al., 2020, La Scola et al., 2020, Gniazdowski et al., 2021, Basile et al., 2021) and by the limit of detection (10^7 viral particles/mL) of the DVC assay. Although there was an overall better concordance between specimens DVC positive and RT-PCR positive with lower Ct, data were somewhat evenly distributed across all RT-PCR Ct values. This finding might suggest that the number of whole virions is only grossly associated with the total viral RNA load.

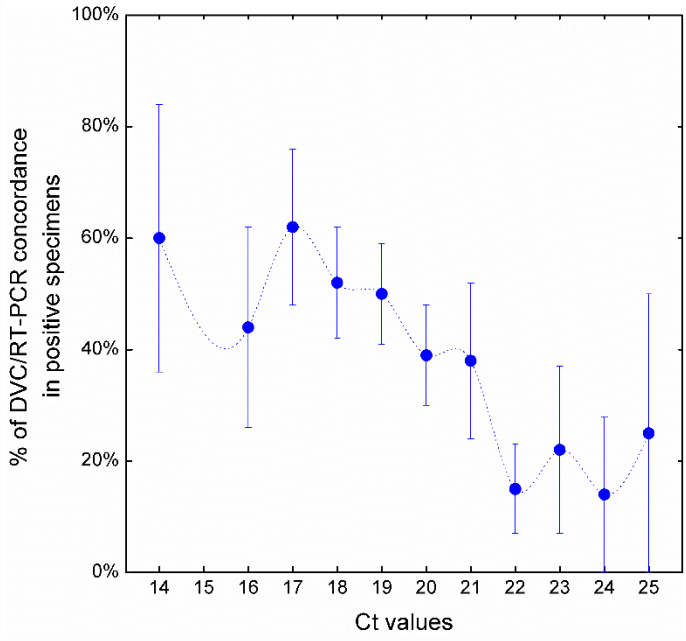


Fig. 4. Graphical evaluation of the DVC assay concordance in % with RT-PCR test based on 170 human specimens resulted positive with Ct values ranging from 14 to 25.

4. Discussion

Here, we present a fast, viral variant independent, label-free biosensor able to detect through light scattering intact SARS-CoV-2 virions in human clinical samples. We showed an expected decrease in concordance between DVC positive test and RT-PCR as Ct values increase and we noticed a large variability of spot intensity within the same Ct values, as reported in the results section. In particular, these findings occur either at low Ct values or high Ct values. While studies have revealed prolonged shedding of SARS-CoV-2 RNA in respiratory and stool samples, viable virus appears to have a short lifespan (Cevik et al., 2021, Piralla et al., 2021, Zou et al., 2020, Aranha et al., 2021, Mercer, T. R., Salit, M., 2021, Tom, M. R., Mina, M. J., 2020). Since the target of the DVC assay is the detection of SARS-CoV-2 virions, the well-known prolonged presence of viral RNA and the virus's short life in biological samples can explain direct non-correspondence between Ct values from RT-PCR and the DVC assay. In fact, the Ct value is not an absolute indicator of infectivity because is not a measure of the number of virions present within a sample (Singanayagam et al., 2020, La Scola et al., 2020, Gniazdowski et al., 2021, Basile et al., 2021). Indeed, the RT-PCR test amplifies and detects specific RNA sequences of SARS-CoV-2 present within the sample in different forms (Yu et al., 2020) and the Ct value is actually considered indicative of the viral load by measuring RNA copies / mL (Suo et al., 2020), but the presence of whole virions, potentially infectious, cannot be inferred solely from this value. It is clear that it will never be possible to achieve 100% concordance with the gold standard technique simply because RT-PCR test provides viral load by Ct value, while the DVC test is able to directly measure the intensity of a spot that is proportional to the number of virions within the swab. Thus, it would be more useful to compare the DVC assay with similar techniques, such as viral culture.

Gathering more information about the symptom onset and the clinical status of the patient at the time when swabbed could be of help to understand the real usefulness of DVC test in clinical practice, however, it was beyond the scope of this study.
Other causes of non-correspondence between Ct values and DVC assay could be caused by different testing modes or systems of sample transport, that can influence either presence and concentration of intact SARS-CoV-2 virions within the human specimens, and thus the true sensitivity of DVC test. Therefore, to carry out the DVC assay, it is important to handle SARS-CoV-2 samples carefully to avoid damaging the virus. In order to limit degradation of spike proteins, rupture of capsids, and in general to minimize loss of virions during collection, a proper conservation and transportation of the swab and good laboratory practices must be adopted by the clinician. Is well known that only intact SARS-CoV-2 virions are capable of entering host cells and replicating, rendering the patient infectious (Jefferson et al., 2021). Based on this understanding, we argue that the detection of intact virions is a critical factor in accurately identifying individuals who may be transmitting the virus (Jefferson et al., 2021). In a commercial context, we hypothesized the deployment of this new assay in an automated process considering DVC as preliminary test, thanks to its simpler and faster approach: this could help to preliminarily screen out up to more than 40% of all patients under investigation resulted positive to RT-PCR, saving costs for the community and, moreover, DVC testing could furnish indications on early treatments and on the control of the viral transmission and its spread in the population. For a potential clinical validation of the DVC assay, further research is required to determine its true and improve its sensitivity is necessary. Since DVC is a label-free method, impurities and non-specific adhesion of virions, or their fragments outside the spot region, can act as background noise, reducing the detection sensitivity. In this regard, antibody-oriented immobilization by use of aptamers may improve the sensitivity of DVC assay, reducing non-specific binding and increasing receptivity of the biosensor, as well as the signal-to-noise ratio of the assay (Gao et al., 2022). Some improvements could be obtained in several ways, such as by increasing concentration of virions by ultracentrifuge. Further matching the spot area to the size of the bottom of the well, we can reduce the sample volume and consequently the dimensions of the well. In fact, the bioactive area of the ACE2 spot (1-2 mm in diameter) covers only a small fraction area of the bottom of the well in which the reaction occurs. The bottom of the well has an area of 28.2 mm^2, about 14 times bigger than the spot area, and since the analyte is ubiquitous in the sample volume, its dispersion is unavoidable, especially if the volume under examination is not reduced to a few microliters (3-5 uL) and if a laminar flow regime is not introduced by means of microfluidics. At the present, all these solutions will be considered for the future of this new unique biosensor, as well as automation of the whole process.

5. Conclusions

The DVC assay detects SARS-CoV-2 virions within swab specimens collected from UTR achieving 40.5% sensitivity, specificity of 90.4% and accuracy of 46% compared to the gold standard RT-PCR assay. Today, for routine clinical detection, viral load refers to the total amount of virus particles in a sample expressed as the number of viral RNA copies per milliliter (Suo et al., 2020). It is important to note that the measurement of viral RNA by RT-PCR includes both infectious and non-infectious particles (Kalamvoki, M., Norris, V., 2022) and the Ct values are not a direct measure of the number of whole virions present within a human swab sample (Singanayagam et al., 2020, La Scola et al., 2020, Gniazdowski et al., 2021, Basile et al., 2021). In fact, the Ct value is indicative of the total amount of viral RNA in the sample, but the presence of only infecting and infectious intact virions cannot be deduced from this value. Nevertheless, the closest result the scientific community could aspire to a clear viral load definition is the detection and counting of all viral particles present in a sample. Moreover, this must be sustained by clear evidence that viral particles are capable of replicating in culture cells to produce its progeny (Jefferson et al., 2021). Comparison tests between DVC assay and viral culture may demonstrate that the virions detected by the NED-VD are infectious, and, by verifying this, the DVC assay could be used also as an alternative to viral culture, due to its rapid and easy preparation assay. Finally, we here propose a new virological method, fast, variant independent and label-free, for detecting SARS CoV-2 virions, thus providing additional information about the direct presence of the whole pathogen and the state of infection of the patient. Lastly, we can assume that the detection of virions through DVC assay can be extended to all spherical viruses having a signal scattering detectable by NED-VD, which means 40 nm in size for virions.

CRediT authorship contribution statement

Alessandro Pennesi: Writing - Original Draft, Data Curation, Visualization. **Guglielmo Ferrari:** Validation, Investigation. **Federica Giardina:** Validation, Resources, Investigation. **Sara Piselli:** Investigation. **Roberto Lo Savio:** Formal analysis, Writing - Review & editing. **Adolfo Carloni:** Conceptualization, Methodology, Writing - Review & editing, Project administration. **Stefania Paolucci:** Supervision, Project administration. **Fausto Baldanti:** Supervision, Project administration.

Declaration of Competing Interest

No competing interests**.**

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