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Clinical Evaluation of a Fast, Sensitive, RT-Lamp SARS-COV-2 Test for the Point-of-Care, Integrating Solid Phase Extraction, with all Reagents Lyophilized

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Abstract

Today, several point-of-care COVID molecular tests are available, but none integrates, on a compact low-cost device, solid-phase RNA extraction, and RT-LAMP amplification, with all reagents freeze-dried on it, a configuration easier to deploy on a large scale. The objective here is to evaluate the sensitivity and specificity of such a test (called 'COVIDISC') in a clinical retrospective study (99 patients), covering a broad range of viral loads (CT varying from 17 to 33, from 5 to 2.106 GC/ µL of the sample). We found a 97% sensitivity and a 100% specificity.

Key words: Point of Care; LAMP; NAAT; Paper microfluidics; SARS-COV 2; RNA; Testing; Diagnostics.

BACKGROUND

Over the last eighteen months, several spread models of COVID have been reported [1-3]. They represent valuable inputs for elaborating efficient testing strategies. One question of interest addressed in these models, and much relevant technologically, is the influence of testing frequency and test sensitivity on the spread rate mitigation of the disease. RT-PCR is the most sensitive test, but, being time-consuming and costly, it cannot be performed at high frequencies and thus may miss a significant number of contaminated cases. On the other hand, antigen tests (such as BinaxNow), are much less sensitive [4], but being low cost and simpler to deploy, can be performed more frequently. Models proposed different trade-offs. From these modeling studies, one may suggest, although no quantitative analysis has been done yet, that the development of new molecular tests, as sensitive as RT-PCR, and whose costs and simplicity of use are comparable to antigen tests, could substantially alleviate the situation. In this spirit, several nucleic acid amplification tests (NAAT) have recently been proposed [5], following up an effort initiated one decade ago [6]. Most of these NAATs are based on isothermal amplification, Loop-Mediated Isothermal Amplification (LAMP), for which, now, large documentation is available [7]. RT-LAMP reaction takes place at a constant temperature (65°C) and thus does not need a thermocycling machine. However, to reach performances comparable to the gold standard (RT-PCR), nucleic acid extraction is required. Today, extraction is traditionally made in spin column-based RNA extraction. The process, involving centrifugation and several pipetting steps, is difficult to accommodate with the low-cost Point of Care

(POC) constraints. To circumvent the difficulty, several RT-LAMP tests, targeting the POC market, have reduced sample preparation to heating or chemical treatment [8,9]. These simplifications are indeed interesting from a POC viewpoint, but in all cases, and unsurprisingly, they led to a drop in sensitivity [10]. By standing significantly below the gold standard, and slightly above the antigen test, the question of the competitive positioning of these tests in the diagnostic landscape is raised.

Here, we develop a new molecular Point-Of-Care (POC) RT-LAMP test, that has the potential to improve significantly or change the situation. The main novelties are the integration of a solid-phase extraction on the device and the lyophilization, on it, of all components needed to perform RT- amplification. The idea is that solid-phase extraction (with fluids driven by capillarity forces, instead of centrifugal forces) allows reaching high sensitivities, while freeze-drying facilitates transportation and storage. In a recent work [11], we tested the concept on a laboratory all-paper system. Since then, we engineered a compact device, called COVIDISC, in which we modified the design and the way how fluids are manipulated, keeping the biological process the same and the cost at a low level. Here we investigate the clinical performances of a series of prototypes of this device. The goal of the present work is thus to assess the clinical performances of this new test, i.e. its sensitivity and specificity, based on a cohort of patients spanning a broad range of viral loads, down to 5 GC/µl of the sample.

METHODS

COVIDISC Description

The COVIDISC consists of two plastic disks, 8.5 cm in diameter, able to rotate around a common axis (see Figure 1). The device performs SARS-CoV-2 RNA detection (Orf1a/b gene) [12] and human 18S RNA detection, thus integrating a positive control. Silica membranes, cut in form of small disks, are placed in lodgings located in the upper plastic disk. These membranes are dedicated to performing nucleic acid extraction. A large absorbing

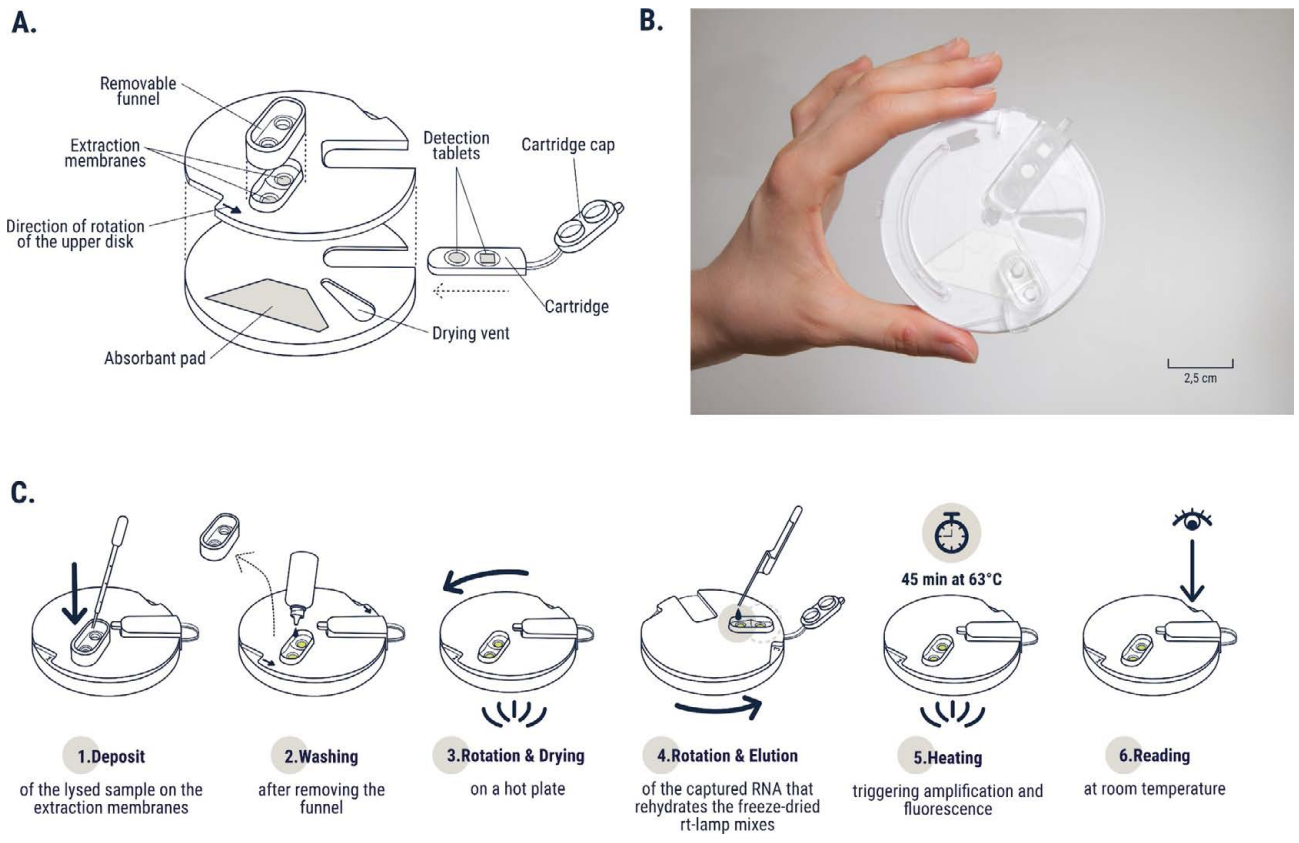


Figure 1: (**A)** Exploded view of the COVIDISC **(B)**. COVIDISC photograph. (**C)**. COVIDISC workflow: 1 – Sample lysis at 65°C before sample injection. 2 – Washing of the extraction membranes. 3 – Upper disk rotation and drying at 65°C. 4 – Upper disk rotation and RNA elution. 5 – Upper disk counter- rotation and closing of the cartridge. 6 – 45 min heating and readout at room temperature.

pad is placed on the lower plastic disk. It is dedicated to pulling, by capillarity, the fluids through the capture membranes, during the extraction step. A removable funnel guides the fluids and inhibits contamination. In another lodging, a capsule contains two additional membranes, called detection or reaction membranes. In them, the RT-LAMP reagents are freeze-dried [11]. The time-life of the lyophilizate is several months (data not shown). One lyophilizate includes the SARS- CoV-2 RT-LAMP mix, deposited on the circular membrane, and the other contains the human 18S RNA RT-LAMP mix, deposited on the square-shaped membrane. Both membranes are placed in a removable capsule (see Supplementary Figure 1A).

COVIDISC Fabrication

The COVIDISC is manufactured by thermoplastic injection molding (Protolabs, France). The lower part of the COVIDISC and the removable funnel is made of white polypropylene. The cartridge and the upper part of the COVIDISC are made of transparent polypropylene.

COVIDISC Protocol

The workflow is shown in (Figure 1) after being mixed, at 65°C, with a chaotropic lysis buffer, the sample is injected in the extraction unit, i.e. the two extraction membranes, placed in contact with the pad, through a funnel that guides the fluids and inhibits wall contamination. After the sample injection is achieved, the funnel is removed, and the membranes are rinsed with 400µL of a 70% ethanol solution, further dried at 65°C for 15 min. Then the disc is rotated, bringing the two extraction or capture membranes (one for the sample, the other for the positive control), in contact with the two reaction membranes. The nucleic acids captured in the extraction membranes are eluted into the reaction membranes, using an aqueous solution. The eluates, driven by capillarity, imbibe the reaction membranes and thus hydrate the freeze-dried RT-LAMP reagents. After heating at 65°C for 45 minutes, the capsule is removed, placed on a visualization device, composed of a blue LED screen and an orange filter (Blue Light Transilluminators), and imaged with a USB camera (Dino-Lite). To avoid issues raised by the colorimetric phenol-red LAMP products (acidic conditions of samples leading to false positives) [13], we coupled our RT-LAMP reaction to a specific fluorescent-probe-based method [14], called QUASR. A minimal fluorescent reader and a smartphone camera are then sufficient for reading the result of the test. With the sample and buffer manipulations included, a total time of, approximately, one hour is needed to perform the test.

Image Analysis

In order to discriminate the positives from the negatives, we developed two algorithms. In algorithm 1, we define, for each image of the reaction membrane, a quantity equal to the average to the 1%

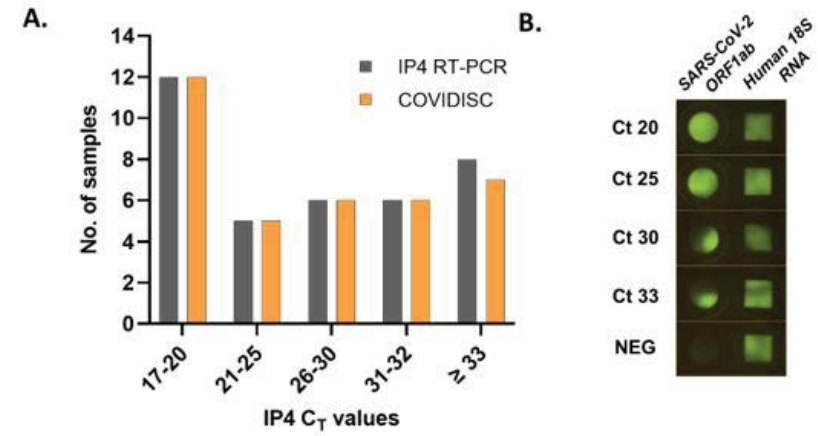
highest intensity levels. By comparing this quantity to the background intensity, we declare the test positive or negative. Between 0.1% and 5%, results were not critically dependent on the fraction of percentiles we choose. This algorithm mimics the direct naked-eyes readout, which is possible with our system, thanks to the high fluorescence intensity of the positive cases. Data obtained with Algorithm 1 is shown in Figure 2, the detail is given in Supplementary Materials, 'Image analysis with Algorithm 1'). In Algorithm 2, we define a Covidness score by analyzing the intensity level distributions emitted by each reaction membrane. This method will be reported later.

Clinical Samples

Clinical samples consist of 99 nasopharyngeal swabs resuspended in a Universal transport medium (UTM, Copan 330C, VPM ImproviralTM, and VTM Sigma-Virocult®). Those samples are obtained from hospitalized patients or from patients consulting emergencies in a period extending from 30th March 2020 and 14th August 2020 (period with a positivity rate between 20 and 50 %) at Robert Ballanger Hospital and stored at -80°C at the day of collection. 99 samples were randomly chosen from the entire collection. RT-PCR of the 99 samples was performed at CNR (Center National of Reference), using a protocol described in [15]. The RT-qPCR analysis showed that the 99 clinical samples included 37 positive samples, with RT-PCR cycle threshold values (Ct) ranging from 17 to 33 for the targeted RNA- dependent RNA polymerase RdRP gene region (designated as "IP4"). The viral load associated with the CT value of 33 was estimated to be close to 2 genome copies/µL of the sample, based on a quantified standard (Vircell amplirun MBTC030).

Clinical Trial

The 99 COVIDISC were prepared a few days before the clinical trial, which took one day. The 99 samples were defrosted at 4°C. Two 200µL aliquots were prepared for each sample. 8 COVIDISC were simultaneously run.



C.

IP4 Ct Category	COVIDISC (%, 95% CI)	Total
Total positive	36 (97.3, 85.8-99.9)	37
Low (>30)	14 (93.3, 68.1-99.8)	15
Medium (20-30)	16 (100, 79.4-100)	16
High (<20)	6 (100, 54.1-100)	6
Negative	62 (100, 94.2-100)	62

Figure 2: A: Comparison between COVIDISC and IP4 RT-qPCR [15]: Histogram of the tests declared positive by PCR (dark gray bars) and COVIDISC (orange bars), as a function of the cycle threshold (Ct) values provided by IP4-qPCR [15]. For clarity, samples have been clusterized in groups

Ethical Statement

The study was approved by National Institute of Health and Medical Research (INSERM) Ethics Evaluation Committee, the Institutional Review Board (IRB00003888).

Characterization of the analytical Sensitivity and Specificity of the COVIDISC on nasopharyngeal and saliva matrices.

The analytical limit of detection (LOD) of the COVIDISC was determined by using a quantified control (Vircell amplirun MBTC030), in which inactivated viral particles of known concentrations were spiked in a nasopharyngeal matrix. The analytical LOD was estimated to be 3.2 genome copies per µl (GC/µl) (see Supplementary Figure 1A). The specificity against a series of viruses, on all-paper systems, using the same workflow as the COVIDISC, was 100% XXX. Similar characterizations carried out in nasopharyngeal (Supplementary Figure 1B) and saliva matrices (Supplementary Figure 1C) led to sensitivities equal to 3.5 ± 1.5 genome copies per µl (GC/µl) of the sample.

RESULTS

(Figure 2A) shows the essential result of our work, i.e. the comparison between COVIDISC and RT-PCR. On the figure, the CT values of the 37 positive samples, obtained by IP4 RT-PCR, span from 17 to 33, i.e. from 2.106 to 2 GC/µl. Important to note, the set of samples shown in Figure 2A includes a substantial number of low viral loads. To establish (Figure 2A), we used Algorithm 1, whose results are consistent with the naked eye. COVIDISC detects 36 positive samples out of 37, leading to a clinical sensitivity of 97.3%. The false negative is close to our analytical LOD (Ct 33, around 5 GC/µL); this probably explains why we did not detect it. The remaining 62 samples, declared negative by real-time RT-PCR, were also found negative by the COVIDISC, leading to a specificity of 100%.

To provide more detail on the test, (Figure 2B) shows the fluorescence emitted by the reactive disk, after amplification, for positive samples of various viral loads (CT values of 20, 25, 30 and 33) and, for the sake of comparison, one negative sample. The RNA 18S positive control (square-shaped) are unambiguously positive. In

the disks (which contain the samples), and in the positive cases, the fluorescence signals are sufficiently strong be visualized with the naked eye. The samples with the largest viral loads (associated to RT-PCR CT values of 20 and 25) produce an emission of fluorescence homogeneously spread on the reaction disk area. For samples with the lowest viral loads (associated to RT-PCR CT values of 30, 33), the fluorescence is still high, but it is localized inside spots occupying a fraction of the disk area. This fraction decreases with the viral loads. We hypothesize that the phenomenon is due to a nucleation process: when the viral load is small, a small number of RNA strands are present in the reaction disk (we estimated this number between 10 and 30 at CT=33), and, consequently, the spreading of the reaction throughout the entire area gets subjected to statistical uncertainty. In all cases, whether spotty or homogeneous, the detection of the positives can be clearly done, either by eye, or by algorithm 1. Table 1 summarizes the results of our study, in which the statistical errors, due to the limited size of the cohort, are calculated.

DISCUSSION

To summarize, the retrospective clinical study reported here shows that the COVIDISC clinical sensitivity is 97% (85.8-99.9) and its specificity is 100% (94.2-100). In brief, what we show here is that COVIDISC performs as well as RT-PCR platforms. We may add that the device integrates extraction and amplification, uses reagents freeze-dried on the device, and is low cost (the production cost, reagents included, is estimated to 5 €). Important to note, it could also be used with saliva (see Supplementary Figure 2C). These characteristics suggest that COVIDISC could be deployed on a much larger scale than the gold standard (RT-PCR) and, in the meantime, offer much higher sensitivity than antigen tests.

AUTHOR INFORMATION

PT, E.Coz, PG, E.Collin, BR and MM designed the study. E.Coz, PG, EM and DGdN prepared the COVIDISCS. E.Coz, PG, DH, MF, QG and E.Collin, performed the study. E.Coz, PG, SM, J-CO,EBL and A.V analyzed the data. PT, E.Coz and PG wrote the manuscript. EBL, JV, JCM edited the manuscript.

TRANSPARENCY DECLARATION

All authors declared no conflict of interests.

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