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RT-PCR detection of SARS-CoV-2 in nasopharyngeal and salivary specimens: contribution of alternative collection systems and extraction processes to cope with mass screening. Interpretation of low viral loads

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Abstract

Objectives: Due to massive screening of the persistent coronavirus SARS-CoV-2, supply difficulties emerged for swabs and extraction reagents leading to test alternative choices. Quality sampling may have an impact on the result and a low RNA detection may be difficult to interpret because it does not necessarily mean that infectious particles are present in biological samples. There is a need to understand whether the Ct value information is relevant and informative.

Methods: We compared the pre-analytical stability of RNA in saline solution, UTM[®], Amies and Cary-Blair transport media. Expression profile of *E*, *N* and *RdRp* genes was assessed at various concentration levels with the Allplex[™] 2019-nCoV Assay. Factors that may influence the determination of Ct were studied with several extraction reagents coupled to the GSD NovaPrime[®] SARS-CoV-2 RT-PCR testing kit.

Results: Seventy two-hour RNA stability has been demonstrated for all the transport media assessed. A matrix effect was shown, leading to a decrease in the detection of *E* and *RdRp* genes, so that only *N* gene was often found for Ct greater than 35.0. A follow-up over more than 67,000 patients suggests that *N* gene may be a sensitive indicator to detect a new active viral circulation, but establishing a

correlation between a positive threshold and a low risk of infection for a given method remains difficult.

Conclusions: Several transport media and extraction processes are suitable for PCR-based SARS-CoV-2 detection. During periods of active virus circulation, any weakly positive results should be considered.

Keywords: contagiousness; COVID-19; Ct interpretation; RNA; SARS-CoV-2; transport media.

Introduction

The last decade has seen the rise of viral epidemics such as MERS-CoV in the Middle East [1], Ebola virus in West Africa and Zika virus in Latin America [2, 3]. End of 2019, a novel respiratory disease emerged in China with a rapid spread across the globe so that the WHO classified this outbreak as a pandemic in March 2020 [4]. From then on, a race started to develop detection tools for the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in order to manage the patient triage [5, 6].

Coronaviruses are enveloped, positive single-stranded large RNA viruses that infect humans, but also a wide range of animals [7]. The common human coronaviruses NL63, 229E, OC43 and HKU1 are widespread especially throughout the winter months [8]. They are responsible for up to one third of all acute respiratory diseases, typically with mild symptoms. More than 80% of the adult population have antibodies against human coronaviruses. The immunity from previous infections lasts only for a short period of time. Therefore, reinfections with the same pathogen are possible just after one year.

The initial sign of COVID-19 which allowed case detection was pneumonia [9]. But it turned out that the course of the disease is non-specific and varies widely, from asymptomatic courses to severe pneumonia with lung failure and death [10]. End of 2020, the introduction and

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increased spread of new SARS-CoV-2 variants first identified in the United Kingdom (Alpha), South Africa (Beta) and Brazil (Gamma) raised concerns. At the beginning of June 2021, the follow-up of screening results has evolved to replace the notion of variant by the broader notion of mutation. The latter are common to a wide variety of variants and three mutations that may be responsible for an increase in contagiousness or immune escape are currently being investigated in France: the E484K mutation, the E484Q mutation present in the kappa variant identified in India, and the L452R mutation detected in kappa and delta, another Indian strain that has become the majority in Europe in particular [11–13].

The generalization of screening in city and hospital requires substantial material resources. As early as the spring of 2020, shortages of swabs and extraction reagents appeared, which severely limited patient management and screening capabilities. It was essential to rapidly implement accurate diagnostic tests with a quick turnaround time to combat a fast-evolving global pandemic like COVID-19.

RT-PCR is the reference method and has a very high sensitivity to detect very low amounts of viral RNA in clinical samples [14]. However, this sensitivity raises questions about the possible risk of contagiousness of these low viral loads. The weakly positive results involve in particular important issues in terms of patient care in healthcare and healthcare institutions, what to do for asymptomatic individuals, individuals previously tested positive and clinically cured, and patients requiring admission to hospital or care.

The goal of this study was to (i) test alternative transport media to increase sampling possibilities; (ii) assess several combinations extraction/PCR testing kits which answer the speed, scale and high throughput needs; (iii) discuss the signification of low viral loads that can help in the early detection of a new active virus circulation.

Materials and methods

Samples and procedures

All samples were handled under a microbiological security station. Whole salivary specimens were diluted 1:5 (volume per volume) in sterile Amies medium (eSwab™, Copan, Brescia, Italy) and homogenized. Two hundred microliters were used as starting material for RNA extraction. Saliva is a non-invasive, easy-to-use sampling method that is less uncomfortable for the patient and particularly useful for self-sampling applications and the testing of children. For nasopharyngeal swabs, 200 µL of the substrate were taken for analysis.

For the performance evaluations, extracted RNA were tested via one-step RT-PCR using the Allplex™ 2019-nCoV Assay (Seegene, Seoul, South Korea) targeting *E*, *N* and *RdRp* genes, and the GSD

NovaPrime® SARS-CoV-2 (GeneScan, Freiburg, Germany) testing kit which targets two specific regions of the *N* gene. MS2 phage genome for the former and the human ribonuclease P (RNase P) for the latter were used as an internal amplification and sample adequacy control. Amplification of the viral targets with a threshold cycle of less than the appropriate limit of detection (LOD) was considered SARS-CoV-2 positive. Samples were classified as negative when no target gene was amplified whereas the internal control used was successfully detected with Ct of less than 35.0 for GSD NovaPrime® and 40.0 for Allplex™. Samples were considered invalid in other cases.

For the follow-up of patient samples analyzed with the Allplex™ 2019-nCoV Assay between March and December 2020, values were inclusive of any detected Ct measures up to 40.0 (LOD). For a qualitative comparison, data for the whole of France have been collected and are available on the website www.gouvernement.fr/info-coronavirus.

Performance evaluations

The stability of SARS-CoV-2 RNA was evaluated by means of 20 positive specimens in different buffers at room temperature, approximately 21 °C. Four storage media were tested: UTM® (Thermo Scientific™, Illkirch, France), Cary-Blair medium (Sigma Transwab®, Corsham, UK), Amies medium and NaCl 0.9% RNase free (Laboratoire Aguetant, Lyon, France). Tenfold dilutions of positive specimens were contrived by spiking SARS-CoV-2 reference material into a tested negative matrix (in UTM® medium) on the one hand, and into sterile Cary-Blair, Amies, NaCl 0.9% media on the other hand. Extraction step was performed with the multi-channel automated liquid handling system Microlab NIMBUS™ (Hamilton, Bonaduz, Switzerland) which operated with the STARMag™ Viral DNA/RNA 200 C (Seegene, Seoul, South Korea) with a final elution volume of 100 µL. RT-PCR analysis were performed with the Allplex™ 2019-nCoV Assay coupled to the analyzer CFX96™ (Biorad, Marnes-la-Coquette, France).

Several extraction kits have been tested with a magnetic-bead based approach that can easily be automated on most open-ended liquid handling platforms: the Mag-Bind® Viral DNA/RNA (Omega Bio-tek, Norcross, GA, USA), the MGIEasy Nucleic Acid Extraction (MGI Tech, Qingdao, China), the GSD NovaPrime® IVD RNA Extraction AE1 and AE2 (GeneScan, Freiburg, Germany), and the STARMag™ Viral DNA/RNA 200 C mentioned above. Apart from the latter kit, samples and master mix were distributed by the Microlab STARlet (Hamilton, Bonaduz, Switzerland) while the actual extractions were performed either by the MGISP-960 (MGI Tech, Qingdao, China) or by the AU1001 (BioTeke, Wuxi City, China). In addition, the protocols for isolation of viral RNA were adapted from the supplier's proposals, in particular the lysis and elution volumes (90 µL for the latter). Performed with the GSD NovaPrime® SARS-CoV-2 testing kit, RT-PCR analysis were carried out either by the CFX96™ or by the AriaDx™ thermocyclers (Agilent, Santa Clara, CA, USA). The PCR curves were analyzed either with the Biorad Maestro™ or with the FastFinder (Ugentec, Hasselt, Belgium) softwares. There was a manual setting of the threshold line for the former while the Ct was determined by a mathematical algorithm for the latter.

Data analysis

Standard deviations, mean sample Ct values and their 95% confidence intervals (CI) were calculated with the appropriate degrees of freedom

using the spreadsheet application Microsoft Excel®. To interpret a paired two-sided Student's *t*-test, we considered *p* values of less than 0.05 to be statistically significant.

A non-parametric test Kappa allowed to measure the inter-rater agreement for categorical items. Statistical significance has been characterized in the literature [15, 16]. Sensitivity, specificity, accuracy, predictive values and their 95% CI were calculated using the software MedCalc®.

Ethical approval

The research related to human use has been complied with all the relevant national regulations, institutional policies and is in accordance with the tenets of the Helsinki Declaration.

Results

The stability study comparing the Universal Transport Medium (UTM®) with the Cary-Blair and Amies media, NaCl 0.9%, showed satisfactory storage of SARS-CoV-2 at room temperature for at least 72 h (Table 1 and Figure 1).

Due to the shortage of some materials at the beginning of the pandemic, dilutions in reference UTM were performed using pools of samples from tested negative patients. In contrast, the study of the Cary-Blair and Amies substrates was conducted using sterile swabs. Simultaneous detection for *E*, *N* and *RdRp* genes using the Allplex™ testing kit revealed a different profile for low viral loads, Ct close to 35.0, between the pool-UTM medium on the one hand and the Cary-Blair and Amies media on the other. For the highest dilution, the *E* gene was almost never detected in pool-UTM medium, whereas sensitivity remained excellent with the two others. The detection of the *RdRp* gene was satisfactory for up to 24 h for all three media, however the detectability decreased significantly after 24 h in pool-UTM medium with 0 and 1 detection out of 5 on days 1 and 2, respectively. PCR curves corresponding to serially diluted specimens are shown Figure 2.

The detection of the *N* gene offered the best performance with an excellent sensitivity of up to 72 h for the three media mentioned above, as well as for NaCl 0.9%.

Table 1: Comparative Ct of target genes using tenfold dilutions of a viral suspension in UTM®, Amies and Cary-Blair transport media. Means of Ct values are given with 95% interval of confidence (replicates of 5).

Target genes	Hours after spike-in											
	0			24			48			72		
	<i>E</i>	<i>RdRp</i>	<i>N</i>	<i>E</i>	<i>RdRp</i>	<i>N</i>	<i>E</i>	<i>RdRp</i>	<i>N</i>	<i>E</i>	<i>RdRp</i>	<i>N</i>
UTM® 10-1	23.6– 23.8	24.9– 25.3	24.5– 25.3	23.7– 23.9	25.3– 25.7	25.1– 26.5	23.7– 23.9	25.0– 25.2	24.7– 24.9	23.7– 23.9	26.0– 26.4	25.5– 27.1
Amies 10-1	23.0– 23.2	24.9– 25.3	24.6– 26.0	23.4– 23.6		25.1– 26.7	23.2– 23.4	25.1– 25.5	24.5– 26.5	23.3– 23.5	24.9– 25.3	24.6– 25.2
Cary-Blair 10-1	23.3– 23.5	25.3– 25.7	24.2– 25.6	24.0– 24.2	26.0– 26.4	24.9– 26.5	23.9– 24.1	25.8– 26.2		24.2– 24.4	25.6– 26.0	25.7– 26.3
UTM® 10-2		28.1– 28.5	27.6– 29.4	27.0– 27.2	28.4– 28.6	27.9– 28.7	26.4– 26.8	28.3– 28.7	27.4– 29.4	26.4– 26.8	29.0– 29.6	28.6– 29.8
Amies 10-2	26.5– 26.9	28.4– 28.6	28.1– 28.5	26.8– 27.0	28.5– 29.1	28.0– 29.8	26.3– 26.9	28.5– 28.9	28.1– 29.3	26.7– 27.1	28.3– 28.7	27.9– 29.7
Cary-Blair 10-2		28.3– 28.5	28.0– 28.4	27.3– 27.5	28.9– 29.5	27.9– 29.7	27.0– 27.6	28.9– 29.3	28.3– 29.5	26.9– 27.3	28.5– 28.9	27.7– 29.5
UTM® 10-3	29.1– 29.7	30.5– 31.3	30.2– 32.2	29.4– 30.0	31.4– 31.6	30.7– 32.3	28.9– 30.3 ^a	31.2– 31.6	30.9– 31.3	28.9– 30.3 ^a	32.2– 34.8	32.0– 33.4
Amies 10-3	30.1– 30.7	31.6– 32.2	30.7– 32.9	30.6– 31.0	31.9– 32.5	31.5– 32.7	30.6– 31.0	32.0– 32.4	31.4– 33.6	30.0– 30.6	31.4– 32.0	30.8– 32.8
Cary-Blair 10-3	30.3– 30.5	31.7– 32.1	31.4– 32.2		31.8– 32.6	31.8– 32.4	30.5– 31.1	32.1– 32.9	31.3– 32.1	31.4– 32.2		
UTM® 10-4	(na)	34.9– 35.7 ^a	34.4– 35.0	34.7 ^b	35.3– 35.9	34.7– 34.9	(na)	(na)	34.4– 35.0	(na)	35.3 ^b	34.9– 36.5
Amies 10-4	33.6– 34.0	34.8– 37.0	34.2– 37.0	33.7– 34.7	35.0– 36.4	35.2– 37.0	33.9– 34.9	35.3– 36.5	34.5– 36.7	33.6– 34.0	34.8– 37.0	34.2– 37.0
Cary-Blair 10-4	33.7– 34.3	35.4– 36.2	35.6– 36.8	35.3– 36.5	35.8– 37.4	35.9– 36.1	34.5– 35.3	35.9– 38.1	35.7– 36.3	34.2– 35.8	34.6– 35.2	35.1– 35.9

^an=4; ^bwhen statistical analysis cannot be performed (n=1); na, non applicable.

Figure 1: Comparison of the SARS-CoV-2 stability using tenfold dilutions of a viral suspension evaluated in duplicate in NaCl 0.9% and Cary-Blair medium.

Between March and December 2020, the period encompassing the first two waves of COVID in France, nearly 67,000 PCR tests were carried out in the laboratory for *E*, *N* and *RdRp* genes (Figure 3). The vast majority of positive tests showed the three targets at the start of the first containment. Then viral loads gradually decreased from mid-April to mid-June and only the *N* gene was found almost exclusively, Ct often greater than 37.0, after the end of the first containment (11 May 2020).

After a 15-day period in June during which the test positivity rate was close to zero, a resurgence of positive samples was observed in which only the *N* gene was found again. Then, in mid-August, samples with higher viral loads appeared with two or three positive target genes. But at the peak of the second wave in early November, the profile with only the *N* gene remained the majority among

the positive results. It should be noted that the rate of screening increased during the second wave of the outbreak and that the number of positive cases detected in the laboratory changed at the same rate as in France as a whole (Figure 4).

To increase capacity and meet the growing demand for testing, the lab deployed several pairs of extraction kits/PCR GSD NovaPrime® targeting the nucleocapsid (*N*) gene. Comparisons between methods showed consistent and satisfactory performances. The same applied when the CFX96™ and AriaDx™ thermocyclers were evaluated using a same analytical method. Regarding samples, the sensitivity and specificity of SARS-CoV-2 salivary screening compared to pharyngeal sampling were not different (95% CI, Table 2).

On the other hand, there were statistically significant differences in Ct values when comparing some extraction kits, as well as thermocyclers with a given method. The same finding was made regarding the results of salivary tests vs. nasopharyngeal swabs. Of note, the measurement of Ct uncertainties were satisfactory and homogeneous for the different extraction kits/PCR pairs studied (Table 3).

Discussion

During the early stages of the COVID-19 pandemic, high demand for testing reagents necessitated evaluation of alternate specimen transport media like Cary-Blair medium, a common substrate for stool transport. We sought to assess the loss of sensitivity observed up to 72 h of storage

Figure 2: *RdRp* gene PCR curves corresponding to serially diluted specimens towards the LOD.

Figure 3: Profile of SARS-CoV-2 detected genes, year 2020.
*Positive detection of the *E*, *N* and *RdRp* targets with Ct<40.

at ambient temperature, which represents a maximum pre-analytical period beyond which the result would be medically meaningless.

We evaluated the stability of virus-spiked samples at three copy number levels, from moderately high to very low, stored in four different buffers. Detection of

Figure 4: Comparative evolution of the number of SARS-CoV-2 positive cases, year 2020.

SARS-CoV-2 *N* gene was consistent across all media and viral loads. Variation in detection signal for *E* and *RdRp* genes with pool of patient samples tested negative, UTM medium in our study, suggested the possibility of a matrix effect. It should be noted that this reduced detection only occurred in spiked samples with low copy numbers. In our experience these results corroborated observations made from a large number of clinical samples, although improved analytical sensitivities have been reported targeting the detection of *E* and *RdRp* genes [17, 18].

RNA-dependent RNA polymerase (*RdRp*) catalyzes the synthesis of a complementary RNA strand from a matrix

RNA strand and is essential for the replication of SARS-CoV-2. The lack of detection of the gene encoding this enzyme could not be an argument for a lack of contagiousness since an inhibitory effect of clinical samples could be involved, at least with the Allplex™ kit used.

We looked at the role that low viral loads might play in spreading the virus, and finally how to interpret positive results that do not necessarily implies the presence of infectious particles in clinical samples.

Analysis of the first epidemic wave in our lab showed that copy levels were moderately high to high when the first containment was introduced on 17 March 2020, whereas they were lower with a relative peak for the single *N* gene detected during the deconfinement. A low copy number may be a witness of a viral scar without contagious risk and a viral RNA carrier for up to 50 days after contamination is well documented [19]. We observed during follow-up in hospitalized patients that Ct values increased after several days until only one target gene was detectable, which was almost always the *N* gene (Figure 5).

Symmetrically, the same low levels of the *N* gene alone were detected early in the second wave. But the dramatic increase in this outcome profile, quickly followed by the detection of higher copy number, favoured active infections rather than viral scars. Li Y et al. showed that viral loads may be low enough in the early stages of infection to account for up to 63% of false-negative tests [20]. In the study by Ai et al.,

Table 2: Performance of SARS-CoV-2 molecular detection with several extraction reagents and PCR methods.^a

Comparisons	Number of assays	Positive samples			Sensitivity	Specificity	Positive predictive value	Negative predictive value	Accuracy	Kappa
		n	Statistical difference for Ct ?	p-Value						
Mag-Bind®-GSD vs. STARMag™-Allplex™	44	36	Yes	0.02	97.3 (92.1–100)	100	100	87.5 (64.6–100)	97.7 (93.3–100)	54.6 (27.9–81.2)
Mag-Bind®-GSD vs. AE2-GSD	94	13	No	0.1	92.9 (79.4–100)	98.7 (96.2–100)	92.9 (79.4–100)	98.7 (96.2–100)	97.9 (95.0–100)	91.6 (80.1–100)
Mag-Bind®-GSD vs. MGIEasy-GSD	94	21	Yes	<0.001	95.5 (86.8–100)	100	100	98.6 (95.9–100)	98.9 (96.8–100)	97.0 (91.1–100)
AE1-GSD vs. AE2-GSD	94	9	Yes	0.02	90.0 (71.4–100)	98.8 (96.5–100)	90.0 (71.4–100)	98.8 (96.5–100)	97.8 (94.8–100)	88.8 (73.5–100)
AriaDx™ vs. CFX96™ (performed with AE2-GSD)	176	22	Yes	<0.005	84.6 (70.7–98.5)	99.3 (98.0–100)	95.7 (87.4–100)	97.4 (94.9–99.9)	97.2 (94.8–99.6)	88.2 (78.0–98.3)
AE1-GSD (salivary) vs. AE1-GSD (nasopharyngeal)	65	10	Yes	<0.001	83.3 (62.2–100)	100	100	96.3 (91.3–100)	96.9 (92.7–100)	89.1 (74.3–100)

^aValues are % (95% CI); n, number of positives samples.

Table 3: Capacitary, Ct uncertainties and limit of detection.

Methods	STARMag™–Allpex™	AE1-GSD	AE2-GSD	Mag-Bind®-GSD	MGEasy-GSD
Capacitary	72 tests/4 h	376 tests/4 h			
Ct measurement uncertainty 95% CI	±1.1	±1.3	±1.1	±1.4	±1.1
LOD, Ct	40.0	38.0

Figure 5: A case report: tracking of detected genes over time in a patient.

Sainte Marie Hospital, Nice, France.

which had the largest cohort of 1,014 patients with COVID-19, 42% of the RT-PCR results obtained at the initial patient submission were false negative [21].

The epidemiological context thus provides valuable insight for the interpretation of weakly positive cases. In the event of high community-based transmission, the probability of non-contagiousness cannot be low, even in the absence of specific epidemiological risk symptoms or exposures.

However, these low viral loads involve significant challenges in terms of patient management in healthcare and healthcare facilities, the conduct to be followed by asymptomatic individuals (contact cases, screening around clusters), and patients requiring hospital admission without COVID-19 indication. Several studies have been conducted to try to distinguish between high and low risk situations and thus prioritize the efforts and precautions to be put in place. In September 2020, the French National Reference Center (CNR) for Respiratory Viruses evaluated many RT-PCR kits under various analytical systems and compared them to their reference technique, called IP4, targeting the *RdRp* gene. Using the abacus of Ct values obtained in comparison with this reference technique, several levels of viral excretion were defined and recommendations were made by the French Society of Microbiology (SFM) for interpretation of the results: negative ($Ct \geq 37$), positive ($Ct \leq 33$) and weak positive [22]. According to a virological analysis carried

out by a French team in particular, there would be a relationship between the viral load and the infectious potential of individuals with COVID-19. La Scola et al. found a significant relationship between Ct and the isolation of SARS-CoV-2 in culture. Results showed that 100% of samples with Ct between 13 and 17 rings produced positive cultures compared to 12% for samples with Ct of 33 rings [23]. Nevertheless, the authors conceded that these results could not be extrapolated to other laboratories and we have shown that there can be significant variations in Ct depending on the nature of the samples analyzed ($p < 0.001$), the extraction kits ($p < 0.001$ to $p = 0.02$) and the thermocyclers ($p < 0.005$) used for a given PCR testing kit. Moreover, if the laboratory uses software to interpret PCR curves, an additional variation in the result should be taken into account as Ct values are often determined by a mathematical algorithm.

All these observations therefore make it difficult to apply a table of correspondence with a reference method for determining a positivity cutoff. In addition, it has not been established that a person is not infectious if his or her PCR positive specimens cannot be grown.

The interpretation of low viral loads may require consideration of other factors that may underestimate the results. Samples taken from individuals suspected of COVID-19 must be inactivated according to certain protocols and analytical methods to protect laboratory staff. Based on the work of Pan et al., thermal inactivation would significantly reduce the amount of SARS-CoV-2 detectable by RT-PCR ($p = 0.017$) [24]. It was shown that in samples with low viral load, the increase in the detection limit following thermal inactivation was greater than in samples with high copy levels ($p = 0.02$). Viral inactivation by guanidinium-based lysis would have fewer effects on the detectable amount of SARS-CoV-2 than thermal inactivation. Lastly, it should be recalled that pre-analytical elements are likely to undervalue viral load, in particular an inadequate collection procedure [25].

Overall, many factors may influence the determination of Ct, and the establishment of a positive threshold associated with a low risk of infection for a given method remains a challenge for the laboratory. During periods of active virus circulation, any weakly positive case should be considered with the Ct result mentioned in the analytical

report, bearing in mind that is a relative value and cannot be compared with the result from another laboratory.

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