ORIGINAL ARTICLE

Journal of
Applied Microbiology sfam

Flexible multiplex PCR to detect SARS-CoV-2, coronavirus OC43 and influenza A virus in nasopharyngeal swab samples

Eduardo Pelegri-Martinez[1] | Xabier Guruceaga[1] | Leire Martin-Souto[1] |
Ana Abad-Diaz-de-Cerio[1] | Aitor Rementeria[1] | Alazne Dominguez-Monedero[2] |
Mikel Gallego[2] | Oscar Martinez[2] | Eunate Arana-Arri[2] |
Maitane Aranzamendi[2] | Andoni Ramirez-Garcia[1]

[1]Fungal and Bacterial Biomics Research Group, Department of Immunology, Microbiology and Parasitology, Faculty of Science and Technology, University of the Basque Country (UPV/EHU), Leioa, Spain

[2]Biocruces Bizkaia Health Research Institute, Hospital Universitario Cruces, Bilbao, Spain

Correspondence
Aitor Rementeria and Andoni Ramirez-Garcia, Fungal and Bacterial Biomics Research Group, Department of Immunology, Microbiology and Parasitology, Faculty of Science and Technology, University of the Basque Country (UPV/EHU), Barrio Sarriena s/n, 48940 Leioa, Spain.
Email: aitor.rementeria@ehu.eus and andoni.ramirez@ehu.eus

Funding information
Eusko Jaurlaritza, Grant/Award Number: 2020333042 and IT1362-19; University of the Basque Country

Abstract

Introduction: Quantitative reverse transcription PCR (RT-qPCR) is the leading tool to detect severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Given that it will almost certainly continue to coexist with other respiratory viruses in the coming years, our study aimed to design a multiplex PCR system not affected by supplier outages and with reduced cost compared to the existing commercially available kits.

Methods and results: In this study, combinations of four primers/probe sets were used to construct a flexible RT-qPCR assay which is capable of discriminating between SARS-CoV-2 and the seasonal human coronavirus HCoV-OC43, or even influenza A virus. Additionally, the human RPP30 gene was used as an internal control. To demonstrate the robustness of the assay, it was applied to a collection of 150 clinical samples. The results showed 100% sensitivity and specificity compared to the automatized system used at the hospital and were better when indeterminate samples were analysed.

Conclusions: This study provides an efficient method for the simultaneous detection of SARS-CoV-2, HCoV-OC43 and influenza A virus, and its efficacy has been tested on clinical samples showing outstanding results.

Significance and impact of the study: The multiplex RT-qPCR design offers an accessible and economical alternative to commercial detection kits for hospitals and laboratories with limited economic resources or facing situations of supply shortage.

KEYWORDS
coronavirus, influenza A virus, multiplex RT-qPCR, OC43, SARS-CoV-2

INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerged as a novel virus, discovered in Wuhan, China in December 2019 (Wu et al., 2020). The virus is the causative agent of the disease known as coronavirus disease (COVID-19) and was the trigger for the pandemic currently being suffered throughout the world. Up to

March 2022, 456 million cases had been confirmed, including 6.1 million deaths, making COVID-19 an unprecedented global health problem (WHO Coronavirus Dashboard, 2022). During this time, the scientific community has made great efforts to understand the full biology of the pathogen, design diagnostic systems, develop vaccines and find new treatments to halt the pandemic. With regard to the diagnostic systems, the World Health Organization (WHO) recommends mass diagnostic testing to monitor the evolution of the pandemic, especially in view of the uneven distribution of vaccines around the world (Sawal et al., 2021). Thus, many diagnostic methods have been designed, with the detection of nucleic acid as the gold standard method (Chams et al., 2020; Hu et al., 2020; Orooji et al., 2021) recommended by WHO for the diagnosis of COVID-19. Among the great variety of kits available, the most popular are those targeting ORF1a/b, N (Nucleocapsid), E (Envelope) or S (Spike) genes, some of which can detect more than one gene simultaneously (Corman et al., 2020; Jang et al., 2021; Kudo et al., 2020).

Most hospitals and microbiological services use commercially available kits and automatized devices to perform the diagnosis using molecular techniques. While these methods offer speed and efficiency, they depend on the manufacturer's availability. Moreover, they are not affordable for many hospitals with limited resources and areas of the world with less access to healthcare, which can lead to worse COVID-19 outcomes (Jimenez-Solem et al., 2021; Tai et al., 2021). Therefore, it would be of interest to develop an economical alternative to commercial detection kits, which can also be used in situations of supply shortage and for hospitals and laboratories without the necessary resources.

The measures adopted against the virus (primarily mask-wearing and social distancing) have been shown to be very useful not only for restricting the pandemic but also for curbing infections by other micro-organisms such as the influenza A virus, rhinovirus, respiratory syncytial virus (RSV) and other seasonal coronaviruses (Baker et al., 2020; Li et al., 2020; Yeoh et al., 2020). Given that the SARS-CoV-2 is unlikely to be eradicated in the next few years, it will be of paramount importance to have detection tools capable of distinguishing the etiological agent causing respiratory diseases in each patient in order to manage them correctly in the event that the severity of disease requires specific treatment.

Multiplex PCR systems can only detect a maximum of four genes simultaneously. Therefore, in this study, among the epidemiologically relevant respiratory viruses that can coexist with SARS-CoV-2, we have selected two with high prevalence and similar symptomatology to SARS-CoV-2. On the contrary, HCoV-OC43 was the most prevalent coronavirus before the pandemic and it is associated with mild infection of the upper respiratory tract (Dijkman et al., 2012; Graham, 1990; Nickbakhsh et al., 2020). On the other hand, the influenza A virus is the only type A virus known to have caused pandemics. Influenza viruses cause 3–5 million severe illnesses every year worldwide (Krammer et al., 2018). Therefore, a new flexible multiplex RT-qPCR has been designed to detect SARS-CoV-2, HCoV-OC43, and influenza A virus. This will provide a wide diagnostic capability for several diseases in a single, easily accessible, and affordable assay.

MATERIALS AND METHODS

Sample collection and hospital laboratory testing

Samples and data from patients were provided directly by the Basque health system (Osakidetza) and by the Basque Biobank (www.biobancovasco.org). A part of the sample volume was processed using standard operational procedures. Specifically, 150 nasopharyngeal swab samples clearly classified as negative (50) and positive (75) for SARS-CoV-2; and 25 samples classified as indeterminate, as per the hospital criteria, were collected in Universal Transport Medium® (Copan), were first diagnosed for SARS-CoV-2 at the Microbiology Service of the Hospital Universitario Cruces (Barakaldo, Spain).

Sample management at the hospital began with RNA extraction, which was carried out using the semi-automatic system 5× MagMAX Pathogen RNA/DNA kit (Applied Biosystems) and the KingFisher™ Flex Magnetic Particle Processor extractor (ThermoFisher Scientific). The entire process followed the manufacturer's protocol, with the only modification being to reduce the two isopropanol and two ethanol washes to one each. Subsequently, RT-qPCR was performed using the TaqPath COVID-19 CE-IVD RT-PCR Kit (Applied Biosystems) on the QuantStudio 5 Real-time Thermal Cycler (ThermoFisher Scientific), following the manufacturer's protocols. The target genes used by this commercial kit are the N, S, and ORF1a/b.

Prior to transport to the research laboratory, the remaining volume of the samples was inactivated with COBAS omni lysis buffer (Roche Diagnostics GmbH). All samples were anonymized using an alphanumeric code and subsequently registered and transported by the Basque Biobank. All processes were approved by the Ethics Committees of the University of the Basque Country (M30_2020_200) and of the Basque Government (PI+CES-BIOEF 2020–15).

RNA extraction

RNA extraction was carried out using the classical 2-propanol precipitation technique optimized for

SARS-CoV-2 (Guruceaga et al., 2020). Briefly, an aliquot of 600 μl of the inactivated sample was centrifuged for 10 min at 12,000 g and 4°C and the supernatant was collected in a new 1.5 ml tube. The same volume (1:1) of pre-cold (−20°C) 2-propanol (PanReac) was then added, mixed by inversion, and incubated for 10 min on ice. The sample was subsequently centrifuged for 10 min at 12,000 g (4°C) and the supernatant was discarded. The pellet was washed in 500 μl of pre-cold (−20°C) ethanol 75% (PanReac) and centrifuged for 5 min at 12,000 g (4°C). Finally, the pellet was air-dried for 5–10 min and resuspended in 40 μl of RNAse-free molecular grade water (PanReac).

CoV-Multiplex and Flu/CoV-Multiplex

Two multiplex RT-qPCR systems were designed: CoV-Multiplex and Flu/CoV-Multiplex. In the first, CoV-Multiplex, primers and probes specific to the nucleocapsid (N) and spike (S) genes of SARS-CoV-2 (Figure 1a) and RNA-dependent RNA polymerase (RdRP) of seasonal coronavirus HCoV-OC43 were designed. For the second multiplex PCR, named Flu/CoV-Multiplex, we exchanged the S gene of SARS-CoV-2 for the primers and probe specific to the matrix gene (M1/M2) of influenza A virus, enabling us to detect HCoV-OC43, SARS-CoV-2 and influenza A virus in the same assay. In addition, both systems included primers and probes for the human Ribonuclease P protein P30 gene (RPP30). This set acts as an internal control of the test and, due to the design of a specific primer located at the binding site of two exons, serves to monitor the correct performance of RNA extraction and reverse transcription (Figure 1d). Four genes can be detected per reaction, which is the maximum recommended by all multiplex PCR guidelines.

Design of primers and probes

The location of the amplicons of the two genes selected for SARS-CoV-2 detection corresponds to the amino acid sequence A262-T302 (S gene) and A208-T247 (N gene) to avoid interference with the most relevant known mutations of the SARS-CoV-2 genes, as we explained in detail in the discussion (Figure 1b,c).

The genomes of SARS-CoV-2 (GenBank accesion: NC_045512), influenza A virus (NC_026431) and HCoV-OC43 (NC_006213) were downloaded from the NCBI Virus database (National Center for Biotechnology Information, www.ncbi.nlm.nih.gov/labs/virus), which collects information from RefSeq, GenBank and other repositories. Primer3 and IDT qPCR design tool PrimerQuest™ Tool (Integrated DNA Technologies Inc., USA) were used for primer and probe design. ClustalW2.0 (www.ebi.ac.uk/Tools/msa/clustalw2/),

and SnapGene software (Insightful Science; www.snapgene.com) was employed to perform the sequence alignments and search for genome areas that meet the test conditions. All primers were analysed using NCBI BLAST (Basic Local Alignment Search Tool, https://blast.ncbi.nlm.nih.gov/Blast.cgi) to check their specificity for the target gene. In addition, both the Multiple Primer Analyzer Tool (ThermoFisher Scientific) and IDT OligoAnalyzer Tool were used to analyse the primers and probes to avoid the probability of dimer formation, nonspecific amplifications, hybridization between primers or secondary structure formation.

The five primer and probe sets (Table 1) were synthesized by IDT, resuspended in PCR-quality water and aliquoted in 100 μM stocks. Primer/probe mixes for each gene were made in 100 μl stocks with a 400 nM concentration of each diluted in TE (Tris-EDTA, 1X Solution, pH 7.4) (ThermoFisher Scientific). Primers were purified by standard desalting and probes by high-performance liquid chromatography. The probes were labelled with different fluorophores and quenchers for detection as required in multiplex qPCR assays, and the choice was studied carefully to avoid signal crosstalk and possible non-specific detections. The labelling was as follows: HCoV-OC43 (RdRP): HEX-ZEN/IB®FQ; SARS-CoV-2 (N): FAM-ZEN/IB®FQ; SARS-CoV-2 (S): ROX-IB®RQ; Influenza A virus (M1/M2): ROX-IB®RQ; Human (RPP30): Cy5-TAO/IB®RQ.

Positive controls

Human RNA specific for Taqman reactions TaqMan™ Control Total Human RNA (Applied Biosystems) at a concentration of 50 ng/μl was used as a positive human control. The virus-positive controls were designed by our group as 1000 bp containing the sequence fragment selected as the target and synthesized by GenScript Biotech. Specifically, the HCoV-OC43 construct was created with the GenParts™ Elite DNA Fragments tool and the controls for the M1/M2 of influenza A virus and the N and S of SARS-CoV-2 were synthesized with the DNA Synthesis Services tool. Both constructs were tested using NGS (Next Generation Sequencing) by Eurofins Genomics. All constructs were amplified and purified with PureLink™ Quick Gel Extraction & PCR Purification Combo Kit (ThermoFisher Scientific). To calculate the limit of detection, nCoV-2019 viral RNA at a concentration of 10,000 copies/μl (Institute of Virology) was used.

PCR conditions

All tests were performed with Bio-Rad® CFX96 System and CFX Manager™ Version 1.6 software using 96-well

FIGURE 1 (a) Genome of SARS-CoV-2. F, forward primer; P, probe; R, reverse primer. (b) Amino acid sequence showing the corresponding amplification region of the primers and the probe of the multiplex systems. (c) SARS-CoV-2 variants with the most characteristic mutations of each. Alpha, B.1.1.7 (UK); Beta, B.1.351 (South Africa); gamma, P.1 (Brazil-Japan); Delta, B.1.617.2 (India); epsilon, B.1.427/B.1.429 (USA). (d) Exon-exon design of the reverse primer in the human RPP30 gene present in the CoV-multiplex and flu/CoV-multiplex systems. 1–14, exon region of the gene.

plates MicroAmp® Optical 96-Well Reaction Plate and MicroAmp® Optical Adhesive Film (Applied Biosystems). For the reverse transcription and quantitative PCR process, we used the NZYSupreme One-Step RT-qPCR Probe Master mix (2×) (NZYTech). PCR reactions were performed in triplicate in 10 µl of which 5 µl corresponded to NZYSupreme Mastermix, 2 µl to all primers and probes (0.5 µl of each gene assay ×4) and 3 µl to extracted RNA. Nuclease-free water was used as a negative control. The following cycling conditions were applied: cDNA synthesis step (20 min/55°C), polymerase activation (3 min/95°C), and subsequently 40 cycles of denaturation (5 s/95°C) and annealing/elongation (50 s/55°C).

For CoV-Multiplex analysis, the samples were considered positive for SARS-CoV-2 when the cycle threshold (Ct) value was ≤35 in both N and S genes, negative when there was no amplification or Ct > 35 in both genes, and

finally, indeterminate when only one of the two genes was positive. Similarly, for the Flu/CoV-Multiplex system, samples were considered positive for SARS-CoV-2 when the Ct value ≤35 in the three replicates of the N gene, negative when Ct > 35, and indeterminate when one or more replicates did not match. This cut-off value was chosen based on previous studies with Bio-Rad® CFX96 System and the individual performance of each primer/probe set (data not shown).

Sample priming

In order to determine the detection capability of our systems, human samples from healthy individuals were primed with the synthesized positive controls. For this purpose, a total of 15 samples were divided into three

TABLE 1 Selected design of primers and probes

Organism	Target gene	Primer	Sequence (5′–3′)	Length (bp)	Tm (°C)	Amplicon (bp)	Localization
HCoV-OC43	RNA-dependent RNA polymerase (RdRP, ORF1ab)	Fw	TATGGTGGTTGGGACGATATG	21	57.2	98	(15.086–15.183)
		Rv	GGCATAGCACTATCACACTTAG	22	56.4		
		Probe	TGTTGACAATCCTGTACTTATGGGTTGGGA	30	67.7		
SARS-CoV-2	Nucleocapsid (N)	Fw	GCTAGAATGGCTGGCAATGG	20	59.3	119	(28.894–29.013)
		Rv	GTGACAGTTTGGCCTTGTTGT	21	59.5		
		Probe	TGCTTGACAGATTGAACCAGCTTGAGAGCA	30	68.3		
SARS-CoV-2	Spike (S)	Fw	GCTGCAGCTTATTATGTGGGT	21	58.7	123	(22.346–22.467)
		Rv	CGTACACTTTGTTTCTGAGAGAG	23	57.2		
		Probe	ACAGATGCTGTAGACTGTGCACTTGACC	28	66.7		
Influenza A virus	Matrix protein (M1)	Fw	CTTCTAACCGAGGTCGAAACGTA	23	60.1	121	Segment 7 (7–127)
		Rv	TGAGAGCCTCAAGATCTGTGTTC	23	60.1		
		Probe	TCAGGCCCCCTCAAAGCCGAG	21	66.5		
Human	Ribonuclease P protein P30 (RPP30)	Fw	CTTCAGCATGGCGGTGTTTG	20	60.4	101	10q23.31 (90.871.979–90.874.880)
		Rv	CTGAATAGCCAAGGTGAGCG	20	58.7		
		Probe	TTCTGACCTGAAGGCTCTGCGCG	23	67.2		

groups and a known concentration of the corresponding positive control was administered to each group. After the RT-qPCR assay had been performed, the detection capability and specificity of the positive control using the designed techniques were tested.

Limit of detection

The detection limit was determined by performing serial dilutions of SARS-CoV-2 RNA (Institute of Virology, Charité). SARS-CoV-2 viral material was provided at a concentration of 10,000 copies/μl, from which the following serial dilutions were prepared: 10,000, 2000, 400, 80, 16, 3.2 and 0.6, and the limits of detection of the N and S genes were studied. To avoid possible degradation of the RNA, RNAase-free tubes were used. Viral RNA was analysed in triplicate and both multiplex RT-qPCR assays. The limit of detection was defined as the concentration of the lowest dilution that can be detected with a probability of >95% and was determined by probit analysis.

Amplification efficiency

Amplification efficiency (E) was determined for all primer/probe sets using the slope of the linear regression line obtained by plotting Ct values versus the logarithm of the concentrations ($\log_{10}$). For this purpose, serial decimal dilutions of each of the genes from the two multiplex PCR systems were performed. An E of 100% means that the number of target sequence molecules is doubled during each replication cycle, although efficiencies of between 90% and 110% are considered acceptable. The E value was calculated using the following equation:

$$E = 10^{(-1/a)} - 1$$

E, amplification efficiency; a, slope of the standard curve, plotted with the Y axis as Ct and the X axis as log (quantity). Results were then multiplied by 100 to express them in percentage.

Statistics

The linear regression analyses used for standardization of the CoV-Multiplex and Flu/CoV-Multiplex systems were performed with GraphPad Prism 7 software. The methods described by Mackinnon (2000) were used to determine the sensitivity (Sn), specificity (Sp), positive predictive value (PPV) and negative predictive value (NPV) of the multiplex RT-qPCR systems. Finally, to study the limit of detection of N and S genes with our systems, probit analysis was used, as per the procedures of Vaks for calculating the limit of detection in molecular diagnostic methods (Vaks, 2018).

RESULTS

In this study, specific primers and probes were designed for the N and S genes of SARS-CoV-2, the RdRP of the

HCoV-OC43, and the M1/M2 of influenza A virus, as well as for the human RPP30 gene as a control. As explained before, combinations of four of these were used to construct the CoV-Multiplex and Flu/CoV-Multiplex assays.

Standardization of CoV-multiplex

The CoV-multiplex system was designed to discriminate the SARS-CoV-2 from the most epidemiologically relevant seasonal coronavirus, HCoV-OC43 since the symptomatic similarity of the two can lead to erroneous diagnoses. For this purpose, the system designed includes primers and probes for the RdRP (HCoV-OC43), N and

S (SARS-CoV-2) and RPP30 (human) genes. To standardize the system, serial dilutions of the positive controls of RdRP, N, S and RPP30 genes were performed and tested in both singleplex and multiplex conditions. These assays were also used to calculate the amplification efficiency (E) of each of the primer/probe sets (Figure 2).

The results obtained showed that there were no evident variations in Ct values in either condition. In addition, we also observed excellent results in efficiency ($100\% \pm 10$) and coefficient of determination (R^2) values in the four genes. Indeed, primer/probe sets performed almost identically in both singleplex and CoV-Multiplex systems. This shows that the probe/primer sets are perfectly suited for multiplexing over a wide range of concentrations.
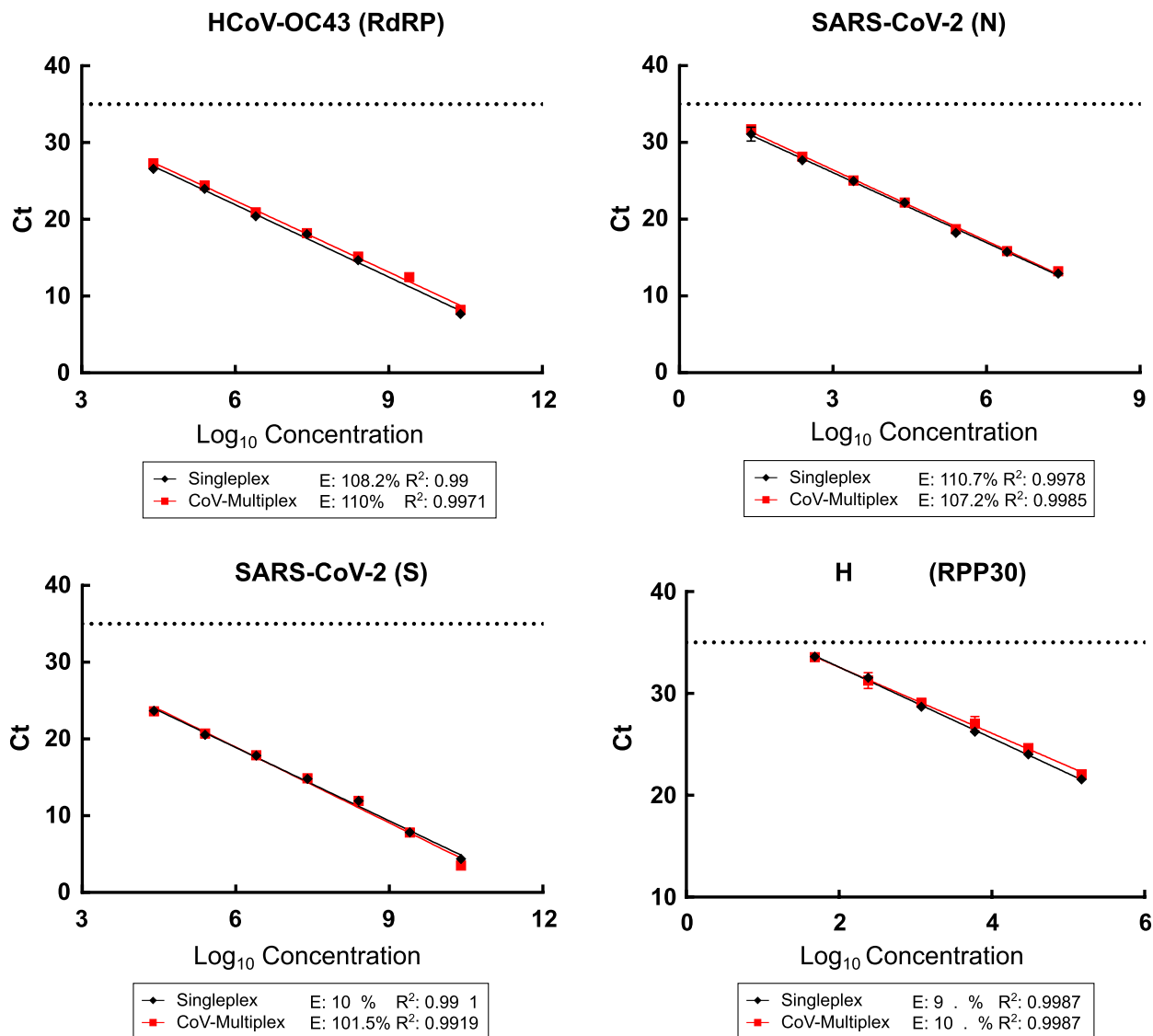

FIGURE 2 Amplification study of RdRP, N, S and RPP30 genes in both singleplex and multiplex PCR (CoV-multiplex), using serial decimal dilutions (initial concentration: 0.25 ng/µl) of RdRP, N and S genes and serial dilutions 1:5 for RPP30 gene (initial concentration: 50 ng/µl). Efficiency (E) and coefficient of determination (R^2) values obtained from linear regression analysis of the data are shown.

Analysis of clinical samples using CoV-Multiplex

To validate the CoV-Multiplex system, a total of 150 samples ($n = 150$) were studied. The samples were previously analysed in the hospital with automatized standard methods for the diagnosis of COVID-19. The procedure for validating the CoV-Multiplex system was, therefore, to compare the results obtained with those of the hospital. To this end, all samples were run, and the Ct values of the two systems were compared (Figure 3a). Using the CoV-Multiplex system, we detected as being positive the 75 samples that had previously been classified as positive by the hospital service. Likewise, the Ct values were equal to or even lower than those from the hospital. Although the Ct differences found could be due, at least in part, to the different RNA extraction methods used, the results suggest that the CoV-Multiplex system is at least as efficient as the reference method and potentially better. Indeed, with regard to the N gene, the CoV-Multiplex system detects the target gene several cycles earlier, achieving differences of up to 10 cycles (Ct 17 vs. Ct 27). For the S gene, the results are very similar to those reported by the hospital, and we found a cycle latency (≥ 3 cycles) in only 5% of the positive samples. Although samples classified as positive in the hospital were those with both genes amplified before Ct 38, all the positive samples in the study showed Ct lower than 31. This fact could be a limitation for our study, but our system showed the ability to also detect Ct 32–38 without problem, as it happened several times in the samples classified as indeterminate by the hospital service.

As regards the 50 negative samples, as expected, their analysis with CoV-Multiplex only showed amplification in the human RPP30 gene used as control (data not shown). The design of one of the primers for this gene, located at the junction between two exons (Figure 1d), ensured that only RNA was detected, but not DNA.

Taking into account the positive and negative samples classified by the hospital, the sensitivity, specificity, and positive and negative predictive values achieved by the CoV-Multiplex system are shown in Table 2.

In addition, there were 25 samples classified as indeterminate by the hospital, in which only one gene was positive (N or S), or which showed amplification beyond the Ct 38 value. After analysis with the CoV-Multiplex system, nine samples were also undetermined. However, six samples were clearly positive for the N and S genes of SARS-CoV-2 and 10 were negative. All these patients were followed up in the hospital with repeated PCR and/or serology tests in the following weeks (maximum 1 month). The final diagnosis showed that 12 samples showed the same result as that obtained with the CoV-Multiplex system and only four samples differed. These samples yielded negative results with the CoV-Multiplex system, but the patients finally proved to be positive.

To complete the CoV-Multiplex validation, the limit of detection was calculated. This parameter was defined as being the lowest dilution concentration that can be detected with a probability of 95%. For this purpose, SARS-CoV-2 RNA was used, and the N and S genes were analysed. The results were 100.4 ± 5 copies per µl for the N gene and 133.9 ± 6.7 copies per µl for the S gene. The results were obtained from a probit analysis, which converts amplification/non-amplification data into probability; therefore, large-scale studies including more dilutions, repeats and even different viral RNAs would help to define the limit of detection more precisely.

Standardization of Flu/CoV-Multiplex

The Flu/CoV-Multiplex system is designed to extend the screening capability offered by the CoV-Multiplex system due to the exchange of the SARS-CoV-2 S gene for the influenza A virus M1/M2 gene, as mentioned above.
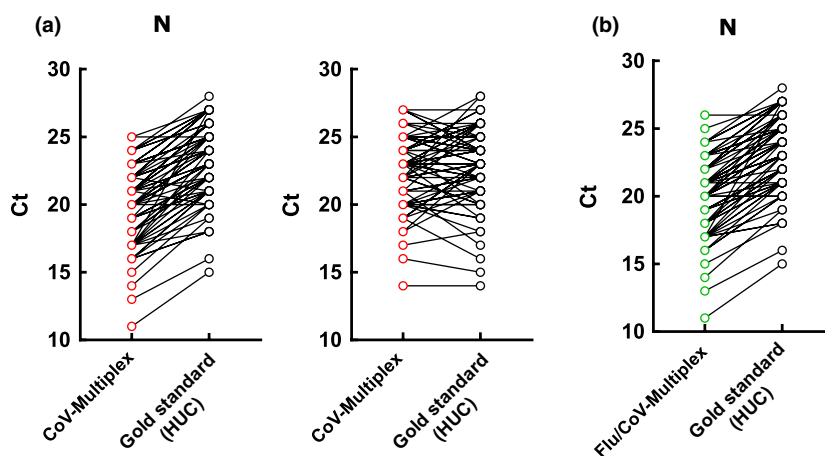

FIGURE 3 (a) Comparison of the Ct values of the N and S genes of SARS-CoV-2 between the CoV-multiplex system and the reference method (gold standard) used at hospital Universitario cruces (HUC). (b) Comparison of SARS-CoV-2 N-gene Ct values between the flu/CoV-multiplex system and the gold standard method.

TABLE 2 Summary of the analysis of the results of the clinical samples performed with the CoV-multiplex

Target gene	Samples				Index values				Concordance with gold standard
	+/+	+/−	−/+	−/−	Sn	Sp	PPV	NPV	
N	75	0	0	50	100	100	100	100	100%
S	75	0	0	50	100	100	100	100	100%

Note: Index values: 0–1×100 (%). +/+, true positive (TP); +/−, false positive (FP); −/+, false negative (FN); −/−, true negative (TN). Sn, sensitivity (TP/TP+FN); Sp, specificity (TN/TN+FP); PPV, positive predictive value (TP/TP+FP); NPV, negative predictive value (TN/TN+FN).
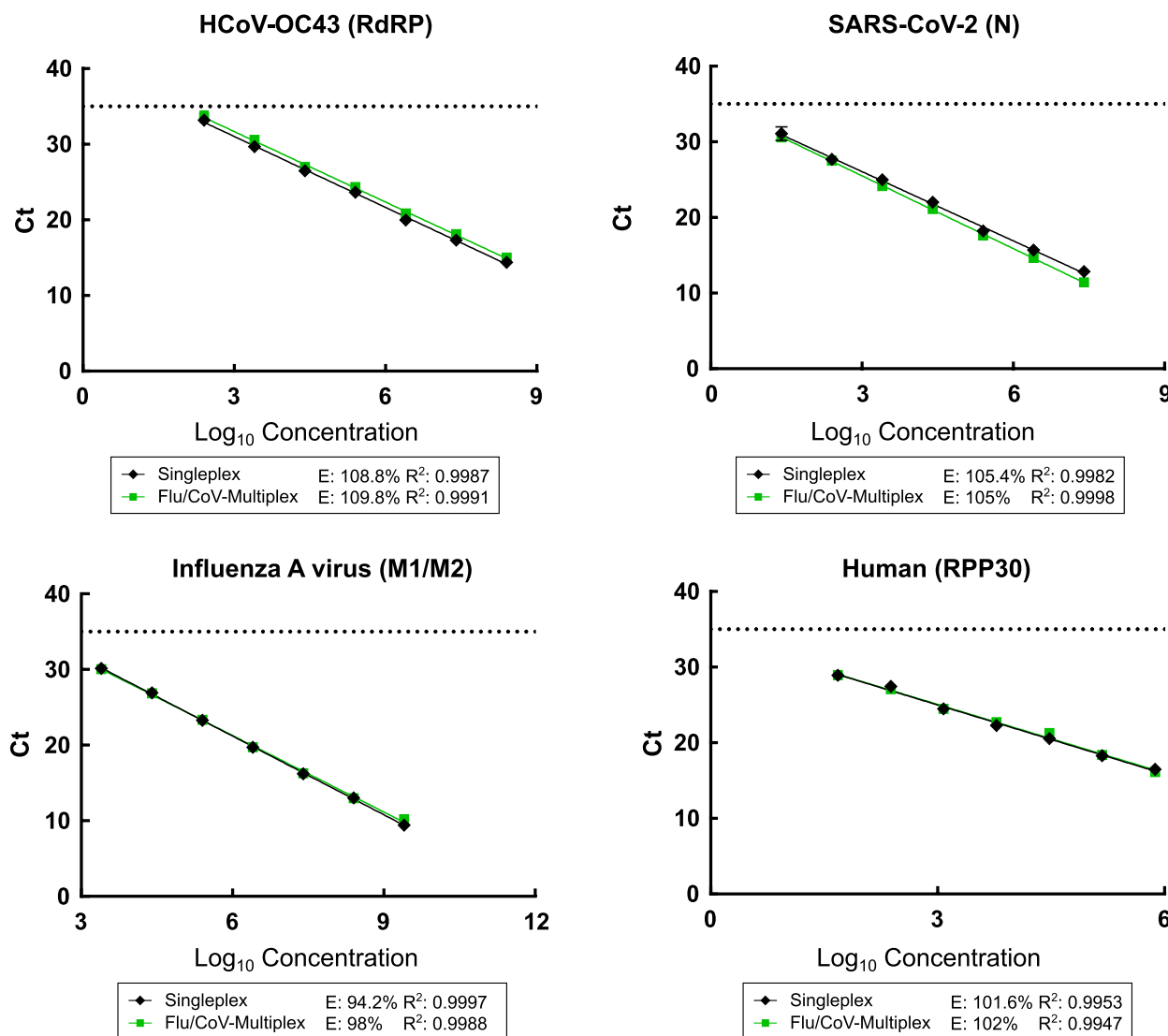
FIGURE 4 Amplification of the RdRP, N, M1/M2 and RPP30 genes in both singleplex and multiplex PCR (flu/CoV-multiplex), using serial decimal dilutions (initial concentration: 0.25 ng/µl) of the RdRP, N and M1/M2 genes and serial dilutions 1:5 for the RPP30 gene (initial concentration: 50 ng/µl). Efficiency (E) and coefficient of determination (R^2) values obtained from linear regression analysis of the data are shown.

The primer/probe set in both systems is the N gene set since it is a more stable gene and less subject to mutations than the S gene (Dutta et al., 2020) (Figure 1c). This system, therefore, includes the primers and probe for RdRP (HCoV-OC43), N (SARS-CoV-2), M1/M2 (influenza A virus) and RPP30 (Human). Standardization was carried out in the same way as the CoV-Multiplex system (Figure 4). The E and R^2 values demonstrate the correct performance of the primers and probes in the Flu/CoV-Multiplex system.

Analysis of clinical samples using Flu/CoV-Multiplex

As with the CoV-Multiplex system, for validation of the Flu/CoV-Multiplex system the same 150 samples were analysed. In the case of the SARS-CoV-2 positive patients, the Ct values obtained show the same tendency as the CoV-Multiplex system to detect the N gene several cycles earlier than those obtained in the hospital (Gold standard) (Figure 3b). Furthermore, the analysis of the negative samples provided the same results as the CoV-Multiplex system (Table 2), giving a result of 100% for sensitivity, specificity, PPV, and PPN. This was the expected outcome, as the difference lies in the fact that this system uses only the N gene to detect SARS-CoV-2. In both cases values of 1 (100%) were obtained and, therefore, the influenza A virus gene introduced in this multiplex does not interfere with the results.

The study of the undetermined samples also reveals the same results as those offered by the CoV-Multiplex system, resulting in a correct diagnosis of 12 out of the 25 undetermined samples.

Study of samples primed with HCoV-OC43 and influenza A virus RNA

To overcome the low availability of samples from patients infected with HCoV-OC43 and influenza A virus, an alternative procedure was used to validate the correct performance of the primer/probe sets selected for the CoV-Multiplex and Flu/CoV-Multiplex systems. Specifically, three known concentrations of positive controls were added to 15 nasopharyngeal swab negative samples (five samples

for each concentration), covering the entire detection range of the system. Subsequently, PCR assays were performed (analysing each sample in triplicate) to demonstrate the detection capability of the system (Figure 5).

The results show that the CoV-Multiplex system is capable of detecting the HCoV-OC43 positive control in real human samples primed at the three concentrations of control used. Likewise, Flu/CoV-Multiplex system detected both HCoV-OC43 and influenza A virus positive controls in the detection range of the system. In addition, the comparison with the linear regression line obtained during the standardization process showed a remarkable fit with the Ct values.

DISCUSSION

Since the beginning of the COVID-19 pandemic, the scientific community has made great efforts to design a wide variety of diagnostic methods for detecting the SARS-CoV-2 virus, ranging from serological tests to nucleic acid detection systems such as ddPCR (Droplet Digital PCR), multiplex PCR or RT-LAMP (Jang et al., 2021; Liu et al., 2020). With the same goal, PCR systems have also been developed to discriminate SARS-CoV-2 from other respiratory viruses with which it is currently coexisting (Mancini et al., 2021). In the present study, we offer a system not affected by supplier outages and with reduced cost compared to the commercially available kits. In fact, it would make clinical centres less reliant on automated extraction kits and all-in-one primer systems, meaning that they would need only a small investment to purchase an enzyme and primer/probes to run the test. To that, we have developed two multiplex RT-qPCR systems, CoV-Multiplex and Flu/
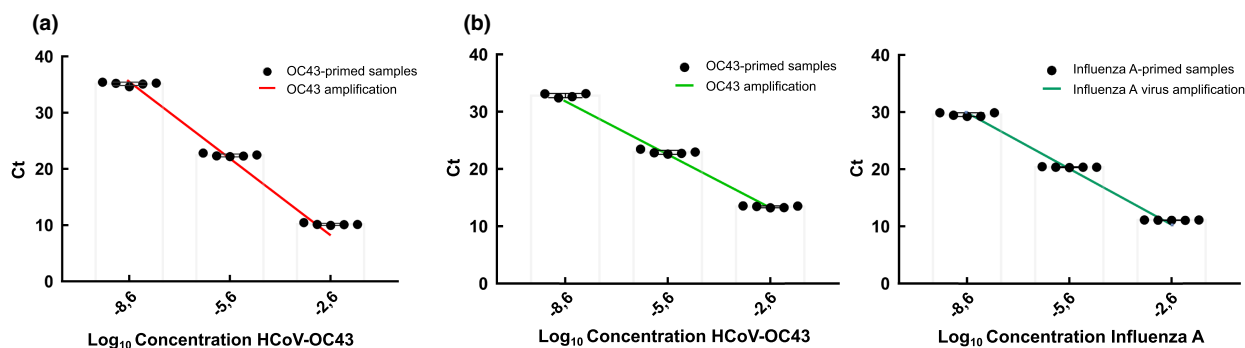

FIGURE 5 Analysis of samples primed with the positive control HCoV-OC43, and influenza a virus performed with both multiplex systems. (a) Analysis of HCoV-OC43-primed samples performed with the CoV-multiplex system (concentrations: 2.5×10^{-9}, 2.5×10^{-6}, $2.5 \times 10{-3}$ ng/μl). (b) Analysis of samples primed with the positive controls HCoV-OC43, and influenza a virus performed with the flu/CoV-multiplex system (concentrations: 2.5×10^{-9}, 2.5×10^{-6}, 2.5×10^{-3} ng/μl). ●, average of the Ct values of each sample (error bars: standard deviation). ——, representation of the linear regression line of HCoV-OC43 obtained from the amplification data ($y = -3.102x + 41.05$). ══, linear regression line representation of the HCoV-OC43 ($y = -3.274x + 43.56$) and influenza a virus ($y = -3.371x + 41.5$) controls, respectively, were obtained from the amplification data.

CoV-Multiplex. Both share the ability to detect SARS-CoV-2 and HCoV-OC43, but in the Flu/CoV-Multiplex system, only the N gene is used for SARS-CoV-2 detection and the M1/M2 gene of influenza A virus was included instead S gene of SARS-CoV-2, providing a higher screening capacity. In this study, even RNA extraction was carried out without using commercial extraction kits, by the classical 2-propanol precipitation technique optimized for SARS-CoV-2 (Guruceaga et al., 2020), although automated or other extraction methods could also be used if the quality of the RNA obtained is adequate.

The restrictive conditions required to correctly design a multiplex PCR (e.g. the choice of primers and probes [location, melting temperature, %GC, etc.], the need to avoid secondary structures, the choice of fluorophores and the size of the amplicon) can enormously hinder the process of optimization. In addition, we had to take into account SARS-CoV-2 undeniable capacity for mutation, which has resulted in the generation of numerous variants that continue to cause alarm among the population, due to possible improvements in the virus capacity for infection or simply a possible loss in effectiveness of leading detection methods and vaccines (Akkiz, 2021; Planas et al., 2021; Plante et al., 2021). For this reason, the primers and probes in this study have been designed outside the most relevant known mutations of the SARS-CoV-2 genes. In the S gene, there is no mutation of relevance within our amplicon. In contrast, we see that the Alpha variant tends to the S235F mutation of the N gene, and the Delta variant tends to G214C and G215C. After verification, we saw that S235F and G215C mutations did not affect the sequence of the primers or the probe and, therefore, the detection capability would not be affected. Regarding the Delta variant, it was the most prevalent variant when the samples used in this study were taken. It tends to G214C mutation, however, only one nucleotide was affected by this change, although it should be confirmed, it is unlikely to affect the ability of our system to detect it. In addition, the genetic evolution of SARS-CoV-2 place Omicron and their lineages as the unique epidemiologically relevant variant of the virus and none of their characteristic mutations affect the primers/probe sets.

Furthermore, the reverse primer designed for the human RPP30 gene was located at an exon-exon junction. This generates very restrictive conditions to ensure that the primer only has an affinity for the mRNA, which is very susceptible to degradation. Thus, detection of this gene helped us to check the quality of the RNA extraction, which is a critical step for proper functioning of PCR reactions. Other authors use different strategies as a control method, such as the RNA spike-in (Reijns et al., 2020; Smyrlaki et al., 2020). Our method, as well as avoiding the introduction of a source of variability, such as a sequence foreign to the target gene, provides an internal control for proper analysis of human samples and information on RNA extraction.

After ensuring all these determining factors, we probed that all the primers/probe sets have an excellent amplification capability in singleplex and multiplex. At the same time, analysis of clinical samples from SARS-CoV-2 infected patients determined that both systems are capable of detecting the virus with 100% sensitivity and specificity with respect to the automatized reference method for diagnosis of COVID-19 (used in the hospital). Moreover, our analysis of the undetermined samples showed a positive/negative result for 16 out of the 25 hospital undetermined samples. A follow-up on these patients showed that in 12 of the cases, our systems had been able to predict the final result, and only differed in four samples. In all of these four, the samples were negative, but the patients finally proved positive. We hypothesize that the patients were sampled too early in the disease or that they were infected after sampling. Although a larger battery of samples would help to define the improvement of our system over the gold standard, our system appears to clarify, at least, almost 50% of the indeterminate samples, increasing the sensitivity and specificity rates with respect to the reference method.

With regard to the ability to detect HCoV-OC43 and influenza A viruses, the lack of samples from infected individuals led to the development of the sample priming strategy used to validate the systems. Other authors have used similar strategies for the validation of their diagnostic systems (Queiroz et al., 2021; Toptan et al., 2020). After testing with CoV-Multiplex and Flu/CoV-Multiplex, the data obtained showed the effectiveness of both systems, making this strategy a good approximation of the result that would have been obtained with infected samples.

In spite of the contributions, this study has also some limitations derived from the need to test clinical samples from HCoV-OC43 and influenza A. First, to design primers/probe sets of each virus, the reference and the most conserved sequences have been used. In fact. The aim of the project was to design a universal primers/probe set in the RdRP gene (ORF1a/b) for all human seasonal coronavirus (OC43, 229E, NL63 and HKU1), but their variability made it a hard challenge. In preliminary results (data not shown), the similarity of HKU1 with OC43 seems to allow the detection by the system, although further studies are required. Regarding the M1/M2 gene used for influenza A virus detection, it is also used in other studies (Ni et al., 2021) and even recommended by WHO (WHO, 2018). However, in future studies, clinical samples should be analysed, including different subtypes of the influenza A virus. Additionally, multicenter studies are recommended to verify that SARS-CoV-2 circulating variants, HCoV-OC43 and influenza A virus can also be detected. Finally, it would be highly desirable to design

primer/probes sets for other respiratory viruses such as RSV or rhinovirus to combine with those designed in this study according to the specific needs.

In the near future, thanks to the action of vaccines and early diagnosis, the number of cases of COVID-19 will stabilize, and it will thus become a persistent disease. However, other micro-organisms capable of producing respiratory diseases—such as other seasonal coronaviruses or influenza viruses—will continue to circulate among the population and we will need to distinguish them from SARS-CoV-2. For this purpose, we present the CoV-Multiplex and Flu/CoV-Multiplex systems as moldable, rapid, cost-effective, efficient, and non-commercial kit-independent tools for discriminating SARS-CoV-2, influenza A virus and HCoV-OC43. In addition, we have also taken a first step in the challenge of creating a multiplex RT-qPCR tool containing primer and probe sets for a much broader group of viruses, which can be flexible and customized for each patient.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

ORCID
Eduardo Pelegri-Martinez https://orcid.org/0000-0001-6512-7772
Xabier Guruceaga https://orcid.org/0000-0003-3258-2482
Leire Martin-Souto https://orcid.org/0000-0001-8540-5629
Ana Abad-Diaz-de-Cerio https://orcid.org/0000-0001-6022-1751
Aitor Rementeria https://orcid.org/0000-0003-0478-1038
Mikel Gallego https://orcid.org/0000-0002-2273-1985
Eunate Arana-Arri https://orcid.org/0000-0001-9759-333X
Maitane Aranzamendi https://orcid.org/0000-0003-2432-7078
Andoni Ramirez-Garcia https://orcid.org/0000-0003-2696-162X

REFERENCES

Akkiz, H. (2021) Implications of the novel mutations in the SARS-CoV-2 genome for transmission, disease severity, and the vaccine development. *Frontiers in Medicine*, 8, 1–10. https://doi.org/10.3389/fmed.2021.636532

Baker, R.E., Park, S.W., Yang, W., Vecchi, G.A., Metcalf, C.J.E. & Grenfell, B.T. (2020) The impact of COVID-19 nonpharmaceutical interventions on the future dynamics of endemic infections. *Proceedings of the National Academy of Sciences of the United States of America*, 117(48), 30547–30553. https://doi.org/10.1073/pnas.2013182117

Chams, N., Chams, S., Badran, R., Shams, A., Araji, A., Raad, M. et al. (2020) COVID-19: a multidisciplinary review. *Frontiers in Public Health*, 8, 1–20. https://doi.org/10.3389/fpubh.2020.00383

Corman, V.M., Landt, O., Kaiser, M., Molenkamp, R., Meijer, A., Chu, D.K.W. et al. (2020) Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. *Eurosurveillance*, 25(3), 1–8. https://doi.org/10.2807/1560-7917.ES.2020.25.3.2000045

Dijkman, R., Jebbink, M.F., Gaunt, E., Rossen, J.W.A., Templeton, K.E., Kuijpers, T.W. et al. (2012) The dominance of human coronavirus OC43 and NL63 infections in infants. *Journal of Clinical Virology*, 53(2), 135–139. https://doi.org/10.1016/J.JCV.2011.11.011

Dutta, N.K., Mazumdar, K. & Gordy, J.T. (2020) The nucleocapsid protein of SARS-CoV-2: a target for vaccine development. *Journal of Virology*, 94(13). https://doi.org/10.1128/JVI.00647-20

Graham, N.M.H. (1990) The epidemiology of acute respiratory infections in children and adults: a global perspective. *Epidemiologic Reviews*, 12(1), 149–178. https://doi.org/10.1093/OXFORDJOURNALS.EPIREV.A036050

Guruceaga, X., Sierra, A., Marino, D., Santin, I., Nieto-Garai, J.A., Bilbao, J.R. et al. (2020) Fast SARS-CoV-2 detection protocol based on RNA precipitation and RT-qPCR in nasopharyngeal swab samples. *medRxiv*, 1–22. https://doi.org/10.1101/2020.04.26.20081307

Hu, B., Guo, H., Zhou, P. & Shi, Z.L. (2020) Characteristics of SARS-CoV-2 and COVID-19. *Nature Reviews Microbiology*, 19, 141–154. https://doi.org/10.1038/s41579-020-00459-7

Jang, W.S., Lim, D.H., Yoon, J., Kim, A., Lim, M., Nam, J. et al. (2021) Development of a multiplex loop-mediated isothermal amplification (LAMP) assay for on-site diagnosis of SARS CoV-2. *PLoS One*, 16(3), e0248042. https://doi.org/10.1371/journal.pone.0248042

Jimenez-Solem, E., Petersen, T.S., Hansen, C., Hansen, C., Lioma, C., Igel, C. et al. (2021) Developing and validating COVID - 19 adverse outcome risk prediction models from a bi - national European cohort of 5594 patients. *Scientific Reports*, 11, 1–12. https://doi.org/10.1038/s41598-021-81844-x

Krammer, F., Smith, G.J.D., Fouchier, R.A.M., Peiris, M., Kedzierska, K., Doherty, P.C. et al. (2018) Influenza. *Nature Reviews Disease Primers*, 4(1), 1–21. https://doi.org/10.1038/s41572-018-0002-y

Kudo, E., Israelow, B., Vogels, C.B.F., Lu, P., Wyllie, A.L., Tokuyama, M. et al. (2020) Detection of SARS-CoV-2 RNA by multiplex RTqPCR. *PLoS Biology*, 18(10), 1–9. https://doi.org/10.1371/JOURNAL.PBIO.3000867

Li, X., Xu, W., Dozier, M., He, Y., Kirolos, A., Theodoratou, E. et al. (2020) The role of children in transmission of SARS-CoV-2: a

rapid review. *Journal of Global Health*, 10(1), 1–2. https://doi.org/10.7189/JOGH.10.011101

Liu, X., Feng, J., Zhang, Q., Guo, D., Zhang, L., Suo, T. et al. (2020) Analytical comparisons of SARS-COV-2 detection by qRT-PCR and ddPCR with multiple primer/probe sets. *Emerging Microbes and Infections*, 9(1), 1175–1179. https://doi.org/10.1080/22221751.2020.1772679

Mackinnon, A. (2000) A spreadsheet for the calculation of comprehensive statistics for the assessment of diagnostic tests and inter-rater agreement. *Computers in Biology and Medicine*, 30(3), 127–134. https://doi.org/10.1016/S0010-4825(00)00006-8

Mancini, F., Barbanti, F., Scaturro, M., Fontana, S., di Martino, A., Marsili, G. et al. (2021) Multiplex real-time reverse-transcription polymerase chain reaction assays for diagnostic testing of severe acute respiratory syndrome coronavirus 2 and seasonal influenza viruses: a challenge of the phase 3 pandemic setting. *Journal of Infectious Diseases*, 223, 765–774. https://doi.org/10.1093/infdis/jiaa658

Ni, M., Xu, H., Luo, J., Liu, W. & Zhou, D. (2021) Simultaneous detection and differentiation of SARS-CoV-2, influenza a virus and influenza B virus by one-step quadruplex real-time RT-PCR in patients with clinical manifestations. *International Journal of Infectious Diseases*, 103, 517–524. https://doi.org/10.1016/j.ijid.2020.12.027

Nickbakhsh, S., Ho, A., Marques, D.F.P., McMenamin, J., Gunson, R.N. & Murcia, P.R. (2020) Epidemiology of seasonal coronaviruses: establishing the context for the emergence of coronavirus disease 2019. *The Journal of Infectious Diseases*, 222(1), 17–25. https://doi.org/10.1093/INFDIS/JIAA185

Orooji, Y., Sohrabi, H., Hemmat, N., Oroojalian, F., Baradaran, B., Mokhtarzadeh, A. et al. (2021) An overview on SARS-CoV-2 (COVID-19) and other human coronaviruses and their detection capability via amplification assay, chemical sensing, biosensing, Immunosensing, and clinical assays. *Nano-Micro Letters*, 13(1), 1–30. https://doi.org/10.1007/s40820-020-00533-y

Planas, D., Bruel, T., Grzelak, L., Guivel-Benhassine, F., Staropoli, I., Porrot, F. et al. (2021) Sensitivity of infectious SARS-CoV-2 B.1.1.7 and B.1.351 variants to neutralizing antibodies. *Nature Medicine*, 27(5), 917–924. https://doi.org/10.1038/s41591-021-01318-5

Plante, J.A., Liu, Y., Liu, J., Xia, H., Johnson, B.A., Lokugamage, K.G. et al. (2021) Spike mutation D614G alters SARS-CoV-2 fitness. *Nature*, 592, 116–121. https://doi.org/10.1038/s41586-020-2895-3

Queiroz, J.A.D.S., Rampazzo, R.C.P., Filho, E.B.D.S., Oliveira, G.S., Oliveira, S.D.C., Souza, L.F.B. et al. (2021) Development of a quantitative one-step multiplex RT-qPCR assay for the detection of SARS-CoV-2 in a biological matrix. *International Journal of Infectious Diseases*, 104, 373–378. https://doi.org/10.1016/j.ijid.2021.01.001

Reijns, M.A.M., Thompson, L., Acosta, J.C., Black, H.A., Sanchez-Luque, F.J., Diamond, A. et al. (2020) A sensitive and affordable multiplex RT-qPCR assay for SARS-CoV-2 detection. *PLoS Biology*, 18, 1–20. https://doi.org/10.1371/journal.pbio.3001030

Sawal, I., Ahmad, S., Tariq, W., Tahir, M.J., Essar, M.Y. & Ahmed, A. (2021) Unequal distribution of COVID-19 vaccine: a looming crisis. *Journal of Medical Virology*, 93(9), 5228–5230. https://doi.org/10.1002/JMV.27031

Smyrlaki, I., Ekman, M., Lentini, A., Rufino de Sousa, N., Papanicolaou, N., Vondracek, M. et al. (2020) Massive and rapid COVID-19 testing is feasible by extraction-free SARS-CoV-2 RT-PCR. *Nature Communications*, 11(1). https://doi.org/10.1038/s41467-020-18611-5

Tai, D.B.G., Shah, A., Doubeni, C.A., Sia, I.G. & Wieland, M.L. (2021) The disproportionate impact of COVID-19 on racial and ethnic minorities in the United States. *Clinical Infectious Diseases*, 72, 703–706. https://doi.org/10.1093/cid/ciaa815

Toptan, T., Hoehl, S., Westhaus, S., Bojkova, D., Berger, A., Rotter, B. et al. (2020) Optimized qRT-PCR approach for the detection of intra-and extra-cellular SARS-CoV-2 RNAs. *International Journal of Molecular Sciences*, 21(12), 1–11. https://doi.org/10.3390/ijms21124396

Vaks, J.E. (2018) New method of evaluation of limit of detection in molecular diagnostics. In: *Joint statistical meetings*. Section on Medical Devices and Diagnostics. Alexandria, VA: American Statistical Association, pp. 529–543.

WHO. (2018) *Information for the molecular detection of influenza viruses*. WHO, pp. 1–63. Available at: https://www.who.int/teams/global-influenza-programme/laboratory-network/eqa-project [Accessed 28th August 2022].

WHO Coronavirus (COVID-19) Dashboard (2022). Available at: https://covid19.who.int/ [Accessed 18th March 2022].

Wu, F., Zhao, S., Yu, B., Chen, Y.M., Wang, W., Song, Z.G. et al. (2020) A new coronavirus associated with human respiratory disease in China. *Nature*, 579(7798), 265–269. https://doi.org/10.1038/s41586-020-2008-3

Yeoh, D.K., Foley, D.A., Minney-Smith, C.A., Martin, A.C., Mace, A.O., Sikazwe, C.T. et al. (2020) Impact of coronavirus disease 2019 public health measures on detections of influenza and respiratory syncytial virus in children during the 2020 Australian winter. *Clinical Infectious Diseases*, 1–4. https://doi.org/10.1093/cid/ciaa1475