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Positive control synthesis method for COVID-19 diagnosis by one-step real-time RT-PCR



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ABSTRACT

Backgrounds: The coronavirus disease 2019 (COVID-19) pandemic is still ongoing. Real-time reverse transcription polymerase chain reaction (real-time RT-PCR) is regarded as a gold-standard method for the diagnosis of COVID-19. However, unexpected contamination of synthesized positive control samples included in COVID-19 test kits have increased the inconclusiveness of disease interpretation. Therefore, it is important to establish new methods for the preparation of reliable positive controls that are not affected by contamination for the accurate for diagnosis of COVID-19, but it still remains a challenge.

Methods: A new approach for producing synthetic positive controls using synthetic positive template (SPT) oligonucleotides was designed. SPT oligonucleotides contain probe binding and virus-irrelevant regions were used as templates for real-time PCR to evaluate the expression level of SARS-CoV-2 genes (RdRP, E, and N). The limit of detection (LOD) for individual SARS-CoV-2 genes by Ct values with different concentrations of SPT templates and genomic RNAs from SARS-CoV-2 infected samples was determined.

Results: LODs with SPT templates were $> 10^{-15}$ (atto) M for RdRP, 10^{-12} (femto) to 10^{-13} (100 atto) M for E gene, and 10^{-12} to 10^{-14} (10 atto) M for N gene, respectively. Real-time RT-PCR assay using serially diluted genomic RNAs prepared from SARS-CoV-2 virus infected cultures showed that picogram quantities of RNAs is resulted in the LOD. The sensitivity of RdRP and E genes based on Ct values was less than that of N gene with this platform.

Conclusion: This method significantly reduces the risk of false-positive reactions resulting from contamination in the synthesis procedures of positive control materials. Therefore, this approach could be integrated into the currently available COVID-19 test kits and will provide a general method for preparing positive controls in the diagnosis of emerging RNA virus infections.

1. Introduction

Emerging and re-emerging pathogens are global public health challenges [1]. For instance, the outbreak of coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2) infection is highly transmissible and remains an ongoing issue worldwide due to its rapid expansion rate, since its discovery in December 2019. The clinical manifestations of most patients include fever, cough, shortness of breath, and myalgia. Radiographic evidence of infection presents as pneumonia with multiple mottling and ground-glass opacity [2]. A fraction of patients with serious conditions will experience complications that include acute respiratory distress syndrome (ARDS) and cytokine storms, which may account for COVID-19-

related deaths [3,4].

The development of new drugs and therapeutic strategies for COVID-19 is still underway. Therefore, a timely and accurate disease diagnosis is crucial for guiding clinical decisions related to the management of emerging infections, before new strategies are implemented.

Many diagnostic tools have been developed for COVID-19. Among the available platforms, nucleic acid testing using real-time reverse transcription polymerase chain reaction (real-time RT-PCR) has been regarded as the gold standard for COVID-19 diagnosis [5,6] since the SARS-CoV-2 sequence information became publicly available [7].

From sequence alignment analyses of a number of SARS-related viral genome sequences [8], a set of primers and probes has been

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Table 1

Analysis of dose-dependent SPT positive controls in the COVID-19 multiplex real-time RT-PCR assay. Data shown are cycle threshold (Ct) values from three independent experiments. ND indicates that the Ct values were not detected. The data represents the average values of $n = 3$ ($P < 0.05$). f: femto; a: atto; z: zepto; ND: not determined. Cy5, FAM, HEX, and ROX indicate the fluorescent dyes that were used for the multiplex real-time RT-PCR.

Gene (Fluorescent dye)	Cycle threshold (Ct) values SPT concentration (M)							No template
	10f	1f	100a	10a	1a	100z	10z	
RdRP (FAM)	13.57 ± 0.80	17.22 ± 0.62	20.76 ± 1.10	25.88 ± 1.65	ND	ND	ND	ND
E (Cy5)	12.36 ± 0.33	16.55 ± 1.45	19.87 ± 0.98	24.62 ± 1.96	28.16 ± 1.27	32.61 ± 0.28	ND	ND
N (HEX)	11.28 ± 0.24	15.09 ± 1.16	18.59 ± 0.98	23.07 ± 1.83	26.89 ± 1.16	ND	ND	ND
Internal control-ADH-1C-m allele (ROX)	29.60 ± 0.14	29.55 ± 0.41	29.61 ± 0.48	29.33 ± 0.86	29.65 ± 0.41	30.30 ± 0.35	30.22 ± 0.55	ND

designed for three regions with conserved sequences: (1) RdRP gene (RNA-dependent RNA polymerase gene) in the open reading frame ORF1ab region, (2) E gene (envelope protein gene), and (3) N gene (nucleocapsid protein gene). However, multiple problems related to this SARS-CoV-2 targeting technology remain [9]. These issues include low sensitivity with respect to the sampling amount [10], inconclusiveness of diagnosis compared to chest CT [11], and the primers, probes, and reagents used for assay, especially with regards to primers that are designed to amplify target regions of the SARS-CoV-2 genome. Notably, the mutations and recombinations that occur in the RNA virus reduce the sensitivity of real-time RT-PCR when using designed primers [9,12]. Moreover, a general nucleic acid testing workflow for SARS-CoV-2 that uses a one-step real-time RT-PCR assay and provides quantitative information on viral loads has been developed [13]. Laboratories and companies are often faced with the problem of not having a reliable source or having an insufficient quantity of positive control materials when developing or validating real-time RT-PCR assays for emerging viruses [14]. To overcome this problem, a new efficient method for preparing positive controls for COVID-19 detection kits needs to be developed that can be applied to the detection of emerging viruses, such as influenza A (H1N1), human metapneumovirus (HMPT), and SARS-CoV [14,15].

COVID-19 test kits have been reported to generate false-positive results, due to CDC contamination, at 24 of the first 26 public health labs that tested them before analyzing actual patient samples. Since the false-positives have been confirmed to occur due to contamination during test kit manufacturing and bulk reagent material processing at the respiratory virus lab, they were most likely exposed to positive control (nCoVPC) [13] preparations [16]. Therefore, a new preparation design that provides contamination-free positive controls for COVID-19 real-time RT-PCR testing is urgently needed.

Here, we present a new approach for producing synthetic positive controls using synthetic positive template (SPT) oligonucleotides that contain probe binding and virus-irrelevant regions as templates for detecting SARS-CoV-2 genes by real-time RT-PCR. Specifically, we designed real-time RT-PCR oligonucleotides for RdRP, E, and N SARS-CoV-2 genes, respectively, and determined reliable real-time RT-PCR conditions. Since our approach applies irrespective of whether the virus is present in the experimental laboratory, it is suitable for preparing positive controls without contamination. Therefore, our new approach can be incorporated into current molecular diagnostic tests for COVID-19 and can be applied to prepare positive controls for the diagnosis of emerging RNA virus infections.

2. Materials and methods

2.1. Oligonucleotides

To design initiation primer template (IPT), SPT, and bridge primers oligonucleotides and conduct *in-silico* evaluations, we downloaded sequences encoding RdRP, E, or N genes from 15 or 22 different SARS-CoV-2 or SARS-CoV species, respectively, available at GenBank [17],

with accession numbers shown (Fig. S1 & Table S1). The sequences were aligned by Clustal Omega [18], and the alignment was used to design bridge primers, SPT, and IPT that were used in the assay (Fig. S1 & Tables S2, S3). The forward/reverse primers and probes for E and N were newly designed, but those for RdRP were adapted from the Charité/Berlin protocol [19]. For an internal control, a portion (+1084 to +1110 regions) of the *Zea mays* alcohol dehydrogenase (ADH-1C-m allele) gene (GenBank Accession No. M32984.1) with 5' ROX and 3' BHQ-1, -2, or -3 modifications were used. The oligonucleotides used in this study are listed in Table 1. Bridge primers, SPT, probe, and IPT were synthesized from Bionics, Genotech, Nanoprobe, and IDT, respectively.

2.2. One-step multiplex real-time RT-PCR

All assays used the same conditions. Primer and probe sequences are shown in Table S3 and used in the assays. A 20 μ L reaction using One-step RT-qPCR Master mix (Solis BioDyne, Estonia) was setup containing 10 μ L of 2 \times Master mix, 1 μ L of primer and probe (2 pmol) for each, 1 μ L of IPT (1 pmol), 1 μ L of bridge primer (0.2 pmol), and templates [10^1 to 10^7 -fold diluted genomic RNAs (i.e. 10^{-15} to 10^{-9} g/ μ L concentration) prepared from SARS-CoV-2 virus infected cultures (National Culture Collection for Pathogens, NCCP) or 10^{-18} to 10^{-12} M of SPT]. To reduce the time and convenience, three different target genes and internal control were mixed together in a single tube and analyzed by multiplex PCR with the slight modification of protocols described in Chamberlain [20] and Henegariu [21] using CFX96 Real time PCR Detection System (Bio-Rad, USA). Thermal cycling was performed at 50 $^{\circ}$ C for 15 min for reverse transcription, followed by 95 $^{\circ}$ C for 3 min and then 40 cycles of 94 $^{\circ}$ C 20 s, 60 $^{\circ}$ C 60 s.

3. Results

3.1. Selection of amplicon targets

Genes encoding RdRP, E, or N proteins have been widely used for the diagnosis of COVID-19 by real-time RT-PCR. Therefore, we performed sequence alignment of 15 or 22 different SARS-CoV-2 or SARS-CoV strains, respectively, to select amplicon targets for our assay. We identified oligonucleotide binding regions with less variation between species (Fig. S1; Tables S2 & S3). These regions are already used in the diagnosis of COVID-19 [19].

3.2. Assay design

The main feature of the new real-time RT-PCR system is that both positive controls and multiple COVID-19 target genes are amplified at the same time using TaqMan-based multiplex real-time RT-PCR analysis. Notably, the positive controls are not prepared by oligo synthesis, but are rather produced by a cascade reaction of synthesized oligonucleotides. A schematic representation of the preparation of positive controls for the target genes and components of the real-time RT-PCR

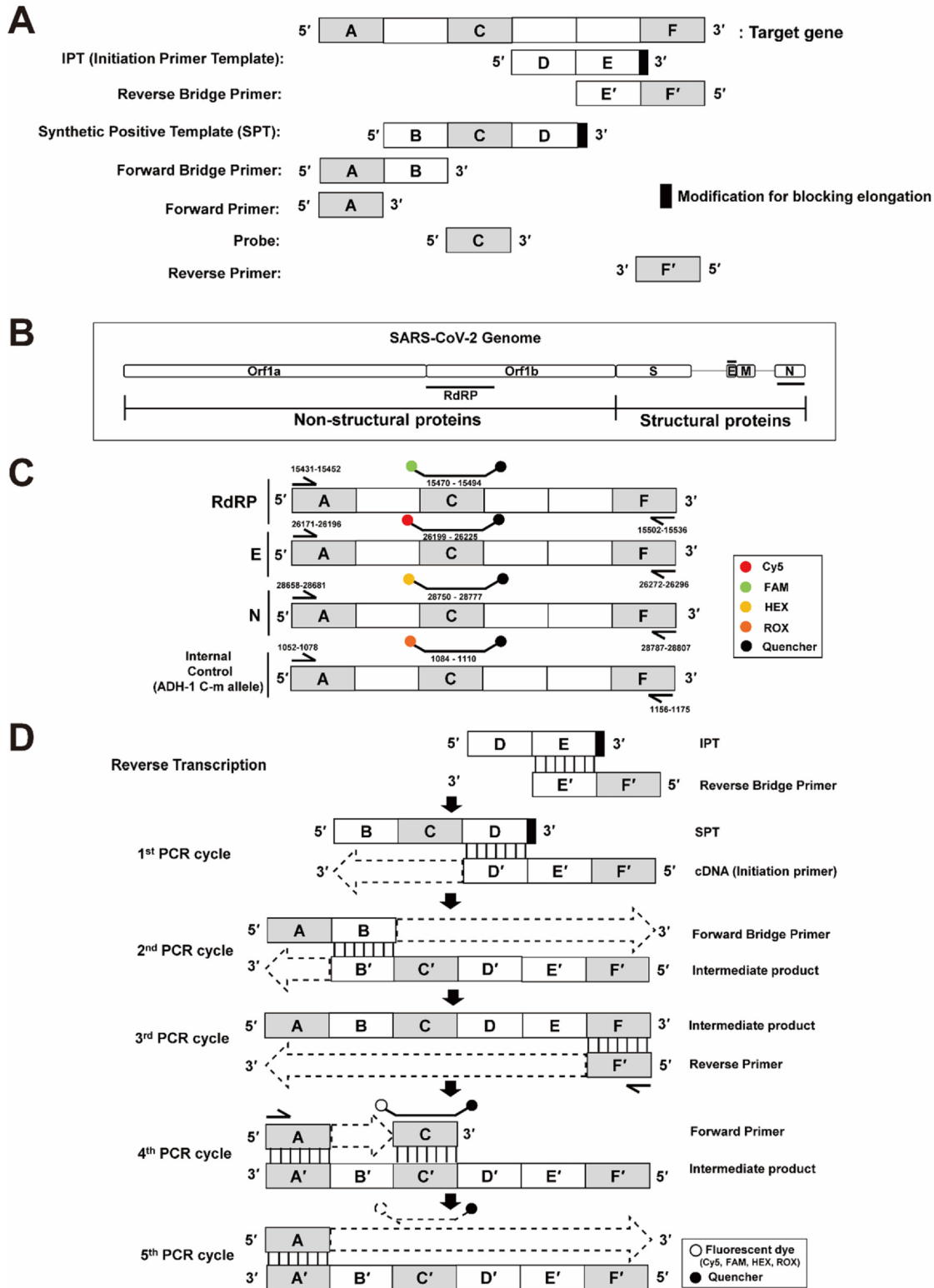


Fig. 1. Schematic assay components and procedures for the COVID-19 real-time multiplex RT-PCR assay. (A) A schematic representation of the synthetic positive template (SPT) system. Regions (A, C, and F) shown in gray bars indicate target gene specific regions, while the other regions are target independent. (B) Schematic representation of SARS-CoV-2 Genome. (C) Regions of amplicons and probes. Numbers indicate regions of primers and probes according to the GenBank accession No. NC_004718. (D) Real-time multiplex RT-PCR procedures using the SPT system. (1) Reverse transcription step: The SPT system uses an RNA oligo as the initiation template in an COVID-19 real-time multiplex RT-PCR assay. The reverse bridge primer (or reverse primer) is used to generate cDNA from the RNA initiation primer template (IPT) during the reverse transcription step. (2) 1st PCR cycle: The cDNA (initiation primer) extends over the SPT, which creates a complementary strand to the SPT and generates the forward bridge primer target site. (3) 2nd PCR cycle: The forward bridge primer extends to create a complementary strand to the 1st PCR cycle product to generate the reverse primer target site. (4) 3rd PCR cycle: The reverse primer hybridizes to its respective target. (5) 4th and 5th PCR cycles: Extension of the forward primer cleaves the TaqMan probe through DNA polymerase 5' nuclease activity.

Table 2

COVID-19 multiplex real-time RT-PCR assay using genomic RNAs from SARS-CoV-2. Genomic RNAs from the National Culture Collection for Pathogens (NCCP) at stock concentrations of around 90 ng/mL were diluted 10^1 - to 10^7 -fold and used for multiplex real-time RT-PCR. ND indicates that cycle threshold (Ct) values were not detected. One representative dataset from the $n = 3$ experiment is shown.

Target gene (Fluorescent Dye)	NCCP No.	Cycle threshold (Ct) values Fold of RNA dilutions from the stock							No template
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	
RdRP (FAM)	43326	14.58	17.77	22.36	26.30	ND	ND	ND	ND
	43328	15.53	18.42	22.86	28.22	38.08	ND	ND	ND
	43330	15.35	18.44	23.76	29.22	ND	ND	ND	ND
E (Cy5)	43326	14.78	17.89	22.72	26.16	30.51	34.74	ND	ND
	43328	15.41	18.39	22.96	27.19	31.02	34.37	ND	ND
	43330	15.31	18.32	23.69	27.75	31.01	33.65	36.69	ND
N (HEX)	43326	17.16	19.73	23.98	27.23	31.96	36.56	ND	ND
	43328	18.29	19.97	24.63	28.44	32.33	36.01	39.61	ND
	43330	17.79	19.30	24.64	28.78	31.79	34.69	39.29	ND
Internal control-ADH-1C-m allele (ROX)	43326	29.60	29.07	29.44	29.78	29.66	29.78	29.94	29.44
	43328	29.35	29.25	29.40	29.42	29.44	29.54	29.51	29.06
	43330	29.46	29.24	29.08	29.39	29.46	29.63	29.93	29.10

COVID-19 diagnostic test is shown in Fig. 1. The TaqMan-based real-time RT-PCR [22] system requires three target gene-specific oligonucleotides, including the forward/reverse primers and a probe, to amplify amplicons. This assay is composed of two different steps: reverse transcription and PCR cycles, as in other real-time RT-PCR methods (Fig. 1). However, this assay does not require any RNA templates from the patient or oligo synthesis for RT-PCR positive controls. Instead, this assay requires additional oligos (IPT and bridge primers) in the reverse transcription step and an SPT in the first PCR cycle. In the reverse transcription step, a reverse bridge primer (E') is hybridized to E sequences in the IPT, a synthetic RNA oligo that is composed of target RNA-independent sequences (D and E), and generates cDNA (initiation primer) that consists of D' , E' , and F' sequences. In the first PCR cycle, an SPT, that contains a TaqMan probe region (C) and target RNA-independent sequences (B and D), is used as a template for the initiation primer by hybridizing $D-D'$, which results in an intermediate product that harbors sequences $B'C'D'E'F'$. In the second PCR cycle, the forward bridge primer is hybridized to the bridge region of the first PCR product and amplified to generate an intermediate product. In the third PCR cycle, reverse primer F' was used for the amplification of product ($ABCDEF$). In the fourth and fifth steps, the PCR cycles result in forward primer extension and TaqMan probe cleavage (C) through DNA polymerase 5' nuclease activity.

3.3. Limit of detection of the one-step real-time RT-PCR SPT system

We performed multiplex real-time RT-PCR using an SPT (positive control) as a template in a concentration-dependent manner, to determine the limit of detection (LOD) for individual SARS-CoV-2 genes. To this end, we used different fluorescent dyes for individual targets (Table S3). Using different dyes, we performed real-time RT-PCR assays, and Ct values were measured with 10×10^{-12} (femto) and 10×10^{-18} (zepto) molar (M) concentration ranges when using SPT templates. The results (Table 1) showed that the LODs were $> 10^{-15}$ (atto) M for RdRP, 10^{-12} (femto) to 10^{-13} (100 atto) M for E gene, and 10^{-12} to 10^{-14} (10 atto) M for N gene, respectively. However, Ct values were not measured in the absence of SPTs, which indicates that the detection was solely dependent on the presence of SPTs, in a concentration-dependent manner. Then, we further analyzed the real-time RT-PCR assay using genomic RNAs prepared from SARS-CoV-2 infected cultures in a concentration-dependent manner. Thus, we used serially diluted genomic RNAs at concentrations of 10^{-15} to 10^{-9} g/ μ L in multiplex real-time RT-PCR assays. With regard to the Ct values (Table 2), we found that the Ct values that were obtained from picogram quantities of RNAs prepared from COVID-19 virus cultures resulted in the LOD. However, sensitivity, based on Ct values, differed

among target genes of different SPTs. Notably, RdRP and E genes were detected to a lesser extent than the N gene, which indicates that RdRP and E are less sensitive to this platform than N gene in the diagnosis of COVID-19. Therefore, diagnoses based on RdRP alone might increase the inconclusiveness of COVID-19 and false-negative ratios.

4. Discussion

Real-time RT-PCR molecular diagnoses are used as a gold standard for diseases due to viral infections. However, medical professionals need to be cautious when diagnosing emerging viral infections such as COVID-19, which lacks a method to confirm the molecular diagnosis.

Here, we developed a simple method for preparing positive controls for real-time RT-PCR, a primary method for diagnosing COVID-19. This method obviates the contamination that has obscured the preparation of positive controls for COVID-19 diagnosis and reduces the false-positive rates of diagnoses based on nucleic acid testing.

In previous studies, the Uni-Control method for preparing real-time RT-PCR positive controls has been developed as a generic approach for diagnosing viral infections [22]. This method used two synthetic control oligonucleotides and irrelevant viral nucleic acids as an initiator template. Therefore, it provides an interim platform into which real-time PCR assays can be rapidly introduced for emerging viruses in the absence of wild-type control material [22]. In their reported method, parainfluenza type 2 virus RNA and equine herpes virus DNA were used as initiator templates for influenza A (H1N1) and human metapneumovirus (HMPV) RT-PCR assays, respectively.

However, there is a major limitation of the Uni-control method. Specifically, the primer that contains the real-time PCR probe hybridization site that is used in the Uni-control system produces a background noise through dimer formation. Therefore, Whiley et al. [15] suggests that positive control Ct values should be set to 24–28. Furthermore, this problem will be worse for diagnosing COVID-19, which requires multiplex real-time RT-PCR for measuring the expression levels of multiple target gene. Compared to the Uni-control system, in our newly designed approach, the problematic sequences reside in the positive control SPT, and this reduces such non-specific reactions. Moreover, certain modifications, such as 3'-end phosphorylation in the SPT and 3'-hexandiol in the IPT, reduce potential primer complications in these regions.

Considering the data in Table 1, the use of different amounts of target gene SPTs to obtain similar Ct values should be considered to facilitate positive control comparisons. In Tables 1 and 2, we expected the RdRP gene primer and probe to present relatively lower reactivity than those of other genes. This result was also observed in the report of Kim et al. [23], where N gene sensitivity was 43-fold higher than that of

the RdRP gene. In our report, we found that N gene sets were much reactive and performed 10^2 -fold better than those of RdRP. Therefore, if we consider Charité/Berlin [19], lower amounts of viral infection will be possibly regarded as false-positives. In addition, the low sensitivity of the RdRP and E genes is a unique feature that requires additional use of the N gene, which is very sensitive for detection. However, N gene should be used cautiously since it shows a high mutation rate.

5. Conclusion

Since COVID-19 is highly infectious, we expect that our new approach will eliminate diagnostic contamination problems, accelerate the development of accurate COVID-19 diagnostic kits, and reduce the viral infection spread.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.cca.2020.10.001>.

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