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Rapid and Accurate Detection of the SARS-CoV-2 Omicron Variant with a CRISPR-Cas12a Reaction in the RT-qPCR Pot

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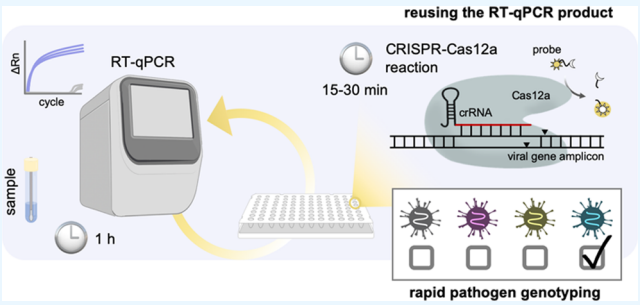
ABSTRACT: Gene sequencing in back of reverse transcription-quantitative polymerase chain reaction (RT-qPCR) is the current approach for discriminating infections produced by different severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) variants in the clinic. However, sequencing is often a time-consuming step, which hinders the deployment of a very fast response during a pandemic. Here, we propose to run a CRISPR-Cas12a reaction after completing the RT-qPCR and in the very same pot to detect with high specificity genetic marks characterizing variants of concern. A crRNA was appropriately designed to detect the S gene of the SARS-CoV-2 Omicron BA.1 variant. A significant response with >20-fold dynamic range was obtained for the Omicron BA.1 S gene, while the Delta S gene did not produce any detectable signal. The sensitivity of the method was analyzed with a series of diluted samples and different Cas12a nucleases. A correlation between the RT-qPCR C_T values and the CRISPR-Cas12a reaction signals was observed. Variant discrimination with the CRISPR-Cas12a reaction was possible in some minutes with high accuracy from patient samples. In conclusion, CRISPR-Cas systems seem ready to be exploited in the clinic to boost personalized diagnoses and accelerate epidemiological surveillance in a cost-effective way.



INTRODUCTION

The huge public health crisis caused by the emergence and spread of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)[1] has underscored the importance of the readiness of fast and precise diagnostic systems. In the clinic, reverse transcription-quantitative polymerase chain reaction (RT-qPCR) is the gold standard diagnostic technique for viral infections due to its high sensitivity and specificity.[2] However, due to the rapid evolution of viruses,[3] it is important to know not only the detection of the infectious agent but also the underlying genotype (variant or strain), in order to anticipate acute clinical courses[4] and adopt confinement measures to avoid superspreading events.[5] In this regard, viral genome sequencing (partial or total) has been widely carried out as a diagnostic backup after a positive signal by RT-qPCR.[6,7] Nonetheless, sequencing requires expensive equipment and specialized staff, so it is usually a time-consuming step (about 1 day in the case of Sanger sequencing and 5 days of whole-genome sequencing if the service is externalized).

To overcome this issue, we have designed appropriate primers and probes to run RT-qPCRs aimed at detecting specific SARS-CoV-2 variants. These approaches comprise target amplification failure,[8] allele-specific amplification and probing,[9,10] and melting curve analysis.[11] Yet, these methods are not fully insensitive to spurious amplifications, and they often require extensive screening of concentrations and annealing temperatures. Here, we propose a novel and simple approach to detect virus variants with ultraspecificity by means of a CRISPR-Cas12a assay[12] on top of the routine RT-qPCR (Figure 1; CRISPR stands for clustered regularly interspaced short palindromic repeats). CRISPR-Cas systems have been applied to detect a variety of pathogens, including SARS-CoV-2, envisioning point-of-care interventions (see ref 13 for a review). In this regard, they are usually coupled to isothermal amplification methods and pursue minimally instrumented signal monitoring. In contrast, the innovation presented in this work is in the ability to interface the CRISPR-Cas12a system with RT-qPCR for rapid pathogen genotyping in addition to detection (i.e., running a CRISPR-Cas12a reaction in the RT-qPCR pot after its completion as a shallow sequencing step), which might be adopted straightforwardly into the clinic. Notably, we applied this approach to detect the Omicron variant[14] without the need for sequencing at a time in which this variant was first being introduced in many countries worldwide.

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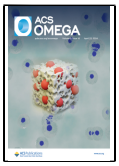

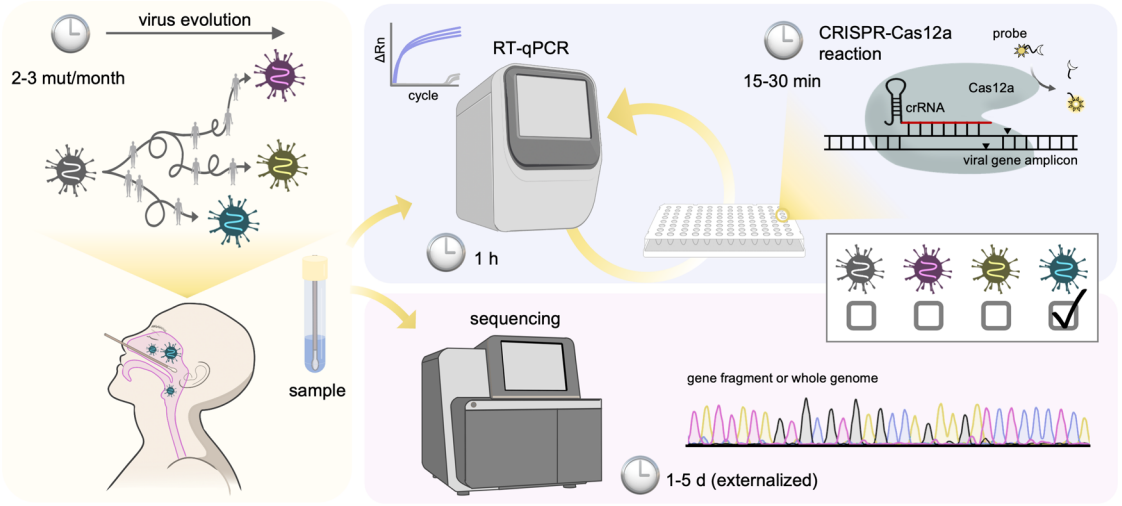

Figure 1. Schematics of the clinical approach followed in this work. Upon sample collection, virus detection can be performed by RT-qPCR (typically in 1 h) and variant discrimination by sequencing (typically in 1 day for a gene fragment and in 5 days for the whole genome). A CRISPR-Cas12a reaction can be run after completing the RT-qPCR for rapid and accurate mutation identification, thereby allowing variant discrimination with less resources.

MATERIALS AND METHODS

Patient Samples. Nasopharyngeal swab samples corresponding to 15 patients with symptoms compatible with coronavirus disease 2019 (COVID-19) were obtained from the Hospital Universitario Marqués de Valdecilla in Spain between late 2021 and early 2022. The ethics committee of the hospital approved this study. Samples were anonymized and inactivated by heat shock before performing any assay. Samples were purified by an RNA clean and concentrator kit (Zymo).

SARS-CoV-2 Detection by RT-qPCR. The TaqPath one-step RT-qPCR master mix, CG (Applied) was used with the Centers for Disease Control and Prevention (CDC) N2 primers to amplify the SARS-CoV-2 N gene (kit provided by IDT) and custom design primers to amplify the S gene (i.e., to perform a multiplexed amplification; Table S1). In a microplate (Applied), 1 μL of sample was mixed with 200 nM of N2 primers, 50 nM of S primers (unless otherwise specified), 50 nM of qPCR probe [single-stranded DNA (ssDNA) labeled with carboxyfluorescein (FAM) and a quencher], and the master mix for a total volume of 20 μL. The microplate was placed in a real-time PCR system (QuantStudio 3, Applied) to measure fluorescence (FAM channel) with the following protocol: 2 min at 25 °C for uracil-*N*-glycosylase incubation, 10 min at 53 °C for RT, 2 min at 95 °C for polymerase activation, and then 40 cycles of 3 s at 95 °C for denaturation and 30 s at 60 °C for annealing and extension. Samples with cycle threshold (C_T) values lower than 40 were considered positive for SARS-CoV-2. Oligonucleotide sequences are provided in Table S1.

DNA Detection by CRISPR-Cas12a. Synthetic double-stranded DNA (dsDNA) molecules mimicking the Omicron BA.1 and Delta S genes were generated for testing purposes (hybridizing two oligonucleotides chemically synthesized by IDT; Table S1). The CRISPR RNA (crRNA) was generated by in vitro transcription with the TranscriptAid T7 high-yield transcription kit (Thermo) from a synthetic DNA template (sequence in Table S1). This element was then purified in a column (Zymo) and quantified in a spectrophotometer (NanoDrop, Thermo). Cas12a from *Lachnospiraceae bacterium* was a commercial preparation (NEB; some experiments were also performed with a preparation from IDT). The CRISPR-Cas12a ribonucleoprotein was formed by incubating crRNA and Cas12a in a suitable buffer (NEBuffer r2.1) for 30 min at room temperature. In a microplate (Applied), 10 nM synthetic DNA (unless otherwise specified) was mixed with 50 nM CRISPR-Cas12a ribonucleoprotein and 500 nM CRISPR probe [ssDNA labeled with carboxytetramethylrhodamine (TAMRA) and a quencher] for a total volume of 20 μL. The microplate was placed in the real-time PCR system to measure fluorescence (TAMRA channel) for 1 h at 37 °C. Represented fluorescence values correspond to absolute signals minus the background signal of the CRISPR probe in the buffer.

Omicron Detection by CRISPR-Cas12a after RT-qPCR. After completion of the RT-qPCR with the primers targeting the SARS-CoV-2 N and S genes, the microplate was supplemented with 50 nM CRISPR-Cas12a ribonucleoprotein and 500 nM CRISPR probe (in total, 2.5 μL was added per well). The microplate was placed back in the real-time PCR system to measure fluorescence (TAMRA channel) for 1 h at 37 °C. Represented fluorescence values correspond to absolute signals minus the background signal of a CRISPR-Cas12a reaction on top of an RT-qPCR without the template.

Gel Electrophoresis. Nucleic acid amplification from a commercial viral genome control (Vircell) or patient samples was confirmed by gel electrophoresis. Samples (10 μL of amplified product) were loaded on a 3% agarose gel prepared with 0.5× TBE buffer, which was run for 30 min at room temperature (120 V). Gels were stained by using RealSafe (Durviz). The GeneRuler ultralow range DNA ladder (10−300 bp, Thermo) was used as a marker.

Virus Sequencing. Amplified DNA from the SARS-CoV-2 S gene generated by RT-PCR was sent to Eurofins Genomics for Sanger sequencing with the SQF primer.

RESULTS

In a region of less than 20 nt of the S gene (coding for the spike protein), Omicron BA.1 carries the Δ211/L212I and ins214EPE mutations,[14] which represent a distinguishable genetic mark with respect to other SARS-CoV-2 variants. We

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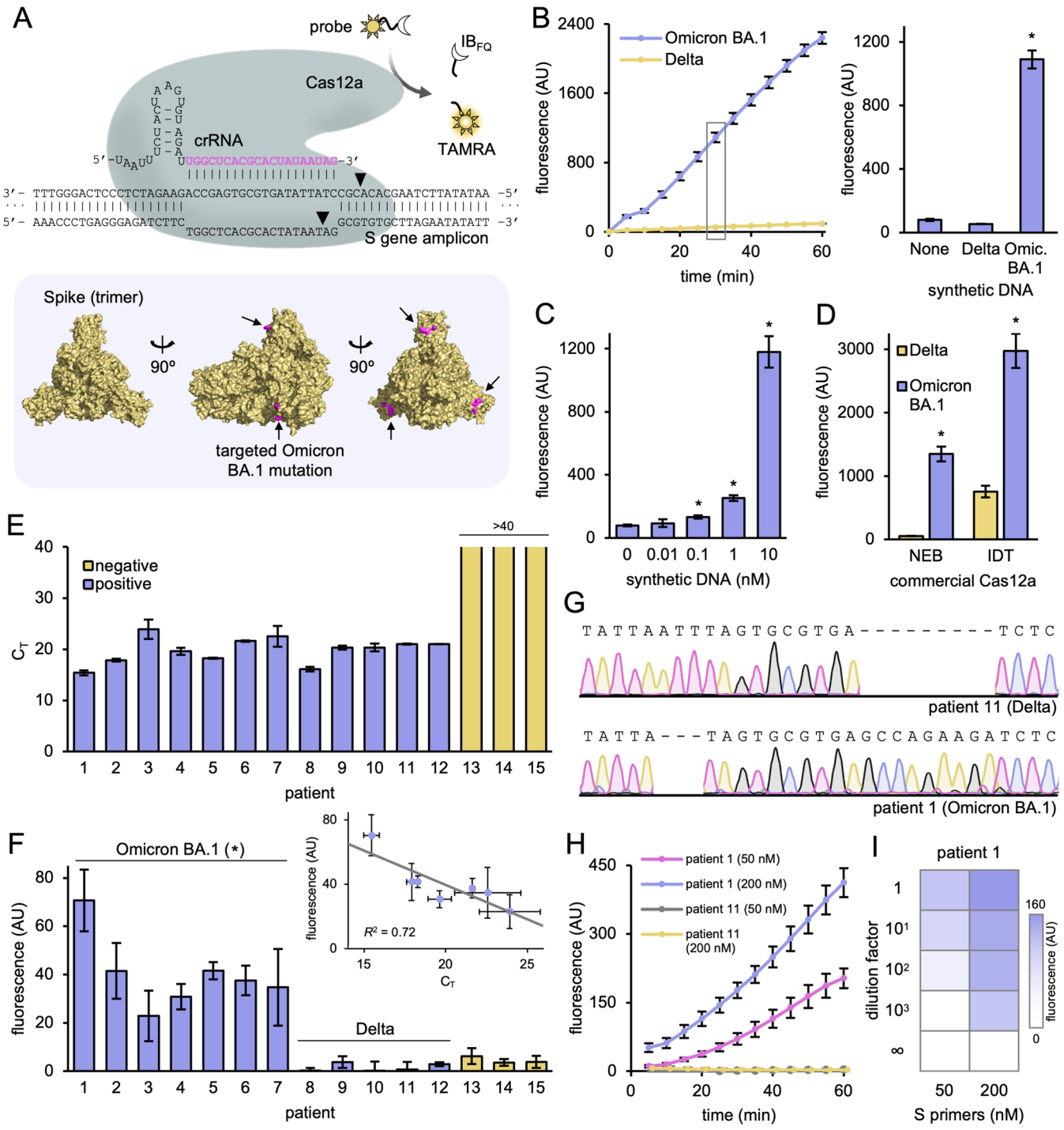

Figure 2. Combination of RT-qPCR and CRISPR-Cas12a to detect the SARS-CoV-2 Omicron BA.1 variant. (A) Scheme of the CRISPR-Cas12a ribonucleoprotein targeting the S gene amplicon (spacer of the crRNA shown in magenta). On the bottom, structural model of the spike protein (Δ211/L212I and ins214EPE mutations colored in magenta). (B) Temporal fluorescence-based characterization of the CRISPR-Cas12a reaction with synthetic DNA as a target. On the right, results at 30 min (*against Delta). (C) Effect of the synthetic DNA concentration (*against 0). (D) Effect of the commercial preparation of Cas12a (*against Delta). (E) Multiplexed RT-qPCR results with primers targeting the N and S genes from patient samples. (F) Detection of the Omicron BA.1 S gene by a CRISPR-Cas12a reaction in the RT-qPCR pot (*against Delta, pooled). In the inset, linear correlation is shown between fluorescence and C_T. (G) Sequence chromatograms from patient 1 (infected by Omicron BA.1) and patient 11 (infected by Delta) of the S gene targeted region. (H) Temporal fluorescence-based characterization of the CRISPR-Cas12a reaction with two different concentrations of the S primers using the samples from patients 1 and 11. (I) Sensitivity analysis by diluting the sample from patient 1. Overall, results obtained at 30 min. Error bars correspond to the standard deviations (n = 3). Statistical significance was assessed by Welch's t-test, two-tailed $P < 0.05$. AU, arbitrary units.

exploited the fact that ins214EPE introduces a reverse complementary noncanonical protospacer adjacent motif (PAM) in the viral genome to design a suitable crRNA (Figure 2A). This PAM (CTTC) can be recognized by Cas12a with sufficient affinity.[15] Using synthetic dsDNA molecules mimicking potential amplified products from Omicron BA.1

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and the ancestral genotype, we show that a CRISPR-Cas12a ribonucleoprotein specifically detected the intended target, producing a significant fluorescence readout (Figure 2B). The collateral catalytic activity of Cas12a upon dsDNA target recognition served to cleave a fluorogenic ssDNA probe to set apart the attached quencher, thereby obtaining a quantifiable readout. The detection was possible even in 5 min.

To study the responsiveness of the system, we varied the concentration of the target DNA. Concentrations above 0.1 nM started to produce a significant readout (Figure 2C), thereby determining the minimal level to be reached in the preceding amplification step. Moreover, we tested two commercial preparations of Cas12a (one from NEB and another from IDT) obtained from the same bacterium. The IDT nuclease showed greater activity (2.2-fold in maximal fluorescence signal), but it displayed a smaller dynamic range to discriminate viral genotypes (24.5-fold at 30 min for the NEB nuclease and 3.9-fold for the IDT nuclease; Figure 2D).

Motivated by these pilot results, we collected a series of nasopharyngeal swab samples from patients with symptoms compatible with COVID-19 in order to detect the virus by RT-qPCR and identify the variant via a subsequent CRISPR-Cas12a reaction. This was done in a time period in which the different national public health systems were concerned by the emergence of a new variant in South Africa with presumed higher transmissibility.[14] According to the data from Nextstrain,[16] the Delta variant (clade 21J) was displaced by Omicron BA.1 (clade 21K) in Spain during the development of this work (between November 2021 and January 2022; Figure S1). To detect the presence of SARS-CoV-2 in the samples, we relied on the CDC N2 primers and probe, which were specifically designed for RT-qPCR-based diagnoses in the clinic. Here, we designed a custom pair of primers to amplify the S gene at the mutated region, and we checked their suitability to perform a multiplexed amplification (Figure S2). By means of RT-qPCR, we identified 12 out of 15 patients infected by the virus (C_T > 40 for 3 patients; Figure 2E).

The silent S gene amplicon in the multiplexed RT-qPCR was subsequently used to run a CRISPR-Cas12a reaction, which occurred in the very same RT-qPCR pot after the addition of a ribonucleoprotein-containing microdrop. The CRISPR probe was labeled with TAMRA to be orthogonal to the FAM-based qPCR probe. Importantly, we found that 7 out of the 12 infected patients carried the Omicron BA.1 variant (Figure 2F), which produced a substantially different response to Delta (24.9-fold on average). Moreover, a correlation between the RT-qPCR C_T values and the CRISPR-Cas12a reaction signals was observed. We confirmed these results by Sanger sequencing of the S gene (Figure 2G; see also Figure S3). To enlarge the dynamic range of the response and achieve confident variant discrimination in less time, the concentration of the primers targeting the S gene was increased (response of 55.1-fold on average at 30 min; Figure 2H). Finally, we analyzed the sensitivity of the method using dilutions of a patient sample, showing discrimination for C_T < 30 (Figure 2I; see also Figure S4).

DISCUSSION

CRISPR-Cas systems have been developed and applied for point-of-care testing together with isothermal amplification methods,[17] and a direct detection of the viral genome has even been accomplished with an RNA-targeting effector bypassing the preamplification step.[18] In the clinic, CRISPR-Cas systems may also be useful due to their easy implementation and high interoperability with already approved protocols by competent authorities. Through the use of a CRISPR-Cas12a reaction after completing multiplexed RT-qPCR, we were able to discriminate infections produced by a particular SARS-CoV-2 variant. In less than 15 min, this supplementary reaction was able to provide a suitable indication, which saves valuable time compared to sequencing and allows information to be provided to the patient at diagnosis. Intriguingly, this procedure may even be complementary to those RT-qPCR-based methods developed for virus variant determination[8−11] to double-check the results or detect additional mutations of interest.

The crRNA employed here was designed to recognize mutations carried by the Omicron BA.1 lineage. However, this subvariant was later displaced by other Omicron lineages that did not carry the targeted mutations,[19] which stresses the need for a continued design process of crRNAs during the real time of pandemic (note that, overall, SARS-CoV-2 evolves by accumulating 2−3 mutations per month[20]). Sequence discrimination by CRISPR-Cas12a relies on both the interaction of the spacer of the crRNA with the target[21] and the PAM recognition by the Cas12a nuclease,[22] thereby offering enough flexibility to target many different mutations occurring in the viral genome. In addition, the use of the CRISPR-Cas12a system is competitive in economic terms. We estimate a cost of $0.2 per reaction, while Sanger sequencing costs about $3 per sample. All in all, CRISPR-Cas systems are increasingly being proven to be ready to complement current diagnostic procedures in the clinic. Our procedure may be of great utility for future personalized diagnoses and epidemiological surveillance.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c09717.

Nucleotide sequences of the elements used in this work (Table S1); information about virus evolution (Figure S1); viral sequence alignments from patient samples (Figure S3); and additional experimental results (Figures S2 and S4) (PDF)

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Author Contributions

G.R. designed the research. R.R. and R.M.M. performed the experiments. A.D.G., D.P.M., M.G., J.C.M., and J.N. collected the patient samples and performed the initial diagnoses in the hospital. R.R., R.M.M., and G.R. analyzed the data. R.R. and G.R. wrote the manuscript. All authors revised the manuscript.

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Notes

The authors declare no competing financial interest.

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