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ALTERNATIVES TO VIRAL TRANSPORT MEDIUM FOR USE IN SARS-COV-2 SAMPLE PREPARATION

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14. ABSTRACT

The COVID19 outbreak has severely impacted laboratory supply chains for all materials required for testing. This supply chain concern has begun to impact the reagents needed for sample collection and transport. In a news article released by National Public Radio, the apparent decrease in new tests seen by contract laboratories like Labcorp and Quest can partially be attributed to a limited supply of VTM. To address this gap in sample transport supplies before it becomes a critical point within the MHS, GEIS requested USAFSAM/PHT perform a bridge study to evaluate the performance of saline and RNAlater as alternative transport media. We performed a six-point dilution series over three days and a freeze-thaw cycle to determine the performance the CDC SARS-CoV-2 assay in samples near the limit of detection prepared in RNAlater or buffered saline. Our results indicate that samples prepared in buffered saline and frozen encounter no reduction in assay sensitivity or increase in variability, but even after only 24 hours of refrigeration the saline samples begin to degrade. In contrast, RNAlater successfully stabilized refrigerated samples with no change in performance over 72 hours of refrigeration, but freezing RNAlater-stabilized samples resulted in decreased assay performance for concentrations near the lower limit of detection. We recommend advising GEIS partner network and MHS clinical labs to collect NP/OP swabs in RNAlater or buffered saline and for sample transport on dry ice to USAFSAM and updating EUA submissions with reference to this bridge study demonstrating suitability of RNAlater and saline as alternative sample collection and transport materials.

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1 SUMMARY

The aggressive nature and rapid spread of coronavirus infections in the 2019 SARS-CoV-2 pandemic resulted in severe strain on the global supply chain for acquiring and testing clinical samples. Within the Department of Defense, many laboratories utilize the US Air Force School of Aerospace Medicine's Epidemiology Laboratory (EpiLab) for their reference testing and supplier for upper respiratory pathogens. During normal operations, the EpiLab maintains sufficient supplies to accomplish the winter workload anticipated for seasonal flu and colds.

However, during the COVID19 outbreak, the supply of viral transport media (VTM) was significantly reduced and became limiting. Labs require VTM in order to stabilize samples (nasopharyngeal swabs, nasal wash, or oropharyngeal swabs) for transport from the site of collection to the EpiLab at Wright Patterson AFB. The dwindling supply of VTM led the DoD's Global Emerging Infection Surveillance (GEIS) partner network to evaluate alternative stabilization methods through the US Air Force School or Aerospace Medicine's Applied Technology and Genomics Laboratory (ATG). Both EpiLab and ATG are part of the Schoolhouse's Public Health and Preventive Medicine Department, which enables efficient test development, validation, and transition between the two laboratories.

In the current study, VTM was a reference for a time-course using phosphate-buffered saline (PBS) and RNAlater as alternatives. ATG tested six concentrations at the low end of the Center for Disease Control and Prevention's (CDC) real-time reverse transcriptase polymerase chain reaction (rRT-PCR) assay which had Emergency Use Authorization (EUA) from the Food and Drug Administration. To simulate shipping conditions, positive control RNA provided by the CDC was diluted and stored under refrigeration for up to 3 days or frozen on dry ice.

Upon dilution, both RNAlater and PBS exhibited expected performance consistent with rRT-PCR assay verifications in VTM. Over the 72-hour time course and after a dry-ice freeze-thaw cycle, RNAlater performance metrics were identical with the initial dilution results. As expected, samples diluted and stored in PBS exhibited degradation over time and after a single freeze-thaw cycle.

In conclusion, the results from ATG demonstrate that RNAlater and PBS are suitable alternatives to VTM for shipping frozen clinical specimens. This research team recommends inclusion of RNAlater or PBS as a sample collection method in the next update to the CDC's EUA and its inclusion in future molecular testing sample collection EUA applications..

2 INTRODUCTION

The late fall and winter of 2019 saw the global pandemic outbreak of a novel coronavirus strain emerging from Wuhan, China [World Health Organization, 2020], testing the readiness for widespread molecular diagnostic testing. Fundamental to controlling and understanding the spread of the virus throughout the community is a well-established laboratory testing and surveillance program. During the course of the outbreak, the United States Food and Drug Administration issued numerous Emergency Use Authorizations to the clinical laboratory network, including a real-time reverse-transcriptase polymerase chain reaction (rRT-PCR) assay developed by the US Centers for Disease Control and Prevention (CDC). This assay is now the primary test used throughout the Department of Defense [CDC, 2020a] and numerous civilian sector healthcare and public health facilities.

Briefly, the lab test relies upon a nasal or oral swab to collect viral particles from a suspected patient's upper airway or a saline rinse of the nasal passages. The collecting provider then places the swab into a stabilizing medium, commonly called a viral transport medium (VTM). Those samples are then shipped to molecular laboratories for processing and clinical diagnostic testing. In the Department of Defense, the primary central reference laboratory is the US Air Force School of Aerospace Medicine's Epidemiology Laboratory (EpiLab), located at Wright Patterson AFB, Ohio. The global network of public health laboratories across the Department ship samples to this centralized facility, leveraging a standardized process for supply chain management. However, this standardized supply chain process is shared commonly with the global civilian testing centers since all clinical labs must adhere to the FDA guidance. The global supply chain for all sample collection, processing, and testing materials has been severely degraded during the COVID19 pandemic [REF]. The supply chain issues extend beyond just the molecular testing supplies to include the VTM required to ship samples from hospitals to labs [REF].

The current CDC guidance lists Amies transport medium and sterile saline as the only alternatives to VTM [CDC, 2020b]. With only three viable alternatives, there is a clear need to investigate other options. Additionally, sterile saline does not contain any stabilizing agents and the viability of samples for testing after prolonged shipping times poses a concern. Recent evidence suggests that the stabilization agent RNAlater may be used to prolong RNA virus stability for downstream molecular testing [Kohl et al, 2017]. Furthermore, viral infectivity remains high when samples are stored in RNAlater for over 30 days at room temperature, 4°C or -80°C, suggesting the highly protective and stabilizing nature of RNAlater [Pham et al, 2018]. RNAlater has also been shown to be protective of the H5N1 highly pathogenic avian influenza virus in fecal homogenate [Forster et al, 2008].

Here we report the results of our efforts to complete a bridge study evaluating RNAlater as a viable sample transport alternative to VTM. We employed methods taken from the Centers for Disease Control and Prevention's Diagnostic Panel Emergency Use Authorization [CDC, 2020a] to ensure method standardization for clinical utility. We compared RNAlater to phosphate buffered saline over a course of 3 days at 4°C and included a single freeze-thaw cycle to simulate shipping. We also used assay verification results performed in negative patient VTM samples as a reference comparator. We assessed the PCR performance of the SARS-CoV-2 assays in the respective media using two-way analyses of variance and goodness of fit comparisons from non-linear first-order exponential regression fits.

3 MATERIALS AND METHODS

3.1.1 Sample Preparation

The in vitro transcribed SARS-CoV-2 RNA standard available from the CDC (10,000 copies/µL) was direct diluted to 300 copies/µL in respective medium (VTM, PBS, or RNAlater) and then diluted serially 1:3 to 0.9 copies/µL in respective medium. Final amounts of RNA in the reactions were 75, 25, 8.3, 2.8, and 0.9 copies. The CDC indicates a limit of detection for the assays as one copy. The 5-point concentration series was then aliquoted into 500 µL samples and stored at 4°C for RNA extraction at 0 hr, 24 hr, 48 hr, and 72 hr post-dilution. An additional 500 µL sample was placed on dry ice immediately following dilution to simulate overnight shipping. We also included a medium-only sample for each time point to serve as a negative process control. The VTM used was the Remel MicroTest M4RT.

3.2 RNA Extraction

We extracted RNA from the samples using a single QIAamp Viral RNA Mini kit (Qiagen, Cat #52906) following the manufacturer's instructions. Our input volume was 140 µL and each sample was extracted in triplicate. Extractions were performed independently, and the extracted RNA was stored at -80°C until use for PCR. This ensured that all samples received identical treatment throughout the process.A Fourth Level Heading That Further Describes the Model

3.3 Thermocycling Preparation and Parameters

We performed thermocycling reactions under conditions listed in the EUA. After manual extraction with the EUA approved kit, RNA was introduced to the version 2 EUA assays purchased from IDT. We did not include N3 assay in our study as the version 3 EUA instructions-for-use eliminated this assay from recommended testing. We used the EUA approved TaqPath 1-step RT-qPCR Master Mix, CG (Thermofisher, A15299) for all samples in 20 µL final reaction volumes (8.5 µL nuclease-free water, 1.5 µL primer/probe mix, 5 µL Master Mix, and 5 µL sample RNA). We included three pre-cycling stages: 2 min at 25°C, 15 min at 50°C for reverse transcription, 2 min at 95°C for heat inactivation of reverse transcriptase and heat activation of the DNA polymerase. Following the hot-start stage, 45 cycles were used of 3 sec at 95°C and 30 sec at 55°C in standard mode on an ABI 7500 FAST (Thermo Fisher). Although the master mix did include ROX as a passive reference dye, in accordance with CDC guidance, we did not use a passive reference in data acquisition. Each plate also included a No Template Control (NTC) well for each assay to serve as an amplification control. In accordance with the CDC EUA guidelines, we characterized amplification as positive if the Ct was below

3.4 Data Analysis and Statistics

We compared PCR performance of PBS and RNAlater against the reference material of VTM. We used Ct values for each of the triplicate concentration-condition samples to obtain an average and deviation. Each primer in the EUA was compared independently using GraphPad Prism 7.0c. We fit concentration versus Ct data using a first-order exponential regression ($Ct = (Ct0 – A) e^{-K*[RNA]} + A$), where A is a fit parameter relative to the plateau and gap, and K is a fit parameter relative to the concentration in the experiment. We calculated differences in performance over time using a repeated measures two-way ANOVA correcting for multiple comparisons for each alternative medium and assay with the immediate addition (0 hours) as the

reference and a 5% false discovery rate approach according to the two-stage step-up method [Benjamini et al, 2006]. When necessary for use in the two-way ANOVA, we imputed missing values with non-linear regression predicted values. We report mean values with the standard deviation.

4 RESULTS

4.1 PCR Results for VTM

Our PCR results for VTM indicate an issue with the lot of VTM (lot 850127, expiration 24FEB21). Upon dilution of the positive control, our initial PCR resulted in undetectable signals. The only concentration for which we obtained measurements was 75 copies. The triplicate Ct values for N1 were 34.0, 34.9, and 34.9; for N2 were 36.2, 34.4, and undetectable; and for Rp were 34.2, 34.4, and undetectable. The expected Ct for 75 copies is between 25 and 26.

In an independent verification of automated extraction methods, the EpiLab encountered the same problem with this lot of VTM as our lab. Their average Ct values (with deviations) for N1, N2 and Rp assays at 75 copies were 34.6 ± 0.6, 34.6 ± 0.1, and 34.3 ± 1.3, respectively. An assay verification from March 2020 in their laboratory showed an average Ct of 28.7 ± 0.3, 30.4 ±1.2, and 28.7 ± 1.4, respectively, at 25 copies. In our study here, we observed initial 25 copy Ct values of 27.4 ± 0.1, 28.6 ± 0.4, and 27.4 ± 0.7, respectively, in RNAlater. The March EpiLab verification assay included 250 and 25 copies tests, whereas neither their extraction study nor our VTM alternatives study included a measurement at 250 copies. Collectively, our results from both labs and RNAlater indicate that the VTM is inhibiting the reactions, whereas the positive control is performing as expected.

We ran a follow up test using a second lot of VTM (lot 448205, expiration 10JUN20) and two lots of universal transport medium (UTM) manufactured by BD Diagnostics (lots 2002004 and 2005168, expiration JUL21 and SEP21, respectively). These tests were performed using only three concentrations: 250, 75, and 25 copies to replicate two data points obtained here using the initial lot of VTM (75 and 25 copies) as well as to replicate two data points used in the EpiLab assay verification study (250 and 25 copies). The average Ct values of triplicate 75 copy samples in VTM lot 448205 were 33.5 ± 0.4, 35.0 ± 0.9, and 34.1 ± 0.3 for N1, N2, and Rp, respectively. The same values for UTM lot 2002004 were 28.1 ± 0.9, 31.5 ± 0.3, and 31.1 ± 0.3 and for UTM lot 2005168 were 31.0 ± 0.6, 33.3 ± 1.2, and 32.3 ± 1.0.

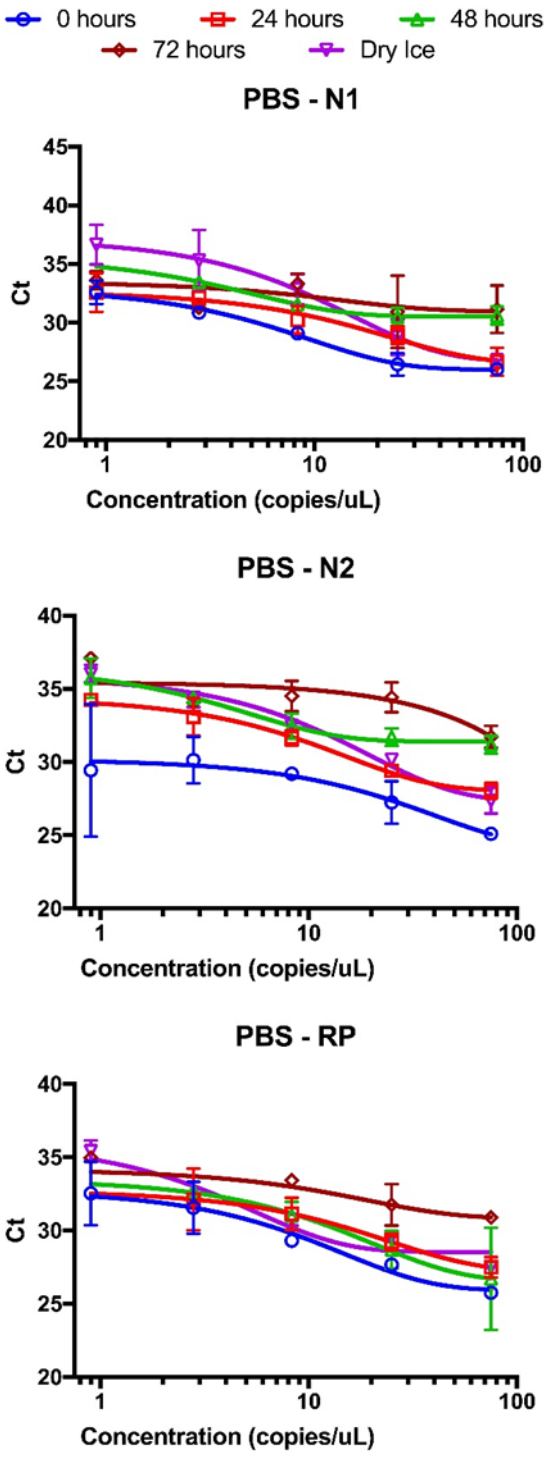

Figure 1 Ct value vs. RNA copies for samples in PBS.

****All three assays exhibited degraded reliability and analytic sensitivity from 0 hours (blue circles) to 72 hours (brown diamonds). Samples exposed to a freeze-thaw cycle (dry ice, purple inverted triangles) also performed poorly compared to 0 hours.**

4.2 PCR Results for PBS

Samples diluted into PBS performed as expected for all three assays immediately after preparation. The Ct values for 75 copies of RNA were 26.0 ± 0.3, 25.1 ± 0.3, and 25.8 ± 0.3 for N1, N2, and Rp, respectively. When fit to a first order exponential curve, the curves visually fit well (Figure 1, blue circles) and the goodness of fit r2 values for N1 and Rp were 0.95 and 0.83, respectively, but the goodness of fit r2 for N2 was only 0.60. The Ct values observed for 0.9 copies included an outlier of 26.2 compared to one at 32.6 and a failed third replicate. The triplicate values for 2.8 copies were 31.9, 28.9, and 29.5, so masking the low Ct value from 0.9 copies resulted in a goodness of fit r2 value of 0.83, indicating a well performing assay condition. The performance of the three assays exhibited significant time-dependence, as after 24 hours the Ct values began to shift upward (Figure 1) and the data became more variable (discussed below in greater detail). The negative control extractions and NTCs did not amplify in any condition for any assay.

4.3 PCR Results for RNAlater

Samples diluted in RNAlater displayed similar behavior as with PBS immediately upon dilution. The Ct values for 75 copies of RNA were 25.4 ± 0.1, 26.7 ± 0.2, and 25.5 ± 0.6 for N1, N2, and Rp, respectively. Additionally, the exponential curve fits were visually tight (Figure 2, blue circles) and the goodness of fit r2 values were 0.94, 0.96, and 0.95, respectively. Assay performance was largely unaffected by exposure to refrigeration at 4°C over three days and freeze-thaw. The exceptions were the low RNA copy values near the limit of detection after exposure to dry ice. The negative control extractions and NTCs did not amplify in any condition for any assay

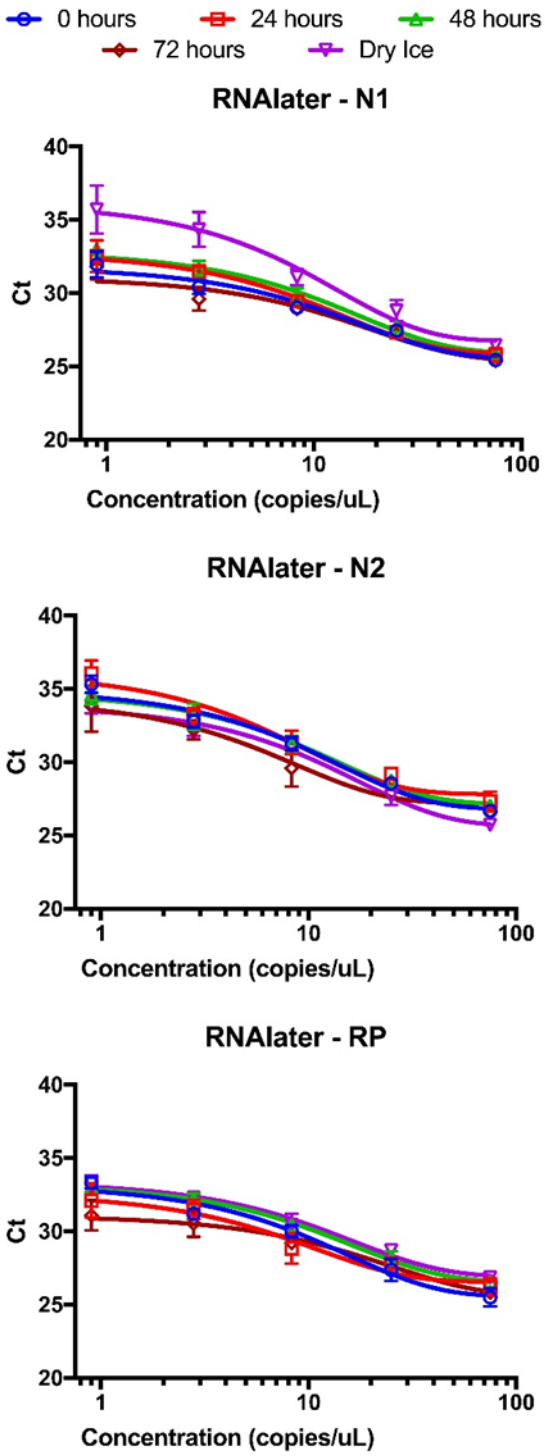

Figure 2 Ct value vs. RNA copies for samples in RNAlater.

****All three assays exhibited consistent reliability and performance over time. Only the N1 assay with low amounts of RNA was negatively impacted a freeze thaw cycle (top panel, purple inverted triangles).**

5 DISCUSSION

As expected, the performance of the PCR assay became highly variable over time in samples prepared in PBS. Figure 2 above shows this variability visually, but the degree to which the data quality becomes degraded is most obvious when assessing the fit parameters. Specifically, comparing the goodness-of-fit values clearly demonstrates the increased variability, and therefore the decreased reliability, of samples prepared in PBS compared against those in RNAlater is the goodness-of-fit (Table 1). The average goodness-of-fit coefficient of variation for RNAlater samples across time is consistently below 5%, whereas the CVs seen in PBS samples is consistently above 11%. Only for the freeze-thaw cycle are the fits consistently good for samples in PBS. Furthermore, when one considers the assay-specific variability over time, we see that for RNAlater the fits are consistently good within assays (average r2 = 0.94, CVs < 5%), whereas for PBS the fits are poor and variable (r2 between 0.7 and 0.81, CVs >15%). These observations suggest that the variability of the raw data is large across time in the PBS samples and that RNAlater permits time-independent reliable data acquisition.

Table 1 Goodness-of-fit comparison by assay, medium, and time.

					Time-dependent Variation		
		N1 r^2	N2 r^2	RP r^2	Average r^2	Deviation	CV
RNAlater	0 hr	0.943	0.959	0.953	0.95	0.01	0.8%
	24 hr	0.957	0.923	0.885	0.92	0.04	3.9%
	48 hr	0.973	0.961	0.985	0.97	0.01	1.2%
	72 hr	0.895	0.866	0.928	0.90	0.03	3.5%
	Dry Ice	0.931	0.979	0.960	0.96	0.02	2.5%
PBS	0 hr	0.953	0.603	0.834	0.80	0.18	22.3%
	24 hr	0.848	0.943	0.746	0.85	0.10	11.7%
	48 hr	0.764	0.867	0.639	0.76	0.11	15.1%
	72 hr	0.026	0.688	0.617	0.44	0.36	81.9%
	Dry Ice	0.884	0.958	0.886	0.91	0.04	4.6%
	Assay-dependent Variation						
RNAlater	Average r^2	0.94	0.94	0.94			
	Deviation	0.03	0.04	0.04			
	CV	3.1%	4.8%	4.0%			
PBS	Average r^2	0.70	0.81	0.74			
	Deviation	0.38	0.16	0.12			
	CV	54.7%	19.5%	15.8%			

Our ANOVA results further corroborate the high variability in the PBS data and the consistency of the RNAlater data. For PBS, the time/condition factor accounted for 20.7%, 44.3%, and 17.0% of the variance in the data for N1, N2, and Rp assays, respectively. In contrast, the effect of time was much less in RNAlater samples. The respective values were 11.6%, 2.95%, and 4.50% of the variance could be explained by time/condition. For the N1 assay in RNAlater, multiple comparisons revealed that the variance is mostly affected by the dry ice condition.

When those data were masked from the ANOVA, time accounted for only 2.60% of the data variance and we observed significant differences as measured by a 5% false discovery rate at 0.9, 2.8, and 8.3 copies. These observations are consistent with the visual difference between the dry ice curve and all others in RNAlater N1 (Figure 2), and suggest that the limit of detection of the assay is being affected by a freeze-thaw cycle.

Our results showing that a sample frozen on dry ice immediately following preparation in PBS works as well as PBS diluted samples not frozen is consistent with other research [Kohl et al, 2017; Mutter et al, 2004; Wang et al, 2006]. Next generation sequencing assays are often more sensitive to RNA degradation than RT-PCR assays, and snap frozen Sendai virus samples were able to be sequenced successfully [Kohl et al, 2017]. When sequenced, a naïve sample (unextracted) resulted in 4 reads covering 6% of the genome. In contrast, the snap frozen PBS sample yielded more than 136,000 reads covering 88.1% of the genome. Samples stored in RNAlater at room temperature for 7 days yielded better performance than snap frozen RNA (more than 198,000 reads, 98.8% genome coverage). Taken together, the Sendai virus sequencing results and our SARS-CoV-2 rRT-PCR results establish a consistent evidence-base for using RNAlater or snap frozen PBS for sample collection and transport.

6 CONCLUSIONS AND RECOMMENDATIONS

Our results show that RNAlater and buffered saline are suitable alternatives to VTM for transporting frozen samples from medical treatment facilities to reference laboratories. If long-term or non-frozen shipping is desired, then RNAlater is a suitable alternative to VTM and buffered saline is not appropriate. We recommend including RNAlater or saline addition as an alternative method for sample preparation in all EUAs submitted to the FDA for use in COVID19 response. Furthermore, we recommend including language in EUA submissions stating that if samples are not collected in RNAlater, they be treated with RNAlater in accordance with manufacturer's guidelines to stabilize the samples for transport. Finally, we recommend that clinical laboratories update their sample submission, preparation, and accession guidelines to include optional, though recommended, addition of RNAlater to all samples prepared for viral testing in suspected upper respiratory infection cases.

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