Removal of SARS-CoV-2 viral markers through a water reclamation facility

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Abstract
There have been multiple reports of COVID-19 virus, SARS-CoV-2 RNA presence in influent wastewater of water reclamation facilities (WRFs) across the world. In this study, the removal of SARS-CoV-2 RNA was investigated in a WRF by collecting samples from various stages related to hydraulic retention time (HRT) and analyzed for viral RNA (N1 and N2) gene markers and wastewater characteristics. SARS-CoV-2 RNA was detected in 28 out of 28 influent wastewater and primary effluent samples. Secondary effluent showed 4 out of 9 positive samples, and all tertiary and final effluent samples were below the detection limit for the viral markers. The reduction was significant (p value < 0.005, one-way analysis of variance [ANOVA] test) in secondary treatment, ranging from 1.4 to 2.0 log10 removal. Adjusted N1 viral marker had a positive correlation with total suspended solids, total Kjeldahl nitrogen, and ammonia concentrations (Spearman's ρ = 0.61, 0.67, and 0.53, respectively, p value < 0.05), while demonstrating a strongly negative correlation with HRT (Spearman's ρ = −0.58, p value < 0.01).

Practitioner points
- Viral RNA was present in all samples taken from influent and primary effluent of a WRF.
- SARS-CoV-2 gene marker was detected in secondary effluent in 4 out of 9 samples.
- Tertiary and final effluent samples tested nondetect for SARS-CoV-2 gene markers.
- Up to 0.5 and 2.0 log10 virus removal values were achieved by primary and secondary treatment, respectively.

KEYWORDS
COVID-19, RT-qPCR, SARS-CoV-2 gene markers, wastewater reclamation

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INTRODUCTION

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is responsible for the current global pandemic of coronavirus disease (COVID-19). Belonging to the genus Betacoronavirus, SARS-CoV-2 is a positive-sense single-stranded RNA virus (Chan et al., 2020; Lai et al., 2020; Lu et al., 2020; Zhu et al., 2020). Multiple studies have reported the presence of SARS-CoV-2 in the feces of infected patients (Sun et al., 2020; Wang et al., 2020; Wu et al., 2020). Subsequently, the presence of SARS-CoV-2 in untreated wastewater has been reported by many recent studies globally (Ahmed et al., 2020; la Rosa et al., 2020; Medema et al., 2020). However, the survivability and fate of the virus or virus markers during wastewater treatment have not been adequately investigated from full-scale water reclamation facilities (WRFs).

While less is known about the fate of SARS-CoV-2 RNA through wastewater treatment systems, results from several studies have documented that temperature, biological activity, and wastewater matrix composition greatly influence the persistence of enveloped viruses in wastewater (Aquino de Carvalho et al., 2017; Bibby et al., 2015; Bivins et al., 2020; Casanova et al., 2009; Ye et al., 2016). It is possible that wastewater matrix composition may affect virus persistence in WRFs as well. Matrices with higher organic content showed increased persistence of enveloped viruses, which has been attributed to viral aggregation to suspended particles, creating protection, and resulting in increased resistance to stressors (Aquino de Carvalho et al., 2017; Bibby et al., 2015). Accordingly, it has been demonstrated that coronaviruses (CoVs), like feline infectious peritonitis virus (FIPV) and human coronavirus 229E (HCoV), persist longer in the unfiltered primary than unfiltered secondary treated wastewater; this was attributed to the protection level provided to virions by solids attachment from predation and inactivation (Gundy et al., 2009). While solid attachment may provide protection, this phenomenon could be beneficial for virus removal in the settling steps of a WRF, as primary and secondary treatment focus on the removal of total suspended solids (TSSs).

Several studies report monitoring data for the presence of SARS-CoV-2 gene markers in both influent and effluent. Results demonstrated viral RNA presence in influent and nondetect in the effluent (Balboa et al., 2021; Haramoto et al., 2020; Randazzo et al., 2020; Rimoldi et al., 2020; Sherchan et al., 2020). However, the presence of SARS-CoV-2 RNA varies for wastewater treatment processes, in a few studies that included monitoring throughout the treatment process train of a WRF, creating uncertainty on removal mechanisms for the virus. Although some variance can be attributed to nonoptimized virus concentration methods, there remains an information gap on the fate of SARS-CoV-2 RNA in the treatment stages of WRFs. Variance in positive results following primary treatment warrants further research to determine the routes of virus removal throughout different treatment processes. Therefore, the objectives of this study were to (1) monitor SARS-CoV-2 RNA at a WRF after different treatment stages; (2) determine which treatment process has the highest removal rates between primary, secondary, tertiary, and disinfection processes; and (3) determine the relation between wastewater characteristics and operating conditions and viral RNA removal. In this study, aside from centrifugal ultrafiltration, a two-step concentration method, hollow fiber ultrafiltration (HFUF) followed by polyethylene glycol (PEG) precipitation, was used for large volume samples grabbed from secondary, tertiary, and final effluent. This method was successful in SARS-CoV-2 RNA detection in the secondary treatment effluent samples.

METHODS

Collection of wastewater samples

Wastewater and treated wastewater were collected after different treatment stages from a WRF in Washoe County, Nevada, USA, with the capacity of 167 × 10^3 m³/day (44 million gallons per day [MGD]) and an average operating flow rate of 114 × 10^3 m³/day (30 MGD) during the study period. The WRF treatment train included screening, grit removal, primary sedimentation, secondary treatment with enhanced biological phosphorus removal (EBPR) activated sludge process, tertiary nitrification and denitrification, post aeration, sand filtration, and final disinfection using chlorine (Lacroix et al., 2020). Samples were grabbed from five different process locations throughout the WRF daily from November 9 to November 22, 2020. As demonstrated in Figure 1, locations are as follows: (1) primary influent (after grit removal), (2) primary effluent, (3) secondary effluent, (4) tertiary effluent, and (5) final effluent post chlorination.

Grab samples were collected across all locations over a period of 2 weeks. HRTs for the treatment process were calculated to account for the daily flow rate. Sample collection at Location (1) began consistently between 9:45 a.m. and 10:30 a.m., and the final sample was taken 14–20 h after. It must be noted that the SARS-CoV-2 RNA gene signal may potentially depend on the grab versus composite sample type as a ~10-fold increase in concentration was reported in the composite sampling of
influent and primary effluent as relative to corresponding grab primary effluent samples (Gerrity et al., 2021). In this study, for the purpose of following the wastewater through the facility based on the HRT values of each treatment process, grab sampling collection was performed. Influent (1) and primary effluent (2) samples were collected in 1-L sterile bottles; 10-L volume samples were collected for secondary (3), tertiary (4), and post-disinfection (5) effluent. Water temperature, pH, and dissolved oxygen (DO) measurements were measured on-site immediately during sampling. Samples were stored at 4°C and transported to the lab within 24 h for processing. Samples were processed to concentrate SARS-CoV-2 RNA gene markers within 3 days. Other water quality parameters such as total Kjeldahl nitrogen (TKN), TSS, total dissolved solids (TDSs), ultraviolet (UV) absorbance measurements at 254 nm (UVA254), and dissolved organic carbon (DOC) were measured according to Standard Methods (APHA, 2018).

**Viral RNA concentration**

Primary influent and effluent samples (Locations 1 and 2, shown in Figure 1) were concentrated using centrifugal ultrafiltration; 100 mL of a homogeneous mixture was centrifuged at 3000g for 15 min to remove particles and debris. Vacuum filtration was conducted on the supernatant using 1.5-μm filter paper (Whatman, Marlborough, MA, USA), 0.8-, and 0.45-μm membrane filters (Merck Millipore, Burlington, MA, USA) in sequence. A total of 30 mL of the resulting wastewater sample was concentrated by a 100-kDa Amicon® Ultra-15 Centrifugal Filter Cartridge Units (Millipore Sigma, St. Louis, MO, USA) and centrifuged at 3500g until 200–300 μL was maintained in the filter. Samples were refrigerated until RNA extraction within 48 h.

Considering the possible low presence of SARS-CoV-2 in secondary, tertiary, and final effluent sampling, this study used a two-step concentration method, combining HFUF and PEG precipitation, to process large volumes of those samples to enrich more. First, it is necessary to remove excess particles in the samples (Locations 3–5, shown in Figure 1). The samples were filtered by 1.5-μm membranes. Subsequently, samples were recovered by HFUF (REXCEED-25S, 30 kDa, Asahi Kasei Medical Co., Japan). First, the sterile blocking buffer (0.1 g of sodium hexametaphosphate in 1-L reagent grade water) was passed through the filter from the inlet (top cap) to the outlet (bottom cap) and then ~10-L samples were filtered. Next, 100 mL of ultrafiltration elution buffer (0.1 g of hexametaphosphate, 5 mL of Tween 80, and 10 μL of Antifoam A in 1-L reagent grade water) was cycled through the filter to elute the virus and protein particles for 5 min. Finally, the concentrated sample was collected into a sterile 250-mL bottle. Concentrated HFUF volumes of 65–195 mL were further processed using 8% PEG 8000 (v/v) and 3% 3-M NaCl (v/v) for precipitation. The mixture was refrigerated at 4°C and shaken slightly for 18 h. To collect the pellet, the mixture was centrifuged at 6700g for 30 min at 4°C. The supernatant was discarded, and the resultant pellet was resuspended in 5-mL TRIzol (Thermo Fisher Scientific, Carlsbad, CA, USA) and vortexed until fully dissolved. Samples were aliquoted into 2-mL tubes and then stored at −80°C until RNA extraction.

**RNA extraction and RT-qPCR**

Total RNA was extracted by the AllPrep PowerViral DNA/RNA kit (QIAGEN, Inc., Germantown, MD, USA) according to the provided user’s manual. To quantify SARS-CoV-2 RNA, quantitative reverse transcription
polymerase chain reaction (RT-qPCR) methods were used. RT-qPCR assay used N1 and N2 primers and probes as per the Centers for Disease Control and Prevention (CDC) protocol (Centers for Disease Control and Prevention, 2020, Table S1). RT-qPCR runs were conducted using 5 μL of Reliance One-Step Multiplex Supermix (BioRad, Hercules, CA, USA); 0.4 μL of each forward, reverse primer, and probe; 8.8 μL of RNase-free water; and 5 μL of RNA template for a final volume of 20 μL in each reaction. RT-qPCR was conducted by the CFX96 Touch Real-Time PCR Detection System (BioRad, Hercules, CA, USA). The PCR conditions were as follows: initial 10 min at 50°C for reverse transcription, initial denaturing for 10 s at 95°C followed by 48 cycles of 3 s at 95°C for denaturation, 30 s at 55°C for annealing, and a final 30 s at 75°C for probe amplification.

Positive and non-template controls (NTCs) were developed for every qPCR assay. For all calibration curves, correlation coefficients (R²) > 0.99 were obtained with amplification efficiencies of 90–110%.

SARS-CoV-2 recovery efficiency and PCR inhibitor identification

Due to the similar enveloped structure with SARS-CoV-2, human coronavirus OC43 strain (HCoV-OC43) was used as a surrogate to investigate the SARS-CoV-2 recovery rate in the concentration methods. In this study, 40- to 100-μL HCoV-OC43 strain was spiked into samples and then the same concentration method of Amicon ultrafiltration and HFUF and PEG was conducted. The quantification of HCoV-OC43 was done according to the method by Uppal et al. (2021). RT-qPCR assay used OC43-SF 5′-GGCTTATGTGGCCCCTTACT-3′ and OC43-SR 5′-GGCAAATCTGCCCCAAGAATA-3′ primer set (for more details, see Tables S5 and S6). The recovery rate was calculated according to Equation 1.

\[
\text{Recovery Rate} \, (\%) = \frac{(\text{HCoV} - \text{OC43 Recovered}/\text{HCoV} - \text{OC43 Spiked}) \times 100.}
\]

The impact of potential PCR inhibitors in the RNA extracts from the wastewater samples was investigated. In this study, all the RNA elutions have been diluted by −10- and −100-folds, and the difference of Ct values of OC43 was determined, respectively (Li et al., 2020; Pei et al., 2006). Additionally, internal amplification control (IAC) performance measures have been applied to detect the presence of RT-qPCR inhibitors according to SARS-CoV-2 RT-qPCR Systems kit (Promega, Maddison, WI, USA). As a process control, presence of pepper mild mottle virus (PMMoV), an RNA virus typical of wastewaters, has been analyzed to evaluate the concentration and virus recovery method according to SARS-CoV-2 RT-qPCR Systems kit (Promega, Maddison, WI, USA). Wastewater samples should have PMMoV amplification with Ct values of less than 40. NTCs were used to analyze the IAC. Amplification curves should be present for IAC related probes, but if fluorescent curves related to SARS-CoV-2 and PMMoV in respective NTC wells pass the limit of quantification, then contamination is present (Promega, Maddison, WI, USA). Additionally, if the IAC Ct varies significantly as compared with the NTC well, then contamination is present and results should not be analyzed quantitatively (Promega, Maddison, WI, USA). If a sample exhibits IAC and PMMoV amplification with no detectable amplification of SARS-CoV-2, it can be concluded that SARS-CoV-2 is not detectable in the respective sample, not due to the presence of inhibitions or improper virus recovery methods.

Statistical analysis

The relationship between viral RNA concentrations and the sample’s metadata was assessed by calculating Spearman’s rho correlation coefficients. All the data were standardized by calculating zero scores to remove the individual variable’s bias. To minimize the impact of non-detect data, the analysis included only the data concerning the influent and primary effluent samples in addition to secondary samples from the second week. The remaining nondetect values were replaced with the half of the detection limit to calculate the rho correlation coefficient.

To compare the virus RNA removal between each treatment process, one-way analysis of variance (ANOVA) analysis was performed. The analysis was done by different approaches to establish the sensitivity of the results to different methods of handling the nondetect data that included substitution with detection limit, half of the detection limit (Farnham et al., 2002; Poma et al., 2019) or applying the rank-sum method according to Helsel (2011). Before performing any analyses, all the data were adjusted based on the recovery efficiency of the method.

RESULTS AND DISCUSSION

Virus recovery efficiency and qPCR inhibition

The average recovery efficiency of HCoV-OC43 in the ultrafiltration method used for primary influent and
effluent samples was 18% ± 7%. The recovery for HFUF followed by PEG precipitation used for secondary samples was found out to be 7% ± 5%. Because there were no positive results for tertiary and final effluent samples, only the secondary samples were spiked.

While measuring the recovery efficiency of the spiked virus is needed to adjust the data for virus RNA removal comparison through the different processes of the WRF, it has been reported that recovery efficiencies for a certain method could vary depending on the sample type, type and concentration of the spiked virus, and chemical characteristics of wastewater (Kantor et al., 2021). Therefore, the originally measured concentration data have been reported alongside the adjusted data based on the recovery values rather than reporting the latter only.

According to the amplification of PMMoV, Ct values fell in the range of 23.1–37.7, which means the methods used were reliable to recover viruses in wastewater (Table S7). Regarding inhibitor detection, all ΔCt values between −10- and −100-fold dilutions were in the range between 3.2 and 4.0. Moreover, the IAC Ct values ranged from 24.4 to 25.3 (Table S7). No RT-qPCR inhibitor has been identified.

### Fate and removal of SARS-CoV-2 RNA in the WRF

Partial virus removal occurred during the 2.7-h average HRT of primary treatment at the WRF. Because there was no significant difference (one-way ANOVA test; p value = 0.396) in SARS-CoV-2 RNA (gc/L) between the influent and primary effluent samples, the primary treatment is not considered a critical step of SARS-CoV-2 removal. After primary treatment, the wastewater went through the EBPR treatment. More than 96% (1.56 log10) removal was observed between primary and secondary effluent. It must be noted that the quantified data have been adjusted based on the recovery efficiencies to calculate the log reduction value. The reductions of the N1 gene after primary and secondary treatment are visualized in Figure 2. To better address the nondetect data, the comparison of the two groups was performed under multiple approaches, including assuming the detection limit and half of the detection limit for the nondetect value and using the rank-sum method to rank the data before the analysis. A significant removal (p value < 0.005) can be seen at secondary treatment compared with influent or primary effluent samples for the N1 gene, under all circumstances.

The removal rates following secondary treatment suggest that secondary treatment plays a pivotal role in viral removal. SARS-CoV sorption to organic matter and flocs can be a removal mechanism during secondary treatment (Bogler et al., 2020; Chaudhry et al., 2015; Ye

### Presence of SARS-CoV-2 RNA in wastewater samples at each treatment stage

All 14 of the influent samples tested positive, as shown in Tables 1 and S2. In this study, the concentration for SARS-CoV-2 RNA markers measured in WRF influent ranged from $4.56 \times 10^4$ to $4.31 \times 10^5$ gc/L (adjusted values: $2.54 \times 10^5$–$2.4 \times 10^6$ gc/L). Primary effluent samples reported positive (14 out of 14) for both N1 and N2 genes where the concentration for N1 and N2 ranged from $4.0 \times 10^4$ to $9.21 \times 10^5$ gc/L (adjusted values: $2.22 \times 10^5$–$5.12 \times 10^6$ gc/L). Out of all the samples taken from secondary, tertiary, and final effluent, only four positive results were found for the secondary effluent samples. Concentration values for N1 and N2 ranged from $7.58 \times 10^2$ to $2.36 \times 10^3$ gc/L (adjusted values: $1.08 \times 10^4$–$3.36 \times 10^4$ gc/L). The adjusted values based on per cent recovery for each sample are shown in Table S3. No viral RNA concentrations were detected in tertiary or final effluent samples, indicating the effectiveness of these treatment processes in eliminating the virus marker signal.

### Table 1 Statistical summary of observed and adjusted viral RNA concentrations (gc/L)

| Stage           | Group | Positive samples frequency | Mean ± SD            | Adjusted mean ± SD | Process log reduction value (average) |
|-----------------|-------|----------------------------|----------------------|--------------------|--------------------------------------|
| Influent        | N1    | 14/14                      | $(1.98 \pm 0.77) \times 10^5$ | $(1.10 \pm 0.43) \times 10^6$ | -                                    |
|                 | N2    | 14/14                      | $(1.63 \pm 1.24) \times 10^5$ | $(9.04 \pm 6.92) \times 10^5$ | -                                    |
| Primary effluent| N1    | 14/14                      | $(2.05 \pm 2.16) \times 10^5$ | $(1.14 \pm 1.20) \times 10^6$ | 0.21                                 |
|                 | N2    | 14/14                      | $(1.55 \pm 1.72) \times 10^5$ | $(8.59 \pm 9.58) \times 10^5$ | 0.28                                 |
| Secondary effluent| N1 | 4/9                       | $(1.14 \pm 0.5) \times 10^3$ | $(1.63 \pm 0.71) \times 10^4$ | 1.70                                 |
|                 | N2    | 1/9                        | $2.36 \times 10^3$          | $3.36 \times 10^4$           | 1.45                                 |

*Calculated with adjusted data.
et al., 2016). The enveloped surface of the virus (E protein) is hydrophobic, facilitating interaction with other hydrophobic surfaces (Gundy et al., 2009; Lon et al., 2020; Mohapatra et al., 2020). Ye et al. (2016) reported that enveloped viruses behave according to a noninstantaneous quasi-equilibrium adsorption kinetic model in wastewater, where adsorption to solids will reach an equilibrium state but not instantly. Results showed up to 26% viral adsorption (or reduction in the liquid phase) when equilibrium is reached with TSS of 235 mg/L or medium-strength municipal wastewater. Kocamemi et al. (2020) confirmed the presence of SARS-CoV-2 RNA in 9 out of 9 sludge samples. Considering the high removals seen in this study across the aeration tank, additional factors may contribute to SARS-CoV-2 RNA removal in secondary treatment. Previous studies demonstrated that microbial predation and the action of proteases and nucleases could contribute to virus removal in secondary treatment (Bogler et al., 2020; Chaudhry et al., 2015; Gundy et al., 2009; John & Rose, 2005; Lv et al., 2006). Viral RNA concentrations are consistently shown to decrease with increasing bacteria concentrations, especially floc forming strains (Deng et al., 2014; Kim & Unno, 1996; Olive et al., 2020).

The retention time was taken into consideration, with optimal timelines for both solid adsorption and microbial predation averaging an hour. Ye et al. (2016) found 99% equilibrium conditions were reached in 0.4–2.9 h, while 90% equilibrium concentrations occurred after only 0.3–1.5 h. In this study, HRT for secondary treatment at the WRF was about three times the primary treatment, averaging 8–9 h. The longer retention time, in combination with increased opportunities for solid phase adsorption and microbial predation, could likely be the mechanisms for the significant removal of SARS-CoV-2 RNA in secondary treatment in this study.

Tertiary and final effluent samples that were tested during the 14-day sampling period returned nondetect results of SARS-CoV-2 genetic material. These results demonstrated efficient removal of the SARS-CoV-2 RNA by wastewater treatment at the WRF using chlorination as a final disinfection method. These results, as well as similar reports and recommendations by the World Health Organization (WHO), suggested that chlorine-based disinfectants were effective against enveloped viruses and SARS-CoV-2 (Collivignarelli et al., 2020; Saawarn & Hait, 2021; WHO, 2020). Meanwhile, an organic matter covered SARS-CoV-2 may be more resistant to oxidation damage from chlorination (Bogler et al., 2020; Geller et al., 2012; Gundy et al., 2009; Ye et al., 2016). Zhang et al. (2020) reported a high level of SARS-CoV-2 genetic material, \((0.5–18.7) \times 10^3 \, \text{gc/L}\), in hospital wastewater after disinfection with sodium hypochlorite, indicating the need to evaluate concentration dose and contact time. The effluent showed nondetect results for SARS-CoV-2 viral RNA after being overdosed with sodium hypochlorite, but high levels of disinfection by-products were reported, as expected. These analyses indicated that although chlorination can be a necessary step in virus removal, supporting secondary treatment as the main step in virus removal could help mitigate the reliance on the chlorination process. Consequently, there was no indication that current disinfection doses should be adjusted to remove any remaining viral load after secondary treatment.

### Statistical analysis on environmental and operational factors with virus removal during water reclamation

Spearman correlation was used to highlight the environmental and operational characteristics that had the strongest correlation with N1 in the WRF samples. Parameters such as TSS, TKN, ammonia, DOC, UVA, and HRT were used in the analysis. By calculating the Spearman coefficients (Table S4), adjusted N1 was seen to be strongly correlated with TKN (Spearman’s \(\rho = 0.667, \, p < 0.0005\)), TSS (Spearman’s \(\rho = 0.609, \, p < 0.0005\)), ammonia (Spearman’s...
CONCLUSIONS

The COVID-19 pandemic highlighted the importance of determining the fate of SARS-CoV-2 across a WRF. In this study, the objective was addressed by sampling at different stages of the WRF based on the HRTs of each treatment step.

The presence of viral RNA was confirmed in all samples taken from influent and primary effluent. Viral RNA removal was found out to be not significant after primary treatment \( (p = 0.396) \). However, secondary treatment showed significant removal \( (p = 0.005) \) of SARS-CoV-2 RNA. Up to 2.0 log_{10} removal value was achieved during secondary treatment. A lack of positive detection at tertiary treatment and final effluent stages confirmed the effectiveness of the combined tertiary nitrification/denitrification and filtration/chlorine disinfection to inactivate further and/or remove the SARS-CoV-2 RNA concentration to nondetectable levels. The total log_{10} SARS-CoV-2 RNA removal level from influent to secondary treated water ranged from 1.5–1.8 during the monitoring period. Even though a few samples were found to be positive after the secondary treatment step, the fact that the current treatment train was capable of removing the viral markers to a nondetect level provides confidence not only to the WRF personnel but also to the public regarding the effectiveness and control of SARS-CoV-2 markers by water reclamation for environmental discharge. Calculating the Spearman’s rho correlation coefficients demonstrated that adjusted N1 was positively correlated with TKN, TSS, ammonia, DOC, and UVA_{254} and negatively correlated with HRT \( (p < 0.05) \).

Considering the hydrophobicity of the SARS-CoV-2 envelope and the correlation between adjusted N1 and TSS, there is a need to examine SARS-CoV-2 removal in liquid and solid phases of the biological treatment process further, as its effectiveness could mitigate any reliance on chlorination.

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CONFLICT OF INTEREST

The authors have no conflict of interest in this research or the manuscript.

AUTHOR CONTRIBUTIONS

Niloufar Gharoon: Data curation; formal analysis; investigation; methodology. Aimee Dewan: Data curation; formal analysis; investigation; methodology. Lin Li: Data curation; formal analysis; investigation; methodology. Laura Haak: Data curation; formal analysis; investigation; methodology. Lauren Mazurowski: Investigation; methodology. Tatiana Guarin: Data curation; formal analysis; investigation; methodology. Krishna Pagilla: Conceptualization; funding acquisition; supervision.

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