RESISTANCE OF SARS COV-2 TO SEAWATER

EMRAH AY, NIZAMI DURAN

Mustafa Kemal University, Medical Faculty, Microbiology Department, Hatay-TÜRKİYE, nizamduran@hotmail.com; emrah_ay@hotmail.com

SARS CoV-2, which is the cause of Covid-19 disease, has become the only and most important agenda of the world due to its mortality and morbidity that globally affects the whole world. The virus has profoundly affected life all over the world. The lifestyles of people have changed due to the virus. This study is planned to understand how important sea water is in SARS-CoV-2 transmission. The study aimed to determine whether there is a risk of sea water in SARS-CoV-2 transmission. The effectiveness of seawater on SARS CoV-2 viability has been investigated in different dilutions of seawater in different time periods. Experiments were carried out in three different titrations of SARS CoV-2 in Vero cell lines. Viral replication has been investigated by detecting morphological changes occurring in cells, cell viability, and the RT-PCR method. Seawater has been found to be highly potent inhibitory on SARS CoV-2 about time and dose. Especially within 300 seconds, seawater has been found to inhibit viral replication up to 1/32 dilution. These results show that viral transmission through seawater is quite difficult for people swimming in the sea during the pandemic. Seawater-mediated spread of SARS-CoV-2 is out of the question. However, these results should not be interpreted as the prophylactic activity of saline against viruses, which are obligate intracellular parasites.

Keywords: resistance, SARS CoV-2, seawater

INTRODUCTION

Coronaviruses are enveloped, positive-sense RNA viruses containing the largest known RNA genomes up to approximately 30 kb in length, that belong to the family Coronaviridae, suborder Cornidovirineae. The Orthocoronavirinae subfamily contains four genera, Alphacoronavirus, Betacoronavirus, Gammacoronavirus, and Deltacoronavirus. Coronaviruses, which have a very large host, cause respiratory infections in humans (Letko *et al*., 2020).

The SARS CoV-2 strain, which is the cause of Covid-19 disease, belongs to the genus betacoronavirus (Perlman and Netland, 2009). Coronaviruses have a single-stranded, non-segmentation RNA genome with positive polarity, and virion has 4 major structural proteins. Nucleocapsid (N) protein, transmembrane (M) protein, envelope (E) protein, and Spike (S) protein. The virus has a single-stranded RNA genome containing 29,903 nucleotides encoding 9860 amino acids (NCBI, 2020). For some types of coronavirus, it has been reported that not all structural proteins are required to create an infectious virion (Schoeman and Fielding, 2019; Vlasova *et al*., 2007).

When the phylogenetic pedigree was examined, it was found that SARS CoV-2 significantly overlapped with the SARS virus sequence isolated in 2015. In structural analysis, SARS CoV-2 is thought to result from the mutation in the "spike" glycoprotein and nucleocapsid protein of the SARS virus (Song *et al*., 2018; Xu *et al*., 2020). SARS-CoV, SARS-CoV-2, and MERS-CoV are coronaviruses that can cause different clinical severity infections that show respiratory and extra respiratory symptoms in humans (Letko *et al*., 2020). Mortality rates with SARS-CoV and MERS-CoV have been reported to be around 10% and 35%, respectively. It is in the category of betaCoVs with SARS-CoV and MERS-CoV with significant mortality and SARS-CoV-2. The virus is spherical or elliptic 60-140 nm in diameter, like other CoVs, and it is sensitive to ultraviolet rays and heat (Wu *et al*., 2020). In addition, the virus has been reported to be very sensitive to lipid solvents containing chloroform except for ether (75%), ethanol, chlorine-containing disinfectant, peroxyacetic acid, and chlorhexidine. It has been reported that SARS-CoV-2 and SARS-CoV-1 have similar stability (van Doremalen *et al*., 2020; Ong *et al*., 2020). In this study, it was planned to determine whether there is a risk of seawater in SARS-CoV-2 transmission.

https://doi.org/10.24264/icams-2022.III.2

MATERIAL AND METHOD

Virus Isolation

In the study, primary rabbit kidney cell culture, Vero E6 (African green monkey kidney cell line), Vero CCL-81, and Beas 2B (human lung epithelial cell line) cell lines were used for viral isolation. Viral isolation was successfully performed in all of these cell lines. Viral growth was confirmed both by determining the presence of cytopathological effect and by the Real-Time PCR method.

The formation of CPE in the cell lines was detected on the 3rd day in the primary rabbit kidney cell line, on the 4th day in the Vero cell lines (Both Vero E6 and the Vero CCL-81 cell lines), and in the Beas 2B cells on the 5th day. After the detection of cytopathological changes in cell cultures, cells were collected from the culture dish after the seventh day of incubation. Cells collected by centrifugation were centrifuged again at 4000 rpm for 20 minutes after the freezing-thawing process (-80°C to 37°C). For the SARS CoV-2 verification in the sample from the supernatant, the sample was studied by RT-PCR method. The presence of viral growth was performed comparatively with cells treated with different dilutions of seawater and control groups without seawater.

Cell Culture

In-vitro tests were performed on Vero E6 cells. Cell cultures were maintained with 10% fetal calf serum RPMI-1640 medium containing 10 mM HEPES, 4 IM glutamine 100 IU/ml penicillin/streptomycin. Incubation of cells was carried out at 37°C in an incubator with 95% air with 5% CO_2. Cell density was adjusted to $1x10^5$ cells per ml. Experiments were carried out by adding cell maintenance medium to 10% of the culture dish in culture dishes. Incubation of cell cultures continued for 96 hours. Cells were removed from the culture vessels with 0.25% trypsinization solution, then collected in 50 ml centrifuge tubes by centrifugation at 1250 rpm for 10 minutes, at the end of incubation. Cell number and viability were determined by hemocytometer with 1% trypan blue dye prepared in 0.9% NaCl.

Proliferation Experiments

Proliferation experiments in cell culture were performed on 24-well flat-bottom microplates. The wells were prepared with RPMI-1640 medium containing 10% fetal calf serum and $1x10^5$ cells were added per ml.

Titration of the Virus

Stock viral strains (maintained at -80°C) were rapidly dissolved in 37°C water (in a bain-marie) and re-activated in cell lines (Vero E6 cell line). The cells were then exploded by “freezing and thawing”. Cells were collected from the culture dish and centrifuged at 4000 rpm for 20 minutes, and the supernatant virus was collected as a stock solution. Also, as in our previous study, the $TCID_{50}$ of the virus was calculated (Yildirim *et al*., 2016). In the present study, three different titers of SARS CoV-2 (1, 10, and 100 $TCID_{50}$) were chosen as the doses to be studied.

Activity Studies

Preparation of Cell Cultures

The seawater sample was first sterilized by passing through membrane filters. Then, different concentrations (1, 1/2; 1/4, 1/8, 1/16, 1/32, and 1/64) were obtained by diluting with seawater phosphate buffer solution. The effectiveness of these different seawater dilutions on SARS CoV-2 was investigated.

https://doi.org/10.24264/icams-2022.III.2

Antiviral Assay (Plaque Reduction Assay)

Experiments were carried out according to the method described by Hayden *et al.* (1980) and Abbas *et al.* (2018). In experiments, the cell density was adjusted to be $1x10^5$ cells/ml. After the cells were inoculated into the plates, they were incubated for 24 hours at 37°C. The virus was titrated in three different doses (1, 10, and 100 $TCID_{50}$). Different concentrations of seawater (1: 1, 1/2, ¼, 1/8, 1/16, 1/32, and 1/64) were distributed to the wells. From each dilution to 8 wells, 100 µl of SARS CoV-2 virus suspensions at 1, 10, and 100 $TCID_{50}$ concentration was inoculated in all wells. Plates were incubated at 37°C for 1 hour for viral adsorption and penetration. Then 3 ml of RPMI-1640 medium containing 2% agarose was added. At the end of the incubation, 10% formalin was added to the wells. The plates were then incubated for two hours, at the end of the incubation, the plates were stained with 0.1% crystal violet prepared in distilled water. Cell culture wells without virus inoculations were selected as the control group. Viral plaques formed at the end of the test were counted and the percentage reduction in plaque formation compared to the control wells was calculated according to the following formula:

% inhibition=[viral count (untreated)-viral count (treated)]x100/(viral count (untreated) (1)

The experiments were repeated in triplicate.

Evaluation

Cell culture plates were evaluated daily for the presence of CPE in the cells. The presence of cells in atypical morphology such as cell rounding, constriction narrowing, and aggregation was evaluated as cytopathological changes. All microscopic evaluations were evaluated comparatively with the control group without seawater. The presence of viral reproduction was also verified by the MTT method in which cell viability can be evaluated as quantitative (Mossman, 1983). In the case of viral reproduction in the wells containing different concentrations of seawater, the determination of a decrease in cell viability was considered as the presence of viral replication, since cell viability would decrease.

Real-Time PCR

The presence of SARS CoV-2 growth in cell culture was also verified by the Real-time PCR method. In comparison with the control group using the real-time PCR method, the presence or increase of viral replication was described as an increase in the number of viral copies.

RESULTS

Effects of the Sea Water on the Replication of SARS CoV-2

In the study, the effects of different dilutions (1/1; 1/2; 1/4 1/8; 1/16; 1/32, and 1/64) of seawater on the replication of SARS CoV-2 have been investigated. In the experiments, the inhibitory effect of all dilutions of seawater against SARS CoV-2 was detected within seconds. This effect of seawater against SARS-CoV-2 was found to be directly related to your water concentration.

All dilutions of seawater were found to inhibit viral replication within 60 seconds, except for 1/32 and 1/64 dilutions against SARS CoV-2 at 100 $TCID_{50}$ titers. In all experimental groups, the presence of viral replication in each dilution was evaluated by the RT-PCR method compared to the control group. Virus replication was evaluated as a decrease or increase in terms of the number of copies. In the control group, cells were treated with SARS CoV-2 only, seawater was not added to these cell cultures.

https://doi.org/10.24264/icams-2022.III.2

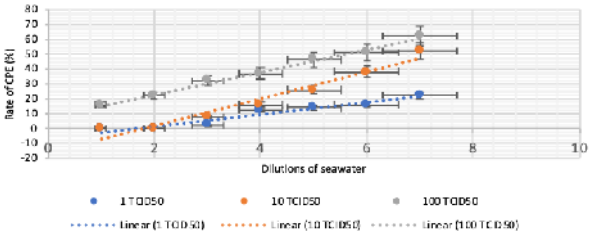

Figure 1. CPE rates were detected in cells after 60 seconds of exposure to SARS CoV-2 with different concentrations of seawater

The effectiveness of SARS CoV-2 in three different titers was investigated. After 60 seconds of exposure to seawater, it was found that the virus in 1 and 10 $TCID_{50}$ titers were completely inhibited at 1/1 and 1/2 dilutions. Decrease in seawater dilution results in an increase in viral inactivation rate. If the virus titer is 100 $TCID_{50}$, a low level of CPE was detected in the cells at 1/1 and 1/2 dilutions of seawater. When the viral titer is elevated ($1TCID_{50}$), it has been found that viral inactivation occurs to a large extent if the virus is exposed to seawater for 60 seconds (Fig. 1).

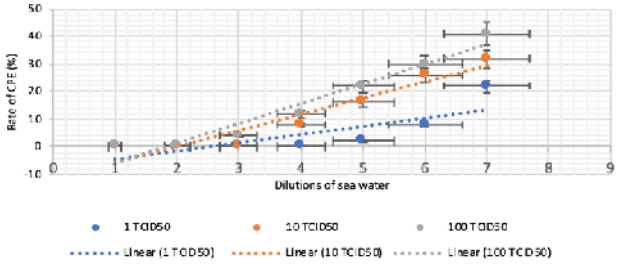

Figure 2. CPE rates were detected in cells after 120 seconds of exposure to SARS CoV-2 with different concentrations of seawater

When the exposure time to seawater was increased to 120 seconds, virus inactivation at $1TCID_{50}$ titer was 0 at 1/1, 1/2, ¼, and 1/8 dilutions. In experiments in which SARS CoV-2 was run at 10 $TCID_{50}$ titers, viral inactivation was found to be 0 in the first three dilutions (1/1, ½, and 1/4). At lower titrations of seawater, the presence of CPE was detected in cells, albeit at low levels. When the virus titer was increased to 100 $TCID_{50}$, it was found that SARS CoV-2 was fully inactivated at 1/1 and 1/2 dilutions. Whereas, in the titers of seawater less than 1/2, the presence of low rates of CPE in the cells was observed (Fig. 2).

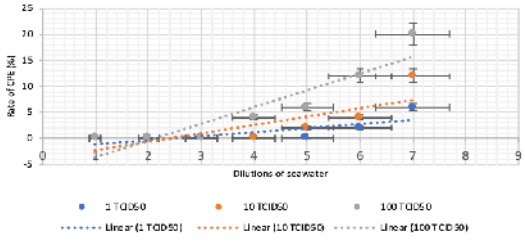

Figure 3. CPE rates were detected in cells after 180 seconds of exposure to SARS-CoV-2 with different concentrations of seawater

https://doi.org/10.24264/icams-2022.III.2

As seen in Figure 3, when the exposure time to seawater was increased to 180 seconds, it was found that the SARS CoV-2 in 1TCID$_{50}$ titer was inactivated in all dilutions from 1/1 to 1/16. When the viral titer was elevated, it was found that viral inactivation titers also increased. It was determined that the inactivation of 10 TCID$_{50}$ titers virus was seen in all dilutions from 1/1 to 1/8. It was determined that the virus with a titer of 100 TCID$_{50}$ was inactivated in all dilutions from 1/1 to 1/4 (Fig. 3).

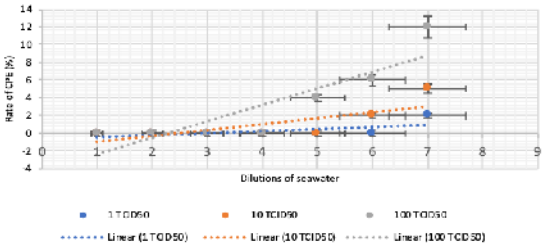

Figure 4. CPE rates were detected in cells after 240 seconds of exposure to SARS-CoV-2 with different concentrations of seawater

When the exposure time to seawater was increased to 240 seconds, it was determined that the full inactivation of the virus in 1 TCID$_{50}$ titer occurred in all dilutions from 1/1 to 1/32. Complete inactivation was observed against SARS CoV-2 in 10 TCID50 titers, from 1/1 to 1/16, while in dilutions from 1/1 to 1/8 against the virus in 100 TCID$_{50}$ titers (Fig. 4).

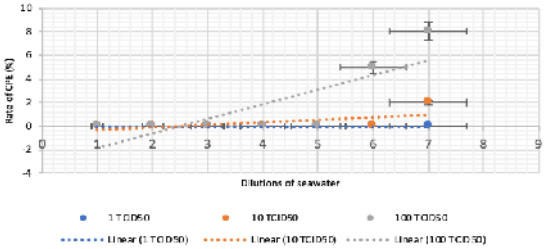

Figure 5. CPE rates were detected in cells after 300 seconds of exposure to SARS-CoV-2 with different concentrations of seawater

When the exposure to seawater was increased up to 300 seconds, viral inactivation was detected in all dilutions of 1 TCID$_{50}$ titer of the virus from 1/1 to 1/64 of the dilution. When the viral titer was chosen as 10 TCID$_{50}$, only a 1/64 dilution of seawater showed a very small amount of CPE in the cells. When the viral titer was determined as 100 TCID$_{50}$, viral inactivation occurred in all dilutions from 1/1 to 1/16 of seawater. However, at 1/32 and 1/64 dilutions, 5 and 8% CPE was detected in the cells, respectively (Fig. 5).

DISCUSSION

SARS CoV-2 pandemic has profoundly affected people all over the world. People's lifestyles have undergone major changes to prevent viral transmission. The main transmission path of SARS-CoV-2 is contact. Carrying out studies on direct or indirect transmission is extremely important in terms of developing new strategies to protect against SARS-CoV-2. For this reason, in our planned study, we have reached important findings regarding the survival of SARS-CoV-2 in seawater. It is a known fact that SARS-CoV-2 is an infectious agent that can easily spread in contact with contaminated surfaces or materials. In this study, which investigated the subject of transmission of SARS CoV-2

https://doi.org/10.24264/icams-2022.III.2

by seawater, it was determined that transmission from infected individuals would not be possible through seawater. This study has shown that seawater can inactivate SARS CoV-2 in seconds even at very low concentrations (up to 1/64 dilution).

CONCLUSION

As a result, it can be said clearly that SARS-CoV-2 cannot be transmitted through seawater. This shows the results that solutions with a high salt concentration can be used effectively in SARS-CoV-2 disinfection. It is important to keep in mind that gargling with high salt concentration solutions may not be important in preventing infection. Because when the virus reaches the mucosal surfaces, it quickly enters the epithelial cells for replication. For this reason, it should be remembered that garaging with solutions with high salt concentrations cannot be prevented viral replication. High salt concentration solutions can only be used safely to eradicate viral contamination in inanimate environments.

REFERENCES

Abbas, S.Y., El-Bayouki, K.A.M., Basyouni, W.M. and Mostafa, E.A. (2018), "New Series of 4 (3H)-quinazolinone Derivatives: Syntheses and Evaluation of Antitumor and Antiviral Activities", *Medicinal Chemistry Research*, 1-12, https://doi.org/10.1007/s00044-017-2083-7.

Hayden, F.G., Cote, K.M. and Douglas, R.G. Jr. (1980), "Plaque Inhibition Assay for Drug Susceptibility Testing of Influenza Viruses", *Antimicrobial Agents and Chemotherapy*, 17, 865-870, https://doi.org/10.1128/AAC.17.5.865.

Letko, M., Marzi, A. and Munster, V. (2020), "Functional Assessment of Cell Entry and Receptor Usage for SARS-CoV-2 and the Other Lineage B Betacoronaviruses", *Nature Microbiology*, 5, 562-569, https://doi.org/10.1038/s41564-020-0688-y.

Mosmann, T. (1983), "Rapid Colorimetric Assay for Cellular Growth and Survival: Application to Proliferation and Cytotoxicity Assays", *Journal of Immunological Methods*, 65, 55-63, https://doi.org/10.1016/0022-1759(83)90303-4.

National Center for Biotechnology Information (2020), "Severe Acute Respiratory Syndrome Coronavirus 2 Reference Genome", Accessed on 2 August 2020.

Ong, S.W.X., Ong, S.W.X., Tan, Y.K., Chia, P.Y., Lee, T.H., Ng, O.T., Wong, M.S.Y. and Marimuthu, K. (2020), "Air, Surface Environmental, and Personal Protective Equipment Contamination by Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) from a Symptomatic Patient", *JAMA*, E1-E3, https://doi.org/10.1001/jama.2020.3227.

Perlman, S. and Netland, J. (2009), "Coronaviruses Post-SARS: Update on Replication and Pathogenesis", *Nature Reviews Microbiology*, 7, 439-450, https://doi.org/10.1038/nrmicro2147.

Schoeman, D. and Fielding, B.C. (2019), "Coronavirus Envelope Protein: Current Knowledge", *Virology Journal*, 16, 2-22, https://doi.org/10.1186/s12985-019-1182-0.

Song, W., Gui, M., Wang, X. and Xiang, Y. (2018), "Cryo-EM Structure of the SARS Coronavirus Spike Glycoprotein in Complex with its Host Cell Receptor ACE2", *PLoS Pathogens*, 14(8), 1-19, https://doi.org/10.1371/journal.ppat.1007236.

Xu, X., Xu, X., Chen, P., Wang, J., Feng, J., Zhou, H., Li, X., Zhong, W. and Hao, P. (2020), "Evolution of the Novel Coronavirus from the Ongoing Wuhan Outbreak and Modeling of its Spike Protein for Risk of Human Transmission", *Science China Life Sciences*, 63, 1-4, https://doi.org/10.1007/s11427-020-1637-5.

Wu, F., Wu, F., Zhao, S., Yu, B., Chen, Y.M., Wang, W., Song, Z.G., Hu, Y., Tao, Z.W., Tian, J.H., Pei, Y.Y., Yuan, M.L., Zhang, Y.L., Dai, F.H., Liu, Y., Wang, Q.M., Zheng, J.J., Xu, L., Holmes, E.C. and Zhang, Y.Z. (2020), "A New Coronavirus Associated with Human Respiratory Disease in China", *Nature*, 579, 265-269, https://doi.org/10.1038/s41586-020-2008-3.

van Doremalen, N., Bushmaker, T., Morris, D.H., Holbrook, M.G., Gamble, A., Williamson, B.N., Tamin, A., Harcourt, J.L., Thornburg, N.J., Gerber, S.I., Lloyd-Smith, J.O., de Wit, E. and Munster, V.J. (2020), "Aerosol and Surface Stability of SARS-CoV-2 as Compared with SARS-CoV-1", *The New England Journal of Medicine*, 382, 1564-1567, https://doi.org/10.1056/NEJMc2004973.

Vlasova, A.N., Vlasova, A.N., Zhang, X., Nagesha, H.S., Haynes, L.M., Fang, Y., Lu, S. and Saif, L.J. (2007), "Two-way Antigenic Cross-reactivity between Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) and Group 1 Animal CoVs is Mediated through an Antigenic Site in the N-Terminal Region of the SARS-CoV Nucleoprotein", *Journal of Virology*, 81, 13365-13377, https://doi.org/10.1128/JVI.01169-07.

Yildirim, A., Duran, G.G., Duran, N., Jenedi, K., Bolgul, B.S., Miraloglu, M. and Muz, M. (2016), "Antiviral Activity of Hatay Propolis Against Replication of Herpes Simplex Virus Type 1 and Type 2", *Medical Science Monitor*, 22, 422-30, https://doi.org/10.12659/MSM.897282.