DOI 10.1007/s10517-022-05574-4
Cell Technologies in Biology and Medicine, No. 2, August, 2022

Cell and Organism Technologies for Assessment of the SARS-CoV-2 Infectivity in Fluid Environment

A. A. Shelemba[1], E. A. Kazachkova[1], Yu. V. Kononova[1], E. I. Kazachinskaya[1], M. Yu. Rukavishnikov[2], I. N. Kuvshinova[2], M. I. Voevoda[1], A. M. Shestopalov[1], and A. A. Chepurnov[1]

Translated from *Kletochnye Tekhnologii v Biologii i Meditsine*, No. 2, pp. 73-77, June, 2022
Original article submitted December 20, 2021

Under conditions of COVID-19 pandemic, considerable amounts of SARS-CoV-2 contained in household, municipal, and medical wastewaters inevitably reach natural water bodies. Possible preservation of virus infectivity in liquid environment is of a paramount epidemiological importance. Experiments demonstrated that SARS-CoV-2 is resistant to multiple freezing/thawing cycles and retains its infectivity in tap and river water for up to 2 days at 20°C and 7 days at 4°C. In natural milk, its viability is preserved in a refrigerator for 6 days. The exposure of aquarium fish to the virus-containing water fails to cause any infection.

Key Words: *COVID-19; contaminated wastewaters; rate of virus inactivation in wastewaters and natural fluids; tolerance to freezing/thawing*

The development of COVID-19 pandemic has attracted attention of many researchers to the fact that not only the pulmonary system, but also the gastrointestinal tract is involved in the infectious process. This leads to the presence of SARS-CoV-2 in human excrements and, therefore, in wastewater and water bodies.

At the first stage of the pandemic, the researchers focused on the disease transmission via a direct contact with an infected person or an indirect contact with an infected surface followed by virus transmission to the mouth, nose, or eye mucosae [8]. In particular, the Center for Disease Control (USA) reported the possibility of a human infection with SARS-CoV-2 via a contact with infected surfaces; however, this risk is rather low and is not the main route of disease transmission (https://www.cdc.gov/coronavirus/2019-ncov/more/science-and-research/surface-transmission.html). The major detected epidemiological points were that the virus survived to 14 days retaining its infectivity on the surfaces under standard conditions and that the smooth surfaces, such as plastic and wood, are more favorable for its survival [5]. A COVID-19 infected person sheds SARS-CoV-2 RNA for a mean period of approximately 14 to 21 days, and the magnitude of shedding ranges between 10^2 and 10^8 RNA copies per gram [6].

Although inhalation of infected drops or aerosols is the main infection route for SARS-CoV-2, other potential transmission routes are considered, including the fecal-oral route [4]. First, SARS-CoV-2 was detected in fecal samples and anal swabs of some patients [8], then, SARS-CoV-2 RNA was detected in untreated wastewater, medical wastewater, secondary purified wastewater, river water, municipal wastewater, silts of sewage treatment plants, and wastewater of cruise ships and planes [11]. It is known that many enveloped viruses retain their infectivity in fluid environment from several days to several months [9]. This raised the concern about the possible direct fecal-oral SARS-CoV-2 transmission as well as the transmission via secondary aerosols in sewage systems with subsequent airborne transmission [12], especially in the

[1]Federal Research Center of Fundamental and Translational Medicine, Siberian Division of the Russian Academy of Sciences, Novosibirsk, Russia; [2]JSC "Vector-Best", Novosibirsk, Russia. ***Address for correspondence:*** alexa.che.purnov@gmail.com. A. A. Chepurnov

case of a poor sewage with improper ventilation, drain traps, and so on.

The presence of SARS-CoV-2 in human excrements and wastewaters has serious consequences for both wastewater treatment [10] and purification of tap water. The load of SARS-CoV-2 in the excrements of COVID-19 patients falls into the range of 10^4-10^8 copies/liter depending on the stage of infection [4,6,10]. The liquefaction of feces in wastewater decreases the virus load to 10^2-10^6 copies/liter [3,4,10]. This and many other studies have been performed in natural or model experiments with their conditions differing from the regional conditions. In particular, the experiments with model (not natural) fluids reproduced the already known results [1].

Evidently, the period of coronavirus survival in water environment significantly depends on temperature, water properties, concentrations of particulate and organic substances, pH of solution, and dose of a used disinfectant. The WHO asserts that the process currently used for disinfection of drinking water is able to effectively inactivate the majority of bacterial and viral communities present in water. Note that SARS-CoV-2 is sensitive to the disinfectants, such as free chlorine. A large-scale epidemic will be accompanied by the release of the virus into sewage; its subsequent presence in technical and drinking waters is also evident. An important point is for how long the virus retains its ability to infect, *i.e.*, its "viability" in a virological sense. In this work, we focus on the most important point, namely, the survival of SARS-CoV-2 in bathing ponds, water accumulation basins, drinking water reservoirs, and natural liquid products, for example, milk, under different working conditions (for example, temperature and water composition). Inevitably, this issue includes the question on the SARS-CoV-2 adaptation to the ichthyofauna of water bodies.

Our aim was to assess the period during which SARS-CoV-2 retains its infectivity in fluid environment on a regional level and the potential of its adaptation to the ichthyofauna of these water bodies.

MATERIALS AND METHODS

SARS-CoV-2. The virus isolate recovered from a nasopharyngeal lavage sample of a patient in Novosibirsk in 2020 was used in the work. The strain is deposited with the collection of the 48th Central Research Institute, Ministry of Defense of the Russian Federation, as SARS-CoV-2/human/RUS/Nsk-FRCFTM-1/2020 [2]. The strain was expanded in Vero cell culture using an infective dose of 0.01 CPE/cell. On day 5, a total CPE and separation of 100% cells were observable. The virus titer (5×10^6 CPE/ml) was determined by endpoint dilution technique.

Cell culture. Continuous Vero cell culture was used. The cells were cultured in Eagle's MEM supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic (Gibco).

Assessment of the virus inactivation during freezing/thawing. The virus aliquots (1 ml) in Eppendorf tubes produced as the master virus pool and stored at -70°C, were frozen/thawed 1, 3, 5, and 7 times. Each sample after the corresponding freezing/thawing cycles was stored at -20°C until titrated. All samples were simultaneously thawed; 10-fold dilutions of each sample (to and through 10^{-7}; 100 μl each) were placed into wells of a 96-well culture plate simultaneously with Vero cell culture grown under the same conditions. The eighth well of each row was used as the control for the absence of CPE.

Virus titer was determined by endpoint dilution technique. For this purpose, the corresponding wells of a 96-well plate with Vero cell culture were infected with 10-fold dilutions of the virus in Hanks' solution. The result was recorded on day 5 after infection by the presence/absence of CPE in each well.

The water was sampled from the Ob River artificial reservoir in summer (water temperature 20-22°C) and late fall (water temperature 4-6°C) into sterile glass containers (200 ml in each). In addition, samples of cold tap water (200 ml) and goat milk (200 ml) were taken. The virus sample (20 ml) with a titer of 5×10^6 CPE/ml was used for infection. The sample of the Ob water harvested at 20-22°C was kept at a room temperature and the remaining samples, in a refrigerator (4-8°C). Each variant was sampled 15 min, 1 and 5 h after infection, and then daily (3 aliquots of 1 ml for each variant and time point) and frozen at -70°C. On completion of the experiment, each sample of each variant was thawed and titrated in a tenfold sequence. Each dilution was placed into 3 wells of a 96-well plate containing Vero cell culture. The results were visually recorded on day 6 using an inverted microscope. The titration was repeated for the second series of samples. In the case of an ambiguous result, the corresponding sample of the third series was titrated.

Study of potential SARS-CoV-2 carriage by ichthyofauna. We tried to answer this question using the marble gourami as the model object. The marble gourami (an aquarium fish species) was used as the model object. A container with aquarium water was supplemented with 4 ml of the virus-containing fluid with a titer of 2×10^5 CPE/ml (final dilution, 2×10^3 CPE/ml). Six marble gourami individuals were placed into the container with the virus suspension and exposed for 40 min; then the fishes were sponged with sterile gauze and transferred to common aquarium water (400 ml). The exposure was repeated for 10 min and the fishes were sponged and transferred to a 5-liter

aquarium aiming to wash them from the virus. Any changes in their behavior were unobservable. On the next day, two individuals were used to prepare a 10% homogenate, which was added to Vero cell monolayer and assayed with PCR for the presence of SARS-CoV-2 RNA. The reference preparation was a sample of aquarium water where the fish individuals were exposed to the virus. The analysis was repeated using two individuals 1 day later and, using two additional fishes, after 21 days.

PCR. RNA was extracted using a RealBest Extraction 100 kit (Vector-Best) according to the manufacturer's protocol. The SARS-CoV-2 RNA was detected with a RealBest RNA SARS-CoV-2 kit (Vector-Best) following the manufacturer's protocol in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad).

Statistical analysis. The data were statistically processed using analysis of variance with the help of Microsoft Excel software package; the differences were regarded as significant at $p<0.05$.

RESULTS

Initially, the effect of freezing/thawing on the virus infectivity was assessed. This is an important issue for a practicing virologist since a decrease in the virus titer during freezing/thawing can influence the results of experiment. Another important point is the effect of weather conditions on the survival of virus in the water bodies of cold countries, such as Russia, where the water temperature can decrease to lower temperatures, more favorable for survival of the virus, and even to freezing. For this purpose, the virus aliquots (1 ml) in Eppendorf tubes were frozen/thawed 1, 3, 5, and 7 times. After the corresponding number of freezing/thawing cycles, the samples were assayed using a simultaneous titration. The multiple freezing/thawing fails to decrease the titer of SARS-CoV-2 within the range of the studied number of cycles. Both the initial virus titer and the titer after 1, 3, 5, and 7 freezing/thawing cycles remained in the range of $3\text{-}7\times10^5$, which is insignificant and suggests the absence of inactivation effect caused by freezing/thawing of the virus suspension.

The temperature regime of a water body undoubtedly has most significant effect on the rate of virus inactivation there. Therefore, we examined the summer (20-24°C) and fall-winter (4-6°C) temperature regimes. The latter regime was also applied to tap water and milk since the temperature of tap water, typically running at a depth of 3 m, and milk, kept in a refrigerator, is 4-10°C. In this experiment, PCR was used to confirm the specificity of CPE. The fact of specific cell death of SARS-CoV-2 was recorded only when the PCR signal exceeded the background signal of the virus dose introduced by 4-6 cycles.

The virus in the river water at 20°C retained its activity for 2 days and at 4°C and for 6 days (Table 1). As for tap water (4°C), SARS-CoV-2 remains infective for even longer period, which is rather difficult to explain. Unlike tap water, the river water by definition contains considerably more protein as microorganisms, protists, algae, fish, metabolic products of the living world, and so on. The proteins in suspensions prevalently act as virus protectors, elevating their viability. In addition, tap water contains chlorine, used during water purification, which should enhance the inactivation of the virus there. Nonetheless, the virus for a longer period retained its infectivity in this particular variant. SARS-CoV-2 also remained infective for a long time in milk. Thus, the primary data published by several researchers [1,3,4], who assessed the survival of SARS-CoV-2 in water as low because of high seasonal water temperature, require correction and the issue in general requires more attention.

In one of the most comprehensive study [3], the duration of persistence of SARS-CoV-2 RNA signal in water and its infectivity were analyzed. At 20°C, the SARS-CoV-2 with initial titers of 10^3 and 10^5 CPE_{50}/ml remained infective for 3 and 7 days, respectively. It was also shown that SARS-CoV-2 RNA was considerably more stable than infective SARS-CoV-2, which suggests that detection of virus RNA alone does not necessarily prove the risk of infection. Presumably, this study fits best the real conditions; however, the authors assayed the water samples from a water purification plant, whereas we used the real natural water

TABLE 1. Time Interval of SARS-CoV-2 Infectivity in Assayed Samples

Sample		Time interval										
		15 min	1 h	5 h	1 day	2 days	3 days	4 days	5 days	6 days	7 days	8 days
River water 20°C	CPE/PCR	+/+	+/+	+/+	+/+	+/+	-/-	-/-	-/-	-/-	-/-	-/-
River water 4°C	CPE/PCR	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	-/-	-/-
Tap water 4°C	CPE/PCR	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	-/-
Goat milk 4°C	CPE/PCR	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	-/-	-/-

from the Ob River artificial reservoir. Moreover, we used the samples harvested under the corresponding temperature regimes and, thus, containing the accompanying microflora and mineral composition. It cannot but be mentioned that it is necessary to study the SARS-CoV-2 stability in the cold (winter) season of the year. This regime is the most poorly studied. Hokajärvi, *et al.*, [7] report a long-term stability of SARS-CoV-2 RNA at -20°C, *i.e.*, at a winter temperature of environment, in the region close to Russia in its climate. Unfortunately, this study also relies on RNA assays, which does not reflect an actual situation with the preservation of infectivity.

The virus retains its infectivity for several hours and days when entering water bodies; thus, the question arises on the possibility of infection of local ichthyofauna with SARS-CoV-2 and its carriage. We tried to answer this question using the marble gourami as the model object. On days 2, 4, and 21, two fish individuals were used to prepare a 10% homogenate, which was added to Vero cell monolayer and assayed for the infectivity of SARS-CoV-2 in the fish bodies. The reference preparation was a sample of aquarium water where two fish individuals were exposed to the virus. The presence of SARS-CoV-2 RNA on the Vero cell monolayer with 10% fish homogenate was assayed by PCR. The results in all cases were negative, suggesting that this fish species is insusceptible to the coronavirus. However, it cannot be excluded that another fish species may be more susceptible to the virus. At least, our attempt to infect FHM (caudal peduncle of the fathead minnow) cell culture has shown that an abortive infection is possible (data not shown), which suggests the danger of a potential ichthyological focus.

Thus, temperature regime is the main factor determining the preservation of SARS-CoV-2 infectivity in natural water bodies and technological devices. No signs suggest that ichthyofauna can become the focus of SARS-CoV-2 in natural water bodies.

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