Investigating of the Occurrence of Pathogenic Viruses in Drinking Water Sources and Their Reduction Efficiencies in Drinking Water Treatment Processes

病原ウイルスの水道原水における存在実態及び浄水処理性の詳細把握

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To control waterborne diseases and to ensure a stable supply of safe drinking water, it is essential to understand the occurrence of pathogenic viruses in drinking water sources and their reduction efficiencies in drinking water treatment processes. Here, we improved and optimized a method that combines a photoreactive intercalating dye with a PCR assay for virus quantification, and developed a novel virus concentration method using two membranes, making it possible to investigate the occurrence of pathogenic viruses and to discuss the presence or absence of infectious viruses in drinking water sources. By applying the developed virus concentration method to water samples collected at actual drinking water treatment plants, we successfully evaluated the virus treatment properties in full-scale drinking water treatment processes. Furthermore, we prepared virus-like particles (VLPs) of human norovirus, which is difficult to culture, and developed a method to quantify them in high sensitivity, and then successfully evaluated the removal efficiencies of human norovirus particles in drinking water treatment processes. In addition, we established a method for producing purified solutions of human sapovirus at high concentrations, and a method for evaluating its infectivity, and then successfully evaluated the removal and inactivation efficiencies of human sapovirus in drinking water treatment processes.

病原ウイルスによる水系感染症を制御し，安全な水道水を安定的に供給するためには，水道原水における病原ウイルスの存在実態，並びに浄水処理工程におけるウイルスの処理性を把握することが必要不可欠である。本研究では，光反応性色素とPCR法を組み合わせた手法をウイルス定量用に改良・最適化すると共に，2種類の膜を組み合わせた新たなウイルス濃縮法を開発し，水道原水における病原ウイルスの存在実態の把握と感染力有無の議論を可能とした。また，開発した濃縮法を実際の浄水場に適用することで，ウイルスの浄水処理性の把握にも成功した。更に，培養が困難なノロウイルスのウイルス様粒子を作製し，高感度定量法を開発することにより，浄水処理工程におけるノロウイルスの除去特性を把握することに成功した。加えて，サポウイルスの高濃度精製溶液の調製法及び感染力評価法を確立し，浄水処理工程におけるサポウイルスの除去・不活化特性の把握にも成功した。

Introduction

Water supply is a key infrastructure indispensable for people to lead healthy and cultured lives, and at the same time, it plays an important role in the control of infectious diseases. On the other hand, climate change is expected to bring about problems that threaten the safety and security of people's lives in the future in terms of both the quality and quantity of water, such as the increased risk of drought and floods and deterioration of water quality in rivers and lakes, which are the raw water for drinking water, due to frequent extreme low and high rainfall events. In particular, waterborne infectious diseases caused by viral contamination of water are an international water problem that still occurs not only in developing countries but also in developed countries including Japan, where sanitary conditions have improved due to advances in medical and pharmaceutical sciences and the

spread of water supply and sewage systems. Control of this problem is an essential issue for the use/reuse of low-quality water with high levels of pathogenic viral contamination, which is expected to increase in the future. In Japan, unintentional reuse of wastewater for drinking is widely practiced, in which surface water including treated wastewater with high virus concentrations discharged upstream is used as raw water for drinking water downstream (commonly referred to as *de facto* reuse), a situation that cannot be avoided in the future. Under these circumstances, to ensure a stable supply of safe and reliable drinking water in the future, it is essential to understand the occurrence of pathogenic viruses, especially infectious viruses, in drinking water sources, and their removal and inactivation efficiencies in the drinking water treatment processes. Based on these findings, it is extremely important to apply effective and efficient drinking water treatment to reduce the risk of waterborne infection by pathogenic viruses to an acceptable level.

Pathogenic viruses that cause waterborne diseases are more infectious than pathogenic bacteria, and infection can be established at doses as low as 1 to 100 virus particles, therefore the World Health Organization (WHO) estimates an acceptable concentration of 1 virus particle/90,000 L (approximately 10^{-5} virus particles/L) in drinking water[1]. Although it is ideal to evaluate the reduction efficiencies of pathogenic viruses in actual drinking water treatment plants, it is virtually impossible to evaluate the efficacy of drinking water treatment processes to reduce pathogenic viruses based on direct quantification of those because the concentrations of pathogenic viruses in treated water after drinking water treatment are extremely low and are usually below the quantification limit of PCR assay even when >1,000 L of water are concentrated to several milliliters. Therefore, it is realistic to estimate the concentration of pathogenic viruses in treated water including drinking water using the concentration of pathogenic viruses in drinking water sources obtained from investigation of the occurrence of pathogenic viruses in drinking water sources and the removal and inactivation efficiencies of pathogenic viruses in drinking water treatment processes evaluated through lab-scale drinking water treatment experiments using artificially propagated virus-spiked water. However, although the PCR assay is widely used to investigate the presence of viruses in the water environment including drinking water sources, since it is fast, highly sensitive, and highly specific, the PCR assay detects and quantifies the viral DNA/RNA of both infectious and inactivated viruses, and does not discriminate the presence or absence of infectious viruses, which is important for accurately assessing the risk of infection in the water environment. In addition, for some of the pathogenic viruses such as human noroviruses, it is difficult to propagate a large number of virus particles enough to conduct lab-scale virus-spiking experiments because of the lack of an effective *in vitro* cell-culture system. Therefore, little is known about the behaviors of difficult-to-culture pathogenic viruses including human norovirus during the drinking water treatment processes.

Against this background, we focused on the viability PCR, which combines the photoreactive intercalating dye such as propidium monoazide (PMA) and DNA/RNA quantification by PCR assay, used to determine whether bacteria are alive or dead, and tried to improve and optimize it as a method to determine viral infectivity. Also, we attempted to develop a novel virus concentration method that can effectively concentrate a wide variety of viruses from large volumes of water samples, and combine with the improved and optimized assay to investigate the occurrence of infectious pathogenic viruses in drinking water sources. In addition, we focused on pepper mild mottle virus, a plant virus that is present at high concentrations in drinking water sources, and investigated its effectiveness as a potential surrogate for pathogenic viruses in physical and physicochemical drinking water treatment processes, and then attempted to investigate the reduction efficiencies of the indigenous pepper mild mottle virus in actual drinking water treatment plants by applying the developed virus concentration method. Furthermore, we focused on virus-like particles (VLPs), which can be produced in a large number of particles without relying on a cell-culture system (i.e., without waiting for the establishment of an effective *in vitro* cell-culture system) and are morphologically and antigenically the same as native virus particles, and tried to prepare VLPs of human norovirus that is difficult to culture. Also, we attempted to develop a novel virus quantification method that can quantify VLPs with high sensitivity, and combine with VLPs to investigate the reduction efficiencies of human norovirus particles in drinking water treatment processes through lab-scale virus-spiking experiments. In addition, we tried to prepare the purified solution of human sapovirus, which belongs to the same family as human norovirus, by applying an *in vitro* cell-culture system for human sapovirus. Also, we attempted to develop a virus quantification method that can quantify the infectivity of human sapovirus, and combine it with the purified solution of human sapovirus to investigate the reduction efficiencies of human sapovirus in drinking water treatment processes through lab-scale virus-spiking experiments.

Occurrence of pathogenic viruses in drinking water sources

We focused on the integrity of the viral capsid, which is one of the main factors that determine whether the virus is infectious or not, and customized the viability PCR to determine whether the viral capsid is damaged. The

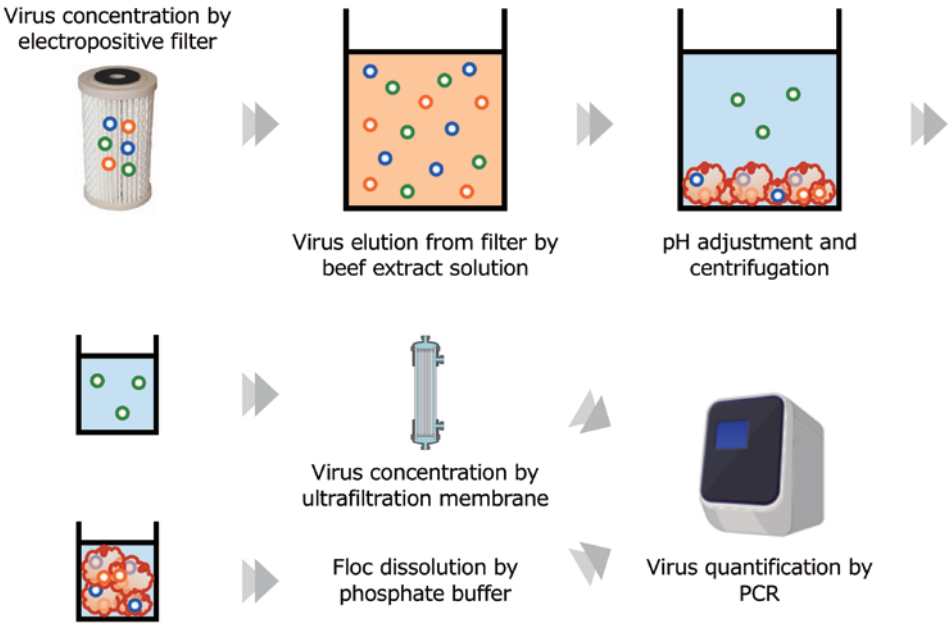

Figure 1 Schematic diagram of a novel virus concentration method using a combination of an electropositive filter and an ultrafiltration membrane.

PMAxx-Enhancer-PCR, which combines PMAxx, a newly improved version of PMA, and PMA Enhancer for Gram Negative Bacteria, was used and optimized the type and concentration of photoreactive intercalating dyes, reaction time, and duration of visible light irradiation, to improve the ability of the viability PCR to discriminate between infectious and inactivated viruses. In addition, to effectively concentrate a wide variety of viruses from large volumes of water samples, we developed a novel virus concentration method (a method combining an electropositive filter and a tangential-flow ultrafiltration membrane; Figure 1). By combining PMAxx-Enhancer-PCR and the developed virus concentration method, we were able to investigate the occurrence of pathogenic viruses (i.e., adenoviruses, astroviruses, noroviruses, sapoviruses, enteroviruses, parechoviruses, hepatitis A virus, hepatitis E virus, and rotaviruses: all nine viruses listed in the WHO *Guidelines for Drinking-water Quality* as pathogenic viruses transmitted through drinking water[1]) in drinking water sources all over Japan, and discuss the presence or absence of infectious viruses based on the integrity of the viral capsid. Indeed, adenovirus, human noroviruses GI, and rotavirus present in drinking water sources tended to have damaged capsids and to be inactivated by damaging the viral capsid. In contrast, astrovirus, human norovirus GII, and enteroviruses present in drinking water sources tended to have intact capsids and to be potentially infectious. We also found that pepper mild mottle virus was ≥100 times more abundant than pathogenic viruses (Figure 2a), and that pepper mild mottle virus is highly likely to present in an infectious without damage to its viral capsid, making it the usefulness of pepper mild mottle as a target virus to determine the virus reduction efficiencies in actual drinking water treatment plants.

Investigation of virus reduction efficiencies in actual drinking water treatment plants

We focused on pepper mild mottle virus and demonstrated that pepper mild mottle virus appears to be a potential surrogate for pathogenic viruses such as adenovirus, norovirus, sapovirus, and enteroviruses in the conventional drinking water treatment processes, coagulation-sedimentation–rapid sand filtration processes, and in next-generation water purification technologies, coagulation–microfiltration processes, through lab-scale virus-spiking experiments[2]-[4]. In addition, we found that the developed virus concentration method using a combination of an electropositive filter and a tangential-flow ultrafiltration membrane can effectively concentrate pepper mild mottle virus from water samples of ≥1,000 L. By focusing on pepper mild mottle virus and applying the developed virus concentration method, we were able to investigate the reduction efficiencies of indigenous pepper mild mottle virus in multiple actual drinking water treatment plants with different treatment processes. Indeed, the concentrations of indigenous pepper mild mottle virus in raw and treated water samples were always above the quantification limit of the PCR assay. We therefore were able to determine the reduction ratios of pepper mild mottle virus: 0.9–2.7-$\log_{10}$ in full-scale coagulation-sedimentation–rapid sand filtration processes and 0.7–2.9-$\log_{10}$ in full-scale coagulation–microfiltration processes (the reduction ratios at Plant A and B were 1.0 ± 0.3-$\log_{10}$ and 2.2 ± 0.6-$\log_{10}$, respectively; Figure 2b, c)[5].

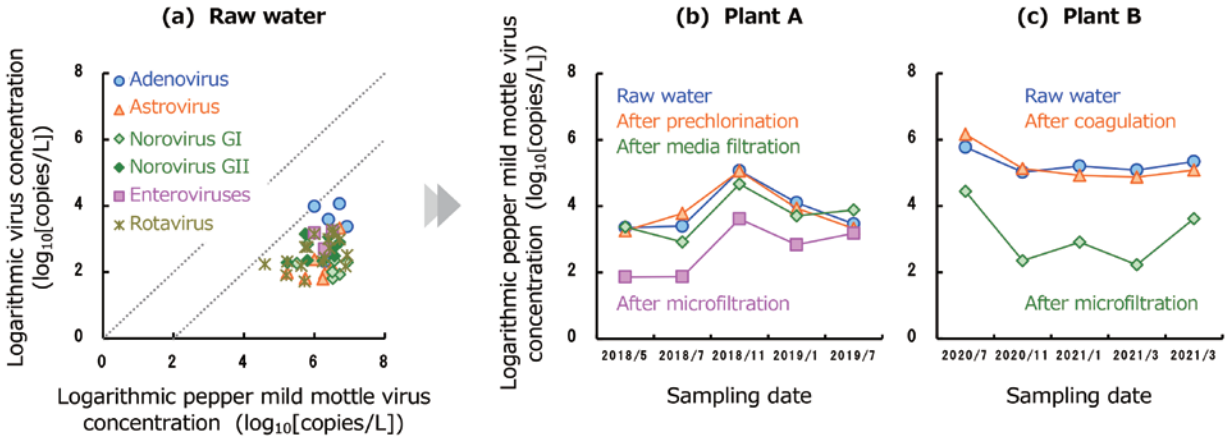

Figure 2 Relationship between the concentrations of indigenous pepper mild mottle virus and pathogenic viruses in drinking water sources (a), and concentrations of indigenous pepper mild mottle virus in raw and treated water at Plants A (b) and B (c). Virus concentrations were determined by PCR.

Investigation of reduction efficiencies of human norovirus in drinking water treatment processes

For human noroviruses, which are difficult to culture, we successfully evaluated the removal efficiencies of human norovirus particles in the coagulation-sedimentation–rapid sand filtration processes, by applying recombinant human norovirus VLPs, which were prepared using the genome sequence of human norovirus and a baculovirus-silkworm protein expression system (Figure 3). Approximately 3-$\log_{10}$ removals were observed for human norovirus VLPs in the coagulation-sedimentation–rapid sand filtration processes[6]. In addition, to increase the sensitivity of VLP quantification, we developed an immuno-PCR (a method combining antigen-antibody reaction and DNA tag quantification by PCR assay; Figure 3) that is 1,000 times more sensitive than the conventional enzyme-linked immunosorbent assay. By combining VLPs and the developed immuno-PCR, we were able to evaluate the removal efficiencies of human norovirus particles in membrane filtration processes. Whereas microfiltration processes with a nominal pore size of 0.1 μm could not remove human norovirus VLPs, approximately 4-$\log_{10}$ removals were obtained by ultrafiltration processes with a molecular weight cutoff of 1 kDa. In addition, >4-$\log_{10}$ removals of human norovirus VLPs were achieved by a combination of coagulation and microfiltration, i.e., coagulation–microfiltration processes[7]. To the best of our knowledge, this is the first study assessing the efficacy of drinking water treatment processes for the removal of human norovirus particles through lab-scale experiments by applying VLPs without waiting for the establishment of an effective *in vitro* cell-culture system for human norovirus.

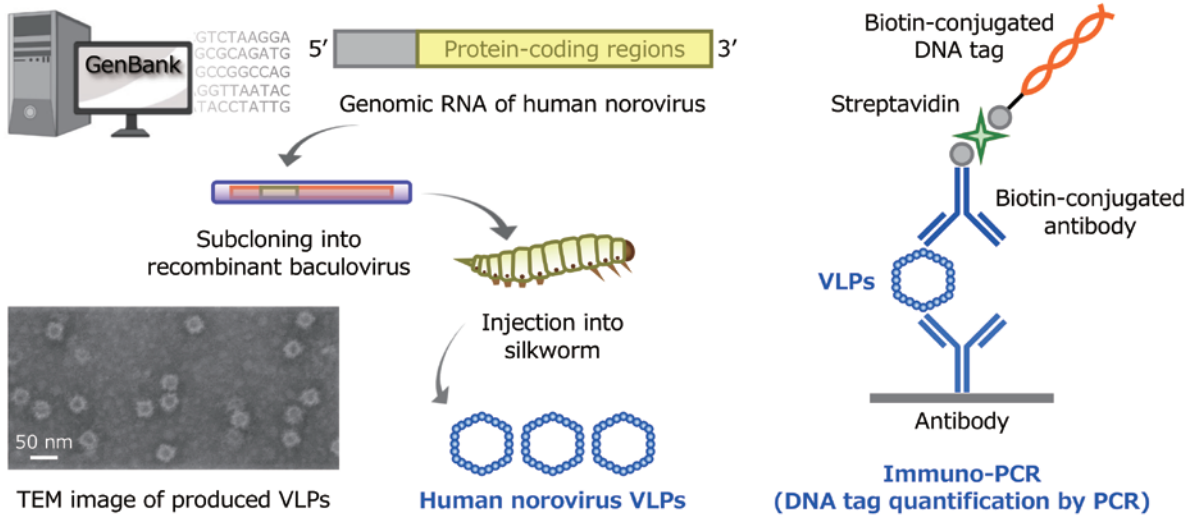

Figure 3 Schematic diagrams of preparation of human norovirus VLPs and VLP quantification by an immuno-PCR.

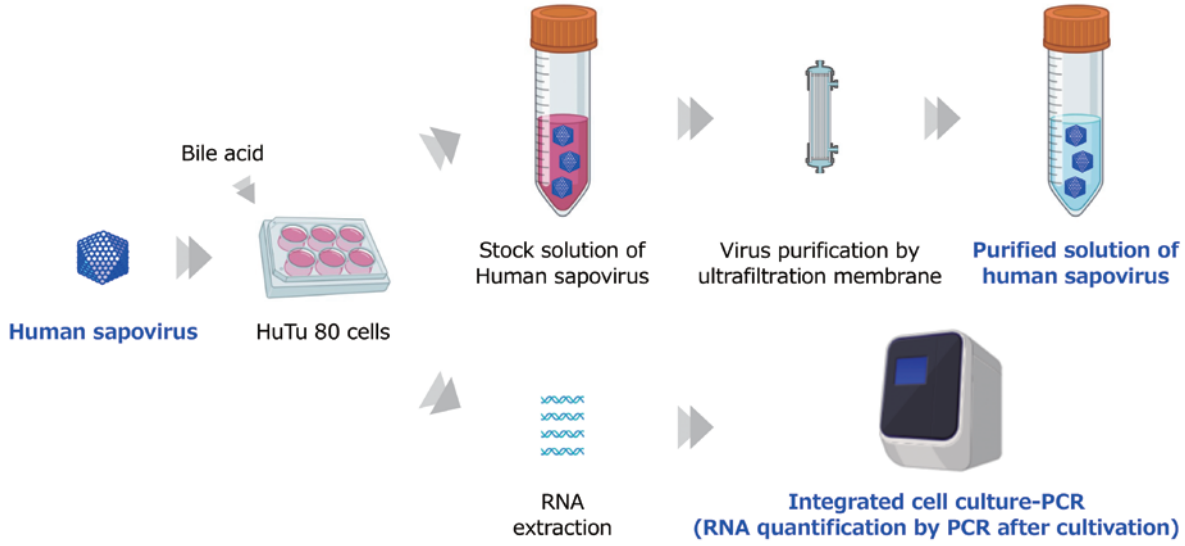

Figure 4 Schematic diagrams of preparation of purified human sapovirus solution and human sapovirus quantification by an integrated cell culture-PCR.

Investigation of reduction efficiencies of human sapovirus in drinking water treatment processes

For human sapoviruses, which were difficult to culture and belong to the same Caliciviridae family as human noroviruses, an *in vitro* cell-culture system using commercially available cell lines and bile acid was discovered in 2020. We prepared the purified solution of human sapovirus (Figure 4) containing virus concentration high enough to conduct lab-scale virus-spiking experiments, and developed an integrated cell culture-PCR (a method combining cell culture and DNA/RNA quantification by PCR assay ; Figure 4) that can quantify the infectivity of human sapoviruses, by applying an *in vitro* cell-culture system. By combining the purified solution of human sapovirus and the developed integrated cell culture-PCR, we were able to evaluate the removal efficiencies of human sapovirus in coagulation-sedimentation–rapid sand filtration processes and membrane filtration processes as well as the inactivation efficiencies of human sapovirus in free-chlorine disinfection processes. In the coagulation-sedimentation–rapid sand filtration processes and the coagulation–microfiltration processes, human sapovirus removals of approximately 2–3-$\log_{10}$ and >4-$\log_{10}$, respectively, were observed[4]. When the efficacy of chlorine treatment was examined by using the developed integrated cell culture-PCR, approximately 4-$\log_{10}$ inactivation of human sapovirus was observed at a CT value (free-chlorine concentration [C] multiplied by contact time [T]) of 0.02 mg-Cl_2·min/L[4]. To the best of our knowledge, this is the first study assessing the efficacy of drinking water treatment processes for the removal and inactivation of human sapovirus through lab-scale experiments by applying an *in vitro* cell-culture system for human sapovirus.

Conclusions

We investigated the occurrence of pathogenic viruses in drinking water sources and their reduction efficiencies in drinking water treatment processes by applying novel virus quantification and concentration methods developed in the present study.

The improved and optimized viability PCR, i.e., the PMAxx-Enhancer-PCR, can determine the presence or absence of viral infectivity quickly and easily (in a few hours), without the use of cell culture. The novel virus concentration method, which can effectively concentrate a wide variety of viruses from a large volume of water samples, can be applied to various types and volumes of water samples, including environmental water and treated drinking water. Therefore, the evaluation method for the presence of infectious pathogenic viruses in environmental water using the PMAxx-Enhancer-PCR and the novel virus concentration method has the potential to be widely used as a method that does not depend on cell-culture methods.

By focusing on pepper mild mottle virus and applying the novel virus concentration method, it is possible to evaluate virus treatability in various actual drinking water treatment plants with different raw water quality, treatment processes, treatment capacity, etc., thereby providing evidence for the safety of drinking water against pathogenic viruses. This approach has the potential to contribute to the establishment of a framework for risk management and control of pathogenic viruses in sustainable drinking water use and the water cycle.

VLPs do not require virus culture using host cells and can be produced in large amounts based on the viral genome

sequence, making them applicable to viruses for which an effective *in vitro* cell-culture system has not yet been established as long as the genome sequence data is available. With the rapid development of genome analysis technology, the metagenomic analysis of viruses in water environments will accelerate in the future, and the prevalence of a wide variety of pathogenic viruses in various water environments around the world will become clear, and their genome sequence data will be accumulated in databases. VLPs are morphologically and antigenically the same as native virus particles, but do not have any genes. Therefore, by applying the new method that combines VLPs and the immuno-PCR, it is possible not only to clarify the treatability of difficult-to-culture pathogenic viruses in drinking water treatment processes, but also to produce VLPs of various virus species, genotypes, and strains, which will provide many findings, such as the differences in behaviors of viruses during drinking water treatment processes among genotypes and strains, and the elucidation of genetic factors (e.g., differences in the amino acids that make up proteins) that affect the differences in behaviors of viruses. The combined use of VLPs and the immuno-PCR has the potential to be used not only for drinking water treatment, but also for understanding the treatability of difficult-to-culture pathogenic viruses in wastewater treatment and water reclamation treatment, clarifying the treatment mechanism, and clarifying the behaviors of difficult-to-culture pathogenic viruses in environmental water.

By applying the purified solution of human sapovirus and the integrated cell culture-PCR, it is possible not only to clarify the treatability of human sapoviruses in various water treatment processes, but also to isolate new strains of human sapoviruses from water samples, develop new, rapid, and easy methods for the quantification of human sapoviruses, the identification of infection receptors, and the development of new disinfectants, vaccines, and infection inhibitors. Therefore, it has cross-disciplinary development potential not only in the field of water environment, but also in the fields of medicine and pharmacology.

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