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# Concentration of MS2 phage in river water by a combined ferric colloid adsorption and foam separation-based method, with MS2 phage leaching from ferric colloid

Yoshihiro Suzuki,[1,*] Takumi Kobayashi,[1] Masateru Nishiyama,[2] and Tomoya Kono[3]

*Department of Civil and Environmental Engineering, Faculty of Engineering, University of Miyazaki, 1-1 Gakuen Kibanadai-Nishi, Miyazaki 889-2192, Japan,[1] Department of Environment and Resource Science, Interdisciplinary Graduate School of Agriculture and Engineering, University of Miyazaki, 1-1 Gakuen Kibanadai-Nishi, Miyazaki 889-2192, Japan,[2] and Department of Biochemistry and Applied Biosciences, Faculty of Agriculture, University of Miyazaki, 1-1 Gakuen Kibanadai-nishi, Miyazaki 889-2192, Japan[3]*

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**The concentration of MS2 phage as a model RNA virus in river water using a combined ferric colloid adsorption and foam separation-based method was examined. The MS2 phage concentrations were determined by the plaque-forming unit (PFU) method and reverse transcription quantitative PCR (RT-qPCR) analysis. When ferric colloid adsorption was performed prior to foam separation, MS2 phage was effectively removed from river water and concentrated in the generated foam within 7 min. The removal efficiency was >99% at the optimum iron and casein concentrations of 5 mg-Fe/L and 10 mg/L, respectively. Furthermore, based on the analysis of the collected ferric colloid dissolved using deferoxamine, the MS2 concentration in the colloid-dissolved solution was 190-fold higher than that found in raw water according to RT-qPCR analysis. This is a novel method for concentrating RNA viruses to facilitate their detection in river water using coagulation and foam separation combined with chelate dissolution of ferric flocs.**



[**Key words:** Concentration method; Ferric colloid absorption; Foam separation; MS2 phage; Detection]

Ongoing progress in microbiological analysis techniques involves the continuous discovery of new pathogenic viruses. Recently, it has become necessary to perform health risk assessments for waterborne diseases caused by pathogenic viruses. Pathogenic viruses, such as noroviruses, enteroviruses, and hepatitis viruses, have been detected not only in sewage (1) and urban river water (2,3) but also in tap water (4), bottled water (5), and seawater (6). Therefore, it is very important to accumulate more information about the pathogenic viruses that cause waterborne diseases. Previously, large numbers of gene sequence for many pathogenic viruses that are considered to be connected with waterborne diseases have been accumulated in the medical and etiological microbiology fields, where the pathogenic viruses collected from patients can be identified using molecular biological techniques such as the polymerase chain reaction (PCR) and gene sequencing analysis. However, it is difficult to capture pathogenic viruses from water bodies because the concentration of pathogenic viruses is extremely low in surface waters. Thus, to obtain information about pathogenic viruses in water samples, an appropriate amount of virus must be concentrated and recovered efficiently from large volumes of water before subjecting the concentrate to PCR analysis and/or gene sequencing analysis.

The most important processes during the detection and identification of pathogenic viruses are their concentration and recovery from surface water. The main concentration and recovery processes are as follows: filtration using positively (7) or negatively (4,6) charged membrane filters, polyethylene glycol precipitation (8), and hollow-fiber ultrafiltration (9). In recent studies, membrane filtration has been applied to the virus concentration process. However, various suspended substances are found in surface waters, so the filtration procedure used to concentrate viruses from the requisite water sample volume using a membrane or a cartridge could result in fouling with suspended substances. In particular, it is difficult to filter a large volume of turbid water because fouling would occur. Furthermore, it is necessary to recover the viruses attached to the membrane or cartridge by elution, which leads to variations in the recovery rate. It has been reported that the recovery rates for norovirus GII type were 12% (10) and 35% (5) in two different studies, whereas in other studies based on filtration, the recovery rate for hepatitis A virus was only 0.32% (11) and that for rotavirus was 3.5% (12). Thus, it is necessary to develop a new method for concentrating pathogenic viruses from surface water, which should be easy to use, rapid, and efficient.

As a possible method of concentrating viruses without filtration, we have focused on foam separation using dispersed bubbles (13) and previously developed a combined ferric colloid adsorption and foam separation-based method (14) for detecting a shrimp disease virus called white spot disease virus (WSDV), which is a DNA virus. When ferric colloid adsorption was performed prior to foam separation, WSDV was effectively removed from seawater and concentrated in the generated foam. Next, to analyze the dissolution of the collected ferric colloid in 0.1 M hydrochloric acid (HCl), the WSDV concentration in the colloid-dissolved solution was 200-fold higher than that found in raw water. Initially, it appeared that this concentration method using bubbles would also be suitable for important RNA virus such as noroviruses, but only a low copy

* Corresponding author. Tel.: +81 985 58 7339; fax: +81 985 58 7344.
*E-mail address:* suzuki@civil.miyazaki-u.ac.jp (Y. Suzuki).

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number was detected in the colloid-dissolved solution, even under the optimum conditions for obtaining a high removal efficiency of noroviruses from polluted water. Thus, the concentrated virus in ferric colloid was considered to be decomposed by the HCl solution. Therefore, to apply the combined ferric colloid adsorption and foam separation-based method for the pathogenic RNA viruses, the effective leaching of virus from ferric colloid may be a break through solution.

In this study, we tested the foam concentration of MS2 phage as a model RNA virus in river water using dispersed bubbles and milk casein, with the pre-adsorption of MS2 onto a ferric colloid. In addition, the recovery of MS2 from the foam-collapsed water, which concentrated the MS2 with ferric colloid, was performed to facilitate MS2 leaching from ferric colloid.

## MATERIALS AND METHODS

**MS2 phage-polluted artificial river water** MS2 phage (NBRC 102619) and the bacterial host *Escherichia coli* (NBRC 13965) were obtained from the Biological Resource Center (NBRC), National Institute of Technology and Evaluation (NITE), Japan. MS2 phage was grown on the *E. coli* host at 37°C for 24 h. MS2 phage stocks were subjected to centrifugation at 3000 ×*g* for 30 min at 4°C to remove any cellular debris. The supernatants were filtered through a 0.45 μm pore size membrane filter. Enumeration of MS2 was performed according to the plaque-forming unit (PFU) method and the stock was quantified as $10^{10}$ PFU/mL. The MS2 stock was stored at 4°C. Sterilized artificial river water (5000 mL; $NaHCO_3$ 96 mg, $CaSO_4 \cdot 2H_2O$ 60 mg, $MgSO_4$ 60 mg, KCl 4.0 mg in 1000 mL of distilled and ion exchanged water; pH 7.5; autoclaved at 121°C for 15 min) was stored at 4°C and inoculated with 500 μL of the MS2 phage stock solution (15). The final concentration of MS2 phage was $10^6$ PFU/mL. The MS2 phage-polluted artificial river water was used as raw water in the combined ferric colloid adsorption and foam separation-based method.

**Ferric colloid adsorption and foam separation-based method** The basic conditions for colloid adsorption and foam separation were those used for the treatment of wastewater, as determined in our previous study (13). The concentration of ferric chloride solution to form the adsorbent, which controls the treatability, was adjusted in the range of 0–5 mg-Fe/L to determine the optimum dosage for MS2 phage removal. Ferric chloride ($FeCl_3 \cdot 5H_2O$, reagent grade; Wako Pure Chemical Industries, Japan) was dissolved in 0.01 M HCl to a final concentration of 10,000 mg/L. A sample of polluted river water (200 mL) was treated with the ferric chloride solution and mixed rapidly (150 rpm) for 3 min using a magnetic stirrer. The added ferric ions rapidly formed ferric hydroxide colloid particles in the polluted river water. After mixing to stimulate the formation of ferric colloid particles, the stock casein solution was added to the sample at a concentration of 10 mg/L casein. The stock casein solution (milk casein, reagent grade; Wako Pure Chemical Industries) was dissolved in 0.01 M NaOH at a final concentration of 10,000 mg/L. Each sample was mixed rapidly (150 rpm) for 1 min. Foam separation was performed by transferring the samples to a cylindrical column (height, 50 cm; diameter, 2.6 cm) in a batch flotation device. Dispersed air was supplied from the bottom of the column via a glass-ball filter (pore size range, 5–10 μm; Kinoshita Rika, Japan). The foam generated on the water surface was drawn into a trap bottle using an aspirator. The recovered foam was then de-foamed and designated as foam water. The processing time required for foam separation was 3 min and the air supply flow rate was 0.3 L of air/min. Samples of the treated water were obtained from the drain. All of the recovered foam water was transferred to a 50 mL centrifuge tube and centrifuged at 3000 ×*g* for 30 min. The supernatant was discarded with a micropipette leaving approximately 1 mL of the sediment liquid at the bottom of the tube, which included the accumulated ferric colloid. The residual suspension was transferred to a 1.5-mL centrifuge tube and centrifuged at 15,780 ×*g* for 5 min. Orange ferric colloid accumulated at the bottom of the tube in pellet form, which was easily separated from the liquid phase using a micropipette. All of the procedures without foam separation were performed in an incubator at 4°C. Raw water (polluted artificial river water), treated water, the supernatant of the foam water, and the collected ferric colloid were analyzed for MS2 phage using the PFU method and reverse transcription quantitative PCR (RT-qPCR) analysis within 6 h. The tests were performed in triplicate using identical polluted artificial river water samples. The total processing time required for foam separation with pre-adsorption was 7 min.

To confirm the removal of iron as ferric flocs in the separating foam, the residual iron concentration in the treated water was measured by absorptiometry using ferrozine (PDTS, Dojindo Laboratories, Japan). We followed the procedure proposed by Stookey (16) using a spectrophotometer (UV-2450, Shimadzu, Japan) at a wavelength of 562 nm with a 100-mm glass cell. The detection limit for iron in this study was 0.005 mg/L.

**Quantification of MS2 phage by RT-qPCR analysis** MS2 RNA was collected using a QIAamp MinElute Virus Spin Kit (Qiagen, Netherlands), according to the manufacturer's instructions. cDNA was synthesized from 2 μg of RNA using a High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, USA) as a template for PCR. Initially, PCR was performed using the cDNA prepared above with the primers: MS2 Fw primer: 5′-CGGACGGTGAGACTGAAGAT-3′ (MS2 genome 194–213; accession no. NC_001417) and MS2 Rv primer: 5′-TAGA-GAGCCGTTGCCTGATT-3′ (MS2 genome 1287–1306), which were designed for the region that included the assembly protein, thereby allowing the amplification of the MS2 genome. The conditions for all of the PCR analyses were: one cycle at 95°C for 5 min, 45 cycles at 95°C for 1 min, 50°C for 1 min, and 72°C for 2 min, followed by one cycle at 72°C for 5 min. The amplification process was performed using GoTaq Green Master Mix (Promega, USA) in a 20 μL reaction volume, which contained 10 μL of GoTaq Green Master Mix, 2 μL of each primer set (Fw and Rv; 5.0 μM), 5 μL of nuclease-free water, and 1 μL of MS2 cDNA. The PCR products obtained were ligated into the pGEM-T Easy vector (Promega) and transfected into competent *E. coli* DH5α (Toyobo, Japan), where the recombinants were identified based on red-white color selection when grown on MacConkey agar (Sigma–Aldrich, USA). Plasmid DNA from at least three clones was extracted using a PureYield Plasmid Miniprep System (Promega) according to the manufacturer's instructions and sequenced using a CEQ8000 Automated Sequencer (Beckman Coulter, USA). The sequences generated were analyzed to determine their similarity with other known sequences using the BLAST suite of programs.

To determine the absolute copy number of the target gene, a cloned plasmid DNA for the assembly protein gene of MS2 was used to generate a standard curve. The concentration of plasmid DNA samples was confirmed using a NanoDrop spectrophotometer, ND-1000 (Thermo Scientific, Wilmington, DE, USA). The reaction mixture was of same composition as described above. The primer sets used to construct the standard curve were: qMS2 forward (5′-GTCGCGGTAATTGGCGC-3′) and qMS2 reverse (5′-GGCCACGTGTTTTGATCGA-3′) (17). The amplification program comprised 30 s at 94°C, followed by 30 cycles at 95°C for 1 min, 50°C for 1 min, and 72°C for 2 min. The PCR products were purified using Microcon centrifugal filter devices (Millipore, Bedford, MA, USA). The product copy numbers were calculated according to the molecular weights of the products and then converted into the copy numbers based on Avogadro's number (1 mol = $6.022 \times 10^{23}$ molecules).

Real-time PCR of the cDNA specimens and DNA standards for MS2 phage was performed using TaqMan Universal PCR Master Mix (Applied Biosystems). All of the real-time PCR analyses were performed in the following conditions: 12.5 μL of 2× TaqMan Universal PCR Master Mix, 2.5 μL of 4 μM of each primer set (qMS2 Fw and Rv), 2.5 μL of 2.5 μM qMS2 TaqMan probe (5′-AGGCGCTCCGCTACCTTGCCCT-3′), 1 μL of template DNA (50 ng), and 4 μL of nuclease-free water. The amplification program was as follows: 2 min at 50°C and 10 min at 95°C, followed by 40 cycles at 95°C for 15 s and 60°C for 60 s. Thermal cycling and fluorescence detection was conducted using a 7300 Fast Real-Time PCR System (Applied Biosystems), where the samples were detected in triplicate. The threshold cycle (CT) represents the PCR cycle during which an increase in the reporter fluorescence above a baseline signal is first detected. The MS2 RNA quantities were determined from the standard curve.

**Leaching of MS2 phage from ferric colloid by mixing with glass beads** The ferric colloid-adsorbed MS2 phage was collected from the foam water, which was obtained under the optimum conditions for MS2 removal from the polluted artificial river water. The ferric colloid was mixed with 0.1 mg of sterilized glass beads (0.105–0.125 mm diameter, As One Co., Japan) and 1.0 mL of sterilized physiological saline solution (PSS) for 1 min using a mixer (3000 rpm, Disruptor Genie, Scientific Industries). After mixing, the suspension was centrifuged. The supernatant was then analyzed as a leaching solution to detect MS2 phage by the PFU method and RT-qPCR analysis. In addition, to improve the leaching effect on MS2 phage, a similar experiment was performed using 0.01 mol/L acetic acid solution instead of PSS.

**Leaching of MS2 phage from ferric colloid by chemical dissolution** The ferric colloid-adsorbed MS2 phage was dissolved with four different solutions, as follows: beef extract solution, oxalate solution, deferoxamine solution, and 1% elution solution (PET). The sterilized beef extract solution (6%, pH 7.5), which has been used previously to elute Qβ and MS2 phages from aluminum colloid (18), was made of beef extract (Difco, BD Biosciences, MD, USA). The oxalate solution (0.1 M oxalate, 0.05 M sodium citrate, pH 8.0) has been used to remove iron adsorbed on the surface of phytoplankton (19). The deferoxamine solution (0.076 M) was made by dissolving deferoxamine mesylate (FW 656, Sigma–Aldrich), which is a strong chelating reagent with ferric ions that is used as an antidote during iron poisoning, as a dissolution agent for ferric colloid. The 1% PET solution is used to elute *Cryptosporidium* oocysts from membranes (US EPA method 1623). The stock PET solution was made by dissolving 20 g sodium pyrophosphate ($NaP_2O_7 \cdot 10H_2O$, Wako Pure Chemical Industries), 30 g ethylenediaminetetraacetic acid (EDTA·3Na, Wako Pure Chemical Industries), and 10 g Tween-80 (Wako Pure Chemical Industries) in 1000 mL of distilled and ion-exchanged water. The ferric pellet, which was assumed to contain concentrated MS2 phage, was collected from the foam water under the optimum conditions and mixed with 1 mL of each type of solution using a vortex for 5–10 min until the ferric pellet was dissolved. The colloid-dissolved solution was analyzed to detect MS2 phage using the PFU method and RT-qPCR.