に示す。膜差圧は、膜の一次圧と二次圧の差とし、25℃で補正した。

3時間に1回膜の物理洗浄を実施しているため、設定した3時間毎に膜差圧の回復が見られている。従来型PAC系と高塩基度PAC系を比較すると、示したデータは3日間という短期的な挙動では、凝集剤（塩基度）の違いによる明確な差異は見られなかった。

図-7をもとに、3時間に1回行っている物理洗浄の終了から次の物理洗浄の開始までの間に上昇した膜差圧をプロットしたものを図-8に示す。その結果、高塩基度PAC系膜差圧のほうが従来型PAC系膜差圧より、上昇幅が小さい傾向を示した。実験②では、膜モジュールを新しい膜モジュールに交換して行ったが、同じ傾向を示した。このことから、物理洗浄間で上昇した膜差圧で生じた差異は、膜モジュールの性能の違いによるものではなく、膜沪過までの前処理の違いにより生じたものと考えられた。

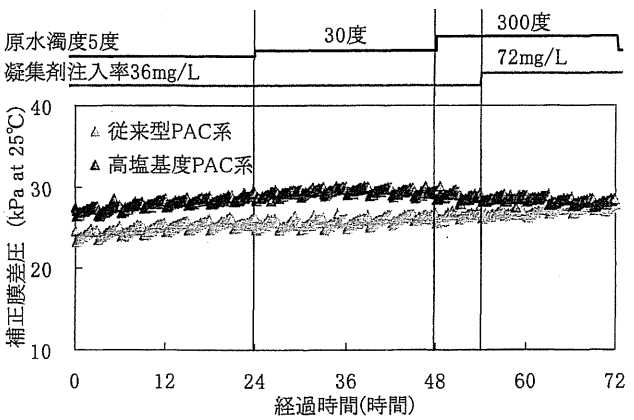


図-7　膜差圧の挙動（25℃補正）（実験①）

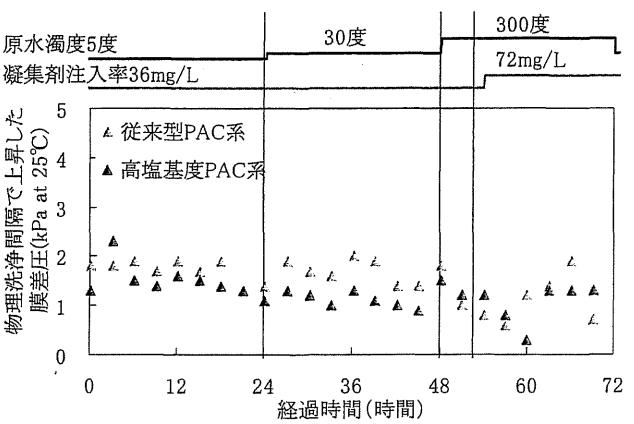


図-8　物理洗浄間隔で上昇した膜差圧（実験①）

凝集条件により、膜差圧の挙動や膜のファウリングが影響を受けることが知られており[23]、膜沪過原水のアルミニウム濃度[24]やフロックのゼータ電位[25]及び粒径分布[26]が影響する可能性があるとの報告もある。本研究では、膜沪過原水である沈澱水において、高塩基度PAC系と従来型PAC系でこれらの項目に差異が生じていることから、物理洗浄間で上昇した膜差圧で生じた差異の原因は、沈澱水中フロックにおける、ゼータ電位や粒径分布などの物性の違いや残留アルミニウム濃度が影響しているものと考えられた。詳細については今後の検討課題と考えられる。

加藤らは、34日間にわたる内圧式モノリス型セラミック膜を用いた実験で、高塩基度PACと従来型PACの膜沪過性を検討し、高塩基度PACは膜差圧上昇が抑制されることを示している[27]。酢酸セルロース膜を用いた本研究の結果や加藤らによる研究結果から考慮すると、高塩基度PACの使用による膜沪過性への負の影響はないものと推定されるが、今後は膜の材質や孔径の影響、長期的な膜沪過運転を行った場合のファウリングへの影響などについて、検討する必要があると考えられる。

3.3　アルミニウムの残留性

実験原水中の総アルミニウム濃度は原水濁度と共に上昇したが、溶解性アルミニウム濃度は0.001mg/L以下となり、定量下限値以下だった。

図-9に、実験①における従来型PACを使用した場合と高塩基度PACを使用した場合の沈澱水中総アルミニウム濃度と沈澱水中溶解性アルミニウム濃度の関係を示す。沈澱水中総アルミニウム濃度の分布は、両系統とも100μg/L～500μg/Lの範囲であった。高塩基度PAC系総アルミニウム濃度のほうが低くなる傾向もみられるが概ね同程度の分布を示し、沈澱水濁度と同様の傾向だった。その一方で、沈澱水中溶解性アルミニウム濃度の分布ついては、従来型PAC系沈澱水は30μg/L～70μg/Lの範囲だったのに対し、高塩基度PAC系沈澱水は10μg/L以下となり、高塩基度PAC系のほうが低くなった。

続いて、図-10に従来型PACを使用した場合と

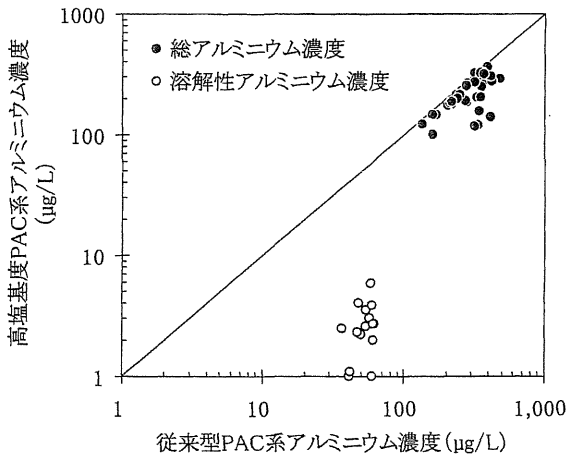


図-9　凝集剤による沈澱水中アルミニウム濃度の比較（実験①）

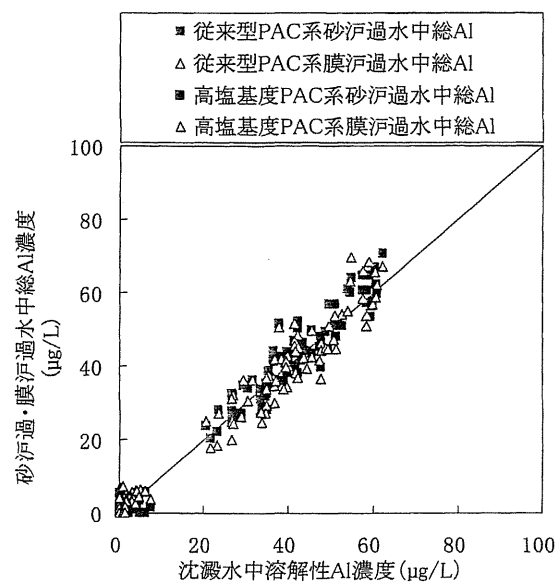


図-10　砂ろ過水中総アルミニウム濃度と膜沪過水中総アルミニウム濃度の関係（実験①）

高塩基度 PAC を使用した場合の沈澱水中溶解性アルミニウム濃度と砂沪過水及び膜沪過水中総アルミニウム濃度との関係を示す。砂沪過水及び膜沪過水中総アルミニウム濃度は20μg/L～70μg/Lの範囲で両者は同程度で、沈澱水中溶解性アルミニウム濃度と同程度だった。すなわち、砂沪過及び膜沪過により沈澱水中の懸濁態で存在するアルミニウムは除去できたが、溶解性のアルミニウムは除去できなかった。このことから、水道水中のアルミニウム濃度を低減するには、沈澱水中溶解性アルミニウム濃度を低減することが不可欠であることが示された。

以上のことから、高塩基度 PAC は従来型 PAC と同一の運転条件において、残留アルミニウムの低減効果があるものと考えられた。

4.　まとめ

地下水にカオリンを添加した実験原水について、パイロットスケールの実験装置にて、高塩基度 PAC の濁度の除去性とアルミニウムの残留性に関して検討した結果、以下の結論が得られた。

1)　高塩基度 PAC を使用した場合の凝集沈澱プロセスにおける濁度の除去性は、従来型 PAC を使用した場合と同等であった。

2)　高塩基度 PAC 系砂沪過水は、従来型 PAC 系砂沪過水より洗浄後の初期漏出濁度の清澄化が早くなった。

3)　高塩基度 PAC 系沈澱水中フロックは、従来型 PAC 系より粒径が大きく、ゼータ電位が至適凝集領域に近かった。これらによって、砂沪過水初期漏出濁度の清澄化が早まるものと考えられた。

4)　膜沪過水濁度は、高塩基度 PAC 系も従来型 PAC 系も、高感度濁度計の検出下限値以下だった。

5)　砂沪過損失水頭及び膜差圧に対する高塩基度 PAC と従来型 PAC の使用の差異は限定的だったことから、高塩基度 PAC を使用することに関する沪過性への影響は小さいと考えられた。

6)　高塩基度 PAC 系沈澱水中に残留する溶解性アルミニウムは、従来型 PAC 系より低かった。また、砂沪過水及び膜沪過水には沈澱水中溶解性アルミニウムと同程度残留した。そのため、水道水中アルミニウムの低減には、沈澱水中溶解性アルミニウムの低減が不可欠であることが示された。

7)　高塩基度 PAC は、従来型 PAC と同一の運転条件にて、水道水中アルミニウムを低減できることが示された。

謝　辞

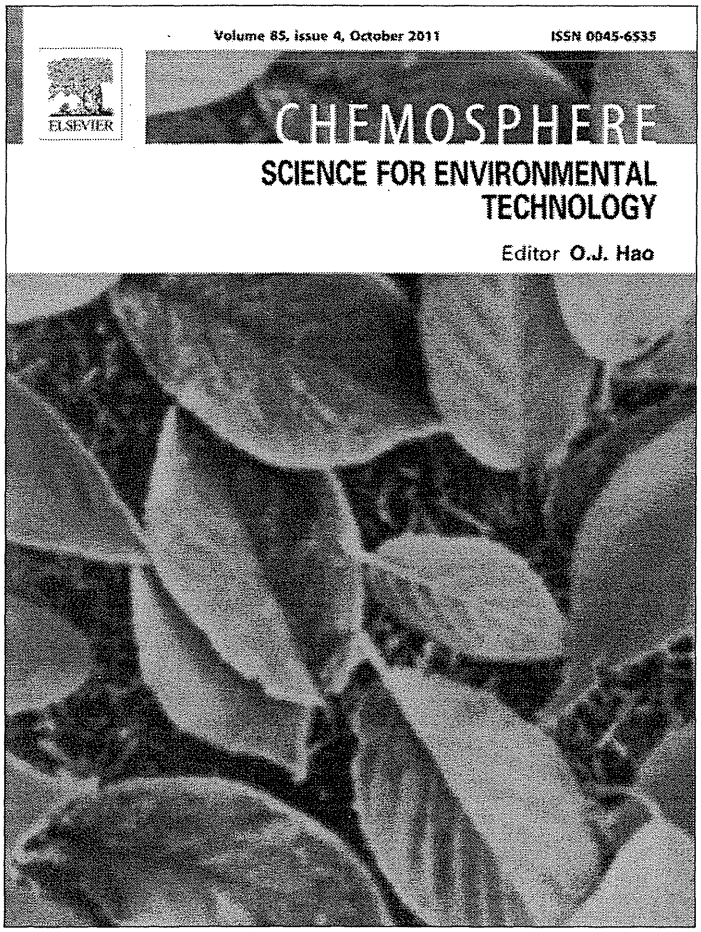
本研究は、厚生労働科学研究費補助金 健康安全・危機管理対策総合研究事業「飲料水の水質リスク管理に関する統括的研究」の一部として行わ

れた。本研究を遂行するにあたりご協力いただいた、平成21年度国立保健医療科学院水道工学研修の研修生である南方則之氏、堀野秀一氏、佐藤研一郎氏に感謝の意を表します。

参 考 文 献

1) 厚生労働省健康局水道課： 水道統計 施設・業務編（平成19年度）
2) Martyn C. N, Barker D. J. P, Osmond C: Geographical relation between Alzheimer's disease and aluminum in drinking water, Lancet, Vol. 1, pp. 59-62 (1989)
3) McLachlan D. R. C, Bergeron C, Smith J. E: Risk for neuropathologically confirmed Alzheimer's disease and residual aluminum in municipal drinking water employing weighted residential histories. Neurology, Vol. 46, pp. 401-405 (1996)
4) Virginie R, Daniel C, Helene J. G, Dartigues J. F: Relation between aluminum concentrations in drinking water and Alzheimer's disease: An 8-year follow-up study, American Journal of Epidemiology, Vol. 152, pp. 59-65 (2000)
5) 厚生労働省健康局水道課：第6回厚生科学審議会生活環境水道部会配付資料水質基準の見直し等について（案）
6) 眞柄泰基、伊藤雅喜、国包章一： 厚生労働科学研究「最新の科学的知見に基づく水質基準の見直し等に関する研究―無機分科会―」分担研究報告書、pp. 19-24 (2005)
7) 内山恵、大野浩一、亀井翼、真柄泰基：PSI を用いたフロキュレーター設計の基礎的研究、水道協会雑誌、第72巻、第6号、pp. 2-11 (2003)
8) 奥田哲士、Phengxay Deevanhxay、長谷川孝雄、西嶋渉、岡田光正：ポリシリカ鉄凝集剤を用いた凝集沈澱―急速沪過処理の特性、水道協会雑誌、第73巻、第11号、pp. 2-10 (2004)
9) 伊藤豊彰： 水田の水環境保全機能 環境保全型水田農業におけるポリシリカ鉄凝集剤（PSI）浄水発生土資源化の可能性、用水と廃水、Vol. 52、No. 1、pp. 76-82 (2010)
10) 海老江邦雄、東義洋、浅香博則、山木暁、萩下隆、凝集処理における攪拌条件の最適化に関する基礎的検討、第52回全国水道研究発表会論文集、pp. 96-97 (2001)
11) 海老江邦雄、東義洋、山木暁：凝集沈澱の処理性改善に関する基礎的研究―GR 値の上昇による濁度と STI の低減化―、水道協会雑誌、第71巻、第9号、pp. 11-21 (2002)
12) 佐藤文彦、巻木康宏、梶早苗、石原俊、松井佳彦：浄水中の残留アルミニウムを低減する新しいポリ塩化アルミニウム、第60回全国水道研究発表会論文集、pp. 108-109 (2009)
13) 木村正興、大野浩一、松下拓、松井佳彦： 高塩基度ポリ塩化アルミニウムを用いた凝集処理時におけるアルミニウム残留性の評価、第60回全国水道研究発表会論文集、pp. 48-449 (2009)
14) 松井佳彦、伊藤雅喜、国包章一： 無機物質分科会分担研究報告書、厚生労働科学研究「飲料水の水質リスク管理に関する総合的研究」研究報告書、pp. 71-97 (2008)
15) Mingquan Yan, Dongsheng Wang, Jianfeng Yu, Jinren Ni, Marc Edwards, Jiuhui Qu: Enhanced coagulation with polyaluminum chlorides: Role of pH/Alkalinity and speciation, Chemosphere, Vol. 71, No. 9, pp. 1665-1673 (2008)
16) WU Xiaohong, WANG Dongsheng, GE Xiaopeng, TANG Hongxiao, YE Changqing: Effect of speciation transformation on the coagulation behavior of Al13 and Al13 aggregates, Water Science & Technology, Vol. 59, No. 4, pp. 815-822 (2009)
17) YAN Mingquan, WANG Dongsheng, QU Jiuhui, HE Wenjie, CHOW Christopher W. K., Relative importance of hydrolyzed Al (III) species (Al_a, Al_b, and Al_c) during coagulation with polyaluminum chloride: A case study with the typical micro-polluted source waters, Journal of colloid and interface science, Vol. 316, No. 2, pp. 482-489 (2007)
18) 石川太了、木村正興、松井佳彦、松下拓、大野浩一：高塩基度 PACl を用いた凝集処理後の残留アルミニウム濃度とその温度影響、第61回全国水道研究発表会論文集、pp. 236-237 (2010)
19) 海老江邦雄、土井克哉：定速砂沪過における凝集フロックの沪層内挙動と沪過水水質の改善、水道協会雑誌、第67巻、第10号、pp. 25-35 (1998)
20) 汚泥処理上からみた合理的浄水方法、汚泥処理上からみた合理的浄水方法に関する研究総括報告書 (1980)
21) 厚生労働省健康局水道課、水道におけるクリプトスポリジウム等対策指針
22) 加藤絵美、村田直樹、川瀬優治、青木伸浩、松井佳彦：高塩基度ポリ塩化アルミニウムによる膜沪過性の検討、第61回全国水道研究発表会論文集、pp. 248-249 (2010)
23) 前田智宏、木村克輝、渡辺義公： 前凝集/MF 膜処理において凝集条件が膜ファウリングに及ぼす影響、第13回衛生工学シンポジウム論文集、pp. 235-238 (2005)
24) 峯岸進一、池田啓一、渡辺義公、山村弘之： 浄水処理における中空糸 UF 膜のファウリング物質の把握、水道協会雑誌、第71巻、第5号、pp. 2-13 (2002)
25) 朴宰亨、滝沢智、片山浩之、大垣眞一郎：生物沪過前処理による精密炉過膜のファウリング制御、水道協会雑誌、第71巻、第3号、pp. 19-31 (2002)
26) 安藤由華、湯浅晶、李富生、松下拓：膜沪過抵抗を抑制させるための凝集処理条件に関する検討、岐阜大学大学院修士論文 (2007)
27) 加藤絵美、村田直樹、川瀬優治、青木伸浩、松井佳彦：高塩基度ポリ塩化アルミニウムによる膜沪過性の検討、第61回全国水道研究発表会論文集、pp. 248-249 (2010)

（平成22年5月6日受付）



Chemosphere 85 (2011) 571–576

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Virus inactivation during coagulation with aluminum coagulants

Taku Matsushita *, Nobutaka Shirasaki, Yoshihiko Matsui, Koichi Ohno

Graduate School of Engineering, Hokkaido University, N13W8, Sapporo 060-8628, Japan

ARTICLE INFO

Article history:
Received 24 March 2011
Received in revised form 21 June 2011
Accepted 21 June 2011
Available online 13 July 2011

Keywords:
Bacteriophage
Inactivation
Virus
Aluminum coagulant
Coagulation process

ABSTRACT

We used the bacteriophages Qβ and MS2 to determine whether viruses are inactivated by aluminum coagulants during the coagulation process. We performed batch coagulation and filtration experiments with virus-containing solutions. After filtering the supernatant of the coagulated solution through a membrane with a pore size of 50 nm, we measured the virus concentration by both the plaque forming unit (PFU) and polymerase chain reaction (PCR) methods. The virus concentration determined by the PFU method, which determines the infectious virus concentration, was always lower than that determined by the PCR-based method, which determines total virus concentration, regardless of infectivity. This discrepancy can be explained by the formation of aggregates consisting of several virus particles or by the inactivation of viruses in the coagulation process. The former possibility can be discounted because (i) aggregates of several virus particles would not pass through the 50-nm pores of the filtration membrane, and (ii) our particle size measurements revealed that the virus particles in the membrane filtrate were monodispersed. These observations clearly showed that non-infectious Qβ particles were present in the membrane filtrate after the coagulation process with aluminum coagulants. We subsequently revealed that the viruses lost their infectivity after being mixed with hydrolyzing aluminum species during the coagulation process.

1. Introduction

Advances in molecular biology have enabled the detection of viruses at low concentrations in environmental waters without the need for virus cultivation. By using these technologies, viruses have been detected in river water, drinking water sources, and in drinking water worldwide. These findings have highlighted the need for treating drinking water to remove viruses. The United States Environmental Protection Agency has included four waterborne viruses on Contaminant Candidate List 3 for drinking water (USEPA); these viruses are currently not subjected to drinking water regulations in the United States but are listed because they require regulation in the future.

Viruses can be removed from drinking water by different treatments, including virus inactivation and physicochemical separation processes. Virus inactivation treatment includes chlorination, ozonation, and UV light irradiation. In these processes, chlorine; an oxidant (ozone), as well as some radicals; and UV light penetrate the virion and reversibly or irreversibly modify the viral genome (Dennis et al., 1979; Camel and Bermond, 1998; Hijnen et al., 2006). The oxidants can also modify the surface protein of the virus (Camel and Bermond, 1998). Modification of the genome or surface protein of the virus abolishes the virus's infectivity. By comparison, in physicochemical separation processes, waterborne virus particles settle out under natural gravity after becoming enmeshed in aluminum/ferric flocs generated during the coagulation process. Alternatively, virus particles are filtered out by ultrafiltration, nanofiltration, or reverse osmosis membranes. In these treatments, viruses are physicochemically separated from the water.

Recently, our group found that the plaque forming unit (PFU)-based concentrations of viruses that had become enmeshed in aluminum floc did not recover to their initial values before coagulation, even after the floc was dissolved under alkaline conditions, suggesting that the aluminum coagulant had virucidal activity (Matsui et al., 2003; Matsushita et al., 2004). However, because we did not confirm whether the floc dissolved completely, it remained unclear whether the change in virus concentration after floc dissolution was the result of the virucidal activity of the aluminum coagulant. To confirm the virucidal activity of the aluminum coagulant, we compared the virus concentration measured by the polymerase chain reaction (PCR) and PFU methods after floc dissolution (Matsushita et al., 2006). The virus concentration determined by the PFU method, which determines the infective virus concentration, was >3 log lower than that determined by the PCR-based method, which determines total virus concentration, regardless of infectivity. This finding suggests that >99.9% of viruses are inactivated by the coagulation process (Matsushita et al., 2006). A similar phenomenon was observed in the

* Corresponding author. Tel./fax: +81 11 706 7279.
E-mail address: taku-m@eng.hokudai.ac.jp (T. Matsushita).

0045-6535/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.chemosphere.2011.06.083

supernatant remaining after the coagulation process (Shirasaki et al., 2009a). However, we did not take into account the effect of aggregation of virus particles on the PFU-based concentration: an aggregate consisting of several virus particles are reported to be able to act as one infectious agent (Floyd and Sharp, 1979; Langlet et al., 2007). The production of aggregates, in addition to virus inactivation, is reported to contribute to the difference between the concentrations of viruses determined by the PFU- and PCR-based methods (Langlet et al., 2007; Teunis et al., 2008). In drinking water treatment plants, the water is treated after the coagulation process by further processes, including sedimentation, sand filtration, membrane filtration, chlorination, and UV irradiation. Enmeshment of viruses in aggregates might enhance or reduce the effectiveness of subsequent treatment processes. It is possible that the aggregated virus particles are removed more readily than the monodispersed particles by membrane filtration. However, these aggregates might form a physical barrier to disinfection by chlorination and UV treatment and thus reduce the efficiency of the disinfection process. The aim of our study was to clarify whether or not the difference between the PFU- and PCR-based virus concentrations was the result of virus inactivation or aggregation.

2. Materials and methods

2.1. Bacteriophages used

Three bacteriophages, Qβ (NBRC 20012), MS2 (NBRC 102619) and T4 (NBRC 20004), were obtained from the NITE Biological Resource Center (NBRC, Chiba, Japan). Qβ and MS2 were used as model viruses, whereas T4 was used to investigate the effect of host cell debris on particle size. The diameters of Qβ and MS2 are approximately 23 nm, whereas T4 has a head shell of approximately 65 × 95 nm and a long tail of approximately 25 × 110 nm with six tail fibers.

Escherichia coli F^+ (NBRC 13965) obtained from NBRC was propagated for 3 h at 37 °C according to the supplier's instructions to prepare an *E. coli* F^+ suspension. The bacteriophages were then propagated for 22–24 h at 37 °C in the *E. coli* F^+ suspension. The respective bacteriophage cultures were centrifuged (2000g, 10 min) and then filtered through a membrane filter with a pore size of 0.45 μm (cellulose acetate; DISMIC-25cs; Toyo Roshi Kaisya, Tokyo, Japan). The filtrate was purified by using a centrifugal filter device (molecular weight cutoff: 100 000; regenerated cellulose, Amicon Ultra-15; Millipore, Billerica, MA, USA) to prepare the virus stock solution.

2.2. Coagulation and filtration procedure for solutions with low virus concentrations

Batch coagulation experiments were conducted at 20 °C in glass beakers with 200 mL of Qβ- or MS2-spiked (approx. 10^8 PFU mL^{-1}) river water (Toyohira River, Sapporo, Japan; turbidity, 1.0 NTU; dissolved organic carbon concentration, 1.0 mg L^{-1}; OD260, 0.031 cm^{-1}). The river water was mixed with an impeller stirrer, and supplemented with polyaluminum chloride (PACl) (PACl 250A; 10.5% Al_2O_3, relative density 1.2 at 20 °C; Taki Chemical, Hyogo, Japan) or alum (8.1% Al_2O_3, relative density 1.3 at 20 °C; Taki Chemical) at 1.08 mg Al L^{-1}. The water was immediately adjusted to, and maintained at, pH 6.8 with hydrochloric acid or sodium hydroxide. The water was stirred rapidly for 2 min ($G = 200\ s^{-1}$, 61 rpm) and then slowly for 28 min ($G = 20\ s^{-1}$, 13 rpm). The water was then left for 20 min to allow the floc particles to settle. Samples were taken from the beaker before addition of the coagulant and after floc particle settling. The virus concentration was measured after passage of the samples through a membrane filter with a pore size of 50 nm (polycarbonate; VMTP, Millipore). We had measured the virus concentration before and after the filtration of coagulant-free Qβ- and MS2-spiked river water, and found that no reduction in the virus concentration was observed by the filtration, confirming that monodispersed viruses had completely passed through the membrane (data not shown).

2.3. Coagulation and filtration procedure for solutions with high virus concentrations

We measured the particle size of the filtrate to confirm whether or not virus aggregates were present after coagulation. PACl coagulation treatment was conducted with 8 mL of Qβ-spiked (approx 10^{11} PFU mL^{-1}) river water in centrifugal tubes at 20 °C. The river water was supplemented with PACl at predetermined concentrations (0, 54, 81, and 108 mg Al L^{-1}). The pH of the water was immediately adjusted to pH 6.8 by adding hydrochloric acid or sodium hydroxide, the amounts of which were determined in preliminary experiments. The water was then vortexed intensely for 1 min and left for 60 min to allow the floc particles to settle. The water was then centrifuged at 2000g for 10 min, and 5 mL of the supernatant was filtered through the 50-nm membrane filter. The particle size was measured with a photon correlation spectrometer (Zetasizer Nano ZS; 532 nm green laser, Malvern Instruments Malvern, Worcestershire, UK) at 25 °C. The same procedure was used for the T4-spiked river water.

2.4. Quantification of bacteriophage concentration

We measured the concentration of infectious bacteriophages by using the agar overlay method (Adams, 1959) with the bacterial host *E. coli* F^+. We determined PFU-based virus concentrations by averaging plaque counts from triplicate plates prepared from one sample.

We quantified the concentration of bacteriophages in samples from extracted viral RNA by the real-time reverse transcription-polymerase chain reaction (RT-PCR) method. This method detects all bacteriophage particles regardless of their infectivity and the existence of aggregates (Langlet et al., 2007). Viral RNA was extracted from 200 μL of sample by using a QIAamp MinElute Virus Spin Kit (Qiagen K. K., Tokyo, Japan). The extracted RNA in a final volume of 20 μL solution was reverse transcribed with a High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems Japan, Tokyo, Japan) at 25 °C for 10 min, 37 °C for 120 min, and 85 °C for 5 s, followed by cooling to 4 °C in a thermal cycler (Thermal Cycler Dice Model TP600, Takara Bio Inc., Shiga, Japan). The cDNA solution was then amplified by using the TaqMan Universal PCR Master Mix with UNG (Applied Biosystems Japan), 400 nM of each primer (HQ-SEQ grade, Takara Bio Inc.), and 250 nM of TaqMan probe (Applied Biosystems Japan). The oligonucleotide sequences of these primers and the probes are listed in Table 1. Amplification was conducted at 50 °C for 2 min, 95 °C for 10 min, and then 40 cycles of 95 °C for 15 s and 60 °C for 1 min in an Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems Japan). The PCR-based viral concentration was expressed as PFU equivalents mL^{-1} as follows. In freshly-prepared stock solutions of the bacteriophages, we assumed that any virus particle possesses infectivity. Accordingly, the PFU equivalent concentration of a sample was determined by converting the number of cycles (C_t value) in the real time RT-PCR amplification of the sample to the PFU concentration according to the relationship between the C_t values and the PFU values for known-concentration freshly-prepared bacteriophage stock solutions.

Table 1
Oligonucleotide sequences of the primers and probes used in real-time RT-PCR quantification of the concentrations of Qβ and MS2 bacteriophages.

Viruses		Oligonucleotide sequence	Position	References
Qβ	Forward primer	5′-TCA AGC CGT GAT AGT CGT TCC TC-3′	49–71	Katayama et al. (2002)
	Reverse primer	5′-AAT CGT TGG CAA TGG AAA GTG C-3′	187–208	
	TaqMan probe	5′-CGA GCC GCG AAC ACA AGA ATT GA-3′	147–169	
MS2	Forward primer	5′-GTC GCG GTA ATT GGC GC-3′	632–648	O'Connell et al. (2006)
	Reverse primer	5′-GGC CAC GTG TTT TGA TCG A-3′	690–708	
	TaqMan probe	5′-AGG CGC TCC GCT ACC TTG CCC T-3′	650–671	

2.5. Electron microscopy

Scanning transmission electron microscopy (SEM, JSM-7400F, JEOL, Tokyo, Japan) was used to analyze the pore size of the membrane used. The pore size of the membrane was expressed as the mean and standard deviation of 16 randomly chosen pores on the electron micrograph.

3. Results and discussion

3.1. Difference in PCR- and PFU-based virus concentrations in filtrate after coagulation

Figure 1 compares the PCR- and PFU-based virus concentrations in the filtrate after passage of the coagulated solutions through the 50-nm membrane filter. Coagulation treatment of Qβ-spiked river water with alum resulted in a 2-log reduction in the viral titer by the PCR-based method from 10^8 to 10^6 PFU equivalents mL^{-1}. By comparison, the same treatment resulted in a 4-log reduction in the viral titer by the PFU-based method to 10^4 PFU mL^{-1}. Thus, the viral concentration in the river water was approximately 100 times (2 logs) as determined by the PCR-based method than by the PFU-based method. When PACl was used as the coagulant, the PFU-based viral concentration of the Qβ-spiked river water was much lower (approx 10^1 PFU mL^{-1}) than when alum was used. However, both coagulants gave the same viral titer with the PCR-based method (approx 10^6 PFU equivalents mL^{-1}). Therefore, coagulation with PACl gave a larger difference (5 logs) in viral titer between the PCR- and PFU-based methods than did alum.

In contrast to our results for Qβ-spiked river water, coagulation treatment of MS2-spiked river water gave a larger reduction in the viral titer by the PCR-based method, to approximately 10^3 and 10^4 PFU mL^{-1} for alum (5 logs) and PACl (4 logs), respectively. However, for the PFU-based method the viral titer for MS2 was approximately 10^2 PFU mL^{-1} for both coagulants; this was almost the same as that obtained for Qβ when PACl was used as the coagulant. These findings agree with those of a previous report (Shirasaki et al., 2009a), in which the PCR-based concentration after in-line coagulation–microfiltration (nominal pore size, 100 nm) was larger for Qβ than for MS2. In summary, we showed that the difference between the PCR- and PFU-based virus concentrations for solutions containing MS2 was smaller than that for solutions containing Qβ, and that for both bacteriophages and coagulants the PCR-based concentration was higher than the PFU-based concentration.

Regardless of the type of virus and coagulant tested, the PFU-based concentration was smaller than the PCR-based concentration. This means that 1 virion detected by the PCR method did not always make one plaque on the host-cell plate in the PFU method. One possible mechanism (Mechanism A) is that some virions became aggregated and behaved as one infectious agent. If this were the case, an aggregate consisting of multiple virions would generate one plaque. In support of this notion Young and Sharp (1977) reported that stocks of poliovirus routinely produced one plaque on host cell monolayers per 100 particles counted. Also Langlet et al. (2007) reported that MS2 exhibited substantial aggregation when the pH was less than or equal to the isoelectric point of 3.9, resulting in the PFU-based concentrations being lower than the PCR-based concentrations; this phenomenon was not observed at pH 6.7. The other possible mechanism (Mechanism B) is that coagulation treatment caused the formation of non-infectious virions. In other words, a part of the virus became inactivated after exposure to hydrolyzing aluminum species during the coagulation process. We previously reported that the virus concentration was lower when determined by the PFU-based method than by the PCR-based method after coagulation with an aluminum coagulant, and we suggested that the virus was inactivated by hydrolyzing aluminum species during the coagulation process (Matsushita et al., 2006; Shirasaki et al., 2007, 2009b).

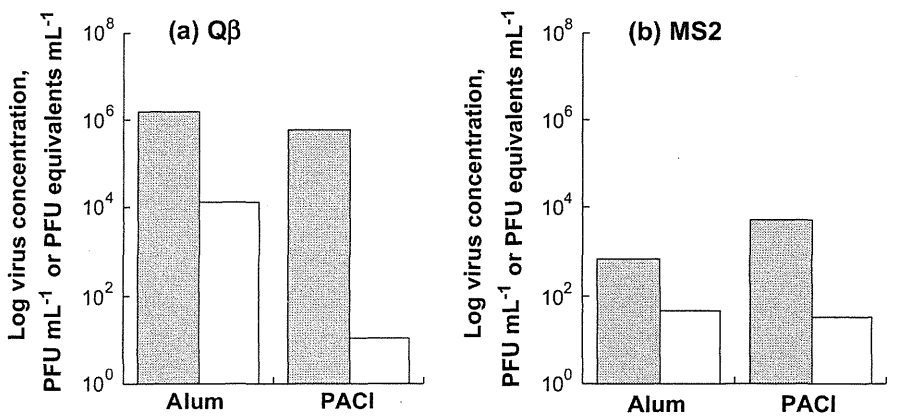


Fig. 1. Comparison of the concentrations of total and infectious viral particles in filtrate (ϕ = 50 nm). Gray and white columns represent PCR-based (i.e., total) and PFU-based (i.e., infectious) virus concentrations. Before coagulation, the initial PCR- and PFU-based concentrations for both the Qβ and MS2 bacteriophages were 10^8 PFU mL^{-1} and 10^8 PFU equivalents mL^{-1}, respectively.

3.2. Aggregate size as an explanation of the difference in PCR-based and PFU-based virus concentrations

If the PFU-based virus concentration is 2 logs lower than the PCR-based virus concentration and the difference between these concentrations can be explained simply by the formation of aggregates that behave as one infectious agent (i.e., Mechanism A), then each aggregate must consist of an average of 10^2 virions. We investigated this hypothesis by calculating the average diameter of the hypothetical aggregate as follows: the ratio of the PCR-based concentration (C_t) to the PFU-based concentration (C_i) after coagulation gave the average number of virions forming one hypothetical aggregate (n).

$$n = \frac{C_t}{C_i} \tag{1}$$

The average number of virions forming one hypothetical aggregate (n) is also expressed as a function of the diameter of the aggregate (R) and that of the virion (r).

$$n = \left(\frac{R}{r}\right)^{D_f} \tag{2}$$

where D_f is the fractal dimension of the aggregate. The following equation is obtained from Eqs. (1) and (2).

$$\frac{C_t}{C_i} = \left(\frac{R}{r}\right)^{D_f} \tag{3}$$

Accordingly,

$$R = \sqrt[D_f]{\frac{C_t}{C_i}}\, r \tag{4}$$

When the fractal dimension of the hypothetical aggregate is 3.0, the aggregate is ideally the most compact and the aggregate diameter is accordingly the smallest. Figure 2 shows the aggregate diameter calculated from the C_i and C_t values in each experiment. For this calculation, we used virion diameters of 23.5 and 22.5 nm for Qβ and MS2, respectively (Shirasaki et al., 2009a), and Df was assumed to be 3.0 (most compact case). We calculated the diameter of aggregates in the PACl-treated solution containing Qβ as 916 nm.

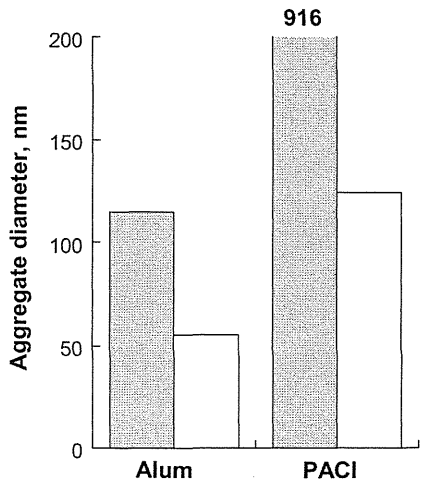


Fig. 2. Aggregate size as an explanation of the difference between PCR- and PFU-based virus concentrations. The fractal dimension of the aggregates is assumed to be 3.0. Gray and white columns represent Qβ and MS2 bacteriophages, respectively.

Because the virus concentration was measured after the samples had been passed through a membrane, the aggregate diameter must have been smaller than the membrane's pore. According to the membrane manufacturer, an electron gun is used to create uniform pores in the membranes, which for the membranes used here were nominally 50 nm. Here, we measured the actual pore of the membrane by using an SEM. Photo 1 is an SEM image of the membrane used for these studies. SEM images of several parts of the membrane revealed that the pores were uniform in size and that abnormally large pores did not exist. We determined the diameter of the pores to be 48.5 ± 3.2 nm, which was consistent with the nominal pore size of the membrane as reported by the manufacturer. Therefore, the 916-nm diameter of aggregates formed by coagulation with PACl was 18 times the 48.5 nm mean pore size of the membrane filter. It is unlikely that aggregates with this diameter could have passed through the membrane's pores, even if they were to change shape because of plasticity. We calculated the diameter of aggregates in the PACl-treated solution containing MS2 and in the alum-treated solution containing Qβ as larger than 100 nm (assuming $D_f = 3.0$); this is more than twice the diameter of the 50-nm pores of the membrane filter. Aggregates of this diameter are also unlikely to pass through a 50-nm membrane filter. This means that the difference in PCR-based and PFU-based virus concentrations of the filtrate was not the result of aggregate formation, or Mechanism A, but was most likely the result of the formation of non-infectious virions by the coagulation process, or Mechanism B. By comparison, the diameter of the aggregates in the alum-treated solution containing MS2 was 55 nm (assuming $D_f = 3.0$), which was almost the same diameter as the pore of the membrane. However, 55 nm was regarded as the minimum possible aggregate size assuming a fractal dimension of 3.0. The fractal dimension of the floc aggregate during the coagulation process changes depending on the operational conditions and is normally smaller than 3.0, with values reported to be 1.5–2.2 (Tambo and Watanabe, 1979; Kim et al., 2001). On the basis of these values, the diameters of the aggregates were 76–135 nm—larger than the pore size of the membrane. Thus, we concluded that, for all four conditions shown in Fig. 1, Mechanism A was implausible, and we propose that Mechanism B explained why the PFU-based virus concentration was lower than the PCR-based virus concentration.

3.3. Measurement of particle size in filtrate after treatment with aluminum coagulant

We provided evidence that any hypothetical aggregate that caused the difference between PCR-based and PFU-based virus concentrations in the filtrate would be larger than the membrane pore. This finding thus suggests that this difference is the result of the formation of non-infectious virions by the coagulation process, or Mechanism B. However, it is also possible that re-aggrega-

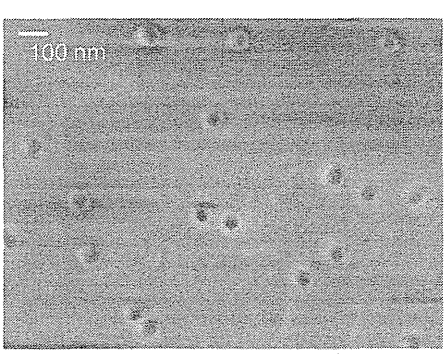


Photo 1. SEM image of the 50-nm membrane filter.

tion of the virus particles occurred after filtration. To investigate this possibility, we directly measured the size of the particles in the filtrate after coagulation treatment with PACl of Qβ- and T4-spiked river water. In these filtrates, the virus concentration of 10^7 PFU equivalents mL^{-1} was below the threshold for detection by photon correlation spectroscopy, so we could not measure particle size. Therefore, we prepared similar solutions with a high virus concentration (approx. 10^{11} PFU equivalents mL^{-1}) and subjected them to coagulation treatment with PACl at a concentration higher than the dose routinely used in the treatment of drinking water. We then filtered these solutions and measured the size of the particles in the filtrate.

Particles in the filtrate can be derived from (i) the colloidal materials present in the river water, (ii) the host cell broth, (iii) the host cell debris, and (iv) the virus. We first measured the particle size of the filtrates of the T4-spiked river water. The T4-stock solution contained the T4 bacteriophage, the colloidal materials from host cell broth, and the host cell debris derived from bacteriolytic action. We found that T4 was completely excluded from the filtrate because its diameter was larger than that of the pores of the membrane (data not shown). The filtrate of the T4-spiked river water therefore contained only particles derived from the colloidal material already present in the river water, the host cell broth, and host cell debris. The particles were found to have a mean diameter of approximately 1 nm (0 mg Al L^{-1} white column, Fig. 3) by photon correlation spectrometry. We therefore measured the particle size of filtrates of coagulant-free Qβ-spiked river water and detected particles with a mean diameter of 16.4 ± 0.6 nm (0 mg Al L^{-1} gray column, Fig. 3). Because the particle size of filtrates of coagulant-free Qβ-spiked river water was different from and much larger than that detected in the T4-spiked river water, the particles detected here were unlikely to be the colloidal material present in the river water, the host cell broth, or the host cell debris, and were accordingly most likely Qβ; however, the extent of host cell lysis by Qβ and T4 might have been different and could possibly have accounted for the particles detected in the Qβ-spiked river water. The diameter of 16.4 ± 0.6 nm for Qβ was slightly smaller than the previously reported value of 23.5 ± 0.8 nm based on TEM observation (Shirasaki et al., 2009a). Because the particle size of coagulant-free Qβ-spiked river water before the filtration was almost the same to that after the filtration (data not shown), the filtration process had not damaged the viruses as they were forced through the membrane. Accordingly, the difference in the diameters between the present and the previous studies was possibly because of the difference in the measurement method employed.

We then measured the particle size of filtrates of PACl-treated T4-spiked river water and identified particles with a mean diameter of approximately 1 nm (54 and 108 mg Al L^{-1} white columns, Fig. 3) by photon correlation spectrometry. The size of these particles suggested that floc particles containing T4 must have been removed by filtration and that they were derived from the colloidal material present in the river water, the host cell broth, or host cell debris. We also measured the particle size of filtrates of PACl-treated Qβ-spiked river water and identified particles with a mean diameter of approximately 16 nm (54 and 108 mg Al L^{-1} gray columns, Fig. 3). These particles were substantially larger than those present in PACl-treated T4-spiked river water (1 nm) and were unlikely to have been derived from the colloidal material present in the river water, the host cell broth, or the host cell debris. The diameter of 16 nm was similar to the size of particles detected in the filtrate of the coagulant-free Qβ-spiked river water, suggesting that the particles were Qβ phage particles. Particles larger than 16 nm were not detected in the filtrate. Taken together, these findings suggest that Qβ phage particles in the filtrate remained monodispersed at the tested PACl doses and did not form aggregates.

We compared the PCR- and PFU-based Qβ concentrations in the filtrates of the PACl-treated Qβ-spiked river water (Fig. 4). Although these filtrates contained monodispersed Qβ, the PFU-based concentrations were always lower than those determined by the PCR-based method at all tested PACl doses. These observations clearly showed that the difference between the PCR- and PFU-based Qβ concentrations in the filtrates after coagulation with PACl was attributable to the presence of non-infectious Qβ particles in the filtrate, because aggregates were not detected. The most likely explanation is that the virus became inactivated after exposure to hydrolyzing aluminum species during the coagulation process. The mechanism of virus inactivation by coagulation remains unclear, but it is likely to differ from that underlying virus inactivation by chlorination, ozonation, and UV irradiation. These processes inactivate viruses by modifying the viral genomes or surface proteins. In contrast, it is possible that intermediate polymers formed during hydrolysis of the aluminum coagulants during the coagulation process with aluminum coagulants adsorb to the viruses and physically interfere with their infectivity of host cells. Further study is needed to clarify the mechanisms underlying the inactivation of viruses by aluminum coagulants.

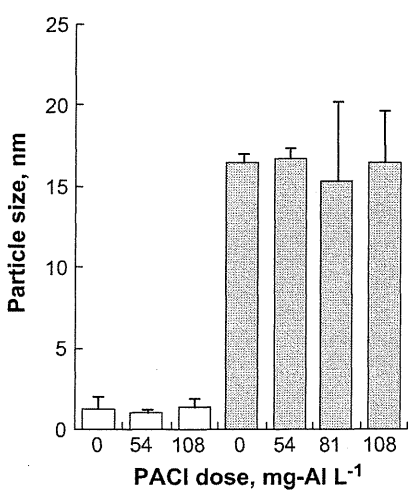


Fig. 3. Mean diameters of particles in filtrate (ϕ = 50 nm) after coagulation with PACl. White and gray columns represent T4- and Qβ-spiked river water, respectively. Error bars represent the standard deviation of 6 measurements.

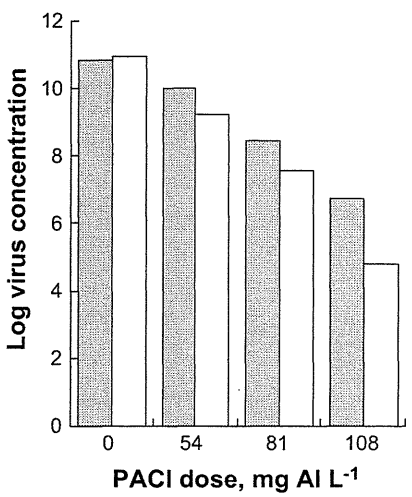


Fig. 4. Comparison of PCR- and PFU-based Qβ concentrations in the filtrate (ϕ = 50 nm) after coagulation with PACl. Gray and white columns represent the PCR- and PFU-based Qβ concentrations, respectively.

4. Conclusions

We used the bacteriophages Qβ and MS2 to determine whether viruses are inactivated by aluminum coagulants during the coagulation process. After filtering the supernatant of the coagulated solution through a membrane with a pore size of 50 nm, the infectious virus concentration was always lower than the total virus concentration. Our particle size measurements revealed that the virus particles in the membrane filtrate were not aggregated but monodispersed, showing that non-infectious Qβ particles were present in the membrane filtrate after the coagulation process with aluminum coagulants. The viruses lost their infectivity after being mixed with hydrolyzing aluminum species during the coagulation process.

Acknowledgments

This research was supported in part by a Grant-in-Aid for the Encouragement of Young Scientists (2010) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan; a Grant-in-Aid (2010) from the Ministry of Health, Labor, and Welfare of Japan; and a Kurita Water and Environment Foundation Research Grant (2009).

References

Adams, M.H., 1959. Bacteriophages. Interscience, New York. pp. 450–454.

Camel, V., Bermond, A., 1998. The use of ozone and associated oxidation processes in drinking water treatment. Water Res. 32, 3208–3222.

Dennis, W.H., Olivieri, V.P., Kruse, C.W., 1979. Mechanism of disinfection: incorporation of Cl-36 into f2 virus. Water Res. 13, 3633–3669.

Floyd, R., Sharp, D.G., 1979. Viral aggregation: buffer effects in the aggregation of Poliovirus and Reovirus at low and high pH. Appl. Environ. Microbiol. 38, 395–401.

Hijnen, W.A.M., Beerendonk, E.F., Medema, G.J., 2006. Inactivation credit of UV radiation for viruses, bacteria and protozoan (oo)cysts in water: a review. Water Res. 40, 3–32.

Katayama, H., Shimazaki, A., Ohgaki, S., 2002. Development of virus concentration method using negatively charged membrane by alkaline elution after acid rinse. J. Jpn. Soc. Water Environ. 25, 469–475 (in Japanese).

Kim, S.H., Moon, B.H., Lee, H.I., 2001. Effects of pH and dosage on pollutant removal and floc structure during coagulation. Microchem. J. 68, 197–203.

Langlet, J., Gaboriaud, F., Gantzer, C., 2007. Effects of pH on plaque forming unit counts and aggregation of MS2 bacteriophage. J. Appl. Microbiol. 103, 1632–1638.

Matsui, Y., Matsushita, T., Sakuma, S., Gojo, T., Mamiya, T., Suzuoki, H., Inoue, T., 2003. Virus inactivation in aluminum and polyaluminum coagulation. Environ. Sci. Technol. 37, 5175–5180.

Matsushita, T., Matsui, Y., Inoue, T., 2004. Irreversible and reversible adhesions between virus particles and hydrolyzing-precipitating aluminum: a function of coagulation. Water Sci. Technol. 50 (12), 201–206.

Matsushita, T., Matsui, Y., Shirasaki, N., 2006. Analyzing mass balance of viruses in a coagulation-ceramic microfiltration hybrid system by a combination of the polymerase chain reaction (PCR) method and the plaque forming units (PFU) method. Water Sci. Technol. 53 (7), 199–207.

O'Connell, K.P., Bucher, J.R., Anderson, P.E., Cao, C.J., Khan, A.S., Gostomski, M.V., Valdes, J.J., 2006. Real-time fluorogenic reverse transcription-PCR assays for detection of bacteriophage MS2. Appl. Environ. Microbiol. 72, 478–483.

Shirasaki, N., Matsushita, T., Matsui, Y., Ohno, K., Kobuke, M., 2007. Virus removal in a hybrid coagulation–microfiltration system—Investigating mechanisms of virus removal by a combination of PCR and PFU methods. Water Sci. Technol.:. Water Supply 7 (5–6), 1–8.

Shirasaki, N., Matsushita, T., Matsui, Y., Kobuke, M., Ohno, K., 2009a. Comparison of removal performance of two surrogates for pathogenic waterborne viruses, bacteriophage Qβ and MS2, in a coagulation–ceramic microfiltration system. J. Membrane Sci. 326, 564–571.

Shirasaki, N., Matsushita, T., Matsui, Y., Urasaki, T., Ohno, K., 2009b. Comparison of behaviors of two surrogates for pathogenic waterborne viruses, bacteriophages Qβ and MS2, during the aluminum coagulation process. Water Res. 43, 605–612.

Tambo, N., Watanabe, Y., 1979. Physical characteristics of flocs—I. The floc density function and aluminum floc. Water Res. 13, 409–419.

Teunis, P.F.M., Moe, C.L., Liu, P., Miller, S.E., Lindesmith, L., Baric, R.S., Pendu, J.L., Calderon, R.L., 2008. Norwalk virus: how infectious is it? J. Med. Virol. 80, 1468–1476.

USEPA, Contaminant Candidate List 3 – CCL <http://water.epa.gov/scitech/drinkingwater/dws/ccl/ccl3.cfm.>.

Young, D.C., Sharp, D.G., 1977. Poliovirus aggregates and their survival in water. Appl. Environ. Microbiol. 33, 168–177.

[日本水処理生物学会誌 第47巻 第1号 9-18 2011]

環境水中のクリプトスポリジウム検出を目的としたRT-LAMP改善法の実用性評価

Validation for an Improved Reverse Transcription-loop-mediated Isothermal Amplification Assay for Detection of *Cryptosporidium* in Water Samples

猪又明子[1]，百田隆祥[2]，泉山信司[3]，勝山志乃[4]，岸田直裕[5]，秋葉道宏[5]，遠藤卓郎[6]

[1]東京都健康安全研究センター環境保健部／〒169-0073 東京都新宿区百人町3-24-1
[2]栄研化学株式会社生物化学研究所／〒329-0114 栃木県下都賀郡野木町大字野木143
[3]国立感染症研究所寄生動物部／〒162-8640 東京都新宿区戸山1-23-1
[4]神奈川県内広域水道企業団水質管理センター／〒243-0424 海老名市社家4587
[5]国立保健医療科学院水道工学部／〒351-0197 和光市南2-3-6
[6]国立感染症研究所細菌第一部／〒162-8640 東京都新宿区戸山1-23-1

AKIKO INOMATA[1], TAKAYOSHI MOMODA[2], SHINJI IZUMIYAMA[3], SHINO KATSUYAMA[4], NAOHIRO KISHIDA[5], MICHIHIRO AKIBA[5], and TAKURO ENDO[6]

[1]Department of Environmental Health and Toxicology, Tokyo Metropolitan Institute of Public Health / 3-24-1 Hyakunin-cho, Shinjuku, Tokyo 169-0073, Japan
[2]Biochemical Research Laboratory, Eiken Chemical Co.,LTD. / 143 Nogi, Nogimachi, Shimotsugagun, Tochigi 329-0114, Japan
[3]Department of Parasitology, National Institute of Infectious Diseases / 1-23-1 Toyama, Shinjuku, Tokyo 162-8640, Japan
[4]Water Quality Management Center, Kanagawa Water Supply Authority / 4587 Shake, Ebina, 243-0424, Japan
[5]Department of Water Supply Engineering, National Institute of Public Health / 2-3-6 Minami, Wako, 351-0197, Japan
[6]Department of Microbiology I , National Institute of Infectious Diseases / 1-23-1 Toyama, Shinjuku, Tokyo 162-8640, Japan

Abstract

An improved reverse transcription-loop-mediated isothermal amplification (RT-LAMP) assay which uses 1μL of RNA extract was evaluated. The original RT-LAMP assay using 5μL of RNA extract showed generally high-sensitive detection of *Cryptosporidium* in water samples; but in some types of water such as pigsty effluents and it's downstream river water, this assay failed to detect *Cryptosporidium*. This failure may be caused by substances in those water samples which block gene amplification. To eliminate the effect of gene amplification-blocking substances, we decreased the quantity of the RNA extract used for the RT-LAMP assay from 5μL to 1μL. The detection limit of the improved RT-LAMP assay was as low as 6×10^{-3} oocysts/LAMP test tube, which theoretically enables us to detect even one *Cryptosporidium* oocyst in a water sample. This improved RT-LAMP assay succeeded in detecting *Cryptosporidium* oocysts much more sensitively not only in pigsty effluents but also in other water samples as well.

1. はじめに

クリプトスポリジウムはヒトに感染すると下痢を主訴とするクリプトスポリジウム症を引き起こす原虫である。クリプトスポリジウム症に効果的な治療法はまだ知られておらず、免疫による自然治癒に依存するため、免疫不全者ではしばしば致死的となる[1,2]。

クリプトスポリジウムの汚染源は主に下水処理場や畜舎であり、これらの放流水が流入した水道原水中よりクリプトスポリジウムが適切な浄水処理で除去されないと、水道水に混入する恐れがある。水道水を介したクリプトスポリジウムの集団感染は1980年代から米国や英国で報告され始め、それ以降毎年報告されている[1,3,4]。わが国では1996年に埼玉県越生町で水道水を介した初めての集団感染が発生し、8,812人が感染する大事故となったことが知られている[5]。

水試料からのクリプトスポリジウムオーシストの主な検出方法は、厚生労働省の「水道における指標菌及びクリプトスポリジウム等の検査方法について[6]」に示されている蛍光抗体染色と微分干渉観察による検鏡法である。しかし、水試料中に存在する夾雑物の中から約5 μmと極めて微小なオーシストを顕微鏡で検出、同定するには、熟練した技術が必要である。また、検査に長時間を要し、多数の検体を迅速に検査することが困難であることから、正確で再現性が高く簡便な代替検査方法が求められている。このような条件を満たす検査方法として多数の遺伝子検査法が検討されているが、未だ実用化には至っていない[7]。

そうした中で、著者らはクリプトスポリジウムのrRNAを標的とした逆転写Loop-Mediated Isothermal Amplification (RT-LAMP) 法を開発し、環境水からのクリプトスポリジウム検出において検鏡法と同様か、あるいはより高感度な検出が可能であることを報告した[8]。しかし、その後様々な環境水についてRT-LAMP法検査を行ったところ、養豚場排水放流水及びその下流の河川水において、検鏡法でクリプトスポリジウムが検出されるにもかかわらずRT-LAMP法で陰性となることを経験した。この原因として、養豚場排水に含まれる強い遺伝子増幅阻害物質、すなわち糞便等の影響が考えられた。

糞便等は強力な遺伝子増幅阻害物質として知られており[9-11]、クリプトスポリジウム等は糞便中に排出されることから、糞便による阻害への対策が必須となる。その一方で、複雑な精製操作は試料のロスと煩雑な作業の増加を招く。そこで、RT-LAMP法の検出感度が6 $\times 10^{-3}$オーシスト相当/反応チューブと極めて高い[8]ことから、阻害物質の影響を回避するために、RT-LAMP反応に用いるRNA抽出液量を従来の5 μlから1 μlに低減するという、極めて単純だが効果的と考えられる改善策を試行し、その結果を評価した。

前提として、RNA抽出液量を従来の1/5量に減らしても十分に検出可能であることが求められるため、本研究で改めてRT-LAMP法の検出感度を確認することとした。次いで、問題の養豚場排水放流水及びその下流の河川水検体、その他の環境水（表流水、湧水、浅井戸水、伏流水）検体、浄水検体について、検鏡法、RT-LAMP従来法（RNA抽出液量5 μl）及びRT-LAMP改善法（RNA抽出液量1 μl）によりクリプトスポリジウム検出を行った。各検査法の結果を比較することでRT-LAMP改善法の実用性を評価したので報告する。

2. 材料と方法

2.1 RNA抽出方法

クリプトスポリジウムオーシストのRNA抽出は先の報告と同様に行った[8]。すなわち、*Cryptosporidium parvum*オーシストを-80℃のドライバス（Micro Cool MC-100, TOMY）と37℃のヒートブロック（Dry Bath Incubator, Fast Gene）で1分間ずつ5回の凍結融解を行い、次に溶解液（10mM Tris-EDTA (pH7.6), 20mM NaCl, 0.1% TritonX-100, 2 mM 1,4-Dithiothreitol, 1.5mAnson-U/ml Proteinase K）20 μlを添加し、60℃で30分間溶解反応を行った。その後2分間の超音波処理を行い、さらに75℃で10分間の追加反応を行った。このRNA抽出液を95℃で5分間加熱してProteinase Kを失活させた後、氷中で急冷した。

2.2 RT-LAMP法

RT-LAMP法の試薬にはLoopampクリプトスポリジウム検出試薬キット（栄研化学）を使用した[8]。試薬キットにはアウタープライマーCryF3及びCryB3各5 pmol、ループプライマーCryLF及びCryLB各20pmol、インナープライマーCryFIP及びCryBIP各40pmol、*Bst* polymerase 8Uが含まれている。これらのプライマーは*Cryptosporidium parvum, Cryptosporidium serpentis, Cryptosporidium andersoni, Cryptosporidium saurophilum*及び*Cryptosporidium hominis*の遺伝子を全て増幅し、一方*Giardia intestinalis, Giardia muris*及び細菌10菌種には反応し

ないことを確認している[12]。さらにReverse Transcriptase (Roche) 1.5Uを添加し、1本のチューブ内で逆転写反応とLAMP反応を実施した。RT-LAMP従来法ではRNA抽出液5 μl、RT-LAMP改善法ではRNA抽出液1 μlと蒸留水4 μlを20μlの増幅反応試薬に加えて計25μlとし、63℃で60分間RT-LAMP反応を行った。濁度の連続測定にはLoopampリアルタイム濁度測定装置 (LA-320C, 栄研化学) を用いた。陽性対照として試薬キットに付属のものを用いた。これは増幅産物のアガロースゲル電気泳動パターンがクリプトスポリジウムとは異なるように配列が設計されており、陽性対照のコンタミネーションを判別できるものとなっている。陰性対照にはキットに付属の純水を用いた。

2.3 RT-LAMP法の検出感度試験

*Cryptosporidium parvum*オーシスト (H8株) は、感染マウスの糞便からショ糖浮遊法及び塩化セシウム浮遊法により精製した[13]。血球計算盤で計数して所定濃度のオーシスト液を調製した後、前述の方法でオーシストからRNAを抽出した。この抽出RNAを感度試験用の鋳型として6×10^{-1}オーシスト相当/ 5 μlから6×10^{-4}オーシスト相当/ 5 μlまで10倍毎の連続希釈濃度に調製し、各濃度段階につき8連でRT-LAMP反応を行った。

2.4 検鏡法、RT-LAMP従来法、RT-LAMP改善法による水試料からのクリプトスポリジウム検出

養豚場排水放流水及びその下流の河川水10検体、その他の環境水 (表流水、湧水、浅井戸水、伏流水) 57検体、浄水13検体についてクリプトスポリジウム検査を行った。なお、これらの検体の一部には既に報告したものが含まれている[8]。養豚場排水放流水は10ml、養豚場下流の河川水は10l又は20l、その他の環境水は20l、浄水は40lを検水量とした。養豚場排水放流水は検水量が10mlと少ないことから、5連で試験を行うこととし、他は1試料ずつ行った。浄水は孔径1 μmのポリカーボネートフィルター、その他の試料は孔径5 μmのPTFEフィルターを用いてろ過濃縮を行った後、フィルター上に捕捉された懸濁物を剥離させて5 mlの濃縮液を得た。濃縮液の半量2.5mlをそれぞれ検鏡法とRNA抽出とに用いた。各検体は採水後冷蔵し、概ね3日以内に検鏡法による検査を行った。多くは検鏡法と同時に遺伝子検査用試料の精製を行ったが、養豚場下流河川水の一部の試料については冷蔵で1カ月保存した後に精製操作を行い、参考試料として使用した。

検鏡法は厚生労働省「水道における指標菌及びクリプトスポリジウム等の検査方法について」[6]に準拠し、一部改良を加えた方法で行った。すなわち、水試料濃縮液から免疫磁気ビーズ (Dynabeads GC Combo, Invitrogen) を用いてクリプトスポリジウムオーシストを捕捉、精製した後、塩酸により免疫磁気ビーズからクリプトスポリジウムを解離した。濃縮液の沈殿物容量が0.5mlを超える場合は、沈殿物量が0.5ml以下になるように試料を分けてから免疫磁気ビーズ法を行った。塩酸解離後のクリプトスポリジウム試料を水酸化ナトリウムで中和し、100℃で5分間加熱処理した。これを観察用PTFEフィルターでろ過し、直接蛍光抗体染色 (EasyStain, BTF) 及びDAPI (4',6-diamidino-2-phenylindole) 染色を行った。染色後のフィルターを封入してプレパラートを作成し、落射蛍光及び微分干渉観察により、クリプトスポリジウムの同定及び計数を行った。

遺伝子検査用試料として各濃縮液の残り半量2.5mlを用い、免疫磁気ビーズ法によりクリプトスポリジウムの精製を行った。上澄液を除去し、オーシストと免疫磁気ビーズとを結合させたまま前述の方法で核酸抽出した。直ちに抽出操作をしない場合は−20℃で保存した。

LAMP法で陽性となった増幅産物各1 μlについて、2%アガロースゲルによる電気泳動を行い、*Cryptosporidium parvum*オーシストのバンドパターンとの比較により、クリプトスポリジウム由来の増幅であることを確認した。

3. 結果及び考察

3.1 RT-LAMP法の検出感度

鋳型RNAを1反応あたり6×10^{-1}オーシストから6×10^{-4}オーシスト相当まで10倍毎に連続希釈し、8連でRT-LAMP法を行った結果をTable 1に示した。オーシスト濃度が低下するに従い、LAMP反応の陽性判定値 (移動平均の微分値が0.07) を越えるのに必要な時間である*Tt*値は大きくなった。鋳型RNA濃度6×10^{-4}オーシストでは8回の*Tt*値の変動係数 (CV値) が16.8%と大きく，6×10^{-3}オーシスト以上の濃度ではCV値0.5〜4.4%であったことから、再現性のある検出感度は6×10^{-3}オーシスト/LAMP test tubeと判断した。この検出感度は著者らが以前に各濃度段階2連で行った結果[8]と同じであった。この検出感度から、RT-LAMP改善法に使用する鋳型RNA量を20μl中の1 μlに減らした場合でも、検水中1オーシストを確実に検出できるものと判断した (1オーシスト/20μl = 5×10^{-2}オーシスト相当/1 μl)。

Table 1 *Tt* values of each diluted sample in the RT-LAMP assay.

	RNA concentration* (oocysts/LAMP test tube)			
	6×10^{-1}	6×10^{-2}	6×10^{-3}	6×10^{-4}
Tt value (min)	22.0	23.7	27.5	41.8
	22.0	24.1	25.2	42.3
	22.3	24.4	28.2	42.0
	22.3	24.0	28.7	33.3
	22.1	24.6	28.5	50.4
	22.2	24.6	26.3	39.4
	22.2	24.2	28.2	31.2
	22.2	24.3	27.5	50.4
Average (min)	22.2	24.2	27.5	41.4
Coefficient of variation (%)	0.5	1.3	4.4	16.8

*RNA concentration was converted to oocyst concentration in a LAMP test tube.

3.2 検鏡法、RT-LAMP従来法、RT-LAMP改善法による水試料からのクリプトスポリジウム検出

2008年1月～2010年2月に採取した養豚場排水放流水及びその下流の河川水、その他の環境水（表流水、湧水、浅井戸水、伏流水）、浄水について、検鏡法、RT-LAMP従来法、RT-LAMP改善法によりクリプトスポリジウム検出を行った。

問題の養豚場排水放流水及びその下流の河川水の結果をTable 2に示した。検鏡法の結果、養豚場A放流水では10オーシスト/5 m*l*、養豚場B放流水では7オーシスト/5 m*l*が検出された。RNA抽出は養豚場A、B放流水について、5 m*l*ずつ5回（A1～A5, B1～B5）行い、それぞれについてRT-LAMP従来法、RT-LAMP改善法でクリプトスポリジウム検査を行った。養豚場下流の河川水8試料については、濃縮液の沈殿物容量が0.5m*l*を超えた試料は2分割（S-1, S-2, 及びK-1, K-2）あるいは4分割（K-1, K-2, K-3, K-4）後にそれぞれ免疫磁気ビーズ精製とRNA抽出を行ったため、検鏡法によるオーシスト数を分割数で除した値を表に示した（例：2分割の場合、検鏡法で9オーシスト検出された試料は4.5オーシストと表記）。養豚場排水放流水及びその下流の河川水26試料（分割試料数を含む）中、RT-LAMP従来法で陽性となったのは7試料（陽性率26.9%）、RT-LAMP改善法で陽性となったのは20試料（陽性率76.9%）であった。26試料のうち、No.13とNo.16は、検鏡法でのオーシスト数がそれぞれ1，0.5と少ないことから、RNA抽出試料にオーシストが含まれなかった可能性が考えられた。検鏡法でのオーシスト数が2以上の23試料では、RT-LAMP従来法で7試料陽性（30.4%）であったのが、RT-LAMP改善法では19試料陽性（82.6%）となり、陽性率が著しく向上した。しかし、No.7の試料ではRT-LAMP従来法で陽性であったのがRT-LAMP改善法で陰性となった。この理由として、鋳型量が少ないために遺伝子増幅が遅れたり不安定になる等の不利な状況となったことや、試料を遺伝子検査に供するために凍結融解を繰り返したことによりRNAが分解した可能性が考えられた。

採水後1ヶ月間冷蔵保存された検体（Table 2 Sample No.18~25）を参考試料として試験した場合、RT-LAMP従来法では多くの試料が陰性となった。一方、RT-LAMP改善法では、分割試料の少なくとも1試料が陽性となったことから、保存中のRNAの分解だけでなく遺伝子増幅阻害の影響も考えられた。

これらの結果から、沈殿物量の多い試料については免疫磁気ビーズ処理時に試料を分割し、それぞれの試料についてRT-LAMP反応を実施することで、検水の陽性率を高めることができると考えられた。また、養豚場排水放流水A,BのRT-LAMP改善法が各5検体中1検体不検出であったことから、阻害が強いと考えられる検体についても磁気ビーズ処理時に複数に分割し、それぞれについてRT-LAMP反応を実施することが有効であると考えられた。検査に使える核酸が少ないことから、複数試料について検査することの必要性はSmith & Nichols[7]によっても指摘されている。なお、10*l*にわずか数オーシスト

Table 2 Detection results for *Cryptosporidium* oocysts in pigsty effluents and it's downstream river water by conventional microscopic observation, original RT-LAMP and improved RT-LAMP

Sample No.	Date of sample collection	Sampling site	Water type	Water volume	Microscopic observation (number of oocysts)	Original RT-LAMP*1	Improved RT-LAMP*2
1	08Jan.2008	Pigsty A1	PE*3	5m*l*	10	−*5	+*6
2		Pigsty A2	PE	5m*l*	10	−	+
3		Pigsty A3	PE	5m*l*	10	−	+
4		Pigsty A4	PE	5m*l*	10	−	−
5		Pigsty A5	PE	5m*l*	10	−	+
6		Pigsty B1	PE	5m*l*	7	+	+
7		Pigsty B2	PE	5m*l*	7	+	−
8		Pigsty B3	PE	5m*l*	7	+	+
9		Pigsty B4	PE	5m*l*	7	+	+
10		Pigsty B5	PE	5m*l*	7	+	+
11	27Feb.2008	S-1	RW*4	5*l*/2	2	+	+
12		S-2	RW	5*l*/2	2	−	+
13		N	RW	5*l*	1	−	−
14		K-1	RW	5*l*/2	4.5	−	+
15		K-2	RW	5*l*/2	4.5	−	+
16	09Feb.2009	S-1	RW	10*l*/2	0.5	−	−
17		S-2	RW	10*l*/2	0.5	−	+
18*7	12Mar.2009	K-1	RW	10*l*/4	133	−	+
19*7	9:00	K-2	RW	10*l*/4	133	−	+
20*7		K-3	RW	10*l*/4	133	−	+
21*7		K-4	RW	10*l*/4	133	−	+
22*7	12Mar.2009	K-1	RW	10*l*/2	200	+	+
23*7	12:00	K-2	RW	10*l*/2	200	−	−
24*7	12Mar.2009	K-1	RW	10*l*/2	55	−	+
25*7	15:00	K-2	RW	10*l*/2	55	−	−
26	07Apr.2009	K	RW	10*l*	3	−	+
Number of total samples (including divided samples)					18 (26)		
Number of positive samples (including divided samples)					−	7 (7)	15 (20)
Positive rate (including divided samples) (%)					−	38.9 (26.9)	83.3 (76.9)

*1 Original RT-LAMP assay was performed using 5μl of *Cryptosporidium* RNA extract.
*2 Improved RT-LAMP assay was performed using 1μl of *Cryptosporidium* RNA extract.
*3 PE : pigsty effluent
*4 RW : river water
*5 − : negative
*6 + : positive
*7 *Cryptosporidium* RNA was extracted after 1 month conservation at 4℃.

の検出を求められることから確率論的なばらつきは避けられず、このようなばらつきは経験的に検鏡法と大差ないと考えられた。

その他の表流水の結果をTable 3に示した。44試料中検鏡法で陽性となったのは4検体（陽性率9.1%）のみであったが、RT-LAMP従来法では9検体（陽性率20.5%）、RT-LAMP改善法では11検体（陽性率25.0%）が陽性となった。RT-LAMP従来法が検鏡法よりも高感度であることは、われわれの既報[8]の結果と同様であるが、RT-LAMP改善法は従来法よりも若干高感度であった。糞便汚染さ

Table 3 Detection results for *Cryptosporidium* oocysts in surface water by conventional microscopic observation, original RT-LAMP and improved RT-LAMP

Sample No.	Date of sample collection	Sampling site	Water volume	Microscopic observation (number of oocysts)	Original RT-LAMP[*1]	Improved RT-LAMP[*2]
1	01Oct.2008	OCO-1	10l/3	0	+[*3]	+
2		OCO-2	10l/3	0	+	+
3		OCO-3	10l/3	0	−[*4]	−
4		OHO-1	10l/2	0	−	−
5		OHO-2	10l/2	0	−	−
6	08Oct.2008	MT	10l	0	−	+
7		OHK	10l	0	−	−
8		OHM	10l	0	−	−
9		OOG	10l	0	−	−
10	09Oct.2008	AM	10l	0	−	−
11		HO	10l	0	−	−
12		TK	10l	0	−	−
13		OHM	10l	0	−	−
14		OOT	10l	0	−	−
15		OTS	10l	0	−	−
16		ONP	10l	0	−	−
17	04Nov.2008	HIK	10l	0	−	−
18		HIM	10l	0	−	−
19	26Nov.2008	IKN	10l	0	+	+
20		IJH	10l	0	+	−
21		IYR	10l	1	+	+
22	01Dic.2008	YRS	10l	0	+	+
23		YR1,2	10l	0	+	+
24		YR3	10l	1	+	+
25		YR4	10l	0	−	−
26		IDY	10l	0	−	−
27	27Jan.2009	INY	10l	0	−	−
28		ITN	10l	0	−	−
29	24Feb.2009	IKN	10l	1	+	+
30	25May 2009	IKN-1	10l/3	0	−	−
31		IKN-2	10l/3	0	−	−
32		IKN-3	10l/3	0	−	−
33	10Jun.2009	IKN-1	10l/2	0	−	−
34		IKN-2	10l/2	0	−	−
35	07Oct.2009	AM	10l	0	−	−
36	14Oct.2009	MT	10l	0	−	−
37	15Oct.2009	HO	10l	0	−	−
38	20Oct.2009	OCO	10l	0	−	−
39		OHO	10l	0	+	+
40		OOT	10l	0	−	−

Sample No.	Date of sample collection	Sampling site	Water volume	Microscopic observation (number of oocysts)	Original RT-LAMP*1	Improved RT-LAMP*2
41		OTS	10ℓ	0	−	−
42		ONP	10ℓ	0	−	−
43	29Oct.2009	TK	10ℓ	0	−	−
44	04Nov.2009	HIK	10ℓ	0	−	−
45		HIM	10ℓ	0	−	−
46	10Nov.2009	OHK	10ℓ	0	−	−
47		OHM	10ℓ	0	−	−
48		OOG	10ℓ	0	−	−
49		IKN	10ℓ	1	−	+
50	09Feb.2010	IKN	10ℓ	0	−	+
Number of total samples (including divided samples)				44 (50)		
Number of positive samples (including divided samples)				4 (4)	9 (10)	11 (12)
Positive rate (including divided samples) (%)				9.1 (8.0)	20.5 (20.0)	25.0 (24.0)

*1 Original RT-LAMP assay was performed using 5μl of *Cryptosporidium* RNA extract.
*2 Improved RT-LAMP assay was performed using 1μl of *Cryptosporidium* RNA extract.
*3 + : positive
*4 − : negative

れた試料に比べると、表流水の汚染は大きく希釈されており、阻害物質の存在量が少ないことから、改善効果が少なくなったものと考えられた。しかし、RT-LAMP改善法によって表流水中の阻害物質の影響が緩和されたことは変わりない。No.20の試料のみ、RT-LAMP従来法で陽性であったのがRT-LAMP改善法で陰性となった。この試料は検鏡法でクリプトスポリジウムが不検出であり、存在するオーシスト数が極めて少ない、あるいは存在するオーシストが壊れる途中で核酸が不安定であったと考えられた。

湧水（9検体）、浅井戸水（4検体）、伏流水（1検体）、浄水（13検体）からは、いずれの方法によってもクリプトスポリジウムは検出されなかった（Table 4）。これらの検体はクリプトスポリジウム汚染がほとんどないと考えられることから、RT-LAMP反応に偽陽性は生じていないと考えられた。

RT-LAMP法で陽性となった増幅産物のアガロースゲル電気泳動を行い、増幅産物が*Cryptosporidium parvum*と同一のバンドパターンであることを確認した。泳動結果の一部をFig. 1（RT-LAMP従来法）及びFig.2（RT-LAMP改善法）に示した。

以上より、一連の増幅結果に偽陽性はなかったと判断した。RT-LAMP改善法は養豚場排水放流水だけでなく、環境水全般についてRT-LAMP従来法よりも検出感度が高く、これまでの検討では偽陽性も認められなかった。本法は検査に使用するRNA抽出液量を減らすだけで阻害が回避できる非常に簡便な改善法であり、検鏡法の結果との対比も得られていることから、RT-LAMP改善法の実用性は十分にあると判断した。本研究に用いた環境水及び検討した核酸抽出方法は限られていることから、検査対象水の阻害物質濃度とクリプトスポリジウム濃度、用いる核酸抽出方法が変われば、最適な核酸抽出液量は変化するものと考えられる。本研究で示した検鏡法との比較のみならず、さらなる核酸抽出法の最適化と、異なる配列を標的とした複数の遺伝子解析法を併用することにより、遺伝子検査法の信頼性の向上が図れると考えられる。顕微鏡による形態観察と遺伝子増幅による標的配列の検出とは原理が異なることから、必ずしも1：1対応はしないものの、遺伝子検査法はクリプトスポリジウム汚染の有無を明らかにする試験目的には十分に有用であると考えられた。今後、多くの検査機関においてRT-LAMP改善法を実施し、評価されることを望む。

4. まとめ

検水中の遺伝子増幅阻害物質の影響を除去するために、RT-LAMP反応に用いるRNA抽出液量を従来の5μlから1μlに低減した改善法を検討し、以下の知見を得た。

1）RT-LAMPの検出感度は6×10^{-3}オーシスト相当/RT-

Table 4 Detection results for *Cryptosporidium* oocysts in finished water, spring water, shallow well water and river-bed water by conventional microscopic observation, original RT-LAMP and improved RT-LAMP

Sample No.	Date of sample collection	Sampling site	Water type	Water volume	Microscopic observation (number of oocysts)	Original RT-LAMP[*1]	Improved RT-LAMP[*2]
1	01Dic.2008	YRS	FW[*3]	20ℓ	0	−[*7]	−
2	24Feb.2009	HBT	FW	20ℓ	0	−	−
3		OKD	FW	20ℓ	0	−	−
4		SEY	FW	20ℓ	0	−	−
5	23Aug.2009	HBT	FW	20ℓ	0	−	−
6		OKD	FW	20ℓ	0	−	−
7		SEY	FW	20ℓ	0	−	−
8	01Dic.2009	OKD	FW	20ℓ	0	−	−
9		SEY	FW	20ℓ	0	−	−
10		YMZ	FW	20ℓ	0	−	−
11	09Feb.2010	HBT	FW	20ℓ	0	−	−
12		OKD	FW	20ℓ	0	−	−
13		SEY	FW	20ℓ	0	−	−
14	15Oct.2008	BRW	SP[*4]	10ℓ	0	−	−
15		SNT	SP	10ℓ	0	−	−
16		OGG	SP	10ℓ	0	−	−
17	22Oct.2008	SND	SP	10ℓ	0	−	−
18	14Oct.2009	SNT	SP	10ℓ	0	−	−
19		NKN	SP	10ℓ	0	−	−
20		BRW	SP	10ℓ	0	−	−
21	15Oct.2009	OGG	SP	10ℓ	0	−	−
22	21 Oct.2009	SND	SP	10ℓ	0	−	−
23	09Dic.2008	OH1	SW[*5]	10ℓ	0	−	−
24		OH2	SW	10ℓ	0	−	−
25	24Feb.2009	OH1	SW	10ℓ	0	−	−
26		OH2	SW	10ℓ	0	−	−
27	01Dic.2008	AMZ	RB[*6]	10ℓ	0	−	−
Number of total samples				27			
Number of positive samples				0	0	0	
Positive rate (%)				0	0	0	

[*1] Original RT-LAMP assay was performed using 5μℓ of *Cryptosporidium* RNA extract.
[*2] Improved RT-LAMP assay was performed using 1μℓ of *Cryptosporidium* RNA extract.
[*3] FW : Finished water
[*4] SP : Spring water
[*5] SW : Shallow well water
[*6] RB : River-bed water
[*7] − : negative

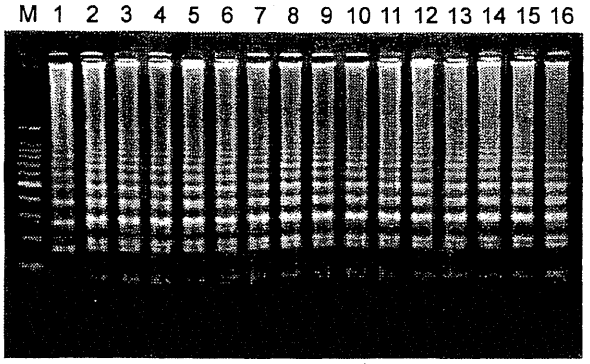


Fig. 1 Analysis of original RT-LAMP products of positive samples by agarose gel electrophoresis.

M : 100bp DNA ladder
1 : Positive control for LAMP reaction[a]
2 : Positive control for electrophoresis[b]
3 : Table 3 Sample No.1
4 : Table 3 Sample No. 2
5 : Table 3 Sample No.19
6 : Table 3 Sample No.20
7 : Table 3 Sample No.21
8 : Table 3 Sample No.22
9 : Table 3 Sample No.23
10 : Table 3 Sample No.24
11 : Table 2 Sample No.6
12 : Table 2 Sample No.7
13 : Table 2 Sample No.8
14 : Table 2 Sample No.9
15 : Table 2 Sample No.10
16 : Table 2 Sample No.11

[a]Positive control for the RT-LAMP assay made of RNA with an artificial sequence. The electrophoretic pattern of this positive control was different from that of *Cryptosporidium parvum* oocysts. If the electrophoretic pattern of samples was the same as lane 1, it was an evidence of contamination by the positive control.
[b]Positive control for electrophoresis which derives from nucleic acid of *Cryptosporidium parvum* oocysts. This positive control was not used in LAMP reactions.

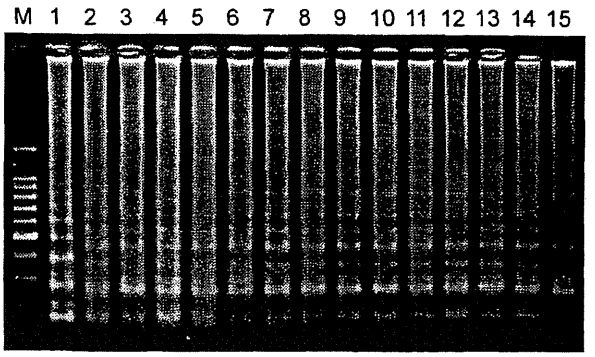


Fig. 2 Analysis of improved RT-LAMP products of positive samples by agarose gel electrophoresis.

M : 100bp DNA ladder
1 : Positive control for LAMP reaction[a]
2 : Positive control for electrophoresis[b]
3 : Table 2 Sample No.1
4 : Table 2 Sample No.2
5 : Table 2 Sample No.3
6 : Table 2 Sample No.9
7 : Table 2 Sample No.10
8 : Table 2 Sample No.11
9 : Table 2 Sample No.12
10 : Table 2 Sample No.17
11 : Table 2 Sample No.22
12 : Table 3 Sample No.19
13 : Table 3 Sample No.29
14 : Table 3 Sample No.39
15 : Table 3 Sample No.50

[a]Positive control for the RT-LAMP assay made of RNA with an artificial sequence. The electrophoretic pattern of this positive control was different from that of *Cryptosporidium parvum* oocysts. If the electrophoretic pattern of samples was the same as lane 1, it was an evidence of contamination by the positive control
[b]Positive control for electrophoresis which derives from nucleic acid of *Cryptosporidium parvum* oocysts. This positive control was not used in LAMP reactions.

LAMP反応であった。この検出感度から、RNA抽出液20 μl中の鋳型RNA液1 μlで1オーシストが検出可能と判断した（1オーシスト/20μl = 5 $\times 10^{-2}$オーシスト相当/1 μl）。

2）RT-LAMP改善法は、検査した養豚場排水放流水、その下流の河川水、その他の表流水全てにおいて、RT-LAMP従来法よりも高感度であり、偽陽性も認められなかった。養豚場排水放流水やその下流の河川水のように阻害が強いと考えられる検体では、検体を複数に分割し、それぞれの試料についてRT-LAMP改善法を実施することで、陽性率を高めることが可能であった。

謝 辞

本研究は平成19～21年度の厚生労働省科学研究費補助金「健康安全・危機管理対策総合研究事業　飲料水の水質リスク管理に関する統合的研究」（H19-健危-一般-012）の補助を受けて実施した。

参考文献

1）金子光美：水道の病原微生物対策，丸善，東京（2006）
2）Guillot, E. and Loret, J. F. : Cryptosporidium. Waterborne pathogens: Review for the drinking water industry, IWA Publishing, London UK (2010)
3）Yoder, J. S., Harral, C., and Beach, M. J. :

Cryptosporidiosis surveillance -- United Sates, 2006 -- 2008, MMRW, Vol.59, No.SS-6 (2010)
4) Smith, A., Reacher, M., Smerdon, W., Adak, G. K., Nichols, G., and Chalmers, R. M. : Review article: Outbreaks of water borne infectious intestinal disease in England and Wales, 1992-2003, Epidemiol. Infect., Vol.134, 1141-1149 (2006)
5) 埼玉県衛生部 :クリプトスポリジウムによる集団下痢症—越生町集団下痢症発生事件—報告書 (1997)
6) 厚生労働省健康局水道課長通知：水道における指標菌及びクリプトスポリジウム等の検査方法について 平成19年3月30日付健水発第0330006号 (2007)
7) Smith, H.V. and Nichols, R.A. : *Cryptosporidium*: detection in water and food, Exp Parasitol. , Vol. 124 (1), 61-79 (2010)
8) Inomata, A., Kishida, N., Momoda, T., Akiba, M., Izumiyama, S., Yagita, K., and Endo, T. : Development and evaluation of a reverse transcription-loop-mediated isothermal amplification assay for rapid and high-sensitive detection of *Cryptosporidium* in water samples, Water Sci. Technol., Vol.60, 2167-2172 (2009)
9) Lantz, P.G., Matsson, M., Wadstrom, T., and Radstrom, P. : Removal of PCR inhibitors from human faecal samples through the use of an aqueous two-phase system for sample preparation prior to PCR, Journal of Microbiological Methods, Vol. 28, 159-167 (1997)
10) Lund, M., Nordentoft, S., Pedersen, K., and Madsen, M. : Detection of *Campylobacter* spp. in chicken fecal samples by real-time PCR, Journal of Clinical Microbiology, Vol. 42, 5125-5132 (2004)
11) Kreader, C. A.: Relief of amplification inhibition in PCR with bovine serum albumin or T4 gene 32 protein, Applied and Environmental Microbiology, Vol. 62, 1102-1106 (1996)
12) 百田隆祥, 小島禎, 池戸正成, 泉山信司, 遠藤卓郎: LAMP法 (Loop-Mediated Isothermal Amplification) を用いたクリプトスポリジウム及びジアルジアの高感度迅速検出, 水環境学会誌. Vol.32, 321-324 (2009)
13) Yagita, K., Izumiyama, S., Tachibana, H., Masuda, G., Iseki, M., Furuya, K., Kameoka, Y., Kuroki, T., Itagaki, T., and Endo, T. : Molecular characterization of *Cryptosporidium* isolates obtained from human and bovine infections in Japan, Parasitology Research. Vol.87, 950-955 (2001)

(受付 2010.8.23)
(受理 2010.11.22)