performance was similar to that of the mechanical coagulation–ceramic MF system. The in-line mixing system has potential as a useful pretreatment for viruses as an alternative to the mechanical mixing system for coagulation. Replacement of the mechanical mixing system with the in-line mixing system will reduce footprint, because there is no requirement for mixing/settling tanks for coagulation.

2. Coagulant dose and coagulation time were important factors controlling the virus removal performance of the in-line coagulation–ceramic MF system: a coagulant dose of 1.08 mg-Al/L and a coagulation time of 1 min were required to achieve high ratios of virus removal, >8.2-log for infectious and >5.4-log for total viruses, which satisfy the USEPA requirement of a 4-log removal/inactivation.
3. The removal performances of the coagulation–ceramic MF process for total Qβ and MS2 were different: the removal ratio for total MS2 was higher than that for total Qβ under all tested coagulation conditions, which was most likely the result of differences between Qβ and MS2 in their interaction with the cake layer.

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リアルタイムRT-PCR法を用いた河川試料水中のクリプトスポリジウムの高感度定量

High-Sensitive Quantification of *Cryptosporidium* in River Water Samples Using a Real-time Reverse Transcription-Polymerase Chain Reaction

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Abstract

Real-time reverse transcription-polymerase chain reaction (RT-PCR) was applied to the detection of *Cryptosporidium* oocysts. Firstly, the sensitivity and specificity of three primer pairs were compared, and the primer pair reported by Miller *et al.* (2006) was considered to be the most suitable one. Secondly, a reverse transcription reaction was added to the real-time PCR assay for increase in sensitivity. The real-time RT-PCR assay revealed that one oocyst contains the 27,400 copies of rRNA, and the quantification limit of the assay was as low as 7.5×10^{-4} oocysts/test tube, while that of normal real-time PCR assay was as low as 2.4×10^{-1} oocysts/test tube. Therefore, the *Cryptosporidium* oocysts can theoretically be detected in reproducible tests using the real-time RT-PCR assay even in water samples containing only one oocyst. Thirdly, the developed real-time RT-PCR assay was applied to detect *Cryptosporidium* oocysts in 14 real river water samples. The concentration was quantified by the real-time RT-PCR assay, and was correlated to the suspected value, but not to the confirmed value, determined by conventional microscopic observation.

Key words: Cryptosporidiosis, *Cryptosporidium*, oocyst, real-time PCR, real-time RT-PCR

1. はじめに

クリプトスポリジウムは温帯・熱帯問わず世界中に広く分布しており、種々の動物の消化管に寄生し、ヒトに重篤な下痢症を引き起こす原虫として知られている[1,2]。また、本原虫のオーシストは浄水処理で用いられる塩素等の消毒剤に対して高い抵抗性を持つために[3,4]、しばしば水道を介した集団下痢症が発生し問題とされている。最も大規模な水系集団感染は、1993年に米国Wisconsin州Milwaukee市で発生した事例であり、推定感染者数40万人以上、確定患者数5千人以上と報告されている[5-7]。我が国では1996年に埼玉県越生町の町民約7割が感染したとされる大規模な集団感染が発生している[8]。近年では、2007年にアイルランドのGalway市にて182人の患者発生が報告されており[9]、クリプトスポリジウムによる水系集団感染は、先進国においても依然として深刻な問題の一つとなっている。このような背景から、浄水処理の徹底、汚染状況の調査が重要視されている。

現在、クリプトスポリジウムオーシスト（以下、オーシストという）の検査で主流となっているのは顕微鏡観察法であり、平成19年3月に厚生労働省健康局水道課より通知された試験法[10]の中でも採用されているが、本手法は技術の習得に多大な時間を要し、また顕微鏡観察はオーシストの判定において個人差が生じる恐れも考えられることから、迅速に検出可能で分析者の負担が少なく、かつ再現性の高い検出法の導入が強く求められている。このような特徴を満たす検査法として、近年注目を集めているのが遺伝子検査法であり、平成22年3月に開催された「水道における微生物問題検討会（厚生労働省健康局水道課水質管理室 主催）」においても、クリプトスポリジウムの検査法が議題に上がり、遺伝子検査法の導入について議論されている。これまでにオーシストの定量を可能とするリアルタイムPCRの検出系は複数報告されているが[11-13]、実用性に関する比較検討はほとんど実施されていない。また、糞便等の臨床試料と異なり、水環境中ではオーシストの存在は低濃度で、10ℓ中にわずか1つのオーシストを検出しなければならないので、感度の問題が無視できない。

そこで本研究では、クリプトスポリジウムの実用的な定量検査系を確立することを目的とした。はじめに、これまでに報告された中から3つのリアルタイムPCR系に着目し、感度や特異性等の比較を行った。次に、最良のリアルタイムPCR系に逆転写反応（RT: Reverse transcription）を組み合わせたリアルタイムRT-PCR法を考案し、定量感度の向上を試みた。rRNAを検出対象とした場合にRT反応による感度向上があることを経験しており[14]、当該研究でも実施を試みた。最後に、構築したリアルタイムRT-PCR法を水環境試料中のオーシスト濃度の測定に適用し、顕微鏡観察を基本とする従来法と比較することで、構築した検査系の実用性を評価した。

2. 材料および方法

2.1 試料

後述するリアルタイムPCR系の比較試験およびリアルタイムRT-PCR法の感度試験には、感染マウスの糞便より精製した*Cryptosporidium parvum*オーシスト（H8株）を用いた[15]。マウスの糞便をショ糖浮遊法及び塩化セシウム浮遊法により精製した後、オーシスト濃度を血球計算盤計数で求めた。希釈調製した所定濃度のオーシスト液から核酸を抽出し、鋳型として用いた。この鋳型は、河川水試料からの検出の際の標準試料としても使用した。

リアルタイムRT-PCR法による河川試料からのオーシストの検出には、利根川水系の河川水試料を用いた。平成21年11月および12月に利根川本川および支川の7地点より各2試料、計14試料の河川水を採取した。親水性PTFEメンブレンフィルター法[10]によって、河川水10ℓを約10mℓまで濃縮した後、免疫磁気ビーズ法[10]による精製・濃縮操作を行い、約110μℓまで濃縮した。濃縮試料の半量（約55μℓ）をリアルタイムRT-PCR法、RT-LAMP（loop-mediated isothermal amplification）法[14]に供し、残りの半量を顕微鏡検査に供した。

2.2 核酸抽出方法

マウスの糞便から精製したオーシストおよび河川水試料からの核酸（DNAおよびRNA）の抽出は、−80℃と室温での凍結融解を5回行い、次に溶解液を添加し（反応チューブ内溶解液終濃度：10mM Tris-HCl（tris-hydroxy methyl-aminomethane, pH7.6），1mM EDTA（ethylene diaminetetraacetic acid），20mM NaCl，0.1%（w/v）TritonX-100，2mM DTT（dithiothreitol），1.5mAnson-U/ml Proteinase K）、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。この核酸抽出液を95℃で5分間加熱し、Proteinase Kを失活させた後、氷中で急冷した。

2.3 リアルタイムPCR法

本研究では、Table 1に示す3つのリアルタイムPCR系を検討対象とした。プライマー濃度、プローブ濃度、PCR条件等の実験条件は既報に従った[11-13]。また、リアルタイムPCR装置としてLightCycler® (Roche) を用いた。蛍光曲線の立ち上がり時間 (Crossing Point：Cp値) は、リアルタイムPCR装置に付属するソフトウェアを用いてSecond derivative maximum法 (2次微分最大値法) で解析した。

2.4 リアルタイムRT-PCR法

本研究では、逆転写反応とリアルタイムPCR法を別々に行う2ステップのリアルタイムRT-PCRを実施した。逆転写反応には、PrimeScript® RT reagent Kit (Perfect Real Time; Takara) を用いた。逆転写プライマーにはTable 1に示したReverseプライマーを用い、反応チューブ内濃度を2.5μMに調整した。サーマルサイクラーを用いて37℃、15分間の逆転写反応を実施した後、85℃で5秒間加熱し、酵素を失活させた。逆転写後の試料は方法2.3のリアルタイムPCR法と同様の操作で、遺伝子定量を行った。

2.5 複数のリアルタイムPCR系の比較試験

3つのリアルタイムPCR系の比較試験には、鋳型を2.4×10^{-1}～2.4×10^{3} oocysts/tubeまで10倍毎の連続希釈濃度で調製し、各濃度段階につき2連でリアルタイムPCR法を実施した。PCR増幅産物は、1.2%アガロースゲルを用いた電気泳動解析に供した。

2.6 オーシスト内部のrRNA遺伝子数の測定方法およびリアルタイムRT-PCR法の感度試験

オーシスト内部のrRNA標的配列のコピー数は、上述の精製オーシストより、人工合成遺伝子を標準試料として測定した。標準試料の人工合成遺伝子は、標的遺伝子配列 (Accession No.: AF161856の187-378まで) を合成し (つくばオリゴサービス (株))、バイオアナライザ (Agilent, 2100 bioanalyzer) を用いて濃度を測定した後、希釈調整した。10～10^{5} copies/tubeに調製した人工合成遺伝子を用いて検量線を作成し、オーシストからの核酸抽出液に対しリアルタイムRT-PCR法を実施することで、オーシスト内部のrRNA遺伝子数を測定した。鋳型を7.5×10^{-4}～7.5×10^{-2} oocysts/tubeの範囲で10倍毎の連続希釈濃度で調製し、各濃度段階につき3連で実施し (計9回)、1オーシストあたりの平均のrRNA遺伝子数を測定した。

また、リアルタイムRT-PCR法の感度試験は、7.5×10^{-6}～7.5×10^{-1} oocysts/tubeの範囲で各濃度段階につき2連でリアルタイムRT-PCR法を実施した。

2.7 リアルタイムRT-PCR法と顕微鏡観察法による河川試料からの検出

濃縮・精製後の河川試料のうち半量に対し、核酸抽出を実施した後、構築したリアルタイムRT-PCR法を用い

Table 1 Nucleotide sequences of PCR primers and TaqMan probes used to quantify Cryptosporidium spp.

Amplification target	Function	Sequence (From 5' to 3') [a]	Length of PCR product	Reference
18S rRNA	Forward primer	AGTGACAAGAAATAACAATACAGG	295	Keegan *et al.*, 2003[11]
	Reverse primer	CCTGCTTTAAGCACTCTAATTTTC		
	TaqMan probe	ACCAGACTTGCCCTCC		
18S rRNA	Forward primer	AGTGACAAGAAATAACAATACAGG	295	King *et al.*, 2005[12]
	Reverse primer	CCTGCTTTAAGCACTCTAATTTTC		
	TaqMan probe	AAGTCTGGTGCCAGCAGCCGC		
18S rRNA	Forward primer	GGAAGGGTTGTATTTATTAGATAAAGAACCA	182	Miller *et al.*, 2006[13]
	Reverse primer	CATTCAAGTTTCTGACCTATCAGCTTTAGACGG		
	TaqMan probe	CTCCCTCTCCGGAATCGAA		

[a] TaqMan probe oligonucleotides were labeled with 6-FAM at the 5'-end and the quencher (TAMRA or BHQ-1) at the 3'-end.

て三連で試験を行った。また、特異性を確認するため、異なるプライマーで別の領域（Accession No.: L16996の413-611まで）を増幅するRT-LAMP法[4]を同時に実施した。本法では、リアルタイムPCR法と同様に18S rRNA遺伝子を対象としているが、増幅される部位が大きく異なっている。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。試薬キットには各5pmolのアウタープライマーCryF3及びCryB3、各20pmolのループプライマーCryLF及びCryLB、各40pmolのインナープライマーCryFIP及びCryBIP、8Uの*Bst* polymeraseが含まれている。さらに1.5UのReverse Transcriptaseを添加し、これにRNA抽出液5μlを加えて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（Realoop-30、モリテックス）を用いた。

顕微鏡観察用の試料は、定法[10]に従い、親水性PTFEメンブレンフィルターを用いて吸引ろ過し、染色操作を行った。すなわちはじめに、メンブレンフィルターの中央に撥水ペンで直径約15mmの円を描き、PBS（Phosphate Buffer Saline）で濡らした後、約200μlのブロッキング試薬（10%ウシ血清アルブミン添加PBS溶液）をメンブレンフィルターの円内全面に行き渡るように滴下した。室温で約5分間作用させた後、残ったブロッキング試薬を吸引除去した。次に、濃縮・精製後の河川試料のうち、半量（約55μl）を円内全面に行き渡るように吸引ろ過した後、約200μlのブロッキング試薬を滴下した。室温で約5分間作用させた後、吸引除去した。その後、メンブレンフィルターをスライドグラス上に移し、湿箱に入れ、60μlの蛍光抗体染色液（EasyStain™、BTF）を円内全面に行き渡るように滴下し、室温で25分間反応させた。さらにDAPI（4',6-diamidino-2-phenylindole）染色液（0.4mg/l）100μlを滴下し、5分間反応させた後、吸引ろ過し、PBS約10mlを用いてろ過洗浄した。最後に、スライドグラス上にメンブレンフィルターを載せ、市販の蛍光試料用水性封入剤（Fluoprep、BioMerieux）を用いて封入処理を行い、プレパラートを作製した。

作製したプレパラートを微分干渉装置付き倒立型蛍光顕微鏡（IX71、OLYMPUS）により観察した。はじめに、B励起光下200倍で観察し、アップルグリーン色の蛍光を発する4～6μmの類円形の蛍光粒子を探索した。G励起光下で赤色の蛍光を発していないことを確認した粒子は、UV励起光下1000倍で観察し、スポロゾイトの核の有無を観察した。さらに1000倍の微分干渉像でオーシストの内部構造の有無を観察した。蛍光観察によってオーシスト壁のみ観察できたものを推定数（Suspected）とし、さらに核やその他のクリプトスポリジウムオーシストに特徴的な内部構造が観察されたものを確定数（Confirmed）とした。

3. 結果および考察

3.1 複数のリアルタイムPCR系の比較

検討した3つのリアルタイムPCR系のいずれにおいても、2.4×10^{-1}～2.4×10^{3} oocysts/tubeの範囲で蛍光曲線の立ち上がりが確認され、初期オーシスト濃度（Log値）とCp値との間に良好な線形関係が見られた（Fig. 1）。一方、2.4×10^{-2} oocysts/tube以下の濃度では、蛍光曲線の立ち上がりが確認されなかった。他の2系と比較すると、Millerら[13]のPCR系は試料毎の変動が少なかった。また、アガロース電気泳動で増幅産物を確認すると、Keeganら[11]とKingら[12]のPCR系では、プライマーダイマーあるいは未反応のプライマーが低分子の領域に認められ（Fig. 2）、効率の良い増幅が行われているか疑問が持たれた。一方、Millerらの系[13]においては、このようなバンドは確認されなかった。また、蛍光曲線の立ち上がり時の傾きが大きく、少ないサイクルで飽和に達し、増幅と検出の効率が比較的高いことが推測された。さらに、PCR終了時の蛍光強度は試料間の変動が小さく強度が揃っており、初期濃度に依存することなく反応が最後まで進行していることが示唆された。特に低濃度のオーシストからの増幅が他の2系と比較すると良好であった。これらのことから、比較した3つのPCR系の中では、Millerらの系が良好と判断された。

3.2 オーシスト内部のrRNA遺伝子数とリアルタイムRT-PCR法の感度試験結果

良好と判断されたMillerらのリアルタイムPCR系を使用して、濃度既知の合成遺伝子を使った検量線を作成し、一方、濃度調整したオーシスト抽出試料に対してはリアルタイムRT-PCRを行い、オーシスト内のrRNAから逆転写反応で得られるcDNAを定量した。その結果、1オーシストあたり約27,400コピー（n=9，標準偏差=1,930）の標的配列、すなわちrRNAが存在することが判明した。オーシスト内のrDNA遺伝子数は20コピー（=ゲノム上5コピー×オーシスト内4スポロゾイト）と計算されるが[16]、逆転写反応を追加してオーシスト内部のrRNAをcDNAに変換することで、標的のコピー数を大幅に増大させ、高感度化が可能であることを改めて確認し

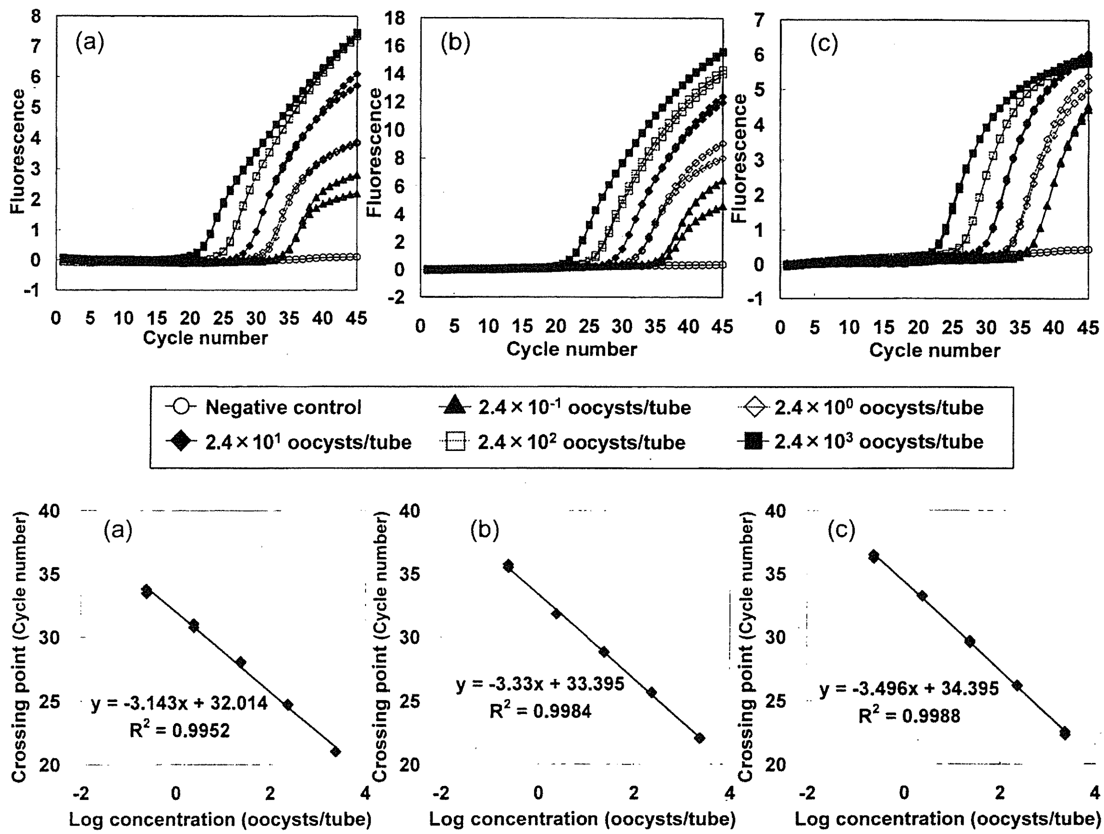

Fig. 1 Amplification plots and standard curves of the real-time PCR assays reported by (a) Keegan *et al.*[11], (b) King *et al.*[12], (c) Miller *et al.*[13] using 10-fold serial dilution of template DNA extracted from *Cryptosporidium parvum* oocysts.

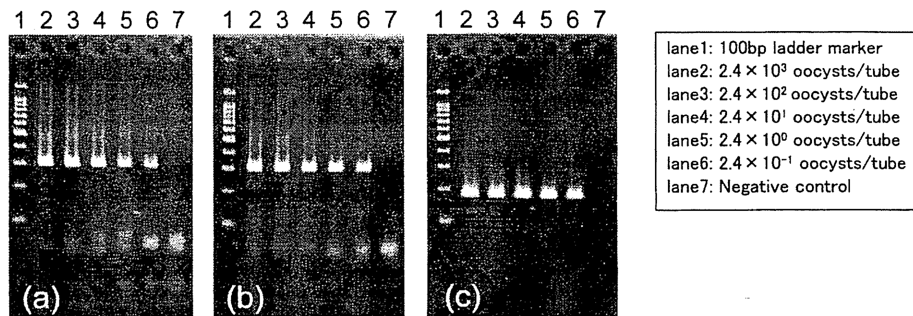

Fig. 2 Agarose gel electrophoresis of PCR products of the real-time PCR assays reported by (a) Keegan *et al.*[11], (b) King *et al.*[12], (c) Miller *et al.*[13] using 10-fold serial dilution of template DNA extracted from *Cryptosporidium parvum* oocysts.

た。

実際に7.5×10^{-6}～7.5×10^{-1} oocysts/tubeの範囲で段階希釈した試料をリアルタイムRT-PCR法に供した結果、7.5×10^{-5} oocysts/tubeの極めて低濃度からも遺伝子増幅を確認できた（Fig. 3、4）。7.5×10^{-6} oocysts/tubeでは、2連で実施した試験のうち、一方では、増幅が確認されなかった。7.5×10^{-5} oocysts/tubeの濃度では2反応とも蛍光曲線の立ち上がりは確認されたものの、Table 2に示すように他の希釈段階に比べてCp値のばらつきは大きかった（変動係数：1.81%）。一方、7.5×10^{-4} oocysts/tube以上の濃度では、Cp値が安定していた（変動係数：0.07-0.52%）。このことから、本研究で開発したリアルタイムRT-PCR法の定量下限は7.5×10^{-4} oocysts/tubeであると判断した。

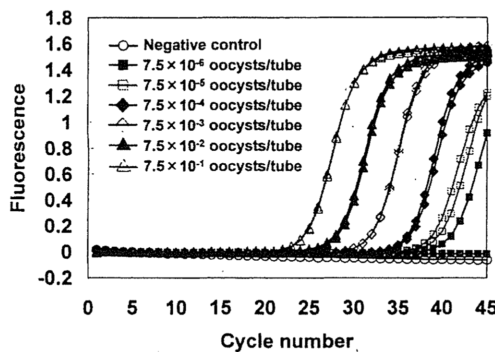

Fig. 3　Quantification limit of the real-time RT-PCR assay using 10-fold serial dilution of template RNA extracted from *Cryptosporidium parvum* oocysts.

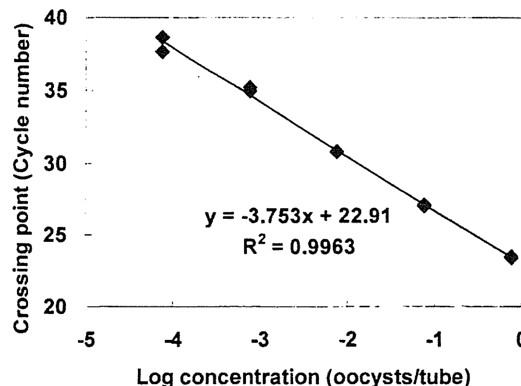

Fig. 4　Standard curve of the real-time RT-PCR assay using 10-fold serial dilution of template RNA extracted from *Cryptosporidium parvum* oocysts.

なお、通常のリアルタイムPCR法においても、先述のように0.24 oocysts/tubeの濃度から検出可能であったが、試料水中のオーシストDNAの全量を1反応チューブに濃縮することは極めて困難であり、一方、試験水量を増やすのは負担が大きく、DNA検査で10lの水試料中の1オーシストを検出するのは実用的ではないと考えられた。一般に遺伝子検査では、試料液量として5μl程度を使用することが多いが、免疫磁気ビーズ法による通常の精製・濃縮を実施しても、100μl程度までしか濃縮することができず、この場合は1 oocyst/100μl＝0.05 oocysts/5μlとなり、検出下限を下回る。仮に濃縮ができたとしても使用可能なDNA抽出液量には限りがあり、繰り返し試験や同じ濃縮抽出試料からジアルジア検査も実施したいことを考えると、感度はできるだけ高いことが望まれる。また、阻害物質が濃縮されて偽陰性になる恐れも否定できない。これまでにクリプトスポリジウム等の遺伝子検出は多数報告されていたが、低感度で複数のオーシストを必要としたり、試料を極限まで濃縮する必要があったり、コンタミネーションが心配されるNested-PCRを必要としたりしていた[17]。当該研究のリアルタイムRT-PCR法はこれまでに報告された従来の遺伝子検査法[17]と比べ、定量感度が著しく高く、より実用的な検査法であると考えられた。

RNAからの検査法としては、hsp70のmRNAを対象としたRT-PCR法も開発されているが[18]、本研究で開発したrRNAを対象としたリアルタイムRT-PCR法と比べて、検出感度は2オーダー以上低く、また、オーシスト由来のmRNAはrRNAと比較して分解速度が高いことが報告されている[19]。このため、浄水場等の現場で安定してオーシストを検出するためには、当該研究のようなrRNAの検出が適していると考えられる。また、高感度な遺伝

Table 2　Cp values of each diluted sample in the real-time RT-PCR assay

Cryptosporidium oocyst concentration (oocysts/tube)	Cp value	Coefficient of variation (%)
7.5×10^{-1}	23.33, 23.47	0.42
7.5×10^{-2}	26.98, 27.08	0.26
7.5×10^{-3}	30.77, 30.80	0.07
7.5×10^{-4}	34.93, 35.19	0.52
7.5×10^{-5}	37.66, 38.64	1.81
7.5×10^{-6}	39.88, —	—

子検出法を使用することで試料を全て使わずに済み、残りの試料からジアルジアの検査を行ったり、別の遺伝子増幅を行ったりすることも可能と考えられる。

3.3 実試料への適用

新しく構築したリアルタイムRT-PCR系を河川水試料に適用した結果、河川試料中のオーシスト濃度を定量することが可能であった（Table 3）。3回の測定値間の変動も小さく、リアルタイムRT-PCR法の定量値は安定していた。また、陰性試料が少なかったものの、リアルタイムRT-PCR法とは異なる増幅領域でプライマーが設計されているRT-LAMP法とも定性的な検出結果が一致したことから、偽陰性・偽陽性等の特異性の問題が生じている可能性は低いと考えられた。

リアルタイムRT-PCR法と顕微鏡観察結果を定性的に比較すると、概ね一致していることがわかるが、14試料中3試料で試験結果が異なった。本研究では、2.1節で述べたように、10ℓの河川水を濃縮・精製した後、試料を2つに分離してリアルタイムRT-PCR法と顕微鏡観察に供しているため、元の試料中のオーシスト濃度が低い場合は、偶然片方の検査試料にしかオーシストが入らない可能性があり、この確率の問題が2手法の定性的結果に差が生じた要因と考えられた。また、顕微鏡観察法では、フィルターろ過や染色工程においてオーシストが損失される作業があり、さらに検査者の判断がオーシスト同定に影響することが要因の一つかもしれない。

リアルタイムRT-PCR法と顕微鏡観察の定量値に関しては、Fig.5に示すように、リアルタイムRT-PCR法による定量値と顕微鏡観察推定値との間に高い相関が見られたことから（寄与率$R^2=0.75$）、新しく構築したリアルタイムRT-PCR法による定量値は妥当なものであると考えられた。一方、顕微鏡観察確定値との間には明確な相関が見られなかった（寄与率$R^2=0.24$）。顕微鏡観察によるオーシストの確定判断には、スポロゾイト等のクリプトスポリジウムに特徴的な内部構造を観察する必要があるが、フィルター上の夾雑物とオーシストの位置や角度によっては、明瞭な内部構造を観察できないこともあり、通常、確定値は推定値より低い値となる。リアルタイムPCR法と顕微鏡観察法の結果を比較するためには、正確な推定および確定判定を行う必要があり、今後の課題と考え

Table 3 Quantification and detection of *Cryptosporidium* oocyst in river water samples.

Time of sample collection	Sampling site[*1]	Microscopic observation (oocysts/5L)		Real-time RT-PCR (oocysts/5L)		RT-LAMP
		Suspected[*2]	Confirmed[*2]	Average	CV (%) [*3]	
Nov-09	A	1	0	0.22	6.6	+[*4]
	B	1	1	–[*4]	–	–[*4]
	C	0	0	–	–	–
	D	2	1	0.91	4.3	+
	E	0	0	0.11	7.6	+
	F	5	3	0.024	23	+
	G	8	3	0.24	14	+
Dec-09	A	0	0	4.8	3.6	+
	B	2	2	7.7	0.71	+
	C	0	0	–	–	–
	D	6	2	20	13	+
	E	7	1	17	4.3	+
	F	15	2	20	17	+
	G	20	3	47	14	+

[*1] Sampling sites A and B are located in Tone River and C, D, E, F, G are located in tributary rivers of Tone River.
[*2] Definition of "suspected" and "confirmed" oocysts is written in section 2.7.
[*3] Coefficient of variation (n = 3)
[*4] + = positive, - = negative

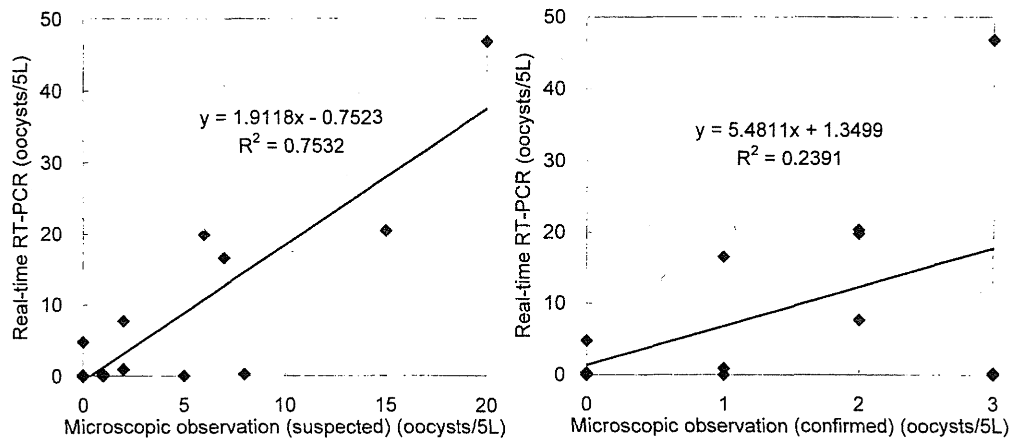

Fig. 5 Correlation of *Cryptosporidium* oocyst concentration in river water samples determined by the real-time RT-PCR assay, compared with that determined by conventional microscopic observation (Left: suspected value, Right: confirmed value).

られた。

また、11月にサイトFおよびGで採取した2試料に関しては、リアルタイムRT-PCR法の定量値が顕微鏡観察法の値より1オーダー以上低くなった。さらに、サイトFから採取した試料では、定量値が0.024 oocysts/5ℓと、極めて小さい値を示した。本研究では、免疫磁気ビーズ法によって試料の精製を行っているが、PCR阻害物質を完全に除去することは困難であるので、一部の試料で阻害の影響が出ることで、定量値が低くなった可能性が考えられる。mRNA遺伝子を対象とした既報のRT-PCR法においても[20]、河川水試料では、井戸水等の清澄な試料に比べ、検出感度が低くなることが報告されている。今後は同じ系統の河川水のみでなく、湖沼水等も含めたより多くの試料に対して検討を進め、顕微鏡観察法とリアルタイムRT-PCR法の定量値を比較していく必要があると考えられる。

4. まとめ

本研究では、水中のクリプトスポリジウムオーシスト濃度を定量可能な、高感度かつ実用的なリアルタイムRT-PCR法を確立した。得られた成果を以下に列挙する。

1）既報の3つのリアルタイムPCR系を比較した結果、MillerらのPCR系は、他の2系と比較して、試料毎の蛍光曲線の挙動の変動が小さく、増幅効率も高いことが推測された。特に低濃度のオーシストからの増幅が良好であると考えられた。さらに他の2系と異なり、非特異産物の生成も確認されなかったことから、MillerらのPCR系が最も実用的であると考えられた。

2）オーシスト内部に大量に存在するrRNAを逆転写反応によってcDNAに変換させることで、増幅対象となるDNA数を1000倍以上増加させることが可能であった。Millerらが報告した系に逆転写反応を組み合わせてリアルタイムRT-PCR法とすることで、感度を大幅に上昇させ、7.5×10^{-4} oocysts/tubeから定量可能な高感度な検査系を構築することに成功した。

3）リアルタイムRT-PCR法は、特異性の問題を生じることなく、実河川試料からオーシストを定量することが可能であり、顕微鏡観察法と同様の定量結果が得られた。リアルタイムRT-PCR法による定量値と顕微鏡観察による推定値との間には高い相関が見られた（R^2 =0.75, n=14）。

5. 謝辞

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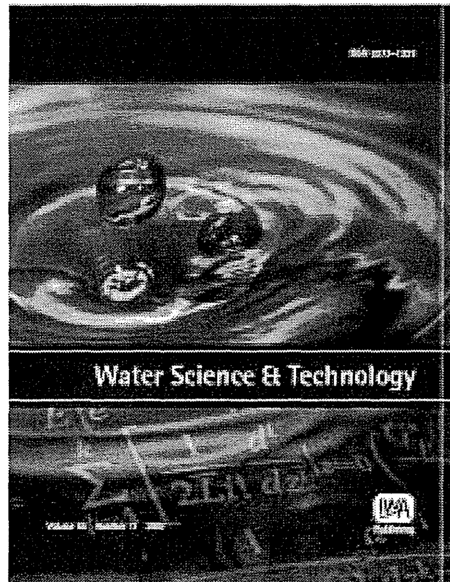

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Source of *N*-nitrosodimethylamine in river waters of the upper Tone River basin in Japan

K. Kosaka, K. Fukui, M. Asami and M. Akiba

ABSTRACT

The presence of *N*-nitrosodimethylamine (NDMA) in the Hirose River and its tributaries, located in the upper Tone River basin, in the Kanto region of Japan, was investigated. NDMA was detected at high levels in the Arato River, one of the tributaries of the Hirose River, at high concentrations (up to 2,100 ng/L). Due to the confluence of the Arato River, NDMA concentration in the Hirose River increased (up to 61 ng/L). The NDMA in the Arato River was due to industrial discharge from a livestock processing plant located near the river. There were three discharges at the plant, with NDMA concentrations of 78, 11, and 33,000 ng/L. The industrial discharges from the livestock processing plant did not contain significant amounts of NDMA precursors on chloramination. On the other hand, sewage effluent was shown to contain NDMA precursors. The amounts of NDMA precursors in the sewage effluent that were rapidly transformed into NDMA were considered to be lower than those slowly transformed into NDMA.

Key words | Arato River, chloramination, drinking water source, Hirose River, *N*-nitrosodimethylamine, Tone River basin

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INTRODUCTION

N-nitrosodimethylamine (NDMA) is a potent carcinogen (United States Environmental Protection Agency [US EPA] 1993). Although no longer used directly in industry, NDMA has been used in the past in a wide range of products, e.g. as an intermediate of unsymmetrical dimethyl hydrazine (UDMH), as an additive of gum products and polymers, and in solvents in the plastics industry (Najm & Trussell 2001). It has also been reported that NDMA was contained in various foods (e.g. meat, meat products, fish, fish products, beer, and milk; San Francisco Public Utilities Commission 2007). At present, NDMA is not listed as a federal standard item in drinking water regulation in the USA. However, NDMA is listed as a contaminant in Contaminant Candidate List 3 (US EPA 2008). The World Health Organization (WHO) Guidelines for drinking water quality (WHO 2008) specifies a target value of NDMA in drinking water of 100 ng/L. In Japan, NDMA was listed as an item for monitoring in 2009, and a target value of 100 ng/L was set in April 2010 (Water Supply Division, Health Bureau, Ministry of Health, Labour, and Welfare 2010).

The occurrence of NDMA in drinking and wastewaters has been investigated in detail in the USA and Canada (Najm & Trussell 2001; Mitch *et al.* 2003; Charrois *et al.* 2007; California Department of Public Health 2009). NDMA concentrations in river water have also been reported (Schreiber & Mitch 2006; Plumlee & Reinhard 2007). NDMA is known to be a chloramination disinfection byproduct (Mitch *et al.* 2003; Mitch & Sedlak 2004; Chen & Valentine 2006). However, NDMA is also formed during ozonation (Andrzejewski *et al.* 2008; Schmidt & Brauch 2008; Asami *et al.* 2009*b*; Tateishi *et al.* 2009).

In Japan, the occurrence of NDMA in drinking and environmental waters has been reported, mainly focusing on two basins located in the Kansai and Kanto regions, i.e. the Yodo and Tone River basins, respectively. The river waters in both basins are used as sources for the local water

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supplies. In the case of the Yodo River basin, NDMA is derived mainly from upstream sewage effluents (Kosaka *et al.* 2009*a*; Tateishi *et al.* 2009). Some sewage effluents were also shown to contain NDMA precursors on ozonation. One sewage effluent, which contained NDMA at high concentrations (several hundred nanograms per liter) employed ozonation. On the other hand, there have been no reports of high concentrations of NDMA precursors on ozonation in the case of the Tone River basin (Asami *et al.* 2009*b*). NDMA was frequently detected at levels of several nanograms per liter in raw water from the middle stretches of the Tone River basin (Kosaka *et al.* 2009*b*). The raw waters also contained NDMA precursors on chloramination. The sources of NDMA precursors were shown to include sewage effluents in the upper Tone River (Kosaka *et al.* 2009*b*), as reported previously (Schreiber & Mitch 2006). Among the samples analyzed in this previous study, NDMA concentrations were relatively high in the Hirose River, one of the tributaries of the upper Tone River. However, the origin of NDMA in the river has not been determined.

The present study was performed to investigate the occurrence and concentration of NDMA in the upper Tone River basin, focusing on the Hirose River and its tributaries. In addition, NDMA formation on chloramination was also investigated in the present study.

MATERIALS AND METHODS

Reagents and solutions

Ultrapure water purified with a Gradient A10 water purification system (Millipore, Bedford, MA) was used in this study. Distilled water purchased from Kanto Chemical (Tokyo, Japan) was used to prepare the mobile phase for use in ultra-high performance liquid chromatography tandem mass spectrometry (UPLC/MS/MS). NDMA and NDMA-d_6 were purchased from Supelco (Bellefonte, PA), and C/D/N Isotopes (Pointe-Claire, QC, Canada), respectively. Chloramine solution was prepared by mixing ammonium chloride solution and hypochlorite solution at a molar ratio of 1.2 of nitrogen to 1 of chlorine (Mitch & Sedlak 2002). Fresh chloramine solution was prepared on each experimental day.

Sampling

Figures 1 a and b show a map of the Tone River basin and schematic of the sampling area with sampling points, respectively. The samples collected were surface waters (i.e. Hirose River, its tributaries, and channels), sewage effluent (i.e. S1), and industrial discharge (i.e. I1, I2, and I3). The Hirose River is one of the tributaries of the Tone River, and is located in the upper Tone River basin. River waters of the Tone River basin are used as sources for the water supply in the Tokyo Metropolitan Area. The Hirose River is used as the source water after the confluence with the Tone River. Connections and pathways of some channels (i.e. C4–C6) are not shown in the figure for ease of representation. Sampling was conducted in May–July 2009. All were grab samples, and those at several sampling points were collected on different days. The samples were filtered with 0.7-μm GF/F filters (Whatman, Florham Park, NJ), and the filtrates were used. Table 1 shows the basic water quality parameters of some samples.

Chloramination

In the present study, NDMA formation on chloramination was investigated by two procedures. The analytical methods for drinking water described by the Japan Water Works Association (2001) describe the formation potentials of disinfection byproducts of chlorination as 1–2 mg/L of chlorine at 24 h. These conditions were used as a reference for chloramination conditions in this study. That is, the chloramination conditions were as follows: reaction time, 24 h; chloramine concentration at 24 h, 3.0 ± 0.5 mg/L; pH 7.0 (5 mM phosphate buffer); temperature, 20°C. These chloramination conditions were designated as chloramination I. Mitch & Sedlak (2004) proposed experimental conditions to evaluate total NDMA precursors on chloramination. Based on their study, chloramination conditions were set as follows: chloramine dose, 2 mM (140 mg/L), reaction time, 10 days; pH 7.0 (5 mM phosphate buffer); temperature, 20°C. These chloramination conditions were designated as chloramination II. Chloramination I was considered only to evaluate the amounts of NDMA precursors rapidly transformed into NDMA on chloramination.

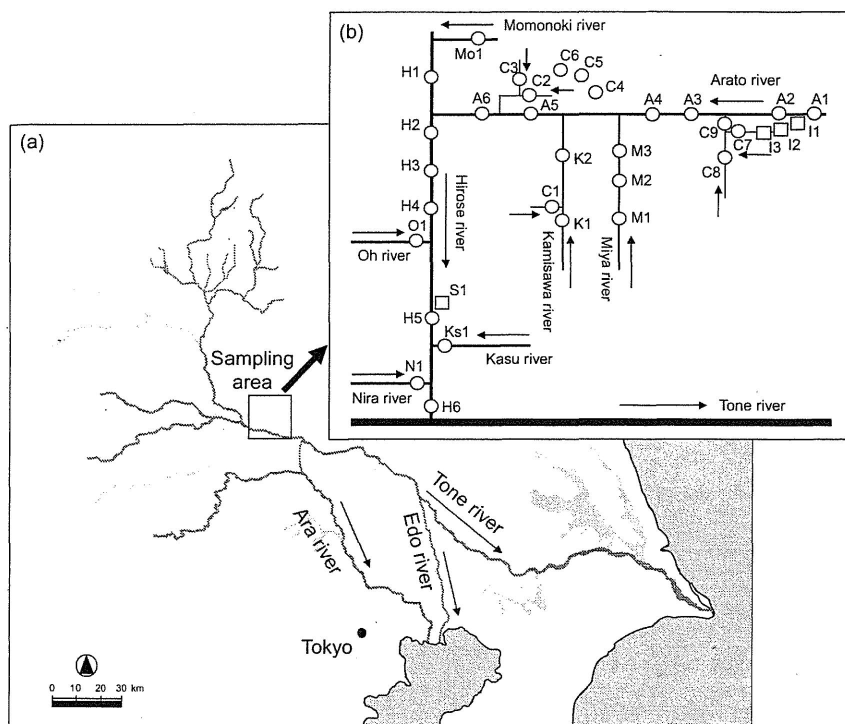

Figure 1 | (a) Map of the Tone River basin and (b) schematic of sampling area with sampling points.

Residual chloramine was quenched by sodium thiosulfate (Wako Pure Chemicals, Osaka, Japan).

Analysis

NDMA concentration was determined by solid-phase extraction (SPE) followed by UPLC/MS/MS. NDMA-d_6 was used as an internal standard. The details of the analytical procedures were described elsewhere (Asami *et al.* 2009*b*; Kosaka *et al.* 2009*a*). Two Sep-Pak® Plus AC-2 cartridges (400 mg × 2; Waters, Milford, MA) were used for SPE, and one Sep-Pak® Vac Florisil® cartridge (1 g; Waters) was used for cleanup. A mixture of dichloromethane and diethylether (1:1 v/v) was used for elution. Sample volume was generally 500 mL, but these were reduced for samples containing NDMA at high concentrations. The concentration factor was 2,500 for a sample volume of 500 mL. Separation was performed using an ACQUITY UPLC system (Waters) with a BEH C18 column (2.1 mm × 150 mm; Waters), and detection was performed using an ACQUITY TQD tandem mass spectrometer (Waters) operated in the electrospray ionization (ESI) positive-ion mode. The multiple reaction

Table 1 | Basic water quality parameters of the Arato River at A3, sewage effluent and industrial discharge

Sampling point	pH	DOC (mg/L)	UV_{260}*	Ammonia (mg-N/L)	Nitrite (mg-N/L)	Nitrate (mg-N/L)	DON (mg-N/L)
A3†	7.7	1.8	0.052	1.7	0.37	3.4	0.72
S1	7.3	3.4	0.073	0.70	0.12	9.1	0.10
I1	7.5	25	0.21	8.7	<0.02	<0.02	6.1
I2	7.9	2.4	0.067	6.3	1.3	4.6	0.35
I3	7.6	6.3	0.11	68	15	31	0.46

*Absorbance at 260 nm.
†Sampling day of A3 was 16 July 2009.

monitoring (MRM) transitions were m/z 74.9 to 43.1 (quantification) and m/z 74.9 to 57.9 (confirmation) for NDMA and m/z 81.0 to 46.0 for NDMA-d_6. The detection limit of NDMA was 1.0 ng/L, which was calculated by repetition analyses (number of repetitions (n) = 5) of known concentration (2 ng/L) in different ultrapure water samples (Asami *et al.* 2009*b*).

Chloramine concentration was determined by titration using *N,N*-diethyl-*p*-phenylenediamine and ferrous ammonium sulfate (APHA *et al.* 2005). Dissolved organic nitrogen (DON) concentration was calculated by subtracting nitrate, nitrite, and ammonia concentrations from dissolved nitrogen (DN) concentration. Nitrate and nitrite concentrations were determined by ion chromatography (IC) (DX-500; Dionex, Sunnyvale, CA). Ammonia concentration was determined spectrophotometrically with phenol (Japan Water Works Association 2001). DN concentration was determined using a TN analyzer (TNM-1; Shimadzu, Kyoto, Japan) connected to an organic carbon analyzer (TOC-V CPH; Shimadzu). Dissolved organic carbon (DOC) concentration was determined using the TOC-V CPH. Absorbance at 260 nm (i.e. UV_{260}) was determined using a spectrophotometer (U-2800; Hitachi High-Technologies, Tokyo, Japan). pH was determined using a pH meter (PHM240 Meter Lab; Radiometer analytical, Lyon, France). Chlorate concentration was determined by IC (ICS-2000; Dionex) coupled with MS/MS (API 3200QTrap; Applied Biosystems, Foster, CA). Analytical conditions of IC/MS/MS were described elsewhere (Kosaka *et al.* 2007).

RESULTS AND DISCUSSION

Occurrence of NDMA in the upper Tone River basin

Table 2 shows NDMA concentrations in the samples. Some samples were extracted and analyzed two or three times (i.e. repetition analyses), and the relative standard deviations (RSDs) were 0.9–19%. Figures 2 and 3 show profiles of NDMA concentrations in the Hirose River and one of its tributaries, the Arato River (Figure 1 b), respectively.

In the Hirose River, NDMA concentration at H1 was low (1.0 ng/L), but increased after the confluence of the Arato River and the concentration of NDMA at H2 was 4.6–61 ng/L. NDMA concentrations decreased downstream and were 1.9–22 ng/L. Sewage effluent containing NDMA (95 ng/L) flowed into the Hirose River, but did not affect NDMA concentration in the river. This was considered to be because the flow rate of the sewage effluent was much lower than that of the Hirose River. NDMA concentration in the Hirose River at H6, just before confluence with the Tone River, was 1.9 ng/L.

NDMA concentrations in the Arato River and its tributaries were investigated to identify the sources of NDMA around H2 in the Hirose River. NDMA concentration in the upstream region (A1) of the Arato River was 2.3 ng/L. The NDMA level increased markedly at A3 (i.e. 370–2,100 ng/L), and decreased gradually in the downstream region. However, NDMA concentration was still high just before confluence with the Hirose River (12–130 ng/L). On the other hand, NDMA concentrations in the Miya and Kamisawa Rivers were 1.3–3.7 ng/L. These results indicated that the NDMA source in the Arato River was located between A2 and A3, and the amounts of NDMA discharge from this point fluctuated. NDMA concentrations in some channels around the Arase River, C2 and C5, were also high because these channels contained water from the Arato River.

There was a plant upstream of A2, from which there were three industrial discharges (i.e. I1, I2, and I3). Therefore, the NDMA concentrations at the three industrial discharge points were investigated. Among the three industrial discharges, I3 was only discharged after treatment (i.e. biological treatment). NDMA concentrations at I1, I2, and I3 were 78, 11, and 33,000 ng/L, respectively. Thus, NDMA was present at high levels in these discharges, particularly at I3. The discharge at I1 flowed directly into the Arato River between A1 and A2. However, NDMA concentration did not change markedly between A1 and A2, as described above. The discharges at I2 and I3 flowed into the channel. This channel (i.e. C7) was confluent with other channels (i.e. C8), and the confluent (i.e. C9) flowed into the Arato River between A2 and A3. Due to the high-level discharge from I3, NDMA concentrations in the channels were high (2,600–9,300 ng/L). These results suggested that the NDMA in the Arato River was mainly due to industrial discharge at I3. The plant is related to livestock processing, and feedstuff and fertilizer are

Table 2 | NDMA concentrations in the samples

Sampling point	Sampling day	NDMA concentration (ng/L)	Sampling point	Sampling day	NDMA concentration (ng/L)
Arato River			M2	03/06/2009	3.5
A1	03/06/2009	2.3 (14)	M3	03/06/2009	1.3
A1	07/07/2009	1.1	Momonoki River		
A2	07/07/2009	5.9 (8.0)	Mo1	14/05/2009	1.0
A3	03/06/2009	830 (12)	Nira River		
A3	07/07/2009	2,100 (0.9)	N1	14/05/2009	13
A3	16/07/2009	370	Oh River		
A4	03/06/2009	160 (8.9)	O1	14/05/2009	1.0
A5	03/06/2009	30	Channel		
A6	14/05/2009	130	C1	14/05/2009	2.0
A6	07/07/2009	12 (1.5)	C2	03/06/2009	93 (18)
Hirose River			C3	03/06/2009	1.3
H1	14/05/2009	1.0	C4	07/07/2009	2.8
H2	14/05/2009	61 (19)	C5	07/07/2009	93 (12)
H2	07/07/2009	4.6	C6	07/07/2009	4.8
H3	14/05/2009	17	C7	16/07/2009	9,300 (10)
H4	14/05/2009	22 (9.8)	C8	16/07/2009	1.4 (12)
H5	14/05/2009	17 (4.9)	C9	16/07/2009	2,600 (5.0)
H5	07/07/2009	1.9	Sewage effluent		
H6	07/07/2009	1.9	S1	07/07/2009	95 (8.5)
Kamisawa River			Industrial discharge		
K1	14/05/2009	2.0	I1	07/07/2009	78
K2	03/06/2009	3.7	I2	16/07/2009	11 (3.0)
Kasu River			I3	16/07/2009	33,000 (4.0)
Ks1	14/05/2009	5.0			
Miya River					
M1	03/06/2009	2.5			

Values in parentheses are relative standard deviation (RSD) (%) ($n = 2$ or 3).

produced from livestock through processes such as heating at this plant.

Next, NDMA load at I3 was estimated to investigate its significance to the Arato and Hirose Rivers. From the NDMA concentration at I3 and its flow rate (about 700–1,000 m^3/day), the NDMA load at I3 was estimated to be about 7.7–11 g/day. In the calculation, the industrial livestock processing plant was assumed to operate only during the day (i.e. 8 h) although information on operating time was not available. Annual mean flow rate of the Arato River upstream of the confluence with the Kamisawa River in 2001–2007 was 1.35 m^3/s (Aisaki 2009). That of the Hirose River near H6 in 2008 was 18 m^3/s (Upper Tone River Office). Note that for the Hirose River, when the values of the measuring instrument were below its lower limit of detection, the values were assumed to be at the lower limit. From these flow rates and NDMA load at I3, the increases in NDMA concentration in the Arato and Hirose Rivers due to NDMA discharge from I3 were estimated to be 66–94 and 5.0–7.1 ng/L, respectively. Based on the fluctuation of NDMA concentration at I3, it was considered that these estimated values were not so different from the NDMA concentrations in the Arato and Hirose Rivers (i.e. A5 and A6, and H6, respectively).

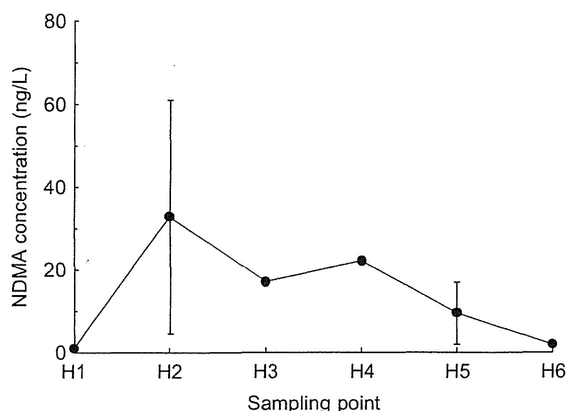

Figure 2 | Profiles of NDMA concentrations in the Hirose River. Mean NDMA concentrations were plotted at the sampling points collected on different days. Error bars show the ranges of NDMA concentration.

Therefore, from the viewpoint of NDMA load, it was confirmed that the industrial discharge at I3 was the main NDMA source in the Arato and Hirose Rivers.

NDMA has been reported in meat and meat products (San Francisco Public Utilities Commission 2007). From the results of the present study, it was not possible to determine why the industrial discharge had such a high concentration of NDMA. However, NDMA may have been formed during livestock processing, and therefore the industrial discharges contained NDMA at high concentrations. Chlorate is present as an impurity in hypochlorite solution, which is widely used as a disinfectant (Asami *et al.* 2009*a*). Therefore, the presence of chlorate in treated water indicates that disinfection using hypochlorite solution was performed in wastewater, although the types of disinfectant existed (e.g. hypochlorous acid/hypochlorite and chloramine produced by the reaction between hypochlorous acid/hypochlorite and ammonia in wastewater) and their levels of consumption could not be determined. Chlorate concentrations in the discharge at I1, I2, and I3 were very low (i.e. < 0.05, 0.05, and 1.5 μg/L, respectively). Therefore, it was considered that hypochlorite solution was not dosed or was dosed in very small amounts, and the presence of NDMA in the industrial discharges was not due to disinfection byproducts. These results suggested that the livestock processing plant was the likely source of NDMA in the river water.

With regard to wastewaters containing NDMA at extremely high concentrations, it was reported that the NDMA concentration in stock solutions of metam sodium used for root treatment was 1,100,000 ng/L, and those in wastewater at printed circuit board (PCB) manufacturing facilities using dimethyldithiocarbamate (DTC)-based chemical treatments ranged from 139 to 56,000 ng/L (Sedlak *et al.* 2005). It was also reported that NDMA concentrations in untreated wastewaters from a drum recycling facility and automobile crankcases were above 750,000 and 350,000 ng/L, respectively (Sedlak *et al.* 2005). Moreover, it was reported that NDMA concentration in wastewater mainly from a clothing factory was 10,000 ng/L (Kosaka *et al.* 2009*a*). Thus, the NDMA concentration in the industrial discharge from the industrial livestock processing plant was lower than in some of these wastewaters and untreated wastewaters reported previously. However, so far, it has not been reported that the industrial discharge from livestock processing contained NDMA at extremely high concentration. In addition, in the previous studies described above, at least some wastewaters were influents to municipal wastewater treatment plants, where they were mixed with other influents and subjected to biological treatment. The removal efficiencies of NDMA by biological treatment processes at municipal wastewater treatment plants are highly variable, ranging from 0 to 75% (Sedlak *et al.* 2005). Therefore, the NDMA concentrations in the influents were considered to be reduced by dilution and biological treatment to some degree before discharge into the environment. In the present study, the NDMA

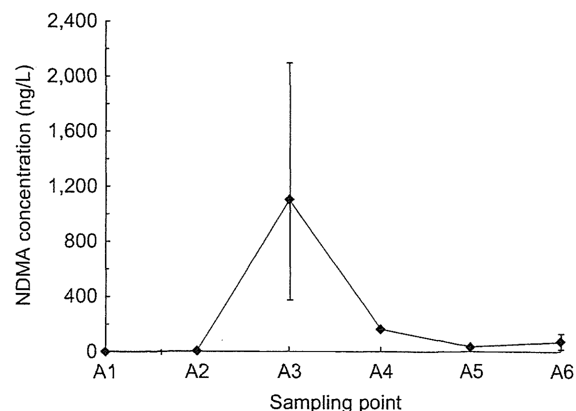

Figure 3 | Profiles of NDMA concentrations in the Arato River. Mean NDMA concentrations were plotted at the sampling points collected on different days. Error bars show the ranges of NDMA concentration.

concentration in the industrial discharge was in the final effluents, which directly impacted the environment. Therefore, NDMA was detected at high levels in some environmental waters (e.g. A3, C7, and C9). NDMA concentrations in the discharges at other similar livestock processing plants were not investigated in the present study, and further investigations are warranted.

NDMA concentrations in some tributaries of the Hirose River other than the Arato River were relatively high, i.e. the NDMA concentrations in the Nira and Kasu Rivers were 13 and 5.0 ng/L, respectively. Therefore, there may be some NDMA sources in the Hirose River in addition to those in common with the Arato River, although the levels of NDMA discharge were lower than that from the discharge at I2.

NDMA formation on chloramination

Figure 4 shows the NDMA concentrations before and after chloramination of the river water at A3, in sewage effluent, and in industrial discharges. Sampling day at A3 was 16 July 2009. As shown in the figure, industrial discharge at I2 was evaluated only by chloramination I.

In the case of I3, NDMA concentrations before chloramination and after chloramination I and II were 33,000, 34,000, and 34,000 ng/L, respectively. Therefore,

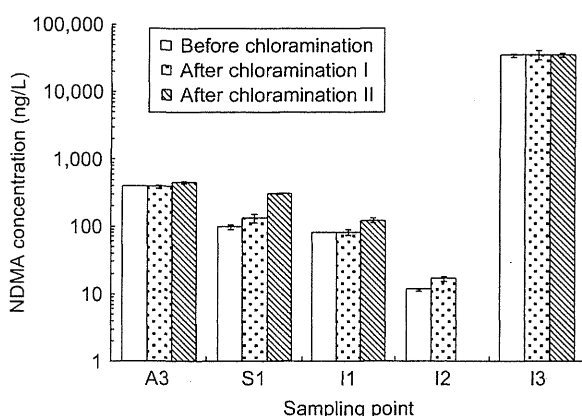

Figure 4 | NDMA concentrations before and after chloramination. Chloramination I conditions: reaction time, 24 h; chloramine concentrations at 24 h, 3.0 ± 0.5 mg/L; pH 7.0 (5 mM phosphate buffer); temperature, 20°C. Chloramination II conditions: reaction time, 10 d; chloramine dose, 2 mM (140 mg/L); pH 7.0 (5 mM phosphate buffer); temperature, 20°C. For I2, only chloramination I was performed. Error bars show standard deviations of repetition analyses.

NDMA was present at high levels in I3, but there were no significant amounts of NDMA precursors by chloramination at I3. Similarly, I1, I2, and A3 did not contain significant levels of NDMA precursors. These results were of interest because DOC was high for I1 and I3 and DON was also high for I1 (Table 1). This was considered to be because industrial discharge from the livestock processing plant did not contain significant amounts of NDMA precursors on chloramination or most of the NDMA precursors were transformed into NDMA during livestock processing. Waste water at PCB manufacturing facilities using DTC-based chemical treatments was reported to contain high levels of both NDMA and NDMA precursors (Sedlak *et al.* 2005). This tendency of the presence of NDMA precursors was different from the observations at the livestock processing plant in the present study.

In the case of S1, NDMA concentrations before chloramination and after chloramination I and II were 95, 130, and 290 ng/L, respectively. S1 contained NDMA precursors. This result was consistent with those of previous studies using municipal wastewater effluents (Mitch & Sedlak 2004; Schreiber & Mitch 2006). The NDMA concentrations after chloramination I and II suggested that S1 had lower levels of NDMA precursors that were transformed rapidly into NDMA than those that were transformed slowly.

CONCLUSIONS

(1) NDMA was detected at high concentrations in the Arato and Hirose Rivers, located in the upper Tone River basin in the Kanto region of Japan. The highest concentrations were 2,100 and 61 ng/L, respectively. The NDMA source in these rivers was industrial discharge from a livestock processing plant located near the Arato River.
(2) The industrial discharges (i.e. I1, I2, and I3) did not contain significant amounts of NDMA precursors. However, S1 did contain NDMA precursors, and the levels of NDMA precursors that were transformed rapidly into NDMA were lower than those that were transformed more slowly.

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