などに留意した対症療法で対処する．幼児の場合は栄養補給にも十分配慮する必要があろう．

AIDS など免疫不全患者の場合は，早期に投薬を開始することが必須である．重症になってからでは治療はきわめて困難である．確実に効く治療薬はないが，完治は期待できなくても，原虫の増殖をある程度抑制して下痢が改善されれば，患者の体力消耗や衰弱を軽減することができる．これまでパロモマイシン，アジスロマイシン，クラリスロマイシンなどが使用されてきたが[10]，現在，パロモマイシンは製造中止で入手できない．

最近，nitazoxanide（NTZ, Alinia™, Lomark Lab., FL, USA）が有望視され臨床試験がなされている．NTZ は腸管寄生性の各種原虫症と蠕虫症に有効とされる新しい駆虫薬である[15]．AIDS の本症下痢患者における治癒率は，1 日 1 g・分 2・2 週間投与で 63%，1 日 2 g・分 2・2 週間投与で 67% であるが[16]，小児 HIV 陽性下痢患者に 1 日 200 mg・分 2・3 日間投与では効果は認められないと報告されている[17]．投与量，投与期間のさらなる検討が必要である．

6）感染予防が大切

確実に効く治療薬がないので，幼児や免疫不全患者では感染を予防することが大切である．ワクチンはない．

予防には，①生水の飲用を避ける：河川，池，井戸などの水はオーシストで汚染されているし，水道水も安全とはいえない．飲用には水道水といえども 1 分間煮沸するか，1 μm 以上の粒子を除去できる家庭用浄水器で濾過したものを使用する，②手洗いの励行：食事前，調理前，トイレ使用のあと，土いじりのあとなどはもちろん，糞便がついたオムツや下着，ペットや家畜に触れたあとは必ず石鹸を使って流水でよく洗う，③口-肛囲接触を伴う性行為を避ける：下痢症状を呈していないときでも，感染者の肛囲にはオーシストが多数付着している．口-肛囲接触を伴う性行為や肛門性交は感染のリスクがもっとも高い，④家畜・家禽・ペットの糞に触れない，⑤プール，海水浴場，公衆浴場などでは口に入った水を飲み込まない，ことなどである．

2 次感染の防止には，①糞便がついたオムツ・下着類は熱湯を掛けてから洗濯する，②感染者の入浴は家族の最後にし，湯を抜いたあとの浴槽・洗面具・洗い場は 90℃ 以上の熱湯をかける，③下痢発症中はもちろん，下痢終息後も 2 週間はプールや公衆浴場の使用を制限する，などに留意する．

何が問題か

1）確実に効く治療薬がない

現在，本症に確実に効く薬剤はなく，免疫不全患者では致死的になる．しかし，免疫機能が回復すれば自然に治癒するので，高 IgM 症候群では骨髄移植，HIV 陽性者では抗 HIV 療法の実施が必須である．また，免疫不全患者においては本症を早期に診断し，重症化する前にアジスロマイシンや NTZ の投与を試みることも欠かせない．いずれにせよ，もっと効果の高い新規治療薬の開発や受動免疫療法の開発は焦眉の課題である．

2）水系感染が起こりやすい

水道水による本症の集団感染例は欧米では 1985 年ごろから毎年のように報告され，1993 年に米国のミルウォーキーで起こった事例では 2 週間で 403,000 人が発症した[18]．わが国でも 1996 年に埼玉県で 1 町営水道の汚染により 8,700〜9,000 人が発症，2,662 人が受診，24 人が入院．学童や幼児も多数感染した[19]．1994 年には神奈川県で 461 人が発症し，124 人が受診，5 人が入院．2002 年には北海道で約 300 人が発症した．

水系集団感染が起こりやすい理由は，①汚染

表　環境水のクリプトスポリジウム汚染状況*
　　（米国）

水の種類	陽性率（%）	オーシスト数/L
下　水	67	1〜120
表流水	9〜100	0.01〜107
地下水	5.6	0.04〜0.9
水道水	3.8〜40	0.001〜0.72
河口付近の海水	62	0.02〜0.44

*：多数の調査報告のまとめ（Rose JB et al, 1991 を
　改変）

源が多い，②浄水処理でオーシストを完全に除去・殺滅することが困難，③オーシストの感染力が非常に強い，ことなどである．患者は1日に数十億個，感染仔ウシでは1日に数百億個のオーシストを排出する．しかも，仔ウシや各種動物における感染率は高いので，環境水は高度に汚染されている[20]（表）．オーシストは小さいので，凝集・沈殿・砂濾過による浄水処理でも，原水に含まれるオーシストの 0.1〜1% は浄水に漏れ出すし，水道水やスイミングプールの塩素濃度ではオーシストはまったく不活化されない．しかも，オーシストは1〜数個の摂取で発症するほど感染力が強い．

　浄水場や下水処理場ではオーシストの除去・殺滅の効率を高めるためのさまざまな対応や技術開発が進められているが，何よりも大切なのは水道の原水となる環境水の汚染をできるだけ低レベルに抑えるための総合的な対策を実施することである．

　海外ではスイミングプールでの集団感染がいくつも報告されており，本症患者は下痢終息後も2週間はプールを使用しないよう指導している．下痢終息後の有形便にも約2週間は数百万個/g のオーシストが排出されるからである．わが国でも市民プールは勿論，幼稚園や学校のプールの安全管理に十分留意する必要がある．

3）検査を実施している施設が少ない

　本症の診断検査は簡単で，しかも検査のでき

る検査技師は全国に多数存在するにもかかわらず，検査を実施している施設は少ない．医師からの指示がなければ検査室ではクリプトスポリジウムの検査は実施しない．医師は小児下痢症，旅行者下痢症，免疫不全患者における下痢症，集団食中毒などの検査オーダーの際に，細菌・ウイルス検査だけでなく，「クリプトスポリジウムを含む原虫の検査」と明記して検体を出す必要がある．

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クリプトスポリジウム
公衆衛生の観点から

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概要

　ヒトのクリプトスポリジウム症は，*Cryptosporidium parvum*のヒト型（genotype I）と牛型（genotype II）が主要な原因病原体である。ヒト型を*C. hominis*という別種とすることが提案されているが，ここでは従来どおり*C. parvum*とする。

　*C. parvum*は1970年代中頃までは家畜に対する病原性が注目され，家畜の寄生原虫とされていた。ところが1976年に免疫機能が正常な女児と免疫不全の下痢患者から*C. parvum*がそれぞれ報告され[1, 2]，ヒトに寄生して病原性を発揮することが初めて明らかになった。その後1980年頃までは主として免疫不全者の重篤な慢性下痢症の原因であるとされていたが，多くの症例が見出されるに至って，小児下痢症，渡航者下痢症，人獣共通感染症あるいは水系感染症の病原体として重要視され，医学あるいは公衆衛生分野において最も関心の高い病原性原虫のひとつとなっている。

　クリプトスポリジウム症は，感染症法の改定により2003年11月5日から5類感染症（全数把握）に規定されており，医師が診断した場合には1週間以内に保健所を通じて都道府県知事などに届け出なければならない。1999年以降，毎年10例前後の散発例の患者発生が報告されているが，報告する自治体に偏りがみられることなどから，実態を反映しているとは考えられない。

ヒトにおける症状および治療

　ヒトのクリプトスポリジウム症の潜伏期は4〜7日程度で，主な症状は下痢（主として水様性），腹痛，39℃を越えない発熱，悪心，嘔吐，倦怠感などであり，下痢は軽度のものから1日に20回以上の激しいものまでみられる。症状は通

【表－1】	クリプトスポリジウム症の臨床的所見		
	エイズ患者 n＝67	その他の免疫不全者 n＝17	健常者 n＝35
症状			
下痢	63	16	31
腹痛	24	6	14
嘔吐（嘔気）	18	4	11
発熱	26	3	11
症状なし	4	1	4
症状（日）			
＜3	0	0	5
4～10	2	1	13
11～20	0	1	9
21～30	1	0	4
＞30	52	1	0
不明	12	3	4
転帰			
治癒	12	8	34
治癒不良	3	1	0
死亡	31	5	0
不明	21	3	1

3) を一部改変して引用

常は1～2週間程度で治まる。成人よりも小児の方が，また初感染の方が再感染よりも症状が重い。エイズ患者などの免疫不全者では慢性，消耗性の重篤な下痢を呈し，ときとして致死的となる。通常は小腸のみに感染するが，エイズ患者では呼吸器や胆道系での感染も報告されている。

公衆衛生上の重大な関心事のひとつは，エイズ患者をはじめとする免疫不全者におけるクリプトスポリジウム症の重篤な経過である。免疫不全者はハイリスクグループであり，健常人が感染しないタイプのクリプトスポリジウムにも感染する可能性がある。症状の種類には患者の免疫状態による大きな差はないが，症状の重篤度と持続する期間および転帰は異なる（表－1）[3]。米国疾病予防センター（CDC）では，エイズ患者においてはCD4＋細胞数が180～200を下回ると症状の長期化や重篤化が起きやすいとしている。

これまでにクリプトスポリジウム症の治療に関する多くの研究がなされてきたが，下痢症患者に投与して効果を示す薬剤が見出されていないため，治療は対症療法となる。免疫不全者に対する有効な治療法は免疫機能を回復することであり，エイズ患者ではCD4＋細胞数の改善を目指した治療が最も効果があるとされている。

感染事例

クリプトスポリジウムの感染は，便とともに排出されたオーシストを経口的に摂取することによる。患者や患畜，保有者（動物）に直接触れたり，便に触ることにより起きる感染には，ヒトからヒトへの感染（家族内感染，託児所（day-care centers）での集団感染，病院内感染，広義の性感染症）が欧米で報告されており，また，ヒト以外の感染動物との接触（ペットや家畜など）もある。例えば，獣医学科の学生が感染した牛の世話をして感染した事例が，わが国の事例も含めて複数ある。胃カテーテルを用いてウサギにエイズ患者由来のオーシストを投与する実験において，ウサギが咳をして投与物が実験者の顔に飛び，これにより感染した事例も報告されている。

食品や水を介した感染には，オーシストに汚染された食物（野菜類，アップルサイダー，牛乳など）を摂取することによる感染や，水道水の汚染による集団発生，河川や湖，プールでの水泳などによる感染がある。

C.parvumの遺伝子型のひとつであるヒト型の宿主はヒトや霊長類などに限られており，ヒトからヒトに感染するという

【表－2】 クリプトスポリジウムの主な水系感染事例

発生年	発生国	地域	確定患者数	飲料水の状態など
'83年	イギリス	Surrey	16人	緩速濾過，塩素消毒
'84年	アメリカ	Texas	117人 2,006人感染	濾過しない塩素消毒のみの水道水 1カ月前にウイルス性下痢症
'85年	イギリス	Surrey	50人	緩速濾過，塩素消毒
'86年	イギリス	Sheffield	104人	水源地周辺の牛が関与
'86年	アメリカ	New Mexico	78人	水泳，託児所も関与？
'87年	アメリカ	Georgia	58人 推計13,000人が感染	水道水（通常処理）の処理不十分
'88年	イギリス	Ayrshire	27人	浄水場周辺で牛糞撒布
'89年	イギリス	Swinden, Oxfordshire	516人	水道水（通常処理）が関与
'90〜91年	イギリス	Thanet	47人	水道水（通常処理）の処理不十分
'91年	アメリカ	Pennsylvannia	551人	塩素消毒のみの水道水
'91年	イギリス	South London	44人	水道水が関与
'92年	アメリカ	Oregon	43人 推計15,000人が感染	水道水（通常処理）の処理不十分
'93年	アメリカ	Milwaukee	285人 推計403,000人が感染	水道水（通常処理）の処理不十分
'93〜94年	アメリカ	Clark county Nevada	103人	水道水が関与 エイズ患者の感染で発覚
'94年	日本	神奈川県平塚市	12人 461人発症	ビルの簡易専用水道が関与 汚水が受水槽に混入
'96年	日本	埼玉県越生町	22人	町営水道（通常処理）が関与 町民の約70％に症状

感染環がある。これに対して，牛型は人獣共通感染症の病原体であり，ヒトとその他の哺乳類を含めた感染環が形成されている。患者から検出されたオーシストの遺伝子型を解析することで，感染経路をある程度推測することも可能である。

1. 食品を介した感染

食品を介して感染する事例は，食品そのものが汚染される以外に，調理者がクリプトスポリジウムを保有していたために食品を汚染したり，オーシストに汚染された水を用いて食品や器具・器材を洗浄することにより発生する。

1996年にアメリカ・ニューヨーク州において，特定の製造所のアップルサイダー（りんごの実入りジュース）に関連したクリプトスポリジウム症の集団下痢症が発生した。製造所ではリンゴや器材を井戸水で洗浄していたが，その製造所の向かいには酪農場があり，井戸水が汚染されていたことが疑われた。

1998年にはワシントンD.C.において，カフェテラスの感染従業者が準備した食品を摂食した人々の間に集団下痢症が発生した。その他の食品として，未殺菌乳の摂取によるクリプトスポリジウム症の発生が報告されている。野菜類や貝類からクリプトスポリジウムを検出したとする報告もあり，こう

した食品を非加熱で摂食することで感染する可能性がある。

2. 水系感染

クリプトスポリジウムについて公衆衛生上最も関心が高い問題は，水系感染による大規模な集団下痢症が発生することであり，他の原虫性感染症とは異なる大きな特徴を有している。水道水の微生物学的安全性は塩素消毒により確保されているが，クリプトスポリジウムのオーシストは塩素耐性を示し，通常の水道水の消毒に用いられている濃度ではまったく効果がない。

クリプトスポリジウムによる飲料水を介した水系感染は，欧米を中心にして世界中で1980年代前半から毎年のように発生したことが報告されている。主な事例を表－2に示した。1993年には，アメリカ・ミルウォーキーにおいて推定40万3,000人が感染・発症したとされる，史上最大の集団下痢症が起きている。わが国では，1994年に神奈川県平塚市の雑居ビルでの400人規模の集団発生と，1996年に埼玉県越生町の町営水道を介した推定9,000人弱の集団下痢症があった。

イギリスでは，毎年3,000〜6,000例のクリプトスポリジウム症が報告されているが，水系感染による患者が多くを占めていることが推測されている。患者の発生は，春と晩夏から

秋にかけてのピークの二峰性を示す。春のピークは牛型が優勢だが，秋のピークではヒト型が優勢である。春のピークは，この時期に牛や羊が出産し，多くの幼獣がクリプトスポリジウムに感染してオーシストを多数排出し，井戸や小規模水道が汚染されることが原因と推定されている。2001年に口蹄疫の大流行があり，約600万頭の牛が処分されたが，この年の春のピークは観察されず，患者に占める牛型の割合は減少した[4, 5]。ちなみに秋のピークが観察されるのは，夏休みに海外で感染し，帰国してからクリプトスポリジウム症の診断を受けるためとされている。

アメリカでもクリプトスポリジウム症発生の情報を収集しており，飲料水由来と親水施設（recreational water）における集団感染は分けて集計されている。CDCの発行するMMWRに掲載された報告によれば，1991〜1994年までの飲料水に関連した症例と親水施設関連の症例がともに8例であったが，1995年から飲料水に関連した事例は減少する傾向にある。しかし，親水施設関連の事例は逆に増えている。親水施設での感染は，プール，河川，湖沼などにおける水泳や，キャンプ場における河川水の飲水などが関連している。

わが国の河川などにおけるクリプトスポリジウムの汚染状況は，種々の調査により調べられているが，多くの河川や湖での汚染が明らかになっている。また，下水や畜産排水におけるクリプトスポリジウムオーシストの分布も調べられており，汚染源は下水や畜産排水が主体であるとされている。その他に野生動物が関わっていることも推測される。

免疫不全者における感染予防

クリプトスポリジウムはエイズ患者などの免疫不全者にとってはときに致死性となる非常に危険な病原体のひとつであり，感染予防に特別の配慮をしなければならない。海外ではエイズ患者の多くがクリプトスポリジウムに感染しており，アメリカでは5〜15%，イギリスでは21%とする報告もある。そのため，クリプトスポリジウムによる下痢が長く続く場合

にはHIV感染の可能性が高いとされ，エイズ発症の指標疾患のひとつとされている。わが国ではエイズ患者およびHIV感染者193例中2例（1.0%）がクリプトスポリジウムに感染していた[6]と報告されている。

CDCではHIV感染者/エイズ患者に対して，クリプトスポリジウム感染の予防方法として1．手を洗う，2．便に接するような性行為は避ける，3．家畜に触れない，4．ペットの便や容器などに触れない，5．食べ物を洗うか加熱調理する，6．水泳や入浴時に水を飲まないように注意する，7．安全な水を飲む，8．旅行中（特に発展途上国）は特別の注意を払う，という項目を挙げている。ペット動物の扱いについて「ペットを飼ってはならない」あるいは「ペットに触れない」とせずに，「ペットの便や容器などに触れない」としているのは，ペットと過ごすという精神面の利点を重視しているからである。

エイズ患者やその他の免疫不全者は，*C. parvum*のヒト型と牛型以外の様々な動物に由来するクリプトスポリジウムに感染する可能性がある。ペットとして飼育される動物の種類は近年ではイヌやネコ，その他の哺乳類にとどまらず，数多くの爬虫類や魚類をも入手することが可能であり，こうした動物に寄生するクリプトスポリジウムも知られている。免疫機能が低下している場合には，幅広い種類の動物との接触や糞便の始末には十分な注意を払う必要がある。

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Title
Quantification and genotyping of *Cryptosporidium* spp. in river water by quenching probe
PCR and denaturing gradient gel electrophoresis

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Abstract
A new detection method was developed for the simultaneous quantification and genotyping of
15   *Cryptosporidium* spp. in river water. Several modifications made to the U.S. EPA Method
1623 enabled high and stable recovery of *Cryptosporidium* from 40 L of river water
(geometric mean = 35%, standard deviation = 8.7%). Quenching probe PCR (QProbe PCR)
was used to quantify the 18S rRNA gene of *Cryptosporidium* spp. This method could
successfully detect single oocyst in a sample, and the lower quantitation limit was as low as
20   2.5 oocysts/sample. In addition, denaturing gradient gel electrophoresis (DGGE) followed by
DNA sequencing was used to identify the genotypes. These methods were applied to detect
Cryptosporidium spp. in the Koyama River, Japan. The positive ratio was 69% (11/16) with
the maximum concentration of 59 oocysts/100L. Seven genotypes including two novel ones
were identified. These results showed that this detection method could provide valuable
25   information of *Cryptosporidium* in river water both in the concentration and in the genotypes,
which is essential for the precise assessment of waterborne risk to human health.

Introduction

Genus *Cryptosporidium* is a pathogenic parasite that causes gastroenteritis in many mammals,
35   including human. Many *Cryptosporidium*-associated outbreaks have been reported in these
two decades (Craun et al., 1998, Solo-Gabriele and Neumeister, 1996, Fayer et al., 2000, Rose
et al., 2002). In Japan, a large outbreak associated with the microorgamism in drinking water
occurred in the town of Ogose in 1996, which forced Japanese water treatment facilities to
take an urgent action against this parasite (Japanese Ministry of Health, Labour and Welfare,
40   2001). It is therefore crucial to evaluate the level of contamination by *Cryptosporidium* in
drinking water.
Thirteen species have been reported in the genus *Cryptosporidium* with a variety of host
species (Xiao et al., 2004). Human infection occurs mostly with *C. hominis* and *C. parvum*,
and their infectivity is different among each species or sub-species (Okhuysen et al., 1999,
45   2002, Engelhardt and Swartout, 2004). Therefore, it is necessary to identify the species or the
genotypes of *Cryptosporidium* in each sample in order to evaluate the threat of waterborne
Cryptosporidium to human health.
Many attempts have done to detect *Cryptosporidium* in water. The U.S. EPA Method 1623

(1999) and the Standard Operating Protocols of Drinking Water Inspectorate (1999) are widely used for the detection. These methods are based on the immuno-fluorescent assays (IFA) and the microscopy-based counting, but these techniques have difficulty distinguishing between species of *Cryptosporidium*. Meanwhile, PCR-based genotyping approaches, such as restricted fragment length polymorphism (RFLP) and DNA sequencing, have been developed for discriminating the *Cryptosporidium* species in water samples. However, these genotyping methods lack in quantify the concentration of *Cryptosporidium* in water samples. There have been no reported methods for simultaneous quantification and genotyping of *Cryptosporidium* in a sample.

In this study, a new detection method was established to determine *Cryptosporidium* spp. in river water both in concentration and in the genotypes. Several modifications of the U.S. EPA Method 1623 (1999) were made to improve the recovery from large volume of river water. MF hollow fiber membrane was used for filtration was used for filtration of river water. Discontinuous density gradient centrifugation in a high density (1.20 g/cm^3) Percoll-sucrose solution was added prior to IMS. A new real-time PCR method, QProbe PCR, was developed to quantify the 18S rRNA gene of *Cryptosporidium* spp. in water samples. QProbe PCR is a real-time PCR method that utilizes a quenching probe (QProbe) (Kurata et al., 2001). This method is based on the characteristic of a fluorescent dye, BODIPY FL, that its fluorescence is quenched when it interacts with guanine (Torimura et al., 2001). To identify the species of *Cryptosporidium* present in the samples, denaturing gradient gel electrophoresis (DGGE) was used to isolate the DNA fragments prior to sequencing. The sequential combination of the QProbe PCR method, the DGGE method and DNA sequencing enabled the simultaneous quantification and genotyping of *Cryptosporidium* spp. in the same water sample.

This method was used in a field survey conducted in the Koyama River, a tributary of the Tone River, one of the main water sources for the drinking water in Tokyo Metropolitan area. *Cryptosporidium* in the river was analyzed both for the total concentration and for their genotype.

Materials and Methods

Sample collection

Water samples (23.8 - 66.1 L) were collected at the Koyama River, a tributary of the Tone River that is a major water source for waterworks in the Tokyo metropolitan area. Sixteen samples were collected from August to November 2004. Each water sample was filtered on site through a MF hollow fiber membrane filter (nominal pore size: 0.1 μm, effective filtration area: 4000cm^2, Mitsubishi Rayon Co., Ltd., Tokyo, Japan).

Concentration, Purification and DNA extraction

The particles remaining in the filter modules were recovered by two times of manually shaking the filter modules for one minute in 200 mL of elution buffer as specified in the U.S. EPA Method 1623 (1999), then the particles were concentrated by centrifugation for 15 min at 1,050 x g. The components of the concentrate were first separated by discontinuous density gradient centrifugation in a high density (1.20 g/cm^3) Percoll-sucrose solution for 10 min at 1,050 x g, followed by immunomagnetic separation (IMS) with an IMS kit (Dynabeads anti-*Cryptosporidium* kit, Dynal Biotech, Tokyo, Japan). The genomic DNA was extracted by five cycles of freezing at -80°C for 5 min followed by thawing at 95°C for 5 min in the presence of 25% w/v of Chelex 100 resin (Bio-Rad, Tokyo, Japan). The resin was removed by filteration through a MF centrifugal filtering devices (GHP Nanosep MF centrifugal device,

Pall, Tokyo, Japan). The extracted DNA was purified using a commercial kit (QIAamp DNA Mini Kit, QIAGEN, Tokyo, Japan) and further concentrated by a UF centrifugal filter unit (Microcon YM-100, Millipore, Tokyo, Japan). The final volume of the concentrate was
100   approximately 10 μL.

Quantification of *Cryptosporidium* spp. by QProbe PCR
QProbe PCR, targeting approximately 1,280 bp of the 18S rRNA gene of *Cryptosporidium* spp. was used to quantify the extracted DNA. Primers were selected from those developed by
105   Xiao et al (1999ab). Since their primers were originally for the nested PCR, the forward primer for the second PCR (5'-GGA AGG GTT GTA TTT ATT AGA TAA AG-3') and the reverse primer for the first PCR (5'-CCT GCT TTA AGC ACT CTA ATT TTC-3') were selected based on the amplification efficiency of preliminary experiments (data not shown). The sequence of the QProbe was developed in this study. Sequences of *Cryptosporidium* spp.
110   that have the identical region to both the forward and reverse primer were listed from the DDBJ database (Miyazaki et al., 2003) using NCBI-BLAST 2.0 (Altschul et al., 1997). The regions that were common to all selected sequences were obtained using ClustalW 1.7 (Thompson et al., 1994). Since QProbe PCR method utilizes the characteristic of BODIPY FL dye that its fluorescence is quenched by interacting with a guanine, QProbe must have a
115   BODIPY FL-labeled cytosine at either of its 5'- or 3'- end. The sequence of the QProbe (5'-CGA ACC CTA ATT CCC CGT TAC CC-BODIPY FL-3') was then developed concerning the melting temperature of the probe and the generative capacity of the primer-dimer. The QProbe was obtained from a commercial laboratory (J-Bio 21, Tsukuba, Japan).
120   The LightCycler System (Roche Diagnostics, Tokyo, Japan) was used for the real-time PCR. The PCR mixture (20 μL) contained 0.4 U of DNA polymerase (KOD -Plus-, TOYOBO, Osaka, Japan), 2 μL of 10 x PCR buffer, 200 nM each dNTPs, 1 mM $MgSO_4$ and 0.25 mg/mL BSA. The concentrations of the forward and reverse primer, and the QProbe, were 600 nM, 200 nM and 100 nM, respectively. PCR conditions consisted of an initial hot-start for 5 min at
125   94°C; 60 cycles of 30 sec at 94°C, 30 sec at 57°C and 75 sec at 68°C; and a final extension of 5 min at 68°C. After PCR, the data were analyzed using a program provided by the QProbe manufacturer (J-Bio 21, Tsukuba, Japan).

Genotyping *Cryptosporidium* spp. by PCR-DGGE and DNA sequencing
130   Nested PCR was conducted for the products of the QProbe PCR, with primers targeting the 18S rRNA gene of *Cryptosporidium* spp. (Morgan et al., 1997). A GC clamp (5'-CGC CCG CCG CGC GCG GCG GGG GCG GGG GCA CGG GGG G-3', Muyzer et al. 1993) was added at the 5'-end of the forward primer (5'-AGT GAC AAG AAA TAA CAA TAC AGG-3'). The reverse primer (5'-CCT GCT TTA AGC ACT CTA ATT TTC-3') was used
135   without any modification. The PCR mixture (50 μL) contained 1 U of DNA polymerase (AmpliTaq Gold, Applied Biosystems, Tokyo, Japan), 5 μL of 10 x PCR buffer, 200 nM each dNTPs and 2 mM $MgCl_2$. The concentration of both the forward and reverse primer was 500 nM. PCR conditions consisted of an initial hot-start for 5 min at 94°C; 30 cycles of 30 sec at 94°C, 30 sec at 60°C and 30 sec at 72°C; and a final extension of 5 min at 72°C.
140   DGGE of the PCR products was performed using the DCode mutation detection system (Bio-Rad, Tokyo, Japan). Denaturant gradient gels (5 to 25%) containing 8% (w/v) acrylamide were formed (100% denaturant is defined as 7 M urea and 40% (v/v) formamide). The gels were electrophoresed at 130 V and 60°C for 10 hours. The gels were stained with Vistra Green (GE Healthcare, Tokyo, Japan) and the stained gels were analyzed using

145 Fluorimager 595 (GE Healthcare, Tokyo, Japan). After DGGE, each band was cut out of the DGGE gel and the DNA fragments were sequenced using ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Tokyo, Japan).

Recovery efficiency of the filtration, concentration and purification methods

150 Three river water samples (40 L each) were collected at the Tone Division Weir. The turbidity of the water was 6.1 NTU. Oocysts of *Cryptosporidium parvum* (ColorSeed, BTF, New South Wales, Australia) were seeded before the filtration. The filtration, concentration and purification methods were described above. As the seeded oocysts were gamma-inactivated, the number of recovered *Cryptosporidium* was counted using a fluorescent microscope (BX60,

155 OLYMPUS, Tokyo, Japan) after staining with EasyStain (BTF, North Ryde, Australia).

Detection limit and quantitation limit of the QProbe PCR method

The detection and quantitation limits of the QProbe PCR method were determined using known concentration (0.83 - 8300 oocysts/PCR tube) of the genomic DNA of

160 *Cryptosporidium parvum* IOWA isolate (Waterborne, New Orleans, Los Angeles). The concentration of oocysts in the stock solution was determined by microscopy (BX60, OLYMPUS, Tokyo, Japan). The genomic DNA was extracted by the method described above, and the extracted DNA was diluted with de-ionized water to the required concentration. The Ct values are usually unstable when the initial concentration is very low, resulting in the

165 low quantifiability. Thus, the quantitation limit of the QProbe PCR method was evaluated separately from the detection limit. The detection limit was defined as the lowest initial concentration at which all samples were successfully detected. The quantitation limit was defined based on the ratio of the detected concentration to the seeded concentration of each sample. The quantifiability at a certain concentration was approved when the confidence

170 interval of the ratio was within 50% to 200%, corresponding to the difference of approximately one PCR cycle.

Discriminating capability of the DGGE method

DGGE was conducted using the genomic DNA of nine species of *Cryptosporidium*, *C.*

175 *parvum* (AF161856), *C. hominis* (AF093491), *C. canis* (AF112576), *C. meleagridis* (AF112574), *C. felis* (AF112575), *C.* sp. strain 938 (AY120913), *C. andersoni* (AB089285), *C. serpentis* (AF093502) and *C. saurophilum* (AF112573), to evaluate the ability to discriminate each species/genotype. The amplification of the DNA and the DGGE method was conducted following the procedure described above.

180

Results and Discussions

Recovery efficiency of the filtration, concentration and purification methods

The recovery efficiency of the filtration, concentration and purification method was 35% (n =

185 3, S.D. = 8.8%). Considering the large volume (40 L) and turbidity (6.1 NTU) of the tested river water, the method developed in this study showed comparable recovery efficiency. Quintero-Betancourt et al. (2003) reported high recovery (57%) of Cryptosporidium from 100 L of tertially treated wastewater with low turbidity of less than 2.5 NTU. Lee et al. (2004) reported that the recovery from reference sediment samples corresponding to 65 - 71 L of

190 surface water was 38.0%. In these reports, Envirochek HV capsule filter (Pall) was used for filtration, and the U.S. EPA Method 1623 (1999) was basically followed.

Detection limit and quantitation limit of the QProbe PCR method

The seeded DNA was successfully detected from all samples regardless of the initial
concentration (8300 - 0.83 oocysts/sample). Therefore, the detection limit of this method was
found to be less than the lowest concentration tested, 0.83 oocysts/sample. This result shows
that single oocyst can be successfully detected by using this method. The confidence interval
of the ratio of the detected concentration to the seeded concentration at each initial
concentration (Figure 1) demonstrated that the lower quantitation limit of this method was 2.5
oocysts/sample. These detection and quantitation limits were comparable to the real time PCR
methods using TaqMan probe and the LightCycler hybridization probes (Higgins et al., 2001,
Fontaine and Guillot, 2002, Limor et al., 2002, Tanrıverdi et al., 2002, Guy et al., 2003).

Discriminating capability of the DGGE method

The bands from all tested species were appeared at the denaturant gradient between 8% and
20% (Figure 2). Since the band positions of nine species were different from each other, it
was approved that this DGGE method could effectively isolate the DNA fragments of
multiple *Cryptosporidium* spp. in a sample. This result also suggests that by using the DNA
fragments from typical species as a marker, the genotypes in a sample could be determined
without the sequencing analysis. Previously, cloning was widely used for isolation of the
DNA fragments of *Cryptosporidium*, but it is not cost-effective, because it often requires
many samples to be sequenced. The DGGE method could reduce the number of samples for
sequencing to identify all genotypes in a sample, because of the preceding isolation of DNA
fragments by the electrophoresis.

Concentration and Genotypes of *Cryptosporidium* in the Koyama River

Cryptosporidium was detected from 11 samples (69%) out of 16 samples collected from the
Koyama River (Table 2). Two samples contained *Cryptosporidium* more than the lower
quantitation limit (2.5 oocysts/sample), and the maximum concentration was 59 oocysts/100L.
Seven genotypes (*C. hominis*, *C. parvum*, *C. andersoni*, *C.* sp. 938, *C.* sp. PG1-26, *C.* sp. t03,
C. sp. t04) including two novel ones (*C.* sp. t03 and *C.* sp. t04) were identified. The most
frequently detected species was *C. andersoni* (7 samples), followed by *C.* sp. 938 (4 samples)
and *C. parvum* (2 samples). The human-specific species, *C. hominis*, was detected from only
one sample, showing that risk assessment based on quantitative data without information on
the genotypes may lead to an overestimation of the risk to human health. Five samples
contained multiple genotypes, suggesting that the direct sequencing of the PCR product may
overlook the existence of "other" genotypes.

Conclusion

This sequential combination of the QProbe PCR method, the DGGE method and the DNA
sequencing enabled the simultaneous quantification and genotyping of *Cryptosporidium* spp.
in the same water sample. It is known that not all species are infective to human, and the
infectivity varies according to the species and sometimes the sub-species. Therefore, from the
viewpoint of assessing waterborne risk to human health, it is essential to determine not only
the concentration of *Cryptosporidium* in water samples but also the species and genotypes.
The detection method developed in this study is promising to obtain the valuable information
for the precise assessment of the waterborne microbial risk to human health.

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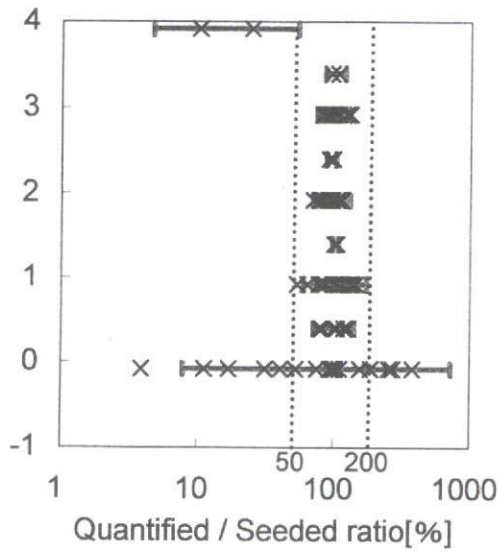
Figure 1. The confidence interval of the ratio of quantified concentration to the seeded concentration. The grey bar shows the 95% confidence interval of the Quantified / Seeded ratio. If this bar was within the dotted lines, the quantifiability at the concentration was approved.
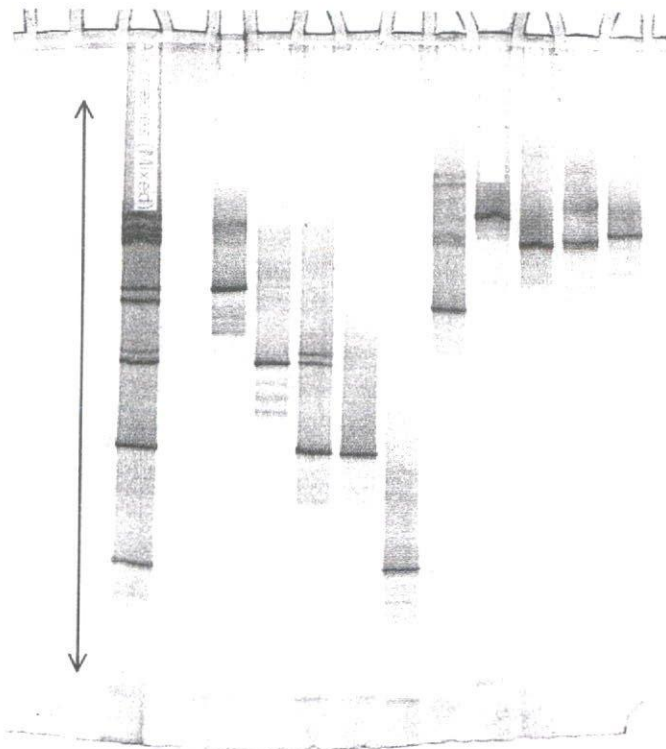
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Figure 2. Discrimination of *Cryptosporidium* spp. by DGGE.

Table 1. The concentration and the detected species in the Koyama River.

Sampling time	Sample volume [L]	Detection [a]	Detected genotypes [b]
3rd Aug., 11:00	45.2	−	
3rd Aug., 12:30	32.0	−	
3rd Aug., 14:00	43.3	+	*C. hominis, C.* sp. 938
3rd Aug., 15:30	33.5	+	*C.* sp. 938
26th Aug., 11:00	56.3	−	
26th Aug., 12:30	43.1	−	
26th Aug., 14:00	37.8	−	
26th Aug., 15:30	65.1	+	*C. andersoni*
16th Sep., 10:30	41.6	+	*C. andersoni*
16th Sep., 12:00	47.4	+	*C. parvum, C.* sp. 938
16th Sep., 13:30	47.4	+	*C. andersoni*
16th Sep., 15:00	66.1	+	*C.* sp. PG1-26
27th Oct., 10:45	30.9	59	*C. andersoni, C.* sp. 938, *C.* sp. t03[c], *C.* sp. t04[d]
27th Oct., 12:15	53.4	+	*C. andersoni*
27th Oct., 13:45	23.8	+	*C. andersoni, C. parvum*
27th Oct., 15:15	44.9	14	*C. andersoni*

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a. − is for samples negative for *Cryptosporidium*, + is for samples positive for *Cryptosporidium*, but the concentration was less than the quantitation limit (<2.5 oocysts/sample) and values represents the quantified concentration in oocysts/100L.

b. The accession number of each species is; *C. parvum* (AF164102), *C. hominis* (AY204231), *C. andersoni* (AB089285), *C.* sp. 938 (AY120913), *C.* sp. PG1-26 (AY271721), *C.* sp. t03 (AB231612) and *C.* sp. t04 (AB231613).

c. A novel genotype with the sequence similar to *C.* sp. 1665 (AY120915, identity = 97%).

d. A novel genotype with the sequence similar to *C.* sp. 1665 (AY120915, identity = 97%).

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Effects of Sunlight, Temperature and Suspended Solids on Inactivation of Poliovirus in River Water

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INTRODUCTION

A huge number of enteric viruses usually exist in sewage. Discharges from wastewater treatment plants would be contaminated with the viruses because removal and inactivation efficiencies of viruses by the conventional wastewater treatment are low. Actually many researchers reported enteric viruses were frequently detected in the river receiving the discharges. Therefore, it is needed to evaluate the risk of infection by the viruses in order to utilize the contaminated river as the water resource. The viral inactivation in the river which is the key factor to evaluate the infectious risk is affected by some water qualities (e.g., suspended solids (SS), organic matters, chlorides) and physical conditions (e.g., water temperature, sunlight, velocity). The objective of this study is to evaluate the inactivation of poliovirus 1 in the river water, focusing on effects of ultra violet (UV) in sunlight, temperature and SS.

MATERIALS AND METHODS

Concentrations of SS and dissolved organic carbon in the river water employed in the experiment were 33.5 and 2.47 mg/L, respectively. Poliovirus 1 (Sabin strain) was inoculated into 15mL sterilized centrifuge tubes (CORNING, USA) with the river water so that the viral concentration was approximately 10^3 PFU/mL. The tubes were exposed to sunlight in the thermostatic water bath (CB-301, AS ONE, Japan) under the condition that the water temperature was 4, 10 or 20°C. The dose of UV in sunlight was measured with the potable UV meter (UV caremate, FUJI ZEROX, Japan). The river water with poliovirus 1 at the same concentration was also inoculated into the tubes covered with aluminum foil and put in the thermostatic water bath to evaluate the viral inactivation under the dark condition. Viral concentrations in the tubes were measured several times during the experiment. Before the measurement, the samples were filtrated with the membrane filter (Millex-FG 0.22µm Filter Unit, Millipore, USA) to remove substances interfering with the viral detection with cell line. Viruses adsorbed to SS were also removed by the filtration. The concentration of viruses in the free state after the filtration was measured with BGM cell line. In the same manner, the inactivation of poliovirus 1 in the river water without SS was also evaluated by using the river water filtrated with the membrane filter (Millex-FG 0.22µm Filter Unit, Millipore, USA). As above-mentioned, inactivations of poliovirus 1 in the river water at 4, 10 and

20°C were evaluated under the following four conditions of SS and sunlight:

RW(L): the experiment in the raw river water under the light condition,

RW(D): the experiment in the raw river water under the dark condition,

FRW(L): the experiment in the filtrated river water without SS under the light condition, and

FRW(D): the experiment in the filtrated river water without SS under the dark condition.

RESULTS AND DISCUSSIONS

Effect of temperature on inactivation of free poliovirus

Figure 1 shows the inactivation of free poliovirus 1 which was not adsorbed to SS in the raw river water. The vertical axis means the viral survival ratio (S) calculated by the following formula:

$$S = C_{R,L}/C^0_{R,L}$$

where, $C_{R,L}$ is the concentration of poliovirus 1 in the raw river water under the light condition (RW(L)) and $C^0_{R,L}$ is the initial concentration. As shown in Figure 1, the survival ratio exponentially decreased with the accumulation of UV dose. This inactivation processes could be expressed with the following function:

$$S = \exp(-kD)$$

where, D is the accumulated dose of UV (J/cm^2) and k is the coefficient of viral inactivation (cm^2/J). The inactivation coefficients were estimated as 0.038, 0.053 and 0.076 cm^2/J at 4, 10 and 20°C, respectively. Based on the estimation, it is concluded that UV doses needed to inactivate 90% of
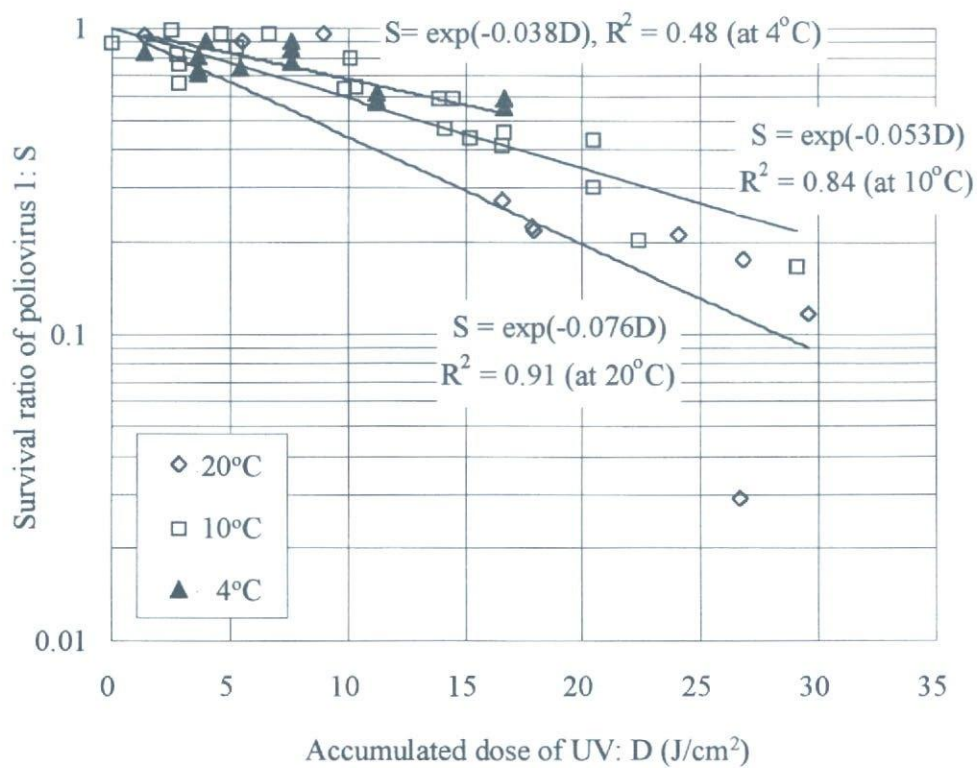

Figure 1. Inactivation of free poliovirus 1 in the raw river water.

poliovirus 1 are 60.6 (4°C), 43.4 (10°C) and 30.3 J/cm^2 (20°C), respectively. The UV dose of 30.3 J/cm^2 at 20°C is equivalent to that accumulated by the summer sunlight only for two hours in the mid-latitude region.

Amount of poliovirus adsorbed to suspended solids

The amount of poliovirus 1 adsorbed could be evaluated as the difference between the viral concentration under the dark condition in the filtrated river water (FRW(D)) and that in the raw river water (RW(D)). The average amount of viruses adsorbed to suspended solids were 1.2 x 10^3, 1.3 x 10^3 and 2.1 x 10^3 PFU/mg-SS at 4, 10 and 20°C, respectively. However, the hypothesis that the water temperature affected the adsorption of poliovirus 1 to suspended solids was rejected by t-test with the significant level of 5%. It was reported that the amount of poliovirus 1 adsorbed to silicate minerals ranged from 64.2 x 10^4 to 89.5 x 10^4 PFU/mg-minerals. The ability of suspended solids in the river to adsorb the virus was about 500 times lower than that of silicate minerals.

Effect of suspended solids on inactivation of free poliovirus

The difference between the concentration of poliovirus 1 in the filtrated river water under the dark condition (FRW(D)) and that under the light condition (FRW(L)) indicates the amount of viruses inactivated by UV without the effect of SS. The amount of viruses inactivated was proportional to the accumulated dose of UV under all conditions of water temperature (Table 1). Also in the presence of SS, the amount of viruses inactivated linearly increased with the accumulation of UV dose. Regardless of the temperature, the viral inactivation rate defined as the slope of the regression line in the presence of SS was lower than that in the absence due to the cutoff of sunlight by SS.

Table 1. Results of linear regression analysis for the inactivation of free poliovirus 1 in the river.

Temperature	River water	Regression line*	R^2
20°C	Raw (with SS)	Y = 22.2D	0.66
	Filtrated (without SS)	Y = 24.2D	0.77
10°C	Raw (with SS)	Y = 15.8D	0.86
	Filtrated (without SS)	Y = 16.7D	0.76
4°C	Raw (with SS)	Y = 15.6D	0.22
	Filtrated (without SS)	Y = 17.8D	0.60

*Y is the amount of viruses inactivated by UV (PFU/mL) and D is the accumulated dose of UV (J/cm^2).

CONCLUSIONS

1) Survival ratio of poliovirus 1 in the river water exponentially decreased with the accumulation of UV dose. Coefficients of viral inactivation at 4, 10, 20°C were 0.038, 0.053 and 0.076 cm^2/J, respectively.

2) Suspended solids in the river could adsorb the amount of poliovirus 1 between 1.2×10^3 and 2.1×10^3 PFU/mg-SS regardless of water temperature. Its ability to adsorb the virus was about 500 times lower that of silicate minerals.

3) In the presence of suspended solids, poliovirus 1 would be protected from the inactivation by UV due to the cutoff of sunlight.