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Cryptosporidium monitoring system at a water treatment plant, based on waterborne risk assessment

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Abstract The water volume required for daily monitoring of *Cryptosporidium* (which can statistically ensure an annual risk of infection below 10^{-4}), was assessed by evaluating the applicability of the Poisson lognormal (PLN) distribution in microbial risk assessment. PLN showed as good a fit to the observed data as to the negative binomial distribution. From the estimated PLN distributions for the source and finished water, the efficacy of the oocyst removal by the conventional water treatment process was estimated to follow log-normal distribution (median = 3.16 $\log_{10}$, 95% CI = 4.27–2.05 $\log_{10}$). The 365 consecutive negative results of daily monitoring for 180 L of finished water were found to be statistically equivalent to the annual risk of infection below 10^{-4}. This research also suggested the possibility of applying a qualitative detection method, such as CC–PCR, as a routine monitoring method for the quantitative risk management.

Keywords *Cryptosporidium*; monitoring system; Poisson log-normal distribution; risk assessment; treatment efficacy

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Introduction

Since the 1990s, cryptosporidiosis has become a worldwide concern as an emerging infectious waterborne disease. In order to manage the risk due to drinking water and to assure its safety, it is necessary to analyse the risk of infection quantitatively. However, quantitative microbial risk assessment has several difficulties. For example, monitoring data for microbes may contain some (sometimes many) "undetected" results, due to very low concentrations of microbes in water. In this study, the monitoring data of *Cryptosporidium* observed at a water treatment plant in Japan were analysed to assess the risk of waterborne infection as well as to estimate the removal efficacy of the conventional water treatment. Poisson log-normal distribution (PLN) was adopted as one of the possible distributions of the counts in a water sample and its usefulness was evaluated from the viewpoint of waterborne risk assessment. Additionally, a volume of water sufficient for daily monitoring was also tested to investigate the possibility of ensuring that the annual risk remained at an acceptable level.

Materials and methods

Observed data for *Cryptosporidium* in surface and finished water

The monitoring data analysed in this study were obtained by Hashimoto *et al.* (2002). Water samples (81) were collected at a water treatment plant in Japan from 1998 to 2002; 27 were collected from the influent surface water and 54 from the finished water (after coagulation, flocculation, sedimentation and rapid sand filtration). The sample volume was <100 L for surface water and 500–4,275.6 L for finished water. Figure 1 shows the log-normal probability plot of the observed data. *Cryptosporidium* was detected from all surface water samples, and its geometric mean was 481 oocysts/1,000 L. However, half of the

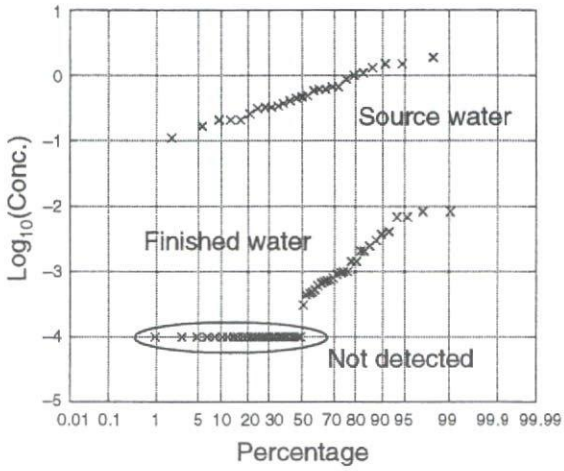

Figure 1 The observed data of *Cryptosporidium*

finished water samples (27/54) had no oocysts detected. The geometric mean of the detected samples was 1.39 oocysts/1,000 L.

Distribution of oocyst concentration in water

The best distribution to characterise the concentration of *Cryptosporidium* in water was selected from among the Poisson distribution, the Poisson log-normal distribution and the negative binomial distribution, using the counted data of the source and purified water. Assuming the oocyst concentration to be constant, and the variability in counts being simply due to the randomness in sampling, the probability that a sample of volume (VCL) contains X oocysts is given by the Poisson distribution. The negative binomial distribution (NB) has also been used to describe the count data of *Cryptosporidium* (Teunis *et al.*, 1997). This distribution was derived as a γ mixture of Poisson distributions (Greenwood and Yule, 1920). The Poisson log-normal distribution (PLN) was selected when the concentration of oocysts was supposed to follow the log-normal distribution. The probability distribution function is:

$$\mathrm{PLN}(n = X) = \int_0^{00} \frac{(mV)^x \exp(-mV)}{X} \frac{1}{m\sigma\sqrt{2\pi}} \exp \frac{-(\ln(m)-\mu)^2}{2\sigma^2} dm \qquad (1)$$

where μ and σ are the parameters for the log-normal distribution and V(L) was the volume of the sample. Since this function has no analytical solution (Shaban, 1988), it was evaluated by numerical integration using Simpson's Rule. A computer-based program was developed to calculate this probability function. The accuracy of the program was confirmed by comparing the results with the table established by Brown and Holgate (1971). Best-fit parameters of each distribution were obtained by the most-likelihood method. Most likelihood estimates (MLEs) were obtained by minimising the following likelihood function.

$$\mathrm{LF}(\theta) = -2\sum \ln(P(x_i, V; \theta)) \qquad (2)$$

where $P(x_i, V_i; \theta)$ is the probability function for the ith sample (x_i oocysts in V_i (L) of sample) with a parameter set θ.

Comparison between PLN and LN

The discreteness of count data caused problems in fitting the observed data accurately to continuous distributions such as log-normal distributions. For example, the concentration

of the sample was calculated by dividing the number of detected oocysts by the volume of sampled water. However, due to the spatial heterogeneity and the discreteness of the counted data, the "calculated" concentration was not always equal to the "real" concentration, especially when the concentration was very low and only a few oocysts were found in the samples. Additionally, the sample for which no oocysts were detected (so-called "not detected" or "ND") cannot be directly applied to the log-normal distribution, because this distribution is only defined for positive values. Accordingly, such data should be substituted by some assumptions (Haas and Scheff, 1990). One possible assumption was to treat ND data as containing 0.5 oocysts in the tested volume. Another possibility was to use the cumulative probability up to the concentration of 1 oocyst/sample as a substitution. The effectiveness of these assumptions was evaluated by comparing the parameters with those derived from PLN. The MLEs were obtained in the same way as the discrete distributions, excluding the case where the "calculated" concentration was assumed to be the "real" concentration.

Annual risk of infection

The annual risk of infection caused by drinking the finished water was assessed using Monte Carlo simulation. The estimated distribution of *Cryptosporidium* concentration in the finished water was used to determine the quality of the drinking water. The amount of water consumed daily was assumed either to follow a log-normal distribution whose median value was 0.153 L/d/person ($\mu = -1.88$, $\sigma = 1.12$) according to Teunis *et al.* (1997) or to be constant at 1 L/d/person. The former was developed to assess the occurrence of infection. The latter was developed to assess the safety of the finished water. It was assumed that consuming 1 L of tap water, which is approximately the 95th percentile of the distribution for the daily consumption of tap water that has not been boiled or heated (Teunis *et al.*, 1997; Yano *et al.*, 2000), should be assured to be safe. All oocysts were assumed to be equally viable and infective to humans, regardless of their genotypes, because no information on the strain or the genotype was available by microscopic count. The effect of chlorination was neglected. The dose-response model was derived from Haas *et al.* (1996). The Monte Carlo simulation was conducted 10,000 times to achieve the distribution of the annual risk of infection.

Required water volume for daily monitoring

The maximum annual risk, assurable by daily monitoring of the finished water, was evaluated to develop the monitoring procedure to assure the safety of tap water. This assessment was based on the results of the risk assessment derived from the constant (1 L/d/person) water consumption model. The acceptable annual risk of infection was set as 10^{-4}. The monitoring was conducted on the finished water every day (365 times/year) to assess the water quality of the year. "All negative" (i.e. 365 consecutive negative results) was adopted as a water-quality criterion. For comparison, the same evaluation was carried out using the log-normal distribution to evaluate the possibility of the application of log-normal distribution instead of PLN. In this case, the cumulative probability <1 oocyst/sample was used as the probability of "no detection".

Results and discussion

Distribution of the oocyst concentration in water

Table 1 shows the MLEs for each distribution, and Figure 2 compares the estimated distribution and the observed data of the source water. Comparing the LF among these three distributions, Poisson distribution showed the poorest fit when compared with the others. It, therefore, indicated that the oocyst concentration in water was not constant but variable.

Table 1 Best-fit parameters and the likelihood function for each distribution

Distribution	Source water (oocysts/L)		Finished water (oocysts/L)	
	Parameter(s)	LF(θ)	Parameter(s)	LF(θ)
Poisson	$\lambda = 0.624$	978.7	$\lambda = 1.23 \times 10^{-3}$	410.1
PLN	$\mu = -0.720$, $\sigma = 0.709$, $\alpha = 0.293$	268.9	$\mu = -8.01$, $\sigma = 1.71$, $\alpha = 3.48 \times 10^{-3}$	207.4
NB	$\beta = 2.14$	270.6	$\beta = 0.333$	205.6

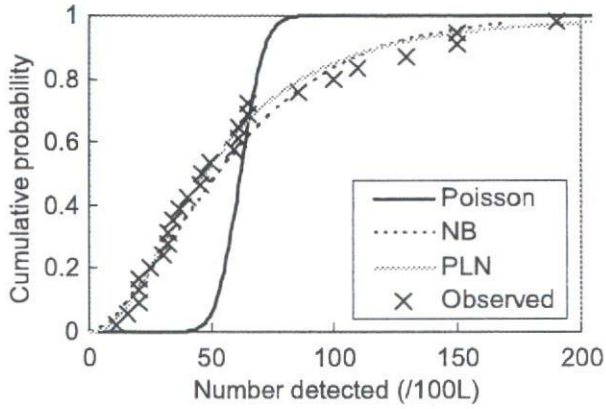

Figure 2 Estimated distributions and the observed data of the source water concentration

However, it was difficult to judge which was better, PLN or NB. In this study, PLN was adopted for the concentration distribution of *Cryptosporidium* in water. The median and the 95th percentile of the concentration of the source water and the finished water were 487, 1,562, 0.33 and 5.56 oocysts/1,000 L respectively.

Comparison between PLN and LN

Table 2 shows the best-fit parameters and the arithmetic mean of each distribution. For the source water there was no difference between the two methods of substitution, because all samples were positive (i.e. there was no need for substitution) and little difference was observed between the results of the LN and PLN. However, the PLN showed a lower median value (μ = –8.01 for PLN and –7.39 or –7.67 for LN) and a higher variability (σ = 1.71 for PLN and 1.14 or 1.46 for LN) for the purified water. This was due to the substitution of all ND samples with the same concentration, which reduced the variability of the concentration. The arithmetic mean of the PLN was found to be larger (1.44 oocysts/1,000 L) than those of the LN (1.18 or 1.35 oocysts/1,000 L) suggesting that the substitution of the concentration could lead to the underestimation of the annual risk of infection.

Efficacy of the treatment

The estimated value of σ (Table 1) for the concentration of source and purified water was 0.71 and 1.71 respectively; this suggested that the purified water showed a greater variabil-

Table 2 Evaluation of the effect of substituting N.D. data

	Source water (oocysts/L)			Finished water (oocysts/L)		
	μ	σ	Arith. Mean	μ	σ	Arith. Mean
0.5 oocyt	–0.731*	0.727*	0.627*	–7.39	1.14	1.18×10^{-3}
Cumul. Prob.				–7.67	1.46	1.35×10^{-3}
PLN	–0.720	0.709	0.626	–8.01	1.71	1.44×10^{-3}

*No difference due to the absnece of N.D. samples.

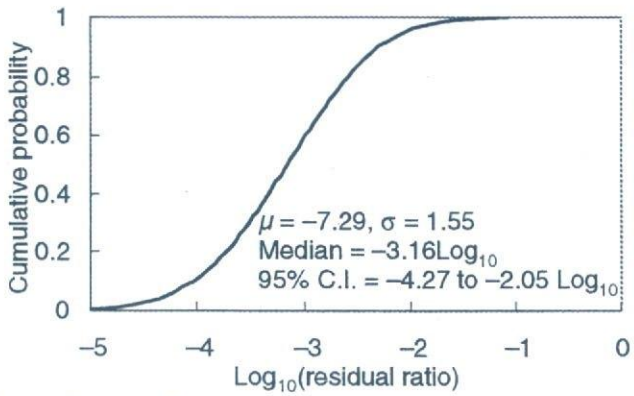

Figure 3 Distribution of treatment efficacy

ity than the source water. Therefore, it can be considered that the efficacy of Cryptosporidium removal by conventional water treatment was also variable. Based on this assumption, the distribution of the remaining ratio was estimated from the difference between the estimated distributions of the source and the finished water concentration.

According to the reproducibility of the normal distribution, the remaining ratio can be assumed to follow log-normal distribution, and the parameters μ and σ can be calculated by those of the concentration of the surface and finished water. Figure 3 shows the estimated distribution of the remaining ratio for the conventional water purification. The parameters were $\mu = -7.29$ and $\sigma = 1.55$. It was clear that the treatment efficacy was very variable (median -3.16 $\log_{10}$, 95% CI -4.27 to -2.05 $\log_{10}$).

Annual risk of infection

Figure 4 shows the distribution and statistics of the natural logarithm of the annual risk of infection for each model. The median and the 95th percentile of the annual risk were found to be (a) -3.26 $\log_{10}$ (1 case in 1,820 people) and -2.58 $\log_{10}$ (1 case in 1,295 people) for the first case (log-normal distributed water consumption) and (b) -2.69 $\log_{10}$ (1 case in 488 people) and -2.58 $\log_{10}$ (1 case in 384 people) for the second case (constant water consumption) respectively. The variability seemed to be very small for both cases, partly because the distribution of the finished water was directly used as an input, i.e. no additional assumptions (such as the efficacy of the water treatment) were required.

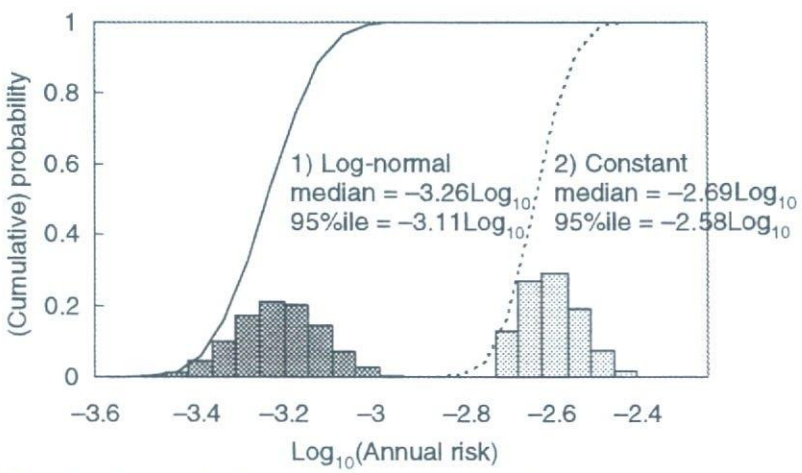

Figure 4 Cumulative frequency distribution of calculated annual risk of infection

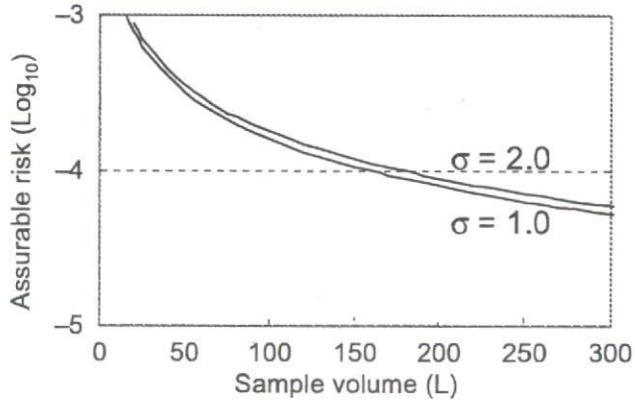

Figure 5 Assurable annual risk of infection by daily monitoring

Required water volume for daily monitoring

In this assessment, the result of the risk assessment derived from the second model (constant water consumption) was applied to calculate the required water volume for the daily monitoring of the finished water. First, the relationships between the parameters of the log-normal distribution (μ and σ) and the expected value of the annual risk of infection were evaluated. The point estimation was conducted because the range of the annual risk of infection was very small (95% C.I. = −2.79 to −2.58 $\log_{10}$). The arithmetic mean of log-normal distributions was given by:

$$\text{A.M.} = \exp(\mu + \sigma^2/2) \tag{3}$$

Thus, the equation of the relationship between the parameters was found to be:

$$\mu = \frac{\sigma^2}{2} \leq -9.64 \tag{4}$$

The range of the parameter σ was set between 1.0 to 2.0 (corresponding to 50 to 2,540 of the ratio of the 95th percentile to the 5th percentile of the distribution), which is supposed to cover all possible variability of the oocyst concentration in water. Under these conditions, the annual risk of infection could be calculated as a function of the sampling volume and the positive ratio.

Figure 5 shows the relationships between the sampling volume and the arithmetic mean of the annual risk without any positive results. As a result, negative results for all 180 L of the finished water samples were found to be needed to assure an annual infection risk of less than 10^{-4}. However, when LN was directly used to calculate the detection probability, the required sample volume increased up to 2,473 L, which was more than 13× as large as the result obtained by PLN. This result indicated that the calculation of the detection probability using a continuous distribution, such as a log-normal distribution, could lead to the overestimation of the volume required for daily monitoring to assure water safety.

Conclusions

In this study, the water volume necessary for daily monitoring was determined to assure that the annual risk of *Cryptosporidium* infection *via* tap water was at an acceptable level. The Poisson log-normal distribution showed just as good a fit to the observed data of *Cryptosporidium* in source and finished water as did the negative binomial distribution. PLN showed a better fit for the source water, while NB was better for the finished water. Comparing the fitted distributions, PLN was found to give higher probability for high con-

centration (Figure 2), suggesting that the PLN may have led to more conservative estimates for the annual risk assessment. The distribution of the treatment efficacy of the conventional water treatment process was found to follow a log-normal distribution with a median value of $-3.16\ \log_{10}$ (95% CI = -4.27 to $-2.05\ \log_{10}$).

Using the PLN distribution fitted to the concentration of the finished water, the maximum annual risk, assurable by 365 successive negative results, was computed. A water volume of 180 L was found to assure an annual risk of infection of below 10^{-4}. For comparison, the same analysis was conducted using the cumulative probability function of the LN. The required water volume was found to be 2,473 L/d, suggesting that the use of cumulative probability of the continuous distribution for the probability of detection could lead to the overestimation of the volume required for daily monitoring.

Microscopic observation of *Cryptosporidium* has sometimes been pointed out to be unfavourable, because little information can be obtained on the viability and the genotype of the detected oocysts, despite considerable investment in terms of skills, time and cost. The monitoring procedure developed in this study does not require any quantitative information, suggesting the possibility of using molecular techniques, such as cell culture PCR, as the routine monitoring method for *Cryptosporidium* in water.

Acknowledgements

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Molecular Characterization of a Single *Cryptosporidium* Oocyst in Sewage by Semi-Nested PCR

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Keywords: Cryptosporidium, Genotype, nested PCR

Abstract This study demonstrates molecular characterization of a single *Cryptosporidium* oocyst isolated from sewage. Using glass capillaries, we manipulated an IFA-stained single *Cryptosporidium* oocyst from purified sewage concentrate under inverted epifluorescence microscopy. Each singly isolated *Cryptosporidium* oocyst was analyzed by 18S rRNA gene based semi-nested PCR and direct sequence. A total of 56 singly isolate oocysts were characterized successfully as 51.8% (29 isolates) of *C. parvum* genotype 1, 17.9% (10 isolates) of *C. meleagridis*, 12.5% (7 isolates) of *C.parvum* genotype 2, 8.9% (5 isolates) of *C. parvum* isolated from pig, 7.1% (4 isolates) of *C. parvum* isolate VF383 and 1.6% (1 isolate) of *C. parvum* isolated from mouse. Results of this study demonstrate that 18S rRNA based semi-nested PCR and direct sequence method can be used to characterize a single *Cryptosporidium* oocyst from sewage with high sensitivity. Furthermore, this method revealed distribution of species and genotypes of *Cryptosporidium* in a water environment.

Introduction

The use of molecular characterization techniques such as PCR-RFLP and PCR-direct sequencing has been adopted in many studies for *Cryptosporidium* from water samples[1–4]. Most of those studies used DNA extracts from water concentrates as PCR templates. Hence, genotyping results indicate the presence of the most dominant genotype. Distributions of respective genotypes or species remain unclear.

To provide the distribution of genotypes or species of *Cryptosporidium* in a water environment, we attempt to investigate molecular characterization of a single *Cryptosporidium* oocyst, isolated under a microscope, from sewage concentrate using 18S rRNA gene based semi-nested PCR and direct sequencing method.

Materials and Methods

Concentration of Sewage and Isolation of a Single *Cryptosporidium* Oocyst

Twice a month, we collected seven sewage samples of 4 L each from a sewage treatment plant located in Tokyo. Samples were centrifuged at 1,500 × g for 15 min. Sediments were meshed through 32 μm metal mesh. The concentrates were purified by sucrose flotation and washed with ethyl acetate. After purification, *Cryptosporidium* oocysts were separated by IMS procedure (Dynabead anti-Cryptosporidium beads; Dynal Biotech, Norway). An immunofluorescent antibody stain was applied to the separated samples using a *Cryptosporidium* antibody kit (Easy stain; BTF Pty. Ltd., Australia).

Stained samples were dropped on a plastic dish; then FITC stained and intact oocyst was manipulated, using a glass microcapillary under inverted epifluorescence microscopy. An isolated single oocyst was dispensed into a 200 μl PCR tube containing 20 μl of lysis buffer (2.5 μl of 10× PCR buffer and 17.5 μl of milli-Q water).

PCR-Semi nested PCR procedure

PCR tubes containing a single *Cryptosporidium* oocyst were subjected to three freeze-thaw cycles and then incubated at 100°C for 15 min with 5 μl of 10% TX-100 for DNA extraction. Initial PCR amplification was performed in 50 μl containing 25 μl of the DNA extract from a single oocyst, 2.5 μl of 1× PCR buffer, 1 μM concentrations of each primer set and 1 U of Taq polymerase (EX Taq HS; TaKaRa Bio Inc., Japan). We used 1 μl of initial PCR product, purified by spin column, as a template for nested amplification. Initial and nested PCR used oligonucleotide primers that are complementary to 18S rRNA gene sequence of *Cryptosporidium* (CPDIAGF1/R for initial PCR, CPDIAGF1/R1 for nested PCR) [5]. Nested PCR products were sequenced on an auto sequencer. Sequence data were compared with GeneBank sequences using BLAST.

Results and discussion

PCR and nested PCR

Table 1 shows results of nested PCR and the sequence of a singly isolated oocyst from sewage. A total of 77 oocysts were isolated singly and subjected to semi-nested PCR and direct sequence. Semi-nested PCR detection rates were 84% (65 isolates positive) for a single *Cryptosporidium* oocyst from sewage. For the 73% (56 isolates) that were sequenced successfully, we compared sequences with the GeneBank database.

The results demonstrate that 18S rRNA based semi nested PCR and the direct sequencing method can be used to characterize a single *Cryptosporidium* oocyst from sewage with high sensitivity and can reveal distribution of species or genotypes of *Cryptosporidium* in a water environment.

18S rRNA gene sequence analysis

Nested PCR products were sequenced to confirm the identification of *Cryptosporidium*. Sequences of the most variable region of known *Cryptosporidium* spp. from GeneBank and isolates from the present study are shown in Fig. 1. Sequences of *Cryptosporidium* from sewage were able to differentiate five genotypes and one species. All sequences were matched GeneBank database as *C.parvum* genotype 1 (AY204235, etc.), *C.meleagridis* (AY166839, etc.), *C.parvum* genotype 2 (AF178700, etc.), *C.parvum* isolated from pig (AF115377, etc.), *C.parvum* isolate VF383 human type (AY030084.1) and *C.parvum* isolated from mouse (AF112571).

Distributions of these six genotypes/species are shown in Table 2. Among the 56 isolates, 51.8% (29 isolates) were *C.parvum* genotype 1 and 7.1% (4 isolates,) *C.parvum* VF383, which have been reported as a human type. Potentially zoonotic types were 17.9% (10 isolates) of *C. meleagridis* and 12.5% (7 isolates) of *C. parvum* genotype 2. Animal genotypes were, 8.9% (5 isolates, pig) and 1.8% (1 isolate mouse). An abattoir in the treatment district is presumed to be

one of the contamination sources of animal type (and zoonotic type), possibly.

Conclusion

This study is summarized as follows:

1) The 18S rRNA based semi-nested PCR and direct sequence method was a useful tool for investigation of molecular characterization and distribution of *Cryptosporidium* genotype in a water environment.
2) Distribution of *Cryptosporidium* genotype in sewage was: 51.8% (29 isolates) of *C. parvum* genotype 1; 17.9% (10 isolates) of *C. meleagridis*; 12.5% (7 isolates) of *C. parvum* genotype 2; 8.9% (5 isolates) of *C. parvum* isolated from pig; 7.1% (4 isolates) of *C. parvum* isolate VF383 (human type); and 1.8% (1 isolate) of *C. parvum* isolated from mouse.

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1) Ward, P.I., et al., (2002), Parasitology, 124, 359–368
2) Xiao, L. et al., (2000), Applied and Environmental Microbiology, 66, 5492–5498
3) Jellison, K.L., et al., (2002), Applied and Environmental Microbiology, 68, 569–575
4) Sturbaum, G.D., et al., (2001), Applied and Environmental Microbiology, 67, 2625–2668
5) Johnson, D.W., (1995), Applied and Environmental Microbiology, 61, 3849–3855

Table 1 Number of samples PCR positive or sequenced successfully

Tested singly isolate oocysts	Nested PCR positive*	Sequenced successfully
77	65(84%)	56(73%)

*:Visually detected by etidium bromide and UV

Table 2 Distribution of isolated *Cryptosporidium* oocyst

	Number of isolates	%
C.parvum genotype 1	29	51.8
C.mereaglidis	10	17.9
C.parvum genotype 2	7	12.5
C.paruvum isolated from pig	5	8.9
C.parvum isolate VF383	4	7.1
C.parvum isolated from mouse	1	1.8

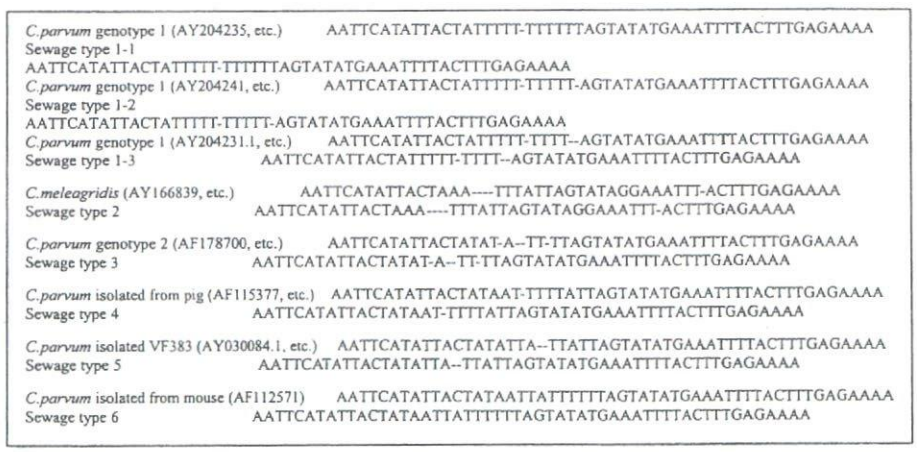

Fig. 1 Sequences of the most variable regions of the 18S r RNA of Cryptosporidium from sewage and known *Cryptosporidium* spp.

Genotyping of single *Cryptosporidium* oocysts isolated from Sewage and River Water

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Abstract

This study demonstrates genotyping of individual single *Cryptosporidium* oocysts in sewage and river water. Using glass capillaries, we manipulated an IFA-stained single *Cryptosporidium* oocyst under inverted epifluorescence microscopy. Each isolated *Cryptosporidium* oocyst was analyzed by 18S rRNA gene based semi-nested PCR and direct sequencing. Most dominant genotype was *C.parvum* genotype 1 in both sewage and river water as 65 and 72%.

INTRODUCTION

The use of molecular characterization techniques such as PCR-RFLP and PCR-direct sequencing has been adopted in many studies for *Cryptosporidium* from water samples[1–4]. Most of those studies used balk DNA extracts from water concentrates as PCR templates. Hence, genotyping results indicate the presence of the most dominant genotype. Distributions of respective genotypes remain unclear. To provide the distribution of genotypes of *Cryptosporidium* in a water environment, we attempt to investigate genotyping of a single *Cryptosporidium* oocyst, isolated individually under a microscope, from sewage and river water concentrate using 18S rRNA gene based semi-nested PCR and direct sequencing method.

MATERIALS AND METHODS

Tested Water Samples

Sewage samples were collected from a sewage treatment plant located in Tokyo, Japan. Eleven sewage samples of 4 L each were collected during May 2003–September 2004. River water samples were obtained from Miyayama, Kanagawa prefecture, Japan, downstream of the Sagami River. Three samples of 200 liters were collected in October 2003, August and November 2004.

Concentration and Isolation of a Single *Cryptosporidium* Oocyst[5,6]

Sewage samples were centrifuged at 1,500 × g for 15 min. Sediments were sieved through 32μm metal mesh and washed with 100 ml of PBS containing 0.1% Tween 80. River water samples were concentrated using the Microbe Sampler[5]. Sewage and river water concentrates were purified by sucrose flotation and washed with ethyl acetate. After purification, *Cryptosporidium* oocysts were separated by IMS procedure (Dynabead anti-Cryptosporidium beads; Dynal Biotech, Norway). An immunofluorescent antibody stain was applied to the separated samples using a *Cryptosporidium* antibody kit (Easy stain; BTF Pty. Ltd., Australia). Stained samples were dropped on a plastic dish; then FITC stained and intact oocyst was manipulated, using a glass microcapillary under inverted epifluorescence microscopy. An isolated single oocyst was dispensed into a 200 μl PCR tube containing 20 μl of PCR buffer.

PCR-Semi nested PCR procedure

PCR tubes containing a single *Cryptosporidium* oocyst were subjected to three freeze-thaw

cycles (-80°C -room temperature) and then incubated at 100°C for 15 min with 5 □l of 10% TX-100 for DNA extraction. Initial PCR with the primer pair CPB-DIAGF1 (5'-GCTCGTAGTTGGATTTCTGTTAA-3', modified from CPB- DiagF [7]) and CPB-DiagR [7] (5'-TAAGGTGCTGAAGGAGTAAGG-3') was used. Initial PCR products were purified by spin column (Ultra Clean PCR Clean-up kit, Mo Bio Laboratories, CA, USA) and finally eluted with 50 μl of Milli-Q water. For nested amplification, 1 μL of purified initial PCR product was used as a template.

The primer pair of CPB-DIAGF1 and CPB-DIAGR1 (5'-CCAATCTCTAGTTGGCATAG-3', modified CPB- DiagR), was used for Semi-Nested PCR. In both amplifications, samples were incubated in a PCR thermal cycler (TP500; TaKaRa Biomedicals, Shiga, Japan) with initial denaturation at 94°C for 5 min, followed by 40 cycles at 94°C for 1 min, 54°C for 30 s, and 72°C for 30 s, and a final extension of 72°C for 7 min.

This nested amplification resulted in approximately 400 bp DNA products containing a polymorphic region enabling discrimination of *Cryptosporidium* species and genotypes [8,9]. Amplification products were purified through gel filtration and sequenced using an auto sequencer (RISA384; Shimadzu Corp., Kyoto, Japan). All sequences were identified using the GenBank database as 18S rRNA gene sequence.

RESULTS AND DISCUSSIONS

A total of 181 oocysts from 11 sewage samples and 81 oocysts from 3 river water samples were isolated. The detection rate of semi-nested PCR was 67% (137/181 oocyst from sewage) and 49% (40/81 oocyst from river water). Among 137 semi-nested PCR positive samples from sewage, 121 samples were sequenced successfully. In river water samples, only 25/40 PCR positive samples were sequenced successfully.

Semi nested PCR products of individual *Cryptosporidium* oocysts from sewage and river water were sequenced to confirm identification of genotype. Sequences of the most polymorphic region of known *Cryptosporidium* spp. from GenBank and isolates from sewage and river water are shown in Table 1.

Among the 121 samples isolated from sewage, the most dominant genotype was *C.parvum* genotype 1 (AY204231 or A204235 or AA204241) as 65% (78 isolates). The *C.parvum* genotype 1 and *C.parvum* VF383(AY 030084) 5% (6) were anthoroponotic type. Potentially zoonotic types were 13% (16) of *C.parvum* genotype 2 (A204238) and 11% (13) of *C.meleagridis*(AY166839). Animal genotypes were 4% (5) of *C*.sp Pig 1(AF108861) , 2% (2) of *C*.sp PG1-26(AY271721) isolated from pig and 1% (1) of *C.parvum* CPM1 mouse type (AF112571). Among genotyped *Cryptosporidium* oocysts from sewage, 93% were anthoroponotic and zoonotic type.

In 25 river water isolates, *C.parvum* genotype 1 represented 72% (18). *C*.sp Pig 1 and *C*.sp PG1-26 were 16% (4) and 4% (1) of the samples, respectively. *C.parvum* genotype 2 of zoonotic type was 8% (2). It is interesting to note that *C.parvum* genotype 1 was the most dominant genotype (72%) in the river water. It suggested contamination of human origin sewage in the river.

ACKNOWLEDGEMENT

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Re-emerging Infectious Diseases from the Ministry of Health, Labour and Welfare, Japan.

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6) EPA Method 1622
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9) Pieniazek, N.J., et al., (1999) Emerging Infectious Disease. 5, 444–449.

Table 1 Sequences of the most variable regions of the 18Sr RNA of *Cryptosporidium* from sewage, river water and known *Cryptosporidium* spp.

C. parvum genotype 1 (AY204235, etc.)	ACTATTTTT-TTTTTTAGTATATGAAATTTTACTTTGAGAAAA
Sewage type 1-1, River type 1-1	ACTATTTTT-TTTTTTAGTATATGAAATTTTACTTTGAGAAAA
C. parvum genotype 1 (AY204241, etc.)	ACTATTTTT-TTTTT-AGTATATGAAATTTTACTTTGAGAAAA
Sewage type 1-2, River type 1-2	ACTATTTTT-TTTTT-AGTATATGAAATTTTACTTTGAGAAAA
C. parvum genotype 1 (AY204231, etc.)	ACTATTTTT-TTTT—AGTATATGAAATTTTACTTTGAGAAAA
Sewage type 1-3, River type 1-2	ACTATTTTT-TTTT—AGTATATGAAATTTTACTTTGAGAAAA
C. parvum isolated VF383 (AY030084.1, etc.)	ACTATATTA—TTATTAGTATATGAAATTTTACTTTGAGAAAA
Sewage type 2	ACTATATTA—TTATTAGTATATGAAATTTTACTTTGAGAAAA
C. parvum genotype 2 (AF178700, etc.)	ACTATAT-A—TT-TTAGTATATGAAATTTTACTTTGAGAAAA
Sewage type 3, River type 2	ACTATAT-A—TT-TTAGTATATGAAATTTTACTTTGAGAAAA
C. meleagridis (AY166839, etc.)	ACTAAA----TTTATTAGTATAGGAAATTT-ACTTTGAGAAAA
Sewage type 4	ACTAAA----TTTATTAGTATAGGAAATTT-ACTTTGAGAAAA
C. sp Pig1 (AF108861)	ACTATAAT-TTTTATTAGTATATGAAATTTTACTTTGAGAAAA
Sewage type 5, River type 3	ACTATAAT-TTTTATTAGTATATGAAATTTTACTTTGAGAAAA
C. sp PG1-26(pig) (AY271721)	ACT--------TTA-CAGTATGTGGAATTTTACTTTGAGAAAA
Sewage type 6, River type 4	ACT--------TTA-CAGTATGTGGAATTTTACTTTGAGAAAA
C. parvum strain CPM1 (mouse) (AF112571)	ACTATAATTATTTTTTAGTATATGAAATTTTACTTTGAGAAAA
Sewage type 7	ACTATAATTATTTTTTAGTATATGAAATTTTACTTTGAGAAAA

(34) 消光型蛍光プローブを用いたリアルタイムPCR法による水中のクリプトスポリジウムの定量および種別判定手法の開発

Development of Quantification and Genotyping methods for *Cryptosporidium* in water by Quenching Probe PCR followed by RFLP

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ABSTRACT;

A new method was developed to quantify *Cryptosporidium* in water. Quenching Probe PCR (QProbe-PCR) technique could successfully amplify approximately 1280bp of *Cryptosporidium* 18S rDNA from a sample with as low as 60 [oocysts/tube] of *Cryptosporidium parvum* bovine genotype. QProbe-PCR showed high accuracy and high sensitivity compared to Real Time PCR with TaqMan probe.

QProbe-PCR has an advantage that the PCR products can be applied for molecular characterization. A restriction fragment length polymorphism (RFLP) technique was used to distinguish *Cryptosporidium* species and genotypes. Five species (*C. parvum* bovine genotype, *C. parvum* human genotype, *C. meleagridis*, *C. felis* and *C. muris*) could be distinguished by the RFLP with restriction enzymes *Ssp* I, *Vsp* I and *Sty* I. The *Sty* I successfully differentiated *C. muris* calf genotype (also known as *C. andersoni*) and *C. muris* mouse genotype. Database-based analysis revealed that 8 species out of 10 could be distinguished by RFLP with these three restriction enzymes.

QProbe-PCR-RFLP techniques can provide information on the genotype as well as the quantity of *Cryptosporidium* from the same sample. This technique can be a useful tool for waterborne risk assessment of Cryptosporidiosis.

KEYWORDS; *Cryptosporidium*, Genotyping, Quenching Probe PCR (QProbe-PCR), Real time PCR, Restriction Fragment Length Polymorphism (RFLP)

1. はじめに

クリプトスポリジウムは水系感染性の病原微生物で、塩素消毒に対して非常に強い耐性を持つことが知られており、先進国においても水道水質管理における重大な問題とされている。1993年、米国 Wisconsin 州 Milwaukee において、水道水中のクリプトスポリジウムにより40万人以上が感染した事例[1]が報告されている。また国内においても、1996年6月に埼玉県越生町において町民約14,000人の7割以上が感染したという事例などがある[2]。

現在、クリプトスポリジウムは分類学上13種存在するとされている[3]。そのうちヒトに感染するのは、主に *C. parvum* と *C. hominis* であるが、他にも *C. muris*[4], *C. meleagridis*[5][6], *C. felis*[6][7], *C. canis*[6][7] が健常なヒトに感

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み合わせが、一番効率良く鋳型 DNA を増幅でき、またプライマーダイマーの生成が少ないことが確認できたため、以降の評価ではこの組み合わせを用いた。

次に、QProbe-PCR 法で用いたプローブの設計を行った。QProbe-PCR 法は、蛍光色素 BODIPY がグアニンと結合することで蛍光が消光する現象を利用するため、プローブのどちらかの末端がシトシンである必要がある。そこで、以下の手順でプローブの設計を行った。はじめに、本プライマーと同じ配列部位を持つ遺伝子を、DDBJ データベース[19]から NCBI-BLAST 2.0[20]により検索し、系統樹作成ソフト ClustalW 1.7[21]を用いて、その全ての塩基配列に共通の部分を抽出した。次に、抽出した塩基配列の中から、プローブの Tm 値がプライマーの Tm 値より 5℃程度高くなること、プローブが二次構造をとりにくいこと、プライマーダイマーを生成しにくいこと、以上3点を基準に、最適なプローブの塩基配列を決定した。Table 1 に選択したプライマーと、設計した Quenching Probe の塩基配列を示す。

Table 1. Primers for amplifying 18S rDNA of *Cryptosporidium* and Designed QProbe

Name	Sequence	Reference
Forward Primer	5'- GGA AGG GTT GTA TTT ATT AGA TAA AG-3'	14), 15)
Reverse Primer	5'- CCC TAA TCC TTC GAA ACA GGA -3'	
QProbe	5'- CGA ACC CTA ATT CCC CGT TAC CC - BODIPY -3'	This study

（3） PCR 条件の決定と QProbe-PCR 法の定量性の評価

DNA の増幅および蛍光の検出には LightCycler (Roche Diagnostics, 東京)を用いた。QProbe PCR 法では、5'–3'エキソヌクレアーゼ活性のないポリメラーゼを使う必要があるため、DNA ポリメラーゼには KOD -Plus-（東洋紡, 大阪）を用いた。反応液の総量は 20μL とした。PCR バッファー、$MgSO_4$、dNTP はポリメラーゼに付属のものを使用し、Mg^{2+}濃度は 1mM、dNTP 濃度は各 200nM とした。プライマー濃度は、Forward プライマーを 1000nM、Reverse プライマーを 300nM とし、また QProbe 濃度は 100nM とした。

定量性の評価の前に、PCR の最適条件を決定するため、アニーリング温度を 55℃～60℃の範囲で変化させて PCR を行い、検出精度と定量下限を調査した。その他の温度条件は、94℃5 分の熱変性の後、94℃30 秒、アニーリング時間 30 秒、68℃60 秒のサイクルを 60 回行い、最後に 68℃5 分で伸長を行った。

得られた最適条件を用いて、QProbe PCR 法の定量性評価を行った。各チューブへの添加 DNA 溶液は、2.1.（1）で作成した DNA 溶液を、1 チューブあたり 6×10^{3}～6×10^{0}[oocysts]となるように段階的に希釈して作成した。

（4） 閾値の決定方法

各サイクルで測定した蛍光強度の変化から検出サイクルを算出するため、以下の手順で、陽性と判定する消光率の閾値(Threshold line)を設定した。まず、各サイクルにおける蛍光の消光率のグラフを作成した。次に、閾値を 1%～99%の範囲内で 1%刻みで順に上げていき、それぞれの境界値から得られた検出サイクルを用いて検量線を作成した。そして、その中から最も高い相関係数を与える値を求め、最終的な閾値とした。この閾値は PCR を行う度に作成した。

2.2. RFLP 法~~および T-RFLP 法~~によるクリプトスポリジウムの種の判別

（1） 供試したクリプトスポリジウムの種

種の判別手法の評価には、国立感染症研究所 寄生動物部の遠藤 卓郎氏より分与していただいた *C. parvum* ウシ型 (Accession No.: AF161856)、同ヒト型 (同 AF093491)、*C. meleagridis* (同 AF112574)、*C. felis* (同 AF112575)、

3. 実験結果

3.1. QProbe-PCR 法による水中のクリプトスポリジウムの定量

アニーリング温度を変化させて、検出感度および定量下限を調査した結果、アニーリング温度が 57℃以下のときは、初期添加量 6×10^3[oocysts/tube]の試料の Ct 値が約 32 サイクル前後だったのに対し、それ以上では 40 サイクル前後まで低下し、また、低濃度の試料では産物の生成が見られなかった。一方、アニーリング温度が 57℃より低いときは、非特異産物の増幅により、プローブの消光率（対象 DNA の産物生成量）が低下した。以上の結果より、57℃の場合が、精度・感度両方の面から見て最適であると判断した。

Figure 1 に、この条件下における、各 PCR サイクルで測定した蛍光の減少量の、初期蛍光強度に対する割合を示す。図中の"Threshold Line"とは、2.1.（4）で述べた手法で作成した「閾値」を指す。また Figure 2 に、この図から得られた検出サイクルと添加した鋳型 DNA 量の関係から得た検量線を示す。

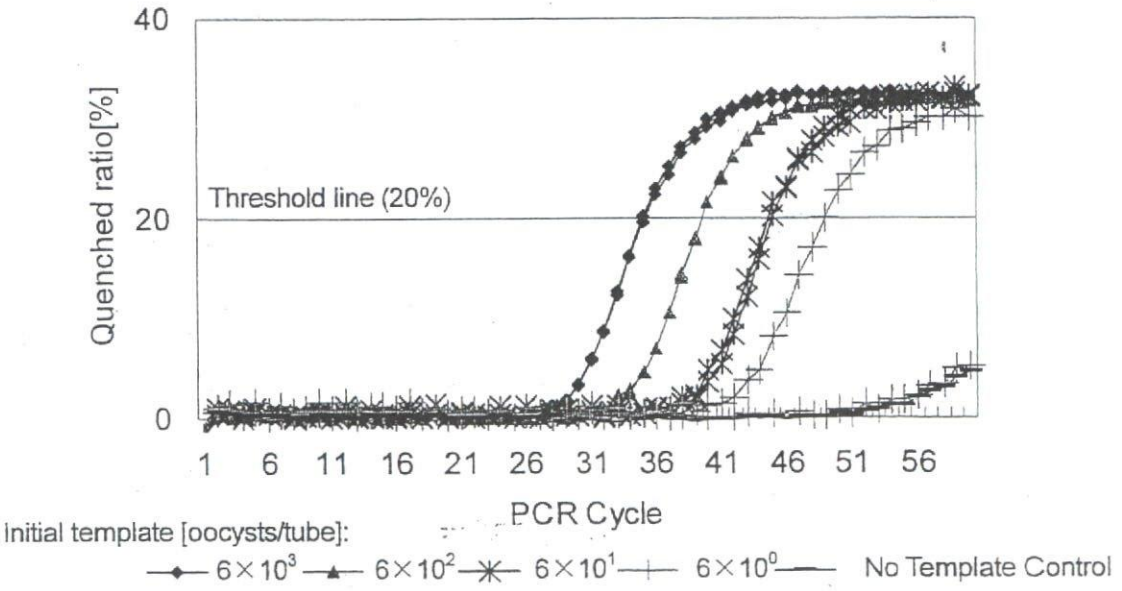

Figure 1. Amplification plots of *Cryptosporidium* 18S rDNA by QProbe-PCR with annealing temperature at 57°C (n=2 for each condition except NTC).

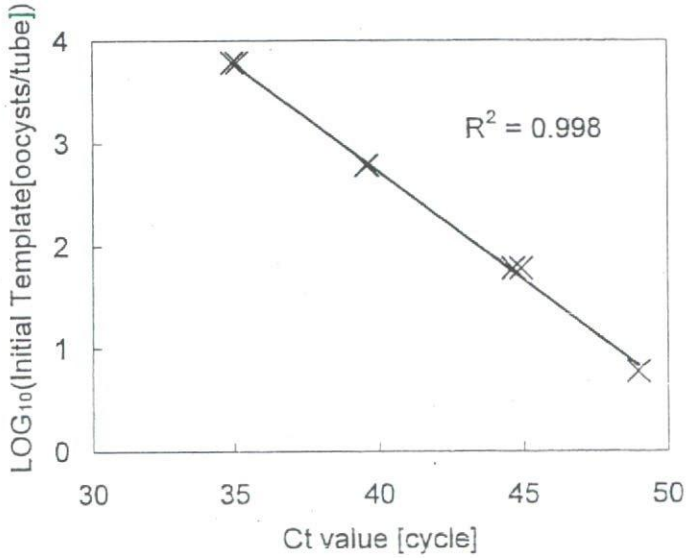

Figure 2. Standard line for QProbe PCR derived from amplification plots in Figure 1.

れぞれ Type C、Type F）を示しており、今回用いた種の中での判別が可能であった。*C.* sp. strain 938 に関しては、今回増幅した領域全域についての塩基配列が得られていないため、理論上の断片長パターンはわからないが、一部の塩基配列に関する情報から、少なくとも 33bp の断片が存在することが予想された。*Ssp* I の断片長パターン（Figure 3 a)）を見ると、1250bp 付近にバンドが見られるだけで、33bp の断片はプライマーダイマーと重なって確認できなかった。以上より、この株に関しては、33bp の断片を生じている可能性はあるが、それ以外の場所では切断されないことがわかった。

Figure 3b) の *Vsp* I による切断断片長パターンを見ると、*C. parvum* ウシ型と *C.* sp. strain 938 で同様のパターン（628bp, 550bp、Type c）が見られた他は、それぞれ独自のパターンを示した。

Sty I による切断断片長パターン（Figure 3c)）は、長い方の断片長が種によって多少異なるものの、*C. muris* を除く全ての種で似ていた（707～747bp と 564～569bp)。*C. muris* は、全ての種で共通な断片長である約 560bp のバンドの他に、450bp 付近と 260bp 付近にバンドが見られた。Table 2 の *C. muris* のウシ型とネズミ型の断片長パターンと比較することにより、この *C. muris* はウシ型である可能性が高いと考えられる。

以上より、制限酵素 *Ssp* I、*Vsp* I を用いた RFLP 法により、遺伝子配列がわかっている 4 つの種または遺伝子型（*C. parvum* ヒト型、ウシ型、*C. meleagridis*、*C. felis*）をそれぞれ区別することができた。また、新たに *Sty* I を用いることで、*C. muris* の遺伝子型（ウシ型、ネズミ型）の判定が可能であることがわかった。

4. 考察

4.1. QProbe-PCR 法によるクリプトスポリジウムの定量について

本研究で開発したクリプトスポリジウムの 18S rDNA を対象とした QProbe-PCR 法は、非常に高い精度（決定係数 R^2=0.998）を持っていることが明らかとなった。また定量下限については、今回の試験結果から 6×10^1[oocysts/tube]としたが、6×10^0[oocysts/tube]の試料からも定量性のある検出結果が得られていること、また、今回はそれ以下の添加量では試験していないことから、実際の定量下限はより低い可能性もある。

比較のため、同じ塩基配列のプライマーおよびプローブを使用して、TaqMan プローブを用いたリアルタイム PCR 法による定量を試みた。このとき、PCR 反応液の作成には PCR Master Mix を使用し、温度条件は QProbe-PCR 法と同じとした。その結果、初期添加量 6×10^3～6×10^0[oocysts/tube]のいずれの試料からも蛍光を検出することができなかった。また、他の遺伝子配列に対する TaqMan プローブを用いた手法[24)25)]の定量下限は、およそ 5[oocyst/tube] であった。したがって、今回開発した QProbe-PCR 法は、検出精度・検出感度の両方の面において、TaqMan プローブを用いたリアルタイム PCR 法と同等であるといえる。

さらに、一般に、TaqMan プローブを用いた手法は、増幅する塩基配列の長さをあまり長くできないと言われている。実際、QProbe 法で用いた増幅部位に対して、TaqMan プローブを用いた手法では意味のある蛍光が得られなかった。一方、QProbe-PCR 法を用いた本手法は、約 1280bp の DNA を対象にすることができた。これは、以下の 2 つの面で優れた特徴であるといえる。まず、得られた PCR 産物を、今回行った RFLP 法やその他の遺伝子工学的手法に適用することで、遺伝子配列に関する情報を得られるという点である。本研究では、この点に関して、RFLP 法によりクリプトスポリジウムの種や遺伝子型の判定を行えることを示した。次に、プローブの設計に際し、TaqMan プローブの配列を決定する場合と比較して、配列選択の自由度が高いという点が挙げられる。これにより、二次構造のとりにくさやダイマーの生成しにくさの面で、より優れたプローブを設計しやすくなる。今回作成した系において 1280bp の長さの配列を対象にできたのも、この特徴に負うところが大きいと考えられる。

ただし、本手法は、既存の TaqMan プローブを用いたリアルタイム PCR 法と同様、試料中のクリプトスポリジウムの DNA を測定対象としているため、検出されたクリプトスポリジウムの生死や、ヒトへの感染力を評価することはできない。

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Molecular Characterization of a *Cryptosporidium* Isolate From a Banded Mongoose *Mungos mungo*

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ABSTRACT: *Cryptosporidium* spp. has been found in more than 150 species of mammals, but there has been no report in mongooses. In this study, we report the isolation of *Cryptosporidium* sp. in a banded mongoose *Mungos mungo*, which was brought from Tanzania to Japan; the isolate was analyzed genetically to validate the occurrence of a new, host-adapted genotype. *Cryptosporidium* diagnostic fragments of 18S ribosomal RNA and 70-kDa heat shock protein genes were amplified from this isolate and compared with the other *Cryptosporidium* species and genotypes reported previously. Analyses showed that the mongoose isolate represents a new genotype, closely related to that of bears.

Cryptosporidium spp. are protozoan parasites found in a variety of vertebrates, including humans. At present, 12 species, i.e., *Cryptosporidium parvum*, *C. wrairi*, *C. felis*, *C. canis*, *C. muris*, and *C. andersoni* in mammals, *C. baileyi* and *C. meleagridis* in birds, *C. serpentis* and *C. saurophilum* in reptiles, *C. nasorum* and *C. molnari* in fish, are recognized as valid (Fayer et al., 1997, 2000, 2001; Alvarez-Pellitero and Sitjà-Bobadilla, 2002). However, molecular studies have shown that *C. parvum* and *C. canis* are composed of genetically distinct but morphologically identical genotypes: human (recently proposed as a new species, *C. hominis* [Morgan-Ryan et al., 2002]), bovine, mouse, monkey, ferret, marsupial, and pig genotypes in *C. parvum*; dog, fox, and coyote genotypes in *C. canis* (Morgan, Xiao et al., 1999; Xiao, Morgan et al., 2000; Xiao et al., 2002). Moreover, isolates that are genetically different from the species or genotypes mentioned above have been found in some animals and are referred to as host-adapted genotypes (Xiao, Limor et al., 2000; Xiao et al., 2002). Although *Cryptosporidium* spp. have been found in more than 150 species of mammals (Fayer et al., 2000), isolates from relatively few species have been characterized genetically. Therefore, it is likely that more host-adapted genotypes occur in mammals. In October 2000, a banded mongoose, *Mungos mungo*, was brought from Tanzania to the Osaka Municipal Tennoji Zoological Gardens in Japan. A fecal sample collected while the animal was in quarantine was found by light microscopy and indirect fluorescence antibody testing using a commercially available kit (Hydrofluor-Combo, Startegic Diagnostic Inc., Newark, Delaware) to contain *Cryptosporidium* sp. oocysts. The oocysts detected were 4–5 μm in diameter and were indistinguishable in size from those of *C. parvum*. Because *Cryptosporidium* sp. has not been found in mongooses previously, we speculated that the isolate might be a new genotype. Therefore, we compared this isolate with other *Cryptosporidium* species or genotypes reported previously to validate the presence of a new host-adapted genotype.

The purification of *Cryptosporidium* spp. oocysts from the fecal sample and the extraction of DNA from oocysts were performed following the method reported previously (Abe, Sawano et al., 2002; Abe et al., 2002a). *Cryptosporidium* diagnostic fragments were amplified by polymerase chain reaction (PCR) with the following primer pairs targeting the different gene loci: 18SiF and 18SiR for the *Cryptosporidium* 18S ribosomal RNA (18SrDNA) gene (Morgan et al., 1997) and chsp1 and chsp4 for the *Cryptosporidium* heat shock protein (HSP70) 70 gene (Gobet and Toze, 2001). The area amplified with each primer pair includes a variable region that can be used to distinguish *Cryptosporidium* species as well as genotypes (Abe, Sawano et al., 2002; Abe et al., 2002b; Abe and Iseki, 2003). PCR amplification was performed under conditions reported previously (Abe et al., 2002b). The DNA of *C. parvum* strain HNJ-1 originating from a patient was used as a positive control for the PCR. This strain was the bovine genotype of *C. parvum* (Abe et al., 2002a). Amplification products were subjected to electrophoretic separation using 3% agarose gels, stained with ethidium bromide, and visualized on a UV transilluminator. The PCR products were gel purified using a QIAquick Gel Extraction kit (QIAGEN GmbH, Hilden, Germany) and sequenced using an ABI PRISM BigDye Terminator Cycle Sequencing FS Ready Reaction kit (PE Applied Biosystems, Foster City, California) on an ABI 310 automated sequencer (PE Applied Biosystems). PCR products were sequenced in both directions using each primer pair mentioned above. Sequences obtained from the mongoose *Cryptosporidium* sp. isolate were aligned with the available nucleotide sequences obtained previously (Morgan, Monis et al., 1999; Xiao, Escalante et al., 1999; Xiao, Morgan et al., 1999; Sulaiman et al., 2000; Xiao, Limor et al., 2000; Xiao et al., 2002) from other *Cryptosporidium* species and genotypes using Clustal-X (version 1.63b). Evolutionary distance between different isolates was calculated by Kimura 2-parameter method. Trees were constructed using the neighbor-joining algorithm (Saitou and Nei, 1987). Branch reliability was assessed using bootstrap analyses (1,000 replicates), and the phylograms were drawn using the NJplot program (Perriere and Gouy, 1996). The partial sequences of 18SrDNA and HSP70 of the mongoose *Cryptosporidium* sp. isolate obtained in the present study have been deposited in the GenBank database under accession numbers AB102769 and AB102771, respectively.

The partial 18SrDNA and HSP70 were successfully amplified in the mongoose isolate. The 18SrDNA fragment was slightly larger than HNJ-1 and was estimated to be approximately 310 bp (data not shown). Abe et al. (2002b) showed that the size of the partial 18SrDNA generated with the primers 18SiF and 18SiR ranged from 288 to 299 bp in the *Cryptosporidium* species and genotypes but was 312 bp only in *C. felis*. However, as shown in Fig. 1, the size of the partial 18SrDNA amplified in the mongoose isolate corresponded to that of *C. felis* (312 bp), and almost all the insertions were found between positions 253 and 275, the same as in *C. felis*, although some of the nucleotides inserted differed between the mongoose isolate and *C. felis* (Fig. 1). As shown in Fig. 2, the size of the HSP70 fragment amplified was 587 bp. In the 587 bp region examined, the mongoose isolate had 71, 74, 73, or 103 bp substitutions compared with the *Cryptosporidium* bear genotype, *C. canis* dog genotype, *C. canis* coyote genotype, or *C. felis*, respectively (Fig. 2). Phylogenetic analysis of the 18SrDNA sequences showed the close relatedness between the mongoose isolate and the *Cryptosporidium* bear genotype: the mongoose isolate was clustered with the *Cryptosporidium* bear genotype (Fig. 3A). Moreover, the close relatedness of the mongoose isolate to the *Cryptosporidium* bear genotype was also reflected in a neighbor-joining tree constructed based on the HSP70 sequences (Fig. 3B). It is necessary for the species differentiation of *Cryptosporidium* parasites to examine the host ranges and infection sites, in addition to the molecular characteristics of the isolates. We again collected fecal samples from this animal a month after the first examination and tried to purify oocysts to infect laboratory animals experimentally. However, we could not find oocysts in the samples necessary to examine the host range of this isolate. Therefore, in the present study we refer to this isolate as the *Cryptosporidium* mongoose geno-