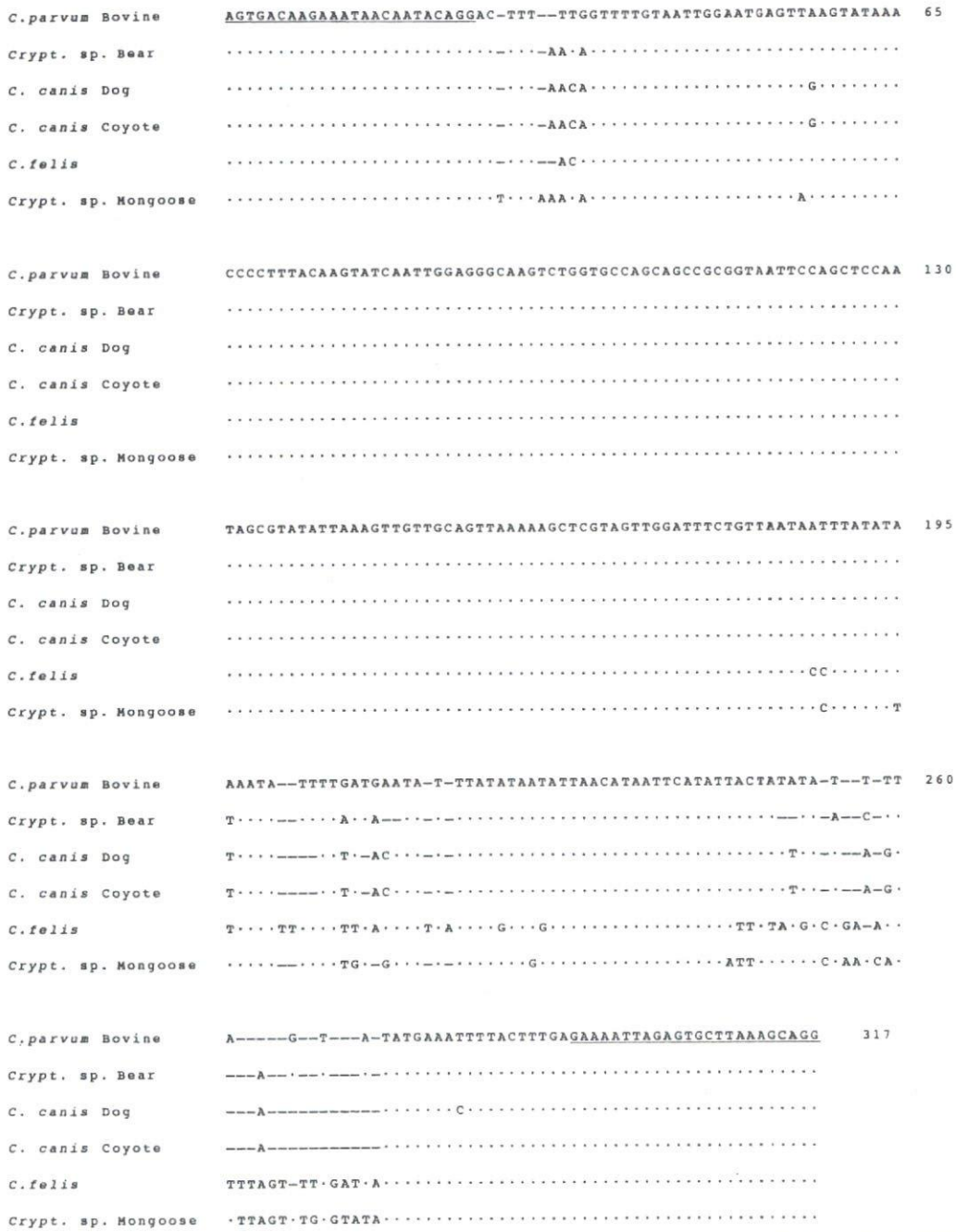

FIGURE 1. Comparison of 18SrDNA nucleotide sequences generated with the primers 18SiF and 18SiR of the mongoose isolate with the available related species and genotypes. The GenBank accession numbers for the *Cryptosporidium* species and genotypes are as follows: *Cryptosporidium parvum* bovine genotype (AF108864), *Cryptosporidium* bear genotype (AF247535), *C. canis* dog (AF112576) and coyote (AY120909) genotypes, and *C. felis* (AF108862). Dots indicate nucleotide bases that are identical to the *C. parvum* bovine genotype bases. Dashes indicate nucleotide base deletions. The sequence of the forward or reverse primer is underlined.

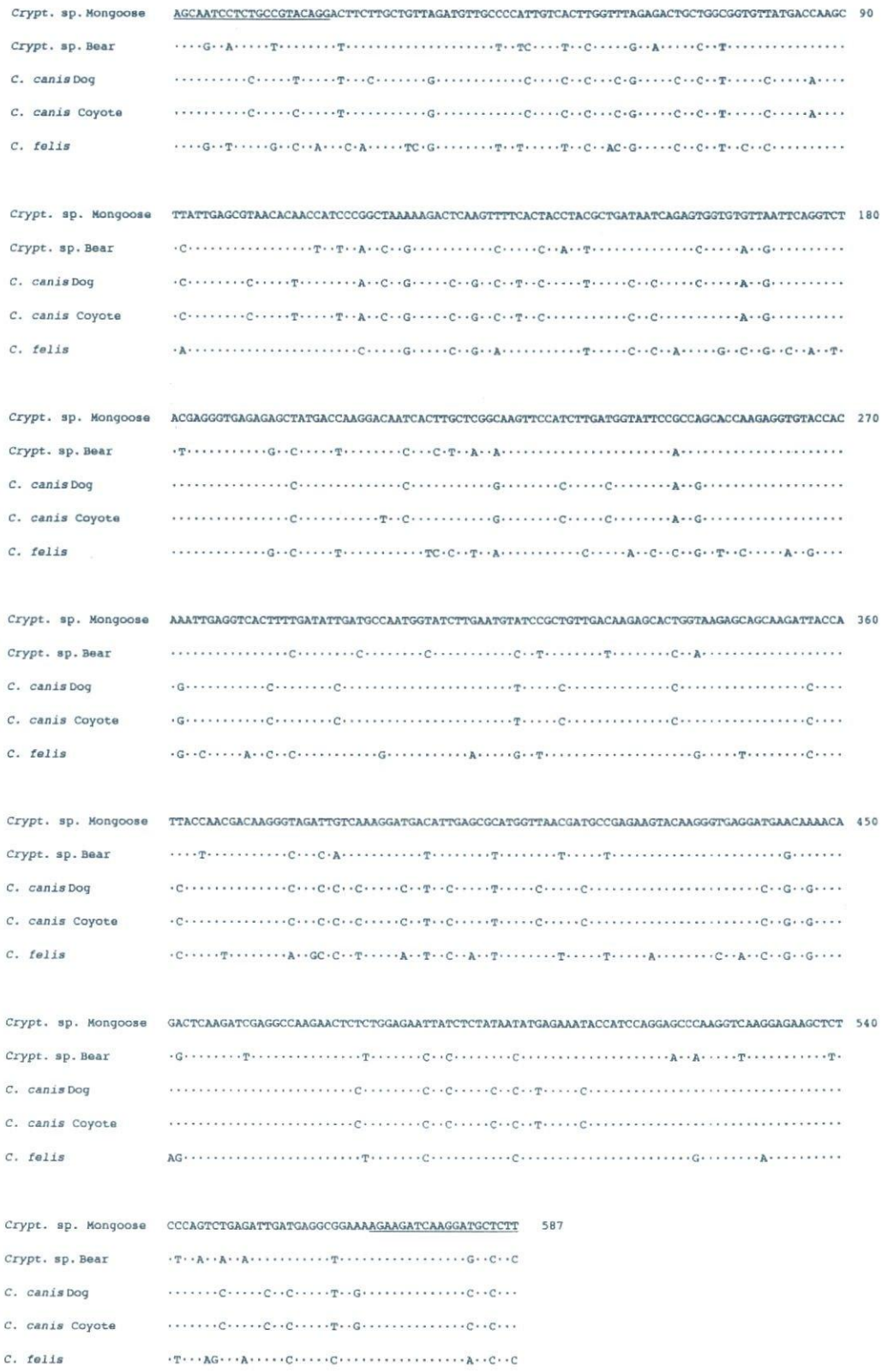

FIGURE 2. Comparison of HSP70 nucleotide sequences generated with the primers chsp1 and chsp4 of the mongoose isolate with the available related species and genotypes. The GenBank accession numbers for the *Cryptosporidium* species and genotypes are as follows: *Cryptosporidium* bear genotype (AF247536), *C. canis* dog (AF221529) and coyote (AY120920) genotypes, and *C. felis* (AF221538). Dots indicate nucleotide bases that are identical to the *Cryptosporidium* mongoose genotype bases. The sequence of the forward or reverse primer is underlined.

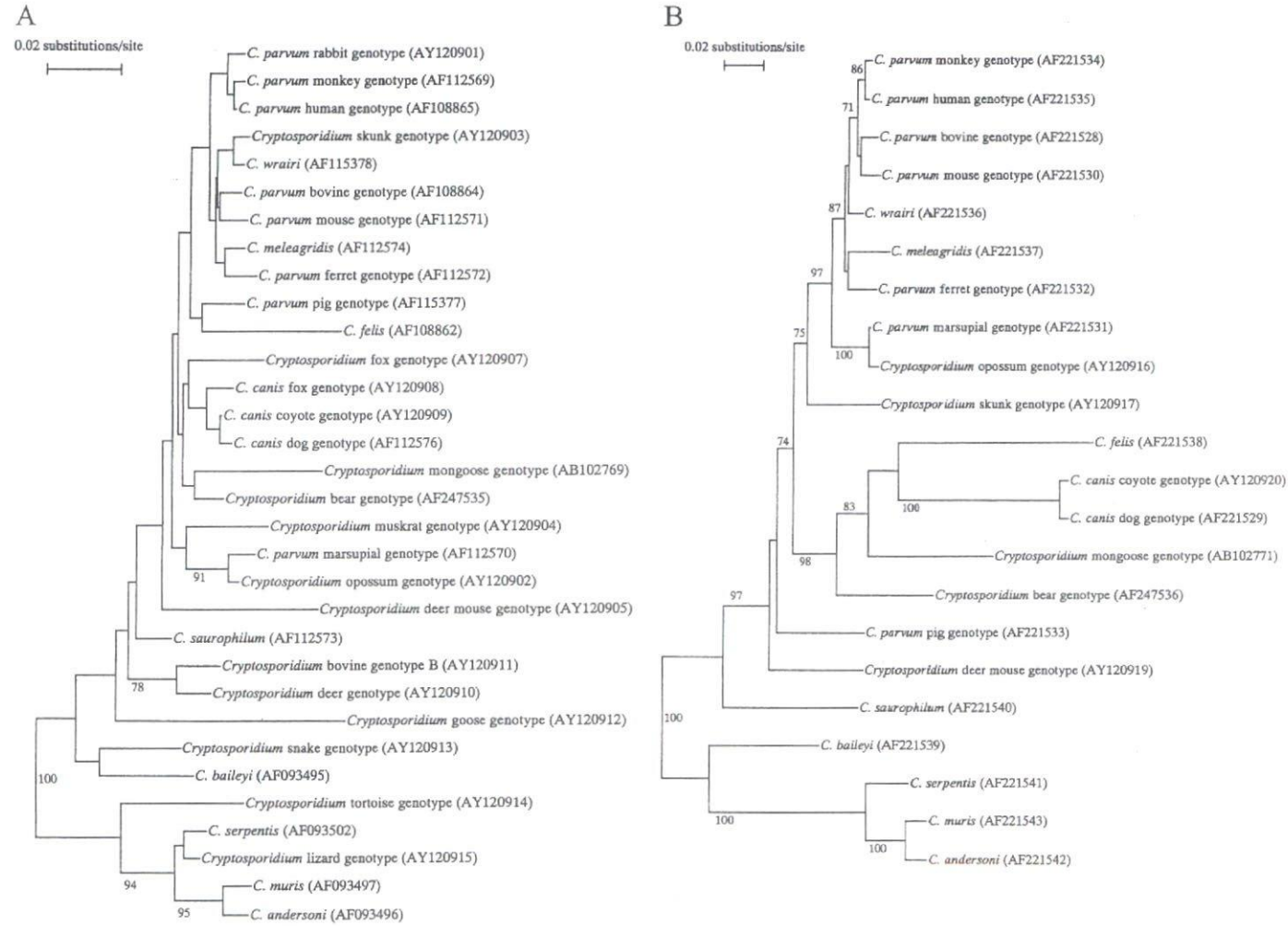

FIGURE 3. Phylogenetic relationship of the mongoose isolate to other *Cryptosporidium* species and genotypes as inferred by neighbor-joining analysis of nucleotide sequences of the 18SrDNA (**A**) and HSP70 (**B**) genes. Additional GenBank accession numbers are shown in parentheses. Bootstrap values of >70% are indicated at each branch.

type, which represents a new genotype that is closely related to the *Cryptosporidium* sp. bear genotype (Xiao, Limor et al., 2000).

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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

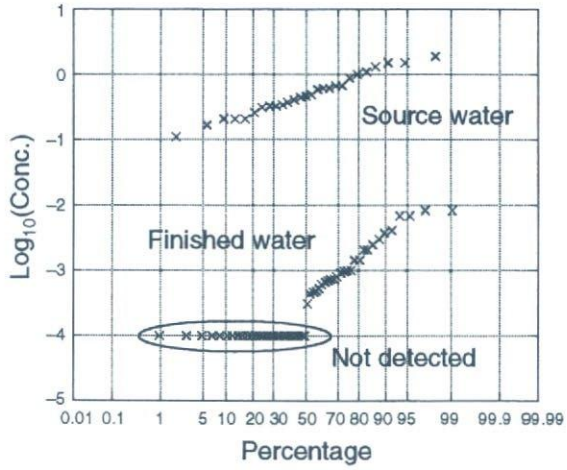

Figure 1 The observed data of *Cryptosporidium*

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

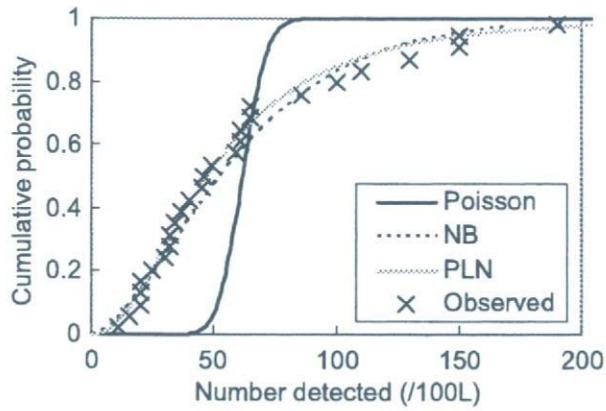

Figure 2 Estimated distributions and the observed data of the source water concentration

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.

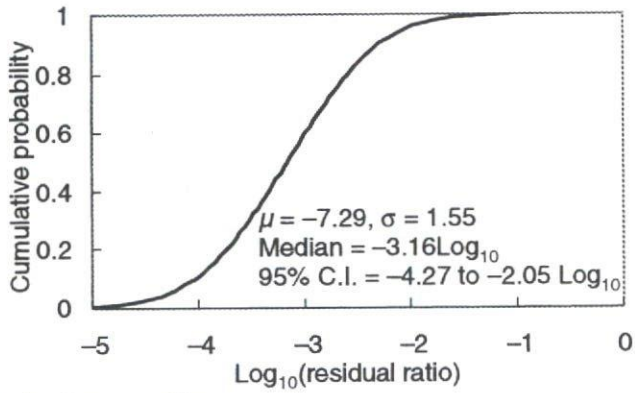

Figure 3 Distribution of treatment efficacy

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.

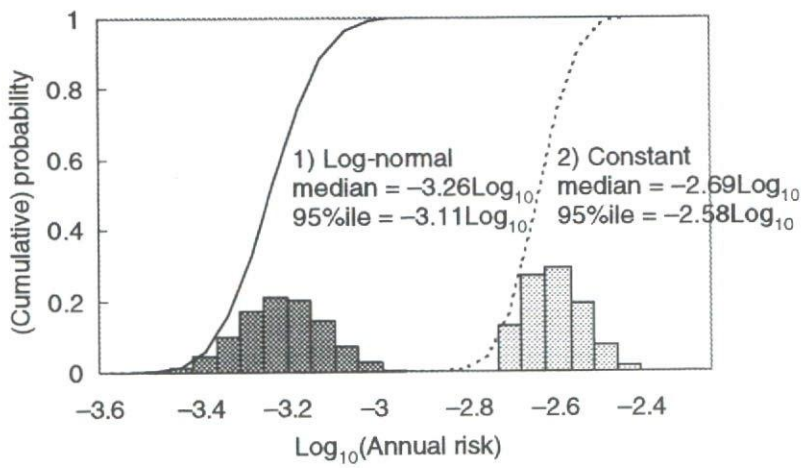

Figure 4 Cumulative frequency distribution of calculated annual risk of infection

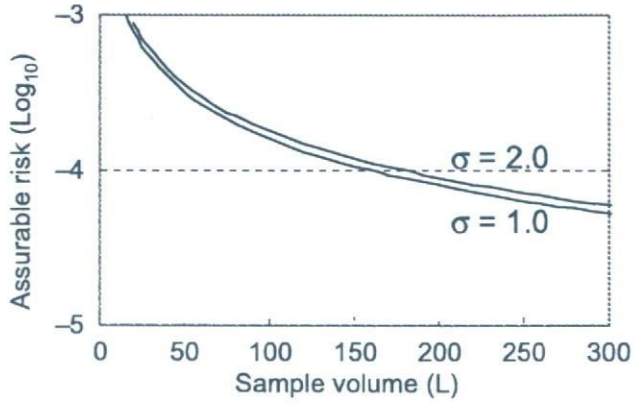

Figure 5 Assurable annual risk of infection by daily monitoring

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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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.

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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

FULL PAPER *Parasitology*

Identification of Genotypes of *Giardia intestinalis* Isolates from Dogs in Japan by Direct Sequencing of the PCR Amplified Glutamate Dehydrogenase Gene

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ABSTRACT. *Giardia* has been detected in domestic dogs in Japan, but the genotype of isolates has remained unclear because identification has relied on conventional microscopy. Here we tried to identify the genotypes of four isolates from dogs in Japan by direct sequencing of the PCR amplified *Giardia* glutamate dehydrogenase (GDH) gene. The primer pair GDHF3 and GDHB5, targeting the GDH gene, was designed to prime a region of the GDH gene sequence conserved in the strains found to have the dog-specific genotype. The specific PCR product (approximately 220 bp), amplified with this primer pair, was only observed when *Giardia* DNA was used as the template. The sequences of the diagnostic fragments were identical among the isolates from dogs, and were differed by 15 bp or 1 bp from the strains, which were found to be the dog-specific genotypes, Assemblage C or D respectively. To verify the identity of the amplified DNA, a phylogenetic analysis was performed. Consequently, the sequence of the isolates from dogs clearly clustered with the strain found to be Assemblage D with neighbor-joining analyses. Therefore, all the isolates from dogs examined were identified as the dog-specific genotype, Assemblage D. In the present study, we revealed the genotype of *Giardia* isolates in Japan, and showed that direct sequencing of the PCR product amplified with the primer pair GDHF3 and GDHB5 was a useful tool for distinguishing between the zoonotic and dog-specific genotypes.

KEY WORDS: canine, direct sequencing, genotype, *Giardia*, PCR.

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The flagellate *Giardia intestinalis* (syn. *G. lamblia, G. duodenalis*) is a well-known intestinal parasite, which causes an enteric disease in humans, livestock, and companion animals. This organism is one of the most common parasites of domestic dogs and a frequently recognized waterborne pathogen. Transmission of this parasite follows ingestion of viable cysts in contaminated food or water. Recent molecular analysis has revealed the genetic diversity within *G. intestinalis* populations, although the isolates from humans and animals are morphologically identical [4, 6, 7, 9–12, 15, 16]. At present, *G. intestinalis* isolates, recovered from humans and animals, fall into two major genotypes, Assemblages A and B, and several animal-specific genotypes such as "dog", "cat", "livestock", "rat", and "muskrat" [4, 6, 7, 9–12, 15, 16]. Furthermore, the dog-specific genotype is divided into two genetically distinct genotypes, Assemblages C and D [7, 11]. The Assemblages A and B have been recognized as zoonotic genotypes because both are found in several animal species including dogs [4, 9, 10, 12]. In contrast, the animal-specific genotypes appear to be host adapted, and there is, as yet, no epidemiological evidence to suggest that they occur frequently in humans. Therefore, these genotypes appear to be restricted to dogs or other animal species, and unlikely to represent a zoonotic risk.

Although *Giardia* has been detected in domestic dogs in Japan, the genotype of isolates remains unclear because identification has been performed with conventional microscopy [2, 3, 8]. Elucidating the genotype of isolates harbored in dogs, which are in close contact with humans, is important if the transmission of *Giardia* infection is to be controlled. In this paper, we reveal the genotype of *G. intestinalis* isolates originating from dogs in Japan by polymerase chain reaction (PCR)-based diagnostic methods.

MATERIALS AND METHODS

Samples of Giardia and the extraction of DNA: *Giardia* cysts were collected from the fecal samples of dogs, which were captured in Osaka city (isolates A and B) or reared in a companion animal shop in Kanazawa city (isolates C and D), by the sucrose centrifugal-flotation method [1]. The dogs showed no clinical symptoms such as diarrhea when the fecal samples were collected. The *Giardia* DNA was extracted and purified as described previously [1]. The DNA of *G. intestinalis* strain Portland-1 was kindly provided from Dr. S. Kobayashi, Department of Tropical Medicine and Parasitology, School of Medicine, Keio University, and used as a positive control for the PCR. This strain has already been found to be of Assemblage A [5, 12].

Restriction fragment length polymorphism (RFLP) analysis of the Giardia glutamate dehydrogenase (GDH) gene: A region of the GDH gene was amplified using the primers GDH1 (forward primer; 5'- ATC TTC GAG AGG ATG CTT GAG - 3') and GDH4 (reverse primer; 5'- AGT ACG CGA CGC TGG GAT ACT - 3') as reported [5]. It is possible to distinguish between Assemblages A and B by RFLP analysis of the amplification product with this primer pair [5]. PCR amplification was performed in a volume of 50 μl containing 1 × PCR buffer, 2 mM $MgCl_2$, 250 μM of each

dNTP, 0.5 μM of each primer, 1.25 units of Ex Taq DNA polymerase (TAKARA Shuzo Co., Ltd., Otsu, Japan), and 5 μl of the DNA sample. We used the PCR buffer and dNTP mixture supplied with Ex Taq DNA polymerase. Reactions were performed on a GeneAmp PCR System 9700 thermocycler (Perkin-Elmer, Foster City, California). Samples were denatured at 94°C for 3 min, and then subjected to 40 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 60 sec, followed by a final extension at 72°C for 7 min. 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 QIAquick Gel Extraction kit (QIAGEN GmbH, Hilden, Germany), and were digested with *Dde* I in a reaction mixture of 2 μl of 10 × buffer, 1 μl of restriction endonuclease (TOYOBO Biochemicals, Japan), 5 μl of purified PCR products, and 12 μl of distilled water to a final volume of 20 μl at 37°C for 2 hr. The digested products were electrophoresed with a 100 bp ladder (Pharmacia Biotech, Uppsala, Sweden) as a size marker in 3% agarose gels. Gels were stained with ethidium bromide, and the approximate sizes of the bands were estimated from photographs.

Detection of the GDH gene amplified with a newly developed primer pair: In the present study, the GDH gene was also amplified using the forward primer GDHF3 (5'-TCC ACC CCT CTG TCA ACC TTT C - 3') and the reverse primer GDHB5 (5'- AAT GTC GCC AGC AGG AAC G - 3'), which were designed to prime a region of the GDH gene sequence conserved in four strains from dogs found to have Assemblage C: Ad-136 (GenBank accession no. U60982), Ad-137 (U60983), Ad-141 (U60984), and Ad-147 (U60985) [11]. Reactions were performed under the following conditions: 94°C for 3 min; then 35 cycles comprising 30 sec at 94°C, 30 sec at 59°C, and 1 min at 72°C; followed by a final extension of 7 min at 72°C. The specificity of the PCR was determined by attempting to amplify a PCR product from dog fecal DNA and other intestinal protozoan DNA (*Isospora canis, Cryptosporidium parvum* strain HNJ-1, *Entamoeba histolytica* strain HM-1:IMSS, *Blastocystis hominis* strain Nand II). The DNA of *E. histolytica* and *B. hominis* were kindly provided from Dr. S. Kobayashi, Department of Tropical Medicine and Parasitology, School of Medicine, Keio University, and H. Yoshikawa, Department of Biological Science, Nara Women's University, respectively. *Isospora canis* was originated from a dog captured in Osaka city, and the oocysts of this organism were collected from the fecal sample by the sucrose centrifugal-flotation method [1]. The DNA of *I. canis* was extracted from the oocysts following the same method described previously [1], and used for confirming the specificity of the PCR.

Direct sequencing of the GDH gene amplified with a newly developed primer pair: In the present study, we sequenced the PCR products amplified with GDHF3 and GDHB5 primer pair for revealing the sequences of GDH gene of isolates from dogs. The PCR products were gel purified using the same kit mentioned above and sequenced using an ABI PRISM BigDye Terminator Cycle Sequencing FS Ready Reaction kit (Perkin-Elmer Corp., U.S.A.) on an automated sequencer (ABI PRISM 310 model; Perkin-Elmer Corp., U.S.A.). PCR products were sequenced in both directions using either GDHF3 or GDHB5. The nucleotide sequences of isolates were aligned by Clustal-X [14], and a phylogram was created using the neighbor-joining method [13].

RESULTS

RFLP analysis of the GDH gene amplified with primers GDH1 and GDH4: Agarose gel visualization of PCR products amplified with GDH1 and GDH4 revealed the same sized diagnostic fragments (approximately 770 bp) among the samples examined (Fig. 1). However, the restriction patterns of the isolates from dogs were all the same, although strictly different from the pattern for Portland-1. Namely, three fragments, ranging from 100 bp to 500 bp, were clearly found in Portland-1, but only two fragments near 400 bp were found in the isolates from dogs (Fig. 2). The restriction pattern of Portland-1 corresponded to that of Assemblage A reported previously [5], but that of isolates from dogs also strictly differed from the restriction patterns of Assemblage B [5].

Detection and direct sequencing analysis of the GDH gene amplified with a newly developed primer pair: The specific PCR product (approximately 220 bp), amplified with GDHF3 and GDHB5, was only observed when *Giardia* DNA was used as the template (Fig. 3). As shown in Fig. 4, sequence analysis revealed two types of sequences. The DNA sequence of the diagnostic fragment of Portland-

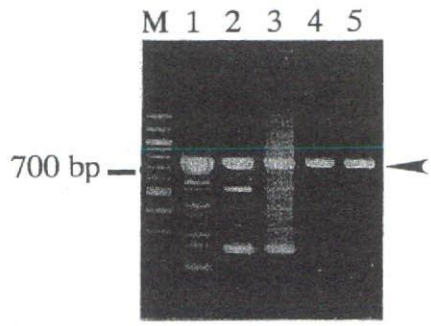

Fig. 1. Detection of the *Giardia* glutamate dehydrogenase (GDH) gene by PCR with the primer pair GDH1 and GDH4. Lanes: M, molecular marker; 1, *G. intestinalis* strain Portland-1; 2 and 3, isolates (A, B) from dogs captured in Osaka city; 4 and 5, isolates (C, D) from dogs reared in a companion animal shop in Kanazawa city. The molecular weight marker was a 100 bp ladder. Arrowhead indicates the diagnostic fragment (approximately 770 bp).

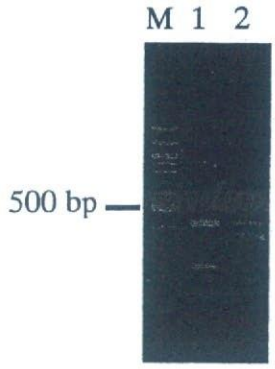

Fig. 2. Restriction patterns of GDH gene products amplified with GDH1 and GDH4. Lanes: M, molecular marker; 1, *G. intestinal* strain Portland-1; 2, isolate (A) from a dog captured in Osaka city. The molecular weight marker was a 100 bp ladder. The restriction pattern of the other isolates (B-D) from dogs was identical to that of the isolate A.

1 or the isolates from dogs examined was 218 bases long. The DNA sequence of Portland-1 was identical to that of the same strain published previously (GenBank accession no. M84604, data not shown) [12], and was also identical to those of the strains (Ad-1, 2) found to be Assemblage A (Fig. 4). On the other hand, the sequences of the isolates from dogs were identical to each other, and were differed by 1 bp from Ad-148 (Assemblage D) or by 15 bp from Ad-136, 137, 141, 147, which were found to be Assemblage C (Fig. 4). To verify the identity of the amplified DNA, a phylogenetic analysis was performed (Fig. 5). Consequently, the sequence of the isolates from dogs clustered with Ad-148 (Assemblage D) with neighbor-joining analyses. Therefore, the isolates from dogs examined were identified as the dog-specific genotype, Assemblage D.

DISCUSSION

In the present study, we examined isolates from dogs in Japan by RFLP analysis to confirm whether these isolates were zoonotic or not. The restriction patterns were strictly different from those of Assemblage A or B, although *Giardia* diagnostic fragments were amplified in all the canine isolates. Therefore, the isolates might have the dog-specific genotype. At present, isolates from dogs fall into zoonotic genotypes (Assemblage A or B) or dog-specific genotypes (Assemblage C or D) based on allozyme electrophoresis or the sequencing of the GDH or the small subunit ribosomal RNA (SSUrRNA) gene [7, 11]. Since the axenization and cultivation of isolates or multiple primer pairs are necessary for the allozyme analysis or amplification of the GDH gene [11], we performed the PCR using the primers reported previously [7] for sequencing the SSUrRNA gene. However, the diagnostic fragment was not amplified in Portland-1 or any isolates from dogs (data not shown). Therefore, we developed a new primer pair (GDHF3 and GDHB5), and successfully amplified fragment of predicted size (approximately 220 bp) in Portland-1 as well as all isolates from dogs, but not in other intestinal protozoa or parasite-negative fecal samples (Fig. 3). These results indicate that a newly developed primer pair, GDHF3 and GDHB5, is specific to *Giardia*.

Although the presence of variant in each genotype, Assemblage A, B, or, C is already known [11, 12], it has not yet been confirmed in Assemblage D because no strains grouped into Assemblage D have been found except for the

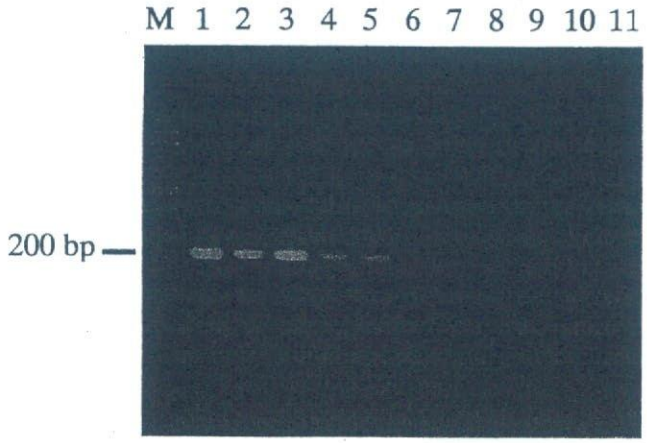

Fig. 3. Detection of the GDH gene by PCR with a newly developed primer pair, GDHF3 and GDHB5. Lanes: M, molecular marker; 1, *G. intestinalis* strain Portland-1; 2–5, isolates (A-D) from dogs; 6, *Isospora canis*; 7, *Cryptosporidium parvum* strain HNJ-1; 8, *Entamoeba histolytica* strain HM-1:IMSS; 9, *Blastocystis hominis* strain Nand II; 10 and 11, Parasite-negative fecal samples from dogs. The molecular weight marker was a 100 bp ladder.