

Quantitative detection of *Cryptosporidium* oocyst in water source by alternatively binding probe competitive polymerase chain reaction (ABC-PCR)

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Aims

Detection of *Cryptosporidium* oocysts, representative health related microorganisms, in water samples has been carried out by direct microscopic visualization or enzyme immunoassays of obtained and/or cultured oocysts with or without fluorescent antibodies. However, these detection methods are labor-intensive, require a large number of oocysts for positive detection and are not suitable for high-throughput processing of samples. Instead of these conventional methods, molecular techniques have been developed for rapid detection of *Cryptosporidium* oocysts from water samples. In particular, real-time polymerase chain reaction (PCR) is widely used for the quantification of *Cryptosporidium* oocysts (Keegan et al., 2003; Miller et al., 2006) because of its high rapidity, sensitivity and reproducibility. However, the real-time PCR requires highly specialized and expensive devices that combine a fluorometer and a thermal cycler for real-time fluorescence measurement. Therefore, alternative simpler molecular quantification techniques are strongly needed for the routine detection of *Cryptosporidium* oocysts.

In this study, we focused our attention on a recently reported novel molecular method for the quantification of nucleic acid sequences named alternately binding probe competitive PCR (ABC-PCR) (Tani et al., 2007) as an improved method for quantification of *Cryptosporidium* oocysts. This method is cost effective because it does not require an expensive real-time fluorescence measurement device but require a simple end-point fluorescence measurement device. We developed an ABC-PCR assay for cost-effective quantification of *Cryptosporidium* oocysts, and applied the assay to the detection from real water samples collected from Japanese rivers. To investigate validity of the ABC-PCR assay, real-time PCR assay and conventional microscopic observation were also performed, and quantification data were compared with each other.

Methods

The sequences of primers and probe of the ABC-PCR assay for the quantification of 18S rRNA gene of *Cryptosporidium* spp. was designed based on those of the previously reported real-time PCR assay (Miller et al., 2006). Serial dilutions of the chemically synthesized oligo-DNA with a length of 192 bp (range, 10¹ to 10⁵ copies per reaction) were used to make a standard curve. Detailed procedure and principle of the ABC-PCR assay is written in the previous paper (Tani et al., 2007). The number of 18S rRNA genes in standard *Cryptosporidium* oocyst was investigated by real-time reverse transcription-PCR, and the number was used for conversion of the number of 18S rRNA genes determined by the ABC-PCR assay to the number of *Cryptosporidium* oocyst.

In total, 14 water samples were collected at 7 sites in tributary rivers of the Tone River basin in Japan where the surface water is utilized for the main production of drinking water for people living in the

Tokyo metropolitan area from November to December 2009. Ten liters of water samples were concentrated by vacuum filtration with polytetrafluoroethylene membrane filters (pore size: 1 μm ; diameter: 142 mm, Advantec, Japan) and purified through immunomagnetic separation (Dynabeads GC Combo, Invitrogen, Japan). Half the volume of concentrated sample solution was used for a conventional assay based on microscopic observation, and the other half volume was used for the ABC-PCR and real-time PCR assays after nucleic acid extraction and reverse transcription. The detailed procedure for sample concentration, purification, microscopic observation, nucleic acid extraction and reverse transcription is written in our previous papers (Inomata et al., 2009).

Results and Conclusions.

Figure 1 shows a typical standard curve of the ABC-PCR assay. Relative fluorescence intensity values obtained from triplicate samples showed good fitting to a rectangular hyperbola with a correlation coefficient (R) of 0.9997. The standard deviation (SD) of triplicate determinations was less than 0.008 for 10^1 to 10^5 copies.

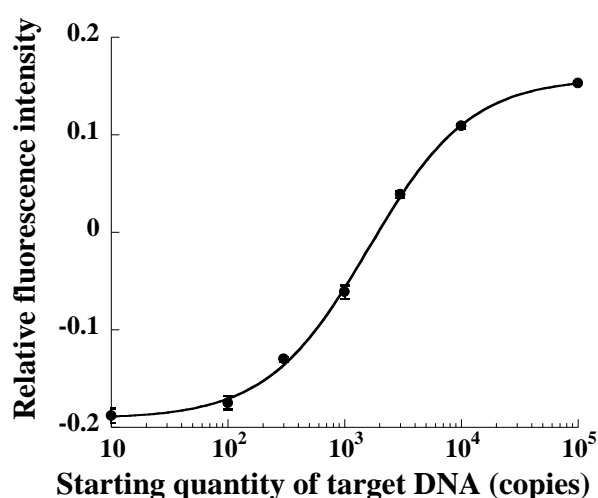


Figure 1 Typical standard curve. A fixed amount of the internal standard (10^3 copies) was coamplified with various amounts of the target DNA in triplicate. The standard curve was obtained by fitting the data points to a rectangular hyperbola. $y = -559.8 / (x + 1602) + 0.1583$; the correlation coefficient (R) was calculated to be 0.9997. The error bars represent the standard deviation (SD) of triplicate determinations per dilution.

Concentrations of *Cryptosporidium* oocysts in river water samples were successfully quantified by the ABC-PCR assay as shown in Table 1. The quantified value by the ABC-PCR assay is similar to that by the real-time PCR assay. The correlation coefficient between the two PCR assays was 0.937. In addition, the quantified concentration in most water samples by PCR assays was comparable to that by conventional microscopic observation although the principle of quantification is different.

Thus, we successfully quantified concentrations of *Cryptosporidium* oocysts in river water samples by the ABC-PCR assay. As the only equipment that is needed for this end-point fluorescence assay is a simple fluorometer and a relatively inexpensive thermal cycler, this method can markedly reduce time and cost to quantify health related microorganisms.

Acknowledgment

This study was financially supported by a Grant-in-Aid for Research Activity Start-up (21860094), Japan Society for the Promotion of Science (JSPS).

Table 1 Quantification of *Cryptosporidium* oocysts in surface water samples by several methods

Sample name	ABC-PCR (oocysts/10L)	Real-time PCR (oocysts/10L)	Microscopic observation (oocysts/10L)
A	0.30	0.44	2
B	8.8	9.6	-
C	0.10	-	2
D	8.8	15	4
E	-	-	-
F	-	-	-
G	0.88	1.8	4
H	50	40	12
I	0.17	0.22	0
J	25	34	14
K	0.76	0.048	10
L	50	40	30
M	1.3	0.48	16
N	65	94	40

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