Development of ATP assay as a surrogate indicator of viability of *Cryptosporidium parvum* oocysts

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**Abstract** Adenosine Triphosphate (ATP) determination was applied to evaluate viability of *Cryptosporidium* oocysts. Three pretreatment methods, such as incubation in acidified Hanks balanced salt solution (HBSS), excystation and sonication were investigated for ATP extraction from oocysts. Incubation in acidified HBSS was insufficient to extract ATP from oocysts, but a linear relationship between the number of oocysts and the concentration of ATP extracted was observed in the test of excystation and sonication treatments. Sonication treatment was able to extract ATP from oocysts more rapidly and precisely than excystation treatment. ATP amount per oocyst by sonication treatment (ATPs) was evaluated to be 2.9 \( \times 10^{-8} \) µg on average, and its detection limit was 500 oocysts/100 µl. Ozone treatment experiments were conducted in batch condition to evaluate differences among ATP concentrations extracted, *in vitro* excystation and DAPI/PI permeability assays. ATPs assay was observed to have a linear relationship with DAPI/PI permeability assay \((R^2=0.98)\). As a result, ATP assay is applicable as a surrogate indicator of the viability of *C. parvum*, and is superior to *in vitro* excystation and DAPI/PI permeability assay, because of its rapid, accurate and simple procedure.

**Keywords** ATP; *Cryptosporidium parvum*; extraction methods; oocysts; sonication; viability

**Introduction** Techniques used for assessing the viability and/or infectivity of *Cryptosporidium parvum* oocysts include *in vitro* excystation (Campbell et al., 1992; Finch et al., 1993), fluorogenic vital dyes permeability (Campbell et al., 1992), infectivity in animal models (Finch et al., 1993) and infectivity with cell culture PCR (Rochelle et al., 1997). Assays of evaluating viability of *Cryptosporidium* oocysts such as the above methods results in some differences in the effect of disinfectants. The fluorogenic vital dye assay assesses the viability of individual *C. parvum* oocysts and correlates well with *in vitro* excystation (Campbell et al., 1992). *In vitro* excystation and DAPI/PI permeability assay is time-consuming and asks for skills and microscopy. Assessment of viability by *in vitro* excystation and infectivity of animals requires organisms in a relatively purified and concentrated suspension. The development of a reproducible, sensitive, simple viability assay would be of value for determining the infectivity of oocysts or the activity of sporozoites within oocysts. ATP is instantly degraded on death of the cells. Therefore, ATP can be used as an indicator of active mass of microorganism (Holm-Hansen and Booth, 1966). There have been numerous attempts to link the ATP content of activated sludge to certain wastewater characteristics or to certain control parameters used in the operation of wastewater treatment plants. The ATP content of activated sludge reflects its viable organism content and it appears this cell constituent can be used as a rapid and convenient indicator of viable organisms in activated sludge because the ATP content/viable cell remains constant (Patterson et al., 1970; Weddle and Jenkins, 1971). The objectives of this research are to determine an effective pretreatment method to extract ATP from oocysts and to assess the applicability of ATP assay as an indicator of oocyst viability.

**Materials and methods**

**Source and purification of oocysts**

*C. parvum* HNJ-1 strain used in this study were obtained from Dr. M. Iseki (Osaka City University Medical School, Osaka, Japan), who originally isolated from it an immunologically
normal Japanese patient with diarrhoea. The oocysts used in this study were produced in 5-week-old female severe combined immunodeficiency (SCID) mice. Each mouse was kept in a separate cage and fed on the commercial mouse food. The feces were collected on trays filled with tap water.

Fecal samples were sequentially passed through 200-, 150-, 100-, 75-μm sieves by agitating and washing the sieves with 0.01% (vol/vol) aqueous Tween 20. Concentration of the particulates from the sieved feces was by centrifugation 1,100 × g for 10 min. The oocysts were purified from the particulates by sucrose density gradient centrifugation with 1.2 and 1.15 specific gravity of cold sucrose solutions sequentially. The pellet was washed twice in pure (Milli-Q®, Millipore co.) water resuspended in 30 ml of 0.2% cold Tween 80 and overlaid with 6 ml of diethyl ether. After thorough mixing, the suspension was centrifuged at 1,100 × g for 5 min, and fat and supernatant layers were discarded. The pellet was washed twice in pure water before further purification by resuspension in 10 ml of cold sucrose solution (specific gravity of 1.1) which was overlaid with 3 ml of pure water. The interface was recovered, diluted with pure water and washed. Purified oocysts were stored in pure water containing antibiotics at 4ºC (Jenkins et al., 1997). Oocyst concentration in stock suspension was determined with a direct hemocytometer count and it was diluted tenfold to prepare low oocyst concentrations, when it was used for experiments.

**Pretreatment for ATP extraction**

The reliability of the ATP method depends upon an extraction procedure, in which complete ATP transfer into solution and no ATP destruction are preferable (Holm-Hansen and Booth, 1966). Since direct ATP measurement used for ordinal microorganisms is not suitable for oocysts due to their robust wall (Kim et al., 1999), three pretreatment methods were investigated for ATP extraction from oocyst; these are incubation in acidified HBSS, excystation and sonication. Oocyst suspensions were diluted to 10^3–10^7 oocysts/ml to evaluate the effectiveness of these three methods for ATP extraction.

In incubation in acidified HBSS of oocysts was intended to increase the permeability of the oocyst wall, while excystation pretreatment was expected to extract ATP from excysted sporozoites. For acid pretreatment, 1 ml of sample in 1.5-ml microcentrifuge tubes was centrifuged at 1,100 × g for 10 minutes and then washed once by resuspending the pellet in 1 ml of acidified HBSS (pH 2.75). The tubes were incubated at 37ºC for 1 hr in the dark. Oocyst suspensions were centrifuged at 1,100 × g for 10 minutes, and the pellets were washed twice with 0.025 M 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES, pH 7.75) solution to make a final oocyst sample of a volume of 100 μl for ATP measurement. Excystation pretreatment protocol was the same method as that of in vitro excystation, which will be described later in details. Oocyst suspension was incubated at 37ºC for 30–120 min. After excystation pretreatment, oocyst suspension was washed with HEPES and utilized for ATP assay. Sonication was examined for a more rapid and simple pretreatment for oocysts. Treatment time and intensity were investigated to optimize sonication pretreatment. For ATP extraction in oocysts, 3 ml of oocyst suspension in 10-ml test tube was sonicated with ultrasonic disruptor (200 kHz, Model UR-200P, Tomy Seiko Co. Ltd, Japan), at 90 W for 1 min. The test tube was immediately placed in an ice bath, and stored for ATP assay.

**ATP assay**

The firefly luciferin-luciferase reaction was used to determine the concentration of ATP, which is known to be highly specific (McElroy and Green, 1956). The reaction is based on the measurement of light emission produced during the oxidation of luciferin by molecular oxygen in the presence of ATP and magnesium ions. The light intensity is directly
proportional to the concentration of ATP (Chappelle and Levin, 1968). For ATP analysis of *C. parvum* oocysts, we used the Kikkoman ATP analysis kit (Kikkoman Co., Japan). A luminescence counter (Lucimcounter, Microtech Co. Niti-on 700) was used to measure light emission.

After each pretreatment, the samples were placed in an ice bath, and 100 µl of the sample were transferred to a cuvette containing 100 µl of 0.025 M HEPES (pH 7.75) by micropipette equipped with disposable tips. Then, 100 µl of ATP extraction reagent were added into the cuvette to extract ATP. Extraction time of ATP was exactly 1 minute. The cuvette was set in a luminescence counter. 100 µl of luciferin-luciferase enzyme were added into cuvette. Then the light emission from the cuvette was immediately measured for a given time. Figure 1 shows an example of standard curves for light emission in integrated times of 30 and 60 seconds. ATP concentrations of the standard curve were in the range of 0.01 µg/l to 100 µg/l. Good correlation was obtained between standard ATP concentration and light emission integrated for 30 sec and 60 sec. Because of rapid loss of ATP and variation in the activity of enzyme during the incubation time, standards were always assayed immediately after preparation (Patterson *et al.*, 1970).

The ATP content of any sample assayed was determined on the basis of the standard curve using relative light unit (RLU) integrated for 30 seconds. For an assay, the test tube was placed in an ice bath.

**DAPI/PI permeability**

The DAPI/PI permeability assay was performed according to the method of Campbell *et al.* (1992). Aliquots of sample in 1.5-ml microcentrifuge tubes were centrifuged at 1,100 *g* for 10 minutes and then washed once by resuspending the pellet in 1 ml of acidified HBSS. The tubes were incubated at 37°C for 1 hr in the dark. Oocyst suspensions were centrifuged at 1,100 *g* for 10 minutes, and the pellets were washed twice with HBSS and 100 µl of suspension were collected. Ten-microlitres of solutions 4',6-diamidino-2-phenylindole (DAPI) (2 mg/ml in absolute methanol) and propidium iodide (PI) (1 mg/ml in 0.1 M phosphate-buffered saline (PBS), pH 7.2) were added to the suspension, and were mixed with a vortex mixer. The mixture were incubated in the dark at 37°C for 2 hr. Oocyst suspensions were washed twice with PBS and resuspended in 100 µl with 0.3 M 1,4-diazabicyclo[2.2.2]octane (DABCO) in 0.1 M PBS (pH 7.2) (DABCO-PBS). Ten-microlitre aliquot was pipetted onto a microscope slide and examined using differential interference contrast (DIC) and epifluorescence microscopy, using an UV filter block for DAPI and a green filter block for PI. The sum of impermeable (DAPI- and PI-negative [DAPI- PI-]) and semipermeable (DAPI-positive [DAPI+ PI-]) oocysts was considered as the number of viable oocysts (Campbell, 1992).
In vitro excystation
The oocyst suspensions pretreated in acidified HBSS were washed twice with HBSS to remove residual acid and were resuspended in a volume of 100 µl with HBSS. One hundred microliters of 2% bile solution in HBSS and 50 µl of 0.44% sodium hydrogen carbonate solution were added to each sample. The suspensions were again mixed on a Vortex mixer and then incubated for 2 hr at 37ºC in the dark. After incubation, the samples were again washed in HBSS and then adjusted to a volume of 100 µl. Twenty microliters of sample were placed in a Hemocytometer chamber and examined with a DIC microscope. The percentage of viable oocysts was determined by the difference in the numbers of empty oocysts observed by DIC microscopy between before excystation and after excystation.

Ozonation
Ozone treatment experiments were conducted in a 500-ml reactor at 20ºC. Ozone was produced from ultra pure oxygen with an ozone generator (Model POX-20, Fuji Electric Co.). Ozone was bubbled for 1 hr in a 500-ml gas absorption flask filled with 500 ml Milli-Q® water (Millipore) to obtain designated concentration of ozone. The purified oocyst suspension was injected into the reactor to make a final concentration of approximately 1.0 $10^5$ oocysts per ml. Samples were withdrawn with elapsed time for monitoring of residual ozone and the viability of oocysts by in vitro excystation, DAPI/PI permeability, and ATP measurements after sonication or excystation treatment. Ozone concentration was measured by Indigo method at the wavelength of 600 nm.

Results and discussions
Comparison of three pretreatment methods for ATP extraction of oocysts
Figure 2 shows ATP extracted from oocysts in acid pretreatment (ATPa). The relationship between ATPa and number of oocysts was not linear, and no significant difference was observed between the extraction time of 1 and 3 minutes. The ATPa concentration extracted from a sample containing $10^6$ oocysts/ml was less than 0.4 µg/L. These results showed that ATP from oocysts could not be efficiently extracted by acid pretreatment.

Figure 3 shows the ATP concentrations extracted by excystation pretreatment (ATPe) at 37ºC for 30, 60 and 120 minutes. A linear relationship was observed between extracted ATPe and number of oocysts in the range of $10^4$–$10^7$ oocysts/ml, and highest ATPe concentration was obtained in the excystation pretreatment time of 60 minutes. Although the number of sporozoites after excystation for 120 min was theoretically higher than that after excystation for 60 minutes, the concentration of ATPe for 120 min was less than that of ATPe for 60 min. This result might indicate that sporozoites were partially inactivated between 60 min and 120 min.

Figure 2 ATP concentration (ATPa) extracted from oocysts by incubation in acidified HBSS
In the preliminary tests, the sonication pretreatment at 90W for 2 minutes increased water temperature of samples up to 85°C. However, no meaningful difference was observed in ATP measurement in 20°C and in 90°C. Figure 4 shows the effect of sonication time and sonication intensity on ATP extraction from oocysts. In most cases, there was no apparent advantage in using the longer sonication treatment time of 120 or 180 seconds, and the highest values were obtained at 30 or 60 seconds. High values of ATP from oocysts were obtained in sonication treatment at 90 and 165 W. Sonication power of 30 W was insufficient for ATP extraction from oocysts. When sonication power was 165 W, a part of sample in the test tube flowed out to the outside. Therefore, sonication power of 90 W was adopted for ATP extraction pretreatment from oocysts, while treatment time of 30–60 seconds was adopted. Levin et al. (1975) reported that when ATP was added directly to viable sludge, the ATP began to disappear immediately, presumably as the cells incorporated or degraded it.

Figure 4 shows the concentration of ATP extracted by sonication treatment (ATPs). A linear relation was observed between ATP concentration and number of oocysts in the range of 10^3–10^6 oocysts/ml. The amount of ATP extracted from an oocyst by sonication treatment was one order higher than that by excystation treatment. The average ATPs concentration extracted from an oocyst was 2.9 \times 10^{-8} \mu g, which was about 36 times higher than the value for E. coli (8 \times 10^{-10} \mu g/cell) reported by Levin et al. (1975). When the samples were repeatedly sonicated (1–5 times for 15 seconds), excystation of oocysts and release of sporozoites were observed microscopically. The number of countable intact and excysted oocysts decreased during the sonication treatment. This implied that the destruction and excystation of oocysts occurs simultaneously by sonication treatment.
Feasibility test for ATP assay

Figure 6 shows the results of remaining percent of viable oocysts measured by *in vitro* excystation, DAPI+PI- (or DAPI-PI-) permeability, ATPs (ATP extracted after sonication pretreatment) and ATPe (ATP extracted after excystation pretreatment) of oocysts with elapsed times. The results of ATPs assay and DAPI+PI- (DAPI-PI-) permeability assay had higher values than that of *in vitro* excystation and ATPe assay. In the case of an initial ozone concentration of 1.0 mg/L, excystation function was damaged at the level of more than 90% of oocysts within 2 minutes. Most sporozoites were inactivated in 10 minutes with the viability measured by DAPI/PI permeability assay. The value of ATPe could account for the activity of sporozoites excysted from oocysts, while the value of ATPs accounts for the activity of sporozoites within all oocysts in the sample. ATPe of oocysts in sample was decreased with elapsed time and diminished almost within 10 minutes, while ATPs of oocysts still remained at about 4% of initial concentration. This implies that even though the excystation function is damaged, the sporozoites still remain active.

Correlation of ATPe and ATPs assay with *in vitro* excystation and DAPI/PI permeability assay gave a coefficient of correlation of 0.94 and 0.98 respectively as shown in Figure 7 and 8. The correlation between ATPs assay and DAPI/PI permeability assay had higher correlation coefficient than that between ATPe assay and *in vitro* excystation. Table 1 shows a comparison of ATPs, ATPe, *in vitro* excystation and DAPI/PI permeability assays as indicators of viability of *C. parvum*. The value of ATPe represents the amount of oocysts with
excystation function and active sporozoites. ATPs assay can measure activity of oocysts in sample within five minutes without microscopy. Therefore, ATPs assay is superior to other assays because of its rapid and simple procedure.

Conclusions
A rapid and simple evaluation method by the ATP measurement was developed as surrogate indicator to assess the viability of Cryptosporidium oocysts. The main conclusions obtained in this study are as follows:
1. Incubation in acidified HBSS was insufficient to extract ATP from oocysts.
2. A linear relationship was observed between ATP concentration (ATPe) extracted after excystation pretreatment at 37°C and the number of oocysts in the range of $10^4$–$10^7$ oocysts/ml, and highest ATPe concentration was obtained in the excystation pretreatment time of 60 minutes.
3. To maximize ATP extraction of oocysts by sonication treatment, 30–60 seconds of sonication treatment at 90 W, 200 kHz is recommended.
4. A linear relation was observed between ATP concentration (ATPs) extracted after sonication treatment and number of oocysts in the range of $10^3$–$10^6$ oocysts/ml. Extraction and measurement of ATP in oocysts by sonication treatment was proved to be more effective than other pretreatments, and determined within 5 minutes without microscopy. The average ATP extracted from an oocyst was estimated to be $2.9 \times 10^{-8}$ µg. The detection limit using this method was 500 oocysts/100 µl.
5. The viability of oocysts evaluated by ATPs assay had a linear relation to that by DAPI/PI permeability assay ($R^2=0.98$). ATPs assay is superior to in vitro excystation and DAPI/PI permeability assay because of its rapid and simple procedure.

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References


