Viability of *Cryptosporidium parvum* oocysts in natural waters

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Abstract. Water-borne transmission of the coccidium *Cryptosporidium parvum* Tyzzer, 1912 is frequently responsible for outbreaks of human cryptosporidiosis. One of the most important was reported in 1993 in Milwaukee in the United States, where 403,000 cases were recorded. The determination of the percentage of oocysts excystated is the first step in evaluating their viability, but it alone is not sufficient. This percentage depended on the conditions of storage and also the presence of oxidant or disinfectent agents in water. The percentage of excystation is not always related to viability. Therefore, determination of the viability of excysted sporozoites by determining their infectivity for enterocytic Caco2 cell lines in culture provides information essential for evaluating the risk of contaminated drinking water.

Oocysts of the coccidium *Cryptosporidium parvum* Tyzzer, 1912 are widely distributed in natural waters and water-borne transmission is frequently responsible for human outbreaks of cryptosporidiosis. The most important outbreak of drinking water was reported in 1993 in Milwaukee in the United States, where 403,000 cases were recorded (MacKenzie et al. 1995). *Cryptosporidium parvum* oocysts have been detected in different types of natural waters. Surface and ground water may be contaminated as well as drinking water supplies even after treatment with disinfectants. Detection and counting of oocysts in water have limits in evaluating risk (Fayer et al. 1997). Detection should be accompanied with a study of the viability in order to evaluate the risk of water-borne transmission for humans. The aim of this study was to evaluate excystation and the viability of sporozoites using cell cultures.

MATERIALS AND METHODS

Oocysts of *Cryptosporidium parvum*

Cryptosporidium parvum oocysts, provided by M. Naciri (INRA, Nouzilly, France), were obtained from experimentally infected neonate calves. This strain was isolated from human faeces in 1982 and maintained by serial passage in calves. Oocysts were isolated and purified using a gradient sucrose method (Arrowood and Sterling 1987) (d = 1.088 and d = 1.044, respectively) and washed three times at 4°C in phosphate buffered saline solution (PBS) at pH 7.2.

Untreated oocysts were divided into 4 groups: Groups 1, 2 and 3 consisted of oocysts resuspended in PBS at pH 5, pH 7 and pH 8, respectively. Group 4 consisted of oocysts resuspended in a 2.5% potassium dichromate solution. All groups of oocysts were maintainted at 4°C for 42 days and an aliquot of each was examined for excystation on days 7, 14, 28 and 42. In addition, calf stools were kept at 4°C for further excystation assays.

Oocysts obtained from calf stool kept for one month at 4°C were stored for one week in a 2.5% potassium dichromate solution and purified using the gradient sucrose method cited above. Oocysts were suspended in a 2% sodium hypochloride solution for 10 min and washed three times in PBS at 4°C. Two aliquots of 4×10^5 oocysts were treated for 20 min with a 2% alkaline glutaraldehyde solution or a 1.6% chlorhexidine solution before excystation, respectively.

Counting of oocysts

Oocysts were counted microscopically with the aid of a hemocytometer. They were also enumerated using flow cytometry (EPICS Profile II, Coulter) after labelling of oocysts by an anti-*Cryptosporidium* fluorescent monoclonal antibody (FITC, Diagnostics Pasteur, France).

Excystation assays

Oocysts washed three times at 4°C in PBS were resuspended in a 1% (w/v) taurocholic acid (Sigma, St. Quentin Fallavier, France) solution in Dulbecco's Modified Eagle Medium buffer (DMEM, Gibco, Eragny, France). The oocysts were incubated for one hour at 37°C and placed in an ice bath to stop excystation. Counting of oocysts was performed before and after excystation assays using a hemocytometer and was also performed using flow cytometry. The percentage of excystation was calculated as the difference between the number of oocysts counted before and after excystation, divided by the number of oocysts counted before excystation and multiplied by 100.

Excystation assays were also performed for calf stool samples kept without any treatment at 4°C for 42 days. The assays were performed in a stool aliquot after 7, 14, 28 and 42 days. Oocysts of each stool specimen were separated in a sucrose gradient, treated by a 2% sodium hypochloride solution for 10 min and washed three times in PBS. A control consisted of an untreated stool specimen. Excystation assays were conducted as above in parallel.

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Scanning electron microscopy (SEM)

Four suspensions of oocysts previously treated by a 2% sodium hypochlorite solution for 10 min, in 2.5% potassium dichromate, and in a 2.5% potassium permanganate solution were kept for one week at 4°C in water. Oocysts were fixed using fresh 2% glutaraldehyde for one hour and rinsed in sodium cacodylate buffer and post fixed for one hour at 4°C with a 1% solution of osmium tetraoxid in sodium cacodylate buffer. Suspensions were progressively dehydrated. The preparations were coated with gold salts and examined with a scanning electron microscope.

Culture of parasite on cell culture monolayers

Caco2 cells from 7-day-old cultures were trypsinized, washed three times in Hanks' balanced salt solution. Cultures of Caco2 cells (10^4 cells/cm^2) were grown in glass coverslip 6-well cluster plates (Nunc, Noisy le Grand, France) in DMEM supplemented with non essential amino acids, antibiotics and 20% (v/v) fetal calf serum (FCS) (Eurobio, Les Ulis, France) in a CO_2 atmosphere (5% in air) at 37°C. Confluent monolayers were infected with 4×10^5 sporozoites per well obtained by filtration through a 5 µm filtre (Millipore, Molsheim, France). Two hours after sporozoites were added, medium was removed and replaced by DMEM supplemented by antibiotics, 4-para-amino benzoic acid (Sigma, France), ascorbic acid (Sigma, France), 25 mM HEPES buffer (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) (pH = 7.4) (Sigma, France), glucose and insulin (Novo, Nordisk, Boulogne-Billancourt, France) and 20% FCS. After a 48 hrs culture, coverslips were fixed in acetone and incubated with anti-*Cryptosporidium* human serum (1 : 200) for 30 min at 37°C. Parasites were detected using a second antibody consisting of sheep anti-human immunoglobulin (IgG, IgA, IgM) FITC-conjugated antibody (Sanofi, Diagnostics Pasteur, France) (1 : 100 in a 10% Evans blue solution) for 30 min at 37°C. After washing, coverslips were microscopically examined and the different stages of the developing parasites were counted in ten oil microscopy fields ($\times$ 1,000) (Favennec et al. 1994).

Statistical analysis was performed using the Student's *t*-test, or the non-parametric correlation test of Spearman.

RESULTS

The mean values of oocyst excystation obtained five days after collecting the stool samples (D0) were 44.8% (range 34.7-59.2%). Results in Table 1 showed seven days later (D7) a percentage significantly higher excystation rate for treated oocysts, reaching 98.1% and 96.8% respectively, than for untreated oocysts (38.1%) (p < 0.001). This percentage did not decrease significantly between D7 and D42 in the different groups of experiments.

The influence of pH during storage of oocyst suspensions on the percentage of excystation was also

Table 1. Effect of the duration of storage at 4°C of oxidant treated and untreated *Cryptosporidium parvum* oocysts on the percentage of excystation.

Days	7	14	28	42
Water (pH 5)	38.1 ± 11.5	33.6 ± 17.1	33.3 ± 13.1	34.2 ± 13.1
NaClO (2%)	98.1 ± 1.4	99.3 ± 0.8	98.6 ± 0.1	93.3 ± 0.8
K$_2$Cr$_2$0$_7$ (2.5%)	96.8 ± 2.8	94.3 ± 1.3	96.8 ± 1.1	88.9 ± 1.8

Table 2. Influence of the pH and duration of storage of *Cryptosporidium parvum* oocysts on the percentage of excystation.

PH	Day 7	Day 14	Day 28	Day 42
5	38.1 ± 11.5	33.6 ± 17.1	33.3 ±13.1	34.2 ± 13.1
7	41.3 ± 15.8	29.2 ± 4.7	31.7 ± 6.7	27.2 ± 3.8
8	38.1 ± 4.1	18.6 ± 2.9	28.8 ± 17.4	23.7 ± 8.1

Table 3. Influence of disinfectants on the percentage of excystation of *Cryptosporidium parvum* oocysts.

| Disinfectant	Concentration	Excystation (%)
Glutaraldehyde	2%	9.6 ± 0.7
Chlorheximide	1.6%	88.1 ± 2.8
None	0	76.3 ± 8.4

studied with untreated oocysts stored at 4°C in water buffered at pH 5, 7 and 8 during the same period as above. Table 2 showed no significant difference between oocysts stored at pH 5 and pH 7 and a non significant decrease between oocysts stored at pH 5 and pH 8.

The influence of treatment of oocysts with a 2% alkaline glutaraldehyde solution or a 1.6% chlorhexidine solution prior to excystation was assayed. Glutaraldehyde inhibited excystation by 89% compared to the control and no inhibition was found with oocysts treated with chlorhexidine solution (Table 3).

The percentage of excystation could be evaluated more easily using flow cytometry than microscopic counts. The results of the two methods were strongly correlated using the Spearman test (r' = 0.95).

Changes in the structure of oocyst walls were observed when oocysts were treated by oxidants such as potassium permanganate, sodium hypochlorite and potassium dichromate (Figs. 2-4) compared to the control (Fig. 1).

The viability of sporozoites excysted from oocysts was tested using Caco2 cells showing that most of the sporozoites were infecting for cells.
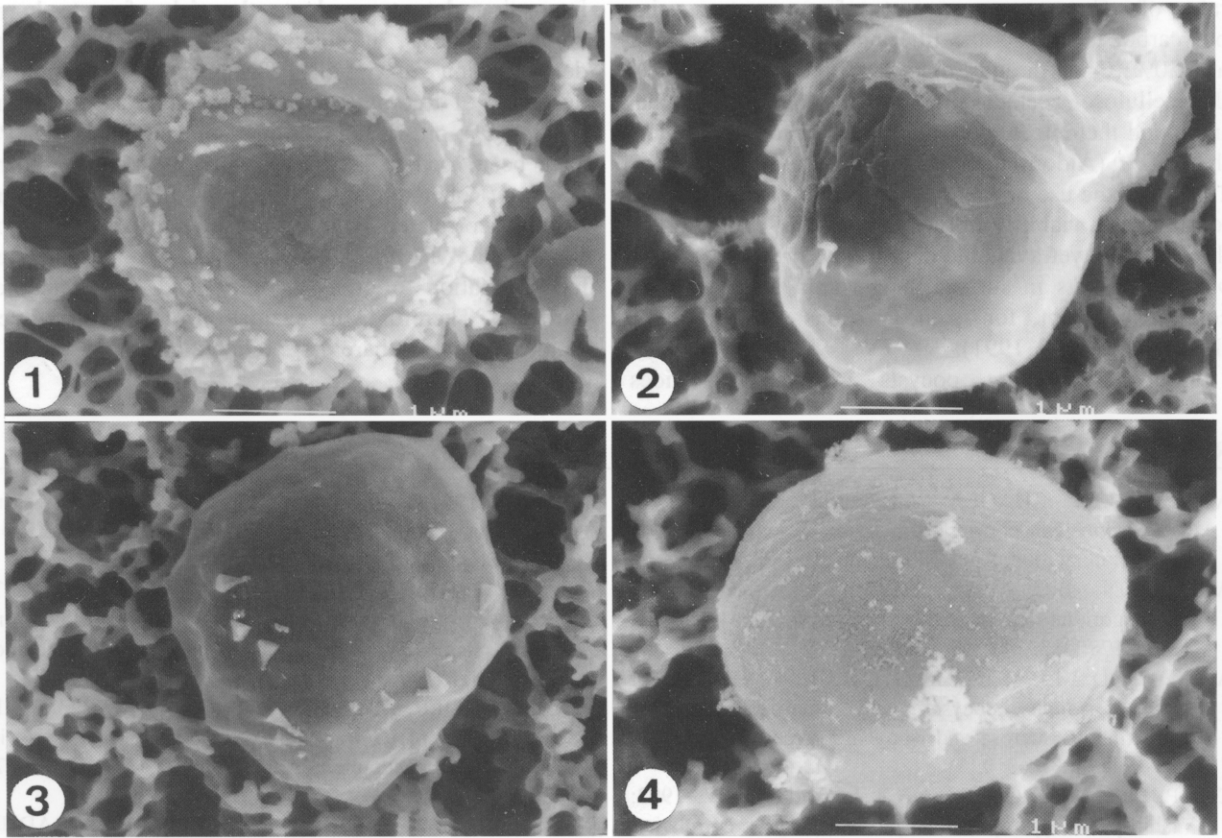
Figs. 1-4. Scanning electron microscopy of *Cryptosporidium parvum* oocysts (× 20,000). **Fig 1.** Oocysts exposed to 2.5% potassium permanganate. Oocyst wall shows numerous echinulations. **Fig. 2.** Oocysts exposed to 2.5% potassium dichromate. Wall of oocyst appears teared. **Fig. 3.** Oocysts exposed to 2% sodium hypochlorite. Shape of oocyst becomes irregular. **Fig. 4.** Oocysts unexposed to oxidants. Wall of oocyst is regular and narrow striated.

DISCUSSION

The agricultural sources of water pollution, especially from dairies and grazing lands, are probably more important than pollution from human sources. An important reservoir consists of infected calves which may excrete oocysts daily for several weeks with a total output approaching 10^{10} oocysts (Anderson 1981). *Cryptosporidium parvum* oocysts may remain viable for as long as one year at 4°C (Smith and Rose 1990) and are strongly resistant to the most commonly used disinfectants. The chlorination of drinking water is not sufficient to prevent *C. parvum* infection (Fayer et al. 1997).

The excystation of sporozoites from oocysts is the first indicator of their possible viability. The conditions of treatment of oocysts may enhance or reduce excystation of sporozoites and thereby influence the risk of infectivity. Storage of oocysts directly in water reduces the ability of oocysts to excyst by 2.57 times compared to oocysts treated by a 2% sodium hypochlorite solution (Table 1). Chlorination of water as

well as treatment with other oxidants such as potassium dichromate or potassium permanganate facilitate excystation of sporozoites and therefore could be considered to enhance the risk. Storage of oocyst suspensions at 4°C, at pH 8 reduced excystation compared to those maintained at pH 5 or pH 7. Woodmansee (1987) obtained lower percentages of excystation at pH 8 than at pH 7 or pH 5.

Excluding the water-borne cryptosporidiosis outbreaks, the risk of contamination for immuno-competent individuals has been evaluated. DuPont et al. (1995) have found that the 50% infective dose was 132 oocysts and the 100% infective dose was 10^2 oocysts. In immunocompromised patients, particularly in those infected by HIV, the infective dose is not known and is likely much lower. In addition, the tests of viability *in vivo* and *in vitro* showed that all oocysts found in water were not alive, depending on the conditions and time of storage before consumption as drinking water.

The occurrence of *C. parvum* in water is greatly underestimated due to the limit of the methods currently used to recover oocysts in the samples. The recovery

rate of *C. parvum* oocysts from water using polycarbonate membrane filtering ranged between 5% and 50% (Ongerth and Stibbs 1987). The recent use of the cellulose acetate filtre dissolution method has improved the detection rate of oocysts recovering more than 70% (Aldom and Chagla 1995, Graczyk et al. 1997) and the use of acetone and alcohol does not seem to alter their viability (Graczyk et al. 1997). However, there are not yet available data on the prevalence of *C. parvum* oocysts in surface and ground water in most countries except for some regions of the USA (LeChevalier et al. 1991).

Although counting oocysts in water provides interesting data to evaluate the risk of water-borne cryptosporidiosis, it should be associated with the determination of viability. The tests of inclusion or exclusion of fluorogenic vital dyes such as propidium iodide (PI) or 4',6-diamidino-2-phenylindole (DAPI) have been used to assess the viability of water-borne oocysts (Robertson et al. 1992). Although results are strongly correlated with the percentage of excystation (Campbell et al. 1992), excystation is only the first step for evaluation of the viability and is sometimes unrelated. Therefore, the evaluation of viability in cell culture or in animal are more reliable models.

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