Quantification of *Cryptosporidium parvum* in anaerobic digesters treating manure by (reverse-transcription) quantitative real-time PCR, infectivity and excystation tests

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Abstract The survival of *Cryptosporidium parvum* oocysts in anaerobic digesters treating manure was investigated for mesophilic, thermophilic, and a combined treatment (mesophilic–thermophilic–mesophilic) under different retention times of oocysts in the reactors. *C. parvum* DNA was extracted with an optimised protocol, and its amount determined by quantitative real-time PCR (qPCR). Results indicated noteworthy differences in DNA content after the different treatments. DNA was not degraded during the process. However, excystation and infectivity tests showed a reduction of viable oocyst numbers of ≥ 2 and ≥ 5 log units after the thermophilic treatment in two different experiments. Thus qPCR-targeting DNA can overestimate the number of oocysts that survive and remain viable after anaerobic digestion. However, targeting DNA is suitable to indicate the presence or absence of oocysts. Reverse transcription qPCR (RT-qPCR) targeting *C. parvum hsp70* mRNA successfully indicated the presence of viable fraction of oocysts.
Keywords *Cryptosporidium parvum*; DNA; hygiene; manure; mRNA; quantitative real-time PCR

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Introduction

Animal manure is a potential source of pathogenic microorganisms, which might include *Cryptosporidium parvum* parasites. Applying manure or slurry directly to the land without previous treatment can contribute to pathogen contamination of land, freshwater and groundwater (Burton and Turner, 2003). *Cryptosporidium parvum* oocysts are highly resistant to environmental stress, they appear to survive under a wide range of temperatures (Jenkins *et al.*, 2003; Robben and Sibley, 2004), and can remain viable and infectious in water for many months. They have been found responsible for major waterborne disease outbreaks worldwide (Smith and Rose, 1998) but also play a significant role in food-borne human infections (Duffy and Moriarty, 2003). These organisms are therefore of particular interest in hygiene control of potentially contaminating organic sources. Agricultural farms have been applying anaerobic digestion of manure and slurry for energy recovery and for reducing the number of pathogens in the substrate. Anaerobic digestion is particularly effective for hygienization at higher (than mesophilic) temperatures, and the time the substrate is retained in the digesters plays a dominant role for pathogen reduction (Kearney *et al.*, 1993). *Cryptosporidium* oocysts are not significantly affected by mesophilic anaerobic digestion (Burton and Turner, 2003); thus their detection and quantification in manure before spreading is important for preventing human and animal infections, and monitoring their activity guarantees the sanitising effect of the anaerobic process.

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Standard detection methods for detecting *Cryptosporidium parvum* in environmental samples (e.g. water samples) based on isolation and identification of oocysts are time consuming and difficult to apply for manure samples, due to the structural composition of manure, which can hinder detection. Purification steps, such as density gradients, can introduce bias when applied for manure. Direct immunofluorescence assays may not be reliable due to non-specific fluorescence and fluorescent background due to debris and other materials (Ongerth and Stibbs, 1987). Several other molecular techniques described for the detection of *C. parvum* in different environmental samples are not yet optimised for manure (e.g. Kaucner and Stinear, 1998; Vesey *et al.*, 1998). A sensitive method is needed to obtain reliable data from environmental samples. The method should be able to detect low numbers of oocysts, as the minimum infectious dose for producing infection is small.

In this work, we applied quantitative real-time PCR (qPCR) in combination with an optimised bead beating nucleic acid extraction method (Garcés *et al.*, 2004) for the rapid and specific detection and quantification of *Cryptosporidium parvum* oocysts in samples from anaerobic digesters treating manure at different temperature conditions. Our aim was to apply qPCR for optimised DNA extracts to evaluate the applicability of the method for monitoring oocysts in anaerobic digesters, and to determine the reduction of oocyst numbers under the different treatments investigated (mesophilic, thermophilic and a system constituted by a mesophilic–thermophilic–mesophilic digester chain (Effenberger *et al.*, 2003). We also aimed at evaluating the method for detecting low numbers of oocysts. DNA was first used due to its higher stability compared to mRNA, and to support reliable detection. A further approach based on quantification of induced *hsp70* heat shock protein transcripts (mRNA) was performed for detecting the viable fraction of oocysts. The ability to produce mRNA has been correlated with the viability of an organism (Mahbubani *et al.*, 1991; Stinear *et al.*, 1996). The heat shock proteins are reported to be synthesized with a high level of efficiency and the transcripts are present particularly in stressed organisms (Lindquist and Petersen, 1990). Preliminary data obtained with reverse transcription qPCR (RT-qPCR) from mRNA extracts are presented in this work, and discussed along with results from qPCR, total oocyst counts, infectivity and excystation tests.

Materials and methods

Oocysts, sampling and reactor configuration

Suspensions of *Cryptosporidium parvum* oocysts were obtained from artificially infected cattle and from wild infections. After purification, they were counted in an Abbe–Zeiss counting chamber with a phase contrast microscope. Suspensions were stored at 4 °C.

Samples for the sentinel chamber experiments (see below) were obtained from a model biogas plant treating liquid cattle manure (concentration 7.6% total solids). The model plant consisted of a chain of three anaerobic digesters (mesophilic 1, 38 °C; thermophilic, 55 °C; and mesophilic 2, 38 °C), with working volumes of 200, 240, and 600 litres for the Meso1, Thermo, and Meso2 digesters, respectively. The model plant was used instead of the full-scale system operated near Rosenheim (Germany) (Effenberger *et al.*, 2006). Experiments with *C. parvum* were carried out in this model plant. Relevant process parameters were monitored to control reactor performance and the stability of the process (not shown).

Sentinel chamber experiments

To evaluate the fate of *Cryptosporidium* oocysts in the digesters, we used sentinel chambers made of polycarbonate and filters of 0.4 μm pore size. Sentinel chambers

(ca. 3 mL volume) were filled with a mixture of pre-quantified pure oocysts suspension (1 mL) and 1.8 mL of digester content (digested manure).

We performed two different experiments. For experiment 1 (Figure 1) we used pure digester content to fill the sentinel chambers, whereas for experiment 2 (Figure 2) supernatant after centrifugation of the digester content for 5 min at 2,500 g was used. This modification was done to reduce the particulate material in the manure and to facilitate subsequent infectivity tests, but to maintain the osmotic pressure and (potentially toxic) composition of the fermenter material.

Filled sentinel chambers were introduced into the anaerobic digesters and maintained inside for different retention times (from 1 to 12 h) by using a removable tray. The retention time (1 and 4 h) corresponded with the feeding interval applied in the corresponding pilot-scale system (Effenberger *et al.*, 2006) and in the model plant.

For experiment 1, the different treatments investigated and the total time the sentinel chambers were maintained inside were as follows: (a) mesophilic (Meso1) for 1 h, (b) thermophilic (Thermo) for 4 h, (c) mesophilic + thermophilic (Meso1 + Thermo) for 5 h, (d) mesophilic + thermophilic + mesophilic (Meso1 + Thermo + Meso2; reactor chain) for 6 h. For experiment 2, sentinel chambers were maintained in the mesophilic, thermophilic and in the reactor chain for 4, 4 and 12, and 20 h respectively. After each processing time, sentinel chambers were removed from the reactors and processed immediately. Chamber contents were recuperated by carefully washing with 1 × phosphate buffer saline (PBS) and concentration by centrifugation for 5 minutes at 2,500 g. The supernatant was removed and the pellet resuspended in 1 mL volume as initially used for spiking the sentinel chambers with the oocyst suspension.

For positive control samples, manure was also spiked with a known amount of oocysts, and not subjected to anaerobic treatment. Positive controls were performed in parallel to treated samples in every experimentation. In experiment 1, the positive control sample was maintained for 6 h at room temperature before DNA extraction, as indicated in Figure 1. A sample with pure oocysts that were stored at 4 °C was also included in this experiment.

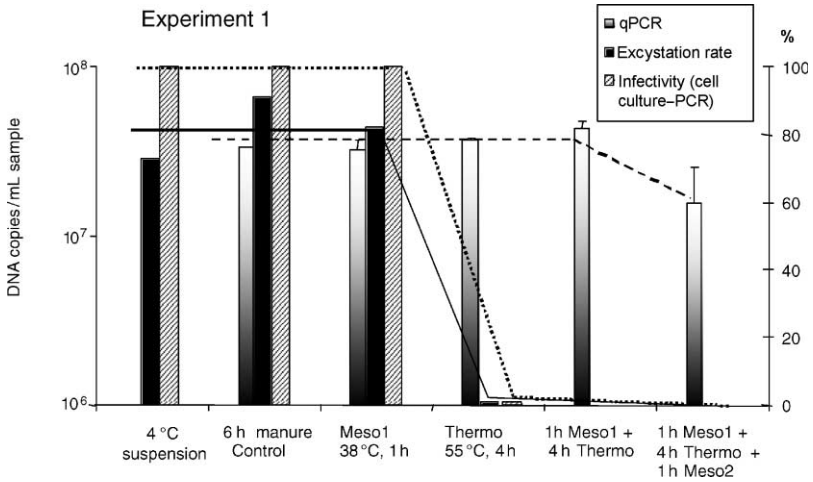

Figure 1 Effect of different anaerobic digestion conditions on *C. parvum* DNA copies detected, excystation ability (%), and percent of infectivity (cell culture–PCR) after NaCl flotation purification; h: hours

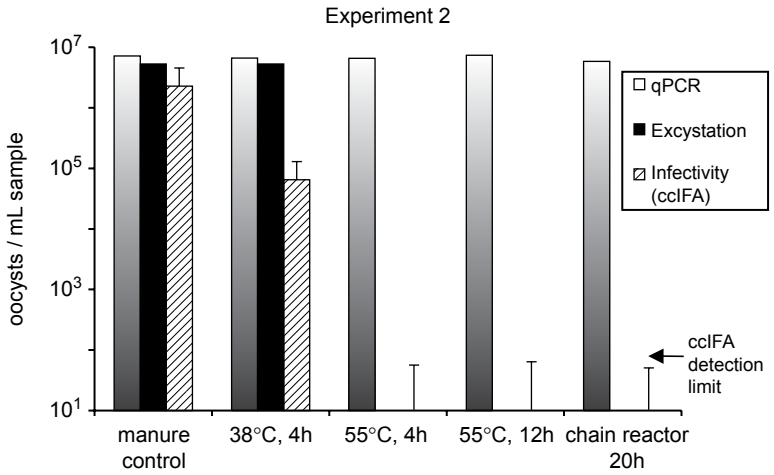

Figure 2 Effect of different anaerobic digestion conditions on *C. parvum* oocysts as determined by qPCR, excystation rate and infectivity test (cell culture immunofluorescence assay) after percoll gradient purification; h: hours

Nucleic acid extraction and (RT)-qPCR

We used 50 μL aliquots from the sentinel chamber contents for DNA extractions. Extraction was performed with the bead beating approach for oocyst disruption and a commercial DNA extraction kit (FastDNA® SPIN Kit for soil) according to our optimised extraction protocol as previously described (Lebuhn *et al.*, 2003; Garcés *et al.*, 2004, 2005).

mRNA extraction from oocysts was done from 100 μL of the sentinel chamber content. Samples were incubated at 45 °C for 20 minutes (heat shock) to induce the production of the *hsp70* mRNA transcripts (Stinear *et al.*, 1996). After heat shock, lysing buffer (Dynal) was added, and the sample subjected to bead-beating treatment for oocyst disruption (for 60 + 60 + 45 seconds in time intervals) as described by Garcés *et al.* (2004, 2005). We used magnetic beads technology (Dynabeads® mRNA DIRECT™ Kit, Dynal Biotech) for the isolation of mRNA. Magnetic separation was performed following the manufacturer's suggestion. Extracted nucleic acids were treated with DNase (TURBO DNA-free™, Ambion) to remove contaminating DNA from the extract as suggested by the manufacturer.

Reverse transcription was done for 30 min at 57.5 °C with 5 μL of the extract using ThermoScript™ Reverse Transcriptase (Invitrogen) to produce cDNA. Negative controls without reverse transcriptase (− RT) and DNase controls were also included.

Primers specific for the *C. parvum hsp70* gene were applied for reverse transcription and qPCR. Quantification by qPCR was carried out applying the standard spiking method as described earlier (Lebuhn *et al.*, 2003, 2004).

Excystation and infectivity test

Prior to excystation and infectivity tests, we purified the samples from the sentinel chamber contents in order to recuperate pure oocysts to avoid interferences in the downstream steps. For experiment 1, purification of the samples was done with the NaCl flotation method, whereas for experiment 2, a percoll gradient was used.

In vitro excystation from pure oocyst suspensions and from the sentinel chamber contents was performed as a measure of vitality, and to compare with (RT)-qPCR results. We used 50 μL aliquots with 25 μL of sodium taurocholate (0.8%) to induce oocyst

excystation. Samples were incubated for 2 hours at 37 °C at mild shaking conditions (Campbell *et al.*, 1992). Excysted and intact oocysts were counted microscopically in a phase contrast microscope. Percent of excysted oocysts was calculated by dividing the number of excysted oocysts (empty oocysts) by the total oocyst number (excysted + intact oocysts).

We also performed infectivity tests from the different samples. For experiment 1, the infectivity test was done with cell culture–PCR as described in Najdrowski *et al.* (2004a, b). In experiment 2, the test was done by an immunofluorescence assay using polyclonal antibodies against the sporozoite cell wall, and infection of HCT-8 human adenocarcinoma cells as described by Wackwitz *et al.* (2004). Infected cells were observed by epifluorescence microscopy. The test was performed to quantify the fraction of infective oocysts after the treatments.

Results and discussion

DNA approach, excystation and infectivity

First experiments were carried out targeting DNA to evaluate the reduction of oocyst numbers after the different treatments. The method detection efficiency (including extraction and PCR detection efficiency) after qPCR was 60–100%. 1–2 copies of DNA per reaction volume could be detected (corresponding to 250–500 oocysts/mL manure).

According to Figure 1, the number of DNA copies per mL manure detected in the different digestion systems was more or less the same as in the control (oocysts in manure) except for the treatment Meso1 + Thermo + Meso2 where we obtained a marginal reduction of 50–60%. Excystation results indicated that viable oocysts were reduced by ca. 2 orders of magnitude after the thermophilic treatment, and more than 3 log units in the connected digester systems (Figure 1). However, excystation results in experiment 1 might not exactly represent the actual fraction of viable oocysts, due to potential overestimation by excystation (Bukhari *et al.*, 2000). In addition, selective enrichment of intact oocysts probably occurred during purification of the sample by NaCl flotation (Najdrowski, not published). This might have produced biased results towards inactivation.

Infectivity results from experiment 1 indicated that oocysts were inactivated by ≥ 2 log units after the thermophilic treatment, and less than 0.1% was obtained for the reactor chain. The relatively small reduction in infectivity was probably mainly due to bias introduced by the NaCl flotation purification (see above). Since lower percentages of infectivity of the remaining oocysts were expected, NaCl flotation was substituted by percoll gradient purification in the following experiments for improving the purification step.

To verify the lack of reduction of DNA content, and to evaluate the excystation and infectivity results from experiment 1, we performed a similar experiment, but this time we used percoll gradient purification of the sentinel chamber contents, and cell culture–inmunofluorescence to assess infectivity. The time the oocysts were maintained in the different treatments was in part extended during this experiment (Figure 2). DNA copies showed almost no significant variation after the different treatments and were comparable to the control (not subjected to treatment). These results for DNA corroborated the previous ones, suggesting that neither the thermophilic nor the chain reactor affected DNA concentrations even at prolonged oocysts retention time. The (almost) unchanged DNA content in the different systems (Figures 1 and 2) indicates that DNA from *C. parvum* oocysts was not degraded in the digesters and that qPCR overestimates viable oocysts by the amount of dead oocysts present. Hence, DNA detected from the thermophilic digesters, Meso1 + Thermo, and Meso1 + Thermo + Meso2 treatments should correspond almost exclusively to non-viable oocysts.

In experiment 2, infectious oocysts were reduced by more than 5 log units after the thermophilic treatment (4 h) in comparison to the control manure. The same was true for the ability to excyst. However, the comparison at the mesophilic treatment indicates that the excystation test overestimated infectious oocysts by 2 log units (Figure 2).

The important difference in infectivity reduction after the thermophilic treatment between experiments 1 and 2 can be explained by the different methodologies used. Percoll gradient purification had a much higher recovery rate (90% in comparison to 30% of NaCl flotation) and appeared not to select specifically for intact oocysts. Also, differences in sensitivity of the detection systems (cell culture–PCR and cell culture immunofluorescence) can explain this difference. In addition, it is possible that the purification steps might have produced changes in the structural composition of some oocysts, thereby some of them might became more susceptible to excystation test, but not being necessarily vital.

Hence, DNA-targeted qPCR is not able to differentiate between dead and viable oocysts, but is an ideal tool for high-throughput screening of samples to determine the presence of oocysts. In the case of positive results, these samples must be further examined for the fraction of infective oocysts.

mRNA approach

In our attempt to determine the viable fraction of oocysts and to supplant infectivity tests for determining inactivation of oocysts, we quantify mRNA-induced transcripts from the *hsp70* gene. No DNA was detected after DNase treatment, and (−RT) controls were always negative. According to Figure 3, cDNA quantified from the manure control sample, which had not undergone treatment, exceeded the spiked DNA copies by more than 1 log unit. This contrasts with the 10^3–10^4 fold induction of heat shock mRNAs observed for *Drosophila* (Lindquist and Petersen, 1990). We assume that detection of heat shock-induced mRNA indicates viability of the oocysts. This assumption was confirmed by the high excystation rate obtained for this sample (ca. 87%).

In the sentinel chambers exposed to thermophilic conditions (50 °C and 55 °C), cDNA quantified was less (ca. 2.2 log units) than the positive control (no treatment) (Figure 3). Detected mRNA was slightly higher (ca. 2 times) after 1 h at 50 °C than after more severe stresses, indicating ca. 2 times higher viability of oocysts after this treatment. However, we observed that mRNA detected in the samples subjected to thermophilic treatment not

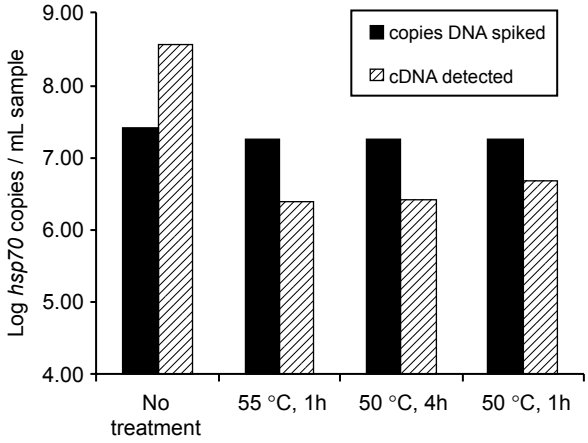

Figure 3 Comparison of *C. parvum* oocysts spiked to manure and *hsp*70 mRNA net production after heat shock of samples from different thermophilic conditions and no treatment (control)

only represents mRNA net production induced by the heat shock, but also residual mRNA induced by the treatments. The temperature in the reactors probably induced *hsp70* mRNA production, which still can be measurable by RT-qPCR as a background. Results from preliminary experiments without heat shock (not shown) support this view.

This first experience showed us that mRNA extraction and quantification by RT-qPCR was feasible even for manure samples, despite the presence of PCR inhibitors (e.g. humic substances) and the low stability of mRNA. At the moment, we are investigating the amount of mRNA produced by the remaining oocysts after anaerobic treatment at different temperatures, and are trying to correlate results with infectivity tests in order to evaluate if RT-qPCR for induced *hsp70* mRNA transcripts could substitute infectivity tests.

Conclusions

DNA copies of *Cryptosporidium parvum* proved to be quantifiable in cattle manure samples by qPCR using our optimised extraction method. Our results suggest that qPCR targeting DNA is a fast, sensitive and specific method to screen environmental samples for *Cryptosporidium parvum* oocysts e.g. in anaerobic digesters during hygiene monitoring. The method is suitable for detecting low numbers of oocysts in difficult environmental samples with chemically complex structure such as manure. Thus, qPCR (target DNA) can provide reliable data and avoid underestimation of oocyst numbers. However, DNA from oocysts was detected even after thermophilic anaerobic treatment. This amount most probably corresponds to dead organisms because they showed no infectivity and were not excystable. Thus targeting DNA can only provide a first hint on the presence of potentially viable oocysts. Positive qPCR results must therefore be confirmed by metabolism-targeted approaches to avoid overestimation of viable oocysts after anaerobic treatment. Such an approach could be the quantification of induced *hsp70* mRNA production.

mRNA transcripts from the *hsp70* gene was shown to be detectable and quantifiable by RT-qPCR in manure samples using extraction by magnetic separation. The control sample showed production of mRNA indicating presence of vital oocysts. A question to be solved is whether the amount of mRNA quantified after the thermophilic digestion was the remaining mRNA induced by the treatment, or if it derived from the fraction of still viable oocysts after the anaerobic digestion. To clarify this, the production rate (mRNA produced per oocyst) must be investigated under different metabolic conditions.

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