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Survival of *Cryptosporidium* species in environments relevant to foods and beverages

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ABSTRACT

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Aims: To provide data on the survival of *Cryptosporidium* oocysts in a range of conditions relevant to foods and beverages.

Methods and Results: *Cryptosporidium parvum* and *C. hominis* oocysts were stored in buffered media at different pH values and with various acids. In addition, neutral solutions with high salt (4·5% w/v), glycerol (20% v/v), sucrose (50% w/v) or ethanol (9 and 40% v/v) were used to determine their effects on survival. After storage periods of between 1 h and 14 days, viability was assessed using sporozoite ratio or infection of MRC-5 cell monolayers (not previously reported for culture of this organism). With all treatments, and with both assay techniques, viable oocysts were found at the end of the storage periods. However, treatments with one of the following additions: high salt, glycerol, sucrose or ethanol showed a negative and statistically significant effect on survival. Decline was noted after 1 day or even 1 h of treatment.

Conclusions: MRC-5 cells are suitable for infection by *C. parvum* and *C. hominis*. Both tissue culture and sporozoite ratio gave broadly similar survival results and the greatest effects were seen with addition of components which reduced water activity.

Significance and Impact of the Study: This study has provided useful additional information to the food industry when considering the risk posed by this organism.

Keywords: *Cryptosporidium*, ethanol, pH, sporozoite ratio, tissue culture, water activity.

INTRODUCTION

The protozoan parasite *Cryptosporidium* came to the fore in the 1980s and early 1990s as a potentially major problem in drinking water supplies. Outbreaks have occurred involving large numbers of people, because of consumption of contaminated water (Anon. 1990, 1995). Although the disease is self-limiting in individuals with healthy immune systems and a full recovery is normally made, the typical symptoms which include diarrhoea, abdominal pain, vomiting, fever and anorexia may lead to hospitalization. It is also a severe and life-threatening illness in the immunocompromised.

As water supplies directly affect the food industry, the implications of the organism contaminating food and beverage products through this route must be considered. Additionally, it is likely that raw fruit and vegetables may occasionally be contaminated with viable organisms via other routes. Consequently these organisms may become incorporated into a range of products and in the absence of pasteurization may survive. It is therefore necessary to understand the likely survival of the organism when applying hazard analysis and critical control point (HACCP)

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and also to decide upon the necessity of stopping production and also potentially recall/destruction of product if water is believed to be contaminated (Dawson 2000). Such incidents affecting the food industry have occurred worldwide: notably in the UK following various boil water notices, during the Milwaukee outbreak in the US, and following a water supply contamination incident in Sydney (Australia) where no outbreak was identified but *Cryptosporidium* oocysts were found in the water supply (Mackenzie *et al.* 1994; McClellan 1998).

Cryptosporidium is known to be an environmentally-resistant organism. It also tolerates a range of disinfectants including chlorine (Weir *et al.* 2002). However, it is not particularly heat resistant (Fayer 1994; Harp *et al.* 1996) and is likely to be susceptible to most heating regimes used by the food industry. Freezing at temperatures of −15 and −20°C also reduces the viability of the organism (Fayer and Nerad 1996). It is known that the organism does not survive desiccation (Deng and Cliver 1999) and it has been reported that reduced water activity decreases viability (Rose and Slifko 1999). This may be relevant to certain food types such as icings, syrups and dry goods. There is also a report on reduction in viability in acidified and carbonated beverages (Friedman *et al.* 1997). However, overall, there is a shortage of published information on its survival in conditions relevant to many food types e.g in conditions of low pH and reduced water activity. The aim of this study was to generate data relevant to such conditions; additionally, the effect of alcohol was assessed.

Cryptosporidium oocysts cannot be cultured on growth media: therefore it is not straightforward to extract the organism from food and enumerate it. To facilitate the generation of data, survival experiments were carried out in tubes containing appropriate media where pH, acid type and water activity were varied. These solutions could then be centrifuged to concentrate the oocysts and their viability assessed.

A variety of techniques has been used to determine *Cryptosporidium* viability: furthermore, although indication of viability is an important factor in determining the potential risk to consumers, the most important consideration is whether or not the organism is still infectious after the treatment. There is considerable evidence that the so-called 'viability techniques' do not always give comparative answers to each other. For example, an oocyst may give a positive viability test, but it may not be capable of infecting a host (Bukhari *et al.* 2000). Such viability techniques encourage interpretation on the side of caution in that the organisms may appear to be viable, but may in fact be damaged and noninfectious. Clearly if there is no indication of viability they are definitely non-infectious.

The work described in this study used two techniques to assess viability. First, sporozoite ratio, which defines the relative numbers of potentially infective sporozoites released from the oocyst and secondly tissue culture, which demonstrates the capability of oocysts to form life cycle stages within monolayers of adherent mammalian cell lines. Sporozoite ratio has been used by various authors (Blewett 1988; Slifko *et al.* 1997a) as a measure of viability and, whilst simple excystation is now known to have limitations as an assay, sporozoite ratio is perhaps the most promising nonculture based method. Slifko *et al.* (1997a) showed sporozoite ratio more closely followed infectivity in HCT-8 cell culture than did excystation, following salt and sugar treatment. Tissue culture methods are now gaining credibility and a study by Rochelle *et al.* (2002) showed equivalence to the 'gold standard' of neonatal mouse infectivity in three cell lines commonly used for growing *Cryptosporidium*, namely HCT-8, Caco-2 and MDCK. The present study was carried out in a routine hospital laboratory which uses particular cell lines for growing other pathogens. Cell lines routinely handled in clinical settings did not include those previously reported to grow *Cryptosporidium*, but preliminary work had shown one cell line (MRC-5) that was routinely used by hospital and pathology laboratories gave reproducible and clear infection with *Cryptosporidium* oocysts. The benefits of using this cell line included the ease of handling and robustness coupled with familiarity with its use in routine screening. A preliminary study showed clear and repeatable infections with inoculations of live oocysts when compared with those that had been heat treated. One aim of this study was to examine potential for a widely available and inexpensive cell line to be used to determine infectivity and provide potentially useful information for risk assessment in the food industry.

Preliminary work had also shown that structures of the correct size and shape for life cycle stages (and clearly different from oocysts) were visible with a simple auramine phenol staining technique, which is also a standard technique in pathology laboratories. These structures showed an intense green fluorescence against the cell monolayer and were absent when heat killed oocysts (70°C, 20 min) were used. Although other techniques such as specific antibody based staining are typically used to allow life cycle stages to be visualized, the quality of the preparations was considered good enough to allow conclusions on infection to be drawn. Also antibodies to life cycle stages are not commercially available.

Although the intention was to generate data with the MRC-5 cell line, the scope of the investigation did not include any statistical comparison between tissue culture and sporozoite ratio. Both *C. hominis* (Morgan-Ryan *et al.* 2002) and *C. parvum* (previously known as *C. parvum* genotypes 1 and 2) were used to generate data.

MATERIALS AND METHODS

Oocysts

Clinical isolates were prepared from *Cryptosporidium*-positive faeces collected by the *Cryptosporidium* Reference Unit (NPHS Microbiology Swansea, Singleton Hospital, Swansea) as part of the National Collection of *Cryptosporidium* oocysts (Anon. 2002). *Cryptosporidium* was confirmed by microscopical inspection of modified Ziehl–Neelsen-stained smears (Casemore 1991) and PCR–RFLP analysis of the *Cryptosporidium* oocyst wall protein (COWP) gene (Spano *et al.* 1997). Briefly, oocysts were separated by flotation from faecal debris using saturated NaCl solution and centrifugation for 8 min at 1600 *g* (Ryley *et al.* 1976). The floated material containing the oocysts was washed with deionized oocyst-free water, the oocysts resuspended in deionized, oocyst-free water and stored at +4°C. To extract DNA, 200 μl oocyst suspension was incubated at 100°C for 60 min and DNA extracted using proteinase K digestion in lysis buffer at 56°C and a spin-column filtration technique (QiAMP DNA mini kit; Qiagen, Crawley, UK). DNA extracts were stored at −20°C prior to use. Primers cry-15 and cry-9 were used to amplify a 550-bp region of the COWP gene, which was then subjected to restriction endonuclease digestion by *Rsa*I. The digestion products were separated by agarose (3% w/v) gel electrophoresis, visualized using ethidium bromide (0·1 mg/100 ml) and recorded using a digital camera and KDS1D analysis software (Kodak UK, Hemel Hempstead, UK). Product sizes were confirmed by comparison with a DNA molecular weight standard marker (Invitrogen, Paisley, UK). Oocyst suspensions confirmed as containing *C. hominis* were resuspended in 1x PBS and those containing sufficient oocysts following visual inspection of a sample by bright field microscopy were used for cell culture. These oocysts were used when <2 months old.

A *C. parvum* isolate originally from deer but passaged in lambs was purchased from Moredun Scientific (Edinburgh, UK). These oocysts were used when <5 months old.

Buffered storage media

Storage media at pH 7 were made by combining stock solutions of 8·8 ml 0·1 mol l^{-1} citric acid and 41·2 ml 0·2 mol l^{-1} Na_2HPO_4. These stock solutions contained either 0·5% w/v NaCl or 4·5% w/v NaCl. Further 0·5% w/v NaCl solutions also contained 20% v/v glycerol or 9% v/v ethanol, or 40% v/v ethanol, or 50% w/v sucrose. Citric acid storage media at pH 2·6, pH 3·6 and pH 4·6 were made by combining stock solutions of 0·1 mol l^{-1} citric acid and 0·2 mol l^{-1} Na_2HPO_4 as follows: 44·5 ml acid/5·5 ml base; 32·25 ml acid/17·75 ml base; 53·25 ml acid/59·75 ml base, respectively. Acetic acid storage medium (pH 3·6) was made with 44 ml 0·2 mol l^{-1} acetic acid and 6 ml 0·2 mol l^{-1} sodium acetate both containing 0·5% w/v NaCl. Lactic acid storage medium (pH 4·6) was made with 10 ml 0·1 mol l^{-1} lactic acid and 30 ml 0·2 mol l^{-1} sodium lactate; both containing 0·5% w/v NaCl. Following sterilization, final pH adjustments of all solutions were made aseptically using sterile solutions of the constituent acids and bases.

Inoculation, storage and recovery of oocysts

Deionized water (25 μl) containing oocysts [$3{\cdot}92 \times 10^4$ (*C. hominis*) or $1{\cdot}75 \times 10^5$ (*C. parvum*)] was added directly to 1 ml volumes of the buffered storage media and stored in 1·5 ml microcentrifuge tubes at 4 or 22°C ± 1°C. At points during the time course, two replicate tubes were assessed for viability either by the sporozoite ratio technique or by tissue culture infectivity. Acidified tubes were first neutralized visually by addition of 50 μl 0·1% w/v phenol red, followed by titration using disodium hydrogen phosphate (Na_2HPO_4). Tubes containing salt, glycerol or ethanol were filled completely with deionized water. Tubes containing sucrose were filled completely with deionized water and the volume split between two further tubes which were then topped up with water. The tubes were then centrifuged (Micro centaur, Sanyo Gallenkamp, Loughborough, UK) at 13 000 *g* for 5 min and the excess buffer removed. No extra wash was carried out as there was a risk of oocyst loss. The oocyst pellets were resuspended either in Medium 199 (for tissue culture) or in excystation medium (see below). Controls were treated similarly i.e. with a centrifugation step, but without the storage period.

Sporozoite ratios

Excystation solution (50 μl) comprising 0·25% w/v trypsin and 0·75% w/v taurocholic acid (Sigma) in phosphate-buffered saline was added to each microcentrifuge tube containing the oocysts pellet from the storage medium and the tubes incubated for 2 h at 37°C with agitation. Two 15 μl aliquots were then removed per sample, placed upon microscope slides and sealed under coverslips using nail varnish. Enumeration was carried out on a computer screen using a compound microscope Model DMLB (Leica, Weltzar, Germany) with Differential Interference Contrast (DIC) (Leica) optics and a 40× lens. The ratio between free sporozoites and empty or partially empty oocysts was calculated as described by Blewett (1988). The theoretical maximum is four.

Tissue culture infectivity

Vials (7 ml) containing 13 mm coverslips (Bobby Sterilin, Stone, UK, product code 129BX/1) were seeded with MRC-5 cells, obtained by trypsinizing a growing culture from the European Collection of Cell Cultures (ECACC; Salisbury) according to the supplier's instructions. These were then

incubated for 2–5 days at 37°C in 1 ml Medium 199 containing Earles Salts; essential amino acids and L-glutamine (Invitrogen) supplemented to give the following final concentrations: 0·075% w/v sodium bicarbonate (Gibco BRL) 10% v/v Foetal Calf Serum (TCS Biosciences, Botolph Claydon, UK) 300 mg l^{-1} benzylpenicillin sodium (Britannia Pharmaceuticals, Redhill, UK) and 16 mg l^{-1} gentamycin (Aventis, Dublin, Ireland). Coverslips were checked for monolayers using an inverted microscope and were considered suitable if they had confluent or subconfluent monolayers. The excess medium was decanted and replaced with 1 ml fresh medium containing the oocysts for viability assay. The replacement medium was as above but containing in addition Fungizone at 2 mg l^{-1} (Bristol-Myers Squibb Pharmaceuticals Ltd, Dublin, Ireland). The entire pellet from each centrifuged storage medium (see above) was added to vials by resuspension in 1 ml of medium and incubated for 5 days at 37°C. Monolayers were stained in the vial as follows: 1 ml of acetone was added gently down the sides of the tube to fix the monolayer. After 5 min, acetone was removed and 250 μl of auramine phenol (Pro-Lab) added and left for 4 min. Excess was then tipped out and 1 ml of deionized water added and then removed. The monolayer was destained with 1% acid alcohol (1 ml) for 4 min (auramine phenol and acid alcohol were prepared according to Collee *et al.* 1996). Acid alcohol was decanted and excess removed by washing with 1 ml deionized water. The deionized water was tipped out and the coverslip dislodged from the bottom of the vial using a hypodermic needle. It was then ejected from the tube by inverting the tube and tapping the base. After air drying, the coverslip was placed (with the monolayer downwards) onto a drop of glycerol-based mountant on a glass microscope slide and observed on a Leica DMLB microscope at 200X or 400X with an I2 filter block.

Life cycle stages appeared as bright fluorescent spherical structures against the background fluorescence of the cells. Numbers per five or 10 fields (depending upon density of infective structures) were counted randomly to gain an estimate of infectivity.

Direct examination of oocysts following storage

Vials containing oocysts were centrifuged (13 000 *g* for 5 min) at various time intervals from 1 h to 14 days and oocyst morphology examined by DIC microscopy.

RESULTS

Sporozoite ratio

Following storage of *C. parvum* oocysts in buffered media based upon citric acid (pH 7·0; pH 3·6 and pH 2·6) and acetic acid (pH 3·6), viable sporozoites were detected during and at the end of 14 days storage at 4°C (Table 1). The lowest sporozoite ratio observed at day 14 was 1·2, indicating the potential for survival in foods under these conditions. These initial results led to further work being carried out at 22°C (Table 1). Again, viable sporozoites were found after 14 days in the solutions described above and also in lactic acid and citric acid at pH 4·6.

Observation of sporozoite ratios from solutions containing one of the following; either 4·5% salt, ethanol, glycerol or sucrose showed a low sporozoite ratio at day 14 after storage at 22°C. A simple linear model was therefore fitted to these ratios assuming no interactions.

Presence of 4·5% salt, ethanol (9 or 40%) and sucrose (50%) were very highly significant in the linear model for sporozoite ratio ($P < 0{\cdot}001$). Glycerol was highly significant ($P = 0{\cdot}004$).

A main effects plot of least squares means for these four factors showed that the model gives almost equal weight to each factor. The presence of these factors had a negative effect on sporozoite ratio. The model suggests that a low sporozoite ratio would be achieved by any one these factors being present.

Additional data (not shown) indicated that with certain treatments, large decreases in sporozoite ratios could be observed after 1 day. Therefore further data were generated to examine whether there was a rapid drop in viability with some treatments. The sporozoite ratio at 1 h was examined as well as at 14 days. With some treatments major changes in sporozoite ratio were observed after 1 h. A plot of Δr (i.e. the change in ratio within 1 h) against ratio at day 14 for a total of 15 observations revealed two distinct populations (Fig. 1). One group comprises batches of oocysts stored at 22°C in neutral media containing one of the following factors: 4·5% NaCl; ethanol at 9% or 40%, or sucrose at

Table 1 Sporozoite ratios at 14 days under different environmental conditions

	Treatment	Sporozoite ratio at 14 days
Citric acid	pH 7	(1·8) (2·3) (2·0) (1·6) 2·8, 3·0, 3·0, 3·8, 4·0
	pH 4·6	3·3
	pH 3·6	(4·0) 3·0
	pH 2·6	(1·8) (2·6) 1·9
Acetic acid	pH 3·6	(1·2) 0·3
Lactic acid	pH 4·6	3·3
High salt	4·5%	0·1, 0·1
Ethanol	9%	0·2, 0·2, 0·1
	40%	0·1, 0·1
Glycerol	20%	(1·5) (1·0) 1·0
Sucrose	50%	0·01, 0·1

Ratios within brackets indicate 4°C storage, otherwise storage was at 22°C.

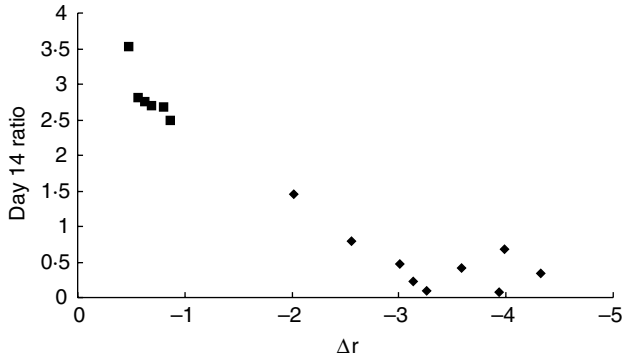

Fig. 1 Sporozoite ratios at day 14 against Δr (change in sporozoite ratio within 1 h) under different environmental conditions. Treatment with either sucrose, ethanol or high salt is (◆) is distinguished from treatment with acid only (■)

50% whilst another comprises batches of oocysts stored in media at pH 7 or 4·6 with citric or lactic acid and 0·5% NaCl. Those with a high Δr (i.e. a large decline in sporozoite ratio within 1 h) are associated with a low final sporozoite ratio, whilst those with a low Δr are associated with a high final sporozoite ratio.

Cryptosporidium hominis oocysts were also used in sporozoite ratio assays. Although at 4°C ratios could be determined, showing some decline by day 7 from an initial value of 4 (data not shown) bacterial growth at 22°C, pH 7·0 made the ratios impossible to determine using the isolate provided.

MRC-5 infectivity

The survival data for the four treatments shown to have a significant effect on sporozoite ratio (high salt, ethanol, glucose and glycerol) gave lower results in MRC-5 infectivity compared with controls performed in parallel and lacking those treatments (Table 2). Survival data on treatments with different acids and at various pH values revealed no obvious effects when compared with citric acid buffer pH 7 controls.

Further study of the effect of a 50% sucrose solution on infectivity of *C. parvum* following 1 day storage period at 22°C was carried out using ten replicates treated with sucrose and nine untreated controls. The mean of life cycle stages counted was 23·6 without sucrose treatment and 4·9 with sucrose treatment. A *t*-test with unequal variances gave a highly significant treatment difference ($P = 0{\cdot}005$).

Examination of oocysts following storage

Using DIC microscopy, morphological changes were apparent in some of the treatments used. Evidence was seen of a shrunken/crinkled appearance in the solutions of low water activity. These were apparent very early on in the sucrose solution (within 1 h). Partial excystation was also observed under some conditions.

Table 2 Percentage reduction in tissue culture infectivity with different treatments

	Treatment	%Reduction*
High salt	4·5% NaCl 8 days at 22°C (1)	77
	4·5% NaCl 9 days at 22°C (2)	57
Ethanol	9% Ethanol 7 days at 22°C (1)	77
	9% Ethanol 8 days at 22°C (1)	66
	40% Ethanol 8 days at 22°C (1)	72
Glycerol	20% Glycerol 7 days at 4°C (1)	57
	20% Glycerol 13 days at 4°C (2)	85
	20% Glycerol 13 days at 22°C(2)	87
	20% Glycerol 14 days at 4°C (2)	53
Sucrose	50% Sucrose 7 days at 22°C (1)	100
	50% Sucrose 8 days at 22°C (1)	86
	50% Sucrose 9 days at 22°C (2)	90

*Percentage reductions are based upon mean counts of life cycle stages (per group of random microscope fields) from two vials seeded with treated oocysts and two vials seeded with oocysts from appropriate control treatments. (1), *C. hominis*; (2), *C. parvum*.

DISCUSSION

This study used two techniques to determine the potential infectivity of *Cryptosporidium* oocysts stored in conditions relevant to foods and beverages. The sporozoite ratio technique represents a relatively easy way to assess viability and can be used to generate useful amounts of data: however its use as a surrogate for infectivity has never been fully investigated and therefore tissue culture is useful in addition to reinforce any conclusions made. A variety of cell lines have been used for culturing *Cryptosporidium* (Rochelle and de Leon 2001). The fibroblast cell line MRC-5 is routinely used by hospital laboratories for culture of herpesviruses, adenoviruses and rhinoviruses. It is easy to handle to create monolayers for infection and has previously been reported as supporting the growth of *Toxoplasma*, a close relative of *Cryptosporidium* (Lavrard *et al.* 1995). The MRC-5 assay worked successfully with both the *Cryptosporidium* species used in this study. This is important because *C. hominis* oocysts cannot be used in mouse infectivity work.

In the present study, a nonspecific fluorescent staining method was used in contrast to immunofluorescent staining as described by Slifko *et al.* (1997a). Preliminary work carried out with live and heat killed oocysts had indicated that life cycle stages were being detected rather than residual oocysts from the initial inoculation. There was also no indication that increasing the incubation time led to further proliferation of life cycle stages as was shown by Slifko *et al.*

(1997a) in the case of HCT-8 cells. Future work would need to compare this technique with immunofluorescence both in MRC-5 cells and in other cell lines. The technique used in this study is easy to use and requires commonly available reagents and, therefore, has potential for a wider use in studies on inactivation of oocysts than those previously described.

Although sporozoite ratios declined in this study, the ratios at the end of 14 days following storage in low pH conditions and with different acids suggested considerable retention of viability. This would mean that short shelf-life foods having equivalent conditions would allow survival of oocysts. Sporozoite ratios of oocyst batches stored in solutions containing high salt, ethanol, glucose or glycerol after 14 days suggested a considerable reduction in viability; however, some sporozoites were still detected. The data from tissue culture infectivity also showed lower values following treatment with high salt, ethanol, glucose or glycerol. As with sporozoite ratio, some survival was apparent at the end of the treatment periods of 7 days or more.

It is possible that loss in viability occurs because of the premature release of sporozoites. This may be the main cause of viability decline as oocysts are placed in different conditions to those in which they have been stored. Slifko *et al.* (1997b) describe spontaneous excystation in pancake syrup by 72 h. Changes in oocyst morphology in sucrose solution (specific gravity 1·18: *ca* 50% w/v sucrose) were noted by Robertson *et al.* (1993). Within a 1 h period, the proportion demonstrating a fold using fluorescence microscopy was *ca* 80%. This effect was reversed if they were subsequently immersed in deionized water.

As stated previously, relatively little work has been carried out on survival in foods or food-type environments. According to Slifko (T.R. Slifko, personal communication) the review of Rose and Slifko (1999) included original data, examining the survival of oocysts in pancake syrup (a_w value 0·85) and 9% sodium chloride/2% dextrose (a_w0·95). Two temperatures were used (7 and 28°C) and the techniques of sporozoite ratio and infectivity in cell culture (HCT-8 cells) to assess viability.

In the case of pancake syrup, after 24 h at 28°C sporozoite levels per oocyst had dropped to 0·07 (a process that took 72 h at 7°C). The NaCl/dextrose samples showed sporozoite ratios of 0·09 after 48 h at 28°C, but 2 weeks were required to give the same level of die off at 7°C.

As regards HCT-8 cells, 99·9% or more reduction in infectivity was seen with pancake syrup at 28 and 7°C after 24 h and 1 week, respectively. A similar reduction was seen with NaCl/dextrose after a week at 28°C and 2 weeks at 7°C.

These data are not directly comparable with our study because of the use of different temperatures and also the solutions are not identical; however both the data of Rose and Slifko (1999) and our data indicate that reduced water activity may have a dramatic effect on sporozoite ratio and also infectivity.

Friedman *et al.* (1997) describe large reductions in the percentage of oocysts with four sporozoites visible in various beverages including beer, cola, orange juice and infant formula over a 24-h period. They also include a pH 4 buffer in their work. The study presents limited data, the methods used were not based upon infectivity and so the interpretation of these data is difficult, but the authors concluded that certain beverages stimulate the release of sporozoites which therefore die in the absence of mammalian host cells. Our results, at least with sporozoite ratio also suggests that some decline in viability may occur over a 24-h period in reduced pH buffers, but there is no indication that viability is reduced to low levels.

Deng and Cliver (1999) studied the survival of *C. parvum* in dairy products. Yoghurt was made from pasteurized low fat milk with a live yoghurt starter by incubation for 48 h at 37°C; this was followed by storage for 8 days at 4°C. Inoculation of *Cryptosporidium* occurred at the start of the process. After the first 48 h there was little decline in viability in spite of the pH of 4·8 and the survival was in general very similar to the control where no starter culture was added. In this work, viability was assessed by microscopy using exclusion of propidium iodide by the sporozoites as a criterion for viability. It is clear from these data that even if viability is being overestimated, over half the oocysts remained completely intact at the end of the 10-day period and therefore did not show sporozoite staining.

Merry *et al.* (1997) examined the survival of *C. parvum* in silages prepared in three different ways; either untreated, inoculated with *Lactobacillus plantarum* or acidified directly with formic acid . After 14 days treatment at 21°C few differences were seen between the treatments, with viability being *ca* 50% as determined by a vital dye method (Campbell *et al.* 1992) similar to that used by Deng and Cliver (1999). Both the acidified and inoculated silage had pH values of 4 after 14 days whereas the pH of the untreated silage had risen above 7. Oocysts continued to show survival at the end of the experimental period (3 months) even with concentrations of acetic, butyric and propionic acids and also ammonia increasing.

Our results are consistent with those of Deng and Cliver (1999) and Merry *et al.* (1997) in not finding major changes where oocysts are exposed to lowered pH treatment in the presence of a range of acids. We used a wider range of acid treatments and pH than these authors but showed no strong negative effects.

It is also likely that different *Cryptosporidium* isolates will give different survival characteristics. Widmer *et al.* (2001) examined the decay of nine different isolates which had been

surface sterilized and incubated in sterile water over a 22-day period at room temperature. Variability between strains was observed, with the Moredun isolate (as used in our study) showing little decline. Age and condition of the isolate may also be a factor. This is an important consideration when comparing survival studies.

If MRC-5 cells are to be used in further infectivity work they should be subjected to comparison with cell lines that have previously been compared with animal infectivity or they should be compared with animal infectivity directly. This will help understanding of their relative behaviour in *in vitro* culture. However, the data presented do indicate that the MRC-5 cell line maybe a suitable line for assessing infectivity of *Cryptosporidium* oocysts. In addition, the data does give some information on likely survival of oocysts under conditions relevant to food and beverages as a preliminary input for use in risk assessment by the food industry.

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