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STUDIES EVALUATING THE APPLICABILITY OF UTILISING THE SAME CONCENTRATION TECHNIQUES FOR THE DETECTION OF PROTOZOAN PARASITES AND VIRUSES IN WATER

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ABSTRACT

In this study concentration techniques regularly used for viral detection, i.e. flat-bed ultrafiltration and Filterite cartridge filtration, were evaluated for their efficacies in the recovery of protozoan parasites from water. Recovery of cysts was studied using tap water seeded with *Giardia muris* cysts and compared to methods designed for the detection of protozoan parasites. Recovery of cysts utilizing 1.2μm membrane filters was 11.1% (4.5-23%) compared to 11.6% (2.7-25.5%) with ultrafiltration (pore size 46-50 Å, with a molecular cut off of 50 000 daltons). Comparison of these methods for the isolation of *Giardia* cysts and *Cryptosporidium* oocysts from environmental water samples also indicated a similar efficacy. The recovery of cysts from 100l of seeded samples using a Cuno wynd cartridge filter was 12.2% (1.6-46%) compared to 13.4% (5-24.2%) using a Filterite cartridge filter. Although the results indicated similar recovery efficacies for these two methods, use of Filterite resulted in a more consistent recovery rate. This study also indicated that the use of cartridge filters for the processing of large volume water samples (100l) showed a slightly better recovery efficacy than the flat-bed filtration technique which limits sample volume to about 10l.

This study shows that concentration techniques utilised for the isolation of enteric viruses can also be applied for the detection of protozoan parasites from water. This procedure allows for co-analysis of both viruses and protozoan parasites and provides a more rapid and cost-effective evaluation of water quality.

KEYWORDS

Giardia, *Cryptosporidium*, enteric viruses, flat-bed filtration, cartridge filter, ultrafiltration

INTRODUCTION

A number of enteric viruses and protozoan parasites are excreted in the faeces of infected individuals and are known to be transmitted by the faecal-oral route. Their presence in water is often a result of contamination by human and to some extent animal faecal waste. Enteric viruses and even more so, protozoan parasite cysts and oocysts, have been found to be more resistant to certain water purification processes than other bacterial indicators (Hibler and Hancock, 1990; Rose, 1990; Sorber, 1983). The presence of these pathogens in water even in very low numbers, poses a high risk to the consumer. To avoid such risks, suitable methodologies enabling the detection of very low numbers of enteric viruses and/or protozoan parasites in

water have long been sought after. The speed, precision, efficiency and sensitivity of such analytical methods are of great importance.

Due to the limited sensitivity of most of the enumeration techniques available for these pathogens, pre-concentration of the water sample under examination is often required. Both the sample volumes and concentration methods used differ from country to country and even between different laboratories. Sample volumes vary from 1–400l of drinking water and depend on existing guidelines and regulations. Concentration techniques include a wide range of flat-bed filtration, and adsorption-elution procedures.

Most laboratories utilise separate concentration techniques for the enumeration of enteric viruses and protozoan parasites, such as *Giardia* or *Cryptosporidium*, from water. This is mainly due to their different characteristics, especially the differences in size between viral particles and protozoan parasite cysts or oocysts. However, the use of different procedures for sample preparation for the detection of enteric viruses and protozoan parasites, respectively, is time consuming, costly and labour intensive.

In this study concentration methods used for the detection of enteric viruses in water have been studied for their ability to recover protozoan parasite cysts and/or oocysts from the same sample. The efficacy of these methods is compared with concentration methods specially devised for the detection of cysts and/or oocysts in water.

MATERIAL AND METHODS

Giardia cysts and viruses

Giardia muris cyst stock was kindly provided by Dr Schaefer (US-EPA). Two to three week old female C3H/HE mice, obtained from the South African Institute for Medical Research, were inoculated by intubation of the inoculum (1 000 cysts/0.2 ml) into the lower oesophagus or stomach. The cysts were harvested on the seventh day, post inoculation, when cyst shedding is at its maximum. The infected faecal matter was cleaned, homogenized and sieved using 0.01% Tween-20 solution. The sieved faecal slurry was concentrated by centrifugation (500xg, 6 min) and pellets were resuspended in 0.01% Tween-20 solution. The cysts were separated from other faecal debris using sucrose flotation (1.0 M sucrose solution, 800xg, 10 min). The interface cyst layer was collected, centrifuged (800xg, 10 min) and washed twice in 0.01% Tween-20 solution. This procedure was repeated twice in order to obtain a clean cyst yield. Clean cyst suspensions supplemented with penicillin and streptomycin were stored at 4°C. Poliovirus type 2 vaccine strain (P712) was obtained from the National Institute of Virology, South Africa and cultivated as described by Nupen *et al.* (1980). Viral stocks were kept at -70°C.

Evaluation of concentration techniques

All experiments were carried out using *Giardia muris* cysts isolated and purified in our laboratory. Cyst viability was observed under phase contrast microscopy using fluorogenic dyes prior to seeding and only viable cysts were counted. The cyst concentration in the seeded samples was kept very low to allow for the simulation of levels which may occur in polluted surface or drinking water. This resulted in a final concentration of between 10-30 cysts per ml of seeded water. The same cyst concentration per ml was used for the 10l and the 100l seeding studies.

Membrane filtration: Seeded tap water samples (10l) were passed through a flat membrane (Millipore, diameter : 142mm, pore size: 1.2µm, surface area: 158cm^2) using a Sartorius filter holder and air pressure. Filtration was carried out within approximately 3 min per 10l of water sample. After filtration, all remaining liquid was collected, the filter was removed and placed in the collected liquid, sonicated for 10 min and further cleaned using a rubber policeman. The deposit was further concentrated by centrifugation (2 100 rpm for 6 min). The pellet was investigated for the recovery of *Giardia* cysts.

Ultrafiltrations: Seeded water sample (10l) were passed through an Amicon XM50 ultrafilter (150mm diameter, pore size 46-50 Å, molecular cut-off 50 000 daltons and surface area 177cm^2) using positive nitrogen pressure of 1.3 bar. The remaining liquid was collected, added to the eluent (Eagle's minimum essential medium with Earle's salts) which was used to remove the cysts from the membrane using a rubber policeman, and centrifuged (2 000 rpm, 1 min). The pellet was used to study the percentage recovery of the seeded cysts. For the recovery of enteric viruses and *Giardia* cysts using the same concentrate, phosphate buffered saline (PBS) was seeded with both poliovirus type 2 vaccine strain (P712) and *Giardia muris* cysts and the final supernatant was used for evaluating virus recovery.

Filterite DFN 0.45-10µm cartridge filter: One hundred litres of the seeded water sample was passed through the Filterite cartridge filters (effective surface area: 5226cm^2, pore size: 0.45µm) at a rate of approximately 3l per minute using an operating pressure of 0.5 bar, while introducing a solution containing 6.66 g of aluminium chloride ($AlCl_3$), 7,9 g of sodium thiosulphate ($Na_2S_2O_3$) and 20 ml hydrochloric acid (HCl) per 100l. Thereafter a backwashing procedure was carried out using glycine eluent (11.36 g/3l, pH 9.5). The resulting fluid was subsequently passed through an Amicon XM 50 ultrafilter which was eluted with Eagle's minimum essential medium (Earle's salts), using a rubber policeman, and centrifuged for (2 000 rpm, 1min). For the recovery of poliovirus type 2 vaccine strain (P712) and Giardia cysts using the same concentrate, PBS was seeded with both poliovirus and *Giardia* muris cysts and the final supernatant was used for evaluating virus recovery.

AMF Cuno wynd (DPPPY1) cartridge filters: Seeded water sample (100l) were passed through the filter at an approximate rate of 3l/min. After filtration the filters were removed from their housings and all residual liquid left in the holder was collected. The procedure described by LeChevallier *et al.*, (1991) was followed with minor modifications. The filters were cut longitudinally, segments were teased apart, and thereafter placed in a Sputnik MKII manual washing machine along with the residual sample from the housing with additional aliquots of 10 ml of Tween-80 (1%) and sodium dodecyl sulphate (1%). This was followed by manual rotation of the machine for 30 seconds. The use of the Sputnik washing machine indicated it to be as efficient for processing the filters as the commonly used stomacher and shortens the sample preparation time as well as the initial cost of building the necessary facilities for protozoan detection. The special freezer bags used in the stomacher are also relatively costly. The entire mass of cut filter segments and residual water was spun dry (Zyliss Salad Spinner). The residual water collected at the bottom of the Zyliss was centrifuged (2 400 rpm, 10 min). The whole wash process was repeated several times until the cut filter segments were considered clean. Great care was given to this washing procedure as studies indicated that, irrespective of the filter cartridge used or the specific elution protocol followed by different laboratories, the first and potentially greatest loss of cysts occurs during this washing procedure (Jakubowski *et al.*, 1978; Madore *et al.*, 1987). The final pellet was evaluated for cyst recovery.

Evaluation of environmental water samples: The recovery efficacy of membrane filtration was compared with ultrafiltration for the enumeration of both *Giardia* cysts and *Cryptosporidium* oocysts from surface water samples (10l) utilized as feed water to a purification plant.

Viral and cyst recovery

Percentage recoveries were calculated as the number of cysts or viruses recovered after concentration, divided by the original number seeded, multiplied by 100. Cysts were counted before and after concentration using a haemocytometer and observing viability under phase contrast. Viruses were cultivated in BGM cells and 50% tissue culture infective dose was evaluated according to Nupen *et al.* (1981).

RESULTS

On average 10^7-10^8 cysts were harvested from 20 infected mice in one day. Cyst viability upon harvesting was very high as observed under phase contrast microscopy and using fluorogenic dyes.

Table 1. Recovery of *giardia* cysts utilizing various concentration methods

Concentration method	% Recovery of cysts			
	Membrane filtration	Ultra filtration	Cuno wynd	Filterite
Average	11,1	11,6	12,2	13,4
Minimum	4,5	2,7	1,6	5,0
Maximum	23,0	25,5	46,0	24,2
Sample volume (ℓ)	10	10	100	100

Cyst concentration was between 10-30/ml. The results are representative of at least 6 repetitions.

Recovery of cysts utilizing 1,2µm membrane filters was on average 11,1% ranging from 4.5% to 23.0% compared to a range between 2.7% to 25.5% and an average of 11.6% obtained using ultrafiltration (Table 1). Comparison of these methods for the isolation of *Giardia* cysts and *Cryptosporidium* oocysts from environmental water samples also indicated similar efficacies (Figs. 1 and 2).

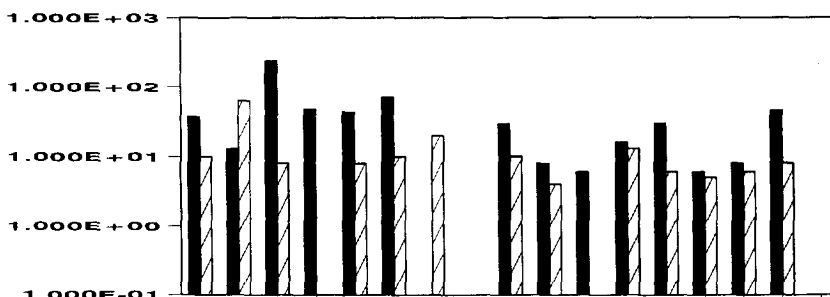

Figure 1. Comparison of membrane filtration and ultrafiltration methods for the detection of *Giardia* cysts in surface water.

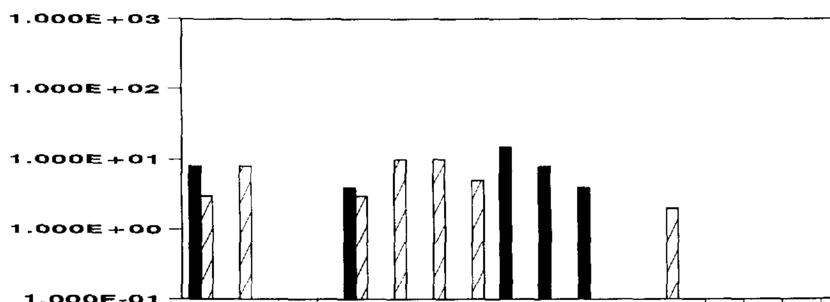

Figure. 2. Comparison of membrane filtration and ultrafiltration methods for the detection of *Cryptosporidium* oocysts in surface water.

As shown in Fig. 1, the use of ultrafiltration was as efficient as using membrane filtration and the numbers of cysts isolated per sample were similar. On one occasion cysts were only found after concentration by ultrafiltration. Both concentration methods were also equally efficient for the enumeration of *Cryptosporidium* oocysts from surface water with a slight increase in the number of positive samples using

the ultrafiltration method. However, there was very little difference in the numbers of oocysts enumerated per water sample (Fig. 2).

The recovery of cysts from 100l of seeded tap water samples using a Cuno wynd cartridge filter was on average 12.2% and ranged between 1.6% to 46% (Table 1). In comparison the recovery of cysts using Filterite was on average 13.4% ranging from 5 to 24.2% (Table 1). Although the results indicated similar recovery efficacies for these two methods, utilization of Filterite resulted in a more consistent recovery.

Ultrafiltration gave the best recovery for enteric viruses in 10l water tested. Recoveries of poliovirus ranged from 63% to 100% with an average of 82%. Recoveries of *Giardia* cysts ranged between 10% and 18% with an average of 14% (Fig. 3).

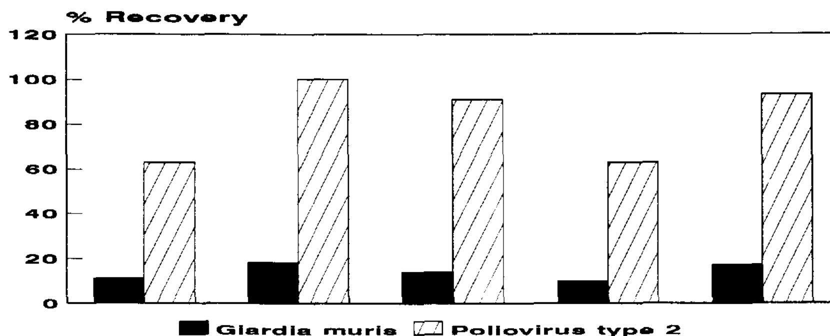

Figure 3. Percentage recoveries of enteric viruses and protozoan parasites using ultrafiltration Aliquots of 1ml cyst stock (10^8/ml) and 1ml of poliovirus stock (10^6/ml) were seeded in PBS. The result of five repetitions are given.

The percentage recovery of poliovirus using Filterite ranged between 37% and 93% with an average of 54% (Fig. 4). *Giardia* cyst recoveries were lower and ranged between 10% and 21% with an average of 14%. Higher recoveries of cysts were obtained from seeded PBS in comparison to those obtained from seeded tap water, probably due to the buffering capacity of the PBS.

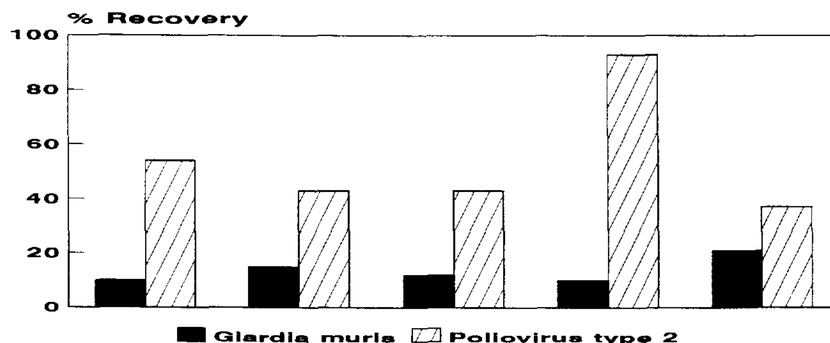

Figure 4. Percentage recoveries of enteric viruses and protozoan parasites using Filterite cartridge filters. Aliquots of 1ml cyst stock (10^8/ml) and 1ml of poliovirus stock (10^6/ml) were seeded in PBS. The result of five repetitions are given.

DISCUSSION

The results of this study indicate that concentration techniques utilised for the isolation of enteric viruses can also be applied to the detection of protozoan parasites in water. This procedure allows co-analysis for both

viruses and protozoan parasites and provides a more rapid and cost-effective evaluation of water microbial quality.

In this study ultrafiltration was shown to be highly efficient for the recovery of poliovirus. In other reports this method was shown to give the highest recoveries for a range of enteric viruses from water (Nupen *et al.*, 1981) and was found to be as efficient as membrane filtration for the enumeration of protozoan parasite cysts and/or oocysts as shown by the results of both the seeding experiments and the analyses of polluted surface water. A flat-bed system was also used for the enumeration of protozoan parasites from water by Wallis and Buchanan-Mappin (1985) and Ongerth and Stibbs (1987).

Ultrafiltration is not cost effective when only testing for protozoan parasites. However, for samples which require co-analyses for both viruses and protozoan parasites, ultrafiltration will be the concentration method of choice. The sole disadvantage of this method is that only 10l of sample can be filtered before flow difficulties become evident due to clogging of the filter.

Cuno wynd filters are the most commonly used for concentration of large volume samples for the enumeration of protozoan parasites (Whitmore and Carrington, 1993). The recovery range found for the Cuno wynd filter in this study was similar to that reported in the literature (Holman *et al* 1983; Rose *et al.*, 1991). It should be noted that the number of reported laboratory seeding studies in which the recoveries of *Giardia* cysts using various concentration techniques are compared, is limited. Erratic recoveries, as found in this study, have previously been reported for *Cryptosporidium* oocysts using Cuno wynd filters (Ongerth and Stibbs, 1987; Musial *et al.*, 1987). Other studies using Cuno wynd filters have also reported similar average recovery ranges for *Cryptosporidium* (Vesey *et al.*, 1991).

The use of the Filterite cartridge filter for the co-processing of large-volume samples for the enumeration of both protozoan parasites and enteric viruses was shown to result in similar recoveries to those found for the Cuno wynd filter. Virus recovery was on average lower than that found for poliovirus using ultrafiltration but similar to other recovery rates reported for Filterite (Dahling and Wright, 1987; Farrah *et al.*, 1976). The results of this study indicated that, where the presence of both viruses and protozoan parasites are to be investigated, the same cartridge filter can be used, thus shortening the time of sample preparation and reducing the cost per analysis. Payment *et al.* (1989) have also reported using a single concentration technique for the enumeration of bacteria, coliphages, enteric viruses and *Giardia* cysts.

By maintaining the same concentration of cysts but varying the sample volume this study showed the significance of using larger volumes of sample for the detection of viruses and protozoan parasites. Similar or even slightly lower recoveries obtained using methods catering for large volumes of water have an advantage over methods in which only a limited volume can be processed due to flow problems. If 20% of the seeded cysts or viruses are to be recovered from concentrates of 10l and 100l, respectively, the number of cysts recovered when concentrating 10l will be 10 times lower than that recovered from 100l. Although higher recoveries of poliovirus were obtained with ultrafiltration compared to those obtained using Filterite, the total number of viruses which will be isolated from 100l water samples will be on average higher than those obtained from 10l samples. The concentration of large volume water samples using Filterite is therefore of great importance especially for drinking water where very low numbers of viruses and protozoan parasites is expected. Risk assessment studies assessing fitness for use of both drinking water and recreational water have also indicated the need for increasing the volumes of water samples tested for protozoan parasites and enteric viruses (Rodda, 1993).

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