Acta Tropica 169 (2017) 119–121

Contents lists available at ScienceDirect

Acta Tropica

journal homepage: www.elsevier.com/locate/actatropica

Cryptosporidium oocysts and *Giardia* cysts—A practical and sensitive method for counting and manipulating small numbers

Jerry E. Ongerth*, Frhat M.A. Saaed

Civil, Mining, & Environmental Engineering, University of Wollongong, Wollongong, NSW 2522, Australia

ARTICLE INFO

Article history:
Received 1 November 2016
Received in revised form 19 January 2017
Accepted 8 February 2017
Available online 10 February 2017

Keywords:
Cryptosporidium oocysts
Counting
Giardia cysts
Manipulating
Small numbers

ABSTRACT

A rapid and inexpensive method is described for accurate and reproducible counting and manipulating small numbers of *Cryptosporidium* oocysts and *Giardia* cysts. From a suspension of oocysts or cysts at concentration from 1000 to 5000/mL (1–5/μL), replicate 5 μL droplets are micro pipetted onto the edge of a microscope slide. Unstained oocysts or cysts in each droplet can be counted in a few minutes and replicated for statistical strength. The concentration of the suspension can then be verified by pipetting desired volumes containing approximately desired numbers onto confined 13 mm membranes for IFA staining and counting with replication as desired. Requiring only a micropipette, analytical balance, and a microscope, this provides a useful tool accessible to virtually any microbiology laboratory.

1. Introduction

Counting and manipulating small numbers of *Cryptosporidium* oocysts and *Giardia* cysts is an essential and critical tool in conducting research and in quality assurance aspects of routine monitoring of these organisms in environmental media. Both organisms are of public health significance requiring monitoring of their presence in drinking water (USEPA, 2006). Previous studies have reported that *Cryptosporidium* and *Giardia* in surface water used for public water supply range in concentration from 0.01 organisms/L to as many as 10 organisms/L (Hansen and Ongerth, 1991; Ongerth, 1989; Ongerth, 2016). Use of quality control tools such as carefully seeded positive controls is an essential part of any method for this type of monitoring (Ongerth, 2013). This is particularly important recognizing that the most frequent result of analysing 10 L surface water samples is zero (Crainiceanu et al., 2003).

The most widely used method for monitoring *Cryptosporidium* and *Giardia* is the USEPA Method 1623 (USEPA, 2005). The method stipulates QA procedures requiring measurement of the number of organisms recovered from at least 10 L sample volumes following addition of 100–500 cysts and oocysts. Use of a haemocytometer is not practical for numbers in this range as the least count provides for accurate definition of minimum concentrations ca. 10^5/mL and dilution from higher concentrations involves significant statistical variability. Due to the lack of a published procedure for practical and reliable counting of small numbers of organisms without sophisticated instrumentation, e.g. a flow cytometer, this requirement is typically met by use of commercially available vials of flow cytometer-counted organisms, typically 100 of each (e.g., Waterborne, 2010). Although this commercial product is available it is not inexpensive and not necessarily readily accessible. Our experience shows that any lab capable of routine microbiology and confidant in the recognition of *Cryptosporidium* oocysts and *Giardia* cysts can produce accurate and reproducible counting and manipulation without flow cytometry assistance.

The objective of the method described below is to enable accurate and reproducible counting of small numbers of *Giardia* cysts and/or *Cryptosporidium* oocysts. A further requirement was to enable simple manipulation of the counted organisms such as transferring them quantitatively to test volumes of water. We have used the procedure described here over the last 20 years in water and wastewater related laboratory activities requiring use of precise numbers of these organisms ranging from a single cyst or oocysts to as many as 100. As desired numbers exceed about 50, relative accuracy requirements, e.g. coefficients of variation (c.v.) at or below 5%, can be achieved by the procedure described here. The principle of the procedure is simple: a suspension of cysts or oocysts can readily be prepared at a dilution providing approximately 1–5 per μL (1000–5000/mL), based on preliminary haemocytometer counts. Using a micropipette, 5 μL droplets can be deposited near the edge of a glass microscope slide, and then examined under

* Corresponding author at: CME Bldg 4 EEC, University of Wollongong, Wollongong, NSW 2522, Australia.
E-mail address: jongerth@uow.edu.au (J.E. Ongerth).

http://dx.doi.org/10.1016/j.actatropica.2017.02.008
0001-706X/

Table 1
Replicate weights of 2 μL of distilled water delivered by micropipette as volume calibration check, for n = 12, mean = 0.00196 g ± 0.0000793 g; coefficient of variation = 4.05%).

Replicate number	Weight delivered (g)	Volume delivered (μL)
1	0.0019	2
2	0.0019	2
3	0.002	2
4	0.0018	2
5	0.002	2
6	0.002	2
7	0.002	2
8	0.0019	2
9	0.0021	2
10	0.0019	2
11	0.002	2
12	0.002	2

Table 2
Replicates of *Cryptosporidium* oocysts and *Giardia* cysts counted in 10 μL droplets with means, standard deviations, & coefficients of variation (c.v.): *Cryptosporidium* c.v. = 4.8%; *Giardia* c.v. = 4.3%.

Replicate number	*Cryptosporidium* oocysts counted	*Giardia* cysts counted
1	10	10
2	11	9
3	11	13
4	10	11
5	13	10
6	10	10
7	11	11
8	10	12
9	15	8
10	10	11
Average	11.1	10.5
s.d.	±0.53	±0.45

bright field illumination at magnification 250× for *Giardia* or 400× for *Cryptosporidium*. Unstained cysts and oocysts are sufficiently refractile that they are easily visible without staining. The procedure is simple and rapid permitting replicate counting to provide statistical strength. Numbers of organisms delivered in practical application can also be checked independently by replicated pipetting directly onto confined membrane filters, then IFA-stained for counting.

2. Materials and methods

Cryptosporidium oocysts and *Giardia* cysts were obtained commercially, Waterborne Inc., New Orleans, LA. The approximate concentration in each suspension was determined by haemocytometer (Bright-Line, Reichert, Buffalo, NY). A working suspension of each organism was prepared by dilution based on haemocytometer counts to have a concentration of approximately 1000 cysts or oocysts per mL. A micropipette, 0–20 μL (P20, Gilson, Middleton, WI) was calibrated by pipetting droplets ranging from 1 to 10 μL on a top-weighing electronic balance (A200S, Sartorius GMBH, Goettingen) (1 μL = 1 mg). Droplets of a volume corresponding to the desired number of organisms (e.g. 5 μL for 5 cysts or oocysts) were deposited near the edge of a clean glass microscope slide. Location of the droplets near the edge of the slide facilitates rapid location of the droplet and focussing of the microscope to minimise counting time. The exact number of organisms can then be determined by counting at 250× for *Giardia* or 400× for *Cryptosporidium* using bright field illumination (Nikon, Japan). If the organisms are to be transferred for further processing the organisms can either be rinsed from the slide into the intended container using a wash bottle or the entire slide can be placed into the container assuring

Table 3
Replicates of *Cryptosporidium* and *Giardia* concentration checks by pipetting volumes from 10 to 200 μL from a nominal 1 organism/μl suspension directly to 13 mm dia. 2 μm pore dia. membranes with IFA-microscopy enumeration.

Pipet Vol.	10 μL		20 μL		50 μL		100 μL		200 μL	
Replicates, n	8	11	4	3	15	15	4	3	34	30
Organisms counted	*Crypto* Oocysts	*Giardia* Cysts	*Crypto* Oocysts	*Giardia* Cysts	*Crypto* Oocysts	*Giardia* Cysts	*Crypto* Oocysts	*Giardia* Cysts	*Crypto* Oocysts	*Giardia* Cysts
Mean No.	10.3	11.3	20.3	21.7	53.1	48.5	95.8	100	209.1	199
s.d.	1.2	0.62	1.41	1.45	1.59	1.18	1.11	4.04	2.55	3.75
c.v.%	11.6	5.5	6.9	6.7	3.0	2.4	1.2	4.0	1.2	1.9

complete transfer. Alternatively, the droplet can be recovered in the pipette to transfer it for further processing. The droplet location on the microscope slide can be rechecked to verify visually that the organisms have been recovered. Minimising counting time is important to preclude drying of the organism droplets if manipulation of the organisms is desired.

Numbers of organisms transferred by pipetting as described above can be checked independently by replicated transfer of desired volumes (e.g. 5 μL or 10 μL) to 13 mm dia., 2 μm pore dia. etched-pore polycarbonate filters (Sterlitech) confined in inline filter holders (Millipore), stained using FITC-labelled monoclonal antibodies (Waterborne Inc., New Orleans), and enumerated microscopically as described elsewhere (Hansen and Ongerth, 1991).

3. Results

An example of micropipette calibration is provided in Table 1. Clearly, this is a rapid and easily performed check on the calibration status of the micropipette. It should be performed periodically using droplets of the volume intended in current sample processing. Examples of drop counted *Cryptosporidium* oocysts and *Giardia* cysts are provided in Table 2. Repetition of the counting procedure any number of times can quickly establish the concentration of organisms in the stock suspensions. Maintaining a continuing record of concentrations in stock suspensions that may be kept in the lab refrigerator for use over a period of months can be compared over time to monitor the stability of the organisms. Replication of the 13 mm in-line filter concentration check procedure for pipetted volumes from 10 μL to 200 μL produced consistent numbers for each volume used, Table 3. The coefficients of variation for from 3 to 34 replicates ranged from 1 to 11% averaging 4.4%, Table 3.

4. Discussion

The drop counting method for establishing exact numbers of *Giardia* cysts or *Cryptosporidium* oocysts, specifically in the range of 1–10 or more organisms, is practical and easy to perform by any reasonably well trained microbiological technician having working familiarity with *Cryptosporidium* oocysts and with *Giardia* cysts. While droplet to droplet variation occurs as a reflection of the relatively low concentration in the stock suspension and the small volumes used (Ongerth and Saaed, 2013), it is of no consequence here as the exact numbers are being verified. Indeed, the important feature of the method is ability produce visually verified numbers of organisms that are intrinsically accurate and to have at hand a manipulable volume containing the exact number of organisms actually counted by the analyst.

In performing this procedure the analyst may find that the heat of the microscope illuminator will cause the droplets to dry during counting. We have found that droplets of 5–10 μL are practical to count without being adversely affected as long as the stock suspension is prepared in the range of ca. 1–5 organisms per μL. The analyst having a need for specific numbers of organisms can quickly adjust the concentration of the stock suspension and droplet volumes to achieve desired and readily verified numbers of organisms.

A critical application for exact numbers of organisms is to the preparation of positive controls to measure recovery efficiency in the routine analysis of water samples to enable determining the concentrations of *Cryptosporidium* and *Giardia* in samples collected from the environment (Ongerth, 2013). Confidence in ability to count and manipulate small numbers of these organisms can be facilitated using the procedure described above. This in turn will provide a tool that may find many uses related to quality control when performing analyses like USEPA Method 1622/1623 that has multiple steps, each with potential for loss of the target organism. The procedure described here is accessible to labs of any size performing microscopic procedures using *Cryptosporidium* oocysts and or *Giardia* cysts, avoiding the need for a flow cytometer, the expense of purchasing cytometer-counted organisms commercially, or the restriction to numbers of organisms available in commercially counted preparations.

References

Crainiceanu, C.M., Stedinger, J., Ruppert, D., Behr, C., 2003. Modelling the U.S. national distribution of water borne pathogen concentrations with application to *Cryptosporidium parvum*. Water Resour. Res. 39 (9), 1235.

Hansen, J., Ongerth, J.E., 1991. Effects of time and watershed characteristics on the concentration of *Cryptosporidium* oocysts in river water. Appl. Environ. Microbiol. 57 (10), 2790–2795.

Ongerth, J.E., Saaed, F.M.A., 2013. Distribution of *Cryptosporidium* oocysts and *Giardia* cysts in water above and below the normal limit of detection. Parasitol. Res. 112 (2), 467–471.

Ongerth, J.E., 1989. *Giardia* cyst concentrations in river water. J. Am. Water Wks. Assoc. 81 (9), 81–86.

Ongerth, J.E., 2013. The concentration of *Cryptosporidium* and *Giardia* in water–the role and importance of recovery efficiency. Water Res. 47 (7), 2479–2488.

Ongerth, J.E., 2016. *Cryptosporidium* and *Giardia* in Water: reassessment of occurrence and significance. J. Environ. Eng., on-line Sept 13, 2016, http://dx.doi.org/10.1061/(ASCE)EE. 1943-7870.0001161.

U.S. Environmental Protection Agency (USEPA), 2005. Cryptosporidium and Giardia, Available from: https://www.epa.gov/sites/.

U.S. Environmental Protection Agency (USEPA), 2006. National primary drinking water regulations: long term 2 enhanced surface water treatment rule. Fed. Regist. 71 (3), 654–686.

Waterborne, Inc, 2010. AccuSpike™-IR Catalog Number: PACIR. Waterborne, Inc., New Orleans, LA.