Dead-End Ultrafiltration (DEUF) for the Detection of *Cyclospora cayetanensis* from Agricultural Water

Date: 09/23/2019

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1. Introduction

Cyclospora cayetanensis is a protozoan parasite that causes human diarrheal disease called cyclosporiasis (1). The symptoms of cyclosporiasis include explosive watery diarrhea, weight loss, cramping, bloating, increasing gas, nausea, fatigue and loss of appetite. Although no fatal cases of this disease have been reported, the infection can lead to hospitalization. Outbreaks and sporadic cases occur annually associated with the consumption of fresh produce such as basil, romaine, cilantro and berries and since 2013, there has been a surge in the number of reported cases of cyclosporiasis. In 2018, 2299 cases of domestically acquired cyclosporiasis were reported to the CDC with approximately one-third of the cases associated with two large multistate outbreaks linked to prepackaged vegetable trays and salads (2). It is not clear how produce becomes contaminated, but it is possible that agricultural water may play a major role in contaminating crops during the irrigation process. Therefore, laboratory methods that can be used to detect the parasite in food and water are essential to identify potential sources of infection and provide critical support for outbreak investigations.

A method for recovering *Cyclospora* and *Cryptosporidium* oocysts from water was published in the FDA BAM Chapter 19A (11) in 2004. The methodology described in Chapter 19A relies on the use of Envirochek™ sampling capsules to recover *Cyclospora* oocysts from large volume water samples by filtration and is based on a protocol published by the EPA, Method 1623 (3), for isolation of *Cryptosporidium* and *Giardia* from drinking water. In 2013, the US FDA was involved in outbreak investigations which demanded the testing of turbid agricultural water collected from farms that harvested crops epidemiologically linked to cyclosporiasis outbreaks. The Envirochek® filters employed in this method clogged rapidly during the filtration and no effective analysis could be performed on the samples collected. In 2015, a study was initiated to define and compare performance characteristics of dead-end ultrafiltration (DEUF), continuous flow centrifugation (CFC) and the EPA Method 1623 for recovery of *Cyclospora* from agricultural water. It was demonstrated that the hollow fiber filters employed in the DEUF method was less prone to clogging in low quality water such as agricultural water having significantly high amounts of small debris and sediment. The DEUF method is also more practical for field applications and the filters are most cost effective.

In July of 2019, a multi laboratory validation study for a new method based on DEUF for recovery and detection of *Cyclospora* oocysts from agricultural water samples was approved. In the validation study, it was demonstrated that the approach is very sensitive and specific for the detection of *C. cayetanensis*, with a detection limit as low as 6 *C. cayetanensis* oocysts in 10 L of agricultural water samples. The analysis protocol is executed using the following methodology: 1. Recovery of oocysts by DEUF, 2. Disruption of oocysts and extraction of *Cyclospora* DNA, 3. Molecular detection by a real-time PCR assay targeting the *C. cayetanensis* 18S rRNA gene according to BAM Chapter 19B with minor modifications.

2. Supplies and Equipment List

2.1. Equipment and Supplies

i. Geopump™ Peristaltic Pump Series II Package (includes easy-load II pump head and a portable battery), Geotech, Cat No. 91352123

ii. Pump Assembly (or equivalent assembly):

a. DIN adapter (for End Port), Molded Products, Cat. No. MPC855 NS.375

b. Blood port (End) cap, Molded Products, Cat. No. MPC-40

c. Dialysate Port (Side) cap, Molded Products, Cat. No. MPC-60D

d. L/S 36 tubing (9.7mm ID), Cole Parmer, Cat. No. EW-96410-36 or EW-06434-36

e. SNP-8 hose clamps (for DIN adapter), Cole Parmer, Cat. No. EW-06832-08

iii. Hollow fiber ultrafilter Rexeed-25S (Asahi Kasei Medical Co.)

iv. Turbidity Meter (optional)

v. 500 ml plastic bottle for turbidity measurements

vi. Flow meter

vii. Scissors

viii. Pliers

ix. Autoclavable biohazard waste bags

x. Appropriate Personal protective equipment (PPE)

2.2. Reagents

i. Sodium thiosulfate, Fisher Cat. No S446 (Only needed for chlorinated water)

3. Procedures

Note: The filtration protocol can be executed in the field.

3.1. Ultrafiltration Procedure for the Detection of *Cyclospora cayetanensis* using Ultrafilters

A. Ultrafiltration Setup

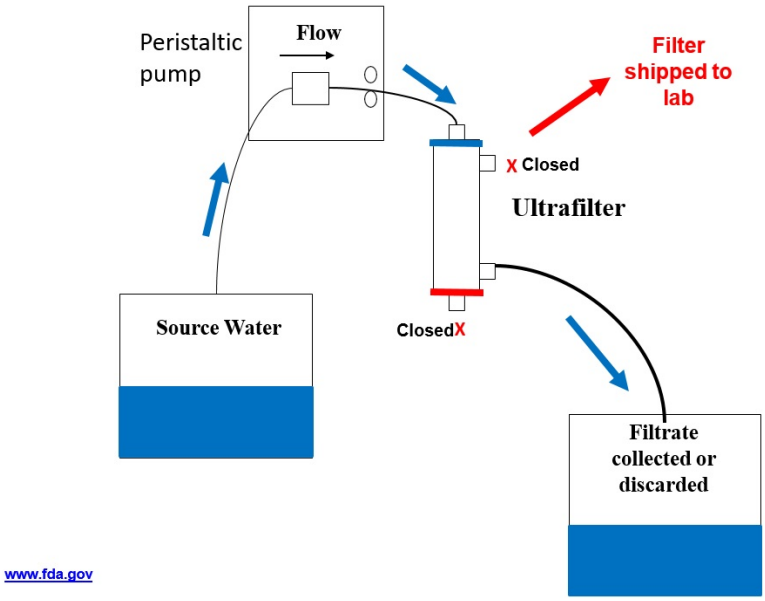

Figure 1 Diagram of the filtration system.

B. Ultrafiltration Protocol

Note: If the water to be collected contains free chlorine, immediately after the filtration process, treat the filter with a 1% sodium thiosulfate solution following the procedure described in section iii.

i. Check turbidity of water

a. Collect approximately 100 ml of the of water in a 500 ml bottle.

b. Allow any debris to settle out of the water for approximately one minute.

c. Pour water into the turbidimeter according to the manufacturer's instructions. Do not transfer the settled debris into the turbidimeter.

d. Allow any debris in the sample in the turbidimeter to settle for approximately one minute.

e. Set aside a sample of approximately 500 ml for turbidity measurements. If a turbidity meter is available, measure the turbidity according to the manufacturer's instructions.

f. If turbidity is higher than 40 Nephelometric Turbidity Units (NTU), check the effluent rate during the filtration. If the rate decreases abruptly, stop the filtration and note the volume of water collected for the sample. If the turbidity is less than 40 NTU, the filter should not clog before filtering a 10-liter sample.

ii. Ultrafiltration Procedure

a. Select the number of filters to be used. The filters cannot be used under freezing conditions.

b. Assemble the filtration system with one filter as shown above, using the supplies and equipment listed above. Position the peristaltic pump in a straight surface or stand lift platform. Place the filter in the stand (or leave it in the surface) and make sure the **blue** end of the filter is on the top.

c. Remove the end port cap from the blue end of the filter.

d. Screw in DIN adapter to the blue end port.

e. Push influent L/S 36 tubing onto the DIN adapter and secure with a SNP 8 tubing clamp. Use the pliers to secure the clamp in place if needed. Use the scissors to cut the extra amount of tubing.

f. Screw in blood port cap to the other end port.

g. Feed the influent tubing through the pump head and close the pump head using the lever.

h. Push effluent L/S 36 tubing onto the open side port (close to the red end) as shown above (no clamp is needed).

i. If a flow totalizer meter will be used to measure the volume of water filtered, screw a 3/4" GHT adapter to each end, cut tubing attached to orange side port and push tubing onto both sides of the meter (ensure directional flow of the meter is correct), no clamps are needed.

j. Place the influent tubing into the body of water and ensure the end of the tubing will stay below the surface of the water and away from plant material or other large debris, which may be present. Be cautious not to disturb the water significantly near the filtration site.

k. Plug in the appropriate power cord into the outlet in the back of the pump and the other end of the power cord into the power source. The power source can be any external 12-18 V DC @ 70 watts or 90-260 V AC 47-65 Hz. Place the battery in a place where it will not get wet.

l. Determine the desired direction of flow and set the toggle switch for the flow direction. Ensure the speed dial is set to zero before starting the pump.

m. Turn the pump “ON” (the black filled circle), record the start time of filtration

n. Once pumping has begun, the speed dial can be adjusted to gradually increase the speed to the maximum setting.

o. As soon the volume of 10L is filtered, turn the pump “OFF” record the stop time of filtration.

p. If the water is chlorinated follow the instructions in item iii below to treat the ultrafilter with 1% sodium thiosulfate. If the water is not chlorinated, skip this step.

q. Remove all tubing from the ultrafilter. Screw a blood port cap into the influent port and place an end port cap or storage port cap on the side cap. Influent tubing can be discarded in the biohazard bags after collecting each sample. Effluent tubing can be re-used for subsequent samples. All tubing clamps and adapters that can be reused should be sterilized by autoclave for future use.

r. Label the ultrafilter with sample name, sample collection date and time.

s. Place the filter in a cooler for transport to the laboratory. Use ice packs to keep the cooler cold if necessary, for the target/suspected microbes collected.

iii. Procedure to treat ultrafilters with 1% sodium thiosulfate solution.

a. Fill a 500-mL bottle with non-chlorinated water and add 5 g sodium thiosulfate, shake to dissolve.

b. Place the influent tubing into the 1% sodium thiosulfate solution and pump the entire volume through the filter without pumping air into the ultrafilter.

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