(Continuation of) Analysis of the presence of *Cyclospora* in waters of the Mid-Atlantic States and evaluation of removal and inactivation by filtration

Contact

Kali Kniel, PhD
Department of Animal and Food Sciences
University of Delaware
kniel@udel.edu

Authors

Kalmia (Kali) E. Kniel

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Summary

DNA analysis by sequencing and PCR was performed on samples from Mid-Atlantic surface waters. Pooled samples ensured sufficient volume and continuity. It is likely that *Cyclospora* was already at low levels and not equally represented in all fractions of samples. Reagent specificity was previously identified as a critical piece of *Cyclospora* detection. QuantiFast (reagent kit) has been discontinued, and in the study QuantiNova (reagent kit) provided nearly identical results in the analysis of 10 presumptive positive samples. An important factor to consider is the low sample number, which was out of our control. Here deep sequencing did not yield direct results, due to lack of specific biomarkers, although it was detected at likely low levels. Digital polymerase chain reaction (dPCR) may be advantageous over qPCR and deserves further consideration.

Objectives

In working on previous parts of our original project (January 2020–October 2021), it became apparent that additional questions regarding the detection of *Cyclospora cayetanensis* should be addressed by further analysis of the samples used in our study. The preliminary findings of *C. cayetanensis* oocysts in surface water from the Mid-Atlantic States region of the United States is unique and warranted further exploration. In part these experiments sought to 1) further understand and assess the sequence analysis of these samples, and 2) attempt to assess the issue of reagent specificity in the detection of *C. cayetanensis*.

Methods

Three different approaches were taken to explore sequence analysis and reagent specificity:

1. Deep sequencing (1Gb) on an Illumina HiSeq platform was combined with taxonomic profiling. Total DNA was purified from the water samples which were previously presumptive positive (by FDA BAM 19b primers) for the presence of *C. cayetanensis*.
2. Samples were analyzed using quantitative PCR with primers from FDA BAM 19b using Qiagen QuantiFast Multiplex PCR Kit (Cat No. 204654, noted in FDA BAM 19b) and Qiagen QuantiNova Probe PCR Kit (Cat No. 208254).
3. Samples were analyzed by digital PCR (dPCR) with FDA BAM 19b primers. Detection was performed in triplicate using 1.25 µl template in the 12.5 µl reaction in a 96-well 8.5K micro-partition plate using the Qiagen QIAcuity dPCR instrument.

Results to Date

Analyses included detection in triplicate from ten pooled samples (P1–P10) from the previous positives (**Table 1**) and 3 pooled negatives (P11–P13).

- Deep sequencing indicated complicated-mixed samples. The most abundant protozoan was *Hammondia hammondi*, related to *Toxoplasma gondii*. Bioinformaticians identified a very weak signal to *Cyclospora_cayetanensis_strain*_CHN_HEN01, with difficulties due to lack of unique biomarkers (data not shown).
- At least one replicate was positive with QuantiFast and QuantiNova (**Table 2**). dCt values (differences between raw Ct values) were used in analyses due to a significant number of replicates with no *Cyclospora* detected (**Figure 1**).
- *C. cayetanensis* was detected in four samples (3, 4, 5, 10) (**Figure 2, Figure 3**), collected from the same pond between June–October 2017. DNA from *Eimeria* was used as negative controls and they were not detected.

Benefits to the Industry

At this time several questions remain regarding sequence and PCR analysis in the accurate and timely detection of *C. cayetanensis* from surface waters. It is likely that detection will include PCR and sequencing. Organisms can only be detected in sequences if they exist in a database, and in this case adequate unique biomarkers do not exist. dPCR allows for improved sensitivity and precision. In dPCR faint genetic signals can be identified even against a strong background, instead of a single positive being lost in a dense pool of negatives. dPCR is advantageous over qPCR and deserves further consideration for detection of *C. cayetanensis* in complex samples. These studies provide new considerations to further the development of methods for detection of *Cyclospora* in surface water.

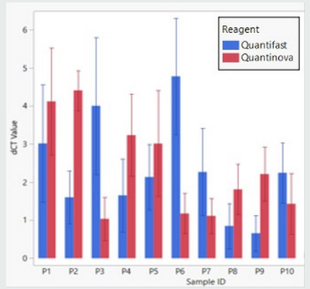

Figure 1. Detection of *C. cayetanensis* using qPCR with QuantiFast (blue) and QuantiNova (red) reagents. Data are presented as dCT values (number of cycles at which detection occurs prior to the terminal cycle (38), e.g., Ct value of 35 is a dCt value of 3).

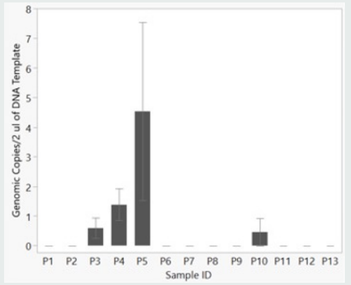

Figure 2. Detection of *C. cayetanensis* using dPCR assay and QIAcuity Probe PCR reagents. Detection for pooled samples (n=13) was performed in triplicate. The mean genomic copies per 2 ul of template are shown with the associated standard error bars.

Figure 3. Detection of *C. cayetanensis* visual output in QIAcuity software. Sample P5 in well F2 of the plate is magnified to show the positive partitions in that well.

Sample ID (P indicates pooled samples)	Sample Location/Water Type
P1	River
P2	Pond
P3	Pond
P4	Pond
P5	Pond
P6	Reclaimed
P7	River
P8	Reclaimed
P9	Pond
P10	Pond

Table 1. Sample identification and water type for sequencing analysis and PCR

Sample ID	Percent of positive replicates (*n* = 6)	
	QuantiFast	**QuantiNova**
P1	50% (3)	83% (5)
P2	67% (4)	100% (6)
P3	100% (6)	50% (3)
P4	50% (3)	67% (4)
P5	83% (5)	50% (3)
P6	67% (4)	67% (4)
P7	50% (3)	67% (4)
P8	50% (3)	67% (4)
P9	33% (2)	67% (4)
P10	67% (4)	50% (3)

Table 2. Percent of *Cyclospora*-positive replicates by Reagent