U.S. FOOD & DRUG ADMINISTRATION
Human Foods Program

# Source Tracking *Cyclospora cayetanensis*: A Targeted Amplicon Sequencing Approach

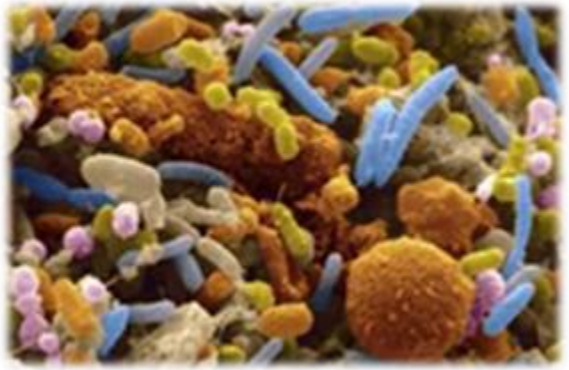
**GenomeTrakr Monthly Meeting**
**November 4, 2026**

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U.S. Food and Drug Administration

# Genetic comparisons among *Cyclospora cayetanensis* strains is complex

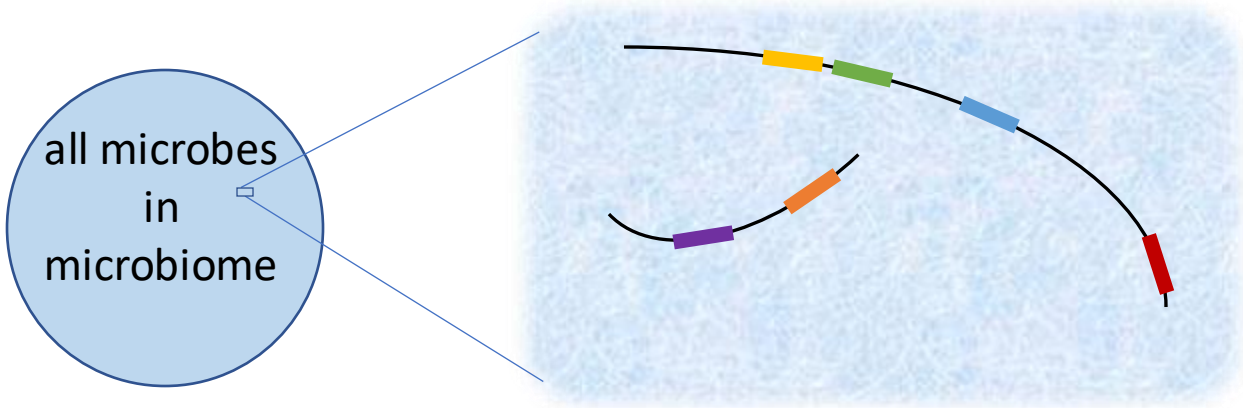
- Currently, cannot be propagated outside the human host, either *in vivo* or *in vitro*
- Whole genome sequence comparisons on a routine basis are not an option due to difficulty of obtaining purified oocysts for sequencing
- Diploid genome results in possible genetic heterozygosity at loci in the nuclear genome

**Targeted approach using culture independent metagenomic samples**



Food, environmental, or fecal microbiome containing *C. cayetanensis* oocysts

DNA extraction



# An improved method for genotyping *Cyclospora cayetanensis* was developed

**Research Collaboration Agreement between FDA and Chapter Diagnostics to develop a multiplex PCR targeted amplicon sequencing (TAS) genotyping method with:**

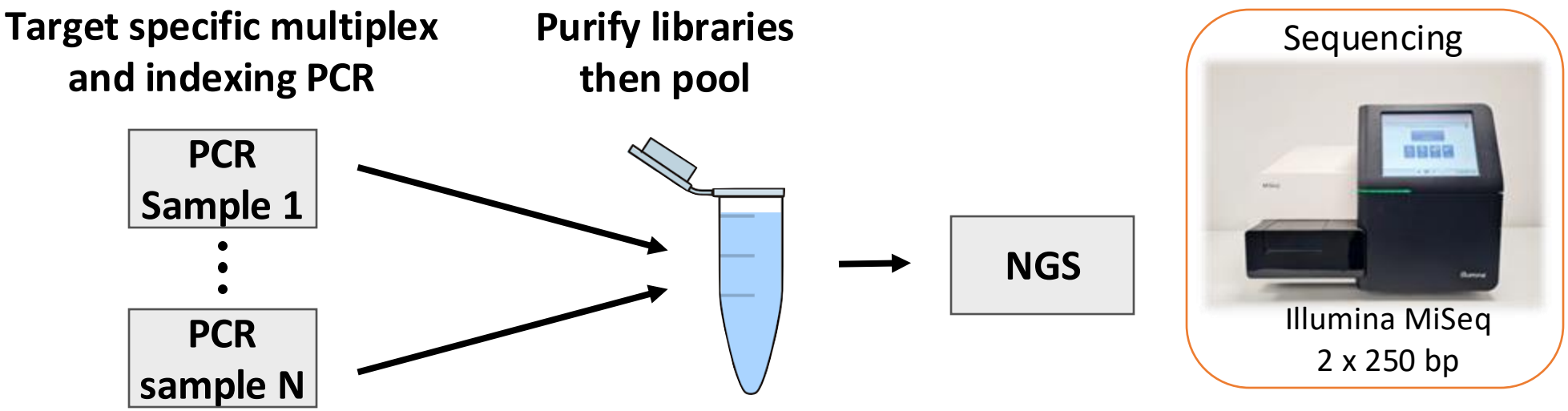
- Sensitivity for genotyping from contaminated fresh produce or environmental samples
- Increased genetic resolution compared to existing methods



**Multiplex Targeted amplicon sequencing method developed**

- Genomic loci chosen by FDA and ChapterDX as targets for markers based on optimum ability to genetically distinguish among *C. cayetanensis* strains (52 targets)
- Multiplex PCR uses lower volume of DNA than individual PCRs for each marker
- Addition of a capture hybridization step (bait-capture) was added for increased sensitivity

# Streamlined protocol for genotyping *C. cayetanensis* in clinical specimens





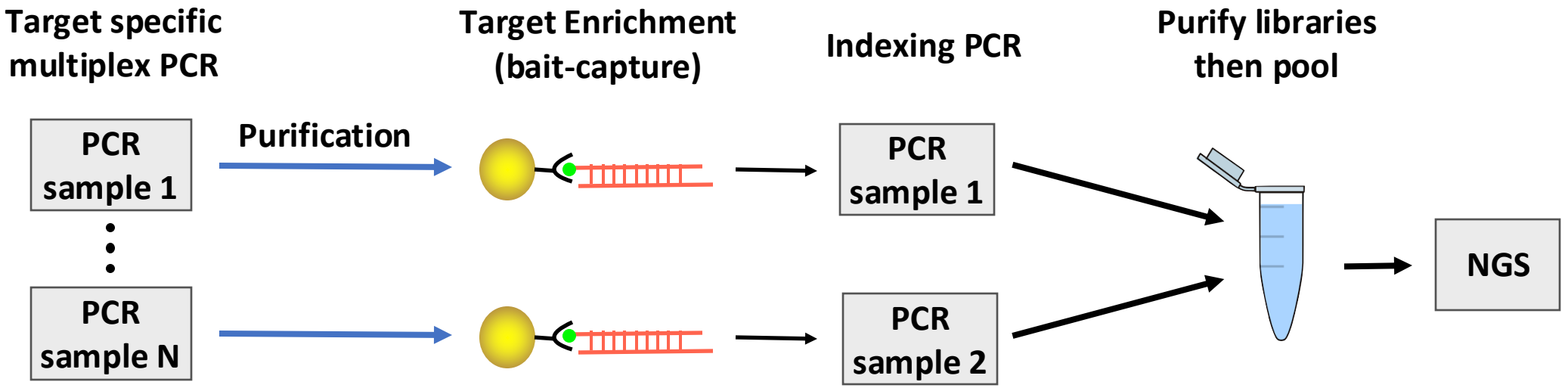

- TAS assay has sensitivity required for most clinical fecal specimens from cyclosporiasis illnesses
- Hybridization capture step added to workflow for samples with low parasite loads

Susan R. Leonard, et. al. 2023. *Frontiers in Microbiology*. DOI:10.3389/fmicb.2023.1212863

# Workflow for low parasite loads is straightforward



DNA ⇨ library can be accomplished in one day




Susan R. Leonard, et. al. 2023. *Frontiers in Microbiology*. DOI:10.3389/fmicb.2023.1212863

# Sensitivity of the genotyping method was evaluated

| Sample matrix | Oocysts inoculated | RT-PCR $C_T$ (mit1C) | Markers haplotyped* |
|---|---|---|---|
| Lettuce | 200 | 30.3 | 47 |
| Lettuce | 200 | 30.3 | 42 |
| Lettuce | 100 | 30.8 | 38 |
| Lettuce | 100 | 30.8 | 32 |
| Lettuce | 50 | 32.0 | 28 |
| Lettuce | 50 | 32.0 | 35 |
| Lettuce | 20 | 31.9 | 24 |
| Lettuce | 20 | 31.9 | 28 |
| Lettuce | 10 | 34.6 | 37 |
| Lettuce | 10 | 34.6 | 37 |
| Salad | 200 | 27.6 | 50 |
| Basil | 200 | 27.5 | 51 |
| Cilantro | 20 | 33.6 | 41 |
| Lettuce mix | 10 | 35.8 | 30 |
| Lettuce mix | 10 | 35.8 | 40 |
| Blackberries | 10 | 32.7 | 44 |

*TAS v1 contains 52 markers

- Fresh produce samples inoculated with oocysts
- 35 min hybridization (bait-capture step)
- Sequence for at least 24 markers obtained even at very low inoculation levels



Susan R. Leonard, et. al. 2023. *Frontiers in Microbiology*. DOI:10.3389/fmicb.2023.1212863

# Genotyping performed on raspberries inoculated with fecal specimen

| Sample | Inoculation | $C_T$ (RT-PCR) | Markers* |
|---|---|---|---|
| CDC04 | NA | 19.5±0.02 | 51 |
| CDC04R1 | 50 uL undiluted | 24.3±0.1 | 51 |
| CDC04R2 | 50 uL of 1:10 | 25.3±0.1 | 51 |
| CDC04R3 | 5 µL of 1:100 | 34.8±0.3 | 29 |
| CDC16 | NA | 23.6±0.05 | 51 |
| CDC16R1 | 50 uL undiluted | 28.0±0.4 | 51 |
| CDC16R2 | 50 uL of 1:10 | 28.4±0.3 | 51 |
| CDC16R3 | 5 µL of 1:100 | 35.7±1.5 | 21 |
| CDC43 | NA | 18.9±0.1 | 51 |
| CDC43R1 | 50 uL undiluted | 23.5±0.1 | 52 |
| CDC43R2 | 50 uL of 1:10 | 23.6±0.3 | 52 |
| CDC43R3 | 5 µL of 1:100 | 30.6±0.2 | 50 |

*Total of 52 markers in TAS v1 assay

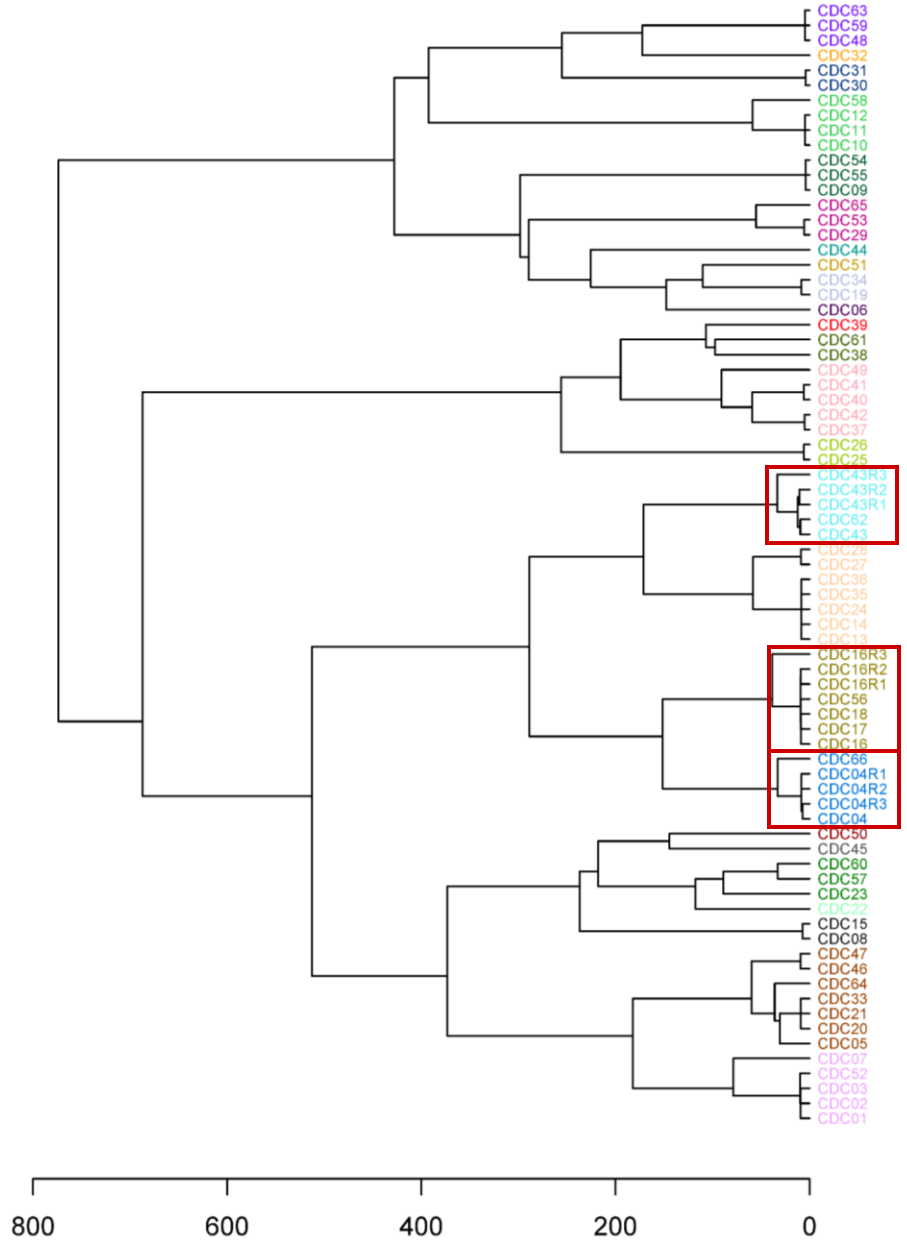
*C. cayetanensis* was genotyped from DNA extracted from raspberries inoculated with fecal specimens



RT-PCR results suggested lowest specimen dilution for two of the samples corresponded to ~10 oocysts/50 g raspberries

Susan R. Leonard, et. al. 2024. *Microorganisms*. doi.org/10.3390/microorganisms12050848

# Genetic linkage of contaminated raspberry samples with clinical specimens




- Hierarchical cluster dendrogram was constructed based on genotype results from both clinical specimens and inoculated raspberries
- The contaminated raspberry samples were assigned to clusters with the corresponding clinical specimen, demonstrating the utility of the TAS assay for traceback efforts



Susan R. Leonard, et. al. 2024. *Microorganisms*. doi.org/10.3390/microorganisms12050848

# TAS marker panel was optimized and version 2 created

**Results from genotyping the 66 clinical specimens were used to evaluate the marker panel**

Most and least genetically informative markers identified

Markers amplified less often when not all markers amplified were identified

In collaboration with CDC, the FDA chose markers for removal (n = 4) and addition (n = 15)

TAS assay marker panel version 2 includes 63 markers

**TAS assay v2 performed as well or better than v1 on inoculated fresh produce samples**

# *C. cayetanensis* reference genotype database is in progress

FDA and CDC collaborated to build a reference genotype database using TAS v2 assay

PRJNA1130490



Genetically, geographically, epidemiologically diverse strains

‘Recurrent’ genetic clusters represented



Cyclosporiasis fecal specimens genotyped by CDC (n = 241) and FDA (n = 107)

# *C. cayetanensis* was genotyped from a naturally contaminated food sample

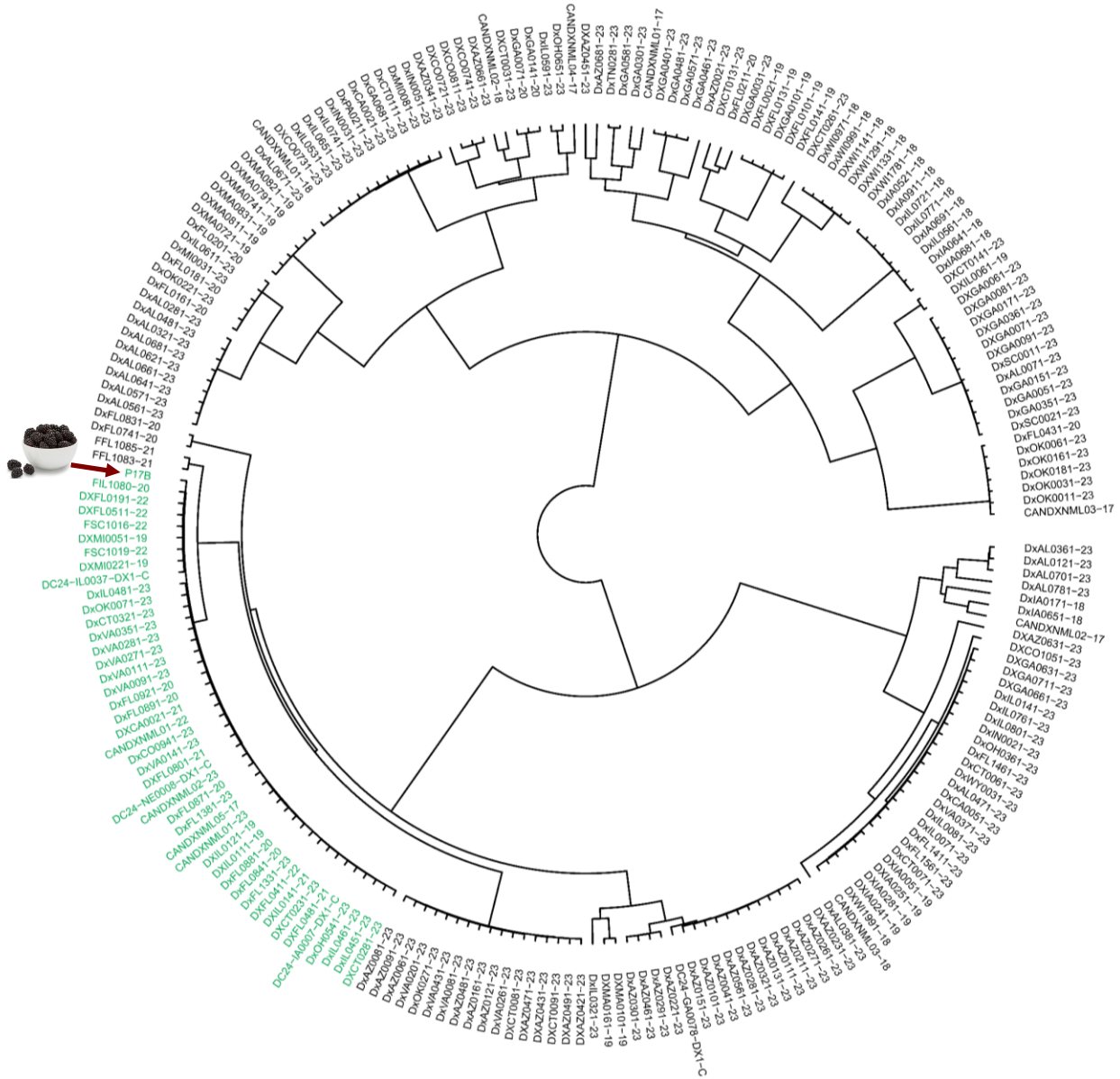
- Canadian Food Inspection Agency (CFIA) detected *C. cayetanensis* in a blackberry sample imported from USA to Canada in 2017
- FDA recently genotyped the *C. cayetanensis* strain contaminating the blackberry sample from the DNA extracted by CFIA in 2017
  - TAS v2 assay with hybridization capture used for genotyping
  - 62 of the 63 markers could be used for genotyping
- Collaboration on genetic linkage of blackberry sample with clinical specimens, including CFIA, Public Health Agency of Canada (PHAC), and CDC







# *C. Cayetanensis* strain contaminating berries clusters with clinical specimens




- Hierarchical cluster dendrogram generated using genotyping results from reference dataset (2018-2024), specimens genotyped at PHAC, and the blackberry sample
  - 220 clinical specimens + blackberries
  - Dendrogram contains 44 clusters
- Blackberries clustered with 43 clinical specimens that included specimens collected in both Canada (n=4) and the USA (n=39)
  - Intracluster genetic similarity high with 99.95-99.99% nucleotide identity across ~16,000 bp (2-8 SNPs)

# *C. cayetanensis* was genotyped from a contaminated water sample

- Water samples were collected in Mexico in an FDA outbreak traceback investigation
- One water sample consisting of five 10L subsamples from a wastewater aeration tank was positive
- Genotyping performed from extracted DNA using FDA TAS method v2 (63 amplicons) with hybridization capture

| Subsample | Amplicons |
|---|---|
| 1 | 43 |
| 2 | 49 |
| 3 | 42 |
| 4 | 36 |
| 5 | 34 |







# *C. cayetanensis* was genotyped from a contaminated sediment sample

- Sediment samples were collected in Mexico in an FDA outbreak traceback investigation
- *Cyclospora cayetanensis* was detected in one sediment subsample
- Genotyping performed from extracted DNA using FDA TAS method v2 (63 amplicons) with hybridization capture
- Sequence data for 49 amplicons obtained







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