Molecular Detection of *Cyclospora cayetanensis* in Fresh Produce:

FDA BAM CHAPTER 19B

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A New Protozoan Causing Diarrheal Illness in Humans: 1993
New Species: *Cyclospora cayetanensis*: 1994

CYCLOSPORA SPECIES — A NEW PROTOZOAN PATHOGEN OF HUMANS

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**Abstract**

Background. Organisms referred to as "cyanobacterium-like bodies" have now been identified worldwide in the feces of both immunocompetent and immunocompromised patients with diarrhea. Organisms with a similar appearance have been isolated from Peruvian patients since 1985. From 1986 to 1991 we studied prospectively two cohorts of infants and young children infected with this organism. We now attempt to identify it.

Methods. Fecal samples were collected weekly from the children and examined with the use of acid-fast staining and staining with a monoclonal antibody specific for cryptosporidium. Stools positive for cyanobacterium-like bodies were preserved in potassium dichromate and exposed to conditions allowing coccidian sporulation and excystation. Both unsporulated and sporulated oocysts were fixed by freeze-substitution techniques and then examined by electron microscopy.

**Results.** Organisms isolated from the feces of Peruvian patients and two patients from the United States were identified as belonging to the coccidian genus cyclospora, after sporulation and excystation of the oocysts according to standard techniques. Complete sporulation occurred within 5 to 13 days in oocysts maintained in potassium dichromate at 25 or 32°C. Complete excystation resulted in the liberation of two sporozoites from the two sporocysts within each oocyst (cryptosporidia have four naked sporozoites within each oocyst). The presence of organelles characteristic of coccidian organisms was confirmed by electron microscopy.

**Conclusions.** We have identified organisms of the genus cyclospora that are remarkably similar to cryptosporidia in their morphologic features and the diarrheal disease that they produce in humans. The complete life cycle and epidemiology of this new protozoan parasite remain to be described. (N Engl J Med 1993;328:1308-12.)

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In the U.S., since the mid-1990s, cyclosporiasis has occurred linked to various types of fresh produce:

- raspberries
- basil
- snow peas
- mesclun lettuce
- cilantro
- bagged mixed salad
Cyclospora cayetanensis

- A coccidian protozoan parasite that causes a severe intestinal illness called cyclosporiasis.
  - diarrhea, nausea
  - cramping, bloating
  - fatigue, weight loss
- The only species of genus *Cyclospora* known to infect humans.
- Most commonly occurs when food or water contaminated with feces containing the parasite’s sporulated oocysts are consumed.
- Found in many countries and it is most common in tropical and subtropical regions.
- Multi-state cyclosporiasis outbreaks have occurred in the U.S. since the 1990’s linked to consumption of fresh produce.
Biology of *C. cayetanensis*

*Cyclospora* is not transmitted directly from one person to another

- Fresh produce and water contaminated with sporulated oocysts serve as vehicles for transmission when ingested.
- Oocysts excyst in the gastrointestinal track, releasing sporozoites which invade the epithelial cells of the small intestine and undergo sexual and asexual maturation processes to form unsporulated oocysts.
- Unsporulated (non-infectious) oocysts are shed by infected individuals into the environment in feces.

Sporulation only occurs in the environment.
- formation of two sporocysts (each containing two sporozoites)
- environmental factors are not fully understood
C. cayetanensis Infections Globally

Distribution of cyclosporiasis in developing regions

Source: Leonor Chacín-Bonilla, May 2017 *Cyclospora cayetanensis*, Global Water Pathogen Project, Section III. Protists
http://www.waterpathogens.org/cyclospora-cayetanensis
Cyclosporiasis outbreaks
Distribution worldwide

Countries that have reported epidemics:

Also fits into “orange” category

Source: Leonor Chacín-Bonilla, May 2017 Cyclospora cayetanensis, Global Water Pathogen Project, Section III. Protists
http://www.waterpathogens.org/cyclospora-cayetanensis
US Cyclosporiasis Outbreaks - 2018

A total of 2,299 domestically acquired lab confirmed cases of cyclosporiasis from 33 states

- Multiple sub-clusters identified in 6 states – some identified cilantro as a vehicle of interest.
- A total of 14 cases reported consumption of meals that included basil in 2 states.
- 250 laboratory-confirmed cases from 4 states linked to vegetable trays containing broccoli, cauliflower, carrots, and dill dip.
- 511 laboratory-confirmed cases from 15 states linked to consumption of salads from a quick-service restaurant chain in the Midwest.
A total of 2,408 domestically acquired cases from 37 states, DC, and NYC

- Multiple clusters of cases associated with different restaurants or events were investigated by state public health authorities, CDC, and FDA.

- Approximately 10% of illnesses were associated with a multistate outbreak of *Cyclospora* infections linked to fresh basil imported from Siga Logistics de RL de CV of Morelos, Mexico.

- Many cases of cyclosporiasis could not be directly linked to an outbreak, in part because of the lack of validated molecular typing tools for *C. cayetanensis*. 
Analysis of 2013 TX outbreaks highlighted major gaps in laboratory methods for *Cyclospora*

1. Improved laboratory methods to perform surveillance studies and identify sources of infection are needed.

2. Molecular epidemiology tools have not been available to assist in outbreak investigations.

- 2013-631 cases in 25 states
- largest number since 1997
- describes restaurant cluster investigations in TX
  - state with highest number of cases
  - cilantro as the most likely vehicle of infection

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**2. Molecular epidemiology tools have not been available to assist in outbreak investigations.**

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Health and the produce industry. The specific challenges posed by *Cyclospora* include under-detection of cases, lack of subtyping methods to link cases to each other or to specific food items, and the absence of practical tools to detect the organism in food and potential sources of contamination in the environment (e.g., soil and insanitary irrigation water). Advances in investigations. The outbreaks of cyclosporiasis in 2013 underscore the need for molecular subtyping to complement evidence from epidemiological investigations, potentially assisting in identifying the number of outbreaks in a given season and suggesting links between clusters, and facilitating source tracking.
MDVIP governs processes for regulatory use of analytical laboratory methods.

- To support the FDA Foods Program regulatory mission
- To ensure FDA laboratories use properly validated regulatory methods

**Foods Program Compendium of Analytical Laboratory Methods: Microbiological Methods**

- **Bacteriological Analytical Manual (BAM)**
  - preferred methods, MLV validated
  - main component

- **other methods not yet added to the BAM**
  - methods approved but not yet posted to the BAM
  - methods not validated to the MLV level

available on FDA's public website at: https://www.fda.gov/food/laboratory-methods-food/foods-program-compendium-analytical-laboratory-methods
BAM 19b: Molecular Detection of Cyclospora cayetanensis in Fresh Produce Using Real-Time PCR

Authors: Helen R. Murphy, Sonia Almeria and Alexandre J. da Silva
Contact: Helen Murphy

BAM 19b replaces all aspects of the *Cyclospora* methodology for produce found in BAM Chapter 19a.

Available at: https://www.fda.gov/food/laboratory-methods-food/bam-19b-molecular-detection-cyclospora-cayetanensis-fresh-produce-using-real-time-pcr
Challenges for Detection of *C. cayetanensis* in Foods

- Enrichment and selection of *C. cayetanensis* in culture is **not possible**.
- Requires isolation of **low numbers of oocysts** from food samples.
- **Microscopic identification is challenging** in food samples.
  - cannot distinguish *C. cayetanensis* from other *Cyclospora* species
- Sensitive **species specific molecular methods** must be used.
1. Improved produce washing
   - 0.1% Alconox wash solution
2. DNA extraction using a commercial kit
   - bead beating using a high-speed benchtop homogenizer to disrupt oocysts.
3. Molecular detection by real-time PCR.
   - ease of execution and rapid analysis
   - internal amplification control (IAC) to monitor for PCR inhibition
PRODUCE WASH PROCEDURE

- 25 g leafy greens or 50 g berries
- 100 ml 0.1% Alconox wash solution
- Gentle agitation in a filter bag
- A second rinse step included
PRODUCE WASH PROCEDURE (cont’d)

- Centrifugation of wash solutions
- Aspiration of wash supernatant
- Wash debris pellet containing recovered Cyclospora oocysts

50 ml tubes

15 ml tubes

2 ml lysing tube
DNA EXTRACTION PROCEDURE

1. disrupt oocysts by bead beating
   - MP BIO FastPrep-24 Homogenizer

2. commercial kit to purify DNA
   - MP BIO FastDNA™ SPIN Kit for Soil
MOLECULAR DETECTION

TaqMan Real-time PCR

- Amplification of a series of serial dilutions of the synthetic positive control target.
- Limit of Detection is a SINGLE COPY of the *C. cayetanensis* 18S rRNA gene target.

Applied Biosystems
7500 Fast Instrument

Duplex reaction

18S = *C. cayetanensis* 18S rDNA
IAC = Internal Amplification Control
PERFORMANCE CHARACTERISTICS

BAM 19B Method

- Appropriate for detection of *C. cayetanensis* in a variety of leafy greens, berries, and vegetables.

- The analytical sensitivity of the method is 5 oocysts on seeded cilantro (25 g) and raspberries (50 g).
MLV Study Results

Detection limit is 5 oocysts.

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Seeding Level</th>
<th>Positive results (N=80)</th>
<th>% positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>cilantro</td>
<td>0</td>
<td>0</td>
<td>0.0%</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>25</td>
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<td></td>
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<td></td>
<td>200</td>
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<td>100.0%</td>
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<tr>
<td>raspberries</td>
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<td>0.0%</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>40</td>
<td>50.0%</td>
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<td></td>
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<td>72</td>
<td>90.0%</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>80</td>
<td>100.0%</td>
</tr>
</tbody>
</table>
The method has been validated for other matrices in extension studies.

- shredded carrots
- basil
- parsley
- romaine lettuce
Method modifications (Not Validated)

- coleslaw
- guacamole
- Pico de Gallo, salsa
DNA CLEAN-UP

Cilantro + soil ➔ PCR inhibition

DNA Clean-up Step
(Not validated)
Detection of *Cyclospora cayetanensis* in Water Using Dead-End Ultrafiltration (DEUF)

- It is possible that agricultural water plays a significant role in contaminating crops during the irrigation process leading to *C. cayetanensis* cases and outbreaks.

- Water testing may be necessary during farm and facility inspections to identify potential sources of infection and provide critical support for outbreak investigations.

- Recently, FDA validated a new method for detection of *C. cayetanensis* in agricultural water by combining DEUF with the DNA extraction and qPCR protocols used in BAM 19B.
Water Method Workflow

Collect large volume water samples using medical grade dialysis filters which can effectively trap parasites, bacteria, viruses, large toxins.

A process called Dead-End Ultrafiltration (DEUF).

In the Field

Sample agricultural water

Filter by DEUF

Ship to the lab

In the Lab

Backflush filters

Concentration

DNA Extraction and qPCR
Recovery and Detection of *Cyclospora cayetanensis* from Agricultural Water

FDA completed multi-lab validation of the *Cyclospora* water method and the method was approved for use in 2019

- Detection limit is 6 *C. cayetanensis* oocysts per 10L of agricultural water.
- Method posted to the Compendium pending addition to the FDA BAM.

Available at: https://www.fda.gov/food/laboratory-methods-food/foods-program-compendium-analytical-laboratory-methods
BAM CHAPTER 19B
IMPLEMENTATION

Implementation Through Laboratory Training

FDA ORA Food and Feed Laboratories

Domestic

International

North and Central American Agencies
Increasing the lab capacity for *Cyclospora* testing of produce in the US

**FDA/ORA Labs:** ARKL, DENL, NFFL, PNL, PSSFL, SANFL, SFFL

- Multiple analysts at all FDA ORA micro labs are capable of executing BAM Chapter 19B.

**DOD**

- FADL-Food Analysis and Diagnostic Laboratory, Fort Sam Houston, TX

**FERN Labs**

- Minnesota Department of Agriculture, Laboratory Services Division
- North Carolina Dept. of Agriculture & Consumer Services
- California Department of Public Health
- Michigan Department of Agriculture and Rural Development
- Washington State Department of Agriculture, Food Safety & Consumer Services Division
- **MD Dept Health***
- **HI Health***
- **WI Ag***
- **NYC Health***
- **UPenn***

*Feb 24-28, 2020*
Currently, no international standards exist for the detection of *C. cayetanensis* in foods.

ISO workgroup has been convened to develop the FDA method as an international standard for detection of *C. cayetanensis* in fresh produce.

- protocol based on FDA BAM 19B
- international ring trial
2019 Proficiency Test
Detection of *C. cayetanensis* in Cilantro

Moffett Proficiency Testing Laboratory, CFSAN
(ISO/IEC 17043 accredited, ANAB certificate AP-2123)

- **Number of participants**: 17
  - seven FDA labs
  - 10 state labs
- **Protocol**: FDA BAM 19b

Cilantro samples for the PT drying in a bio-safety cabinet.
ORA Regulatory Testing for *C. cayetanensis*

- **Fresh Herb Assignment**
  - cilantro, parsley, and basil.
  - import and domestic

- **Cyclospora** interim data as of 7/1/2019
  - 219 domestic
    - 2 positive
  - 197 import
    - 8 positive

- **Extended → March 2020**

https://www.fda.gov/food/sampling-protect-food-supply/microbiological-surveillance-sampling#reports
In Conclusion

Foodborne cyclospororiasis is a problem with **global impact**

- FDA is addressing the analytical challenges associated with *C. cayetanensis* and the safety of fresh produce.
  - Development and validation of improved and streamlined laboratory methods

- FDA continues to further implement standardized methods to detect *Cyclospora* though training.
  - Domestic and international public health partners

To better understand *C. cayetanensis* transmission and reduce outbreaks:

**Identify sources of contamination**

**Effective Control Measures**
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Validation of the *Cyclospora* Water Method

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