Cyclospora cayetanensis and Produce Safety

Association of Food and Drug Officials
Cyclospora Webinar
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Some parasites cannot be distinguished to the species level by microscopy techniques: some different species are morphologically identical; e.g., *Entamoeba dispar* vs. *Entamoeba histolytica*. Microscopy techniques lack sensitivity to detect of low number of parasites eggs, oocysts or cysts in food and environmental samples; e.g., detection of *Cyclospora cayetanensis* in foods.

**Major limitations of microscopy in foodborne parasitology**
DNA-based detection

Distinct pathogens – based on matches with DNA sequence databases

**Sample Type**

- Stools
- Water
- Food
- Slug/Snail
- Animal material
- Plant material

**PCR/Real-Time PCR/PCR+DNA Sequencing**

- Anisakidae
- Angiostrongylus cantonensis
- Entamoeba spp.
- Cryptosporidium spp.
- Cyclospora spp.
- Diphyllobothrium spp.
- Giardia spp.
- Microsporidia
- Trypanosoma cruzi
- Toxoplasma gondii
- Trichinella spp.

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Alice Y. Ho, Adriene S. Lopez, Michael G. Eberhart, Robert Levenson, Bernard S. Finkel, Alexandre J. da Silva, Jacqueline M. Roberts, Palmer A. Orlandi, Caroline G. Johnson, and Barbara L. Herwaldt

An outbreak of cyclosporiasis occurred in attendees of a wedding reception held in Philadelphia, Pennsylvania, on June 10, 2000, in a retrospective cohort study. 54 (68.4%) of the 79 interviewed guests and members of the wedding party met the case definition. The wedding cake, which had a cream filling that included raspberries, was the food item most strongly associated with illness (multivariate relative risk, 5.9; 95% confidence interval, 3.6 to 10.5). Leftover cake was positive for Cyclospora DNA by polymerase chain reaction analyses. Sequencing of the amplified fragments confirmed that the organism was Cyclospora cayetanensis. The year 2000 was the fifth year since 1995 that outbreaks of cyclosporiasis definitely or probably associated with Guatemalan raspberries have occurred in the spring in North America. Additionally, this is the second documented U.S. outbreak, and the first associated with raspberries, for which Cyclospora has been detected in the epidemiologically implicated food item.

Emerging Infectious Diseases • Vol. 8, No. 8, August 2002

FDA Bacteriological Analytical Manual (BAM) Chapter 19 A.
Detection of Cyclospora and Cryptosporidium from Fresh Produce:
Isolation and Identification by Polymerase Chain Reaction (PCR) and Microscopic analysis.

Palmer A. Orlandi, Christian Frazer, Laurenda Carter, and Dan-My T. Chu, 2004c

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2013 multistate outbreaks of *Cyclospora cayetanensis* infections associated with fresh produce: focus on the Texas investigations

F. ABANYIE1, R. R. HARVEY2,3, J. R. HARRIS1, R. E. WIEGAND1, L. GAUL4, M. DESVIGNES-KENDRICK2, K. IRVIN6, I. WILLIAMS3, R. L. HALL1, B. HERWALDT1, E. B. GRAY1, Y. QVARNSTROM1, M. E. WISE5, V. CANTU4, P. T. CANTEY1, S. BOSCH1, A. J. DA SILVA1,6, A. FIELDS5, H. BISHOP1, A. WELLMAN6, J. BEAL6, N. WILSON1,2, A. E. FIORE1, R. TAUXE3, S. LANCE3,6, L. SLUTSKER1, M. PARISE1, and the Multistate *Cyclosporiasis* Outbreak Investigation Team†

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2Epidemic Intelligence Service, Centers for Disease Control and Prevention, Atlanta, GA, USA
3National Center for Emerging and Zoonotic Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, GA, USA
4Texas Department of State Health Services, Austin, TX, USA
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Received 8 October 2014; Final revision 10 February 2015; Accepted 10 February 2015

Cilantro was the most likely vehicle of infection in restaurant A, B, C, and grocery store clusters.

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.
Bacteriological Analytical Manual (BAM)

FDA’s Bacteriological Analytical Manual (BAM) presents the agency’s preferred laboratory procedures for microbiological analyses of foods and cosmetics. AOAC International published previous editions of this manual in a loose-leaf notebook format, and, more recently, on CD-ROM. This online BAM is now available to the public. Some changes have been made to methods since the previous version. A listing of chapters updated since the last hard-copy version (Edition 8, Revision A /1998) can be found in About the Bacteriological Analytical Manual. The members of the BAM Council are listed below. In addition recent changes for most Chapters are documented in a brief Revision History at the beginning of the Method. There is also e-mail contact information for each Chapter. Chapter numbers have been retained from the previous version. However, for this Table of Contents, chapters have been grouped by category. Please send comments to Karen Jinneman.

Jump to:

https://www.fda.gov/food/laboratory-methods-food/bacteriological-analytical-manual-bam
| 19A | Detection of Cyclospora and Cryptosporidium from Fresh Produce: Isolation and Identification by Polymerase Chain Reaction (PCR) and Microscopic Analysis. | P.A. ORLANDI, C. HUANG, L. CARTER, D.T. CHAI (eds.) |
| 26A | Detection and Quantification of Hepatitis A Virus in Shellfish by the Polymerase Chain Reaction | B.B. GODWIN (et al.) |
| 29 | Cronobacter | Y. CHEL, K. LEMPET, T. HAMMACK |

Methods for Microbial Toxins

| 15 | Bacillus cereus Diarrheal Enterotoxin | R.W. BENNETT |

Additional Methods

| 20A | Inhibitory Substances in Milk | L.J. NATURIN (et al.) |
| 20B | Rapid HPLC Determination of Sulfamethazine in Milk | J.D. WEBER (et al.), M.D. SMIDLEY |
| 21A | Examination of Canned Foods | W.L. LANDRY, A.H. SOHN, G.A. LANCETTE (et al.) |
| 21B | Modification of Headspace Gas Analysis Methodology, Using the SP4270 Integrator | W.L. LANDRY, M.J. LUBESE |
BAM 19b: Molecular Detection of Cyclospora cayetanensis in Fresh Produce Using Real-Time PCR

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

Revision History:
- November 2015: Cyclospora Fruiting Body Extension Report has been added.
- September 2017: Modified FastDNA Spin Extraction Protocol section: Duplicate text removed from step C; The word “shaking” has been replaced with the word “inverting” in step G.
- September 2017: Posted a Published Journal Article (PDF) and a Supplemental Data File (PDF).
- August 2017: Cyclospora Basil Extension Report, Cyclospora Parsley Extension Report, and Cyclospora Carrot Extension Report have been added.
- August 2017: Matrix extension study for basil and parsley has been added.
- Six Appendices (PDF format) are available at the end of this method.

Appendices: (PDF Format)
1. Appendix 1: Alcains3: Produce Wash Solution Recipe
2. Appendix 2: Tris EDTA (TE) pH 7.5 Primer Dilution Buffer Recipe
3. Appendix 3: Preparation of the Internal Amplification Control (IAC) Target Working Solution
4. Appendix 4: Preparation of the Positive Control Target Working Solution
5. Appendix 5: AAB 7500 Fast 12.0 or 2.0 Method
6. Appendix 6: AAB 7500 Fast V1.4 Method
F. qPCR Data Analysis Flowchart:

1. Are all replicates of both the HVT and the DNA extraction control (Coy18S target reactions) NEGATIVE?
   - NO: Invalidate Results
   - YES: Proceed to Step 2

2. Are all replicates of the Positive Control (Coy18S target reactions) POSITIVE?
   - NO: Invalidate Results
   - YES: Proceed to Step 3

3. Does the sample produce at least one (1) Coy18S target reaction with an amplification factor (AF) ≥ 30.07?
   - NO: Invalidate Results
   - YES: Proceed to Step 4

4. Does the re-ester sample produce at least one (1) Coy18S target reaction with an amplification factor (AF) ≥ 30.07?
   - NO: Invalidate Results
   - YES: Proceed to Step 5

5. If the sample yield target reaction product underestimates the reference (Coy18S) or an average Cx value more than 0.5 cycles higher compared to the NCPC?
   - NO: Invalidate Results
   - YES: Invalidate Results

Appendices (PDF Format):
1. Appendix 1: Alconox® Produce Wash Solution Recipe
2. Appendix 2: Tris EDTA (TE) pH 7.5 Primer Dilution Buffer Recipe
3. Appendix 3: Preparation of the Internal Amplification Control (IAC) Target Working Solution
4. Appendix 4: Preparation of the Positive Control Target Working Solution
5. Appendix 5: ABI 7500 Fast v1.2.0 Method
6. Appendix 6: ABI 7500 Fast v1.1.4 Method

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<thead>
<tr>
<th>Matrix</th>
<th>Seedling Level</th>
<th>Positive samples (80 tested)</th>
<th>% positive</th>
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<td>0</td>
<td>0</td>
<td>0.0%</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>25</td>
<td>31.3%</td>
</tr>
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<td>40</td>
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<tr>
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<td>100.0%</td>
</tr>
</tbody>
</table>

Also approved for shredded carrots, and basil and parsley.

Detection Limit is 5 oocysts.

Interlaboratory validation of an improved method for detection of Cyclospora cayetanensis in produce using a real-time PCR assay

Helen R. Murphy a, b, Hediyee Nese Cinar c, Copal Gepinath c, Kathy E. Noc d, Lacesha D. Chatman e, Nancy E. Miranda e, June H. Wetherington e, Jason Neal-McGuire f, Gabrielle S. Fries f, Elizabeth Sachs f, Christopher J. Starna f, Cynthia L. Johnson f, Fernanda S. Nascimento f, Monica Santin f, Aleksey Molokov f, Manouk Samadpour f, Harish Janagama g, Amy Kahler g, Candace Miller g, Alexandre J. da Silva g

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5 U.S. Department of Agriculture, Agricultural Research Service, Environmental Microbial and Food Safety Lab, Beltsville, MD 20705, USA
6 CB Laboratory & Consulting, Inc., Lake Forest Park, Washington 98025, USA
7 Centers for Disease Control and Prevention, Division of Foodborne, Waterborne, and Environmental Diseases, Waterborne Disease Prevention Branch, Atlanta, GA 30333, USA
8 Interlaboratory validation of an improved method for detection of Cyclospora cayetanensis in produce using a real-time PCR assay

Also approved for shredded carrots, and basil and parsley.

Detection Limit is 5 oocysts.
A total of 2,299 domestically acquired lab confirmed cases of cyclosporiasis from 33 states with 160 hospitalizations

Multiple sub-clusters identified in 6 states and epidemiological studies conducted 3 states identified cilantro as a vehicle of interest. A total of 14 cases reported consumption of meals that included basil in 2 states.

511 laboratory-confirmed cases from 15 states linked to consumption of salads from a quick-service restaurant chain in the Midwest.

“On July 26, 2018, the FDA completed final analysis of an unused package of romaine lettuce and carrot mix distributed to McDonald’s by the Fresh Express processor in Streamwood, IL. The analysis confirmed the presence of *Cyclospora* in that sample.”


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This outbreak was linked to McDonald’s salads sold in 14 states in the Midwest that contained a romaine lettuce and carrot mix supplied by Fresh Express. The FDA worked with McDonald’s to quickly remove implicated salad from the stores. Testing conducted by the FDA identified the parasite in an unopened package of the bagged salad mix, supporting epidemiologic evidence that the salad mix is the source of the outbreak.

“During our investigation, two samples of domestically grown romaine lettuce were also found to be positive for Cyclospora even though they were not sourced from locations associated with the lettuce that was linked to this outbreak. None of the romaine lettuce associated with these positive test results for Cyclospora went into the marketplace and all of the produce suspected of being contaminated was destroyed, preventing additional Cyclospora illnesses from occurring. However, these findings are important as they represent the second time that Cyclospora has been identified in produce grown in the U.S.”
“On July 26, 2018, the FDA completed final analysis of an unused package of romaine lettuce and carrot mix distributed to McDonald’s by the Fresh Express processor in Streamwood, IL. The analysis confirmed the presence of *Cyclospora* in that sample.”

This sample was reported by the Pacific Northwest Laboratory in one of the subs of romaine lettuce analyzed. This sub was positive using the BAM Chapter 19B method with a Ct of 37.9. The result indicated that the sample had a low concentration of oocysts in the 25g of romaine lettuce tested, e.g., less than 5 oocysts.
Molecular Analysis- PCR and DNA sequencing

1.B - Test DNA samples with mit3PCR which amplifies a 182 bp fragment from C. cayetanensis mitochondrial genome

2.B - DNA sequencing analysis of the 182 bp amplicon produced by mit3PCR

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Microbiological Surveillance Sampling: FY18-19
Fresh Herbs (Cilantro, Basil & Parsley) and Processed Avocado and Guacamole Assignments

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FDA Surveillance to Estimate the Prevalence of *Cyclospora cayetanensis* in Fresh Produce (Using the BAM Chapter 19B method)

**Fresh Herbs Results as of 10/1/2019**

The FDA plans to collect 1,600 fresh herbs samples (761 domestic, and 839 of international origin) under this assignment. As of September 30, 2019, the agency had collected and tested 746 domestic samples (98 percent) and 468 import samples (56 percent) of the totals. The following figures summarize the interim sampling results. As the testing is still underway, no conclusions can be drawn at this time.

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Increasing the lab capacity for Cyclospora testing of produce in the US

FDA/ORA Labs: ARKL, DENL, NFLL, 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
• Maryland Department of Health Laboratories Administration
• Hawaii Department of Health
• State of Wisconsin, Department of Agriculture
• NYC DOHMH Public Health Laboratory
• University of Pennsylvania, New Bolton Center
Reported U.S. cases of laboratory-confirmed, non-travel-associated cyclosporiasis in people with onset of illness during May–August, 2018*

As in 2013, 2014, 2015 and 2017 a large percentage of the 2018 cases could not be linked to any of the outbreaks/clusters identified through epidemiologic studies.

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US Cyclosporiasis Outbreaks - 2018

Approximately 14 cases

114 cases identified through epidemiological studies. Additional 42 cases (identified through interviews).

250 laboratory-confirmed cases

511 laboratory-confirmed cases

1,368 cases that were not linked to the clusters above due to lack of statistically significant commonalities and other surveillance issues. That represented approximately 60% of the 2,299 domestically acquired cases reported in 2018.

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It currently contains 31 whole *C. cayetanensis* genome sequences and 36 sequences from *C. cayetanensis* complete mitochondrial genomes. The sequence data was obtained from DNA extracted from clinical samples from patients diagnosed in the U.S. The content of this public database on NCBI is being provided by both FDA and CDC teams. A high-quality reference whole genome assembly was generated and annotated by FDA is also available from this database.
• A method that combines robust ultrafiltration using hollow fiber filters with sensitive and specific molecular detection was developed.

• This method has a detection limit of 6 *C. cayetanensis* oocysts per 10L of agricultural water.
The MLV study is a validation of the process which begins at the backflush step.

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Molecular Analysis- PCR and DNA sequencing

1.A - Test DNA samples with BAM Chapter 19B qPCR

- 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.

Wells 6 to 19= water seeded with 10,000 to 500 oocysts in 50L water samples

1.B - Test DNA samples with mit3PCR which amplifies a 182 bp fragment from *C. cayetanensis* mitochondrial genome

2.B - DNA sequencing analysis of the 182 bp amplicon produced by mit3PCR

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Research Challenges Ahead

• Develop the new generation of molecular detection and genotyping techniques based on available genomic data (harmonization of those methods among federal agencies; FDA, CDC and USDA)

• Populate “CycloTrakr” with sequences from *C. cayetanensis* clinical and environmental samples obtained from different geographic areas where *C. cayetanensis* has been identified as a public health issue (e.g., U.S., Guatemala, Peru, Mexico, etc.)

• Validate the new generation molecular methods for regulatory detection of *C. cayetanensis*

• Understand how contamination of produce takes place at various levels

• Ascertaining the prevalence of *C. cayetanensis* in food and in certain environmental samples that may impact food safety

• Validation of the BAM Chapter 19B method on a variety of food matrices
Genomics and Molecular Epidemiology

Research Collaboration Agreement between CDC and FDA

• Facilitates the sharing of specimens that are critical to the laboratory methods and tools that each Agency is developing

• Allows both FDA and CDC to develop and improve molecular epidemiology methods to detect clusters of cases in humans as well as detect and trace the parasite in a variety of samples without duplication of efforts

• Harmonize methodologies between the two sister agencies for detection, identification to species and genotype level to facilitate interventions and effective deployment of regulatory tools

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Thank you

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