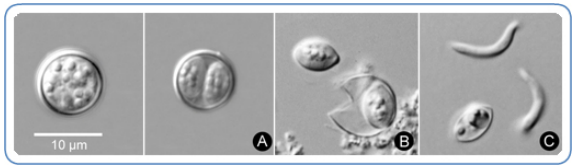
Cyclospora cayetanensis and Produce Safety

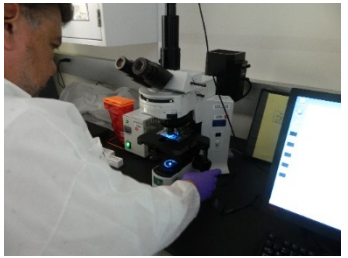
Association of Food and Drug Officials
Cyclospora Webinar
May 15th 2020

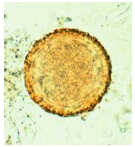
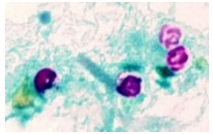
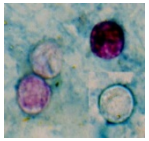
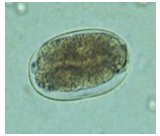
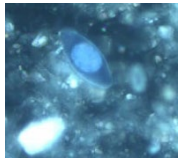


Alexandre J. da Silva, MSc, PhD
SBRS Lead Parasitologist
FDA/CFSAN/OARSA/DVA
Laurel, MD

Foodborne Parasitology - Microscopy






Major limitations of microscopy in foodborne parasitology

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

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.

Outbreak of Cyclosporiasis Associated with Imported Raspberries, Philadelphia, Pennsylvania, 2000

Alice Y. Ho,* Adriana S. Lopez,†‡ Michael G. Eberhart,* Robert Levenson,* Bernard S. Finkel,* Alexandre J. da Silva,‡ Jacquelin M. Roberts,‡ Palmer A. Orlandi,§ Caroline C. 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 Frazar, Laurenda Carter, and Dan-My T. Chu, 2004c

Disclaimer: This is a scientific presentation. Questions about compliance and enforcement will not be answered by the speaker s.

Epidemiol. Infect., Page 1 of 8. © Cambridge University Press 2015
doi:10.1017/S0950268815000370

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

F. ABANYIE[1]*, R. R. HARVEY[2,3], J. R. HARRIS[1], R. E. WIEGAND[1], L. GAUL[4], M. DESVIGNES-KENDRICK[5], K. IRVIN[6], I. WILLIAMS[3], R. L. HALL[1], B. HERWALDT[1], E. B. GRAY[1], Y. QVARNSTROM[1], M. E. WISE[3], V. CANTU[4], P. T. CANTEY[1], S. BOSCH[3], A. J. DA SILVA[1,6], A. FIELDS[6], H. BISHOP[1], A. WELLMAN[6], J. BEAL[6], N. WILSON[1,2], A. E. FIORE[1], R. TAUXE[3], S. LANCE[3,6], L. SLUTSKER[1], M. PARISE[1], and the Multistate Cyclosporiasis Outbreak Investigation Team†

[1] *Center for Global Health, Division of Parasitic Diseases and Malaria, Centers for Disease Control and Prevention, Atlanta, GA, USA*
[2] *Epidemic Intelligence Service, Centers for Disease Control and Prevention, Atlanta, GA, USA*
[3] *National Center for Emerging and Zoonotic Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, GA, USA*
[4] *Texas Department of State Health Services, Austin, TX, USA*
[5] *Fort Bend County Health & Human Services, Rosenberg, TX, USA*
[6] *United States Food and Drug Administration, College Park, MD, USA*

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.

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.

Bacteriological Analytical Manual (BAM)

Laboratory Methods (Food)

Foods Program Compendium of Analytical Laboratory Methods

Other Analytical Methods of Interest to the Foods Program

Foods Program Methods Validation Processes and Guidelines

CFSAN Laboratory Quality Assurance Manual

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:

Content current as of:
02/21/2020

Regulated Product(s)
Food & Beverages

https://www.fda.gov/food/laboratory-methods-food/bacteriological-analytical-manual-bam

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. FRAZAR L. CARTER D.T. CHU (ret.)
19B	Detection of *Cyclospora cayetanensis* in Fresh Produce using Real-time PCR New 06/2017; Updated 10/2017	H.R. MURPHY S. ALMERIA A.J. da SILVA
26A	Detection and Quantitation of Hepatitis A Virus in Shellfish by the Polymerase Chain Reaction	B.B. GOSWAMI (ret.)
26B	Detection of Hepatitis A in Foods New 01/2014	J.W. WILLIAMS-WOODS G. HARTMAN W. BURKHARDT
29	*Cronobacter*	Y. CHEN, K. LAMPEL, T. HAMMACK
Methods for Microbial Toxins		
13B	Staphylococcal Enterotoxins Detection Methods New 06/2017	S. TALLENT R.W. BENNETT J.M. HAIT
15	*Bacillus cereus* Diarrheal Enterotoxin	R.W. BENNETT
Additional Methods		
20A	Inhibitory Substances in Milk	L.J. MATURIN (ret.)
20B	Rapid HPLC Determination of Sulfamethazine in Milk	J.D. WEBER (ret.) M.D. SMEDLEY
21A	Examination of Canned Foods	W.L. LANDRY, A.H. SCHWAB, G.A. LANCETTE (ret.)
21B	Modification of Headspace Gas Analysis Methodology, Using the SP4270 Integrator	W.L. LANDRY M.J. URIBE

https://www.fda.gov/food/laboratory-methods-food/bacteriological-analytical-manual-bam

BAM 19b: Molecular Detection of Cyclospora cayetanensis in Fresh Produce Using Real-Time PCR

Laboratory Methods (Food)

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CFSAN Laboratory Quality Assurance Manual

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

Revision History:

- November 2019: *Cyclospora* Romaine lettuce 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**: 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 v2.0 or 2.3 Method
6. **Appendix 6**: ABI 7500 Fast v1.4 Method

Disclaimer: This is a scientific presentation. Questions about compliance and enforcement will not be answered by the speaker s.

https://www.fda.gov/food/laboratory-methods-food/bacteriological-analytical-manual-bam

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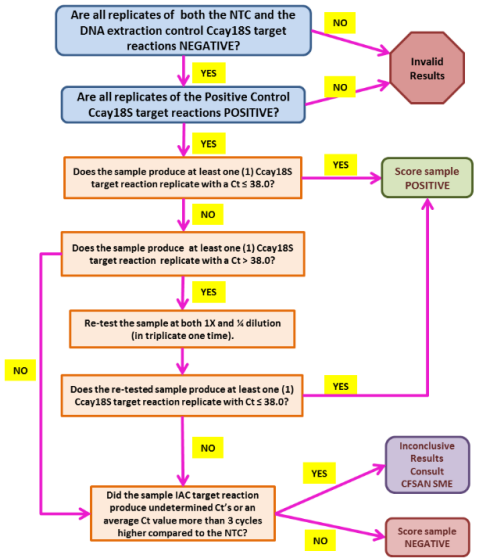
F. qPCR Data Analysis Flowchart::



U.S. FOOD & DRUG ADMINISTRATION

← Home / Food / Science & Research (Food) / Laboratory Methods (Food) / BAM 19b: Molecular Detection of Cyclospora cayetanensis in Fresh Produce Using Real-Time PCR

BAM 19b: Molecular Detection of Cyclospora cayetanensis in Fresh Produce Using Real-Time PCR

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Laboratory Methods (Food)

- Foods Program Compendium of Analytical Laboratory Methods
- Other Analytical Methods of Interest to the Foods Program
- Foods Program Methods Validation Processes and Guidelines
- CFSAN Laboratory Quality Assurance Manual

Authors: Helen R. Murphy, Sonia Almeria and Alexandre J. da Silva
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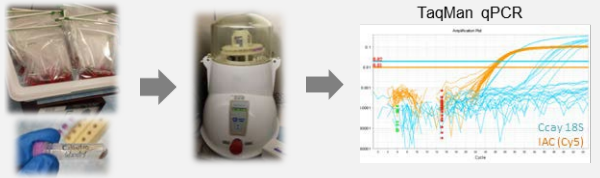
BAM CHAPTER 19B

Matrix	Seeding Level	Positive samples (80 tested)	% positive
cilantro	0	0	0.0%
	5	25	31.3%
	10	64	80.0%
	200	80	100.0%
raspberries	0	0	0.0%
	5	40	50.0%
	10	72	90.0%
	200	80	100.0%

Also approved for shredded carrots, and basil and parsley

Detection Limit is 5 oocysts



Food Microbiology 69 (2018) 170e178

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

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

Helen R. Murphy [a, *], Hediye Nese Cinar [a], Gopal Gopinath [a], Kathy E. Noe [b], Lacresha D. Chatman [b], Nancy E. Miranda [b], June H. Wetherington [c], Jason Neal-McKinney [c, f], Gabrielle S. Pires [c], Elizabeth Sachs [c], Kristopher J. Stanya [c], Cynthia L. Johnson [c], Fernanda S. Nascimento [d], Monica Santin [e], Aleksey Molokin [e], Mansour Samadpour [f], Harish Janagama [f], Amy Kahler [g], Candace Miller [g], Alexandre J. da Silva [a]

[a] U.S. Food and Drug Administration, Center for Food Safety and Applied Nutrition, Office of Applied Research and Safety Assessment, Division of Food and Environmental Microbiology, Laurel, MD 20708, USA
[b] U.S. Food and Drug Administration, Southeast Food and Feed Laboratory, Atlanta, GA 30309, USA
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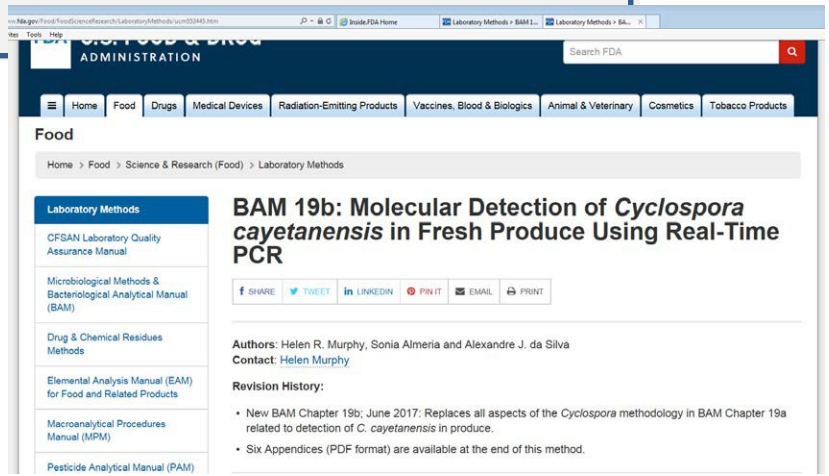
[d] Centers for Disease Control and Prevention, Center for Global Health, Division of Parasitic Diseases and Malaria, Parasitic Diseases Branch, Reference Diagnostic Laboratory, Atlanta, GA 30329, USA
[e] U.S. Department of Agriculture, Agricultural Research Service, Environmental Microbial and Food Safety Lab, Beltsville, MD 20705, USA
[f] IEH Laboratories & Consulting Group, Lake Forest Park, Washington 98155, USA
[g] Centers for Disease Control and Prevention, Division of Foodborne, Waterborne, and Environmental Diseases, Waterborne Disease Prevention Branch, Atlanta, GA 30329, USA



US Cyclosporiasis Outbreaks - 2018

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.

250 laboratory-confirmed cases from 4 states linked to consumption of Fresh Produce vegetable trays containing broccoli, cauliflower, carrots, and dill dip.

"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**."

https://www.cdc.gov/parasites/cyclosporiasis/outbreaks/2018/b-071318/index.html

Disclaimer: This is a scientific presentation. Questions about compliance and enforcement will not be answered by the speaker s.

US Cyclosporiasis Outbreaks - 2018

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News & Events

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FDA Statement

Statement from FDA Commissioner Scott Gottlieb, M.D., on the FDA's ongoing efforts to prevent foodborne outbreaks of *Cyclospora*

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For Immediate Release September 18, 2018

Inquiries

Media

Peter Cassell
240-402-6537

Consumers

888-INFO-FDA

"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."

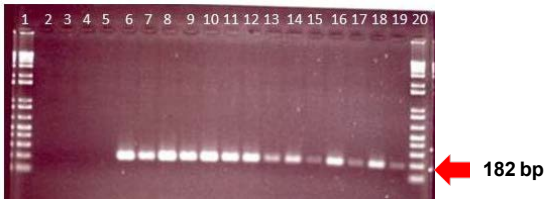
"During our investigation, two samples of domestically grown romaine lettuce were also found to be positive for *Cyclospora* even though they were not sourced from locations associated with the lettuce that was linked to this outbreak. None of the romaine lettuce associated with these positive test results 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."

Preliminary application of mitochondrial markers in 2018 positive samples

"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**."
https://www.cdc.gov/parasites/cyclosporiasis/outbreaks/2018/b-071318/index.html

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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https://www.fda.gov/food/sampling-protect-food-supply/microbiological-surveillance-sampling-fy18-19-fresh-herbs-cilantro-basil-parsley-and-processed

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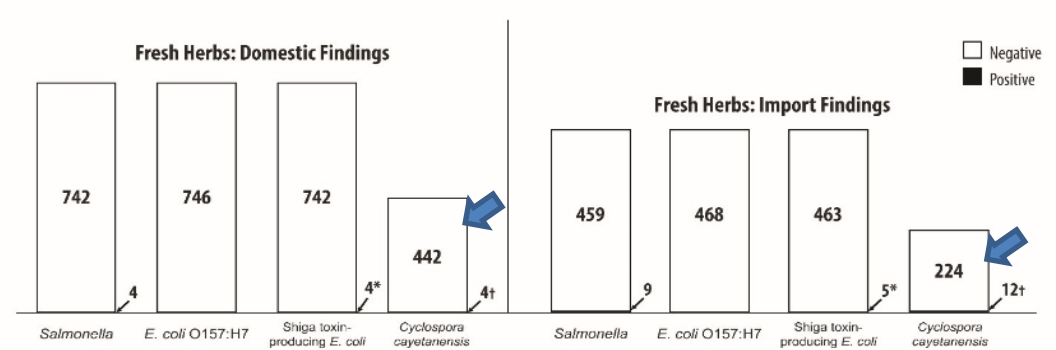
FDA Surveillance to Estimate the Prevalence of *Cyclospora cayetanensis* in Fresh Produce (Using the BAM Chapter 19B method)

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.



* Upon further review, the FDA determined these bacteria did not have the potential to cause severe illness. Analysis showed the bacteria did not possess any of the known characteristics that would enable them to adhere to intestinal epithelium (i.e. the cells in the luminal portion of the intestines), which is essential for severe infections.

† The number of samples tested for Cyclospora is smaller than the numbers tested for the other target pathogens because the FDA began testing for Cyclospora later (i.e., in the summer) when Cyclospora-related illnesses typically occur.

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

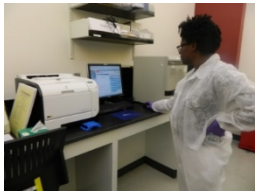
- FADL-Food Analysis and Diagnostic Laboratory, Fort Sam Houston, TX

FERN Labs

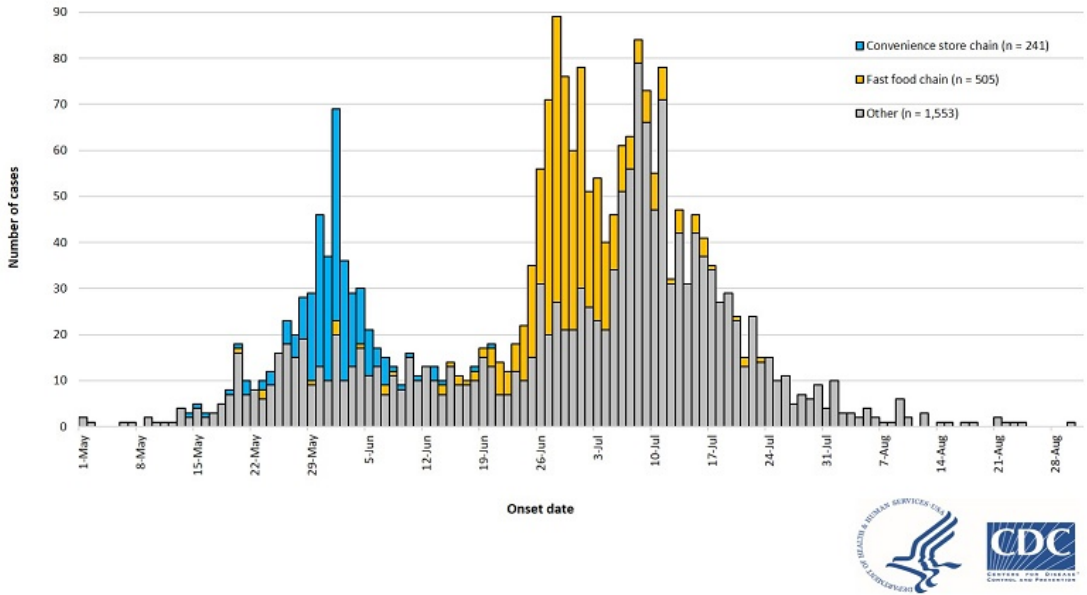
- Minnesota Department of Agriculture, Laboratory Services Division
- North Carolina Dept. of Agriculture & Consumer Services
- California Department of Public Health
- Michigan Department of Agriculture and Rural Development
- Washington State Department of Agriculture
- Maryland Department of Health 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.

US Cyclosporiasis Outbreaks - 2018

511 laboratory-confirmed cases

Approximately 14 cases

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

250 laboratory-confirmed

Molecular Epidemiology

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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Genomics and Molecular Epidemiology

A database to consolidate *C. cayetanensis* genome sequences was registered in 2016 named *Cyclospora cayetanensis* Genome Trakr (CycloTrakr)

https://www.ncbi.nlm.nih.gov/bioproject/357477

Display Settings: Send to:

Cyclospora cayetanensis Accession: PRJNA357477 ID: 357477

CycloTrakr (Cyclospora cayetanensis GenomeTrakr)

This is the UMBRELLA BIOPROJECT for datasets from Cyclospora cayetanensis and related apicomplexan parasites from food, clinical and environmental samples applicable for molecular epidemiology

Accession	PRJNA357477
Type	Umbrella project
Publications	1. Cinar HN *et al.*, "Comparative sequence analysis of Cyclospora cayetanensis apicoplast genomes originating from diverse geographical regions.", *Parasit Vectors*, 2016 Nov 29;9(1):611 2. Cinar HN *et al.*, "The Complete Mitochondrial Genome of the Foodborne Parasitic Pathogen Cyclospora cayetanensis.", *PLoS One*, 2015;10(6):e0128645
Submission	Registration date: 14-Dec-2016 **CFSAN**
Relevance	Food safety

NAVIGATE UP

This project is a component of the GenomeTrakr umbrella project

NAVIGATE ACROSS

6 additional projects are components of the GenomeTrakr umbrella project.

Related information

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BioProject
BioSample
Data projects
Full text in PMC
PubMed
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Recent activity

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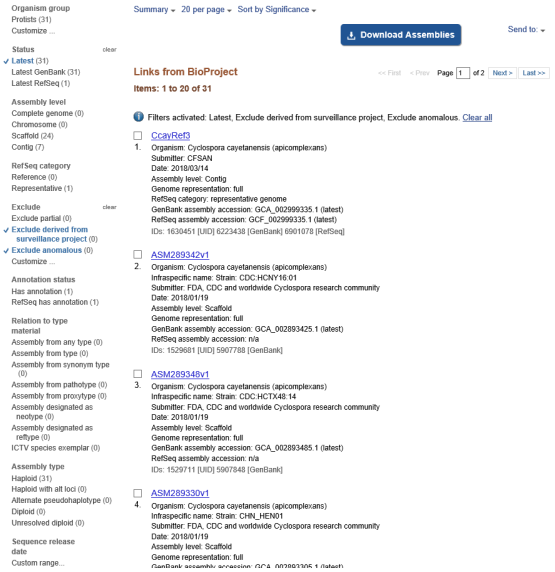
Cyclospora cayetanensis BioProject

cyclospora cayetanensis bioproject (3) BioProject

See more...

Project Data:

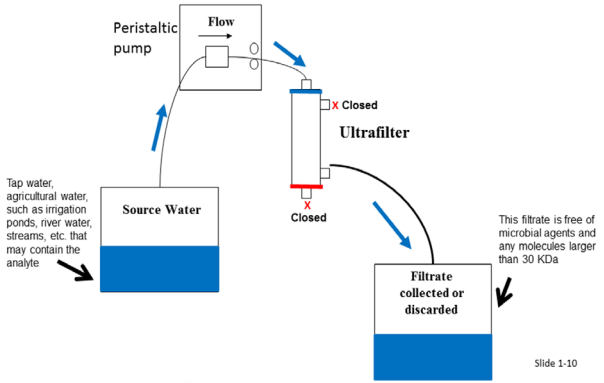
Resource Name	Number of Links
SEQUENCE DATA	
Nucleotide (total)	6631
WGS master	31
Genomic DNA	40
Transcript	5822
SRA Experiments	36
Protein Sequences	6220
PUBLICATIONS	
PubMed	5
PMC	5
OTHER DATASETS	
BioSample	73
Assembly	31



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.

From Bench to Field

- 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



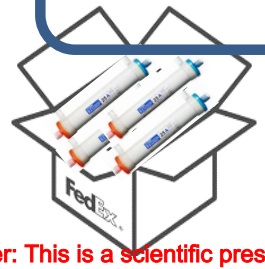
MLV- Methodology and Lab Workflow

Moffett Center

Collect agricultural water

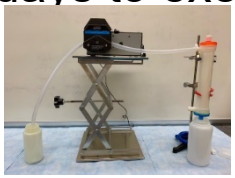
Seed water samples

Filter by DEUF



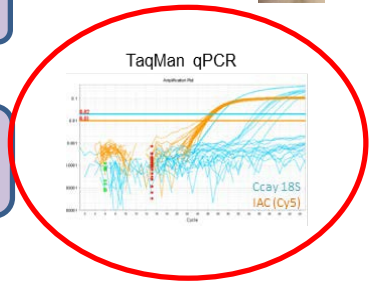
Participating Laboratories (5 days to execute)

Backflush filters



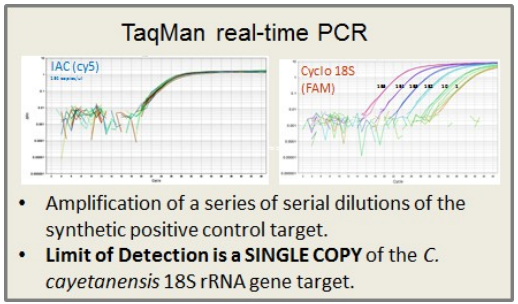
Concentration

DNA Extraction and TaqMan qPCR

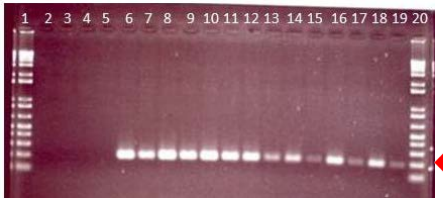


The MLV study is a validation of the process which begins at the backflush step.

Molecular Analysis- PCR and DNA sequencing



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



Wells 6 to 19= water seeded with10,000 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

Disclaimer: This is a scientific presentation. Questions about compliance and enforcement will not be answered by the speaker s.

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
- Ascertain 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

Thank you

FDA U.S. FOOD & DRUG ADMINISTRATION

CENTER FOR FOOD SAFETY & APPLIED NUTRITION

FDA Parasitology Research Team:

Helen Murphy
Hediye Cinar
Gopal Gopinath
Sonia Almeria
Mauricio Durigan
Laura Ewing
Emma Patregnani
Joyce Njoroge
&
Alex da Silva

Validation of *Cyclospora cayetanensis* detection method for regulatory use in different produce types

Association of Food and Drug Officials

Webinar

May 15th 2020

Sonia Almería, DVM, PhD
Research Microbiologist
FDA/CFSAN/OARSA/DVA
Laurel, MD
maria.almeria@fda.hhs.gov

Validation of molecular detection of *C. cayetanensis* BAM Chapter 19b method

Guidelines for the Validation of Analytical Methods for the Detection of Microbial Pathogens in Foods and Feeds

Edition 3.0

U.S. Food and Drug Administration Foods Program

October 2019

- **Regulatory Science Steering Committee**
- **Microbiology Methods Validation Subcommittee (MMVS)**

https://www.fda.gov/media/83812/download

Non-culturable organisms: protozoa, RNA virus

Guidelines for the Validation of Analytical Methods for the Detection of Microbial Pathogens in Foods and Feeds, Edition 3.0

Table 2 - General Guidelines for the Validation of Qualitative Detection Methods for Microbial Analytes - Unique Isolation and/or Enrichment Challenges [a]

Criteria	Single Laboratory Validation Study	Independent Laboratory Validation Study	Multi-Laboratory Validation Study
Participating Laboratory	Originating Laboratory	Collaborator	Collaborators
# of Target Organism (Inclusivity)[b]	[c]TBD	[d]NA	NA
# of Non-Target Organism (Exclusivity)[e]	TBD	NA	NA
# of Collaborators Providing Usable Data[f]	NA	1	Minimum of 5[g]
# of Foods	1 or more[g]	1 or more[g]	1 or more[g]
# of Analyte Levels/Food Matrix	3 levels: Minimum of two inoculated levels (one fractional[h] and one1 log higher[i]) and one uninoculated level	3 levels: Minimum of two inoculated levels (one fractional and one1 log higher) and one uninoculated level	3 levels: Minimum of two inoculated levels (one fractional and one 1 log higher) and one uninoculated level
Replicates per Food at Each Level Tested	≥3[j]	≥3[j]	≤8[j]
Aging of Inoculated Samples Prior to Testing [k]	Yes	Yes	Yes
Addition of Competitor Strain[l]	In 1 food at +1 log>analyte at fractional positive analyte level	In 1 food at +1 log>analyte at fractional positive analyte level	In 1 food at +1 log>analyte at fractional positive analyte level
BAM Reference Method Comparison Requirement[m]	Yes, if available	Yes, if available	Yes, if available

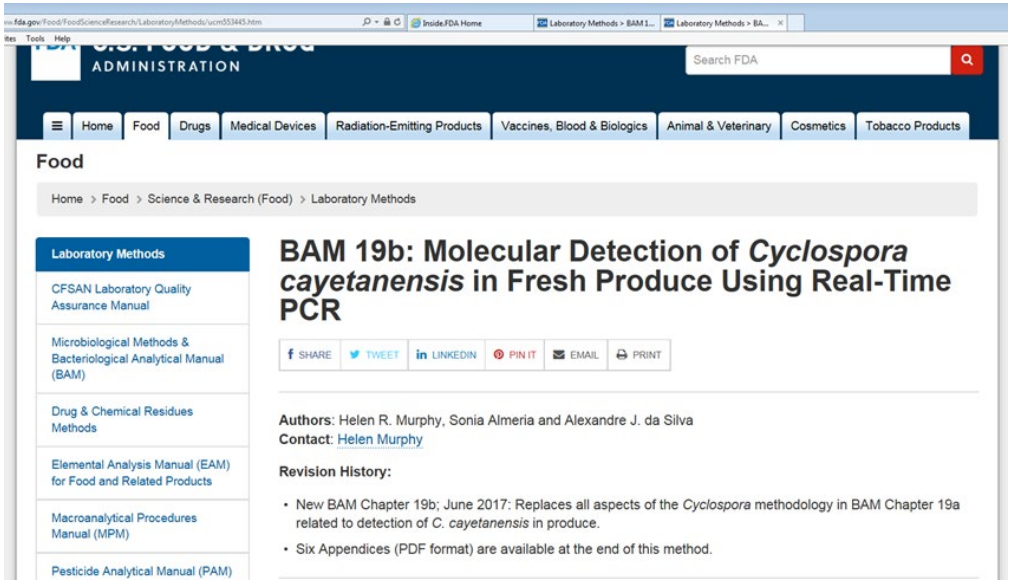
*Analysts should consult with MMVS to determine appropriate statistics before initiating study.

Challenges on detection of *Cyclospora* in produce

- Absence of culture methods (animal models and *in vitro* cell culture) to grow the parasite
- Oocysts from clinical samples are the only source for scientific research
- Small number of oocysts detected in food samples compared to clinical samples
- Detection is challenging but molecular methods are available

- **Recovery (washing) of oocysts from fruits and vegetables is very important**
- **Method development for detection in fresh produce and other foods:**
  - Implies need of multiple replicate tests
  - Different concentrations of the parasite spiked into a range of different matrices
  - Interlaboratory ring test trials
- **Materials and scope of testing is still limited** (genotyping, no viability test to date).

In 2016 the U.S. FDA validated a method for the detection of *C. cayetanensis* in cilantro and raspberries based on SLV and MLV; published in the FDA Bacteriological Analytical Manual (BAM Chapter 19b)



https://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm553445.htm

Imported raspberries from Guatemala

1996: 1465 cases in Canada and 20 states in the U.S.

1997: 1012 cases In Canada and 14 states in the U.S.

2000- Wedding cake-raspberries positive *C. cayetanensis* DNA

Cilantro:

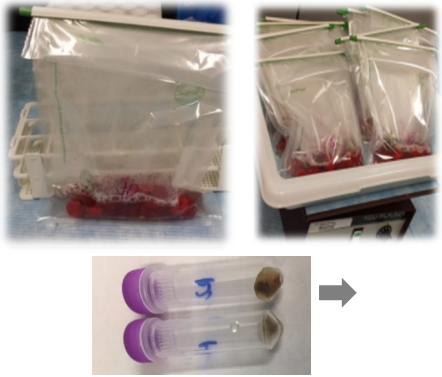
2013: 631 cases of cyclosporiasis in 25 states.

2015: 546 cases of cyclosporiasis in 31 states.

2018: 53 cases

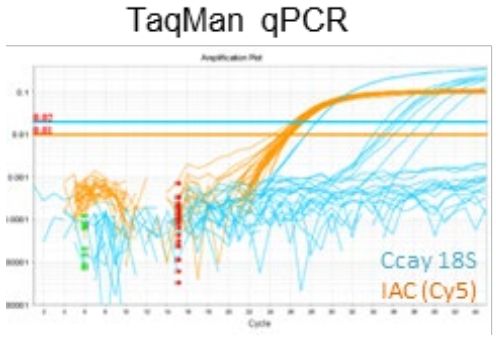
BAM Chapter 19B method protocol

1. **Wash procedure to recover *C. cayetanensis* oocysts**
2. **DNA Extraction from concentrated material**
3. **Identification to the species level by real time PCR amplification**



1

2



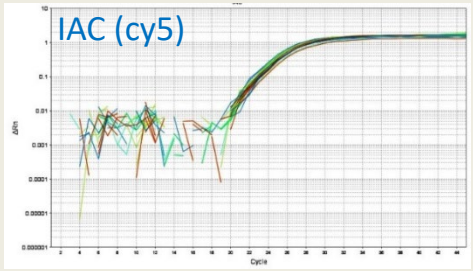
3

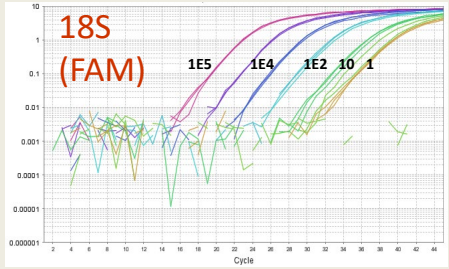
https://www.fda.gov/Food/FoodScienceResearch/LaboatoryMethods/ucm553445.htm

3. Molecular Detection using TaqMan® Real-time PCR

Duplex Reaction

1. Target the *C. cayetanensis 18 S* rRNA gene (Verweij et al., 2003)
2. Internal Amplification Control (IAC)





An internal amplification control (IAC) to monitor for potential matrix derived inhibition of the reaction

Standard curve generated using:

Traceable Synthetic Positive Control

- Amplicon sequencing
- Rule out false positives

Research Paper

Evaluation of an Improved U.S. Food and Drug Administration Method for the Detection of *Cyclospora cayetanensis* in Produce Using Real-Time PCR

HELEN R. MURPHY,* SEULGI LEE,† AND ALEXANDRE J. DA SILVA

Journal of Food Protection, Vol. 80, No. 7, 2017, Pages 1133–1144
doi:10.4315/0362-028X.JFP-16-492

Food Microbiology
journal homepage: www.elsevier.com/locate/fm

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

Helen R. Murphy [a, *], Hediye Nese Cinar [a], Gopal Gopinath [a], Kathy E. Noe [b], Lacresha D. Chatman [b], Nancy E. Miranda [b], June H. Wetherington [c], Jason Neal-McKinney [c, f], Gabrielle S. Pires [c], Elizabeth Sachs [c], Kristopher J. Stanya [c], Cynthia L. Johnson [c], Fernanda S. Nascimento [d], Monica Santin [e], Aleksey Molokin [e], Mansour Samadpour [f], Harish Janagama [f], Amy Kahler [g], Candace Miller [g], Alexandre J. da Silva [a]

Multilaboratory validation (MLV)

Table 2
Comparison of the collaborative study detection rates using nPCR and qPCR for analysis of cilantro and raspberry samples inoculated with *C. cayetanensis* oocysts.

Matrix	No. oocysts inoculated	No. samples analyzed[d]	Detection rate[a] (%)			False-positive rate[b] (%)			Fisher's exact test *P* values[c]		
			nPCR	qPCR A	qPCR B	nPCR	qPCR A	qPCR B	nPCR vs qPCR A	nPCR vs qPCR B	qPCR A vs qPCR B
Cilantro	0	39	–	–	–	2.6 (0.0, 11.5)	0	0	1.0000	1.0000	1.0000
	5	40	22.5 (12.1, 37.7)	30.0 (18.0, 45.5)	32.5 (20.0, 48.1)	–	–	–	0.6120	0.4531	1.0000
	10	40	92.5 (79.4, 98.1)	72.5 (57.0, 84.0)	87.5 (73.4, 95.0)	–	–	–	0.0367[e]	0.7119	0.1600
	200	40	100.0 (89.6, 100)	100.0 (89.6, 100)	100.0 (89.6, 100)	–	–	–	1.0000	1.0000	1.0000
Raspberries	0	40	–	–	–	5.0 (0.5, 17.4)	0	0	0.4937	0.4937	1.0000
	5	40	67.5 (51.9, 80.0)	55.5 (39.8, 69.3)	45.0 (30.7, 60.2)	–	–	–	0.3588	0.0707	0.5026
	10	40	97.5 (86.0, 100)	92.5 (79.4, 98.1)	87.5 (73.4, 95.0)	–	–	–	0.6153	0.2007	0.7119
	200	40	100.0 (89.6, 100)	100.0 (89.6, 100)	100.0 (89.6, 100)	–	–	–	1.0000	1.0000	1.0000

Seeding Level

Detection Rates

— Not applicable.
[a] Percentage of inoculated samples which gave a positive result by nPCR, qPCR analysis A (qPCR A), and qPCR analysis B (qPCR B). Different analysts at each participating laboratory performed qPCR A and qPCR B. Numbers in parentheses are the lower and upper 95% confidence intervals of detection rates.
[b] Percentage of uninoculated samples which gave a positive result with 95% confidence intervals in parentheses.
[c] *P* values calculated using the Fisher's exact test to identify statistical differences in results obtained between nPCR, and qPCR analysis A, and qPCR analysis B. $P \geq 0.05$ is not considered significant.
[d] A total of 40 produce samples were prepared by the originating laboratory at each level for each matrix. One uninoculated cilantro sample was excluded from the study due to a technical error which occurred during the washing procedure.
[e] Statistically significant difference between the two methods.

Multilaboratory validation (MLV) in cilantro and raspberries

Collaborating laboratories: 5

Samples analyzed per replicate: 8; 2 analysts/lab

MLV Validation Results

Seeded samples with known numbers of oocysts submitted to the collaborating laboratories

Matrix	Seeding Level	Positive samples (80 tested)	% positives
cilantro	0	0	0.0%
	5	25	31.3%
	10	64	80.0%
	200	80	100.0%
raspberries	0	0	0.0%
	5	40	50.0%
	10	72	90.0%
	200	80	100.0%

Detection Limit is 5 oocysts

https://www.fda.gov/Food/FoodScienceResearch/LaboatoryMethods/ucm553445.htm

Matrix Extensions

"FDA field microbiology labs analyze a huge variety of food matrices. Even so, methods used in FDA field laboratories for regulatory purposes must be evaluated for *each* food"

Matrix extensions only if no additional modifications to the method have been made

- Method to test a food (or foods) from the same category included in the original validation study
  - Lettuce to spinach

- If the food (or foods) is not within the same category of food included in the original validation study an independent validation study will be required. Shredded carrots

Cyclospora Matrix Extension in carrots

USA

- **2016.** A restaurant-associated sub-cluster of Cyclosporiasis in Texas was epidemiologically **linked to consumption of coleslaw containing shredded carrots** and cabbage.
- Investigations in Iowa and Nebraska indicated that some illnesses within those states may have been caused by a **contaminated salad mix**, containing several types of lettuce, red and green cabbage and **carrots**.
- **2018**.
  - Vegetable trays with cauliflower, broccoli and **carrots**.
  - romaine/**carrots** mix salad

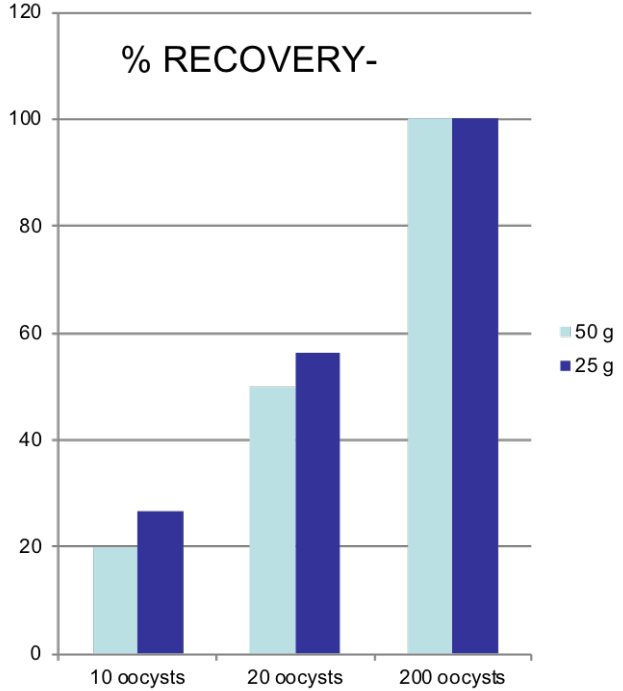
MMVS recommendations for ME:

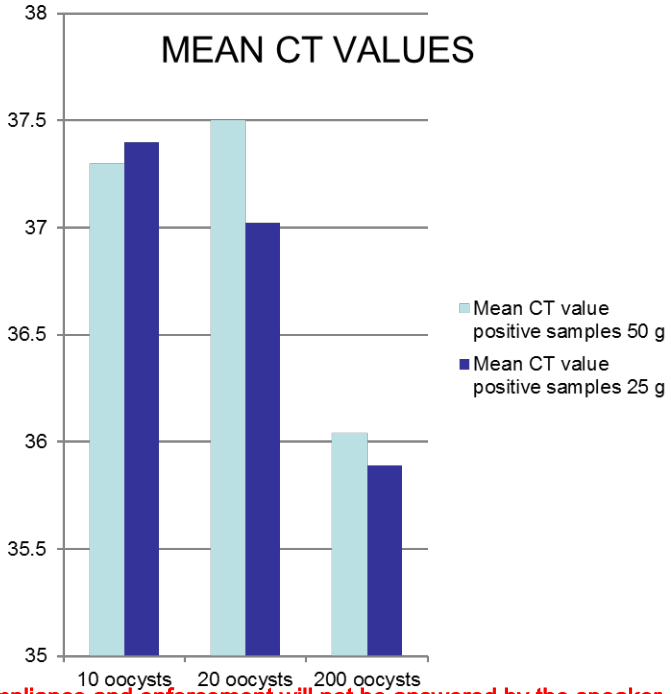
- Ten replicates needed to be tested at the fractional level (25-75% positive samples tested).
- FDA's *C. cayetanensis* BAM method protocol to recover oocysts from produce

Matrix extensions in high-risk fresh produce: CARROTS

SAMPLE SIZE (G) SELECTION

Carrots: 25 gr versus 50 gr seeded samples





# oocysts	18S No. positive qPCR reactions (out of 3 replicates)	18 S C_T value	IAC C_T value*
0	0	Und	25.2±0.1
0	0	Und	24.7±0.2
0	0	Und	25.1±0.1
0	0	Und	25.0±0.2
0	0	Und	25.0±0.0
0	0	Und	24.8±0.2
0	0	Und	25.0±0.1
0	0	Und	25.2±0.0
5	2	36.3±1.3	24.2±0.1
5	2	37.0±1.1	25.8±0.1
5	2	36.9±0.5	26.1±0.2
5	1	35.5	25.9±0.3
5	0	Und	25.7±0.1
5	2	37.2±1.1	25.8±0.1
5	0	Und	25.9±0.1
5	0	Und	25.7±0.1
5	0	Und	25.3±0.2
5	0	Und	25.1±0.1
10	3	35.8±0.04	25.9±0.1
10	2	37.2±0.02	25.8±0.1
10	0	Und	25.7±0.3
10	3	36.1±1.23	24.5±0.05
10	3	35.1±0.2	24.5±0.05
10	0	Und	24.6±0.1
10	3	35.7±0.07	24.3±0.0
10	3	37.0±0.6	24.1±0.2
10	3	35.5±0.7	24.3±0.1
10	0	39.5**	24.2±0.1
20	2	37.0±0.8	25.4±0.0
20	1	37.7	25.6±0.2
20	3	36.5±0.8	25.5±0.1
20	3	37.4±0.4	24.2±0.2
20	3	35.8±0.6	24.4±0.4
20	3	35.8±0.6	24.4±0.0
20	3	35.3±0.2	24.2±0.1
20	3	36.3±0.6	24.0±0.1
20	1	36.3	24.2±0.1
20	3	34.1±0.2	24.2±0.1
200	3	31.5±0.2	25.5±0.1
200	3	32.2±0.4	25.8±0.6
200	3	33.3±0.3	25.4±0.0
200	3	32.1±0.2	24.2±0.1
200	3	31.9±0.2	24.2±0.0
200	3	32.8±0.8	24.1±0.2
200	3	32.7±0.9	24.2±0.2
200	3	33.3±0.4	24.1±0.1

CARROTS-MATRIX EXTENSION

Matrix (Indonesia)	Oocysts seeded	No. of Samples tested	No. of samples positive by qPCR:	
Shredded Carrots (25 grams)	0	8	0	0%
	5	10	5	50.0%
	10	10	7	70.0%
	20	10	10	100.0%
	200	8	8	100.0%

Disclaimer: This is a scientific presentation. Questions about compliance and enforcement will not be answered by the speakers.

Matrix extension in carrots versus MLV results

Matrix (Indonesia)	Oocysts seeded	No. of Samples tested	No. of samples positive by qPCR:	
Shredded Carrots (25 grams)	0	8	0	0%
	5	10	5	50.0%
	10	10	7	70.0%
	20	10	10	100.0%
	200	8	8	100.0%

Matrix	Seeding Level	Positive samples (80 tested)	% positives
cilantro	0	0	0.0%
	5	25	31.3%
	10	64	80.0%
	200	80	100.0%
raspberries	0	0	0.0%
	5	40	50.0%
	10	72	90.0%
	200	80	100.0%

Technique approved for regulatory use on carrots by MMVS in February 2017

Disclaimer: This is a scientific presentation. Questions about compliance and enforcement will not be answered by the speakers.

FRESH HERBS MATRIX EXTENSIONS: BASIL AND PARSLEY

The FDA planned a surveillance sampling for 2018 to determine the prevalence of *C. cayetanensis, Salmonella* and *E. coli* in cilantro, basil and parsley.


U.S. FOOD & DRUG ADMINISTRATION

Home / Food / Compliance & Enforcement (Food) / Sampling to Protect the Food Supply / Microbiological Surveillance Sampling: FY18-19 Fresh Herbs (Cilantro, Basil & Parsley) and Processed Avocado and Guacamole Assignments

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

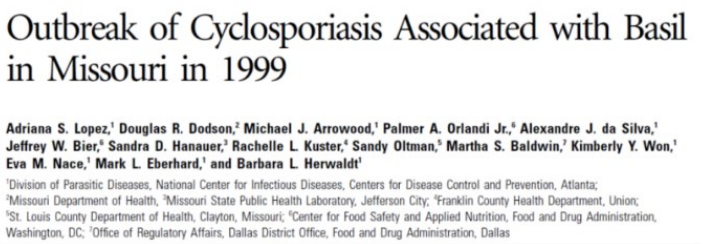
MAJOR ARTICLE

BASIL:

1999. Outbreak in Missouri. Leftovers of chicken pasta salad with basil. Microscopy and PCR positive.

2019: 241 cases in 11 states

PARSLEY: Not linked to *C. cayetanensis* outbreaks in USA


Outbreak of Cyclosporiasis Associated with Basil in Missouri in 1999

Adriana S. Lopez,[1] Douglas R. Dodson,[2] Michael J. Arrowood,[1] Palmer A. Orlandi Jr.,[6] Alexandre J. da Silva,[1] Jeffrey W. Bier,[4] Sandra D. Hanauer,[3] Rachelle L. Kuster,[4] Sandy Oltman,[5] Martha S. Baldwin,[7] Kimberly Y. Won,[1] Eva M. Nace,[1] Mark L. Eberhard,[1] and Barbara L. Herwaldt[1]

[1]Division of Parasitic Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta; [2]Missouri Department of Health, [3]Missouri State Public Health Laboratory, Jefferson City; [4]Franklin County Health Department, Union; [5]St. Louis County Department of Health, Clayton, Missouri; [6]Center for Food Safety and Applied Nutrition, Food and Drug Administration, Washington, DC; [7]Office of Regulatory Affairs, Dallas District Office, Food and Drug Administration, Dallas

	18S No. positive qPCR reactions (out of 3 replicates)	18 S C_T value	IAC C_T value*
0	0	Und	26.1±0.2
0	0	Und	25.0±0.2
0	0	Und	25.4±0.1
0	0	Und	25.2±0.1
0	0	Und	25.3±0.1
0	0	Und	25.0±0.2
0	0	Und	25.5±0.1
0	0	Und	24.7±0.2
5	3	36.0±1.2	25.9±0.4
5	1	37.4	26.0±0.3
5	0	Und	25.8±0.1
5	3	36.2±1.3	25.6±0.2
5	0	39.2**	25.5±0.8
5	3	35.6±0.6	25.8±0.1
5	2	37.04±0.7	25.2±0.2
5	2	35.4±0.1	25.2±0.2
5	2	35.9±0.6	25.6±0.6
5	0	Und	25.6±0.9
10	3	36.1±0.6	25.8±0.1
10	3	32.9±1.9	25.6±0.2
10	3	35.0±0.8	25.6±0.2
10	3	34.1±0.2	25.4±0.2
10	3	35.9±1.3	25.1±0.3
10	3	35.2±0.2	26.4±0.4
10	3	36.13±1.2	25.8±0.9
10	3	35.6±0.7	26.4±0.8
10	3	35.1±0.4	25.4±0.1
10	3	34.1±0.5	25.5±0.2
200	3	30.7±0.1	25.4±0.2
200	3	30.8±0.0	25.9±0.3
200	3	30.9±0.1	25.7±0.2
200	3	30.5±0.2	27.1±0.2
200	3	32.7±0.2	27.2±0.5
200	3	30.0±0.1	24.9±0.1
200	3	32.0±0.4	26.2±0.1
200	3	31.0±0.12	26.2±0.2
200	3	30.1±0.1	25.8±1.1
200	3	35.1±0.5	24.7±0.3

Basil-Matrix extension

Matrix	Oocysts seeded	No. of Samples tested	No. of samples positive by qPCR:	
Basil (25 grams)	0	8	0	0%
	5	10	7	70.0%
	10	10	10	100.0%
	200	10	10	100.0%

Technique approved for regulatory use on basil by MMVS in June 2017

Disclaimer: This is a scientific presentation. Questions about compliance and enforcement will not be answered by the speakers.

Parsley-Matrix extension

Matrix	Oocysts seeded	No. of Samples tested	No. of samples positive by qPCR:	
Parsley (25 grams)	0	8	0	0%
	5	10	8	80.0%
	10	10	9	90.0%
	200	10	10	100.0%

Technique approved for regulatory use on parsley in June 2017

# oocysts	18S No. positive qPCR reactions (out of 3 replicates)	18 S C_T value	IAC C_T value*
0	0	Und	24.8±0.15
0	0	Und	25.3±0.2
0	0	Und	24.5±0.04
0	0	Und	24.8±0.1
0	0	Und	24.7±0.1
0	0	Und	24.7±0.1
0	0	Und	24.7±0.3
0	0	Und	24.4±0.03
5	3	37.3±1.3	25.4±0.2
5	3	36.6±0.7	25.9±0.2
5	2	37.0±0.7	25.3±0.1
5	3	36.6±1.7	25.1±0.2
5	2	35.8±1.1	24.8±0.2
5	2	37.3±0.2	25.4±0.2
5	3	36.9±1.3	25.0±0.05
5	0	Und	25.6±0.3
5	0	Und	25.4±0.2
5	1	37.5	26.5±0.1
10	3	33.9±0.5	25.3±0.02
10	3	35.8±0.9	25.1±0.03
10	3	34.8±0.2	24.7±0.1
10	3	34.3±0.4	25.1±0.3
10	3	36.7±0.9	24.7±0.15
10	3	35.2.3	24.7±0.3
10	1	37.0	24.6±0.4
10	3	34.5±0.1	25.2±0.1
10	0	Und	25.0±0.15
10	3	35.5±1.6	25.3±0.2
200	3	30.9±0.1	25.1±0.3
200	3	30.4±0.1	25.0±0.2
200	3	30.7±0.4	24.6±0.2
200	3	30.3±0.1	24.5±0.1
200	3	30.4±0.2	24.6±0.2
200	3	30.3±0.3	24.8±0.2
200	3	30.1±0.2	24.5±0.02
200	3	30.3±0.2	24.5±0.2
200	3	31.0±1.5	24.1±0.3

Disclaimer: This is a scientific presentation. Questions about compliance and enforcement will not be answered by the speakers.

Bagged pre-cut romaine lettuce salad Matrix Extension Study

Regulatory positive sample in July 2018 in unused romaine/carrot mix (FDA ORA lab)

Matrix	Oocysts seeded	No. of Samples tested	No. of samples positive by qPCR:	
Bagged pre-cut romaine Lettuce (25 grams)	0	8	0	0%
	5	10	8	80.0%
	200	8	8	100.0%

Mixed bagged salads

Lettuce and mesclun salad mix:

1997: USA, Florida
2002: Germany
2013: USA (2 states) 161 cases
2018: USA (16 states) 511 lab confirmed cases linked to a fast food restaurant chain.
2019: clusters of illness (no number)

Technique approved for regulatory use on romaine lettuce in 2018

DISPATCHES

Cyclosporiasis Outbreak in Germany Associated with the Consumption of Salad

Peter C. Döller,* Karl Dietrich,† Norbert Filipp,† Stefan Brockmann,‡ Caroline Dreweck,‡ Reinhard Vonthein,§ Christiane Wagner-Wiening,‡ and Albrecht Wiedenmann¶

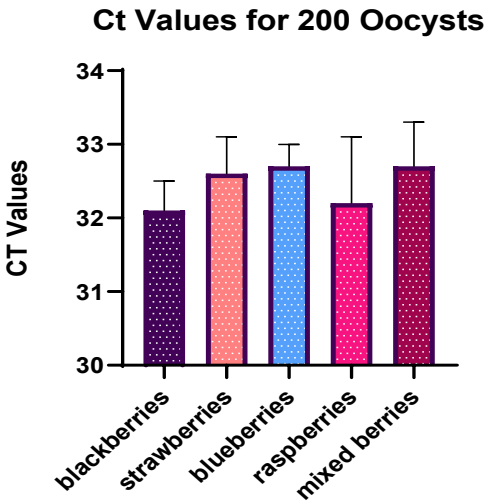
Disclaimer: This is a scientific presentation. Questions about compliance and enforcement will not be answered by the speakers.

Validation in different fresh berries

Matrix	No. oocysts inoculated	Mean C_T	Mean 18S rRNA (copies/reaction)	% recovery
Blackberries	5	36.9±0.7	2.8±1.5	30.6±28.5
	10	35.9±1.0	5.7±3.2	39.5±27.2
	200	32.1±0.4	65.5±20.4	25.1±7.8
Strawberries	5	37.0±0.7	2.3±1.2	25.0±23.2
	10	36.9±0.5	2.7±1.1	20.4±8.4
	200	32.6±0.5	52.41±21.9	20.1±8.4
Blueberries	5	37.0±0.5	2.8±1.0	34.0±22.1
	10	36.8±0.6	3.6±1.7	24.7±15.1
	200	32.7±0.3	52.1±13.22	20.0±5.1
Raspberries	5	37.1±0.7	3.0±2.0	20.3±17.6
	10	35.4±1.2	4.7±3.7	39.7±23.9
	200	32.2±0.9	56.0±32.8	21.5±12.6
Fresh mixed berries	5	37.6±0.5	1.4±0.4	19.9±9.3
	10	36.3±0.9	4.0±2.5	30.8±19.2
	200	32.7±0.6	49.7±19.2	19.1±7.3

Not significant differences in detection among berry types

Technique approved for regulatory use on blackberries by MMVS in February 2020



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Evaluation of the U.S. Food and Drug Administration validated molecular method for detection of *Cyclospora cayetanensis* oocysts on fresh and frozen berries

Angela Assurian[a], Helen Murphy[b], Laura Ewing[b], Hediye Nese Cinar[b], Alexandre da Silva[b], Sonia Almeria[b,*]

The new validated method is appropriate for regulatory detection of *Cyclospora cayetanensis* in:

- Leafy greens such as lettuces, cilantro, parsley and basil
- Soft fruit such as raspberries and blackberries
- Shredded carrots, cabbage. Whole vegetables such as beans or peas

Gathering these scientific data is important as FDA advances with the use of this method to estimate the prevalence of *C. cayetanensis* in different produce and to support outbreak investigations.

https://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm553445.htm

Method analysis in prepared dishes

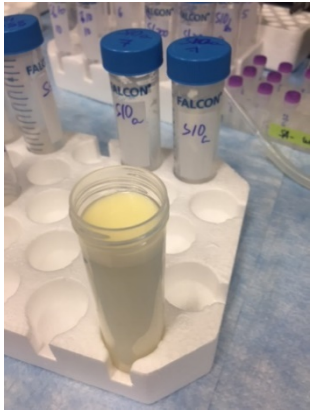
- *Cyclospora cayetanensis* outbreaks are frequently associated with fresh produce and prepared side dishes containing fresh produce and other ingredients.
  - The validated FDA method for detection of *C. cayetanensis* had not been evaluated for use in prepared side dishes with multiple ingredients.
- Methods that are optimized for one food category may not perform well in other food categories.

Examples:

- Coleslaw
- Mexican and Mediterranean dishes containing cilantro, basil and/or parsley

Method modification for detection of *Cyclospora cayetanensis* in coleslaw as a prepared dish

# oocysts	Coleslaw with dressing, washing in 0.1% Alconox®	Coleslaw with dressing, washing in 1% Alconox®
5	4 (0)	4 (2)
10	4 (1)	4 (4)
200	2 (2)	2 (2)



The use of a higher concentration of Alconox improves the recovery of oocysts in coleslaw with dressing

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ELSEVIER

Evaluation of the U.S. Food and Drug Administration validated method for detection of *Cyclospora cayetanensis* in high-risk fresh produce matrices and a method modification for a prepared dish

Sonia Almeria[a,*], Alexandre J. da Silva[a], Tyann Blessington[b], Tami Craig Cloyd[b], Hediye Nese Cinar[a], Mauricio Durigan[a], Helen R. Murphy[a]

Pico de gallo/salsa with cilantro: Method Modification in washing procedure

Matrix	Oocysts seeded	No. of Samples tested	No. of samples positive by qPCR (%)	
Pico gallo 25 grams	0	3	0	0%
	5	11	9	81.8%
	10	12	12	100%
	200	11	11	100.0%

The results highlight the importance of evaluating the performance characteristics of the BAM Chapter 19b method in different food matrices to support outbreak investigations.

Thank you for your attention

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