Detection of *Cyclospora cayetanensis* in mixed bagged pre-cut salads by the FDA Bacteriological Analytical Manual (BAM) Chapter 19b method

Sonia Almeria[1] and Alicia Shipley[2]

[1]Office of Applied Research and Safety Assessment, Center for Food Safety and Applied Nutrition, U.S. Food and Drug Administration, Laurel, MD 20708;

[2]JIFSAN, University of Maryland, College Park, MD, USA

Abstract

Numerous *Cyclospora cayetanensis* foodborne outbreaks affecting hundreds of people have occurred in the U.S. since the mid-1990s. Recently, outbreaks were linked to the consumption of salads containing romaine lettuce and carrots served in Midwest restaurants in 2018, and bagged salad mixes containing iceberg lettuce, carrots and red cabbage sold in stores in the Midwest in 2020. The FDA Bacteriological Analytical Manual (BAM) Chapter 19b method has been validated in carrots and romaine lettuce by matrix extension studies but has not been previously evaluated in mixed pre-cut salads containing these ingredients. In the present study, the BAM Chapter 19b method was evaluated in two ready-to-eat (RTE) mixed salads. Twenty-five-gram samples of pre-cut mixed salad 1 (containing romaine and iceberg lettuce, carrots and red cabbage) and mixed salad 2 (containing romaine and iceberg lettuce, carrots, red cabbage, radish and pea pods) were seeded with 5 and 200 *C. cayetanensis* oocysts. Unseeded produce was used as negative control. The method included washing of the produce, extraction of *C. cayetanensis* DNA, and molecular detection using a Taqman assay targeting the 18S rRNA gene with an internal amplification control (IAC). As few as five oocysts were detected in both mixed salads (n=10 in each type) with positive detection rates of 30% and 60%, respectively for mixed salad 1 and mixed salad 2. All unseeded salad samples were negative, and all salad samples seeded with 200 oocysts (n=7 in each type) were positive. Statistically significant differences were observed in 18S rRNA *C. cayetanensis* Ct values in samples seeded with 200 oocysts between both salads ($p<0.05$). The results showed that the method was robust, reproducible, and able to detect as few as 5 oocysts in RTE salads. The BAM Chapter 19b method should provide reliable results when used in regulatory testing in salads in advance for potential future outbreak investigations. The differences in detection among mixed salads highlights the importance of evaluating the performance of the *C. cayetanensis* detection method in different food matrices.

Introduction

Cyclospora cayetanensis is a protozoan parasite causing an intestinal illness in humans called cyclosporiasis. Human cyclosporiasis is a significant public health concern in the USA where large foodborne outbreaks and sporadic cases have occurred since the mid-1990s. These outbreaks have been frequently associated with consumption of imported fresh produce. Importantly, several cyclosporiasis outbreaks have been linked to consumption of lettuce and salad greens in the USA in recent years. Specifically, an outbreak took place in 2018 and the 511 laboratory-confirmed cases of *C. cayetanensis* (spanning 16 states) were reported from people who had consumed a variety of salads containing romaine lettuce and carrots from fast food restaurants in the Midwest (CDC, 2018). In a second large outbreak in 2020, a multistate outbreak in 14 states (701 laboratory-confirmed cases) was linked to consumption of commercial bagged salad mixes containing iceberg lettuce, carrots, and red cabbage (CDC, 2020). The FDA already has a validated method in the Bacteriological Analytical Manual (BAM) as Chapter 19b in order to detect *C. cayetanensis* in shredded carrots and some fresh herbs, such as cilantro and parsley. However, outbreak events indicate lettuce in salads as a high-risk produce for outbreaks of *C. cayetanensis*, emphasizing the need to extend and validate this method for such commodities. Thus, the present study investigated detection of *C. cayetanensis* oocysts in two types of bagged, mixed salads in advance of potential future outbreak investigations.

Materials and Methods

Two salad mixes were used in this study: pre-cut, mixed salad 1 (containing romaine and iceberg lettuces, carrots, and red cabbage) and pre-cut, mixed salad 2 (containing romaine and iceberg lettuces, carrots, red cabbage, radish, and pea pods).

Each salad mix was weighed into 25 g samples and then seeded with either 0 (used as a negative control), 5, or 200 *C. cayetanensis* oocysts by random spreading of about 10-20 droplets of stored oocyst dilutions across the sample surface with a micro pipet. Both salad mix 1 and 2 were processed by following the typical BAM Chapter 19b methodology, consisting of three main steps:

A) Washing of produce using a 0.1% Alconox detergent solution. Wash water was then concentrated by centrifugation to recover, pool, and concentrate the wash debris that would contain *C. cayetanensis* oocysts.

B) DNA was extracted from the wash pellets using the FastDNA Spin Kit for Soil in conjunction with a FastPrep-24 Instrument (MP Biomedicals, Santa Ana, California).

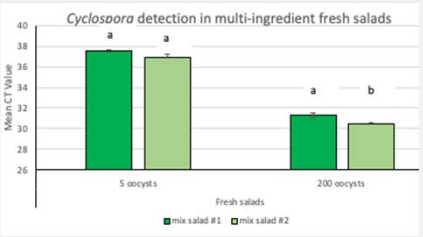
C) qPCR analysis (using the Qiagen QuantiFast Multiplex PCR Kit) in a dual Taqman™ method to target the *C. cayetanensis* 18SrRNA gene and amplification of an internal amplification control (IAC) included for detection of false negative results as well as to monitor for reaction failure stemming from food-matrix-derived PCR inhibitors. The cut off value (Ct value) was no greater than 38.0.

Statistically significant differences between Ct values in the salad mixes were analyzed by "t" test using GraphPad with $P \leq 0.05$ indicating statistical differences.

Results

Matrix	Oocysts seeded	No. of Samples tested	No. of samples positive by qPCR:		Meat Ct value
Mixed salad 1 (romaine and iceberg lettuces, carrots, and red cabbage) **(25 grams)**	5	10	3	30.0%	37.5±0.1
	200	7	7	100.0%	31.3±0.3

Matrix	Oocysts seeded	No. of Samples tested	No. of samples positive by qPCR:		Meat Ct value
Mixed salad 2 (romaine and iceberg lettuces, carrots, red cabbage, radishes, and pea pods) **(25 grams)**	5	10	6	60.0%	36.9±0.2
	200	7	7	100.0%	30.4±0.1



The BAM method allowed for detection of as few as 5 *C. cayetanensis* oocysts per 25g samples in both mixed salad sample types. All unseeded samples were negative (data not shown), and all samples seeded with 200 oocysts were positive. Mean Ct values were similar in both types of samples at the low seeding level ($p>0.05$), but significant differences were observed among both types of mixed salads seeded with 200 oocysts, with Ct values significantly lower (higher detection) in the mixed salad 2, which contained more ingredients. No inhibition was observed in any processed samples based on the internal amplification control (data not shown).

Conclusions

The BAM Chapter 19b protocol was robust and reproducible for detection of *C. cayetanensis* in both salad mixes 1 and 2, allowing for consistent data in the analysis of complex salads. As few as 5 oocysts was the limit of detection achieved in both salad types, which is the standard in the validated BAM Chapter 19b methodology in leafy greens and berries. Differences in detection among mixed salads highlights the importance of evaluating the performance of the *C. cayetanensis* detection method in different food matrices. Since lettuce and salad greens have become increasingly implicated in cyclosporiasis outbreaks, this study proves to be valuable in strengthening laboratory applications for detection of *Cyclospora cayetanensis* in preparation for future outbreak scenarios with these matrices.

FDA Mission Relevance

The objective of this project was to evaluate and validate a regulatory method, encompassing the recovery, detection, and quantification of *C. cayetanensis* oocysts from multiple ingredient pre-cut salads to provide the laboratory science for FDA's regulatory, policy, and compliance and enforcement programs. The reproducibility of this method could prove to be essential if used across multiple facilities throughout the country in outbreak scenarios.

References

BAM 19b: Murphy H. R., Almeria S. and da Silva A. J., 2017a. Molecular Detection of *Cyclospora cayetanensis* in Fresh Produce Using Real-Time PCR. https://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm553445.htm

CDC 2018. Centers for Disease Control. Available at https://www.cdc.gov/parasites/cyclosporiasis/epi.html

CDC, 2020. Centers for Disease Control. Available at https://www.cdc.gov/parasites/cyclosporiasis/outbreaks/2020/index.html