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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

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ABSTRACT

The performance of the U.S. Food and Drug Administration (FDA) validated method for regulatory detection of *Cyclospora cayetanensis* in leafy greens and berries was evaluated in additional high-risk fresh produce items and in a dish prepared with these produce commodities. The method was robust and reproducible in basil, parsley, shredded carrots, shredded cabbage and carrot mix, and could detect as few as 5 oocysts in 25 g samples. Some differences in *C. cayetanensis* detection were found among the fresh produce analyzed. Significantly lower target gene copy numbers per reaction were obtained with shredded carrots, and shredded cabbage and carrot mix compared to leafy greens, which highlights the importance of evaluating the performance characteristics of validated methods in different food matrices. In the prepared dish, coleslaw with dressing, the method was optimized to detect 5 oocysts in a 25 g sample by using 1.0% Alconox® in the washing solution instead of 0.1% as originally described. These data are important to assess the prevalence of *C. cayetanensis* in different produce items and to support outbreak investigations.

1. Introduction

Cyclospora cayetanensis is an intestinal protozoan parasite that causes a diarrheal illness in humans called cyclosporiasis. Symptoms of cyclosporiasis include explosive watery diarrhea, nausea, fatigue, increased gas, weight loss, bloating, and loss of appetite, with symptoms typically beginning an average of 7 days after infection (Herwaldt, 2000). Humans become infected with *C. cayetanensis* after consuming food or water contaminated with the parasite's sporulated oocysts. Infected humans shed non-sporulated oocysts, which require 7–15 days under ideal conditions (23–27 °C) in the environment, to sporulate and become infective (Ortega and Sanchez, 2010).

Cyclosporiasis is an emerging infectious disease in developing and developed countries such as the U.S. and Canada (Dixon, 2016). Global trade of foods may play a significant role in the transmission of *C. cayetanensis* in the U.S., considering that some of the outbreak cases have been traced back to fresh produce imported from developing regions. The food items implicated in these outbreaks included dishes prepared with fresh produce, such as basil, snow peas, berries, cilantro and bagged mixed greens (Abanyie et al., 2015; CDC, 2016; Dixon, 2016; Hall et al., 2012; Herwaldt, 2000; Ho et al., 2002; Kozak et al., 2013; Ortega and Sanchez, 2010). In 2017, there were a total of 1065 cases reported from 40 states in the U.S. with 52 hospitalizations, and at least 597 (56%) of those were domestically acquired (CDC, 2017).

The identification of food items that serve as vehicles in cyclosporiasis outbreaks represents a major challenge. The long incubation period for *C. cayetanensis* infection, the short shelf life of implicated commodities (i.e., fresh produce), and the complex epidemiological investigations required to identify the contaminated produce item present in a dish with multiple ingredients, are among the factors that hamper these investigations. Produce can become contaminated with *C. cayetanensis* in the field and during harvest, storage and transportation. Factors including poor worker hygiene practices and contaminated soil and agricultural water could also play a role in this process (Chacin-Bonilla, 2017). (Chacin-Bonilla, 2017). Recent studies have shown *C. cayetanensis* contamination in ready to eat and pre-packaged/bulked vegetable products in Canada and Europe (Caradonna et al., 2017; Dixon et al., 2013; Lalonde and Gajadhar, 2016a) serving as an indication that the current sanitation processes do not guarantee food safety when dealing with certain parasites of fecal origin (Caradonna et al., 2017).

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Improved methods for detection and characterization of the parasite are essential to identify and track sources of produce contamination and to strengthen surveillance (Abanyie et al., 2015). *Cyclospora cayetanensis* cannot be propagated *in vivo* or *in vitro,* currently making the use of enrichment methods to confirm its presence in foods or environmental samples impossible. The U.S. Food and Drug Administration (FDA) developed and validated a new regulatory method for detection of *C. cayetanensis* in produce (Murphy et al., 2017a, 2018). The method employs an enhanced washing solution to recover *C. cayetanensis* oocysts from produce, a commercially available procedure to disrupt the oocysts and purify their DNA, and a species-specific TaqMan™ real-time PCR assay targeting the *C. cayetanensis* 18S rRNA gene for molecular detection. This FDA method was originally validated for the detection of *C. cayetanensis* in cilantro and raspberries, two of the matrices historically linked to cyclosporiasis outbreaks in North America (Abanyie et al., 2015; Herwaldt, 2000; Ho et al., 2002; Murphy et al., 2017a). However, a variety of other fresh produce have been implicated in outbreaks. For example, basil, in some instances in prepared dishes (CDC, 1997; Kozak et al., 2013; Lopez et al., 2001), was linked to multiple outbreaks in Canada and in the U.S. (Chacin-Bonilla, 2017; Herwaldt, 2000; Kozak et al., 2013; Ortega and Sanchez, 2010). In fact, the first time *C. cayetanensis* was molecularly and microscopically detected in an implicated food was in leftovers from a chicken pasta salad containing basil linked to outbreaks in Missouri in 1999, confirming the original epidemiological data (Lopez et al., 2001). *Cyclospora cayetanensis* contamination has also been reported in basil in fresh produce surveillance studies in Vietnam and Nepal, and parsley was positive for *C. cayetanensis* in samples tested in Egypt (reviewed by Dixon, 2016). In Germany in 2000–2001, butterhead lettuce, mixed lettuce, dill, parsley and green onions were associated with an outbreak involving 34 people who ate salads (Döller et al., 2002). In 2013, an investigation of cyclosporiasis cases in Iowa and Nebraska indicated that some restaurant-associated illnesses may have been caused by a contaminated salad mix (containing several types of lettuce, red and green cabbage and carrots) (FDA, 2013). Additionally, in 2016, a restaurant-associated sub-cluster of cyclosporiasis in Texas was epidemiologically linked to consumption of coleslaw containing shredded carrots and cabbage (Fox, 2017).

It is important to assess the efficacy of the new validated FDA method for detection of *C. cayetanensis* in additional produce matrices and in prepared dishes to identify potential improvements for use during future outbreak investigations or surveillance activities. Method modifications may be needed to strengthen perfomance in various types of fresh produce and, in particular, in prepared dishes which may include multiple fresh produce items and other ingredients. The objective of the present study was to evaluate the performance of the FDA method for detection of *C. cayetanensis* in fresh produce items previously linked to outbreaks, i.e. shredded carrots, shredded cabbage with carrot mix, basil, and parsley, and to evaluate specific modifications developed for optimal use in a prepared dish, coleslaw.

2. Material and methods

2.1. Preparation of oocysts and initial seeding studies

Purified *C. cayetanensis* oocysts originating from a patient from Indonesia, and stored in 2.5% potassium dichromate, were used in these experiments; approximately 50% of the oocysts were sporulated in this preparation. Sporulated oocysts should have higher gene copy numbers than non-sporulated oocysts, but to our knowledge there are no published comparison data on this regard. The use of the oocysts was approved by the institutional review board of the FDA (protocol number 15–039F). The oocysts were washed with 0.85% NaCl and concentrated prior to enumeration. Six replicates of the purified oocysts were counted using a hemocytometer on an Olympus BX51 microscope (Optical Elements Corporation, Dulles, VA, U.S.). Oocysts were then diluted in 0.85% NaCl to contain 20 oocysts/μL and 1 oocysts/μL for seeding experiments.

The oocysts were initially seeded in the validated produce matrices (cilantro and raspberries); the analysis demonstrated that the previously established performance standards for the detection method (Murphy et al., 2017a) were achieved (data not shown). Subsequently, the same preparation of oocysts was used for all seeding experiments in shredded carrots, cabbage and carrot mix, basil, parsley, and prepared coleslaw described in the present study.

2.2. Sample preparation and seeding in fresh produce

The fresh produce analyzed consisted of bagged shredded carrots, bagged shredded green cabbage with carrot mix (commercial classic coleslaw all natural, approximately 95% shredded green cabbage and 5% shredded carrots), sweet basil, and Italian parsley. All produce was fresh, showing no signs of deterioration, obtained from local grocery stores, and stored at 4 °C for no longer than 24–48 h prior to seeding. Individual fresh produce test samples (25 g for each of the commodities) were prepared as described previously (Murphy et al., 2017a).

The samples were seeded with 200, 10, or 5 oocysts by dropwise application of 10 μL or 5 μL of the appropriate oocyst dilution using a micro-pipet to spread the oocysts randomly over multiple surfaces of the sample. Unseeded samples were also included as negative controls and processed together with the seeded samples. Unseeded and seeded samples were allowed to air dry uncovered at room temperature for approximately 2 h. Afterwards, samples were carefully transferred to BagPage +400 filter bags (Interscience Lab Inc., Boston, MA), sealed by securing the folded openings with small binder clips, and held at 4 °C for 48–72 h prior to initiating the produce wash step. No more than 12 samples were processed per experiment which included at least one unseeded sample plus samples with all three seeding levels. Between eight to eleven sample replicates for each matrix were examined unseeded and at each seeded level.

A total of 141 samples of fresh produce (36 shredded carrot samples, 40 shredded green cabbage and carrot mix samples, 38 sweet basil samples, and 37 Italian parsley samples) were analyzed in this study.

2.3. FDA BAM chapter 19B method for detection of C. cayetanensis in fresh produce

The wash protocol to recover the oocysts from fresh produce, the DNA extraction of concentrated oocysts, and the qPCR analysis using a TaqMan™ method targeting the *C. cayetanensis* 18SrRNA gene were performed as described in the FDA's BAM Chapter 19B (Murphy et al., 2017a, 2017b). The wash protocol to recover the oocysts from fresh produce was performed using 0.1% Alconox® detergent and sequential centrifugations to recover, pool, and concentrate the wash debris. After this step, the produce wash debris pellets were stored at 4 °C for up to 24 h or frozen at −20 °C for longer periods prior to DNA isolation. The DNA extraction procedure was performed using the FastDNA SPIN Kit for Soil in conjunction with a FastPrep-24 Instrument (MP Biomedicals, Santa Ana, California) and extracted DNA samples were stored at 4 °C for up to 2 days prior to performing PCR or at −20 °C for longer term storage.

A TaqMan™ dual real-time PCR assay targeting both the *C. cayetanensis* 18S rRNA gene and an exogenous internal amplification control (IAC) was performed on an Applied Biosystems 7500 Fast Real-Time PCR System (ThermoFisher Scientific, Waltham, MA). The IAC reaction was used to monitor for reaction failure due to matrix derived PCR inhibition. The commercially prepared synthetic gBlocks gene fragment (Integrated DNA Technologies, Coralville, CA) was used as a positive control for amplification of the *C. cayetanensis* 18S rRNA gene (Cyc18SrDNA control). Serial dilutions of the positive control target, covering six orders of magnitude ranging from 5×10^4 to 0.5 copies/μL, were prepared. For positive control reactions and standard curve experiments, 2.0 μL of the appropriate positive control dilutions were used as template in real-time PCR reactions to achieve the desired