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Optimization and validation of methods for isolation and real-time PCR identification of protozoan oocysts on leafy green vegetables and berry fruits

Laura F. Lalonde *, Alvin A. Gajadhar

Centre for Food-borne and Animal Parasitology, Canadian Food Inspection Agency, 116 Veterinary Road, Saskatoon, Saskatchewan S7N 2R3, Canada

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ABSTRACT

Leafy green vegetables and berry fruits have vastly different physical and biochemical characteristics, are typically consumed raw with minimal washing, and are potential transmission vehicles for food-borne disease caused by protozoan parasites such as *Cryptosporidium*, *Cyclospora* and *Toxoplasma*. Validation of a generic oocyst isolation and detection method applicable to each leafy green and berry type is required to provide reliable laboratory support for surveillance programs and as necessary for disease outbreak investigations. The objectives of the current study were to optimize and validate the performance of these methods for the isolation of protozoan oocysts from several types of leafy greens and berries. *Eimeria papillata* oocysts were used as a surrogate for coccidia of public health concern to spike produce samples. An artificial stomacher or orbital shaker was used, followed by centrifugation, to isolate and concentrate oocysts, respectively, and a qPCR melt curve analysis (qPCR MCA) was used for detection and identification. Processing methods, wash buffers and storage conditions were evaluated and optimized for five types of berries (blackberries, blueberries, cranberries, raspberries and strawberries), five types of herb (cilantro, dill, mint, parsley, thyme) and green onions. Blackberries, cranberries, raspberries and strawberries were most effectively washed by orbital shaking with an elution solution, while glycine buffer was more effective for blueberries. Stomaching with a glycine buffer was optimal for oocyst recovery in leafy herbs with soft stems, while aromatic woody-stemmed herbs such as thyme required orbital shaking to minimize the release of PCR inhibitors. Oocyst recovery from green onions was highest when processed by orbital shaking with elution solution. Oocyst recovery rates ranged from 4.1–12% for berries and 5.1–15.5% for herbs and green onions. As few as 3 oocysts per gram of fruit, or 5 oocysts per gram of herbs or green onions could reliably be detected using the optimized isolation methods and qPCR MCA.

1. Introduction

The importation of leafy green vegetables (leafy greens) and berry fruits from Central and South America is steadily increasing (Anonymous, 2010). Much of this produce is minimally processed and consumed raw, leading to an increasing risk of exposure to parasites that would normally be controlled by food processing temperatures (Gajadhar, 2015a; Gamble, 2015). Outbreaks of gastrointestinal illness related to the consumption of imported leafy green herbs, vegetables, or berries contaminated with *Cyclospora*

* Corresponding author. Tel.: +1 306 385 7815; fax: +1 306 385 7866.
E-mail address: laura.lalonde@inspection.gc.ca (L.F. Lalonde).

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cayetanensis have occurred sporadically in North America and Europe since 1996 (Dixon, 2015; Doller et al., 2002; Gibbs et al., 2013; Herwaldt and Ackers, 1997; Hoang et al., 2005; Insulander et al., 2010; Lopez et al., 2007). *Cryptosporidium* has been detected in ready-to-eat vegetables and irrigation water in both developing and developed countries (Amoros et al., 2010; Rzeżutka et al., 2010), and *Toxoplasma* has also recently been detected in leafy greens in Poland (Lass et al., 2012). Since they are generally consumed raw and with minimal washing, a wide variety of leafy greens and berries with vastly different physical and chemical characteristics are potential vehicles for food-borne disease caused by *Toxoplasma*, *Cryptosporidium* or *Cyclospora*, therefore a broad-specificity oocyst isolation and detection screening method should be validated as needed for each type.

Several methods have been developed for the microscopic or molecular detection of *Cyclospora* (Shields et al., 2012; Steele et al., 2003) or *Cryptosporidium* (Cook et al., 2006a, 2006b, 2007; Robertson and Gjerde, 2000) oocysts on fruits and vegetables, however most target only a single protozoan oocyst species, and have not been validated for use in multiple types of produce. In order to deliver reliable survey results, to establish accurate baseline data, and to facilitate timely and effective food-borne disease outbreak investigations and response, a validated screening method for the detection of oocysts of multiple species of protozoan parasites is required. Past method development and validation studies have focused primarily on single produce types (such as lettuce or raspberries) or protozoan species (mainly *Cryptosporidium* or *Giardia*) using microscopy to detect oocysts (Cook et al., 2006a, 2006b, 2007; Shields et al., 2012, 2013). Microscopic methods for detection and identification of *Cyclospora* and other protozoan of human health concern are limited due to inconsistent staining of oocysts, time required for oocysts to be sporulated for taxonomic identification, and the need for parasitology experience to distinguish oocysts from fecal or other debris (Gajadhar et al., 2015; Mansfield and Gajadhar, 2004). Although several commercial immunomagnetic separation (IMS) and immunofluorescent antibody (IFA) kits are available for *Cryptosporidium* and *Giardia*, none are currently available for *Cyclospora* or *Toxoplasma*. A real time PCR assay with melt curve analysis (qPCR MCA) has been developed for the simultaneous detection and differentiation of several protozoan oocyst species of public health importance (*Cryptosporidium*, *Cyclospora*, *Sarcocystis*, *Cystoisospora* and *Toxoplasma*) (Lalonde and Gajadhar, 2011). It is capable of detecting and identifying a wide variety of coccidia present in food, clinical, or environmental matrices. The qPCR MCA approach is well suited for high-throughput screening, can differentiate *Cyclospora* from other coccidia, including *Eimeria* (Lalonde and Gajadhar, 2011), and has been successfully applied to detecting zoonotic protozoan species in human fecal samples (Lalonde et al., 2013); however it has not been validated for use in leafy greens or berries.

Processing consists of isolation and detection of oocysts from the food matrix. Adequate validation of the oocyst isolation method is necessary for each type of leafy greens or berries tested, and the processes performance characteristics such as sensitivity and accuracy for these products should be determined. Certain fresh, ready to eat leafy greens and berries have inherent physical or biochemical properties which make removing and detecting protozoan oocysts difficult. Leafy greens and berries often contain phenols, polysaccharides, pectin and other compounds that inhibit PCR (Schrader et al., 2012) and may reduce the sensitivity of molecular assays. Raspberries and blackberries are incredibly delicate and temperature sensitive, resulting in tissue breakdown during processing which can lead to debris that that physically interferes with the recovery of oocysts. They also have fine hair-like projections that may trap oocysts (Kniel et al., 2002), making isolation a challenge. To overcome these physical and chemical challenges and maintain a suitable level of assay sensitivity, method modifications specific for each type of leafy green and berry may be necessary and require validation to ensure that tests used are fit for purpose. The objectives of the current study were to 1) optimize and determine the performance characteristics of methods for isolation of protozoan oocysts from several types of leafy greens and berries, and 2) validate the qPCR MCA assay for use to detect protozoan oocysts in these matrices.

2. Materials and methods

2.1. Oocysts and produce samples

Oocysts of *Eimeria papillata* were used as a surrogate for those of *C. cayetanensis* and other coccidia of public health concern in all recovery experiments, because they are non-pathogenic and fresh oocysts were available. The *E. papillata* oocysts were propagated by passage in mice, and isolated from feces by washing and straining with water and by Sheather's flotation (Levine, 1985). The oocysts were sporulated (72%) and stored at 4 °C in 2.5% potassium dichromate until used. Stocks of *E. papillata* used for spiking produce samples were prepared by triplicate enumeration with a hemocytometer and diluted in an antibiotic–antimycotic solution (Streptomycin, Penicillin, Amphotericin B, Life Technologies) for use within 30 days. For low concentration stocks, triplicate aliquots of oocyst dilutions were dried on microscope slides with concave wells and enumerated using a fluorescent microscope to detect autofluorescence.

Produce samples used for spiking and recovery experiments were purchased at local retail grocery stores and included five types of berries (blackberries, blueberries, cranberries, raspberries and strawberries), five types of herb (cilantro, dill, mint, parsley, thyme) and green onions. Unspiked samples of produce were tested alongside spiked samples from each batch to ensure they had not been naturally contaminated with oocysts. Additionally, samples containing wash buffer only (no produce) were spiked and processed with each batch as positive controls to verify the performance of the processing and flotation procedures. Produce samples were spiked with 5000 oocysts (unless otherwise indicated) ~18 h prior to processing to most closely mimic field contamination. Enumerated aliquots of oocysts were pipetted onto the surface of pre-weighed produce samples in several 5–10 μl droplets, covered and stored at 4 °C or room temperature overnight.