A viability assay for *Cyclospora* and its surrogates *Eimeria*

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Summary

To mitigate human health risks posed by foodborne pathogens, produce growers and regulators require tools for assessing not only their presence but also their viability. The inability to propagate *Cyclospora*, either in *in vitro* or *in vivo*, hinders viability assessment. Many pathogens repel infiltration of certain dyes only when living. Propidium monoazide (PMA) has proven successful as a basis to establish the viability of other pathogens. Digital PCR (dPCR) has proven very useful for absolute quantification of nucleic acids, without requiring standard curves. Hence, we propose to develop a viability assay for *Cyclospora* in produce, combining PMA staining with dPCR. Success would afford the industry, and regulators, a rapid, sensitive, and specific assay to diagnose parasite contamination and test the presence of viable protozoan pathogens.

Objectives

1. Adapt and validate sensitive biomarkers for risk assessments:
   We will target at least ten highly-expressed genes to develop biomarkers for dPCR. After validating the specificity and sensitivity of the dPCRs, we will develop a multiplex assay for simultaneous detection of 4 genes with the highest specificity and sensitivity.
2. Develop quantitative viability assays using dPCR system and PMA treatment:
   We will evaluate PMA treatment to discriminate viable from nonviable parasites and evaluate dPCR as a basis for quantifying live parasites. We will vary several treatment conditions to optimize the PMA protocol and will conduct a spiking experiment to evaluate the sensitivity and specificity of dPCR using experimental and samples from the CPS-funded "AFECCT" project. Finally, we will translate the standardized protocol to evaluate live and dead *Cyclospora*.

Methods

1. We will target ten genes that are highly expressed in mature oocysts. Assay sensitivity will be established by limiting the dilution of parasite oocysts, purification of gDNA, and conducting dPCR. We will seek to develop a multiplex assay for the simultaneous detection of 4 genes with the highest specificity and sensitivity.
2. We will evaluate PMA treatment to discriminate viable from nonviable parasites and evaluate dPCR as a basis for quantifying live parasites using several conditions (**Figure 1**). After PMA treatment, DNA will be isolated. PMA-dPCR will be conducted to discriminate live from dead parasites by spiking experiments varying the proportions of dead and live parasites in mixtures and with samples from the ongoing CPS-funded "AFECCT" project.

Results to Date

To understand fundamental processes related to oocyst maturation and to develop biomarkers, we have recently conducted RNA-sequencing (RNA-Seq) and looked at genes with >1000 TPM (transcripts per million). Preliminary data validate several transcripts as promising biomarkers for viability in *Cyclospora* (**Figure 2**). To develop and access the sensitivity of biomarkers, we developed *Eimeria* 5S-qPCR standard curve, indicating a 100% RT-qPCR efficiency (**Figure 3A**). Using the standard curve of 5S-rRNA qPCR, we validated the PCR inhibitory effect of PMA using serially diluted *Eimeria* gDNAs and PMA-treated dead oocysts. We observed that PMA treatment reduced qPCR intensity drastically for gDNA (**Figure 3B**); however, preliminary data also suggest that the walls protecting *Cyclospora* repel the stain, which we hypothesized might selectively penetrate dead parasites (**Figures 3C**, **Figure 4**).

Benefits to the Industry

A globalized food trade, extensive production, and complex supply chains broaden the reach and stakes of microbiological food safety outbreaks. The CDC estimates ~48 million people in the United States are sickened by a foodborne illness, annually. The estimated cost of food safety incidents for the U.S. economy is around $7 billion per year. Thus, produce growers and regulators urgently need mitigation tools capable of assessing not only the presence of foodborne pathogens but also their viability. If successful, our project will provide the produce industry and its regulators with the means to quantify viable parasites contaminating fresh produce. This will aid risk assessment and mitigation, enhancing the safety of fresh produce and the reputation of the fresh produce industry.

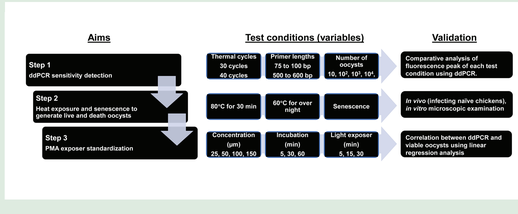

Figure 1. Overview of test conditions and validation methods to demarcate viable from non-viable oocysts using propidium monoazide (PMA) treatment and dPCR.

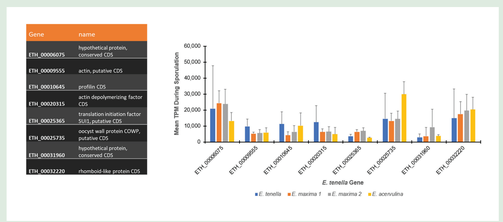

Figure 2. Selecting biomarkers from RNAseq data from three *Eimeria* species in fully sporulated oocysts.

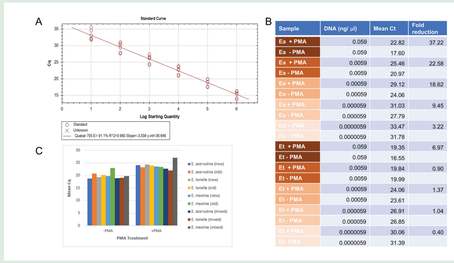

Figure 3. Developing biomarkers and testing with and without PMA-treated parasites. **A**) Developing a standard curve with *Eimeria* 5S-qPCR. **B**) Fold reduction of purified gDNA with and without PMA. **C**) Average Ct value and Ct changes with and without PMA-treated oocysts.

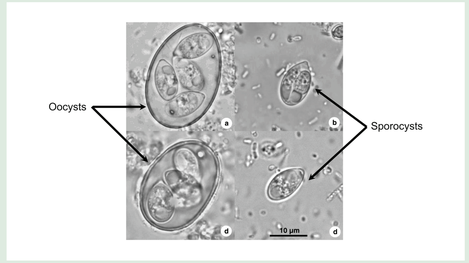

Figure 4. Sporulated oocysts and sporocysts of *Eimeria* species.