The limit of detection of the BioFire® FilmArray® gastrointestinal panel for the foodborne parasite *Cyclospora cayetanensis*

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Abstract

Cyclosporiasis is a foodborne diarrheal illness caused by the parasite *Cyclospora cayetanensis*. The BioFire® FilmArray® gastrointestinal (FilmArray GI) panel is a common method for diagnosing cyclosporiasis from clinical stool samples. The currently published limit of detection (LOD) of this panel is in genome equivalents; however, it is unclear how this relates to the number of *C. cayetanensis* oocysts in a clinical sample. In this study, we developed a technique to determine the LOD in terms of oocysts, using a cell sorter to sort 1 to 50 *C. cayetanensis* oocyst(s) previously purified from three human stool sources. We found the FilmArray GI panel detected samples with 20 *C. cayetanensis* oocysts in 100% of replicates, with varying detection among samples with 1, 5, or 10 *C. cayetanensis* oocysts. This method provides a parasitologically relevant LOD that should enable comparison among *C. cayetanensis* detection techniques, including the FilmArray GI panel.

1. Introduction

Cyclosporiasis is a foodborne illness characterized by gastrointestinal distress and is caused by the coccidian parasite *Cyclospora cayetanensis*. Individuals become infected by ingesting sporulated oocysts via contaminated food or water, with fresh produce as the most common food vehicle (Centers for Disease Control and Prevention [1]). After consumption, the sporozoites are excysted and invade the intestinal epithelium, where they undergo asexual followed by sexual reproduction, resulting in unsporulated noninfective oocysts that are excreted in stool. Under optimal conditions, the oocysts will require one to two weeks to sporulate in the environment to become infective. The only known method of infection is via food or water sources contaminated with sporulated oocysts (Centers for Disease Control and Prevention [1]).

The first attributed outbreak of cyclosporiasis in the United States was in 1990 [2]. Following large multistate outbreaks in the mid-1990s, the disease became nationally notifiable in 1999. Since then, increasing numbers of cases have been reported each year, usually during the summer months [3] with >1000 cases reported annually since 2018 (Centers for Disease Control and Prevention, [4]). The US Centers for Disease Control (CDC) conducts real-time genotyping of *C. cayetanensis* from clinical stool specimens during the summer season to inform outbreak investigations [3,5,6]. Cyclosporiasis outbreaks have been linked to various produce food vehicles, including raspberries, basil, cilantro, and various lettuces and bagged salad mixes. In 2021, for example, 1020 laboratory confirmed cases of cyclosporiasis were reported from 36 states and one jurisdiction, and two multistate outbreaks involving 170 individuals were investigated, though no specific food vehicle was identified (Centers for Disease Control and Prevention [4]).

Enhancements to molecular diagnostic methods, including the development of syndromic panels, may in part explain the increase in laboratory confirmed cyclosporiasis cases reported in recent years [3,5–7]. In 2014 the US Food and Drug Administration (FDA) cleared the use of the BioFire® FilmArray® gastrointestinal panel (FilmArray GI panel) for the diagnosis of cyclosporiasis from clinical stool specimens. The FilmArray GI panel is a culture-independent rapid molecular multiplex panel that tests for 22 unique gastrointestinal pathogens, including *C. cayetanensis*, in a single stool sample. This panel requires 200 μL of stool, which is injected into the prepared reagent pouch and placed into a BioFire® FilmArray® system for rapid PCR-based multiplex detection of the 22 gastrointestinal pathogens.

The FilmArray GI panel may help to detect outbreaks more quickly [8] and can improve patient outcomes and lower costs compared to relying on more complicated and expensive methods for pathogen diagnosis [9]. In a recent outbreak in 2018 in Wisconsin, 71% of associated cyclosporiasis cases were diagnosed using the FilmArray GI panel [10]. The most

common alternative method relied on nonmolecular techniques such as acid-fast/modified acid-fast staining [10]. Similarly, ~53% of all stool specimens received at the US Centers for Disease Control and Prevention (CDC) in 2021 for inclusion in the *C. cayetanensis* genotyping program with known diagnostic methods were from patients diagnosed using the FilmArray GI panel. Acid-fast/modified acid-fast staining was the next most common method used, with 34% of specimens submitted from patients diagnosed using that method.

Given the increasing diagnostic use of the FilmArray GI panel, it is essential to clarify the performance characteristics of the panel in terms that are comparable to other *C. cayetanensis* diagnostic methods. The FilmArray GI panel has been found to be highly sensitive (100%) and specific (100%) when compared to conventional methods of *C. cayetanensis* detection [8]. The limit of detection (LOD) of the FilmArray panel is currently only known in genome equivalents (estimated at 180 Geq/mL) (BioFire Diagnostics, [11]). How this value was calculated exactly, and how it would relate to parasite burden, is not clear. The relationship between number of genomes and number of parasites present is not well understood but is not expected to be one-to-one given the lifecycle of *C. cayetanensis*. This makes it challenging to compare the LOD of the FilmArray GI to methods such as acid-fast/modified acid-fast straining that rely on visualization of the oocyst stage, or to parasite-specific qPCR or other methods that rely on DNA extracted directly from oocysts in stool. Thus, in this study, we sought to establish a methodology to determine the LOD of a diagnostic assay in terms of numbers of oocysts and use it to identify the LOD of the commonly used FilmArray GI panel, as this value provides a more consistent baseline of comparison among *C. cayetanensis* diagnostic and detection assays.

2. Methods

We obtained isolates of *C. cayetanensis* oocysts that had been previously purified from stool collected from individuals from 3 countries: Indonesia, China, and Nepal. These samples were collected for research purposes and selected for this study because they contained sufficient oocysts necessary for the purification and sorting process (CGH protocol #2014–107). The samples were previously obtained from Indonesia in 2014, China in 2011, and Nepal in 1997 and stored refrigerated (2–8°C) in potassium dichromate until purification of the oocysts as part of a genomics study [12]. The oocysts were separated from the stool matrix using discontinuous density gradient centrifugations and further purified by flow cytometry [12]. After purification, the oocysts were stored refrigerated (2–8°C) in phosphate-buffered (PBS) solution. To ensure that these three *C. cayetanensis* oocyst isolates were representative of *C. cayetanensis* that may be encountered during outbreaks in the United States, we genotyped the isolates and compared them alongside a reference population made up of clinical *C. cayetanensis* specimens ($n = 1209$) collected in the United States and abroad between 2016 and 2021 following methods previously described [3,5,6].

To ensure that the oocysts were not infective, all material to be sorted was inactivated by incubating at 55°C for 30 minutes [13] as a safety precaution.

We diluted the inactivated purified oocysts 1:100 or 1:1000 in PBS and sorted them using a BD FACSAria II (BD Biosciences, San Jose, CA) furnished with violet (407 nm), blue

(488 nm), and red (633 nm) lasers. We identified oocysts by size (approximately 8–10 μm) by forward scatter (FSC), their internal complexity by side scatter (SSC) and also by their autofluorescence emission properties which was a result of excitation by a 488 nm laser [12]. The autofluorescence was detected at the fluorescein isothiocyanate (FITC) channel (wavelength range 515–545) and peridinin chlorophyll protein (PerCP) channel (wavelength range 665–685). We sorted using 85 μm nozzle at 45 psi with the sort precision mode set to "purity."

We sorted sets of four replicates of quantified oocysts (range 1–50) from each of the three isolates. We used three of each replicate in the experiment (Table 1) and retained one replicate for backup as needed. To minimize carryover and contamination, we sorted each replicate individually from low (1) to high (50) oocyst quantity into low protein binding tubes (Eppendorf LoBind) preloaded with 50 μL of PBS solution. We vortexed each sample immediately upon removing a tube from the sorter to ensure that the oocysts made it into the PBS solution. In a biosafety hood, we added 150 μL of a parasite-free stool sample in Cary-Blair transport medium to each of the tubes with sorted oocysts, for a total of 200 μL, which is the required sample volume for the FilmArray system. The parasite-free stool sample had previously been analyzed on the FilmArray GI panel (in duplicate) and tested negative for all 22 pathogens that can be detected by this assay: *Campylobacter, Clostridioides, Plesiomonas shigelloides, Salmonella, Yersinia enterocolitica, Vibrio, Vibrio cholerae*, Enteroaggregative *E. coli*, Enteropathogenic *E. coli*, Enterotoxigenic *E. coli* lt/st, Shiga-like toxin-producing *E. coli* stx1/stx2, *E. coli* O157, *Shigella*/Enteroinvasive *E. coli, Cryptosporidium, Cyclospora cayetanensis, Entamoeba histolytica, Giardia lamblia*, Adenovirus F40/41, Astrovirus, Norovirus GI/GII, Rotavirus A, and Sapovirus.

In a biosafety cabinet, we loaded and analyzed all replicates within 72 hours of addition of the stool in Cary-Blair mix to the sorted oocysts. We followed the manufacturer's instructions to transfer the sorted oocysts and stool Cary-Blair mix from the LoBind tubes into the sample collection tube, which were then injected into the FilmArray GI panel pouch using separate loading stations for each pouch. In one case the sample injection failed, and the sample and cartridge were discarded. We used the backup replicate to ensure that 3 replicates of each of the 3 *C. cayetanensis* sources were run at every sorting level.

We removed the pouches from the biosafety hood and transported the loaded cartridges in individual containers to place on the BioFire Torch system which contained eight individual units. We loaded the cartridges on the BioFire Torch system within 30 minutes of sample injection as per the manufacturer's instructions. The BioFire Torch instrument identified all samples as either positive or negative for *C. cayetanensis*.

We defined the LOD as the lowest level at which 95% of samples tested could be consistently detected (US Department of Health and Human Services, 2011), which is the same threshold used by the manufacturer to determine the published LOD (BioFire Diagnostics, [11]). A total of 65 specimens were run on the BioFire Torch system, including the negative stool which was run in duplicate.

3. Results

The genotyping results indicated that the 3 *Cyclospora* isolates used for spiking are genetically similar to specimens from prior cyclosporiasis outbreaks in the United States (Fig. 1). The Nepal specimen fell within a large cluster that contains specimens from throughout the United States, including specimens that have been epidemiologically linked to outbreaks in 2019 [6] and 2020 [5]. The specimen from China clustered alongside specimens from New York and Iowa, and the Indonesia specimen fell within a cluster containing specimens from several states including Texas and New York (Fig. 1).

Using our method of spiking flow-sorted quantified oocysts into stool, we estimated the LOD of the BioFire® FilmArray® GI panel is 20 *C. cayetanensis* oocysts in 200 μL stool (Table 1). All replicates from all 3 sources were positive at the 20-oocyst spike-in level. At the next lowest level tested (10 oocysts) six of the nine (67%) replicates tested were positive for *C. cayetanensis*, which is below the 95% threshold established to determine the LOD. Samples with one and five *C. cayetanensis* oocysts also tested below the 95% threshold, with eight of nine replicates positive at the five-oocyst level, and three of nine replicates positive at the one-oocyst level. There was some variation among the different oocyst sources, with oocysts from the Nepal isolate more consistently detected to a lower limit (100% detection in all replicates from 5 to 50 oocysts). The oocysts from the Indonesia isolate showed a consistent decline in detection below 20 oocysts, while the oocysts from China isolate showed variation (Table 1).

One replicate of 50 oocysts of the Nepal isolate also tested positive for the gastrointestinal pathogen *Campylobacter* in addition to *C. cayetanensis*. All other replicates at all sorted levels were positive only for *C. cayetanensis* or negative for all pathogens.

4. Discussion

We found that using fluorescence-activated cell sorting to sort purified *C. cayetanensis* oocysts into discrete levels was an effective method to determine a LOD of the BioFire GI molecular diagnostic test. The currently established LOD of the BioFire GI panel is in genome equivalents, which is a challenging measure to use as a base of comparison as the relationship between number of genomes and number of parasites present is not well understood but is not expected to be one-to-one given the lifecycle of *C. cayetanensis*. Understanding the LOD of the BioFire GI panel in terms of oocysts can better enable comparison to other diagnostic methods that enumerate the oocyst stage, such as microscopy. Furthermore, work has been done to understand the LOD of methods used to detect *C. cayetanensis* oocysts on various types of produce [14,15]. These studies have also relied on spiking *C. cayetanensis* oocysts onto produce to determine the number of oocysts that can be detected by PCR and multiplex qPCR methods [14,15]. Establishing a consistent base of comparison to use among diagnostic tests and environmental tests may facilitate a better understanding of infection with this parasite.

We found that this panel detected 20 oocysts in 200 μL stool in all nine replicates tested. The reported LOD of the FilmArray panel is 180 Geq/mL, which is equivalent to 36 Geq in

the 200 μL volume used in the FilmArray assay, indicating that indeed the ratio of oocysts to genome equivalents is not exactly one-to-one. While there are no published LODs for other *C. cayetanensis* diagnostic methods, we may be able to draw parallels to other similar parasitic pathogens such as *Cryptosporidium spp.*, which has a similar oocyst stage to that of *C. cayetanensis*. The LOD for acid-fast staining of *Cryptosporidium* sp. from formalin preserved fecal samples is 976 oocysts/mL [16], or roughly 200 oocyst/200 μL stool. This is 10× higher than the LOD for *C. cayetanensis* that we estimated for the FilmArray GI panel in this study. If the LOD for acid-fast/modified acid-fast staining of *C. cayetanensis* oocysts is like that of *Cryptosporidium spp.*, it is probable that the FilmArray GI panel detects much lower levels of *C. cayetanensis* oocysts in stool compared to acid-fast staining.

Since 2018, the CDC Parasitic Diseases Branch has been conducting regular genotyping of clinical *C. cayetanensis* stool specimens during the summer months when cyclosporiasis cases peak in the United States [3,5,6]. In 2021, ~53% of specimens sent for genotyping with a reported diagnostic method were from patients diagnosed with the FilmArray GI panel. CDC's genotyping workflow has a set of inclusion criteria [3] to ensure that only specimens with sufficient DNA coverage are included in clustering analysis, and only 71% of specimens from patients diagnosed with FilmArray GI panel passed the genotyping inclusion criteria in 2021. The lack of successful genotyping from these specimens could be due to two factors: (1) potential false positives with the FilmArray GI panel for *C. cayetanensis*; or (2) differences in the detection limit of the *C. cayetanensis* genotyping workflow relative to the FilmArray GI panel. Given that we found that the FilmArray GI panel can detect even a single oocyst in a reaction, it is likely that the FilmArray GI panel can detect a lower number of oocysts than the CDC genotyping workflow. However, further work needs to be done to clarify the number of *C. cayetanensis* oocysts that are needed for successful genotyping through the CDC workflow to rule out false positives in the FilmArray GI panel.

During this study we analyzed a total of 65 stool samples with or without *C. cayetanensis* oocysts in the FilmArray GI panel. All samples were negative for pathogens other than *C. cayetanensis* except for one, which was positive for the bacterial GI pathogen *Campylobacter*. It is possible that the parasite-free stool specimen used had very small levels of *Campylobacter* present and thus was detected in only 1 out of 65 replicates (1.5%); or this was a false positive detection. While our study did not explicitly investigate the likelihood of detecting false positives for *C. cayetanensis* or any other pathogen with the FilmArray GI panel, false positives with the BioFire GI panel have been reported for rotaviruses and adenovirus [17], and have also been reported for *Campylobacter* [8] at rates higher than those observed in this study. Indeed, the FDA issued a recall of the BioFire GI panel in 2019 due to elevated rates of false positives for *Campylobacter* and *Cryptosporidium* (Class 2 Device Recall FilmArray Gastrointestinal (GI) Panel (fda.gov)); supporting that the detection of *Campylobacter* in 1.5% of replicates in our study was likely a false positive for that pathogen. We did not test how coinfection with other intestinal pathogens may impact the LOD for *C. cayetanensis* in terms of oocysts. However, the published LOD for the BioFire GI Panel found no differences in sensitivity among assays spiked with single pathogens compared to those spiked with four different pathogens (BioFire Diagnostics, [11]).

While we were able to successfully determine the LOD of this diagnostic assay using cell-sorted oocysts, there are some limitations to be considered. First, the oocysts used in this study were from the CDC research collection and were not recently collected. We also heat inactivated the oocysts to ensure that they were no longer viable as a safety precaution. Heat inactivation has been shown to reduce viability and is not expected to impact DNA quality of parasite oocysts [13], though it is not clear how the DNA quality within the oocysts may have decreased, if at all, over time. However, our oldest oocysts were also those that were detected to the lowest number, indicating age of oocysts as used in this study may not have played a significant factor in our results. Obtaining fresh purified *C. cayetanensis* oocysts is challenging as *C. cayetanensis* can neither be cultured in the lab nor propagated in animal models. Thus far, the only source of oocysts is human stool samples. Purification of oocysts from the stool matrix is a time intensive process that also requires a large volume of stool with very high numbers of oocysts present [12], which can be difficult to obtain. Due to the limited oocysts available the numbers of replicates tested in this study were also limited ($N = 9$ at each level).

We also observed some variation in the limit of detection among the three oocyst isolates used in this study. This variation could be due to differences in sequences at the primer binding sites resulting in less efficient amplification in some isolates. The *Cyclospora* target used in the BioFire GI panel is proprietary, so we cannot compare sequences among the three oocysts sources at the target region. However, the three oocyst sources are genetically distinct, with the Nepal and China specimens clustering on a separate branch from the specimens from Indonesia (Fig. 1). Recent work has shown that these three isolates may even represent different *Cyclospora* species: Nepal—*C. ashfordi*, China—*C. henanensis*, Indonesia—*C. cayetanensis* [18]. Thus, it is reasonable that sequence variation among the different isolates used could influence the limit of detection with the BioFire GI panel, though this appears only to be the case at low concentrations of target DNA (<20 oocysts), and further studies would be needed to corroborate this hypothesis.

In summary, we developed a method to measure the limit of detection for diagnostic methods for *C. cayetanensis* and applied it to the BioFire GI panel. The limit of detection for this diagnostic test was 20 oocysts in 200 μL of stool. However, we did not test levels between 10 and 20 oocysts, and it is possible that the LOD may lie within this range. Regardless, this measurement may facilitate meaningful comparison of performance characteristics between different detection methods, including new commercially available diagnostic tests that continue to enter the market. Furthermore, there is growing concern regarding *C. cayetanensis* in food production, resulting in recent efforts to develop tests to detect the oocyst stage of this parasite in produce and agricultural water samples [19,20]. The methods described in this study could also be useful to determine LOD of these environmental sampling methods and could also be adapted for other parasites with an oocyst stage similar to *C. cayetanensis*.

Funding

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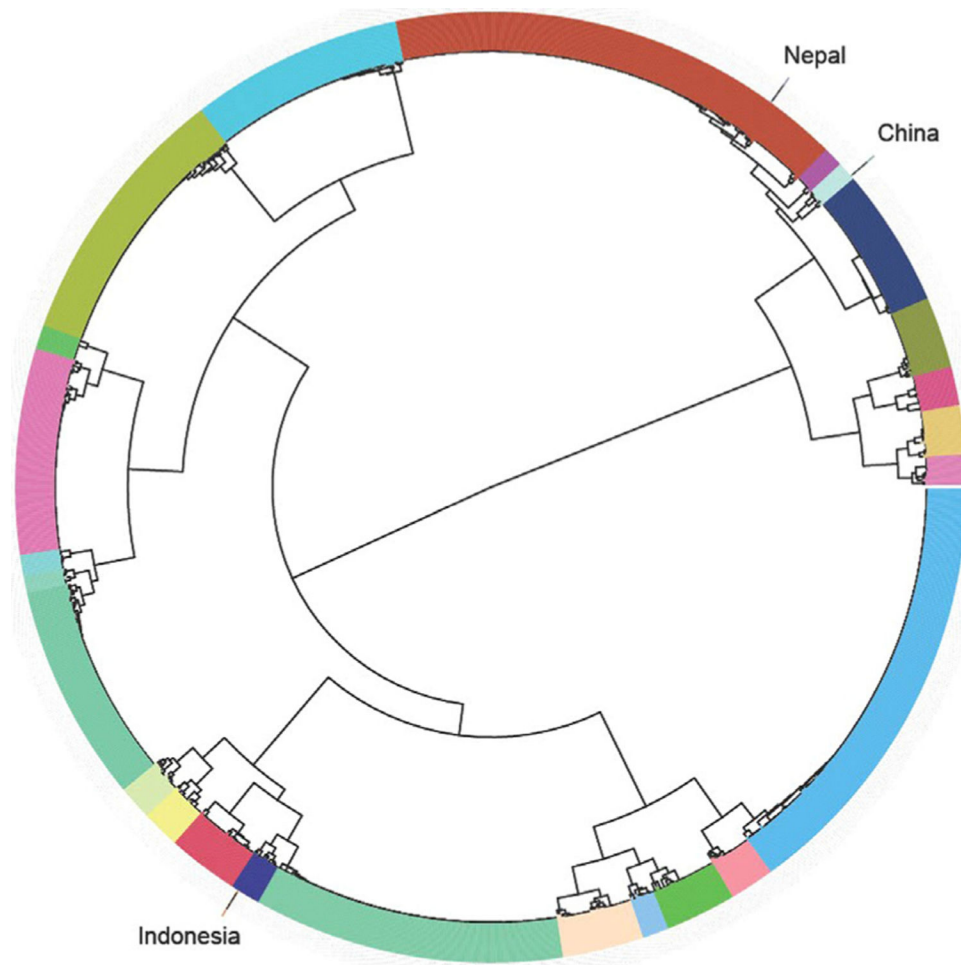
Fig. 1.
Clustering results of the 3 international isolates alongside specimens collected from clinical stool samples from the United States and abroad from 2016 to 2021. The Nepal isolate fell among a large cluster (in red) containing specimens from throughout the Unites States; the China isolate fell among a smaller cluster containing specimens from New York and Iowa (sky blue); and the Indonesia specimen fell within a cluster containing specimens from Texas and New York (navy blue).

Table 1

Results of FilmArray GI Panel for each of the 3 different oocyst isolate sources, from 1 to 50 oocysts.

No. of oocysts	Aggregate		Indonesia		China		Nepal	
	No. of positive (*n* = 9)	% positive	No. of positive (*n* = 3)	% positive	No. of positive (*n* = 3)	% positive	No. of positive (*n* = 3)	% positive
50	9	100	3	100	3	100	3	100
40	9	100	3	100	3	100	3	100
30	9	100	3	100	3	100	3	100
20	9	100	3	100	3	100	3	100
10	6	67	1	67	1	33	3	100
5	8	89	2	67	3	100	3	100
1	3	33	1	33	1	33	1	33