Development of a Rapid Alternative Method for Detection of *Aspergillus flavus, A. fumigatus, A. niger* and *A. terreus* in Cannabis Products Using Real-Time PCR

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Introduction

Due to increasing state-level legislation concerning medicinal and recreational marijuana across the United States and Canada, consumers now have access to cannabis edibles and inhalables for consumption that can be contaminated by yeast and mold like *Aspergillus* spp. These molds are a leading cause of aspergillosis infections in humans. A trend for a microbial pathogen detection system in cannabis and its derivatives is emerging. Due to concerning health impacts, challenges are focused on effective and efficient screening detection. Gold standard methods for yeast and mold testing, such as the FDA BAM - Chapter 18 (1), typically lack quantitative capability, rely on microbiological/ biochemical identification and are labor intensive. These current methods require at least 5 days for results and subjective interpretation for the screening of true positives and are not specific for *Aspergillus* species. Bio-Rad Laboratories has developed a new detection method consisting of an enrichment broth and the iQ-Check *Aspergillus* real-time PCR kit, targeting the four main species (*A. fumigatus, A. flavus* and *A. niger, A. terreus*) linked to aspergillosis that also enables differentiation of *A. terreus* in cannabis products. A complete study including limit of detection and inclusivity/exclusivity panel testing was performed. The performance of this qualitative method is described in this study.

Methods

Method Design

As shown in Figure 1, the *Aspergillus* method includes enrichment of the cannabis matrix in Buffered Peptone Water (BPW), DNA extraction and purification, real-time PCR amplification and detection, and automated data interpretation. The challenge was to develop an efficient enrichment method and a novel multiplex PCR assay capable of simultaneously detecting four unique genetic targets in a single tube reaction. *A. fumigatus, A. flavus* and *A. niger* were screened with primers and probes detected in the FAM fluorophore channel. *A. terreus* was detected in the Texas Red flurorphore channel. An internal control was also designed consisting of a heterologous target sequence (double stranded probe coupled with HEX fluorophore) that is co-amplified with primers identical to *Aspergillus* targets. A positive control with both FAM and Texas Red targets was also included to each PCR run to pass the specifications and validate the run.

Fig. 1. Flowchart of iQ-Check *Aspergillus*.

Inclusivity/Exclusivity

The specificity of the real-time PCR assay was validated with a comprehensive panel of inclusivity and exclusivity strains. Forty-six exclusivity species of yeast and mold represented a large panel of non-targeted *Aspergillus* or non-*Aspergillus* strains. Twenty-one *Aspergillus* species composed the inclusivity panel. Each exclusivity strain was tested at a concentration of 10^5 conidia/µl vs. 10^3 conidia/µl for the inclusivity strains to mimic what may be found in an enrichment. Five microliters of the purified DNA was directly added to the PCR well and analyzed with iQ-Check *Aspergillus* real-time PCR assay.

Limit of Detection

The analytical sensitivity of a PCR method can be expressed as the Limit of Detection (LOD), defined as the lowest concentration consistently detectable in at least 95% of all samples tested. In this study, 10 replicates of a dilution series were analyzed to determine the assay sensitivity. Serial dilutions of each species, *A. fumigatus, A. flavus, A. niger* and *A. terreus* (or *A. niger* and *A. terreus* together), were prepared with a starting concentration equivalent to 3.50 x 10^5, 8.20 x 10^3, 6.40 x 10^4 and 1.40 x 10^4 CFU/ml respectively. Each serial dilution underwent 10 DNA extractions followed by target amplification using the iQ-Check *Aspergillus* real-time PCR kit.

Assessment on Cannabis Matrices

The method was also assessed with cannabis matrices at the Colorado Department of Public Health and Environment laboratories (CDPHE). Five grams of cannabis flower, 5–6 g of infused non-edibles, 5–6 g of infused edibles or 1 g of solvent-based concentrated were tested with the iQ-Check method. Spiked cannabis flower matrices were grown at 24, 30, 40 or 48 ± 3 hr to determine the optimal incubation time. All unspiked cannabis matrices described previously were enriched at 37 ± 1°C for 48 ± 3 hr in BPW + 0.3 g/L of antibiotics. A total of 50 matrices were tested with this protocol using the standard DNA extraction protocol followed by a purification step with iQ-Purification Reagent (iQ-PR).

Proficiency Testing

To evaluate the method on cannabis samples, the Emerald Institute's proficiency test was performed. The objective was to test three different samples and correctly detect the positives. These samples consisted of spiked and unspiked dehydrated hemp flowers. Each sample was resuspended using 1 ml of sterile water. Then, 9 ml of BPW + antibiotic was added and incubated at 37 ± 1°C for 48 ± 3 hr. DNA was subsequently extracted from a 1 ml aliquot of the enriched sample. Five microliters of the DNA extract in 20 µl of iQ-PR (or diluted at 1:10 in distilled sterilized water) was tested with the iQ-Check *Aspergillus* real-time PCR assay. The same aliquot of the enrichment was used to confirm results from all samples using a competitor molecular method and confirmation using FDA BAM recommendations on DRBC or DG18 agar plates.

Results

Inclusivity/Exclusivity

Twenty one inclusivity species were tested and validated. Of the forty six exclusivity species assessed, 5 species were positive in FAM channel (Table 1). *Aspergillus oryzae*, *Aspergillus parasiticus*, *Aspergillus tamarii* were detected with high Cq values contrary to *Aspergillus penicillioides* and *Aspergillus vadensis*. These species have been verified in dedicated studies (2). *A. oryzae, A. penicillioides* and *A. tamarii* are phylogenically close to *A. flavus*. *A. parasiticus* is close to *A. restricti* and *A. vadensis* belongs to *nigri* section. It means that the positive result may indicate a true *Aspergillus* inclusivity species especially for species of *flavi* or *nigri* sections. Furthermore, the aspergillosis disease prevalence of those strains is unknown. Regarding the close phylogenetic link between some *Aspergillus* species (e. g. the five exclusivity species described), the inclusivity/exclusivity study was validated and guarantees functionally the specificity of the *Aspergillus* PCR kit.

Limit of Detection (LOD)

The limit of detection (95%) was determined for each species and a cocktail of *A. niger* and *A. terreus*. The LOD of *A. flavus* was 65 CFU/ml. The LOD of *A. niger, A. terreus* and *A. fumigatus* were 176, 284 and 2633 CFU/ml respectively. The double-inoculum had an LOD of 8 x 10^3 CFU/ml for *A. niger* and 3.6 x 10^5 CFU/ml for *A. terreus* demonstrating the assay's capability to successfully distinguish between the two species of *Aspergillus*.

Assessment on Cannabis Matrices

The CDPHE Laboratory study analyzed four categories of unspiked cannabis matrices with iQ-Check *Aspergillus*. It was found that 48 ± 3 hr was the optimal enrichment time for accurate *Aspergillus* detection (Table 2). This parameter enables detection in FAM or Texas Red channel contrary to the other time parameters that showed detection only in FAM channel. In some of the matrices, *Aspergillus* was detected after 48 hr of enrichment and standard extraction method followed by a purification step with iQ-PR (Table 3 and Fig. 2). These results showed the reliable performance of the method on cannabis matrices and the ability to detect *Aspergillus* in naturally contaminated flowers.

Proficiency Testing

One sample (out of the 4 provided) was detected positive using the iQ-Check *Aspergillus* method. Native and diluted 1:10 DNA enabled the detection in FAM channel indicating the presence of *A. flavus, A. fumigatus* and/or *A. niger* after an enrichment of 48 hr at 37°C. Native DNA was detected at 23.97 Cq values and diluted DNA at 26.62 (Fig. 3 and Table 4). The four replicates for each extract showed similar results with a standard deviation of 0.2 and 0.1 Cq for native and diluted DNA respectively. These results show a good repeatability of the method. Furthermore, cultures on agar plates confirmed these results. All these results were confirmed by the Emerald report.

A

Species		Collection ID	Cq in FAM channel	Cq in Texas Red channel	Cq in HEX channel	Qualitative result
Aspergillus	*terreus*	CECT 20365		31.66	28.00	+
		CECT 20194		38.84	28.14	+/-
		CECT 2808		44.24	28.01	+/-
	fumigatus	CECT 20190	29.32		27.96	+
		CECT 2071	31.63		27.98	+
		CECT 20228	32.67		28.19	+
		CECT 20827	32.74		28.13	+
	niger	CECT 2807	29.17		28.01	+
		CECT 2088	29.89		28.00	+
		CECT 2775	29.44		28.07	+
		CECT 2090	34.80		28.13	+/-
	flavus	CECT 20802	31.41		28.23	+
		CECT 20400	30.58		28.03	+
		CECT 2949	29.36		28.08	+
		CECT 2684	27.26		28.04	+
		CECT 2685	26.86		28.00	+
		CECT 2686	26.46		28.18	+
		CECT 2687	25.01		28.15	+
		CECT 20401	27.97		27.96	+
		CECT 20402	25.45		28.23	+
		CECT 20403	26.86		28.18	+

B

Genus	Number of species tested	Qualitative result
Aspergillus (other than *fumigatus, flavus, niger,* and *terreus*)	28	+ (x3) +/- (x2) - (x23)
Byssochlamys	1	-
Candida	3	-
Cladosporium	1	-
Debaryomyces	1	-
Kluyveromyces	1	-
Paecilomyces	1	-
Penicillium	5	-
Pichia	1	-
Saccharomyces	2	-
Yarrowia	1	-
Zygosaccharomyces	1	-

Table 1. Inclusivity and exclusivity study.
A: Inclusivity study
B: Exclusivity study
+: positive sample with Cq values lower than 33
+/-: positive sample with Cq values higher than 33
-: negative sample
CECT: Colección Española de Cultivos Tipo

Table 2. Comparison of incubation time for spiked cannabis matrices.

Incubatin time (hr)	Min of Cq Target (fla/fum/nig)	Max of Cq Target (fla/fum/nig)	Average of Cq Target (fla/fum/nig)	Min of Cq Target (ter)	Max of Cq Target (ter)	Average of Cq Target (ter)
24	31.91	35.69	33.45			
30	38.83	38.83	38.83			
40	25.71	42.89	32.15			
48	23.33	48.27	32.18	35.50	41.00	38.01

Table 3. iQ-Check *Aspergillus* with unspiked cannabis matrices.

Matrices	Average of Cq Target in FAM channel	Average of Cq Target in Texas Red channel	Average of Cq Internal Control in HEX channel
Flower - Cherry diesel	32.06	44.00	28.08
Flower - Citral Skunk	33.20		28.52
Flower - Composite	33.83		28.55
Flower - Corn Bread	26.93		28.89
Flower - Grape Cola	36.63		28.67
Flower - Grape Stomper	27.11		28.74
Flower - Headband	40.32		27.95
Flower - Kimbo Kush	32.13		28.34
Flower - Strawberry Nightmare	45.88		28.45
Flower - Toxic Koolaid	30.99		28.62
Flower - Gorilla Glue #4	30.48		28.45

Table 4. Emerald proficiency testing. +, positive sample; -, negative sample.

Sample	Dilution	Cq in FAM channel	Cq in Texas Red channel	Cq in HEX channel	Qualitative result	Expected result
1	-	23.97		28.15	+	+
	1:10	26.61		28.11	+	+
2	-			28.37	-	-
	1:10			28.11	-	-
3	-			28.39	-	-
	1:10			28.16	-	-

Fig. 2. **Cannabis matrices detection in FAM channel.** Blue, positive control; Orange, Flower – composite; Green, Flower – headband; Pink, Flower - cherry diesel.

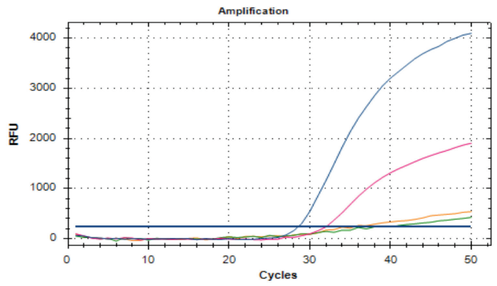
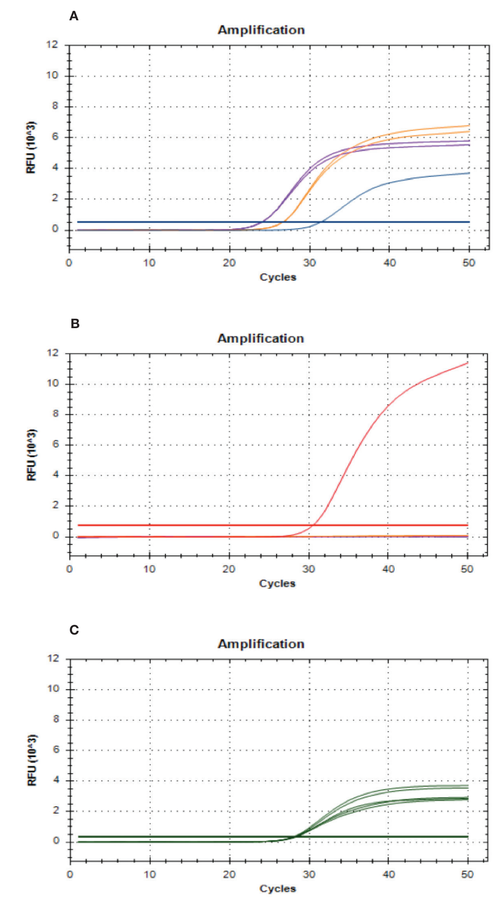


Fig. 3. **Emerald proficiency testing.** Purple, Native DNA with a mean Cq value of 23.97; Orange, DNA diluted at 1:10 with a mean Cq value of 26.62; Blue or Red, positive control; **A**, detection in FAM channel; **B**, detection in Texas Red channel; **C**, internal control in HEX channel.



Conclusions

A novel solution with a simplified real-time PCR assay to qualitatively detect *Aspergillus* species from cannabis matrices in 48 hr using BPW was developed by Bio-Rad. Preliminary results demonstrate that iQ-Check *Aspergillus* is an effective method for the detection of *A. fumigatus, A. flavus, A. niger* and *A. terreus* in a multiplex format. The inclusion of an internal control confirmed the absence of false negative results. Supplementary data obtained with the Emerald proficiency testing and the study conducted by the CDPHE Laboratory showed the efficiency of the *Aspergillus* method in cannabis matrices and significant time savings as compared to the FDA BAM method (1). Furthermore, this method enables the user to distinguish *A. terreus* from *A. flavus, A. fumigatus* and *A. niger.*

References

1 United States Food and Drug Administration. Bacteriological Analytical Manual. April 2001. Chapter 18 Yeasts, Molds and Mycotoxins

2 Klich and Pitt. (1988) Differentiation of *Aspergillus* flavus from *A. parasiticus* and other closely related species. Trans. Br. mycol. Soc. 91 (1), 99–108

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