Cannabis Microbiome Sequencing: Implications for Cannabis Safety Testing

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Acknowledgement

Many thanks to our Chief Science Officer Kevin McKernan for allowing me to use his slides to present on this fascinating and developing topic in cannabis science.
Medicinal Genomics sells reagents and develops qPCR assays for the microbial testing of cannabis.

This presentation will explore the differences in culture based methods vs. molecular methods. It will also point out some of the major pitfalls of culture.

Culture based methods are standard in food testing however cannabis is a very different and unique matrix used by patients that are often immunodeficient and therefore calls for different methodology.
Cannabis Matrix is Unique

**Flower**
- Lipid rich
- Trichomes
- Cannabinoids
- Terpenes
- Endophytes

**Extracts & Concentrates**
- Rosin
- Isolates
- Distillates
- Tinctures
- Bubble Hash
- Hydrocarbon extracts

**MIPS**
- Gummies (gelatin)
- Chocolate (fat)
- Candy (sugar)
- Just about anything
Current US Regulations

**AHP**
- Total Aerobic Count
- Total Yeast & Mold
- Total Coliform
- Bile Tolerant Gram Negative (BTGN)
- *E. coli*
- *Salmonella* spp.

**USP**
- Total Aerobic Count
- Total Yeast & Mold
- Total Coliform
- Bile Tolerant Gram Negative (BTGN)
- *E. coli*
- *Salmonella* spp.
- *Pseudomonas aeruginosa*
- *Staph. aureus*

**BCC (California)**
- Shiga-Toxin producing *E. coli* (STEC)
- *Salmonella* spp.
- *Aspergillus flavus, fumigatus, niger, & terreus*
Traditional Culture-based Methods

Examples
- Plating
- Gram-staining
- Microscopy
- Biochemical test

Advantages
- Cheap
- Sensitive (single colony)
- Reliable

Disadvantages
- Time consuming (days to grow)
- Labor intensive (multiple dilutions)
- Not all microorganisms grow on or in artificial media
Field Fail Rates

Emerald Cup Over Limit (AHPA Guidelines)

- APC: 2.7%
- Yeast and Mold: 14.3%
- Pseudomonas: 0.0%
- Salmonella: 0.0%
- Coliforms: 1.7%
- E. coli: 4.0%
- Total: 14.9%

* SC Labs, CannMed 2016
* Tests performed with 3M Petrifilm
Cannabis Microbiomes with ITS Sequencing

PathoSEEK
Over 300 16S & 18S Microbiomes later

The Medicinal Genomics team has published two different papers on cannabis microbiome sequencing and metagenomic analysis which uncovered many different pathogens and potential toxic byproducts.

Our follow up study examined the differences in microbiological testing platforms compared to molecular methods. This study exposed many discrepancies between commonly used culture based platforms 3M and BioMerieux as well as caveats with these growth mediums and the types of microorganism growth they foster.
PathoSEEK Reveals Microbiomes

qPCR

Sequencing

Microbiome ID
Diverse Microbiomes Discovered
reminder that 95% of microbes don’t culture
The role of potassium BK channels in anticonvulsant effect of cannabidiol in pentylentetrazole and maximal electroshock models of seizure in mice

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Mechanism of citrinin-induced dysfunction

Chagas GM a, Oliveira MA, Campello AP, Kluppel ML.

Author information

Abstract

The effect of citrinin on Ca2+ transport was studied in isolated cells. The mycotoxin significantly inhibited the activity of mitochondria. Citrinin promoted a decrease in the velocity of citrinin acts by a mechanism similar to ruthenium red. In Ca2+ fluxes. Citrinin promoted a marked decrease in the fraction became less affected. All the observed effects
Confirm the Paxilline and Citrinin Genes are also present.
DNA Sequence ≠ Expression

Design an LC-MS/MS method to detect this in a flower matrix.

Orser, Marquez replicate Uhlig et al at DigiPath

Uhlig et al.
Clumping impairs sampling
CFU/g Correlations with qPCR Cq
**Aspergillus & Heterogenous Macro Colonies**

<table>
<thead>
<tr>
<th>CFU</th>
<th>Blank</th>
<th>100K</th>
<th>10K</th>
<th>1K</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cq</td>
<td>Blank</td>
<td>23.3</td>
<td>26.6</td>
<td>29.9</td>
<td>32.3</td>
</tr>
</tbody>
</table>

ATCC stocks diluted on 2 different culture based environments and compared to qPCR.
Aspergillus quantification

Declumping Heterogeneous Macro-colonies

Vortex with 1% Tween-80 and filter through 5um Millipore Durapore PVDF spin column

Unfiltered Conidia

Filtered Conidia

qPCR & Plateing
Your Eyes are Deceiving

Same Genomes  Different Genomes
Both labs tested the same edible marijuana product but used two different microbial testing methods.

<table>
<thead>
<tr>
<th></th>
<th>Lab A</th>
<th>Lab B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testing Method Used</td>
<td>qPCR</td>
<td>Culture based</td>
</tr>
<tr>
<td>Test Used</td>
<td>Species specific Aspergillus qPCR</td>
<td>Plating – Visual confirmation of Aspergillus niger</td>
</tr>
<tr>
<td>Results</td>
<td>PASS</td>
<td>FAIL</td>
</tr>
</tbody>
</table>

Why did lab A pass the sample but lab B did not? Identified as *A. brasiliensis* not *A. niger.*
Mold and Bacterial qPCR testing

- qPCR provides specific microbial risks in under 24 hours with the exception of slow growing pathogens that require enrichment.
- Most culture based systems are failing too much material while **failing to grow important risks**.
- False positive TYM tests encourage improper use of fungicides such as myclobutanil.
- Clostridium botulinum found (Anaerobe and oil loving)

Metagenomic analysis of medicinal Cannabis samples; pathogenic bacteria, toxigenic fungi, and beneficial microbes grow in culture-based yeast and mold tests [version 1; referees: 3 approved, 1 approved with reservations]

- Kevin McKeman1, Jessica Spangler1, Yvonne Helbert1, Ryan C. Lynch1, Adrian Devitt-Lee1, Lei Zhang1, Wendell Orphe1, Jason Warner1, Theodore Foss1, Christopher J. Hudalla2, Matthew Silva2, Douglas R. Smith1

Author details
Grant information
MIPS interfere with 3M TAC chemistry

TAC (regular, count at 48hr)

E. coli alone

E. coli + Candy
MIPS interfere with 3M Coliform chemistry

Coliform (regular, count at 24hr)

E.coli Alone

E.coli + Candy
3M states in their coliform protocol that citrate acts as an inhibitor to their reporter assay.

Citrate is commonly used in cannabis cultivation in the form of Cal-Mag.

Citric acid is also used in the manufacture of many MIPs as a flavoring agent.
**Pseudomonas aeruginosa** is a BTGN Bacteria

**P. aeruginosa ATCC # 9027 24hrs Growth @ 32C**

**P. aeruginosa ATCC # 10145 24hrs Growth @ 32C**

According to 3M™ Product literature on Petrifilm™ Enterobacteriaceae Count Plate, *Pseudomonas* appears as red colonies without gas or acid after 24 hours at 32C.
The bioMerieux TEMPO® platform utilizes a pH sensitive fluorescent dye to monitor the growth curve.
**P. aeruginosa Culture Passed BTGN Analysis**

Actual report from a certified MA 3rd party lab that failed a *Pseudomonas aeruginosa* culture for Total Yeast & Mold but passed it for BTGN.

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Figure 3C. *Pseudomonas aeruginosa* grown in CAMP fails Biomerieux YM assay and AC assay. It does not trigger the EB failure. It is a Bile Tolerant Gram Negative bacteria and should fail that test. Hardy Diagnostic plates deliver different results. Both Pass Pseudomonas.
Questions

1. Do original microbials match post culture microbials?

2. What % of the CFU/g are actually Yeast and Mold?
**Experimental Design Comparing Platforms**

3M & Biomerieux vs. qPCR & Sequencing

- **Capture DNA Before Culture**
- **Culture 2 different ways**
- **Before Culture DNA**
- **After Culture**

**Compare qPCR Before vs After**

- **NextGen Sequence**
Discordant Before vs After data with culture

Raises a Heisenberg Uncertainty problem for regulators

- The act of measuring microbial load, changes the microbial load.

Questions

1. Do the differing culture systems agree with one another?

2. In samples where we see discordance between culture and qPCR what are the underlying differences in the microbiome?
This figure illustrates a big problem with culture based methods.

Culturing produces a completely **distorted** picture the cannabis microbiome.

Same sample, different picture after culturing!
Bacillus

- Bacillus subtilis is another commonly used biocontrol reagent in cannabis grow operations acting as a probiotic and a fungicide
- Demonstrated to reduce harmful contaminants and plant pathogens such as Fusarium
- It’s use will trigger TAC counts but it is completely harmless to cannabis users
Trichoderma

- Trichoderma is a genus of fungi commonly used by organic cannabis growers as a biocontrol agent and probiotic.
- Trichoderma species produce a mixture of antifungal enzymes, including chitinases and β-1,3 glucanases, which work synergistically to break down the cell walls of other fungi.
- Trichoderma is harmless to cannabis users.
TYM Discordance, Failure to grow Aspergillus, & Trichoderma Beneficial Antagonism
Positive Feedback Loop

High false positive microbial tests = More pesticide use less beneficial microbe use

Cyanide inhalation
Pesticides
Fungicides top the list

* SC Labs, CannMed 2016

16S qPCR and Sequencing demonstrate bacteria grow very well in TYM cartridges.
Correlations with Bacteria are weak
18S qPCR specificity

- When 18S qPCR & Sequencing is performed how many times do we see 16S sequence hits?

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<thead>
<tr>
<th></th>
<th>BMX</th>
<th>3M</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S Primers w/ 16S hits</td>
<td>520</td>
<td>334</td>
</tr>
<tr>
<td>18S Primers w/18S hits</td>
<td>194,986</td>
<td>174,320</td>
</tr>
<tr>
<td>% off target</td>
<td>0.266685813</td>
<td>0.191601652</td>
</tr>
<tr>
<td>16S Primers with 16S hits</td>
<td>2,900,912</td>
<td>606,942</td>
</tr>
<tr>
<td>16S Primers with 18S hits</td>
<td>114,646</td>
<td>204</td>
</tr>
<tr>
<td>% off target</td>
<td>3.952067488</td>
<td>0.033611119</td>
</tr>
</tbody>
</table>

*16S in fungal Mitochondria
Experimental Design: In samples where there was pronounced discordance (i.e. where qPCR showed signal while plating showed no growth and vice versa) we picked colonies off these plates and performed 16S & 18S ITS sequencing. In almost all cases of discordant samples we find a bacterium called Ralstonia present.
What is Ralstonia?

Ralstonia is an endofungal bacteria that is both a plant and human pathogen. These bacteria infect and live inside of fungal cells. Some examples of fungal cells infected by Ralstonia include: Penicillium, Fusarium, and Aspergillus.
Why should we care?

TLDR: Ralstonia respiratory tract infections occur in immunocompromised patients. This is a pathogen that culture based methods cannot detect!
Immunocompromised patients aren't the only ones who should worry

CASE REPORT

Cryptococcal meningitis in a daily cannabis smoker without evidence of immunodeficiency

Bryan B Shapiro,¹,² Rebecca Hedrick,² Brigitte C Vanle,² Courtney A Becker,³ Chris Nguyen,³ David M Underhill,³ Margie A Morgan,⁴ Joel D Kopple,⁵,⁶,⁷ Itai Danovitch,² Waguih William IsHak²,⁶

SUMMARY

Cryptococcal meningitis is a life-threatening condition most commonly observed in immunocompromised individuals. We describe a daily cannabis smoker without evidence of immunodeficiency presenting with confirmed Cryptococcus neoformans meningitis. An investigation of cannabis samples from the patient's preferred dispensary demonstrated contamination with several varieties of Cryptococcus, including C. neoformans, and other opportunistic fungi. These findings raise concern regarding the safety of dispensary-grade cannabis, even in immunocompetent users.
Aspergillosis Presenting as Multiple Pulmonary Nodules in an Immunocompetent Cannabis User

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Case Report

Aspergillosis in the Immunocompetent

a large fungus ball with abundant compact and laminated mycelium, consistent with *Aspergillus* species. There was no evidence of parenchymal invasion, infarcts, or vascular thrombi by pathology. No cultures were obtained at the time of biopsy due to the high suspicion for malignancy based on the prior testing. Based on the pathology, the Infectious Diseases (ID) service was consulted for recommendations.

On further questioning, the patient denied prior lung disease. At the time, he did not carry a diagnosis of COPD, had never previously taken inhaled or systemic glucocorticoids, and had no history of tuberculosis. He denied exposures to construction or recent renovations that may have predisposed him to infection. He was not receiving any immunosuppressant therapy. He had undergone recent HIV testing, which was negative. He had no history or findings to support prolonged neutropenia or underlying immune deficiency. The patient was a long-standing smoker, with over a 30 pack/year history. He also reported heavy, daily alcohol use, but had no diagnosis or findings consistent with cirrhosis. He admitted to smoking one pipe of marijuana daily for over 30 years, but denied other recreational drug use.

Figure 1: Computed tomography of the chest demonstrating multiple pulmonary nodules and a spiculated lung mass in the left upper lobe. Characteristic radiologic findings that have been reported with *Aspergillus* infection include ground glass attenuation surrounding a nodule (halo sign), the later crescent sign (air from a cavity surrounding a mass), or cavitary lesions [9].
The causal agent of tuberculosis, *Mycobacterium tuberculosis* is present in the cannabis microbiome and there are documented outbreaks of this pulmonary disease via the “hotboxing” of vehicles.
1. Molecular based methods can capture specific microbial risks while many of these risks fail to be detected by culture based methods due to their slow growth in these mediums or their residence within fungal cells.

2. Culture based methods distort what is actually present on the plant and other matrices and therefore provides an inaccurate picture of microbial risks.

3. Because culture based methods can grow off target organisms this leads to inflated counts that leads to higher failure rates and increased fungicide use.

4. Complexities of different cannabis product matrices affect the readout chemistry of commonly used methods and therefore give inaccurate counts of potential contaminants present.
• If we want to reduce pesticide usage, it’s imperative to have specific microbial testing.
• Beneficial microbes like Trichoderma and Bacillus are known to reduce the need for pesticides via beneficial antagonism and often inflate numbers associated with total count tests
• Targeting specific known threats in the cannabis microbiome will yield much more meaningful results for patient safety.
Let’s get cannabis on the same footing!

**FDA is Moving to Sequence Based Safety Testing**

**GenomeTrakr: Using Genomics to Identify Food Contamination**

The FDA Foods Whole Genome Sequencing Staff is coordinating efforts by public health officials to sequence pathogens collected from foodborne outbreaks, contaminated food products and environmental sources. The genome sequences are archived in an open-access genomic reference database called GenomeTrakr, that can be used: to find the contamination sources of current and future outbreaks; to better understand the environmental conditions associated with the contamination of agricultural products; and to help develop new rapid methods and culture independent tests.

- **GenomeTrakr Fast Facts**
  - Includes updates on the number of pathogens sequenced
- **Video overview of how foodborne pathogens are isolated from a food sample and sequenced**
- **GenomeTrakr: Pushing Back the Frontiers of Outbreak Response**
- **More details about GenomeTrakr**
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Thank you!

Questions?