Pathogenic Enterobacteriaceae require multiple culture temperatures for detection in Cannabis sativa L. [version 1; peer review: awaiting peer review]

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Abstract

Background: Cannabis safety testing requires adequate detection of a broad class of bacteria known as Enterobacteria, from the family of Enterobacteriaceae. These organisms are responsible for many food-borne illnesses including gastroenteritis, and are common targets in the food testing industry. While all these organisms contain 16S DNA, not all of them grow on commercial culture-based platforms at a single culture temperature.

Methods: We assessed four Enterobacteria (Aeromonas hydrophila, Pantoea agglomerans, Yersinia enterocolitica, Rahnella aquatilis) that vary in their preferred culture temperature, human pathogenicity and prevalence in cannabis. We cultured them on two different plating media and compared these results to two different qPCR assays.

Results: All four bacteria grew on one plating medium at 30°C. 75% of them failed to grow at 36°C. Using a different plating medium, 75% grew at 30°C and zero grew at 36°C. Two different commercially available quantitative PCR assays detected 100% of the organisms.

Conclusions: Several Enterobacteria are highly medium- and temperature-sensitive, and can easily evade culture-based detection. Some of these bacteria are known to infect cannabis and may pose a clinical risk to cannabis trimmers or consumers. Quantitative PCR detected all of these species. Quantitative PCR is often criticized for failing to discern live versus dead DNA, but the definition of “live” is dependent on the culture medium and temperature used.

Keywords
Cannabis, Enterobacteriaceae, Microbiome, Whole Genome Sequencing, qPCR, culture
**Introduction**
Cannabis safety testing requires a complex array of proficiencies, including expertise in the detection and quantification of cannabinoids, pesticide residues, heavy metals, volatile compounds, mycotoxins and microbial burden. Each one of these fields of expertise is incredibly nuanced and often suffer from a lack of readily available laboratory standards that can easily cross state lines with the current federal interstate commerce laws in the United States.

The 2018 Farm bill enabled the transport of hemp (<0.3% THC) across state lines. As a result, the cannabis microbial space has recently introduced AOAC Standard Method Performance Requirements (SMPRs) and microbial Certified Reference Standards (CRMs) that contain relevant hemp matrix. These tools are welcomed by the industry; however the microbial testing space remains conflicted over the lack of a gold standard.

Traditional reference methods used in the food industry have long used cellular culture or petri-dish plating technologies to assess microbial burden. However, not all organisms can be cultured and often the organisms that can require different growth temperatures, media and time to propagate. These conditional modes of detection often fail to identify relevant pathogens.

Additionally, culturing human pathogens can present additional risks to laboratory personnel or civilians, given the frequently documented cases of laboratory leaks. Molecular methods using gDNA or RNA can provide more comprehensive surveys. Replication of the organism’s DNA or RNA can reduce and, in some cases, even eliminate the replication of the pathogen. These methods are less reliant on the viability of the organism or its required carbon sources. These techniques are also less susceptible to undercounting viable but not culturable organisms (VBNC) or sublethal injuries that many decontamination or “curing” techniques induce. “Decontaminated” cells are often still metabolically active and require more time to culture as the organism must first repair the 8-oxo-G damage to their DNA prior to replication.

Many of the decontamination protocols used in the cannabis field have limited peer reviewed evidence of the degree of sublethal injury and the shelf life of the decontaminated product. Often, products that pass microbial testing may be found to have excessive mold or bacterial growth at a later date in the supply chain. This often directs growers to ‘lab shop’ for laboratories utilizing methods that are blind to a particular decontamination technique or culturing platform that can’t detect their most common contaminants.

Here we describe one scenario where such lab shopping would improve pass rates for a given grower but expose patients to pathogenic risks. We also investigated the abundance of these microbes in published cannabis microbiome studies.

Four Enterobacteria (Aeromonas hydrophila, Pantoea agglomerans, Yersinia enterocolitica, Rahnella aquatilis) were acquired from the American Type Culture Collection (ATCC) and plated on various media and temperatures; we then compared the quantity of 16S DNA from these organisms using qPCR.

**Methods**

*Aeromonas hydrophila, Pantoea agglomerans, Yersinia enterocolitica, Rahnella aquatilis* were acquired from ATCC (ATCC#7966, ATCC#43348, ATCC#9610, ATCC#833990). ATCC recommends 30°C, 26°C, 30°C, 30°C for the growth of these respective organisms. Since Enterobacteria testing is usually done at 36°C, we plated organisms at 36°C and 30°C. To improve the visibility of some species, each detected colony was manually marked in blue in with a sharpie. We compared these CFUs to Ct values generated using the Medicinal Genomics Entero qPCR assay (#420108) and Medicinal Genomics TAC qPCR assay (#420106).

Organisms were resuscitated from lyophilized stocks by inoculation into 30ml of Tryptic Soy Broth (TSB) without selection for overnight static growth, according to the ATCC recommended growth temperatures listed above. Cells/ml were estimated via serial dilution and plating on both 3M EB Petrifilm and 3M RAC Petrifilm. Once a colony forming unit (CFU/ml) of each growth was observed, these counts were used to make four different dilutions targeting the same final CFU concentration in triplicate (12 total Petrifilm per organism per temperature point). This two-stage process was performed to avoid too numerous to count (TNTC) plates. A 1ml extract of each final dilution was plated on each plate.

For qPCR, a 10-fold serial dilution of each stock growth (into ddH2O) was performed starting at 1/10⁰, 1/10⁰, 1/1,000⁰, 1/10,000⁰ and 1/1,000,000⁰. These were purified according to the manufacturer’s instructions, and subjected to qPCR with two different qPCR assays (Medicinal Genomics TAC assay #420106 and Medicinal Genomics Entero assay #420108).

PCR cycling (according to the manufacturers instructions) was performed with an initial 95°C denaturization for five minutes, 40 cycles of 95°C for 15 seconds and 65°C for 1 minute.

Since many organisms and growth conditions produced no colonies, only the presence or absence of a signal during qPCR was evaluated. These organisms are found in many inclusion and exclusion documentation for culture-based enumeration products currently AOAC approved for use. Many of these accreditation bodies like AOAC look to see Ct data correlated with CFUs. These correlations will be impossible to draw if many organisms fail to form colonies but consistently amplify with qPCR.

Read analysis was performed using the OneCodex bioinformatics platform (Underlying data). The microbiome platform contains 171 public cannabis microbiome libraries. The data includes previously published 16S amplicon sequences and whole genome sequencing of colonies derived from cannabis flowers. Read totals and species abundance calculations were derived from parsing the results of the CSV download of the Complete.
Result Table for each of the 171 classification analyses and counting up ‘Reads with Children’ for each result with the rank of species. Links to these data are supplied in the Extended data\textsuperscript{22}. Libraries containing fungal ITS amplification were omitted from the search.

**Results**

*Aeromonas hydrophila* and *Rahnella aquatilis* failed to grow at 36°C on two different plating media (3M RAC and EB Petri-films) but successfully grew on these media at 30°C. *Yersinia enterocolitica* also failed to grow at 36°C on EB plates but grew successfully at 30°C on EB plates and grew at both 30°C and 36°C with RAC plates. *Pantoea agglomerans* only grew on RAC plates at 26°C and 30°C and did not grow on EB plates at any temperature (Figure 1–Figure 4 and Table 1). Quantitative PCR detected all four organisms (Figure 5).

Previously published cannabis microbiome studies were searched for sequencing read abundance of the four microbes (Underlying data\textsuperscript{24}). Three of 171 samples contained *Aeromonas* sequences...
over 1% read abundance, while *Pantoea agglomerans* was above 1% in 30/171 samples and even consisted of over 87% of the reads in one sample (Figure 6). Four other Pantoea were also found in the cannabis microbiome data, namely *Pantoea ananatis*, *Pantoea dispersa*, *Pantoea stewartii* and *Pantoea cedenensis*. These are all available from ATCC and have recommended growth temperatures of 28°C, 26°C, 26°C, and 30°C, suggesting more organisms native to cannabis may be missed by plating at a single temperature.

**Discussion**

*Aeromonas hydrophila* is responsible for 13% of gastroenteritis cases in the US. It has been detected in 3/171 microbiome sequencing samples (McKernan et al. 2016) at very low read levels (Figure 6). This is consistent with many fecal-oral pathogens that are not native to cannabis plants.23

*Pantoea agglomerans* is less pathogenic than *Aeromonas hydrophila* but is ubiquitously found in multiple independent cannabis microbiome studies with both PCR and culture-based plating19,21,26. It was seen in 30/171 microbiome sequencing samples and is often the most abundant read count even in 16S amplification surveys (Figure 7). Several samples with high *Pantoea* read abundance are a result of whole genome shotgun surveys of isolated colonies from metagenomic surveys performed on TYM studies utilizing potato dextrose agar (PDA) as a growth medium20.

*Pantoea agglomerans* is described as a plant growth-promoting rhizobacteria for Cannabis27,28. Cruz et al. documented 53 pediatric cases of *Pantoea agglomerans* infections, mostly from penetrating trauma from vegetative matter or catheter-related bacteremia29. Seok et al. described a case of *Pantoea agglomerans*-induced bilateral endophthalmitis30. The skin rashes and infections, described by Okwundu et al. may be relevant for trimmers in constant contact with cannabis plant matter and sharp trimming tools31.

McKernan et al. reported *Pantoea agglomerans* growing more frequently on PDA-25°C than PDA with Chloramphenicol
(PDA-CAMP-25°C) or Dichloran Rose Bengal with CAMP (DRBC-25°C)\textsuperscript{19}. These culture media are used for TYM detection and they consistently harbor the off-target growth of a common Enterobacteria found on Cannabis. The failure of Enterobacteria plating media to culture one of the most common Enterobacteria (at 36°C) found in cannabis will lead to continual discordance of molecular methods compared to plating systems.

\textit{Yersinia enterocolitica} is listed by the American Center for Disease Control (CDC) as a pathogen of concern. The CDC stated that \textit{Yersinia enterocolitica} is “responsible for 117,000 illnesses, 640 hospitalizations and 35 deaths every year in the US” and is one of many \textit{Yersinia} that cause yersiniosis\textsuperscript{33}. This is recognized as a fecal-oral transmitted infection, usually from contaminated water on outdoor farms with livestock\textsuperscript{33}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{\textit{Yersinia enterocolitica} plated on RAC and EB 3M Petrifilms, grown for 24 hours at 30°C and 36°C (Colonies were stained in blue for additional contrast).}
\end{figure}
*Rahnella aquatilis* is more commonly found in water supplies\(^{11}\). The CDC discovered their first clinical isolate in 1985 from a burn wound, but it has been detected in various bodily fluids from urine, sputum, stool and bronchial lavage\(^{15}\). Most cases involve immunocompromised hosts. Urinary tract infections and sepsis are documented in the clinical literature\(^{15,36}\).

**Figure 4.** *Rahnella aquatilis* plated on RAC and EB 3M Petrifilms, grown for 24 hours at 30°C and 36°C.
Table 1. Summary table of growth performance of four organisms across multiple media and temperatures. RAC = 3M rapid aerobic count plate. EB = 3M enterobacteria plate. qPCR TAC = qPCR total aerobic count assay. qPCR Entero = qPCR enterobacteria assay.

| Organism                  | RAC 26°C | RAC 30°C | RAC 36°C | EB 26°C | EB 30°C | EB 36°C | qPCR TAC | qPCR Entero |
|---------------------------|----------|----------|----------|----------|----------|----------|----------|-------------|
| Aeromonas hydrophila      | NA       | +        | -        | NA       | +        | -        | +        | +           |
| Pantoea agglomerans       | +        | +        | -        | -        | -        | -        | +        | +           |
| Yersinia enterocolitica   | NA       | +        | -        | NA       | +        | -        | +        | +           |
| Rahnella aqualitis        | NA       | +        | -        | NA       | +        | -        | +        | +           |

Conclusions

There is no universal carbon source or temperature that can capture all pathogenic risks on cannabis. These are inhaled products and should be held to higher standards than orally ingested products.

Regulators may be tempted to default to the pre-existing tools given their long history in the food industry. We described a scenario in the cannabis testing industry where this misplaced trust in traditional methods will harm the patient but improve profits of growers. With the high prevalence of *Pantoea agglomerans* in multiple cannabis microbiome and plate-based surveys of cannabis matrices, defaulting to a platform with known blind spots will lead to further conflict in the industry.

Given that *Pantoea agglomerans* is one of the few published plant-growth promoting organisms in cannabis, and the frequency at which it grows on total yeast and mold medium, as well as its failure to grow at the same temperature as other Enterobacteria, this organism will continue to confuse culture-based microbial detection platforms. It should be noted that the Enterobacteria regulations are often more stringent than total yeast and mold regulations (1,000 CFU/g versus 10,000 CFU/g), and organisms that fail to grow on Enterobacteria plates but do grow on total yeast and mold plates may never trigger a positive test. A range of 1,001 to 9,999 CFU/g of *Pantoea agglomerans* will fail to be detected on Enterobacteria plating tests (at 36°C) while also passing a TYM test using PDA.

*Pantoea agglomerans* is also CAMP-sensitive which explains its reduced prevalence in TYM testing using CAMP selection13. It is possible for CAMP-based TYM testing, 3MEB Petrifilm testing and 3M RAC Petrifilm testing to fail to detect this organism if multiple growth temperatures are not utilized. Higher-specificity molecular methods offer a more parsimonious solution to this problem.

qPCR is often criticised as an inadequate replacement to petri-based enumeration methods because of its lack of concordance to plating. This becomes a circular argument when the plating methods have known and obvious blind spots. In this study, qPCR detected all of the pathogens and plating failed to culture all of them on 3M EB Petrifilm plates at 36°C, and failed to culture 75% of them on RAC plates at 36°C. This is a scenario where qPCR might be accused of detecting non-viable organism, when the organisms are, in fact, viable except on the chosen medium or temperature.

PCR can amplify non-viable organisms’ DNA. Tools are available to remove free circulating DNA from lysed cells using nucleases that can be chemically inactivated prior to PCR15. The efficacy of nucleases on VBNC organisms whose cell membranes or cell walls are still intact is a nascent field that needs further investigation. PCR can detect VBNC organisms, while plating requires much longer incubation times and potentially unique medium to allow these organisms to resuscitate. This brings the shelf life of products that pass short duration culture testing into sharp focus, as only organisms that can actively replicate with the proper temperature and carbon source can be detected. Molecular methods offer a more universal detection platform, as all organisms have DNA and non-viable or lysed organisms’ DNA are nuclease-sensitive and easy to account for. The sub-lethal injuries or VBNC states of microbes on partially decontaminated or cured product requires further discussion regarding the goal of microbial testing. For example, dried foods require longer incubation times to properly adjust for microbial resuscitation that can occur on products with long shelf lives. Cannabis flowers are often dried for two weeks and their microbiome may resemble that often seen in dried foods16,30.

The inability to detect these organisms at temperatures lower than body temperature (30°C versus 36°C) is often justified as being an irrelevant temperature for human health. This is not supported by the clinical literature where these organisms, despite their lower ex vivo culturing temperature, still infect humans. Not all compartments of the human body are at a single temperature or supply a fixed low complexity carbon source. This is highly relevant to viral tropism in the human respiratory pathway and is believed to drive much of the seasonality of influenza and coronaviruses16. We have evidence that these organisms infect humans and that they remain undetected on an inhaled product when using a single medium and temperature for replicative detection. If we continue to demand more modern, more...
Figure 5. qPCR results for DNA purified from cultures of *Aeromonas hydrophila*, *Pantoea agglomerans*, *Yersinia enterocolitica*, *Rahnella aquatilis*. Each qPCR reaction contained a spike-in cannabis internal control in the HEX (green) channel. The target total aerobic count (TAC) or Entero qPCR assay is labelled with a fluorescein amidite (FAM) dye (Blue). qPCR is performed according to the manufacturer’s instructions.
Figure 6. *Aeromonas* was detected in 16 samples at low levels (note log scale of reads on Y axis). 3/172 samples were over 1% of the reads.

Figure 7. Top: *Pantoea* Illumina Read Abundance in 30/171 microbiome samples. Bottom: normalized Illumina read abundance. Whole genome shotgun sequencing of isolated colonies is represented as (*). Other samples are metagenomic sequences from 16S amplification.
sensitive and more specific technologies like qPCR that perfectly emulate previous culture-based technologies which fail to culture specific microbes, we will not only fail to advance the field of clinical microbiology, but we will fail patients as well.

Data availability

Underlying data

Figshare: qPCR data for Pathogenic Enterobacteriaceae require multiple culture temperatures for detection in Cannabis sativa L., https://doi.org/10.6084/m9.figshare.1935075

This project contains the following underlying data:
- Y.Enterocolitica_A.HydropHilla_Dilution_TAC_Entero_assays.csv (qPCR results)

Figshare: Pathogenic Enterobacteriaceae require multiple culture temperatures for detection in Cannabis sativa L., https://doi.org/10.6084/m9.figshare.19346411.v2

This project contains the following underlying data:
- Petrifilm data (culture plate detection images)

Figshare: Pantoea_Aeromonas_OneCodex, https://doi.org/10.6084/m9.figshare.19179140.v1

This project contains the following underlying data:
- pantoeapantoea_agglomerans_submit021520223.xlsx (sequencing read abundance data for the four assessed Enterobacteria)

Data are available under the terms of the Creative Commons Attribution 4.0 International license (CC-BY 4.0).

Extended data

NCBI SRA: Cannabis microbiome sequencing reveals several mycotoxic fungi native to dispensary grade cannabis flowers, Accession number SRX1441690: https://identifiers.org/insdc.sra:SRX1441690

BioProject: Cannabis microbiome evolution in culture, Accession number PRJNA343388: https://identifiers.org/bioproject:PRJNA343388

BioProject: Under Counting of Total Yeast and Mold on Cannabis using DRBC, Accession number PRJNA725256: https://identifiers.org/bioproject:PRJNA725256

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