New York State Department of Health - Wadsworth Center
Laboratory of Environmental Biology
NYS ELAP Laboratory ID 10765

Division of Environmental Health Sciences
Albany, New York

NYS DOH LEB-607

Identification of *Clostridium botulinum* in Medical Marijuana Products

Contents

1.0. Scope and Application

1.1. This method, NYS DOH LEB-607, Identification of *Clostridium botulinum* in Medical Marijuana Products, describes methods for detecting and identifying *Clostridium botulinum* (ELAP Method ID 9976) in medical marijuana samples as required in Title 10 (Health), Subpart 55-2.15 and Chapter XIII, Section 1004.14 of the official Compilation of Codes, Rules, and Regulations, of the State of New York. It is used as a follow-up to NYS DOH LEB-604 section 9.4, or section 9.7 (after verification growth), and applies to sample enrichments showing growth in Reinforced Clostridial Medium under anaerobic conditions at 30-35°C.

1.2. Protocols for the identification of these organisms in samples of medical marijuana products can be found in the NYS DOH LEB-600 series. See Medical Marijuana Microbial Testing Plan flowcharts.

2.0. Summary of the Method

2.1. Medical marijuana samples showing growth in Reinforced Clostridial Medium are subcultured onto Columbia agar plates and incubated at 30-35°C under anaerobic conditions for 48-72 hours. Bacterial colonies are transferred to Blood Agar plates and identified using API® 20A identification strips. Samples producing bacterial colonies on Columbia agar that are identified as *C. botulinum* is are reported as positive.

3.0. Definitions

3.1. RCM stands for Reinforced Clostridial Medium

3.2. COA stands for Columbia Agar

3.3. PBST stands for Phosphate Buffered Saline, pH 7.2, containing 0.1% Tween® 80.

3.4. Polysorbate 80 (Tween® 80) is a nonionic surfactant and emulsifier.

3.5. TSAB stands for Trypticase Soy Agar with 5% Sheep Blood

4.0. Health and Safety Warnings

4.1. Microbiological analyses involve the culturing of potentially pathogenic organisms.

4.1.1. All microbiologically contaminated materials, including media, shall be autoclaved after use.

4.1.2. Laboratory equipment and benches shall be disinfected using either Envirocide®, 10% bleach, 70% ethanol before and after use.

4.1.3. Mouth pipetting is prohibited.

4.1.4. All accidents, particularly those which may result in infection, shall be reported according to laboratory specific procedures.

4.1.5. The analyst must be familiar with any possible hazards from the reagents and standards used for sample preparation and analysis.

4.1.6. Always follow guidelines listed in safety data sheets (SDS) for proper storage, handling, and disposal of samples, solvents, reagents, and standards. SDSs are located within the laboratory. These guidelines must be made available to all personnel involved in microbiological analyses.

4.1.7. Appropriate lab coat, safety glasses and gloves must be worn when performing standard or sample preparations, working on instrumentation, disposing of waste, and cleaning laboratory equipment.

5.0. Shipping Conditions, Receiving, Preservation and Storage

5.1. Sample Shipping Conditions

5.1.1. The medical marijuana products from the Registered Organizations (ROs) are shipped as per manufacturer's specifications and must adhere to all regulatory requirements.

5.2. Sample Receipt

5.2.1. Medical marijuana products from the RO are received, verified and documented ensuring that method, regulatory and Accreditation Body requirements are met.

5.3. Method Holding Times

5.3.1. This procedure is initiated upon completion of the Microbial Presence/Absence Test for Medical Marijuana method (see NYS DOH LEB-604).

5.4. Preservation

5.4.1. Presence-Absence test aliquots that are presumptive positive for *Clostridium botulinum* are stored refrigerated until it has been determined that they are not needed for additional microbiological evaluation.

5.5. Storage

5.5.1. If storage is required prior to analysis, isolates or archived plates are stored refrigerated until it has been determined that they are not needed for additional microbiological evaluation.

6.0. Interferences

6.1. Some components of medical marijuana products, e.g., ethanol, may inhibit the growth of microorganisms.

7.0. Apparatus and Materials

7.1. Equipment

7.1.1. Incubator, set at 30.0-35.0°C
7.1.2. Automatic pipettors and sterile aerosol-resistant micropipette tips
7.1.3. Sharpie or equivalent
7.1.4. Disposable sterile inoculating loops, 10µL
7.1.5. Disposable sterile inoculum spreader, or equivalent
7.1.6. Disposable sterile microfuge tubes
7.1.7. Anaerobic jar
7.1.8. GasPak™ EZ Anaerobe sachet – BD BBL, cat. no. 260678, or equivalent
7.1.9. Dry Anaerobic Indicator Strips – BD BBL, cat. no. 271051, or equivalent
7.1.10. Biosafety cabinet with HEPA filter

7.2. Reagents and Chemicals

7.2.1. Phosphate Buffered Saline pH 7.2, 10 and 100 mL aliquots in tubes/bottles, containing 0.1% Tween 80 (PBST).

7.2.2. COA plates, 15 x 100mm. Ensure that the formulation is in agreement with that specified by USP.

7.2.3. TSAB plates, 15 x 100mm. Ensure that the formulation is in agreement with that specified by USP.

7.2.4. Disinfectants such as Envirocide® (Fisher Scientific cat. no. 19898220), 70% ethanol, and/or Clorox.

7.3. Forms

7.3.1. *Clostridium botulinum* Identification Result Sheet (e.g., LEB-RS-607A).

7.3.2. API® 20A Identification Results Sheet

7.3.3. Walk-In Temperature Record

7.3.4. Cold Room Temperature Record

7.3.5. Refrigerator Temperature Record

8.0. Quality Control/Assurance

8.1. Method Detection Limits

8.1.1. Method Detection Limits are product-specific and are determined in accordance with relevant standards, regulations and Accreditation Body requirements.

8.2. Calibration and Standardization

8.2.1. Incubator temperatures shall be observed and recorded twice daily, separated by at least 4 hours.

8.2.1.1. Temperature of the 30.0-35.0°C walk-in is recorded.

8.2.1.1.1. If the incubator temperature does not stay within 30.0-35.0°C, laboratory-specific corrective actions are followed. Analytical results may be invalidated if the incubator temperature exceeds 35.0°C, at the discretion of laboratory.

8.2.2. Temperatures of the cold room and refrigerators are observed and recorded twice daily separated by at least 4 hours.

8.2.2.1. If the cold room or refrigerator does not stay within 1.0-8.0°C, laboratory-specific corrective actions are followed.

8.2.2.2. The optimum temperature range for a cold room or refrigerator is 1.0-4.0°C

8.2.2.3. If the cold room or refrigerator was in a defrost cycle at the time that the temperature was recorded, and the temperature does not reach 8.0°C, re-testing of media is not required.

8.2.2.4. Media may be re-tested for quality, depending on the number of degrees and the amount of time that the cold room or refrigerator temperature was out of compliance, at the discretion of the laboratory.

8.2.3. Max/min temperatures are recorded when twice-daily temperature measurements are not possible, such as on holidays and weekends.

8.2.4. Thermometers must be verified as prescribed by the Accreditation Body and in accordance with relevant regulations and standards.

8.2.5. The volumetric accuracy of automatic pipetters and serological pipettes is determined as prescribed by the Accreditation Body and in accordance with relevant regulations and standards.

8.2.6. The intensity and efficacy of the UV light in the biosafety cabinet is determined as prescribed by the Accreditation Body and in accordance with relevant regulations and standards.

8.2.7. Biosafety cabinets are certified annually.

8.3. Quality Control

8.3.1. Comparative recovery and sterility between lots of COA, PBST, and TSAB will be determined as prescribed by the Accreditation Body and in accordance with relevant regulations and standards.

8.3.2. Agar plates can be used for up to 2 weeks after the preparation date if stored refrigerated in plastic bags and in the dark.

8.3.2.1. Agar plates can be used after 2 weeks storage if ongoing QC demonstrates no loss in selectivity or growth promotion.

8.3.2.2. Analyses must be completed prior to the expiration date of the media and analyses must not be initiated on the day media expires.

8.3.3. Liquid media shall be stored in tightly-capped bottles in the dark at 4°C for up to 3 months from the date of preparation.

8.3.3.1. Liquid media can be used after 3 months storage if ongoing QC demonstrates no loss in selectivity or growth promotion.

8.3.3.2. Analyses must be completed prior to the expiration date of the media and analyses must not be initiated on the day media expires.

8.3.4. Sterility of disposable loops and spreaders is determined as prescribed by the Accreditation Body and in accordance with relevant regulations and standards.

8.3.5. The use test for reagent water is performed annually, when cartridges are changed, or repairs are made to the deionized water systems.

8.4. Corrective/Preventive Actions

8.4.1. The laboratory will initiate non-conformances and/or corrective/preventive actions in accordance with laboratory-specific procedures and as prescribed by the Accreditation Body and in accordance with relevant regulations and standards.

9.0. Procedure

9.1. General

9.1.1. Aseptic technique is used for all procedures.

9.1.1.1. Aseptic technique can be found in a general microbiology textbook or on-line.

9.1.2. All work surfaces are disinfected prior to subculturing and colony identification.

9.1.3. Subculturing and colony identification are performed in a different location than sample preparation and initial sample analyses to prevent cross-contamination of incoming products.

9.1.4. Pre-reduce the COA and TSAB plates by placing them in an anaerobic container with a GasPak™ sachet and Dry Anaerobic Indicator Strip and incubate them either refrigerated or at room temperature at least overnight.

9.1.4.1. Follow manufacturer's instructions for GasPak™ and Dry Anaerobic Indicator Strips.

9.1.4.1.1. Dry Anaerobic Indicator strips are blue under aerobic conditions and white under anaerobic conditions.

9.1.4.1.2. If the color strip indicates the presence of aerobic conditions after incubation overnight in the anaerobic chamber, transfer the plates to a different anaerobic chamber and replace the GasPak™ and indicator strip.

9.1.4.1.3. If anaerobic conditions are still not achieved, follow laboratory specific corrective actions.

9.1.5. All organisms isolated from turbid EEBM enriched sample must be identified regardless of if they are a regulated organism.

9.2. Subculture

9.2.1. For each turbid RCM sample enrichment produced according to NYS DOH LEB-604 section 9.4, remove either three or five pre-reduced COA plates from the cold room and warm to room temperature.

9.2.1.1. Two COA plates will be used for turbid untreated sample enrichments, two plates for turbid heat-treated samples, and one for either the corresponding matrix spike or positive control.

9.2.1.2. If the RCM for the heat-treated sample enrichment is not turbid, only three COA plates are required.

9.2.2. Use an inoculating loop to streak the sample from the turbid RCM enrichment that has not been heat-treated onto two COA plates.

9.2.3. If turbid, use a separate inoculating loop to streak from the turbid RCM sample enrichment that was inoculated with the heat-treated sample onto two COA plates.

9.2.4. Use a separate inoculating loop to streak the sample from the corresponding turbid matrix spike enrichment onto a COA plate for colony isolation.

9.2.4.1. If there isn't a corresponding matrix spike, streak an isolated colony of *C. sporogenes* ATCC 11437 from the monthly transfer plates (prepared according to lab procedures) as a positive control.

9.2.5. Once the COA plates have dried, invert and place in an anaerobic container with a GasPak™ sachet and Dry Anaerobic Indicator strip and incubate at 30.0-35.0°C for 48-72 hours under anaerobic conditions (see section 9.1.4.).

9.2.6. After incubation, record the results for the untreated sample and heat-treated sample (if applicable) COA plates as "Y" for growth-positive (bacterial colonies are present) or "N" for negative (bacterial colonies are absent) in the "COA Sample Result" section of the *Clostridium botulinum* Identification Results Sheet (e.g., LEB-RS-607A)

9.2.6.1. If there is growth on COA, proceed to 9.3.

9.2.6.2. If there isn't any growth on the COA plates, the sample is negative for the presence of *C. botulinum.*

9.2.6.3. If the turbidity in the RCM was confirmed as growth in LEB-604 section 9.7, but there was no growth on the COA plates, proceed to 9.4 to identify any organisms isolated on nonselective agar plates from NYS DOH LEB-604 section 9.7.

9.2.7. After incubation, record the growth of either the matrix spike or positive control COA plates as "Y" for growth-positive (bacterial colonies are present) or "N" for negative (bacterial colonies are absent) on the "COA M.S./P.C. Result" section of the *Clostridium* Identification Results Sheet (e.g., LEB-RS-607A).

9.2.7.1. If the sample result is negative on COA, it is not necessary to identify colonies on a matrix spike or positive control plate that show morphology typical of *C. sporogenes* ATCC 11437.

9.2.7.1.1. *C. sporogenes* ATCC 11437 appears as buff-colored colonies showing medusa-like morphology.

9.2.7.2. If the matrix spike result is negative on COA, turbidity in the RCM may have been caused by matrix characteristics. The test results are valid if the positive and negative controls for aerobic plate counts and mold plate counts meet QC criteria (see NYS DOH LEB-605).

9.2.7.3. If the matrix spike result is negative on COA, and the positive and negative controls for aerobic plate counts and mold plate counts do not meet QC criteria (see NYS DOH LEB-605), results are considered invalid and the analyses must be repeated.

9.2.7.3.1. Additional testing using USP methods designed to decrease inhibitory effects of product that could result in growth in matrix spike samples may be undertaken using archived samples, at the discretion of the Laboratory Director.

9.2.7.4. If a positive control is analyzed instead of a matrix spike, and the results are negative, the results are considered invalid and analyses must be repeated.

9.3. Colony Identification

9.3.1. Select one or more well-isolated colonies having distinct morphologies from the growth-positive untreated sample, heat-treated sample (if applicable), and matrix spike/positive control COA plates and record the sample sources and colony morphologies on the *Clostridium botulinum* Identification Results Sheet (e.g., LEB-RS-607A).

9.3.1.1. If growth is confluent on any of the COA plates, re-streak for isolation of individual colonies and proceed with 9.3.1.

9.3.2. For each colony being identified, remove one pre-reduced TSAB plate from the cold room and warm to room temperature.

9.3.2.1. The TSAB plate must be pre-reduced according to section 9.1.4.

9.3.3. Use an inoculating loop to inoculate each colony into a separate sterile microcentrifuge tube containing 100µL of sterile PBST.

9.3.3.1. Bacterial colonies can be selected from either of the two COA sample plates.

9.3.4. Vortex each tube to mix thoroughly then spread the 100µL onto a pre-reduced TSAB plate.

9.3.5. Select a well-isolated colony showing characteristics of *C. sporogenes* ATCC 11437 from either the matrix spike or positive control COA plate, place into a sterile microcentrifuge tube containing 100µL of sterile PBST, vortex to mix then spread onto pre-reduced TSAB plates as indicated in 9.3.3-9.3.4.

9.3.5.1. Record the sample source and colony morphology on the *Clostridium botulinum* Identification Results Sheet (e.g., LEB-RS-607A) of individual colonies and proceed with 9.3.4.

9.3.6. Once the TSAB plates have dried, invert and place in the anaerobic container with a GasPak™ sachet and Dry Anaerobic Indicator strip, and incubate at 30.0-35.0°C for 18-24 hours under anaerobic conditions.

9.3.7. After incubation, use the growth on the TSAB plates to perform a gram stain following the instructions given in NYS DOH LEB-613.

9.3.8. Record the results of the gram stain on the *Clostridium botulinum* Identification Results Sheet (e.g., LEB-RS-607A).

9.3.9. Proceed with the API® Identification Test Strip method to identify the organism using the API® 20A Test Strips.

9.3.9.1. Attach all API® 20A Identification Results sheets to the *Clostridium botulinum* Identification Results Sheet (e.g., LEB-RS-607A).

9.3.9.2. The API® 20A test kit is unable to differentiate between *C. botulinum* and *C. sporogenes*. It is important to identify the species to determine if the contamination is endogenous to the sample or due to laboratory contamination. If the sample results indicate *C. botulinum*/*C. sporogenes* proceed to section 9.4 to differentiate between the two.

9.3.10. If the API® 20A Identification Test kit fails to identify the isolate proceed to 9.4.

9.4. Identification of Non-Target Organisms and/or *Clostridium* Speciation

9.4.1. The identification of either isolated *C. botulinum* species and/or non-regulated bacterial contaminants is required.

9.4.2. In cases where there is growth of a non-regulated analyte(s), consultation with the NYS Medical Marijuana Program is required.

10.0. Data Acquisition, Reduction, Analysis, Calculations, Acceptance Criteria and Documentation

10.1. Record the accession number, analyst initials, Gas Pak lot and expiration date, indicator strip lot and expiration date, COA lot date, incubation start and end dates and times, TSAB lot date, incubation start and end dates and times, colony morphology, source of colony (sample, matrix spike, or positive control), Gram stain results, colony identification and results on the *Clostridium botulinum* Identification Results Sheet (e.g. LEB-RS-607A).

10.2. If the identified organism(s) is *C. botulinum*, circle "Positive for *C. botulinum*", otherwise circle "Negative for *C. botulinum*" on the *Clostridium botulinum* Results Sheet (e.g., LEB-RS-607A).

10.3. If an identified organism(s) isn't a regulated analyte, check the comment at the bottom of the form, and record the identified organism on the *Clostridium botulinum* Results Sheet (e.g., LEB-RS-607A).

10.3.1. The comment at the bottom of the results sheet is added as a note in the final report.

10.4. If an identified organism(s) is a regulated analyte that is not *C. botulinum*, the sample is reported as negative for *C. botulinum*, but a note is entered onto the report explaining that the organism was isolated from the *C. botulinum* presence/absence analyses.

10.5. If at any point in the analyses the matrix spike fails to grow in any medium, the test results are valid as long as the positive and negative controls for the aerobic plate counts and mold plate counts meet QC criteria (see NYS DOH LEB-605).

11.0.Method Performance

11.1. Demonstration of Capability

11.1.1. Prior to acceptance and use of this method for data reporting, a satisfactory initial demonstration of capability (DOC) is required. Thereafter, an ongoing DOC is to be performed annually.

11.1.2. An initial DOC shall be made prior to using any method, and at any time there is a change in instrument type, personnel or method or any time that a method has not been performed by the laboratory or analyst in a twelve (12) month period.

11.1.3. All DOCs shall be documented, and all data applicable to the demonstration shall be retained and readily available at the laboratory.

11.1.4. Consult state regulations and standards for additional information on performing a DOC for microbiological contaminants.

11.2. Laboratory Detection Limits

11.2.1. Detection limits are determined in accordance with relevant standards, regulations and accreditation body requirements.

12.0.Waste Management/Pollution Prevention

12.1. It is the responsibility of the laboratory to comply with all federal, state and local regulations governing waste management, particularly the hazardous waste identification rules and land disposal restrictions.

12.2. Bacterial/fungal cultures and contaminated or potentially contaminated disposable materials are disposed of in biohazardous waste cans and autoclaved prior to discarding.

12.3. Dispose of non-hazardous aqueous waste in the laboratory sink followed by flushing with tap water.

12.4. Dispose of glassware in appropriately labeled boxes. Be sure that, whenever possible, the glass has been thoroughly rinsed and is contaminant-free before disposal.

12.5. Consult federal, state and local regulations for additional information or for information on the disposal of products not described in this method.

13.0.References

13.1. APHA. *Standard Methods for the Examination of Water and Wastewater,* 23rd edition.

13.2. United States Pharmacopeia. USP38-NF33, The United States Pharmacopeial Convention, General chapters <61>, <62>, <1111>.

13.3. TNI 2016 Standards – EL-V1M2-2016-Rev2.1: Quality Systems General Requirements

13.4. TNI 2016 Standards – EL-V1M5-2016-Rev2.0: Microbiological Testing

13.5. API® 20A Test Strips Instructions for Use, bioMérieux

13.6. NYS DOH LEB-604, Microbial Presence/Absence Test for Medical Marijuana Samples.

13.7. NYS DOH LEB-605, Aerobic Bacteria and Mold Plate Count

13.8. Title 10 (Health), Subpart 55-2.15 and Chapter XIII, Section 1004.14 of the official Compilation of Codes, Rules, and Regulations, of the State of New York.

13.9. NYS DOH LEB-613, Identification of Thermophilic Actinomycetes in Medical Marijuana Products

14.0. Appendices - Forms

Clostridium botulinum **Identification Results Sheet (LEB-RS-607A)**

Incubate COA for 48-72 hours and TSAB for 18-24 hours anaerobically
(30-35°C Incubator)

Accession Number: _____ Analyst Initials: _____

Final Results (circle one): Negative for *C. botulinum* Positive for *C. botulinum* _____
(list any other organism found)

COA Start Date/Time:	_____	Sample Growth:	Y	N
COA End Date/Time:	_____	M.S./P.C. (circle one) Growth:	Y	N
COA Lot Date:	_____	Heat Treated Sample Growth:	Y	N

TSAB Start Date/Time:	_____	Gram Stain Results:	_____
TSAB End Date/Time:	_____	API® 20A Start Date/Time:	_____
TSAB Lot Date:	_____	API® 20A End Date/Time:	_____
Gram Stain Date/Time:	_____	Stain Positive Control Result:	_____
Gram Stain Lot/Exp Date:	_____	Stain Negative Control Result:	_____

Source (Sample, M.S., or P.C.), Colony Morphology and API® 20A Colony Identification

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All API® 20A Result Sheets are attached. COA = Columbia Agar, M.S. = matrix spike, P.C. = positive control plate used in lieu of a matrix spike. *C. sporogenes* ATCC 11437, used as a matrix spike, appears as beige, raised, with yellowish centers and a flattened periphery with entangled filaments (medusa head colony) on COA.
Gas Pak Lot#/Exp. Date: _____Indicator Strip Lot#/Exp Date: _____
If the contaminant is a non-regulated analyte(s), check below and fill in the organism(s) identified so the text will be entered as a note on the report: ___ "(+) for _____ (not ELAP-regulated analyte)"

Reviewed by: Date: