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**Division of Environmental Health Sciences
Albany, New York**

NYS DOH LEB-612

Identification of *Enterococcus* in Medical Marijuana Products



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1.0. Scope and Application

- 1.1. This method, NYS DOH LEB-612, Identification of *Enterococcus* in Medical Marijuana Products (ELAP Method ID 9979) describes methods for detecting and identifying *Enterococcus* spp. in medical marijuana products to support laboratory testing required by 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 9.5, and applies to sample enrichments showing growth in Trypticase Soy Broth under 5% CO₂.
- 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 Trypticase Soy Broth under 5% CO₂ at 30-35°C are subcultured onto *Streptococcus* Selection Agar and incubated at 30-35°C under 5% CO₂ for 24-48 hours. Bacterial colonies are transferred to blood agar plates and identified using API® 20Strep identification strips. Samples producing bacterial colonies on *Streptococcus* Selection Agar that are identified as *Enterococcus* spp. are reported as positive.

3.0. Definitions

- 3.1. TSAB stands for Trypticase Soy Agar with 5% sheep's blood
- 3.2. TSB stands for Trypticase Soy Broth
- 3.3. PBST stands for Phosphate Buffered Saline, pH 7.2, containing 0.1% Tween® 80.
- 3.4. SSA stands for *Streptococcus* Selection Agar
- 3.5. CoAB stands for Columbia Sheep Blood Agar

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, or a minimum concentration of 70% ethanol before and after use.
 - 4.1.3. Mouth pipetting is prohibited.
 - 4.1.4. Contaminated glassware and plastic ware shall be decontaminated prior to washing.
 - 4.1.5. All accidents, particularly those which may result in infection, shall be reported according to laboratory-specific policies and procedures.
 - 4.1.6. Laboratory safety procedures shall be followed at all times. Regulations required by federal, state and local government agencies shall be implemented and followed.



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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 Presence/Absence procedure (see NYS DOH LEB-604, section 9.5).

5.4. Preservation

5.4.1. Presence-Absence test aliquots that are presumptive positive for enterococci 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 with 5% CO₂
- 7.1.2.** Automatic pipetters 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 – e.g., BD BBL, cat. no. 260678
- 7.1.9.** Dry Anaerobic Indicator Strips – e.g., BD BBL, cat. no. 271051
- 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.** SSA, 15 x 100mm, plates.



- 7.2.3. TSAB or CoAB, 15 x 100mm, plates.
- 7.2.4. Hydrogen peroxide, 3%
- 7.2.5. Microscope slides
- 7.2.6. Disinfectants such as Envirocide® (Fisher Scientific cat. no. 19898220), 70% ethanol, and/or Clorox.

7.3. Forms

- 7.3.1. *Enterococcus* Identification Result Sheet (e.g., LEB-RS-612A).

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 incubator with 5% CO₂ is recorded on the Incubator Temperature Record.
 - 8.2.1.1.1. If the incubator temperature does not stay within 30.0-35.0°C, follow laboratory-specific corrective actions. Analytical results may be invalidated if the incubator temperature exceeds 35.0°C, at the discretion of the laboratory.
- 8.2.2. Temperatures of the cold room and refrigerators are observed and recorded twice daily separated by at least 4 hours on either the Cold Room or Refrigerator Temperature Record.
 - 8.2.2.1. If the cold room or refrigerator does not stay within 1.0-8.0°C, follow laboratory-specific corrective actions.
 - 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 calibrated as prescribed by the Accreditation Body and in accordance with relevant regulations and standards.



- 8.2.5.** Sterility of disposable loops and spreaders is determined as prescribed by the Accreditation Body and in accordance with relevant regulations and standards.
- 8.2.6.** CO₂ levels are tested monthly.
- 8.2.7.** The volumetric accuracy of automatic pipettors and serological pipettes is determined as prescribed by the Accreditation Body and in accordance with relevant regulations and standards.
- 8.2.8.** 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.
- 8.2.9.** Biosafety cabinets are certified annually.

8.3. Quality Control

- 8.3.1.** Comparative recovery and sterility between lots of SSA, and TSAB will be determined. Sterility of disposable loops and spreaders is 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 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.
- 8.3.3.** The use test for deionized water is performed annually, when cartridges are changed, or repairs are made to the deionized water systems and as prescribed by the laboratory, Accreditation Body and in accordance with relevant regulations and standards.

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 TSAB and/or CoAB 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, laboratory-specific corrective actions.

9.2. Subculture

9.2.1. For each turbid TSB sample enrichment produced under 5% CO₂ according to NYS DOH LEB-604 section 9.5 and confirmed to have growth (see NYS DOH LEB-604 section 9.7), remove three SSA plates from the cold room and warm to room temperature while drying in the biological safety cabinet.

9.2.2. Use an inoculating loop to streak the sample from the turbid TSB onto two SSA plates for colony isolation.

9.2.3. Use a separate inoculating loop to streak the matrix spike from the corresponding turbid TSB onto one SSA plate for colony isolation.

9.2.3.1. If there isn't a corresponding matrix spike, streak a loopful of the positive control *Ent. faecalis* ATCC 29212 from the monthly transfer plates.

9.2.4. Once the plates have dried, invert the plates and incubate at 30.0-35.0°C under 5% CO₂ for 24-48 hours.

9.2.4.1. Do not stack plates more than four high.

9.2.5. After incubation, record the growth for the sample on SSA plates as "Y" for growth-positive (bacterial colonies are present) or "N" for negative (bacterial colonies are absent) in the "SSA Sample Result" section of the *Enterococcus* Identification Results Sheet (e.g., LEB-RS-612A).

9.2.5.1. If the sample is growth-positive on SSA, proceed to 9.3.

9.2.5.2. If there isn't any growth on the SSA plates, the sample is negative for the presence of enterococci.

9.2.5.3. Proceed to 9.4 to identify any organisms isolated on nonselective agar plates in NYS DOH LEB-604 section 9.7.

9.2.6. After incubation, record the results of either the matrix spike or positive control SSA plate as "Y" for growth-positive (bacterial colonies are present) or "N" for negative (bacterial colonies are absent) in the "SSA

M.S./P.C. Result” section of the *Enterococcus* Identification Results Sheet (e.g., LEB-RS-612A).

9.2.6.1. If the sample result is negative on SSA, it is not necessary to identify colonies on either the matrix spike or positive control plate that show morphology typical of *Ent. faecalis* ATCC 29212.

9.2.6.1.1. Typical *Ent. faecalis* ATCC 29212 colonies are small, white and are non-hemolytic.

9.2.6.2. If the sample and matrix spike results are negative on SSA, turbidity in the TSB 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 for that sample meet QC criteria (see NYS DOH LEB-605).

9.2.6.3. If the matrix spike result is negative on SSA, 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.

9.2.6.3.1. 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.

9.2.6.4. 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 positive SSA plates and record the sample source and colony morphology on the *Enterococcus* Identification Results Sheet (e.g., LEB-RS-612A).

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

9.3.2. For each colony being identified, remove one TSAB or CoAB plate from the cold room and warm to room temperature while drying.

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

9.3.3. Using an inoculating loop, 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 SSA sample plates.

9.3.4. Vortex each tube to mix thoroughly then spread the 100µL onto the TSAB/CoAB plate.

- 9.3.5.** At a minimum, select well-isolated colonies showing characteristics typical of *Ent. faecalis* ATCC 29212 from either the matrix spike or positive control SSA plate and spread onto TSAB/CoAB plates as indicated in 9.3.3-9.3.4.
- 9.3.5.1.** Record the sample source and colony morphology on the *Enterococcus* Identification Results Sheet (e.g., LEB-RS-612A).
- 9.3.5.2.** If growth is confluent on the SSA plate, re-streak for isolation of individual colonies and proceed with 9.3.5.
- 9.3.6.** Once 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 48-72 hours under anaerobic conditions (see section 9.1.4.).
- 9.3.7.** After incubation, use the growth on the TSAB/CoAB plates to:
- 9.3.7.1.** Perform a gram stain using instructions listed in NYS DOH LEB-613.
- 9.3.7.2.** Perform a catalase test.
- 9.3.7.2.1.** Place the TSAB/CoAB plate in the biohazard hood, take off the lid, and expose the plate to oxygen for at least 30 minutes.
- 9.3.7.2.2.** Transfer a small amount of growth to a clean, dry microscope slide using an inoculating loop.
- 9.3.7.2.3.** Add a drop of 3% hydrogen peroxide to the slide and mix with the inoculating loop.
- 9.3.7.2.4.** A positive result is the rapid evolution of oxygen (5-10 seconds) as evidenced by bubbling.
- 9.3.7.2.5.** Use *Ps. aeruginosa* ATCC 9027 as a positive control and *Ent. faecalis* ATCC 29212 as a negative control.
- 9.3.7.3.** Record the results of the gram stain and catalase test on the *Enterococcus* Identification Results Sheet (e.g., LEB-RS-612A).
- 9.3.8.** If the organisms are gram positive cocci and catalase negative, use the growth on the TSAB/CoAB plates to proceed with the API® Identification Test Strip method to identify the organisms using the API® 20Strep Test Strips.
- 9.3.8.1.** Attach all API® 20Strep Identification Results sheets to the *Enterococcus* Identification Results Sheet (e.g., LEB-RS-612A).
- 9.3.9.** If the organisms are not gram positive cocci and/or catalase positive proceed to 9.4.
- 9.3.10.** If the API® 20Strep Identification Test kits fail to identify the isolate proceed to 9.4.

9.4. Identification of Non-Target Organisms

- 9.4.1.** The identification of 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.



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

- 10.1. Record the accession number, analyst initials, SSA lot date, start and end dates and times, TSA and TSAB/CoAB lot, start and end dates and times, source of colony (matrix spike, positive control, or sample), colony morphology, gram stain results, catalase test results, colony identification and results on the *Enterococcus* Identification Results Sheet (e.g., LEB-RS-612A).
- 10.2. Report samples showing bacterial growth on SSA that result in identification of *Enterococcus* spp. as positive for *Enterococcus*.
- 10.3. Report samples showing growth on SSA that do not result in identification of *Enterococcus* spp. as negative for *Enterococcus*.
- 10.4. Report samples showing no growth on SSA as negative for *Enterococcus*.
 - 10.4.1. A note is added to the final report if unregulated contaminants are identified.
- 10.5. Invalidate the test results for samples lacking growth in the matrix spike at any point in the analysis or from which *Enterococcus* spp. was not identified only if the positive and negative controls for aerobic plate counts and mold plate counts do not 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 relevant standards, regulations and Accreditation Body requirements for additional information on performing DOCs for microbial 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. Bacterial cultures and contaminated or potentially contaminated disposable materials are disposed of in biohazardous waste cans and autoclaved by the Glassware Department prior to discarding.



13.0. References

- 13.1.** United States Pharmacopeia. USP38-NF33, The United States Pharmacopeial Convention, General chapters <61>, <62>, <1111>.
- 13.2.** API® 20Strep Identification Test Kit Instructions for Use, bioMérieux
- 13.3.** 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
- 13.4.** NYS DOH LEB-604, Microbial Presence/Absence Test for Medical Marijuana Samples
- 13.5.** NYS DOH LEB-605, Aerobic Bacteria and Mold Plate Counts for Medical Marijuana Testing
- 13.6.** NYS DOH LEB-613, Identification of Thermophilic Actinomycetes in Medical Marijuana Products



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14.0. Appendices – Forms

Enterococcus Identification Results Sheet (LEB-RS-612A)

Incubate SSA for 24-48 hours and TSAB* for 18-24 hours
(30.0-35.0°C with 5% CO₂ Incubator, 37111-5139)

Accession Number: _____ Analyst Initials: _____

Final Results (circle one): Negative for *Enterococcus* Positive for *Enterococcus* _____
(list organism)

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

TSAB* Start Date/Time: _____	Gram Stain Results: _____
TSAB End Date/Time: _____	Catalase Test Results: _____
TSAB Lot Date: _____	
	API® 20Strep Start Date/Time: _____
	API® 20Strep End Date/Time: _____

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

1	
2	
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9	
10	

All API® Strep Result Sheets (LEB-RS-614B) are attached. SSA = *Streptococcus* Selection Agar, TSAB = Trypticase Soy Agar with 5% sheep's blood, M.S. = matrix spike, P.C. = positive control plate used in lieu of a matrix spike. *E. faecalis* ATCC 29212, used as a matrix spike, appears as small, white colonies on SSA.

*Columbia sheep blood agar can be used in place of TSAB.

Gas Pak Lot#/Exp. Date: _____ Indicator Strip Lot#/Exp Date: _____

Reviewed by _____ Date _____