TITLE:

Protocol for the identification and antimicrobial susceptibility testing of bacteria isolated from playgrounds

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SUMMARY:

The protocol outlines how to evaluate the occurrence of drug-resistant bacteria on various playground equipment elements and the top layer of the ground. This document describes among others, the procedure for the identification of bacteria isolates using the MALDI-TOF MS Bruker Biotyper platform, and the main steps of the disk diffusion susceptibility test.

PROTOCOL:

1. **Collection of bacterial samples from playground equipment and surfacing materials:**

   CAUTION: The collected samples might contain pathogenic microorganisms. Wear appropriate protection - gloves.
   NOTE: Use only sterile reagents and containers.

   1.1. Prepare a sterile swab with a transport tube, sterile saline solution (0.9%, *w/v*), water-resistant marker, and transport refrigerator. Consideration should be given to the selection and delineation of surfaces from which samples will be taken.
   1.2. Moisten a sterile swab with sterile saline solution (0.9%, *w/v*).
   1.3. Rub the selected surface of playground elements with the swab for a minimum 2 minutes.
   1.4. Place the swab in a sterile transport tube, keep it at 4 °C, and transport it to the laboratory immediately.

2. **Collection of soil and sand samples from the top layer of the ground within the playground area:**

   CAUTION: The collected samples might contain pathogenic microorganisms. Wear appropriate protection - gloves.
   NOTE: Use only sterile containers.

   2.1. Prepare sterile 50 mL polypropylene tubes, a water-resistant marker, and transport refrigerator. Consideration should be given to the selection of sites from which samples will be taken.
   2.2. Collect the sand or soil samples from the surface layer at a depth of 1-10 cm directly into 50 mL polypropylene tubes.
   2.3. Gather the material of the same type (for example from the one sandbox) from three different locations, and combine them in one tube.

2.4. Place the tube with soil or sand samples to transport refrigerator (at 4 °C), and transport it to the laboratory immediately.

3. Bioaerosol sampling:

CAUTION: The collected samples might contain pathogenic microorganisms. Wear appropriate protection - gloves.

3.1. Use a portable biological air sampler to collect biological contaminants suspended in the air in the playground area.
3.2. Install sterile cones filled with an appropriate collection liquid.

NOTE: The necessary consumables of the sampler (cones, caps, collection liquid doses) might be purchased from the device manufacturer.
NOTE: Use only sterile reagents and consumables.

3.3. Place the sampler at a height of approximately 80-100 cm above ground level.

NOTE: This height corresponds to the average body height of 2-3-year-old children who frequently use playgrounds.

3.4. Collect the bioaerosol for 10 minutes with an airflow rate of 250 L/min.

NOTE: Each sample contained bioaerosol suspended in 2 500 L of air.

3.5. Secure the collection cones containing liquid with a cap, store at 4 °C, and transport them to the laboratory.

4. Determination of the number of colony forming units (CFU) from swabs:

CAUTION: The collected samples might contain pathogenic microorganisms. Wear appropriate protection, and conduct experiments under the laminar flow cabinet.
NOTE: Use only sterile reagents and consumables.

4.1. Cut off aseptically the cotton tip of the swab containing biological material, and place it in 1 mL of sterile 0.9% (*w/v*) NaCl solution.
4.2. Vortex for 1 minute.
4.3. Prepare 10-fold serial dilutions using a sterile 0.9% (*w/v*) NaCl solution.

NOTE: Make sure that each dilution is intensively vortexed for 15 seconds and that tips are changed after each step.

4.4. Spread 0.1 mL of each diluted sample on a tryptic soy agar plate.
4.5. Incubate the plates at 37 °C for 48 hours.

4.6. Count all colonies from plates with 30 to 300 colonies.
4.7. Express the number of colony forming units per milliliter (CFU/mL).

5. Microbiological cultures from swab and soil or sand samples:

CAUTION: The collected samples might contain pathogenic microorganisms. Wear appropriate protection, and conduct experiments under the laminar flow cabinet.
NOTE: Use only sterile reagents and consumables.

5.1. Cut off aseptically the cotton tips of the swabs containing biological material, and place in tubes containing 5 mL of sterile tryptic soy broth.
5.2. Place the soil samples in flasks containing 100 mL of sterile tryptic soy broth.
5.3. Incubate cultures at 37 °C for 24 hours.
5.4. Using sterile inoculation loops, streak the obtained bacterial suspensions onto plates with selective chromogenic media dedicated to bacteria of interest. For example, *Staphylococcus*, *Enterococcus*, *Salmonella*, *Pseudomonas*, and *Escherichia coli,* etc.

NOTE: Using the selective media did not preclude the growth of other types of microorganisms.

5.5. Incubate plates at 37 °C for 24 hours.
5.6. Following the instructions in the manual for a given medium, use a loop to pick a single isolated colony of the appropriate color, and inoculate it in 5 mL of sterile tryptic soy broth.

NOTE: Growth on a selective medium and the appropriate color of the colony do not guarantee the correct identification of the bacterial isolate. Taxonomic identification should be confirmed by other methods, e.g. MALDI-TOF MS.

5.7. Incubate at 37 °C for 24 hours.
5.8. Prepare bacteria for the procedure of the species identification (see sections 7-8) or add 20% (*v/v*) glycerol and store the bacteria at −80 °C for further use.

NOTE: The glycerol stock of bacteria can be stored at -20 °C, but for a shorter period of time (rather days or weeks, but not years as in the case of -80 °C).

6. Microbiological cultures from bioaerosols:
6.1. Gently vortex the cones with liquid containing the airborne particles.
6.2. Transfer all the liquid into a sterile falcon tube.
6.3. Centrifuge at 8000 rpm for 15 minutes.
6.4. Decant the liquid.
6.5. Culture the residue as was described in section 5.

6.6. Prepare bacteria for the procedure of species identification (see sections 7-8) or secure the bacterial isolates for species identification in the future, as was described in section 5.8.

7. Multiplication of bacteria isolates for species identification by MALDI-TOF MS:

CAUTION: The collected samples might contain pathogenic microorganisms. Wear appropriate protection, and conduct experiments under the laminar flow cabinet.
NOTE: Use only sterile reagents and consumables.

7.1. Thaw glycerol stock of bacteria at room temperature, pick up a sample with a sterile inoculating loop, and streak on a tryptic soy agar plate.
7.2. Incubate plate at 37 °C overnight.

8. Sample preparation for bacterial species identification using MALDI-TOF MS.

CAUTION: Standard personal protective equipment should consist of at least gowns and gloves.
NOTE: All the steps should be performed in a biological safety cabinet.
NOTE: For microorganism identification use overnight growth of a pure bacteria culture.

8.1. Add 150 µL of sterile quality water to each 1.5 mL centrifugal tube.
8.2. Transfer one loopful of microorganisms to a tube with sterile water using a sterile, clear 1 µL inoculating loop.
8.3. Using a 200 µL pipette, pipette the liquid up and down 5-10 times to homogenize.
8.4. Add 450 µL of 100% ethanol to a tube. Homogenize the sample with a pipette (see section 8.3.).

NOTE: Such secured samples might be stored at -80 °C for 2 months before MALDI-TOF MS analysis.

8.5. Centrifuge at 13 000 rpm for 5 minutes.
8.6. Remove the organic solvent.
8.7. Dried the precipitate at 30 °C.
8.8. Add 40-100 µL of 70% (*v/v*) formic acid in ultrapure water into a tube.

CAUTION: Formic acid is flammable, toxic, and corrosive.

8.9. Vortex for 3 minutes.
8.10. Add 40-100 µL of acetonitrile. Vortex for 1 minute.

CAUTION: Acetonitrile is easily ignited by heat. Acetonitrile liquid and vapor can irritate the eyes, nose, throat, and lungs.

8.11. Centrifuge at 13 000 rpm for 3 minutes.
8.12. Add 1 µL of the resulting suspension to a 96-spot reusable stainless steel MALDI target plate in triplicates. Air dry.
8.13. Overlay spots with 1 µL of HCCA matrix.

CAUTION: HCCA is a skin and eye irritant.
NOTE: HCCA matrix refers to a saturated solution of α-cyano-4-hydroxycinnamic acid (HCCA) in a standard solvent (50% acetonitrile, 47.5% ultrapure water, 2.5% trifluoroacetic acid).
CAUTION: Trifluoroacetic acid causes severe skin burns and eye damage.

8.14. Air dry for 2 minutes.
8.15. Add 1 µL of the suspension of Bacterial test standard (BTS) to the open spot on the 96-spot reusable stainless steel target plate.

NOTE: Prepare BTS according to the manufacturer's instructions and store at ≤ -18 °C until ready for use.

8.16. Proceed the steps 8.13 and 8.14.
8.17. Conduct MALDI-TOF analysis.

NOTE: The plate must be run within 24 hours after preparation.

9. Species Identification by MALDI-TOF MS:

NOTE: The protocol was dedicated to the analysis conducted using a Bruker Daltonik GmbH ultraflex MALDI-TOF/TOF spectrometer controlled by flexControl software.
NOTE: The protocol was dedicated to microorganism identification using MALDI BioTyper Compass software.
NOTE: Identification relied on comparing the mass spectra collected for the test sample with a reference spectrum available in the database.

9.1. Insert the MALDI plate into the mass spectrometer.
9.2. Open the flexControl software and calibrate the mass spectrometer working in the MBT method.

NOTE: Follow the software manual.
NOTE: Positive ion mass spectra at m/z range of 2000 - 20,000 Da were acquired in the linear mode.

9.3. Acquire mass spectra for each spot.

NOTE: Follow the software manual.

9.4. Open the BioTyper Compass software. Analyse mass spectra.

NOTE: Follow the software manual.
NOTE: The software allows for the comparison of the mass spectrum of an unknown organism with a library of reference mass spectra.

9.5. Generate MALDI BioTyper report.
9.6. Interpret the results following the instructions included in the generated report.

NOTE: Generally, scores of ≥2.0 are accepted for identification to the species level, and stores in the range of 1.7-2.0 are accepted for genus assignment. Scores below 1.7 should be considered unreliable.
NOTE: If no species identification is made, repeat the sample preparation protocol using a new subculture or the isolate must be identified by another methodology.

10. Disk diffusion susceptibility test protocol:

10.1. Prepare sterile Mueller-Hinton agar plates.

NOTE: Prepare Mueller-Hinton agar following the manufacturer's recommendation (approximately 25 mL of liquid agar for 100-mm plate).
NOTE: Prepare one plate for each organism to be tested.

10.2. Prepare the inoculum.

NOTE: Conduct the assay using bacterial isolates identified with a high score of probability (≥2.0) by MALDI-TOF MS.

10.3. Transfer bacterial isolates to 5 mL of tryptic soy broth.
10.4. Incubate broth at 35 °C for 24 hours.
10.5. Transfer 50-100 µL of bacterial suspension to 3-5 mL of sterile saline (0.9%, *w/v).* Vortex.
10.6. Prepare a densitometer designed to measure the turbidity of the bacterial suspension in McFarland units. Adjust the turbidity of this suspension to 0.5 McFarland units.

NOTE: If the suspension appears more dense than the 0.5 McFarland units, add saline to obtain the appropriate density.
NOTE: Use the suspension within 15 minutes of preparation.

10.7. Dip a sterile swab into the inoculum tube.

NOTE: The swab should not be dripping wet.

10.8. Inoculate the surface of a Mueller-Hinton agar plate with the test bacteria.

NOTE: Streak the swab in a back-and-forth motion very close together. Move across and down the plate. Rotate the plate 60° and repeat this action.

10.9. Discard the swab into a suitable container.

10.10. Allow the surface of the agar to dry for 3-5 minutes but no longer than 15 minutes.

10.11. Lift the lid of the plate, and place 5 antimicrobial-impregnated disks on the Mueller-Hinton agar plate growing bacteria using sterile forceps. Firmly press the disks to ensure complete contact with the agar surface.

NOTE: For selection of antimicrobial agent see CLSI recommendations. Antimicrobial disks can be purchased from any reputable suppliers.
NOTE: Sterilize the forceps with a sterile alcohol pad and allow them to air dry or immerse them in alcohol followed by ignition.
NOTE: Disks should not be placed closer than 24 mm (center to center) on the Mueller-Hinton agar plate.
NOTE: Avoid placing disks close to the edge of the plate.

10.12. Invert the plates, and place them in a 35 °C air incubator for 16-24 hours depending on the type of pathogen.

10.13. Using a ruler or caliper measure the diameter of the bacterial inhibition zone around the disc, to the nearest millimeter.

NOTE: Include the diameter of the disk in the measurement.

10.14. Follow CLSI standards for the accepted limits for the diameters of inhibition and determine the susceptibility or resistance of the organism to each antimicrobial agent tested.

Disclaimer:

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This document serves as an illustrative procedure for isolating and identifying drug-resistant bacteria from playground equipment elements and surfacing materials**.** It is the responsibility of the testing laboratory to ensure content and modify it as necessary to meet applicable regulatory requirements, quality management system standards, and safety requirements.

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