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We use this protocol and it's working

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Abstract

Nontuberculous mycobacterial (NTM) were identified using matrix-assisted laser desorption-ionization time of flight mass spectrometry (MALDI-TOF MS), and tests were performed to determine antibiotic susceptibility, the ability to form biofilms, sliding motility characteristics, and the ability to adhere to and invade pneumocyte cells.

Attachments



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89KB

- 1 NTM species identification
 - 1.1 NTM colonies were taken from LJ media using a 1 mL loop and put into a 1.5 mL microtube containing 0.5 mm of glass beads and 500 μ L of 70% ethanol.
 - 1.2 The vortex was carried out for 15 minutes and then incubated vertically at room temperature for 10 minutes.
 - 1.3 Vortex was carried out again, transferred into 2 mL of round-bottomed microtube, and then centrifuged at a speed of 10,000–14,000 g for 2 minutes.
 - 1.4 The supernatant was removed, 10 mL of 70% formic acid was added to the pellet, and then the vortex was carried out until homogeneous.
 - 1.5 10 mL of acetonitrile was added, and then vortex was carried out until homogeneous.
 - 1.6 Centrifugation was carried out at a speed of 10,000–14,000 g for 2 minutes.
 - 1.7 1 μ L of supernatant was taken, placed on the target spot on the slide, and allowed to dry completely.
 - 1.8 1 μ L of matrix VITEK® MS-CHCA (bioMérieux, USA) was added and allowed to dry until the matrix and isolate crystallized.
 - 1.9 The slide was loaded into the VITEK® MS MALDI TOF machine (bioMérieux, USA), and the sample number was added.
 - 1.10 The results were read using a computer connected to MALDI TOF VITEK® MS (bioMérieux, USA). The identification test results will appear on the monitor in the form of the NTM species name.
- 2 NTM sensitivity test to various antibiotics.

- 2.1 Determination of the concentration of the stock of antibiotics was conducted through the dilution process. The dilution was carried out based on the content of each antibiotic used to obtain a final concentration of 1000 mg/mL.
- 2.2 0.1 mL of Middlebrook 7H9 broth was added to each well microdilution plate using a pipette, and one column was left for negative control with a volume of 0.2 mL.
- 2.3 0.1 mL of antibiotic was added to each column, and serial dilution was carried out in each subsequent column, leaving 1 column for positive control.
- 2.4 Bacterial colonies were taken from 3-5 colonies of LJ medium and transferred to the saline solution using a tube until it reached turbidity of 0.5 McFarland ($1-2 \times 10^8$ CFU/mL).
- 2.5 Diluted suspension of 0.5 McFarland ($1-2 \times 10^8$ CFU/mL) was carried out using the saline solution in a ratio of 1:20 so that the concentration became $5-10 \times 10^6$ CFU/mL, then 0.1 mL was added to each appropriate column, except for the sterile control column.
- 2.6 In each well, around the Microdilution plate, saline was added to keep the atmosphere moist.
- 2.7 The Microdilution plate was wrapped with plastic or plastic tape to prevent dryness.
- 2.8 Incubation was done at a temperature of $35 \pm 2^\circ\text{C}$ for 7 days.
- 2.9 Observation of growth was conducted through the growth of bacteria in wells containing antibiotics compared to growth controls.
- 2.10 The test was repeated 3 times, and the MIC value was determined for each test.
- 2.11 Determination was carried out into sensitive (S), intermediate (I), or resistant (R) groups based on the MIC criteria according to Table 1 (see attachment).
- 3 Biofilm formation
 - 3.1 NTM isolates were grown in Middlebrook 7H9 medium for 24 hours at 37°C .

- 3.2 Each well of a 96-well microtiter polystyrene plate was filled with 198 mL of Middlebrook 7H9 medium (except the wells in columns 1 and 12, rows A and H)
- 3.3 2 mL of NTM isolate suspension was added into each column filled with media and left 1 column for negative control. In the negative control column, isolate *S. epidermidis* ATCC 12228 was added.
- 3.4 The wells in columns 1 and 12, rows A and H, were filled with saline to keep the atmosphere moist. The biofilm formation test using a 96-well microtiter polystyrene plate is shown in figure 1 (see attachment)
- 3.5 Incubation was completed at 37°C for 7 days.
- 3.6 The wells were washed three times with 200 mL PBS.
- 3.7 Adherent cells were stained with 0.1% crystal violet (50 mL), and then washed with distilled water 3 times (using a pipette).
- 3.8 The paint was resuspended with 200 mL of 5% isopropanol acid, and then the absorbance was observed at 595 nm.
- 3.9 The bacterial OD value was calculated based on the absorbance value for 6 repetitions carried out in the B-G well. The OD value of bacteria was obtained by calculating the average absorbance value of at least 3 almost the same.
- 3.10 ODc value was calculated, where ODc = Average OD of the negative control + 3x SD of negative control.
- 3.11 The results were interpreted into categories of strong, medium, weak, and unable to form biofilms (negative), as shown in Table 2 (see attachment).
- 4 Ability to perform sliding motility
 - 4.1 Sliding motility media was prepared, namely Middlebrook 7H9 (without OADC) and 0.3% agar.
 - 4.2 Inoculation of NTM culture was carried out as much as 3 µL at optical density (OD) 600.6 (2.7×10^5 CFU) in the middle of the sliding motility media, and the plate was covered with parafilm.

- 4.3 Incubation was carried out at 37°C in a 5% CO₂ atmosphere for up to 16 days in a humid atmosphere.
- 4.4 Measurement of the length of the NTM growth area was carried out on days 4, 8, 12, and 16 using digital calipers in mm.
- 4.5 The sliding motility test was carried out 3 times.
- 4.6 The average length of growth was calculated from 3 test repetitions.
- 5 Ability to perform adhesion and invasion
 - 5.1 A549 cell line (human type II pneumocyte) culture was carried out.
 - 5.2 Supplement at DMEM (Invitrogen, Australia) was added with 10% FBS (Bovogen, Australia), 100 mg/mL of streptomycin, and 2 mM of L-glutamine.
 - 5.3 Observation of monolayer cell growth was carried out until it reached >95% growth.
 - 5.4 The monolayer cells were washed with PBS, and about 1×10^7 CFU of NTM bacteria were added to each well.
 - 5.5 Incubation of A549 cell line (human type II pneumocyte), which had been infected at 37°C for 4 hours, was carried out.
 - 5.6 The culture medium was removed, and 0.25% trypsin was added to the PBS to remove the A549 cell line (human type II pneumocyte) from the well surface.
 - 5.7 The A549 cell line (human type II pneumocyte), which had been washed using 200 mL of 0.025% sterile Triton X-100, was lysed.
 - 5.8 Dilution was carried out for 1000x

- 5.9 20 mL suspension was put into Middlebrook 7H9 medium to determine the amount of bacterial CFU attached to each well.
- 5.10 Incubation was carried out for 10 days.
- 5.11 Bacterial colonies were counted.
- 5.12 Tests were repeated on each species three times at different times. Each time the test was repeated twice.
- 6 Observation of NTM biofilm cell structure using SEM
- 6.1 Bacterial suspension was prepared equivalent to 1 Mc Farland.
- 6.2 20 mL of bacterial suspension was put into a 12-well microplate filled with a round cover glass and 1980 I of Middlebrook 7H9 media.
- 6.3 Incubation was carried out at 35-37⁰C for 7 days.
- 6.4 Wells overgrown with biofilm were washed with PBS 2 times.
- 6.5 The cover glass was taken and rehydrated using ethanol serially (70% for 10 minutes and then 96% for 10 minutes).
- 6.6 Ethanol was discarded, allowed to dry overnight at room temperature.
- 6.7 The cover glass was taken and coated twice with platinum vanadium using an ion sputter (Bal-Tec SCD 005) for 11 seconds, after which the cover glass was glued to double-side carbon tape to be observed using SEM (JEOL JED-2300, Japan).