Video Article

The MODS method for diagnosis of tuberculosis and multidrug resistant tuberculosis

Mark F Brady[1], Jorge Coronel[2], Robert H Gilman[3], David AJ Moore[4]

[1]The Warren Alpert Medical School of Brown University

[2]Laboratorio de Investigacion de Enfermedades Infecciosas, Universidad Peruana Cayetano Heredia

[3]Department of International Health, Johns Hopkins Bloomberg School of Public Health

[4]Wellcome Trust Centre for Clinical Tropical Medicine, Imperial College London

Correspondence to: Mark F Brady at brady@post.harvard.edu

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Abstract

Patients with active pulmonary tuberculosis (TB) infect 10-15 other persons per year, making diagnosing active TB essential to both curing the patient and preventing new infections. Furthermore, the emergence of multidrug resistant tuberculosis (MDRTB) means that detection of drug resistance is necessary for stopping the spread of drug-resistant strains. The microscopic-observation drug-susceptibility (MODS) assay is a low-cost, low-tech tool for high-performance detection of TB and MDRTB. The MODS assay is based on three principles: 1) mycobacterium tuberculosis (MTB) grows faster in liquid media than on solid media 2) microscopic MTB growth can be detected earlier in liquid media than waiting for the macroscopic appearance of colonies on solid media, and that growth is characteristic of MTB, allowing it to be distinguished from atypical mycobacteria or fungal or bacterial contamination 3) the drugs isoniazid and rifampicin can be incorporated into the MODS assay to allow for simultaneous direct detection of MDRTB, obviating the need for subculture to perform an indirect drug susceptibility test. Competing current diagnostics are hampered by low sensitivity with sputum smear, long delays until diagnosis with solid media culture, prohibitively high cost with existing liquid media culture methods, and the need to do subculture for indirect drug susceptibility testing to detect MDRTB. In contrast, the non-proprietary MODS method has a high sensitivity for TB and MDRTB, is a relatively rapid culture method, provides simultaneous drug susceptibility testing for MDRTB, and is accessible to resource-limited settings at just under $3 for testing for TB and MDRTB.

Video Link

The video component of this article can be found at https://www.jove.com/video/845/

Protocol

1. Prepare stock solutions
    1. Phosphate buffer stock
        1. Mix 950ml of sodium dibasic solution (9.47g of sodium phosphate dibasic dissolved in 1000ml distilled water) with 950ml of potassium phosphate monobasic solution (9.07g of potassium phosphate monobasic dissolved in 1000ml distilled water) and stir; keep back 50ml of each solution to adjust pH if necessary
        2. Adjust pH to 6.8 ± 0.2: add sodium phosphate dibasic solution to raise pH; add potassium phosphate monobasic solution to lower pH
        3. Autoclave at 121-124°C for 15 minutes to sterilize
        4. Plate a 100µl aliquot of the phosphate buffer solution on nutrient agar medium in a Petri dish and incubate at 37°C for 48 hours to confirm sterility
        5. Store in refrigerator at 2-8°C for up to one month
        Note: Each sputum sample requires 10ml of phosphate buffer solution

    2. Decontamination stock
        1. Combine equal volumes of sodium hydroxide solution (8.0g of sodium hydroxide dissolved in 200ml sterile distilled water) and sodium citrate solution (5.8g of sodium citrate distilled in 200ml sterile distilled water)
        2. Mix and autoclave at 121-124°C for 15 minutes
        3. Store in refrigerator at 2-8°C for up to one month
        Note: Each sputum sample requires 2ml of decontamination stock

    3. Culture media stock
        1. Into 900ml of sterile distilled water add 3.1ml of glycerol and 1.25g of casitone and 5.9g of 7H9 medium powder; mix with constant agitation until completely dissolved

2. Autoclave at 121-124°C for 15 minutes
3. Cool and divide the sterile medium into 4.5ml aliquots in sterile 16x 100 mm glass tubes for sample preparation, and 10.8ml aliquots for preparation of antibiotic solutions and internal controls
4. Incubate at 37°C for 48 hours to verify sterility (lack of turbidity)
5. Store at 2-8°C with cap tightly closed for up to one month
   Note: Each sputum sample requires one tube containing 4.5ml of 7H9 medium

4. Antibiotic stock solutions
   1. A. Isoniazid stock (8 mg/ml)
      1. Dissolve 20 mg isoniazid completely in 2.5ml sterile distilled water
      2. Filter with 0.2µm syringe filter for aqueous solvent
      3. Store in 20µl aliquots in sterile micro centrifugation tubes at -20°C for up to 6 months
         Note: Each stored 20µl aliquot is sufficient for up to 100 samples (including wastage)

   2. B. Rifampicin stock (8 mg/ml)

   1. Dissolve 20mg rifampicin completely in 1.25ml DMSO
   2. Add 1.25ml sterile distilled water and mix
   3. Filter with 0.2µm syringe filter for organic solvent
   4. Store in 20µl aliquots in sterile microcentrifuge tubes at -20°C for up to 6 months
      Note: Each stored 20µl aliquot is sufficient for up to 100 samples (including wastage)

2. Prepare working solutions
   1. Decontamination working solution
      1. Dissolve 0.1g of NALC crystals in every 20ml of decontamination solution required
         Note: Each sample needs 2ml of decontamination solution
         Note: Discard any NaOH-NALC decontamination solution that remains unused after 24 hours as NALC loses its mucolytic activity over time

   2. Culture media working solution
      1. Set out:
         1. 1 tube with 7H9 medium for every sputum sample to be processed, plus 1 additional tube for every plate (for the negative control column)
         2. 2 tubes of 7H9 medium for the positive controls (3 if monoresistant strains are used)
         3. 1-2 tubes with 10.8ml 7H9 for antibiotic solution preparation

      2. Add 0.5ml OADC to the 4.5ml of 7H9 in each sample tube
      3. Add 1.2ml OADC to tubes with 10.8ml 7H9
      4. Set aside 2 tubes with 5ml 7H9-OADC for positive controls, and tube(s) with 12ml 7H9-OADC to be used for antibiotic solution preparation (these do not require PANTA)
      5. Reconstitute PANTA and add 0.1ml to each sample tube and to the negative control tubes (7H9-OADC-PANTA: total volume=5.1ml)
         Note: Complete medium with PANTA (**7H9-OADC-PANTA**) is used for sputum samples and negative controls; use 7H9-OADC without PANTA for positive controls and for antibiotic solution preparation

   3. Antibiotic working solutions
      1. 1. See Figure 1 for steps to making the antibiotic working solution in an unused 24-well plate
         Note: For accurate susceptibility results, the final antibiotic concentrations are critical. The following procedure is suitable for laboratories equipped with micropipettes. If micropipettes are not available, tuberculin syringes can be used with a different series of dilutions described in the User Guide Appendix 3 at modsperu.org
         Note: Do not re-freeze or re-use antibiotic working or stock solutions as drug activity may be lost. Discard all unused antibiotic solutions at the end of the processing day Figure 1. Making the antibiotic working solutions (from User Guide at modsperu.org)

3. Sputum sample and plate preparation
   1. Decontamination
      1. Place 2ml of sputum sample into a 15ml centrifuge tube (if less, make up to 2ml with phosphate buffer; if more, use only 2ml)
      2. Add 2ml NaOH-NALC solution
      3. Cap tube tightly and vortex for 20 seconds; invert tube to ensure NaOH-NALC solution contacts the entire interior surface of the tube and lid
      4. Let stand for a minimum of 15 minutes – can prolong by a few minutes if the sample is particularly viscous. To avoid over treatment, should not exceed 20 minutes
      5. Fill the tube to 14ml with phosphate buffer (pH 6.8) to neutralize alkali and terminate the decontamination process, and mix well by inverting the tube 4 times
      6. Centrifuge at 3000 g for 15 minutes (see Appendix 2 in User Guide at modsperu.org for equation of rotations per minute necessary to reach 3000 g)
      7. Carefully pour off supernatant into a liquid waste container with 10% sodium hypochlorite or other suitable disinfectant and retain the pellet

   2. Preparation of final sample suspension and backup

1. Using 7H9-OADC-PANTA (from the tube containing 5.1ml), resuspend the sample pellet in a total volume of 2ml in the centrifuge tube with a Pasteur pipette; mix well
2. Remove 1ml of sample suspension and store in a microcentrifuge tube at 2-8°C as a backup
3. Add the second 1ml of sample suspension to the tube with the remaining 7H9-OADC-PANTA; mix well. This is the final sample solution ready for plating

3. Placing sample suspensions into 24-well plate
    1. Place 900µl of the final sample suspension into each of the 4 wells of a column in the 24-well plate
    2. Repeat with additional samples until all columns of the plate, except Column 3, are filled (or until all samples are plated)
    3. Place 900µl of 7H9-OADC medium without sample in the 4 wells of Column 3 of each sample plate (negative internal controls)

4. Adding antibiotics to samples
    1. Using a multi-channel pipette, carefully fill 4 tips with 100µl from the wells with 7H9-OADC and antibiotic working solutions (Column 2 in antibiotic dilution plate- see Figure 1)
    2. Add the 100µl aliquots to the column 1 wells with 900µl sample solutions without touching plate or sample
    3. Repeat until all columns have received the additional 100µl of medium (drug-free wells) or antibiotic solutions (including negative control column 3)
    4. Close the plate with its lid and place in a sealable polythene (Ziplock) bag and seal (bag is not opened again from this point onwards)
    5. Incubate at 37°C (CO_2 enrichment is not necessary)

4. Quality control
    1. Negative controls are run on each plate. The presence of any growth in any of the wells in the negative control column indicates cross-contamination, therefore the entire plate must be discarded and the samples re-plated.
    2. Positive controls are run on a separate plate at the end of the day**.** One fully drug-susceptible strain is run in a column and a strain resistant to INH and RIF is run in another column. If there is concern about running an MDR strain, both an INH monoresistant strain and a RIF monoresistant strain can be run in the place of the susceptible and MDR strains. All positive controls should have growth in the drug-free wells to demonstrate that the media support growth. To demonstrate that the drug concentrations are correct, the susceptible strain should not grow in the INH or RIF wells, but the MDR strain should grow also grow in the INH and RIF wells.

5. Plate reading
    1. With the day of plating as “Day 0,” plate reading starts on Day 5, and if negative on Day 5 reading is done every day (or alternate days depending on workload) until day 14 and then every 2-3 days from day 15-21. Plates are examined on an inverted light microscope with the 10x objective
    2. A positive culture is one in which there are two or more colony forming units (>2 CFU) in BOTH drug-free wells. Early mycobacterial growth looks like small curved commas or spirals (days 5-9). Colony formation usually progresses to “cords” or “tangles” (see image library on modsperu.org)
    3. On the day a culture is positive in the 2 drug-free wells, the drug-containing wells should be read. Any growth in the presence of a drug indicates resistance to that drug.
    Note: If the reading of the drug-containing wells is done >1 week after a positive culture is noted in the drug-free wells, this may not represent resistance as break-through growth is occasionally seen
    4. Samples that are not positive by Day 21 are considered negative cultures
    5. Cloudiness of the wells is a result of contamination overgrowth, whereas mycobacterial growth does not result in cloudiness
    6. On completion of 21 days cultures may be discarded. Cultures remain in their sealed bags and are placed in autoclave bags and autoclaved at 121-124°C for 45-60 minutes

Discussion

The MODS assay is targeted at resource-limited settings. For the first time, MODS brings the ability for rapid liquid culture detection of tuberculosis and multidrug resistant tuberculosis to resource-limited settings at just under $3 per test. MODS is a non-proprietary, iterative methodology, and the MODS community is always interested in improvements that other laboratories have managed to make.

A recurrent concern is the biosafety of liquid media culture of tuberculosis because liquids can be spilt or aerosolized. We believe that the MODS assay is more biosafe than any assay that involved indirect drug susceptibility testing because indirect drug susceptibility testing involves the manipulation of highly concentrated solutions of mycobacteria with the attendant risks of spillage and aerosolization; in contrast, MODS simply involves the inoculation of a sputum sample into a plate, after which the plate is sealed within a plastic bag and never again opened. This is supported by data from Korea (Kim 2007) which showed that the occupational risk to laboratory workers who plated out sputum samples without doing drug-susceptibility testing was no greater than that in workers doing sputum smear microscopy; in contrast, those doing drug-susceptibility testing had much higher occupational risk for tuberculosis.

We strongly recommend that none of these procedures be undertaken without proper precautions for laboratory workers. This includes N-95 masks for personal respiratory protection, a Class 2 biological safety cabinet with exhausted air filtered through HEPA filters, and a lock on the laboratory door to stop turbulence of airflow while samples are being manipulated.

Standard operating procedures for processing extrapulmonary samples, a photo library of mycobacterium tuberculosis and other mycobacteria and bacterial and fungal contamination, a recommended quality assurance strategy, a procedure for accreditation of laboratories to start using MODS, and an FAQ sheet are available at modsperu.org

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