TB Collaborative: Challenges, Resolutions, & Recommendations

EPA BEAD Workshop

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Efficacy Working Group

- Association of companies working collaboratively with EPA on continuous improvement of UDM and implementation of OECD QCT II.

Ecolab Inc.

Lonza Inc.

Mason Chemical Company

Stepan Company

Reckitt Benckiser

- Administered by Pat Quinn, The Accord Group

Agenda

- Recommended *M. terrae* Culturing & Testing Procedures
- Lab 1 Experiences
- Lab 2 Experiences
- Recommendations for OECD Method Enhancements

M. terrae Collaborative Culturing Procedure

Reconstitution

- Obtain *Mycobacterium terrae* (ATCC 15755) from ATCC
- **Reconstitute culture pellet in 1 ml MADC (Middlebrook 7H9 Broth with 10% ADC enrichment & Glycerol) and dilute with 5 ml MADC**
- Inoculate pre-poured M7H9 or M7H11 plates with 0.1 ml of *M. terrae* suspension
- **Incubate plates for 20-22 days at 36±1°C**

Stock Preparation

- **At end of incubation add 5 ml of MADC to each plate and re-suspend growth sterile glass or plastic spreader. Place suspension in flask. Rinse each plate with an additional 5 ml aliquot of MADC and again add this suspension to flask.**
- **Mix contents of flask thoroughly and then pipette 1 ml + of suspension into separate sterile cryovials.**
- Store vials at ≤-70°C for maximum of 14 months (can be used after 14 months if titer is verified as still being suitable for testing) Discard cryovials if contaminated or if identification is questionable

Bolded items to be reviewed in presentation

M. terrae Collaborative Culturing Procedure

Test Culture Preparation

- Defrost 2-4 cryovials quickly
- Add 1 ml of *M. terrae* suspension from each vial into separate flasks containing 100 ml MADC broth
- **Incubate flasks for 20-22 days at 36±1°C**
- On day of test, remove two 25 ml portions of broth suspension and place each aliquot into separate sterile 50 ml centrifuge tubes
- Centrifuge at 5,000-10,000 g for 20±5 minutes
- Decant supernatant and re-suspend culture pellet in 25 ml of sterile distilled/deionized water. Vortex to completely suspend growth. Repeat centrifugation and re-suspension step 3 times.
- **Resuspend each final culture pellet in 5 ml of sterile DI water. Pool suspension and place in bijou bottle with 10 glass beads. Vortex 5 minutes to break up cell clumps**
- **Adjust suspension spectrophotometrically at a defined wavelength (650 nm) based on a standard curve specific for *M. terrae***
- Add 3 part soil load (BSA, YE, Mucin) prior to carrier inoculation
- For a 4 log reduction performance standard, the dried carrier count should be 4.5-5.5 logs/carrier.

M. terrae Collaborative Test Procedure

Inoculation of Carriers

- Vortex final cell suspension
- **Inoculate each carrier with 10 uL of suspension, avoid contacting/spreading of suspension**
- Dry inoculated carriers in petri dish with top removed in desiccator at a vacuum of 20-25 inches mercury for 60±10 minutes

Exposure of Inoculated/Dried Carriers to Test Substance or PBS (control)

- Evaluate four control carriers and three test carriers/test substance/test system
- Use certified timer to assure a contact time of 5 minutes ±3 seconds per carrier
- Transfer dried carriers to separate capped sterile vials
- Add 50 uL of test substance or PBS (controls) to each vial at timed intervals, leave vials uncapped during contact period

M. terrae Collaborative Test Procedure

Neutralization of Test Substance

- At end of contact period add 10 ml of appropriate neutralizer to each vial at same timed intervals used for test substance/PBS addition. Vortex briefly
- Vortex all vials for 30±5 seconds

Dilution and Recovery of Treated Carriers

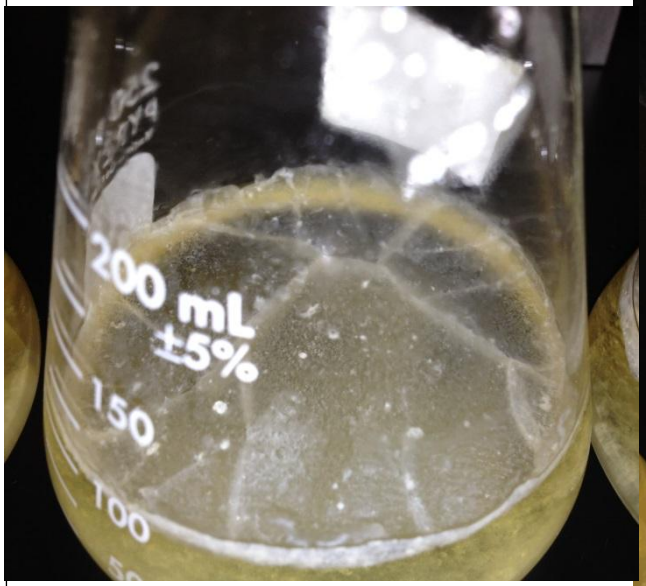
- **Filter all samples, sample and control dilutions using pre-wetted 0.45 um Pall Metricel Black 47 mm dia filters**
- Rinse vials and walls of filter with PBS in accordance with collaborative protocol
- Place sample, sample and control dilution filters on pre-poured sterile M7H11 agar plates
- **Incubate plates for 21-28 days**

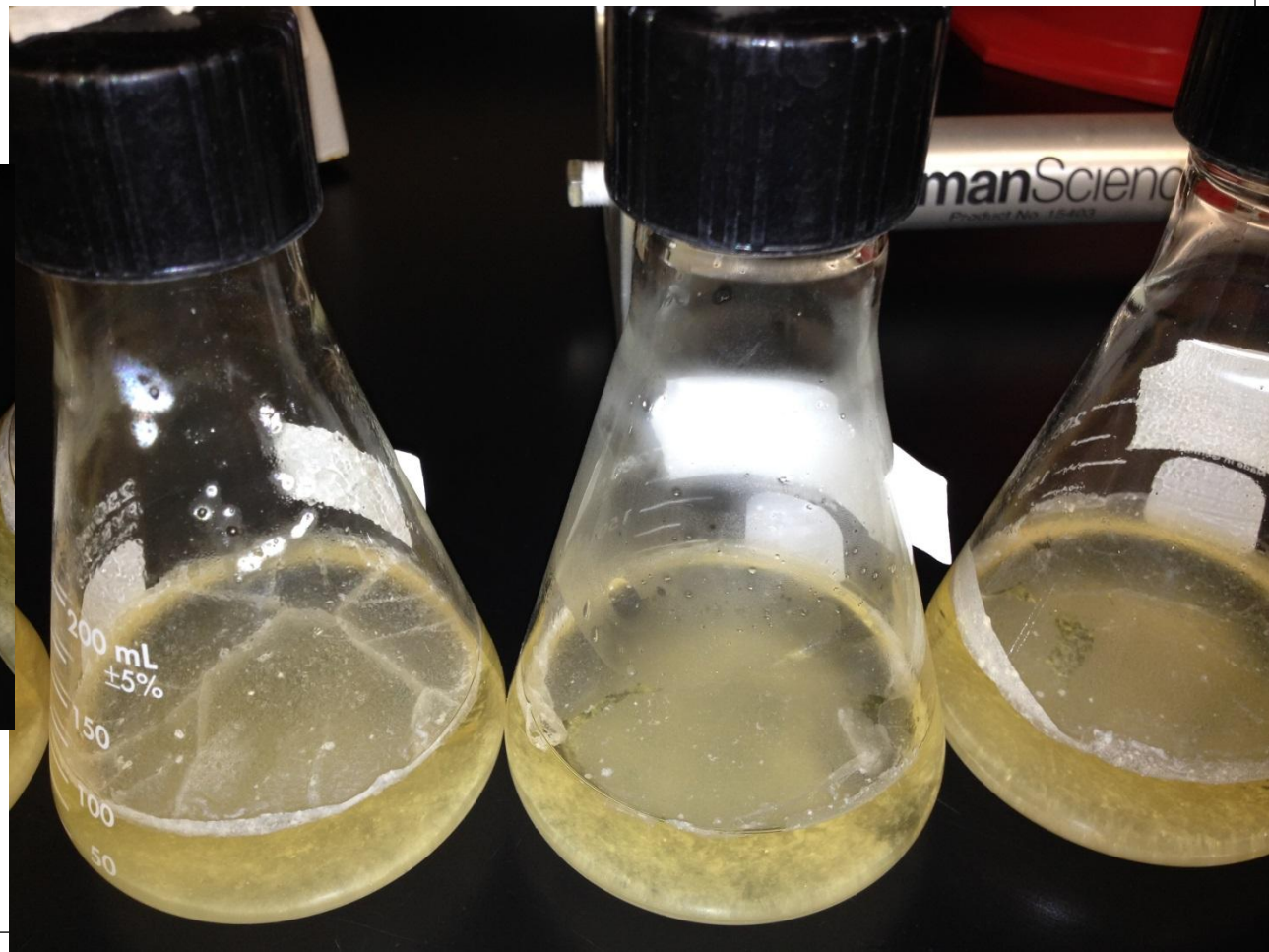
Recording of Test Results and Calculation of Log Reductions

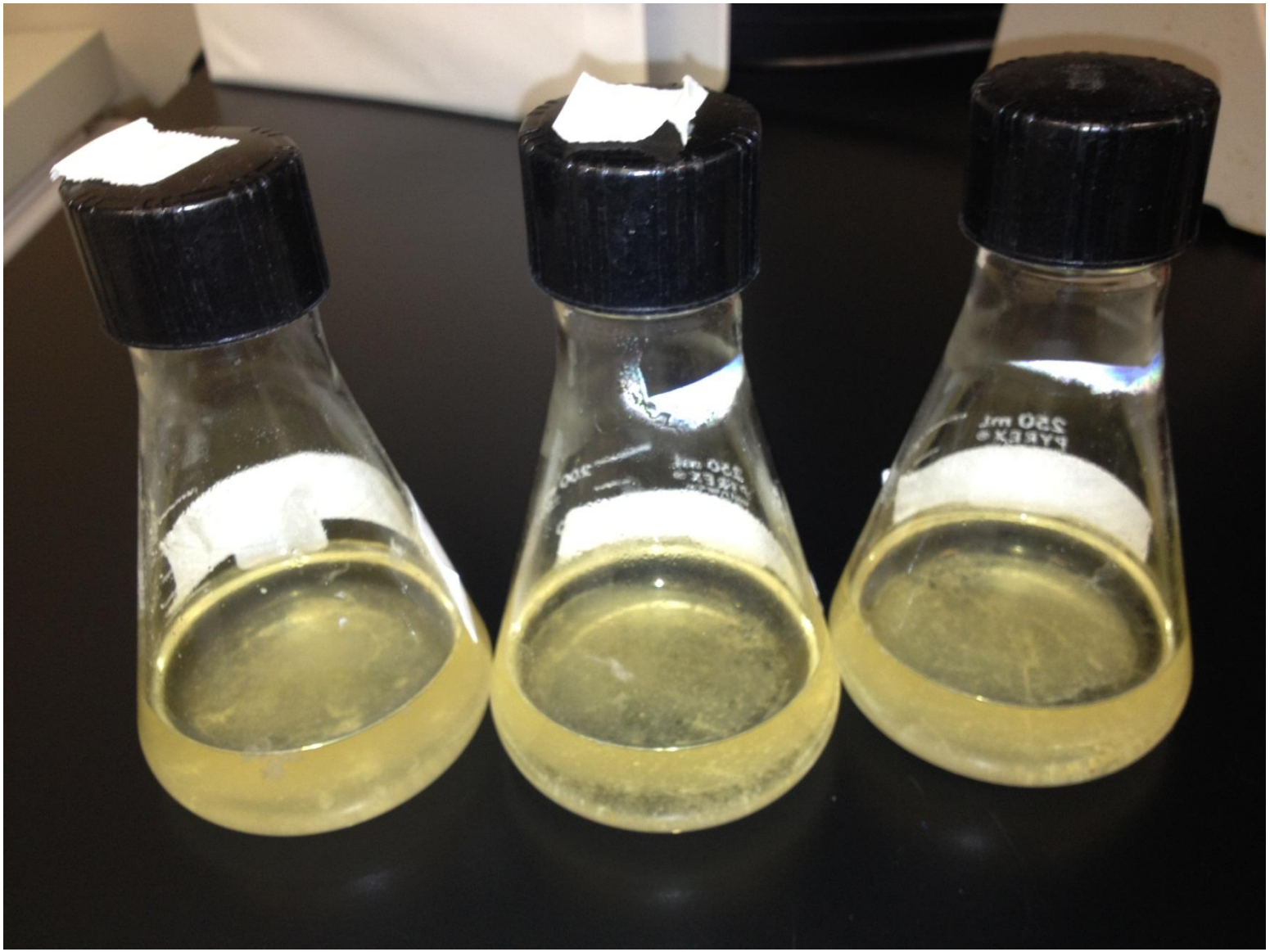
- Record colony counts from filter plates following incubation and calculate log reductions based on control carrier counts

Lab 1: Experiences

- Variable *M. terrae* Growth






250 ml
PYREX

Lab 1: Experiences

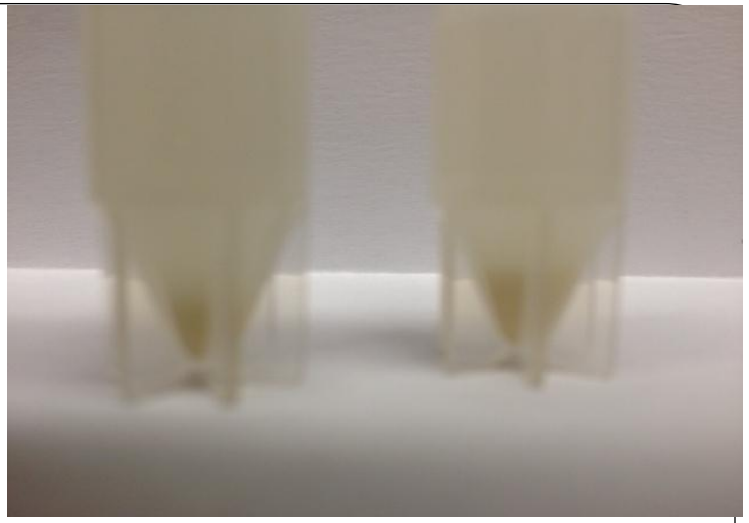
- Variable *M. terrae* Growth seen with
  - Same culture stock
  - Same Incubator and temperature
  - Same media Lot
- Recommend further research to arrive at a consistent test culture
  - May consider:
    - Shaking
    - Alternate Media
    - Review report of EPA Cooperative Agreement on TB (MicroBioTest)

Lab 1: Experiences

- Dried Carrier Count results:
  - 1/3 of test dates did not meet Dried Carrier Count Criteria
  - A target of 1.7 -2.0 OD may achieve a 4.5-5.5LD/carrier
- Recommend OD values be further investigated to arrive at a target for the user to be included in the method.

Optical Density @650nm	Dried Carrier Counts (LD/ Carrier)	Mean LD/Carrier (Std Dev)
1.880	5.54, 5.46, 5.43, 5.46	5.48 (0.05)
1.543	4.53, 4.57, 4.60, 4.57	4.57 (0.03)
1.547	4.60, 4.46, 4.43, 4.46	4.49 (0.08)
2.030	5.24, 5.16, 5.17, 5.11	5.17 (0.05)
1.632	4.20, 4.34, 4.43, 4.36	4.33 (0.10)
1.911	4.84, 4.81, 4.77, 4.76	4.79 (0.04)

Lab 2: Experiences



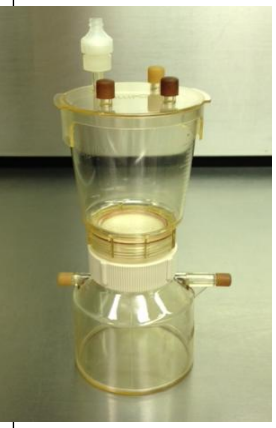
- ***M. terrae Culturing Procedure***
  - MADC broth contains higher glycerol levels (15%) than recommended by media supplier (0.2%)
  - Bagging of plates during incubation not mentioned
  - Collection of growth from incubated agar plates awkward and adds contamination risk
  - Varied solids content of culture suspension in cryovials
  - Test Culture 7H11 Agar Plates and MADC broth appear to be easily contaminated (Fungal)
  - Appearance of cultures set up on the same day and incubated under same conditions was quite different. (Same as Lab 1)
  - Culture suspension solids will settle without agitation

Lab 2: Experiences

- **Incubation of *M. terrae* Broth & Agar**
  - Cross contamination arose when *M. terrae* plates incubated together in plastic bags, incubate plates/flasks separately
  - Crowding of incubators can cause temperature variances
  - Use of convection type incubators maybe a problem
  - Use of forced circulation incubators can help reduce temperature variations
  - Mapping of incubators maybe helpful

Lab 2: Experiences

- **Testing of *M. terrae***
  - Black filters a definite improvement
    - Improved agar adhesion
    - Reduced trapped air bubbles
    - Easier to distinguish growth



  - Open top filter units are a contamination risk even when testing in a LF hood
  - Colony density approaching 200CFU/filter was difficult to count
- Needed to vary dilutions to achieve Dried Carrier Count Criteria (Clumping, varied solids content, temperature variances, static culturing conditions)
- **Neutralization Confirmation Control**
  - Sequence of steps confusing

Lab 2: Dried Carrier Counts

- 46% Test Dates did not meet Dried Carrier Count Criteria
- OD Range did not correlate to Dried Carrier Count Criteria

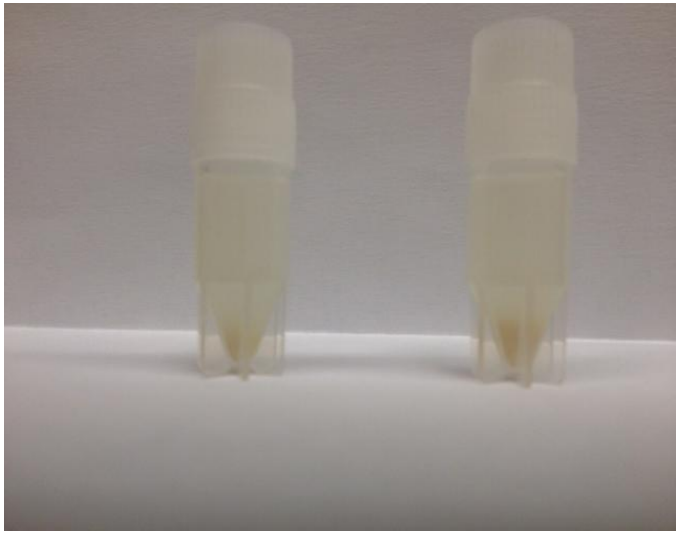
Test Date	Optical Density @650nm	Dried Carrier Counts (LD/Carrier)	Mean LD/Carrier (Std Dev)
7-10-13	0.700	3.85, 4.08, 4.15, 3.95	4.01(0.13)
7-15-13	0.587	3.70, 3.30, 3.95, 3.70	3.66 (0.27)
7-16-13	0.583	4.87, 4.63, 4.63, 4.81	4.74 (0.12)
TARGET	1.3-1.7	4.5 – 5.5	4.5 – 5.5
7-22-13	1.345	5.08, 5.11, 5.04, 5.11	5.08 (0.03)
7-23-13	1.674	6.10, 6.04, 6.13, 6.14	6.10 (0.05)
7-24-13	1.304	4.69, 4.82, 4.67, 4.75	4.73 (0.07)
7-29-13	1.625	5.56, 5.34, 5.49, 5.52	5.47 (0.10)
7-30-13	2.130	6.32, 6.36, 6.35, 6.34	6.34 (0.01)
7-31-13	1.642	5.62, 5.60, 5.51, 5.58	5.58 (0.05)

Lab 2: Recommendations

- Identify purpose of high glycerol levels in MADC. Could this adversely impact *M. terrae* growth?
- Use of LF hood for culturing and testing *M. terrae should be stressed*
- Better procedure for harvesting *M. terrae* from agar plates needed to reduce contamination risk (Use of disposable tissue culture flasks?)
- Use rotary shaker during broth test culture incubation
- Pay attention to incubation conditions/incubator types. Avoid over-crowding. Use of forced circulation incubators and incubator mapping could help.
- Investigate use of a homogenizer for more consistent broth culture suspensions
- Establish a *M. terrae* stock titer level minimum. Also establish a maximum variance allowed between stock cryovials.
- Specify that sterile Whirl-Pak bags or equivalent be used during incubation of broth/agar cultures. Bottles and plates are to be packaged separately to prevent cross contamination.
- More work is needed to identify the ideal optical density range for standardization of *M. terrae* suspensions.
- Cover filters during use to reduce contamination risks

Current Preparation of M. terrae Stock and Test Culture Suspensions

Cryovials Containing *M. terrae* Culture Suspension Collected from M7H11 Plates



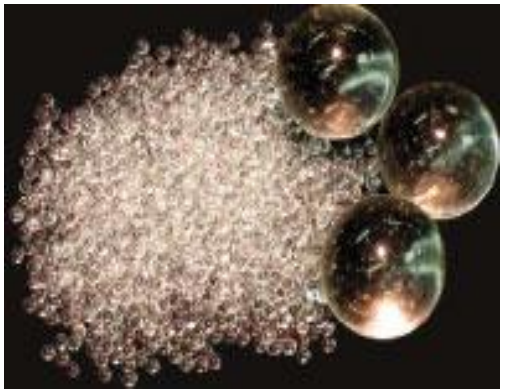
Vortexing of Test Culture in Bijou Bottle

Homogenizers Manual

Motorized Mortar & Pestle Homogenizer

Automated Homogenizer

Varied Size Glass Beads



Summary

- Further research is needed to arrive at a consistent test culture
- Additional procedural steps are needed to reduce contamination
- Further research is needed to establish an OD target to achieve required dried carrier counts

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