Antimicrobial Efficacy Test Methods Workshop Highlights from Workshop Sessions DAY 1: February 18, 2014 – *Clostridium difficile* Test Methods		
Method/Activity	**Aspect**	**Comments/Recommendations**
EPA's guidance on testing *C. difficile*	Towelette Testing	• In this session, an overview of the proposed revisions to EPA's guidance for *C. difficile* claims was provided to participants. See associated ppt presentation for details. • Most notable, testing will be conducted using actual towelettes, but will not be based on the expressed liquid. • **Action Item**: MLB will consult with Antimicrobials Divisions on use of liquids in QCT-2 for towelettes.
Method/Activity	**Aspect**	**Test Microbe**
ASTM E2839-11 (Standard Test Method for Production of *Clostridium difficile* Spores) or BEAD/MLB SOP MB-28-01	Strains	• EPA provided a comprehensive overview of the *C. difficile* strain (in the revised guideline) used in testing. • No comments from participants.
	Diagnostic characteristics	• Typical characteristics of growth on media and spore/cell morphologies were described by EPA. • No comments from participants.
	Specialized equipment, media and regents	• The equipment and supplies necessary for testing of *C. difficile* were discussed by EPA. Also, the maintenance and function of the anaerobic chambers were discussed by EPA. • A question was raised regarding whether or not EPA has seen any difference in using glass tubes vs. plastic tubes for the RCM step? EPA has not conducted a comparison study. • During the session, EPA reiterated the need to maintain fully reduced media for use in recovery assays: ▪ **Action Item:** EPA to clearly identify time frame for reducing liquid and solid media. EPA suggests a minimum of 24 hours under anaerobic conditions. EPA will provide revisions to ASTM E2839-11. ▪ Labs place RCM loosely-capped overnight in anaerobic environment to reduce. • EPA demonstrated use of the Coy Anaerobe chamber and the anaerobe jars.

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		• EPA does not use anaerobe jars, but they are considered adequate if used properly. • Majority of participants use anaerobe jars. ▪ Comment: If jar opened, replace indicator packs before closing. ▪ Over time, anaerobe jars lids (inner lid) may be compromised and/or crack, thus inspection of the lid's seal is necessary prior to use. • Comment: Typical practice for incubation of plates includes sealing the agar plates with parafilm to reduce dehydration; this practice may be employed for incubation of plates in jars for the 5 day period. • Comment: Some labs buy CABA plates from bioMerieux and reduce them on site. Most labs buy from Anaerobe Systems (pre-reduced). • Comment: Media performance of in-house media should be conducted to confirm media quality by use of stock spores of known titer to check media. At a minimum, for purchased media, review and maintain the certification documents. ▪ Verify performance of purchased media if lot to lot differences in quality are suspected. ▪ **Action Item:** EPA will follow-up on the need to include media performance data in the GLP data submissions. • Comment: Consensus on the use of ST80; everyone using for dispersion.
	Establishment of frozen stock	• EPA presented the procedure for generating and storing stock (frozen vegetative stock). • **Action Item:** EPA to add shelf-life of frozen stock to the standard and SOP – EPA suggested 18 months.
Method/Activity	**Aspect**	**Sporulation Protocol**
ASTM E2839-11		• EPA provided a detailed presentation of the spore production protocol. • When using anaerobe jars, MLB recommended the use of a second jar to remove plate(s) for observation during the 7-10 day incubation. • Recommend use of swinging bucket rotor for centrifugation steps, especially for the purification step.

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		• **Action Item:** EPA will add time frame for storage of spore suspension (post heat treatment) prior to purification to SOP and ASTM standard; 2 to 7 days was recommended.
Method/Activity	**Aspect**	**Spore Purity and Resistance Testing**
ASTM E2839-11		• **Action Item:** Revise MLB SOP MB-28-01 to instruct users to bring HistoDenz and spore suspension to room temp prior to purification step, and ensure that liquid in tubes (in heat block or water bath) is at 65°C prior to starting the 10 minute exposure time.
Method/Activity	**Aspect**	**Other Acceptable Sporulation Protocols**
ASTM E2895-13 (Standard Test Method for Producing Spores of *Clostridium difficile* using Liquid Medium)	Mission – One sporulation method	• Another acceptable method, ASTM E2895-13, was discussed; some concerns were raised about the enzymatic process of purifying the spores and its impact on spore resistance. • A new method, Liver broth method, is currently being balloted by ASTM; and according to one stakeholder, the method is comparatively simple and provides a highly pure spore preparation without a separate purification step. • EPA believes it is desirable to have one standard method for spore production. ▪ Users need to consider pros and cons of each method and decide on one method. ▪ **Action Item:** EPA will seek feedback from stakeholders on the use of a single method and will discuss options internally for a long-term resolution. • Participants agreed to provide feedback to EPA on any issues associated with qualifying spores for use.
Method/Activity	**Aspect**	**Product Efficacy Testing – Quantitative Methodologies**
ASTM E2197-11 (Standard Quantitative Disk Carrier Test Method) MLB SOP MB-31	Carriers	• MLB demonstrated QCT-2 in a step-by-step fashion. • Participants expressed concerns that there is only one source of the brushed stainless steel carriers (Pegen). • Currently, the standard method allows re-use of carriers. ▪ **Action Item:** Re-use not advisable. Participants to petition ASTM committee to remove option from method.
	Spore	• Target test spore suspension concentration is 8 to 9 logs/mL. Suspension may be

	suspension	diluted on day of use to reach target of 6 to 7 logs/carrier.
	Inoculum	• Comment: Vortex mix spore suspension every 3 carriers. May use same tip throughout carrier inoculation process. ▪ **Action Item:** EPA to revise SOP MB-31.
	Carrier drying	• Comment: ASTM standard silent on drying of spore-formers like *C. difficile*. • Currently in MLB's SOP: Inoculate carriers, dry in biological safety cabinet for 30 min with lid off, transfer to desiccator to dry (lids off) 2 hr with vacuum. Thirty minute/no vacuum drying step allows user time to check drying of carriers to ensure inoculum remains on carrier/sufficient levels of inoculum before transferring to desiccator.
	PES membranes	• **Action Item:** EPA to provide source and catalog number for PES filters. ▪ Pall Corporation #66234 (individual filters) ▪ Pall Corporation #4806 (microfunnel filter unit, 0.2 µm)
	Vials	• **Action Item:** EPA to provide source and catalog number for vials. ▪ Thermo Scientific #2118-9050 (Nalgene, straight-side wide-mouth jar, polypropylene)
	Vortexing carriers	• Comment: Adequate vortexing is essential to remove inoculum from carrier. MLB uses vortex set on highest setting; carrier should be spinning in mixture during the process. Visually inspect carriers to ensure inoculum is completely removed by conducting additional vortexing if necessary. • Comment: Vacuum should be on during rinsing of vials and dilution tubes. Not specified in MLB's SOP. • **Action Item**: Revise MLB SOP-MB-31 to keep the vacuum during rinsing.
	Filtration	• Comment: Some labs have observed that colonies are not obvious on filters early in the incubation time frame (2 days) for treated carriers; a film of growth on filter is observed after 3-5 days. Subculture reveals presence of *C. difficile*. Additional incubation time frame recommended (to 5 days) for treated carriers. • Comment: Wetness of plates can adversely affect growth of organism on filters. • Comment: If using microfunnel filter units for filtration after treatment, analysts

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		must snap top off the filter unit and continue to apply vacuum to filter remaining liquid through filter.
	Important time frames	• EPA reiterated the need to conduct the work as quickly as possible, from end of contact time to completion of filtration. Surviving spores are vulnerable, risk exposure to oxygen. • For example, dilutions should be made with 30 minutes of neutralization and filtration/plating should occur within 50±10 minutes of preparing the dilutions. • Also, if using an anaerobe jar, load plates into jar within 15 minutes following completion of plating – this was a recommendation and should be incorporated in the lab SOPs if using anaerobe jars.
	Questions to consider	• How to do neutralization? Not discussed, however, neutralization will be conducted in accordance with BEAD/MLB SOP MB-26-00 using the following revisions specific to *C. difficile.* ▪ The spore suspension will be diluted appropriately in ST-80 to obtain 20-200 CFU/filter. ▪ A suspension test using 10µL of the diluted spore suspension will be employed instead of a carrier-based approach. ▪ Contact time will be 10 minutes and neutralization will be considered adequate if the CFU for all treatments are within 50% of the titer control.
AOAC 2008.05 (Three Step Method)	Advantages of method	• MLB demonstrated the TSM in a step-by-step fashion using the consolidation of fractions B and C. • Comments: Carriers are single use only. • The TSM has a good track record, and AOAC validation history with spore formers.
	Disadvantages of method	• Sensitivity concerns due to sampling (10 µl of 1 mL sample)
Comparison of QCT-2 to TSM		• EPA stated that use of a single test method for efficacy evaluations is desirable in the long term. • EPA has not conducted side by side studies to know if products give same results under both methods.

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		• **Action Item:** The development of a reference standard (high and low efficacy treatments) for both methods is desirable; sodium hypochlorite is a viable candidate. EPA will initiate studies.
ASTM E2896-12 (Standard Test Method for Quantitative Petri Plate Method (QPM))	Control of humidity during drying of inoculated plates	• MLB provided a full demonstration of the procedure. • Comment: Humidity may vary during the drying process; however, an environmental monitoring system documents the relative humidity in the incubator.
	Media	• Comment: Consider pre-dispensing all liquid reagents (dilution blanks) and media for ease of use.
	Uniform wiping procedure	• Comment: Important to use consistent pressure, from test to test, during wiping of carrier during neutralization step. • Comment: Avoid releasing all of active ingredient (i.e., liquid) onto the plate; neutralization is negatively impacted by extreme pressure/high liquid volume – more liquid, more neutralization that has to take place.
All Methods	Incubation and recording or results	• Comment: For treated carriers, colonies can be counted on day 3; however, filters with few or no colonies should incubate for up to 5 days. • **Action Item**: Revise SOPs and ASTM standard for incubation periods for control and treated carriers.
	Calculations and interpretation of results	• No comments. • EPA announced plans to conduct a collaborative study on QPM and *C. difficile*. The goal is launch the study in 2014 to fulfill ASTM data requirements. This study is considered to be the first in a series of evaluations designed to determine the method's performance. A number of labs expressed interest in participating in the study. The biggest impediment is treatment (antimicrobial wipes) selection – i.e., use of commercially available products. ▪ **Action Item**: Develop the study protocol and work with stakeholders for laboratory support.