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Quantitative Method to Measure Disinfectant Product Efficacy V.2

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None

This protocol is used for measuring the inactivation of *Pseudomonas aeruginosa* by liquid disinfectants. In brief, stainless-steel discs are inoculated with single 10 μ L drops of a *P. aeruginosa* suspension also containing a three-part soil mixture. The three-part soil consists of bovine mucin, bovine serum albumin and yeast extract. The inoculum drops are dried on the discs and then the discs are transferred into flat-bottom vials for testing. Testing involves overlaying each dried inoculum spot with 50 μ L of a disinfectant working solution or a negative control solution. A contact time is allowed to elapse and then a disinfectant-neutralizing agent is added. Any material still adsorbed on the carrier is eluted by vigorous washing/mixing of the carrier in the neutralizer. The neutralizer eluate and 10-fold serial dilutions of the eluate are filtered through 0.2-micron membrane filters. The filters are transferred to the surfaces of agar plates and incubated. Colony forming units on the filters are counted and log reductions for the disinfectant treatments are calculated.

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protocol

Reproducibility problem with a proposed standard method to measure disinfection efficacy. PLOS ONE [PONE-D-22-07243] - [EMID:120744b88080feae]

Disinfectant, biocide, antimicrobial, efficacy, Pseudomonas

protocol ,

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None

Hardware

1. Carriers, discs (1 cm in diameter) made of AISI Type 304 Stainless Steel with 150 grit unidirectional finish on one side. Thickness is 22 gauge. Passivate by soaking in 7% citric acid for 20 to 30 minutes according to ASTM A967 before cleaning. Clean with a non-ionic detergent, rinse with water and sterilize. Use the screened, brushed surface of the carrier as the testing surface. Carriers are single-use.
2. Centrifuge with rotor capable of achieving 5000g.
3. Centrifuge tubes, conical, 15 mL, for centrifugation of test cultures.
4. Culture tubes, 20 mm × 150 mm glass, with Morton closures, for test culture preparation.
5. Desiccation unit with gauge to measure vacuum and maintain chamber pressure of 0.068 to 0.085 MPa. Use of color indicator desiccant recommended. Sufficient size to hold at least three 100 mm diameter Petri dishes; used for drying of inoculated carriers.
6. Filter sterilization units (with polyethersulfone membrane, 0.2 µm pore size), use to filter sterilize soil components.
7. Forceps, straight or curved, with smooth flat tips to handle membrane filters, and to transfer inoculated carriers into vials.
8. Filter membranes (polyethersulfone) for recovery of test microbe, 47 mm diameter and 0.2 µm pore size.
9. Micropipettes, calibrated (e.g., 200 µL, 1 mL) with 10-100 or 20-200 µL tips, use for deposition of antimicrobial test substance or control substance on carriers and preparing dilutions.
10. Petri plates, 150 mm diameter, sterile.
11. Pipette, calibrated 10 µL positive displacement, with corresponding 10 µL tips, for carrier inoculation.
12. Timer, for tracking of timed events and intervals.
13. Vials with lids, plastic, that are sterile, flat-bottomed, wide-mouthed (at least 25 mm diameter), for holding inoculated carriers and for accommodating neutralizer.
14. Vortex-style mixer, for vortex-mixing of various solutions.

Reagents and Media

1. Bovine Serum Albumin (BSA) stock. Add 0.5 g BSA (radio immunoassay (RIA) grade or equivalent, CAS # 9048-46-8) to 10 mL of PBS, mix to dissolve completely and pass through a 0.2 µm pore size (polyethersulfone) membrane filter, aliquot, and store at -20°C ± 2 °C.
2. Detergent, non-ionic (1% solution), use to clean carriers.
3. Dextrose solution, 10%(w/v). Filter sterilize the solution using a 0.2 µm filter. Added to SB prior to

use.

4. Mucin stock. Add 0.04 g mucin (from bovine submaxillary gland, CAS # 84195-52-8) to 10 mL of PBS, stir or vortex-mix until thoroughly dissolved, and pass through a 0.2 µm pore size (polyethersulfone) membrane filter, aliquot, and store at -20°C ± 2°C. To facilitate dissolving the mucin, add the PBS to a small vessel with a stir bar and place on a stir plate to create a strong vortex. Slowly sprinkle the mucin into the liquid vortex and stir for at least 20 minutes or until completely dissolved.
5. Neutralizer; varies based on the test substance. Confirm adequate neutralization by adding 20-200 CFU of the test organism to mixtures of the test substance and the neutralizing agent (e.g., use Lethen broth with 0.5% Tween® 80 and 0.07% Lecithin for quaternary ammonium compounds) and then plating survivors per the protocol. Confirm lack of neutralizer toxicity to the test organism in a similar manner.
6. Phosphate buffered saline (PBS), pH of 7.0 ± 0.5.
7. Synthetic broth (SB), growth medium for test cultures.
8. Tryptic soy agar (TSA). This medium is available commercially in either pre-poured plates or dehydrated form to prepare according to manufacturer's instructions.
9. Tryptic Soy Broth (TSB), use to rehydrate lyophilized cultures.
10. Tryptic Soy broth (TSB) with 15% (v/v) glycerol, use as a cryoprotectant. Suspend 7.5 g tryptic soy broth in 212.5 mL de-ionized water. Add 37.5 mL glycerol and stir, warm slightly to dissolve. Dispense into bottles and autoclave for 15 min at 121°C.
11. Water, use reagent-grade water free of substances that interfere with analytical methods. Any method of preparation of reagent-grade water is acceptable provided that the requisite water quality can be met.
12. Yeast Extract stock. Add 0.5 g yeast extract to 10 mL of PBS, mix to dissolve completely, and pass through a 0.2 µm pore size (polyethersulfone) membrane filter, aliquot, and store at -20°C ± 2 °C.

Familiarity with microbiology safety laboratory procedures and aseptic technique is required.

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None

Prepare vials of frozen stock culture

1 Prepare vials of frozen stock culture

- 1.1 Rehydrate a lyophilized culture of *Pseudomonas aeruginosa* ATCC 15442 with 5 to 6 mL of Tryptic Soy Broth (TSB)
- 1.2 Mix thoroughly.

- 1.3 Incubate broth culture at $36^{\circ}\text{C} \pm 1^{\circ}\text{C}$ for 24 h ± 2 h.
- 1.4 Streak a loopful of the broth culture onto two Tryptic Soy Agar (TSA) plates to obtain isolated colonies.
- 1.5 Incubate TSA plates for 24 h ± 2 h at $36^{\circ}\text{C} \pm 1^{\circ}\text{C}$.
- 1.6 From the TSA plates, select 3-5 isolated colonies and re-suspend in 1 mL of TSB. Select colonies from each of the two phenotypes present.
- 1.7 Spread plate 0.1 mL of the TSB suspension onto each of 6-10 TSA plates.
- 1.8 Incubate the TSA plates for 24 h ± 2 h at $36^{\circ}\text{C} \pm 1^{\circ}\text{C}$.
- 1.9 Add approximately 5 mL of sterile cryoprotectant solution (TSB with 15% (v/v) glycerol) to the surface of each TSA plate.
- 1.10 Re-suspend the growth in the cryoprotectant solution using a sterile spreader without damaging the agar surface.
- 1.11 Aspirate the suspension from the plate with a pipette and transfer it into a sterile vessel.
- 1.12 Repeat the growth harvesting procedure with the remaining plates and continue adding the subsequent suspensions to the same collection vessel.
- 1.13 Mix the vessel contents and dispense 0.5-1.0 mL aliquots of the harvested suspension into cryovials for freezing.

- 1.14 Confirm the identity and purity of the culture by culture characteristics, biochemical characteristics (e.g., Gram-negative, Oxidase positive), fermentation, enzymatic reactions and/or molecular techniques.
- 1.15 Store the cryovials at -70°C or lower for a maximum of 18 months.

Prepare the test culture (minus the soil load)

2 Prepare the test culture (minus the soil load)

- 2.1 Prepare tubes of Synthetic Broth (SB) by adding 0.1 mL of 10% sterile dextrose (w/v) solution to each 10 mL tube of SB and mix.
- 2.2 Remove a cryovial of the stock culture from the freezer and allow it to thaw at room temperature.
- 2.3 Add 0.1 mL of thawed stock culture to 10 mL of SB + dextrose and mix.
- 2.4 Incubate the inoculated SB + dextrose under static conditions for 24 h \pm 2 h at 36°C \pm 1°C. The 24 h \pm 2 h culture is expected to contain approximately 108 CFU/mL.
- 2.5 Confirm there is a visible pellicle on the surface of the culture. Discard the culture if the pellicle has been disrupted or there are pellicle fragments in the culture.
- 2.6 Remove the pellicle from the surface of the culture and around the sidewalls of the vessel with vacuum suction.
- 2.7 Using a serological pipette, withdraw the remaining broth culture (approximately 7 to 8 mL) avoiding any sediment on the bottom of the tube and transfer it into a 15 mL conical centrifuge tube.

- 2.8 Within 15 minutes of completing the previous step, centrifuge contents of the conical centrifuge tube at $5,000 \times g$ for 20 minutes.
- 2.9 Remove and discard the supernatant without disrupting the pellet.
- 2.10 Re-suspend the pellet in 5 mL to 10 mL Phosphate Buffered Saline (PBS).
- 2.11 Create a homogenous mixture by vortex-mixing. Tap the centrifuge tube briskly if parts of the pellicle remain after mixing – this will assist in disaggregating the pellet. If tapping is necessary, then follow tapping with more vortex-mixing until a visibly homogenous suspension is achieved.
- 2.12 Dilute the 5 mL to 10 mL of resuspended culture in additional PBS, as needed, to achieve a mean control carrier count level of $5 \log \pm 0.5 \log$ CFU/carrier after drying. The culture suspension from this step is then used to prepare the final test suspension (containing the soil load).

Prepare the final test suspension containing soil load

3 Prepare the final test suspension containing soil load

- 3.1 Combine the following three soil components together: 25 μ L BSA stock, 35 μ L yeast extract stock, 100 μ L mucin stock.
- 3.2 Vortex the three-part soil suspension for 10 seconds.
- 3.3 Add 340 μ L of the test culture to the three-part soil, then mix.
- 3.4 Maintain the final test suspension at room temperature until it is used to inoculate carriers. Inoculate all carriers within 30 minutes of final test suspension preparation.

4 Inoculate and dry the carriers.

- 4.1 Place carriers with the brushed side facing upward inside an empty, sterile plastic Petri dish. There should be no more than 20 carriers per dish. Inoculate three (3) carriers for each antimicrobial test condition and three (3) carriers to serve as control carriers. Inoculate additional carriers to serve as extras, in case they are needed (e.g., when a carrier is mishandled).
- 4.2 Using a positive displacement pipette with a 10 μ L tip, withdraw 10 μ L of the final test suspension and deposit it at the center of each carrier, keeping the pipette perpendicular to the carrier during deposition of the final test suspension. Avoid contact of pipette tip with carrier. Do not spread the final test suspension with the pipette tip. To maintain culture consistency, vortex-mix the final test suspension frequently during inoculation of the carriers. The same pipette tip may be used to inoculate all carriers. Discard inoculated carriers if the final test suspension runs over the edge of the carrier.
- 4.3 Transfer the Petri dish(es) with the inoculated carriers into a desiccation unit and remove the lid of the Petri dish. Do not exceed 40 inoculated carriers per desiccator.
- 4.4 Close the desiccation unit and apply vacuum.
- 4.5 Maintain a vacuum of 0.068 MPa to 0.085 MPa for the duration of the drying procedure.
- 4.6 Maintain the inoculated carriers in the desiccation unit (under vacuum) at room temperature for 45 to 60 minutes.
- 4.7 Visually inspect the inoculated carriers to verify that the inoculation spot is visibly dry. Do not use carriers that are visibly wet for testing. Do not use carriers if the inoculum has spread to near the edge of the carrier.
- 4.8 After drying and removal from the desiccation unit, hold carriers in a closed Petri dish at room temperature for up to 30 minutes before a test or control substance is applied. Do not use carriers for testing if they have been stored for longer than 30 minutes.

Expose dried inoculated carriers to the test substance or control substance

- 5 Expose dried inoculated carriers to the test substance (i.e., the disinfectant solution) or control substance. The control substance is PBS.
 - 5.1 Use 3 carriers for each test condition, and 3 corresponding control carriers.
 - 5.2 Using a sterile forceps, transfer each dried carrier with the inoculated side up to a flat-bottom vial and cap the vial. Repeat until all carriers are transferred.
 - 5.3 In a timed fashion with appropriate intervals, sequentially deposit 50 µL of the antimicrobial test substance (at room temperature) over the dried inoculum on each test carrier, ensuring complete coverage. Apply the antimicrobial test substance with a pipet that is held vertically. Use a new pipette tip for each carrier; do not touch the carrier surface with the pipette tip during the application of the antimicrobial test substance or the control substance; replace with new carrier(s) and vial(s) if this occurs. Do not cap the vials. If the antimicrobial test substance or control substance runs off the carrier or does not completely cover the inoculum spot, then discard that carrier and replace with a new carrier. Repeat the same procedure for control carriers last, except use PBS instead of the antimicrobial test substance.

Neutralize the test substance

- 6 Neutralize the test substance
 - 6.1 At the end of the timed contact period, add 10 mL of the neutralizer (Lethen broth with 0.5% Tween® 80 and 0.07% Lecithin) to each vial in the specified order according to the predetermined schedule.
 - 6.2 Vortex-mix each vial for 2 to 3 seconds following the addition of the neutralizer.
 - 6.3 Following the neutralization of the entire set of carriers, vortex-mix each vial for 30 seconds. Ensure that the liquid and carrier are both spinning in the vial during vortex-mix. Do not remove the carrier from the vial.

Dilute and plate the neutralized sample

7 Dilute and plate the neutralized sample.

- 7.1 Within 30 minutes of neutralization, dilute and filter all samples; prioritizing test substance treated carriers first.
- 7.2 Transfer 1 mL of the neutralized sample into 9 mL PBS.
- 7.3 Prepare additional 10-fold serial dilutions as necessary to achieve countable filters (0-200 CFU).
- 7.4 Pre-wet the membrane filter (0.2 μ m pore-size PES membrane) with 10 mL of PBS.
- 7.5 Add samples to filtering units; use a separate membrane filter for each sample. If a carrier falls onto the filter membrane, aseptically remove it using sterile forceps. If the filter membrane is accidentally punctured in the process of removing the carrier, then discard the membrane.
- 7.6 Rinse each empty sample vessel with 10 mL of PBS. Vortex-mix for 4 seconds and pour the vial contents into the same filter unit.
- 7.7 Rinse the interior sidewalls of the membrane filtering unit with 20 mL of PBS.
- 7.8 Apply a vacuum to the filter unit to pull the liquid through the filter.
- 7.9 With the vacuum on, aseptically remove the membrane filter and place it onto the surface of a TSA plate. Apply the filter to the agar surface in such a way to minimize the trapping of air bubbles between the filter and the agar surface.
- 7.10 Incubate all plates at $36^{\circ}\text{C} \pm 1^{\circ}\text{C}$. For control carriers, incubate for 48 ± 4 hours. For treatments, incubate for 72 ± 4 hours.

Count colonies and record results

8 Count colonies and record results.

- 8.1 Count the colony forming units (CFUs) on each plated filter. If the number of colonies on a filter exceeds 200, then record as Too Numerous to Count (TNTC). If no colonies are present, then record as zero.
- 8.2 Verify that colony appearances on the plated filters are consistent with *P. aeruginosa*. Random contamination with non-*P. aeruginosa* CFUs that does not obscure counting of *P. aeruginosa* does not invalidate the test. Randomness of contamination can be confirmed using biochemical tests (e.g., Gram stain, catalase test, oxidase test). Consistent contamination across multiple plated filters indicates a systematic problem and the test should be repeated.

Calculate log densities

9 Calculate log densities

- 9.1 Calculate the log₁₀ CFU on each carrier using the following equation:

$$\text{Log}_{10} \left\{ \left[\frac{\sum_{i=1}^n (Y_i)}{\sum_{i=1}^n (C_i \times D_i)} \right] \times V \right\}$$

- 9.2 Where:
Y = CFU per filter,
C = volume filtered,
V = total volume of neutralizer,

$D = 10^{-k}$,
 k = dilution,
 n = number of dilutions, and
 i = lower limit of summation (the fewest number of dilutions).

9.3 When TNTC values are observed for each dilution filtered, substitute 200 for the TNTC at the highest (most dilute) dilution and account for the dilution factor in the calculations.

9.4 Calculate the mean \log_{10} density (LD) for each set of three treatment conditions and the controls as follows:

Mean LD = $[(\log_{10}\text{CFU on carrier \#1}) + (\log_{10}\text{CFU on carrier \#2}) + (\log_{10}\text{CFU on carrier \#3})]/3$

Calculate \log_{10} reductions

10 Calculate the \log_{10} reduction (LR) for each treatment condition as follows:

10.1 $LR = (\text{Mean LD for Control Carriers}) - (\text{Mean LD for Treated Condition})$

10.2 If no CFUs are recovered from each of the three treated carriers, then the log reduction is greater than or equal to the mean control carrier log density.