A Rapid Method to Determine Disinfectant Efficacy

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Abstract

A method has been developed using the BD™ Oxygen Biosensor System (BD™ OBS) to determine the efficacy of disinfectants. Borosilicate glass cylinders (carriers) are contaminated with a heavy suspension of test organisms. The contaminated cylinders are then exposed to the disinfectant for 10 minutes and placed in a 24-well OBS plate and a neutralizing/growth medium. The plate is incubated for six hours at 37ºC in fluorometer and fluorescence intensity is measured every 15 minutes. Any oxygen consumption as a result of organism growth, such as in positive control well, unquenches the sensor fluorescence, which is then read by the fluorometer as an increase in the fluorescence intensity. In the absence of organism growth, such as after exposure to disinfectant, the fluorescence remains quenched and there is no increase in fluorescence intensity. The efficacy of disinfectant was determined by detecting the curves in wells showing increased fluorescence intensity over time versus curves in wells with no increase in the fluorescence intensity. Using BD OBS plates, the disinfectant efficacy was determined in six hours with minimum manipu-lation, whereas the current methods described by AOAC require 48-54 hours and are time and labor intensive.

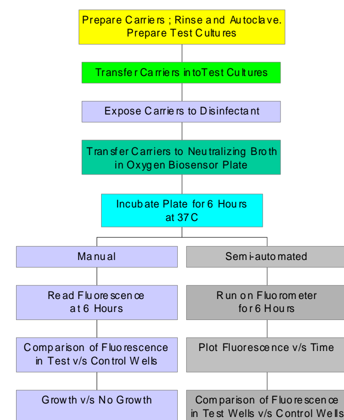
Introduction

The AOAC recommends three methods[1] for testing the efficacy of disinfectants, namely Use-Dilution Method (955.14, 955.15 and 964.02), Hard Surface Carrier Method (991.47, 991.48 and 991.49) and Phenol Coefficient Method (955.11, and 955.12). The AOAC methods in general are tedious, time and labor intensive, technique sensitive[2] and require 48-54 hours of testing with live bacterial cells and up to 72 hours with spores. The efficacy data available is only qualitative and only suggests growth or no-growth at a given disinfectant dilution after exposure.

The objective of this study was to determine the feasibility of using BD Oxygen Biosensor System for testing disinfectant efficacy in a microtiter tray format and to propose a system that is simpler, user-friendly, more reproducible, more cost-and time-effective and more quantitative than the current methods. The BD Oxygen Biosensor System[3,4] consists of a microtiter plate containing a fluorescent compound (tris 1,7-diphenyl-1, 10- phenanthroline ruthenium (II) chloride) at the well bottom. The fluorescence is quenched in the presence of oxygen and unquenched when the oxygen is removed. Organisms growing in the liquid medium in micro-wells consume oxygen. When the rate of oxygen consumption is greater than the rate of oxygen diffusion in to the growth medium, the fluorescence intensity of the fluorophore increases dramatically. The fluorescence is measured by a fluorometer (automated) or with an UV transilluminator (manual) at specific time intervals and the data is recorded and/or analyzed in a spreadsheet. When Time is plotted against the Relative Fluorescent Units (RFU), an "S" shaped curve is observed (positive curve). The "S" shaped curve provides information on the lag time, which is the amount of time required for the organism in a given well to reach the mid-point of the "S" shaped curve 5. The lag time is proportional to the total number of viable organisms present in a given well at time zero.

The purpose of testing various disinfectant dilutions is to ensure the efficiency of the disinfectant at a ready-to-use concentration. The general test protocol involves contamination of test cylinders, exposure of contaminated cylinders to disinfectant followed by placement of the cylinders in oxygen sensor plate containing neutralizing broth for incubation at 35ºC to 37ºC. If an inhibitory substance is present in the medium, the growth of the organisms is inhibited and the lag time is extended. One such inhibitor was Spore-Klenz, the disinfectant for which the efficacy study was carried out. Various concentrations of the disinfectant produced different lag times, indicating the effect of the disinfectant at the concentration in a particular well.

Oxygen Biosensor Method: General Test Protocol



Materials and Methods

BD™ Oxygen Biosensor System

- 96-well Trays: BD Cat. No. 353830, 96-well clear round bottom microtiter plates.
- 24-well Plates: BD Falcon™ 24-well trays, Cat. No. 351147 were prepared in the laboratory (Fig. 1).

Disinfectants

DECON-SPORE™ Ready-to-Use (Veltek Associates, Inc., Phoenixville, PA) and SPOR-KLENZ® Ready-to-Use (Steris Corporation, St. Louis, MO) were used. The active ingredients in both disinfectants are 0.80% Hydrogen Peroxide and 0.06% of Peroxyacetic acid. Both Spore-Klenz and Decon-Spore are broad-spectrum disinfectants used for sterilization and disinfection of equipment and facility surfaces.

Test Organisms

The following ATCC® (American Type Culture Collection, Manassas, VA) organisms were sub-cultured in Trypticase Soy Broth (TSB, BBL 299071) overnight at 37ºC and standardized in saline to 70-75% transmittance (~1 McFarland equivalent) using a colorimeter (Vitek 52-1210):

- *Salmonella choleraesuis* ATCC 10708
- *Pseudomonas aeruginosa* ATCC 15442
- *Staphylococcus aureus* ATCC 6538

Neutralizing and Growth Medium

DE Neutralizing Broth (BBL, 299039) was prepared according to the manufacturer's instructions. The DE Neutra-lizing Broth [7] neutralizes a broad spectrum of disinfectants and preservative antimicrobial chemicals. The active components are Sodium Thioglycolate, Sodium Thiosulfate, Sodium Bisulfite, Lecithin and Polysorbate 80. DE Neutralizing Broth is recommended for neutralization of iodine, chlorine, formaldehyde, gluteraldehyde, quaternary ammonium compounds, phenols, hexachlorophene, formalin, ethanol, hydrogen peroxide and peroxyacetic acid.

Test Protocol for 24-well Plates

- Dispense two mL of DE Broth aseptically in to each well of a 24-well microtiter plate containing biosensor.
- Submerge borosilicate glass cylinders (Bellco, Cat. No. 2091-00808) in 70% Isopropyl Alcohol for 15 minutes. Wash 3x with DI water and sterilize submerged in DI water for 15 minutes at 121ºC in autoclave.
- Expose to test organisms in sterile reagent troughs. Test each organism and disinfectant concentrations (Ready-to-use and 1:50 dilution) in duplicate.
- Remove excess inoculum from cylinders by blotting on sterile filter paper pads. Expose cylinders to disinfectant (test) or saline (positive control) for 10 minutes.
- Blot and place cylinders (exposed to disinfectant or saline) in the 2-mL of DE Broth pre-dispensed in BD OBS.
- Incubate the plate for 6 hours at 37ºC, without shaking.

Test Protocol for 96-well Plates

- Serially dilute the disinfectant from ready-to-use concentration to 0.78% in two-fold dilutions through out the plate using sterile water. Use sterile saline as a substitute for the disinfectant in control wells.
- Spike wells with standardized suspension of test organisms and incubate tray for 10 minutes at room temp.
- Add DE Neutralizing Broth to each well after the 10-minute exposure to disinfectant.
- Incubate the plate on fluorometer for 6 hours at 37ºC, without shaking.

Manual Fluorescence Reading

At the end of the incubation period, place the biosensor plate on an UV transilluminator. The fluorescence in positive wells appears as bright orange glow against dark bluish gray negative wells.

Fluorometer

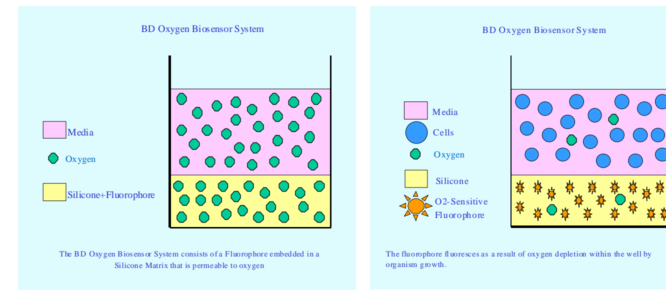
- Read the Relative Fluorescence Units (RFU) in each well of the 24-well and 96-well plate every 10 minutes using 485 excitation and 612 emission wavelengths.
- Collect the data from each well and analyze using a spreadsheet. The RFU for each well is plotted against time. A well with no change in fluorescence intensity is considered "negative" for growth. A well is considered "positive" for growth when an "S" shaped curve is observed due to organism growth. The lag time is the amount of time required for the organisms in a given well to reach the mid-point of the "S" shaped curve.

Disinfectant Efficacy Testing: Current Methods

- AOAC Use-Dilution Methods:
  - 955.14, 955.15 and 964.02
- AOAC Hard Surface Carrier Methods:
  - 991.47, 991.48 and 991.49
- Qualitative
  - Growth/No Growth
- Results in 48-54 Hours
- Labor Intensive
- Technique Sensitive

Disinfectant Efficacy Testing: Oxygen Biosensor Method

- Microtiter Plate-Based Technology
- Uses Oxygen Biosensor in 96 or 24-well Plates
- Rapid Method, Detection in 6 Hours
- Measures Fluorescence Intensity
- User-Friendly
- Manual or Semi-Automated Reading
- Measures the Total Effect of Disinfectant



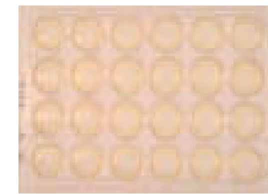


Fig. 1 24-well Oxygen Biosensor Tray

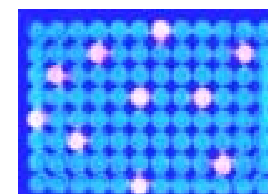


Fig. 2 96-well Oxygen Biosensor plate with a few fluorescing wells. The fluorescence produced is a result of oxygen depletion within the well by organism growth. The fluorescent signal in an amount of time is proportional to the number of organisms inoculated. Since the biosensor is located at the bottom of wells, it is unaffected by condensation and colored or turbid solutions.

Fig. 3 The Effect of Disinfectant Concentration
The method used in this study probes the total effect of disinfectant on microbe population, measuring the effect of injury as well as death (non-viability). The method allows for the routine examination of disinfection kinetics, which provides a greater scientific insight into the process of disinfection than that achieved by the standard qualitative test. In this particular experiment, the effect of disinfectant on three test organisms was clearly reflected by staggered and increased lag times when the disinfectant concentration was low, whereas at or beyond the minimum inhibitory concentration, no growth was observed.

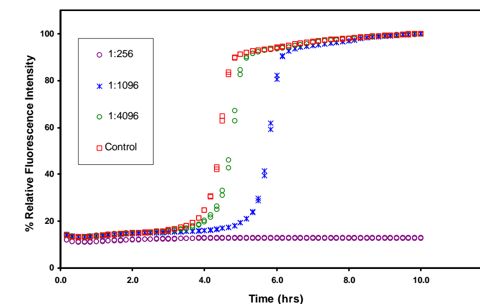


Fig. 4 The Effect of Disinfectant Concentration on *S. choleraesius*.
When the fluorescence intensity is plotted against time, the effect of disinfectant concentration on organism population is quite apparent. The minimum disinfectant concentration that was inhibitory to the organisms showed no increase in fluorescence intensity, whereas the lower concentrations showed an increase in lag time relative to controls. Duplicates at each concentration are shown.

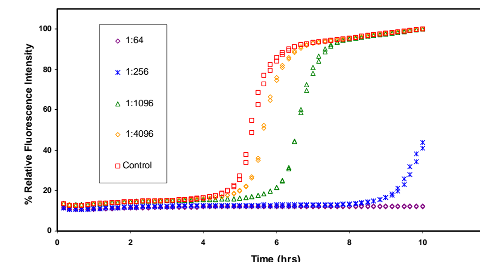


Fig. 5 The Effect of Disinfectant Concentration on *S. aureus*.
S. aureus showed a delayed growth at the 1:@56 dilution of Decon-Spore, showing a time to detection of ~10 hours, as compare dto 5.3 hours for the control. Under these conditions, the number of injured organisms increased relative to the 1:1024 dilution, and a greater fraction of the cells become non-viable. Duplicates at each concentration are shown.

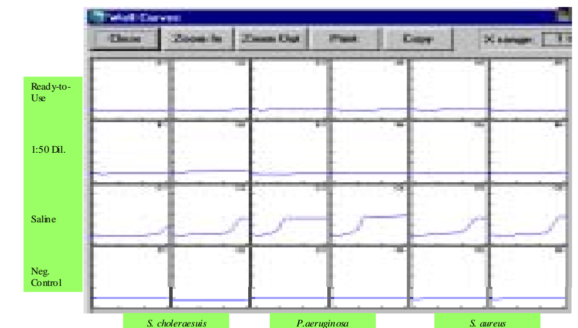


Fig. 6 Determination of efficacy in 6 hours.
Using a slight modification of the general test protocol, the disinfectant efficacy on borosilicate glass cylinders was determined in a 24-well sensor tray. Controls were detected in 3 to 5 hours, whereas no fluorescence increase was observed in test (ready-to-use and 1:50 dilution) and negative control (sterile cylinders) wells. Manual observation of fluorescence intensity showed a strong signal in positive wells, as compared with weak background fluorescence in negative wells.

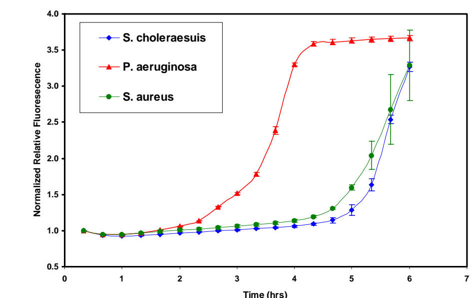


Fig. 7 Determination of efficacy in 6 hours.
When fluorescence was plotted against time, the positive controls showed a typical sigmoidal growth curve, whereas the test and negative control wells showed a flat line. Data are plotted as the average and standard deviation of duplicates at each concentration.

Results

Determination of the Efficacy

The growth curves of a test organism exposed to the disinfectant are compared to its respective positive control exposed to saline.

No Growth

The absence of growth in a disinfectant well versus positive growth in the positive control well suggests the efficacy of the disinfectant at that concentration. The positive control well has an expected lag time of approximately 3-5 hours.

Delayed Growth

- Delayed growth or extended lag time suggests some inhibition or injury inflicted to the organisms by the disinfectant.
- The delayed growth can be a result of disinfectant concentration or the total time of exposure to disinfectant before neutralization.
- Delayed growth is also a result of increase in the number of injured organisms due to disinfectant exposure and decrease in the number of non-injured organisms. The injured organisms are required to repair the injury before growth can be resumed, hence the delay.

Growth

Presence of growth (comparable detection times to control) after exposure to disinfectant suggests that the disinfectant failed to disinfect the organisms at that concentration.

Conclusion

- It is feasible to determine the efficacy of disinfectant in 6 hours using the BD™ Oxygen Biosensor System.
- The method is rapid, reproducible, cost and time effective and simple.
- The method provides qualitative and semi-quantitative data.
- The lag times produced by organisms indicate the potency of disinfectant.
- The method provides a measurement of the total effect of disinfectant on microbe populations, measuring the effect of injury and non-viability.

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