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AIHA LAP #183867
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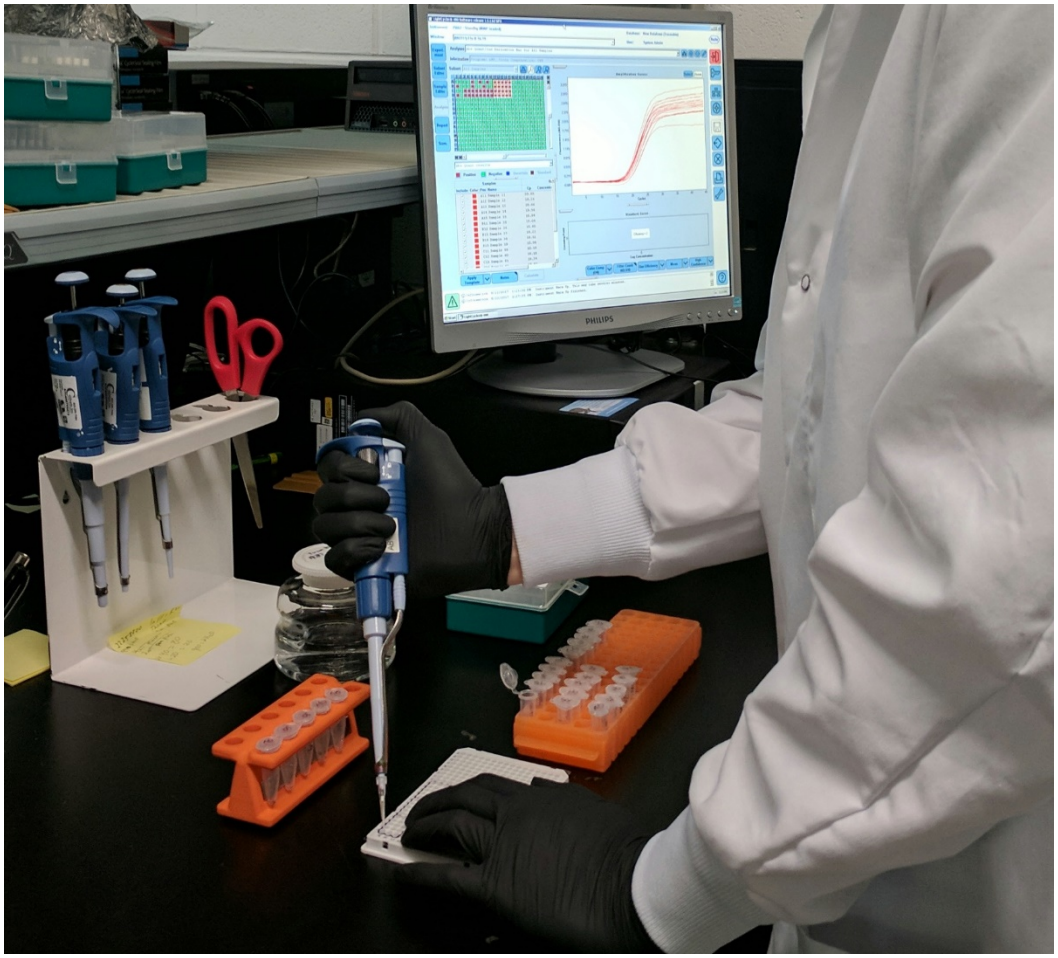
Experimental Briefing: Rapid Degradation of *Clostridium difficile*, *Candida albicans*, *Mycobacterium tuberculosis*, and Influenza B/Lee/40.



Product Testing Lab
August 28, 2017

Overview

- Assured Bio Labs, LLC was contracted by Clean Air Zone, Inc. (CAZ) to conduct a time series analysis to determine the capacity of CAZ Solution to degrade viable *Clostridium difficile*, *Candida albicans*, *Mycobacterium tuberculosis*, and Influenza B.

- Stock solutions

Table 1. Target Organisms.

Organism	Strain	Concentration
Clostridium difficile	ATCC 9689	$3.4x10^8$
Candida albicans	AB 522	$7.2x10^8$
Mycobacterium tuberculosis	ATCC 25177	$1.23x10^8$
Influenza B	B/Lee/40	$3.1x10^5$

The mixtures were inoculated at a 1:100 dilution into three different solutions (see Table 2).

Table 2. Solutions.

Solution	Treatment
80% CAZ Solution Viable or Live	None
80% CAZ Solution Autoclaved	Autoclaved for 30 minutes at 121° C and 19 psi to kill all viable CAZ Solution microbes
Tap Water	None

- The inoculated CAZ and water solutions were aerated during incubation by gently mixing on a Glass Col. multiwell plate mixer at a 65 rpm at room temperature.

- Samples were collected for DNA analysis immediately following inoculation (T_0) and every 12 hours thereafter ($T_1 \ldots T_{10}$) for 120 hour total.

Key Findings

- DNA analysis allowed for the detection and quantification of the specific target organisms when added to the complex microbial CAZ Solution mixture. DNA analysis used in this study utilizes high fidelity quantitative polymerase chain reaction (qPCR) technology that has superior detection range compared to standard microbial culture analysis.

- *Clostridium difficile* DNA was significantly reduced within **48 hours** in **80% CAZ solution** (see Figure 1). *Clostridium difficile* DNA stayed relatively stable at its lowest observed concentration for the rest of the study.

- *Candida albicans* DNA was significantly reduced within **36 hours** in **80% CAZ Solution Autoclaved** solution (see Figures 2). *Candida albicans* DNA increased 84-96 hours after concentration however dropped immediately after. *Candida albicans* DNA fluctuated but did not degrade in Tap Water solution 120 hours after inoculation.

- *Mycobacterium tuberculosis* DNA was significantly degraded **immediately** after inoculation in both **Live and Autoclaved 80% CAZ** solutions (see Figure 3). *Mycobacterium tuberculosis* DNA took 96 hours to degrade in Tap Water.

- Influenza B DNA was significantly degraded **24 hours** after inoculation in **80% CAZ** solutions (see Figure 4). Influenza B DNA fluctuated over time but stayed within low concentration levels during the duration of the study. Influenza B DNA was not affected by Tap Water solution even 120 hours after inoculation.

- The findings indicate that even when the living fraction of the CAZ solution is killed, the enzymatic fraction continues to function and is capable of neutralizing *Candida albicans, Mycobacterium tuberculosis*, and Influenza B.

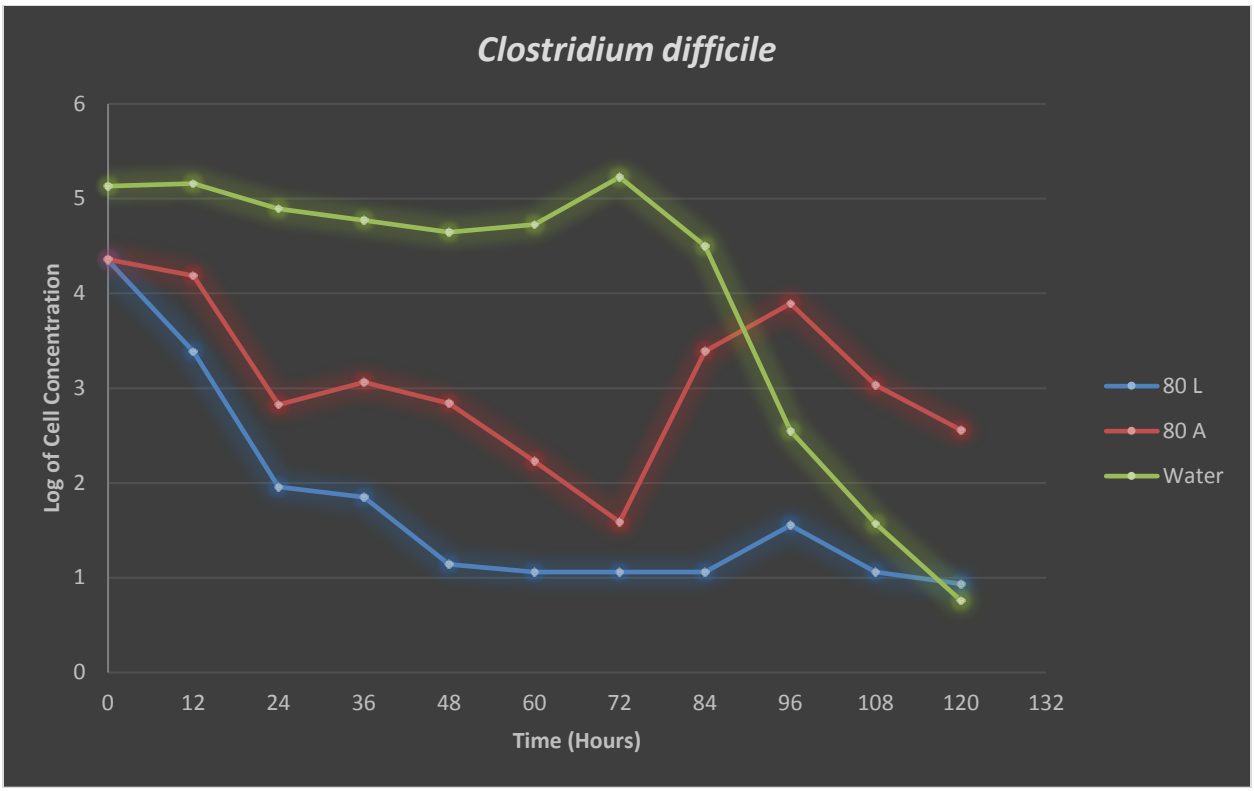
Experiment Methods

Stock Solution. *Clostridium difficile* and *Mycobacterium tuberculosis* were obtained from the American Type Culture Collection (ATCC), *Candida albicans* (AB 522) is from the American Industrial Hygiene Association, EMPAT 55 003F2-2014, and Influenza B virus was purchased from Charles River Laboratories. Six Banana Broth vials were used to grow *Clostridium difficile* for 72 hours at 35°C. The vials were then centrifuged for 5 minutes and after the supernatant was disposed, the bacteria was re-suspended in saline. The culture was quantified using a hemocytometer. *Mycobacterium tuberculosis* was cultured onto six plates of Middlebrook 7H10 media and inoculated into 3 tubes of Middlebrook 7H10 broth, incubated for approximately three weeks at 35°C. Broth culture was pressed through 12 ml luer lock syringe with cheese cloth patch in approximately five times until solution was homogeneous. The culture was then stained with crystal violet and quantifies on a hemocytometer. *Candida albicans* was cultured onto Malt Extract Agar (MEA) and incubated for five days at 37°C. A sterile cell scraper was used to remove the cells from the media plates and prepare the concentration of cells suspended in sterile saline. The stock solution was quantified using a hemocytometer. Influenza B was purchased already pre-quantified.

Inoculations. Three solutions were prepared (see Table 2), 45 ml of each was dispensed into sterile 100 ml screw-top bottles. The bottles were placed in a Bio-level 2 safety cabinet for inoculations. Exactly 0.5 ml of the each organism was dispensed into each of the three bottles. The bottles were shaken for 30 seconds to evenly disperse the cells. Afterwards, 15, 1.5 ml snap top tubes were filled with 250 µl from the bottles, five tubes per bottle. The tubes were immediately frozen and stored at -81°C. Then each bottle was dispensed in 6 ml aliquots into 5 of the 6 wells in a 6-well multiwell plate (Griener). The plates were placed on a mixing device at 65 rpm at room temperature and ambient light. The multiwell plates remained on the mixing device for 120 h. Every 12 hours, 15, 1.5 ml tubes were collected and frozen for the duration of the experiment. Hence, there were five replicates for each 12 hour time period for each of the three solutions.

DNA analysis. One hundred and sixty five samples (n=165) were analyzed using qPCR. The qPCR assays provided both detection and quantification of cell units. Fifteen samples were analyzed for each time sampling period from 0 to 120 hours with a frequency of 12 hours between collections.

Results



NOTE: Logarithm of cell concentration values was taken for better visual representation.

Figure 1. Rapid degradation of *Clostridium difficile* cells is observed in 80% CAZ Solution(blue line) shortly after inoculation. Within 48 hours the number of bacterial cells dropped significantly. A slight increase in cell concentration is observed 96 hours after inoculation followed by quick drop already within next 12 hours. The *Clostridium difficile* cell concentration somewhat fluctuated in Autoclaved 80% CAZ solution (red line), degrading and raising over time with sudden increase 72 hours after inoculation. The observed behavior is either due to bacteria growth or cell clumping during sampling. *Clostridium difficile* started consistently degrading in Tap Water solution (green line) 72 hours after inoculation.

Results

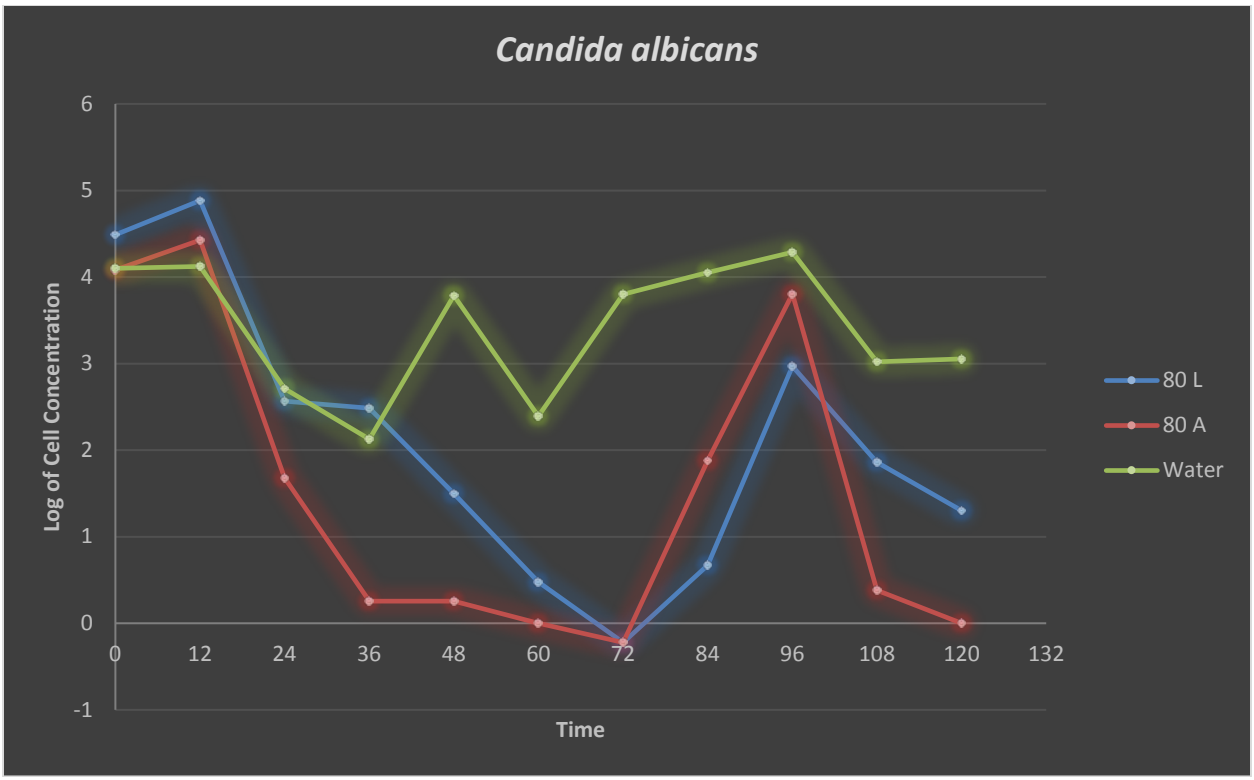


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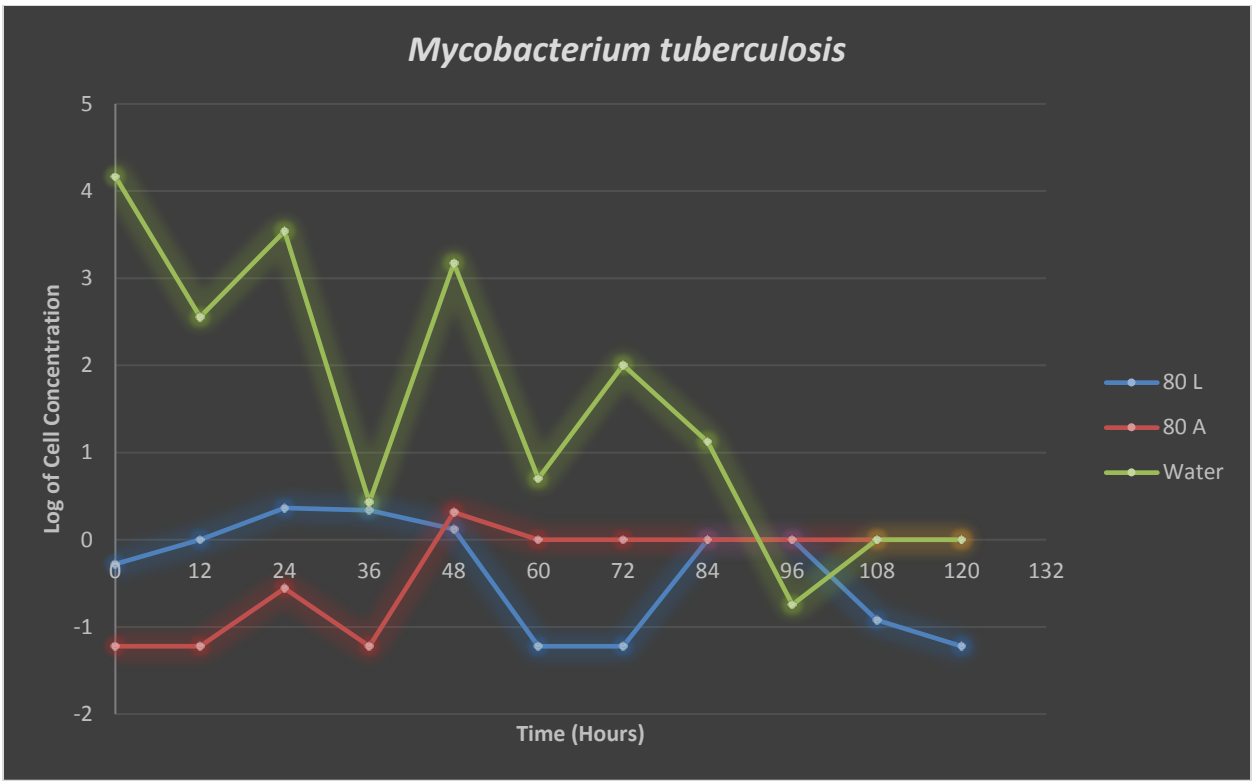
Figure 2. Rapid degradation of *Candida albicans* cells is observed in Autoclaved 80% CAZ Solution (red line) shortly after inoculation. Within 36 hours the number of bacterial cells dropped significantly. A sudden large increase in cell concentration is observed 84-96 hours after inoculation followed by quick drop already within next 12 hours. It took 72 hours after inoculation for *Candida albicans* to degrade significantly in live 80% CAZ solution (blue line). Similar to the behavior observed in Autoclaved 80% CAZ Solution the cell concentration raised rapidly 84-96 hours after inoculation followed by degradation at a slower rate than it is observed in Autoclaved 80% CAZ Solution. *Candida albicans* cell concentration fluctuated in Tap Water solution (green line) with lowest concentration observed at 36 hours after inoculation and highest at 96 hours. *Candida albicans* cell concentration stayed at relatively high level of concentration in Tap Water solution even after 120 hours after inoculation.

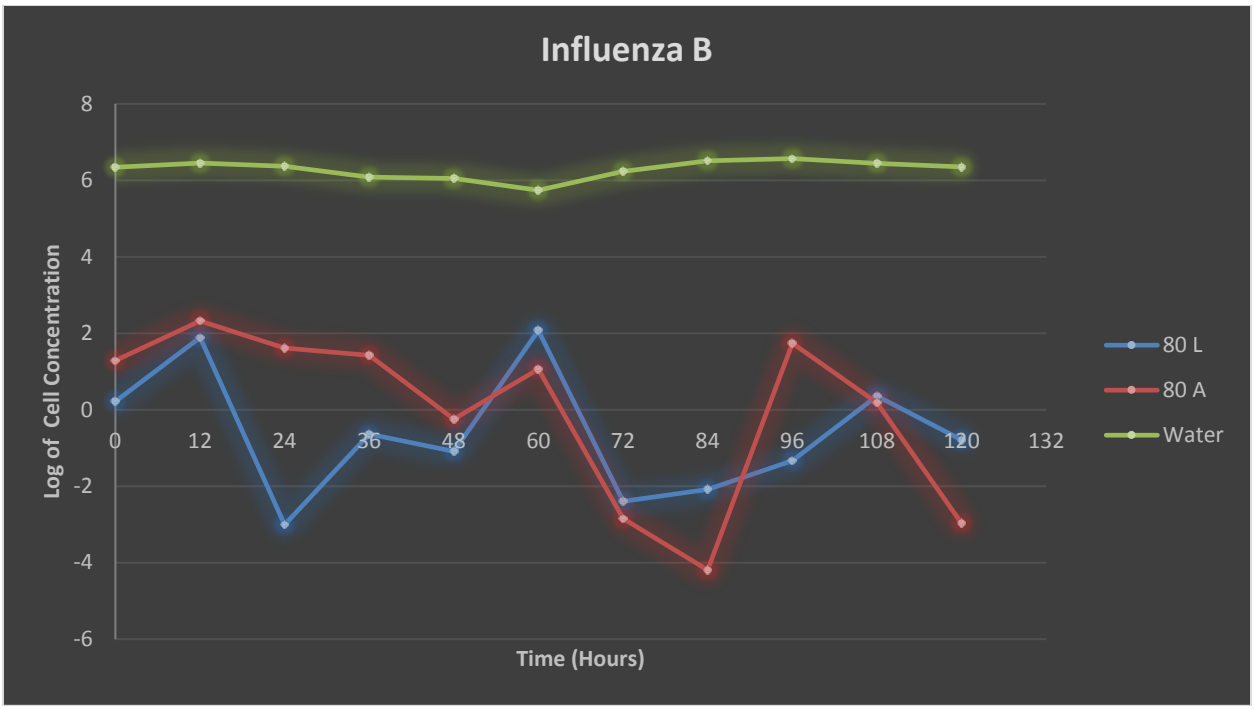
Results



NOTE: Logarithm of cell concentration values was taken for better visual representation.

Figure 3. ***Mycobacterium tuberculosis*** **degradation is observed in 80% CAZ Solution(blue line) and Autoclaved 80% CAZ Solution (red line) immediately after inoculation. A slight increase in cell concentration is observed 24 hours after inoculation in 80% CAZ Solution followed by immediate degradation.** ***Mycobacterium tuberculosis*** **cell concentration fluctuated in Tap Water solution (green line) with large raises and drops every 12 hours, the bacteria degraded completely 96 hours after inoculation.**

Results



NOTE: Logarithm of cell concentration values was taken for better visual representation.

Figure 4. Influenza B cell concentration degradation is observed in 80% CAZ Solution (blue line) and Autoclaved 80% CAZ Solution (red line) shortly after inoculation. The cell concentration fluctuated in both solutions raising highest 60 hours after inoculation in 80% CAZ solution and 96 hours after inoculation in Autoclaved 80% CAZ solution. Influenza B cell concentration stayed relatively stable in Tap Water solution (green line). The virus did not degrade even 120 hours after inoculation.