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Investigation of PMA-PCR technique for rapid detection of antimicrobial drug resistant bacteria

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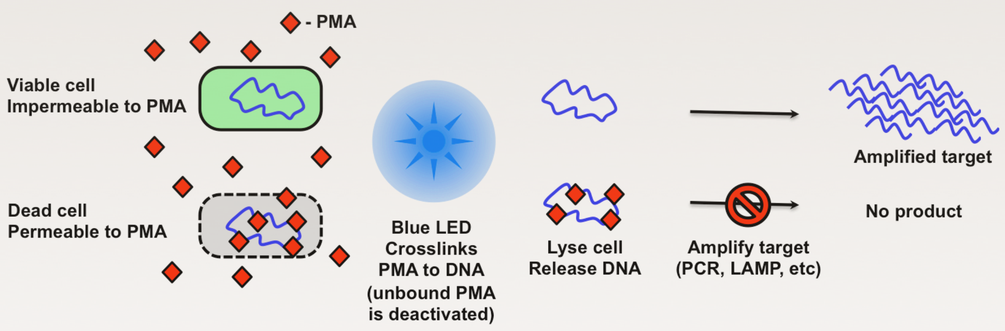
Background

Drug resistance is an emerging threat due in part to overuse of antibiotics, meanwhile, few new antibiotics are being developed. To combat this quickly increasing problem, it is critical to develop a rapid diagnostic for drug resistance. Currently, the most common method of evaluating and quantifying sensitivity of bacteria to drugs is to culture overnight in the presence of drugs (broth microdilution or disk diffusion assays).

Waiting overnight for cell growth in a sample tube has the benefit of being easy to interpret visually. However, the downfall is that it takes more than 12 hours just to see the results, and even so it can be difficult to determine whether cells are killed, or simply growing more slowly.

There is also the option of incubating bacteria with drugs, and running real time PCR compared to a heat-killed control sample. In this case the results of each run would be compared to and subtracted from the heated sample. This method gives information on whether or not cells grow, but it can still take a significant amount of time to tell the difference between cells that are dead, vs growing slowly, vs cells that grow at first, and then are killed slowly by the antibiotic.

In an attempt to find a method that is both faster and more accurate, we decided to test PMA-PCR (also known as "viability PCR"). Propidium monoazide (PMA) is a high affinity photoreactive DNA binding dye that is weakly fluorescent by itself, but becomes highly fluorescent upon binding to nucleic acids. The dye is cell membrane-impermeable and thus can be used to selectively modify DNA from dead cells with compromised membrane integrity, while leaving DNA from viable cells intact to be amplified during PCR. This allows us to quantify how much of the original product has lived or died. In our experiments, PMA-PCR treatment was tested to rapidly determine whether or not varying antibiotics could kill *E. coli* cells.



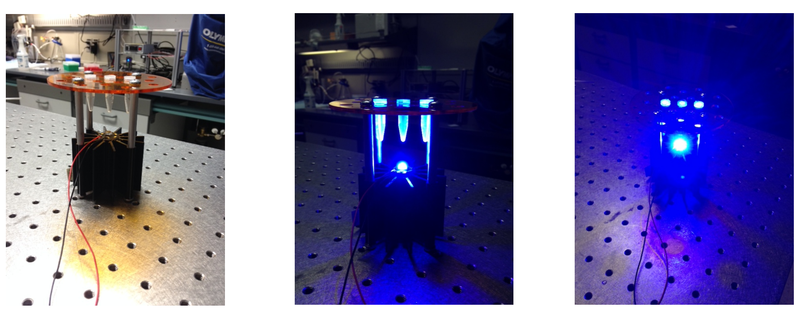
Representation of how PMA treatment inhibits dead-cell DNA from being amplified during PCR.*

Methods

In order to run each experiment in the most efficient way, we conducted preliminary experiments to determine the research standards. The results indicated that using 0.5 µM of ENT primer (broad-range primers that target the 16S gene of *Enterobacteriaceae* family) worked well for further experiments. We chose the broad-range primers because they would work for many other pathogens besides *E. coli*. DNA extraction with a spin column gave better results (lower C(t) values) than simply heat-treating whole cells. The C(t) value for an individual sample is defined as the cycle at which the samples' fluorescence trace crosses the chosen threshold line, which was 0.05.

The main experiments with antibiotics began by taking an overnight culture of either *E. coli* K12 strain ER2420 with plasmids pACYC177 or pACYC184 and diluting it with varying concentrations of several different drugs; Cefotaxime, Kanamycin, or Amoxicillin. *E. coli* 177 should be resistant to Kanamycin and Amoxicillin, whereas 184 should be susceptible to all three. We diluted overnight cultures into broths containing different concentrations of drugs, and incubated them for two hours in a 37°C shaker, along with cells without any drugs as a no-drug control. While those were incubating, a sample of the *E. coli* without any antibiotics was heat killed at 95°C for ten minutes.

After all samples were done incubating, we performed the PMA treatment. The process of a PMA treatment involves adding a PMA dye and PMA Enhancer to a sample of *E. coli* cells, and exposing the samples with blue LED light. From there the samples of *E. coli* both treated with PMA and identical samples not treated with PMA underwent a PureLink™ Genomic DNA Mini Kit extraction to extract DNA and remove PMA, which could inhibit PCR.



Pictures of the PMA LED light source

We set up the PCR with the optimized parameters as described above: 0.5 µM of ENT primers with extracted *E. coli* DNA. SYTO 9 dye (2 µM final concentration) was included for real-time monitoring. The PCR cycle used was a 3-step annealing procedure, beginning with 30 seconds (s) denaturing at 95°C, then annealing for 15 s at 95°C, 20 s at 54°C, and 30 s at 68°C repeated a total of 40 times, and lastly an extension step at 68°C for five minutes. All data was collected from a DNA Engine OPTICON 2.

*Images provided by Dr. Robert Meagher

U.S. DEPARTMENT OF ENERGY
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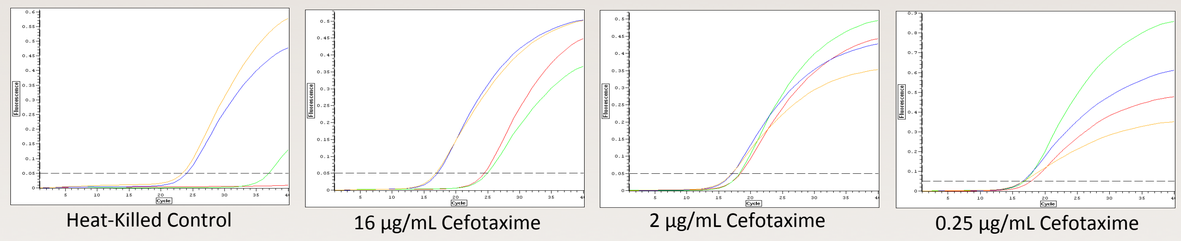
Results

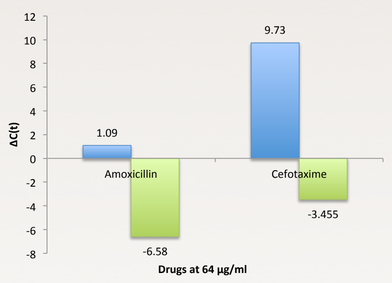
When testing *E. coli* 184 against varying concentrations of Cefotaxime, the PMA treatment gave more reliable results than no PMA to heat-killed comparisons. The 184 heat-killed control C(t) was **23.71**, and the alive no PMA sample averaged to **17.16**.

Without PMA, the 16 µg/mL solution gave a C(t) value of **16.99**, 2 µg/mL gave 17.095, and 0.25 µg/mL gave 16.615. Those same samples treated with PMA had C(t) values of **24.68**, 18.32, and 17.335, respectively. If only comparing the heat-killed (23.17) and no PMA antibiotic treated 16 µg/mL run (16.99), it would show that the *E. coli* had grown significantly in the presence of a lethal dose of Cefotaxime. When also comparing the alive no PMA sample (17.16) to the no PMA antibiotic sample (16.99), it looked as though the cells initially continued to grow even when there was a drug present. However, when comparing the Cefotaxime no PMA run (16.99) to the Cefotaxime PMA run (24.68), there was a significant difference, which shows that the cells did die. All three of these comparisons together show that the *E. coli* initially continued to grow with the antibiotic because the high amount of cells overwhelmed the drug. Then with time the drug began to kill the cells.

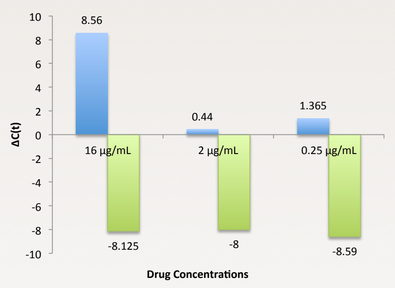
2 µg/mL showed growth from heat killed to Cefotaxime killed, but there was only slight death between PMA and no PMA. Comparing no PMA to PMA of the 0.25 g/mL showed that there was no death. Altogether these results show that 16 µg/mL or more of Cefotaxime would be needed to sufficiently kill a sample of *E. coli* 184. The images below show what this data looks like after PCR.



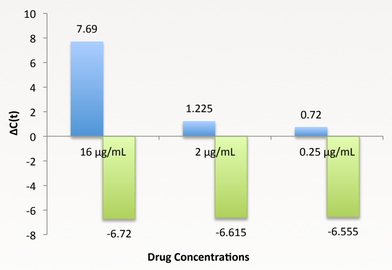
Heat-Killed Control | 16 µg/mL Cefotaxime | 2 µg/mL Cefotaxime | 0.25 µg/mL Cefotaxime



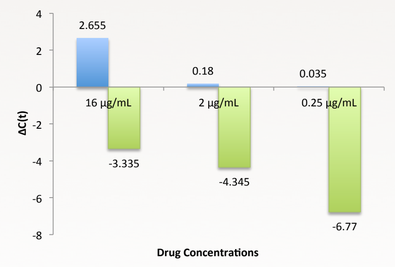
E. coli 184 with 64µg/mL of Amoxicillin and Cefotaxime. (07/02/2014)



E. coli 177 tested against three concentrations of Cefotaxime. (07/03/2014)



E. coli 184 tested against three concentrations of Cefotaxime. This is the experiment explained in the results above. (07/10/2014)



E. coli 184 tested against three concentrations of Kanamycin. (07/11/2014)

No PMA − PMA: Shows how many cells died. A ΔC(t)=1 means ½ of the DNA comes from live cells. ΔC(t)=2 means ¼ of the DNA comes from live, and ¾ from dead…etc.

Heat-Killed − No PMA: Shows how much *E. coli* grew. ΔC(t) should be roughly equal to the number of cell divisions the sample went through.

Discussion

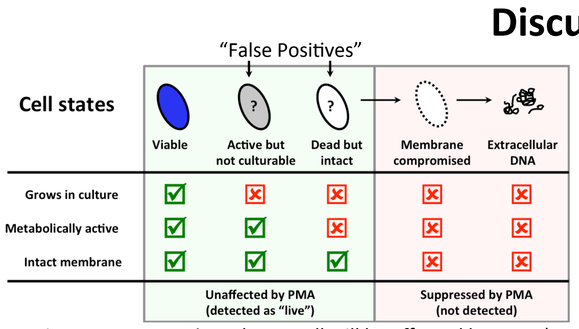


Diagram representing when a cell will be effected by PMA.*

It is to be noted that there are some downfalls to PMA. There is the possibility that it can produce false positives if the dead cell has an intact membrane or if the active cell is not culturable (we observed this during a PMA experiment with Ciprofloxacin). Overall, using PMA and being able to compare the PMA results to the no PMA results (🟦) gave a more accurate comparison of how many cells were able to live or die in the presence of an antibiotic than comparing no PMA to a heat-killed sample (🟩). When the heat-killed, PMA, no PMA, and alive control sample data values are all put together, a complete storyline of how the *E. coli* came to live or die can be put together. It can show us that the *E. coli* originally grew when the antibiotic was first applied to the cells, but they end up dying. This is a very common effect because when there is a high amount of cells, the drug can at first be overwhelmed (so-called "inoculum effect"). After time, the antibiotic will begin to take effect and the cells will die, and their DNA will not be amplified. A higher C(t) value of a PMA run in comparison to a lower value without PMA is proof of successful PMA with cell death.

Future Work

The current study shows proof of concept that PMA can be used to rapidly distinguish susceptible from resistant bacteria for certain drugs, but further work is needed to compare the PMA technique to standard techniques like broth microdilution, for a variety of classes of drugs. The current protocol is time-consuming, particularly the DNA extraction. This could be improved with parallel techniques, such as switching from individual spin columns to 96 well-plate based extraction. We are developing a filter-based microfluidic device which could be used to perform all-in-one culture, PMA activation, and cleanup in a single disposable device.