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Development and Evaluation of a High-Throughput Screening Method for Bacterial Antibiotic Production

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Abstract

Due to the misuse and overuse of antibiotics, the number and species diversity of multi-drug resistant bacteria continues to rise. To fight this growing threat, new methods for discovering novel antibiotics must be developed. Natural products produced by microorganisms continue to be a robust source of novel antibiotics; however, a rudimentary method that can screen natural sources accurately and efficiently has yet to be established. Current methods for screening bacterial libraries for antibiotic production are either highly complex or inefficient and prone to error. Herein, a rapid and robust high-throughput liquid culture screening method for antibiotic production by bacteria is described, which has the ability to screen both single and multi-culture mixtures of bacteria *in vitro*. Over 300 bacterial species were screened in monoculture, and 12% and 15% were found to produce antibiotics capable of ≥30% growth inhibition of *Staphylococcus aureus* or *Escherichia coli* respectively.

1. Introduction

Multidrug resistant bacterial infections are one of the top three threats to global public health. There are many factors that contribute to the upsurge of multidrug resistant bacterial infections. The largest contributing factor is the excessive usage of antibiotics, as they are among the most commonly prescribed drugs in human medicine[1,2]. As the misuse and overuse of antibiotics rise, the resistance to antibiotics also increases. Antibiotic resistance is defined as the ability of microorganisms to grow in the presence of bacteriostatic or bactericidal antibiotics via genetically resistant mechanisms[1,2,3]. Antimicrobial resistance genes are carried on either plasmids, which are circular molecules of double stranded DNA, or transposons, sequences of DNA that can independently move to different positions in the genome[2]. The genes are transferred via horizontal gene transfer which requires donor DNA, mutations, and host acceptance. The rate at which antibiotic resistance spreads can be affected by a microbes' physiology, genetics, interactions, and environmental exposure[3]. Because bacteria have the ability to overcome antibiotics, hard-to-treat-infections are generated. The "ESKAPE" pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter* species) are most commonly associated with such infections[2,4]. The increase in number of these aggressive infections has a direct correlation with the increase of antimicrobial-resistant pathogens; collectively, this has an immense negative impact on today's healthcare systems[4]. The U.S. Centers for Disease Control and Prevention reported that more than 2,000,000 people in the U.S. are affected by multidrug resistant bacterial illnesses annually. The CDC also cited that over 23,000 lives are lost due to antibiotic resistant bacterial infections per year[1]. Mortality and morbidity are not the only effects from antibiotic resistance. Increased treatment costs, diagnostic uncertainties, and lack of trust in orthodox medicine are also negative outcomes[4]. The rise of multidrug resistant bacterial infections is a complex issue that not only affects the individual patient but the broader community. Pharmaceutical corporations have reduced their focus on antibiotic production due to lack of success over the past few decades; this leaves discovery and development up to smaller companies and institutions[5]. Natural product isolation has proven to be the most successful approach to combat the increasing number of multidrug

resistant bacterial infections. Natural products have been used for the past century and continue to help move forward antibacterial therapies by their large role in drug development[6,7].

Antimicrobial natural products consist of unique chemical properties which offer researchers ample opportunities to explore possible drug treatments. Over the last 20 years, natural products and their derivatives accounted for 60% of new small-molecule drugs. These novel products are found in natural sources including land plants, soil, fungi, lichens, endophytes, and marine plants[7]. There are many different methods used to screen natural sources for antimicrobial activity. In 2018, researchers from Rockefeller University in New York found a new class of antibiotic called malacidin. They screened 2,000 soil samples for calcium dependence using high-speed computer processing. This narrow approach was used to specifically look for antibiotics similar to daptomycin, a natural product from a soil bacterium that was isolated in 2003[8]. Another recent class of natural product antibiotics is teixobactin, which was found in 2015 and produced by a soil organism *Eleftheria terrae*. It was discovered using iChip, which allowed the environmental bacterium to grow, isolate, and identify the antibiotic that was produced (Fig. 1)[9].

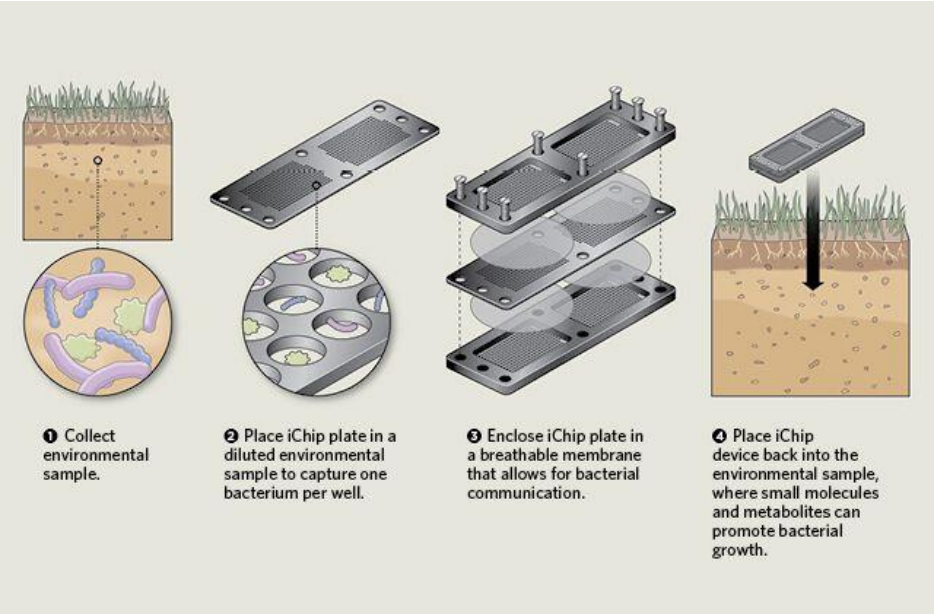

Figure 1. iChip method.

Figure 1 Image courtesy of Piddock (2015), iChip is an instrument that isolates individual bacteria from soil, salt marsh, saliva, mud, etc[9].

Cheaper and more robust methods to screen bacterial samples for antibiotic activity include agar spread plate method and agar overlay method (Fig. 2)[10]. Both of these methods create a lawn of desired pathogen over an agar plate where a single producer is found when a sample bacteria is pipetted onto the agar and a ring of inhibition is produced.

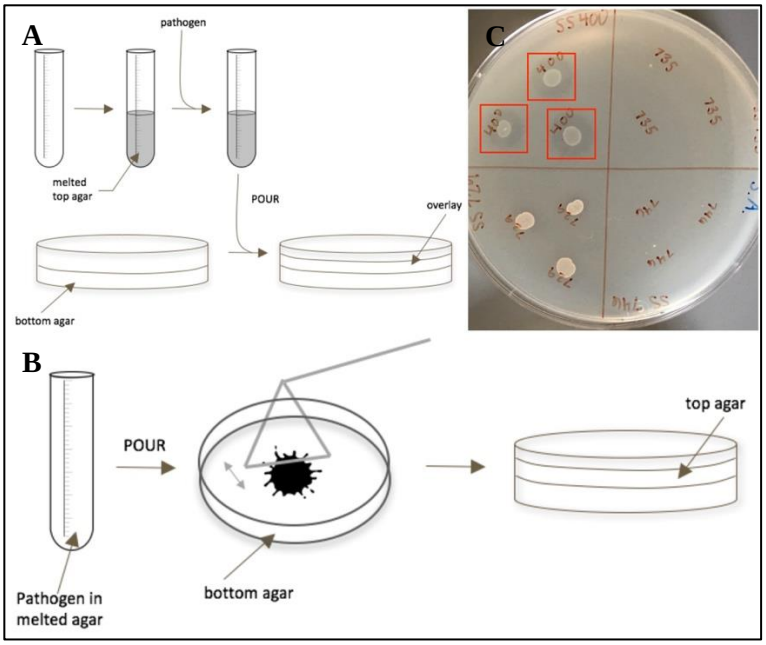

Figure 2 Agar overlay and spread plate methods.

Figure 2 Antibiotic activity measured on agar from A) agar overlay method or B) spread plate method. Both results in C) rings of inhibition on agar.

Other methods are the disk or well diffusion assays and broth or agar dilution assays. These methods are highly prone to error, unpredictable, qualitative, and time consuming compared to the proposed method. Decades before malacidin and teixobactin, no new classes of antibiotics had been discovered. Some argue that natural product isolation only re-isolates already known substances which is a huge disadvantage for the field. In order to find novel compounds, new screening methods that test for antibiotic activity must be developed. Natural products and screening methods are two major factors that lead researchers to the discovery and development of antibiotics[11]. Previously, over 400 bacteria were isolated in the past five years from a variety of environments including bulk and rhizosphere soil, the phytotelmata of *Sarracenia* pitcher plants, desert soil, and freshwater sediment. Most bacteria were previously screened for antibiotic activity against Gram-positive and Gram-negative pathogens in monoculture using either the spread plating method or the agar overlay method. Using these methods, 282 samples were tested for single antibiotic production against *S. aureus*, 22% were found to produce antibiotic activity. There were 325 bacterial samples tested for single production against *E. coli*, 8% were found to produce antibiotic activity. The goal of this research is to introduce a new, high-throughput method of screening bacteria for antibiotic production.

2. Materials and Methods

The following are general steps, unless otherwise noted. All steps were completed with aseptic techniques. All agitation occurred at 160 rpm in a temperature controlled console shaker (Excella E25) at 25 °C. All bacterial samples were grown on 10% tryptic soy agar (TSA) for 36-48 hours at 25 °C. All liquid bacterial cultures were grown in 10% tryptic soy broth (TSB) and shaken in an orbital shaker for 24 hours.

2.1 Agar Spread Plate Method

Seventy µL of overnight target pathogen suspension was pipetted onto a 1.5% Tryptic Soy Agar plate. A sterilized plastic spreader was used to evenly spread pathogen across plate and plates were left to dry. 1.7 µL of desired cell suspension was spotted onto dried plates and monitored up to a week at room temperature for zones of inhibition against target pathogen.

2.2 Agar Overlay Method

Four mL of 1.5% Tryptic Soy Agar (soft agar) was melted via microwave and cooled to 50 °C in a hot water bath. 100 mL of target pathogen overnight suspension was added to the soft agar, inverted and poured onto prewarmed, 37 °C, 1.5% Tryptic Soy Agar plate. Plates were slightly rocked for even coverage and left to dry. 1.7 µL of desired cell suspension was spotted onto dried overlay plates and monitored at room temperature up to a week for zones of inhibition against target pathogen.

2.3 High-Throughput Liquid Assay Method

As shown in Fig. 3, a five-day process began with creating liquid bacterial cultures by inoculating a selected colony into 4 mL of 10% TSB were shaken. On day two, the liquid cultures from day one were used to make 96-well filtration plates (0.22µm hydrophilic low protein binding Durapore® membrane). The filter plates were prepared with 140µL of 10% TSB and 10µL of appropriate sample in each well (n=4). Filter plates were shaken for 48 hrs. On the third day, *S. aureus* and *E. coli* were grown in full strength TSB and shaken for 24 hrs at 37 °C. On the fourth day, 50µL of Gram-positive, *S. aureus*, Gram-negative, *E. coli*, or full strength TSB were pipetted into wells of a sterile 96 well VWR® tissue culture plate. Then, the filter plates were placed directly on top of the newly made plates, and were centrifuged for 10 min at 1,000 x g at 25 °C. This allowed all cellular material to stay in filter membrane while all excreted secondary metabolites were transported into the new plate. Each plate had a known antibiotic producer against the pathogen, TSB with pathogen, and TSB without pathogen as controls. The plates were shaken for 24 hrs.

On the fifth and final day, plates were analyzed for absorbance at 590 nm using Gen5 All-In-One Microplate Reader software with BioTek Synergy. Each plate's data were individually analyzed using Microsoft Excel. The average of TSB without pathogen was subtracted from each well of the 96 well plate to create a standard baseline of "0" so media would not play a factor. The new average of TSB without pathogen represents the absorbance of a well which contains 0% of the pathogen or 100% inhibition. The new average of TSB with pathogen represents the absorbance of a well which contains 100% of the pathogen or 0% inhibition. The average plus two standard deviations of all ratios per bacteria were used to create a 95% confidence interval of total pathogen inhibition. If the bacterial sample had an avg+2SD below 1, then it qualified to have antibiotic activity. The bacterial sample qualified to be a strong producer when at least 30% inhibition occurred against the pathogen.

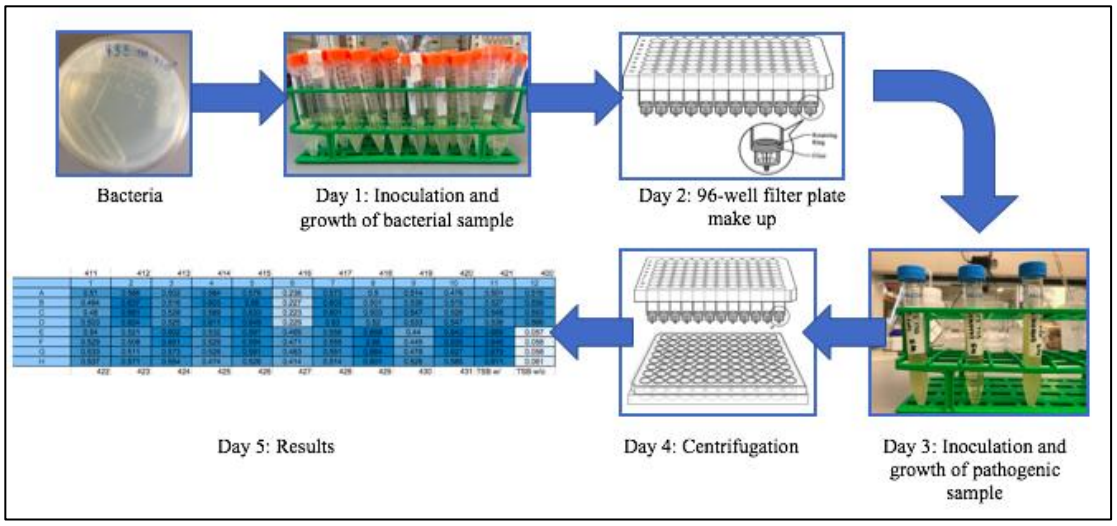

Figure 3. Proposed method.

Figure 3 Workflow of the proposed high-throughput screening method. The five-day process starts after the colonies form on streaked agar plates.

3. Results

There were 387 bacterial samples tested for single production against *S. aureus*, 29% were found to produce antibiotic activity and 12% were found to be strong producers. There were 377 bacterial samples tested for single production against *E. coli*, 32% were found to produce antibiotic activity and 15% were found to be strong producers.

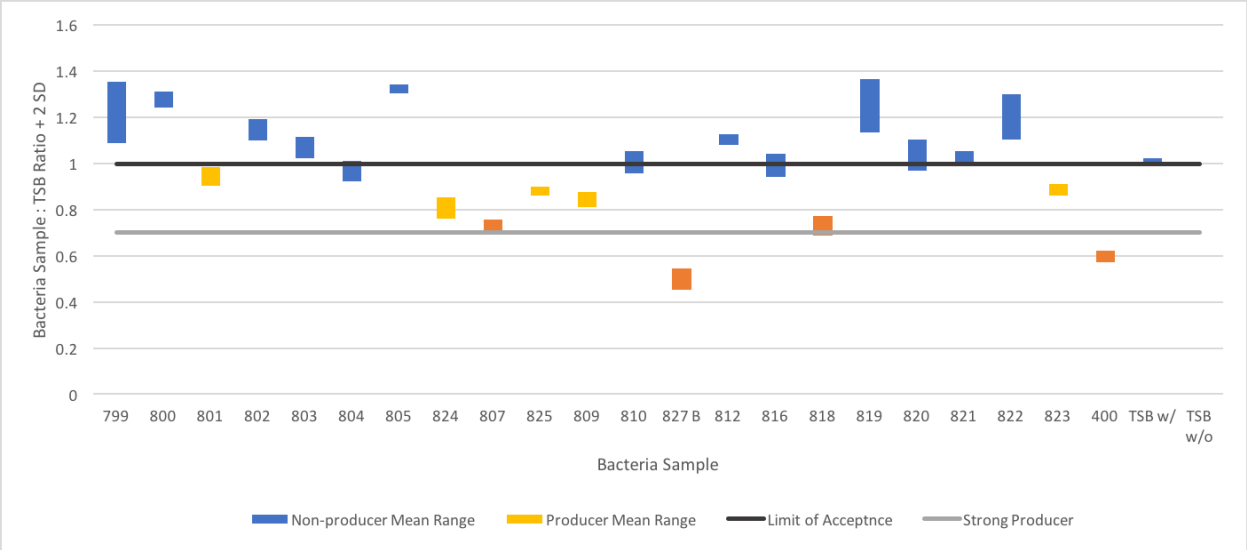

Figure 4. Data analysis graph.

Figure 4 Representation of a stacked column bar graph post excel data analysis which shows the ratio between each average of 21 different bacteria samples to TSB average + 2 SD screened against *S. aureus* for antibiotic activity in pure monoculture.

4. Discussion

The proposed high-throughput liquid assay method is an efficient way of screening many bacteria samples in both time and quantity. Once all of the bacteria are fully grown on agar plates, the proposed procedure obtains results in just five days. In comparison to the current agar overlay or agar spread plate method, this new method decreases the total experimental time by more than a half. Also, the new method is quantitative as the agar overlay method is qualitative. The new robust method is more precise, more rigid, and less sensitive than prior methods as their results are easily misled by growth rate, medium formulation, and agar depth. Most methods assess microbes on agar which lacks certain aspects that would be provided in the microbe's natural state. In the natural environment, a microbial community is made up of a mixture of various species than can chemically and physically interact with each other[12]. The cultivation of two or more microbes can result in secondary metabolite production and excretion. The metabolites act as interspecies or intraspecies signals that can lead to activation of silent operons, small molecule exchange, or a change in cell physiology[13,14].

Research has repeatedly demonstrated that antibiotic production is greater when bacteria are faced with other bacterial species[15]. However, the field lacks a high-throughput screening method to test for antibiotic activity, especially in mixed culture. The proposed method has the ability to screen co-cultures efficiently and effectively. Two co-culture assays have already been tested, however methodology needs some improvement. Perfecting the methodology for screening antibiotic production in mixed-cultured bacteria will greatly advance the field for finding natural products. The method allows researchers to quickly and precisely decide if a sample(s) is worth working with towards natural product isolation. Fortunately, there are many useful methods to isolate natural products. One of the most advanced methods to analyze natural products uses imaging mass spectrometry, which provides the ability to visualize the excreted metabolites in two- and three- dimensions. The visual aid allows the researcher to distinguish which bacteria are producing the metabolites of interest. The ability to image the microbial metabolic exchange will

be of greater help as the number of microbes being screened ncreases[16]. Future work in the lab will incorporate multi-microbial cultures tested by the proposed method and imaging mass spectrometry to analyze the cultures that show antibiotic activity.

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