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Deserted Dry Land Soil: A Promising Source for New Antimicrobials

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Abstract:-Antibiotics are the medications that destroy or slow down the growth of other microorganisms specially bacteria. Each gram of soil may contain approximately 1000 different bacterial species. Many could be capable of producing metabolites that can be use as antibiotics or antimicrobials. When bacteria develop the ability to defeat these antibiotics designed to kill them they become resistant, the most urgent threats to public's health. Influenced by the crowd sourcing initiative of Tiny Earth® we aims to look for possible antibiotic producer strains of bacteria or fungi from the dry land soil. Current study is a baseline study that discusses the results obtained from soil samples collected at different sites within Navajo Reservations, Arizona near Tuba City, Moenave, Rare metals, and Moencopi Wash. There were three goals we targeted in this baseline study i.e. collection of soil samples in Tuba City and surrounding areas, isolation and identification of soil-derived bacteria, and look for antagonistic activity against test strain of ESBL negative *Klebsiella pneumoniae*. Out of a total of 104 selected colonies, only nine (09) gram positive bacterial colonies (rod; spore former), were identified as producer strains against ESBL negative test strain of *Klebsiella pneumoniae*. In next stage of the study, we are going to test the crude extracellular extract of these 09 strains against ESKAPE safe relatives (*Enterococcus raffinosus, Staphylococcus epidermidis, Escherichia coli, Acinetobacter baylyi, Pseudomonas putida* and *Enterobacter aerogenes*). Furthermore, with any positive antibacterial activity, the crude extract will be purified using various chemical methods and the bacterial strains will be identified using 16S ribosomal RNA genotyping.

Keywords:- *Dry Land Soil, ESKAPE Pathogens, Antimicrobials, 16S Ribosomal RNA*

I. INTRODUCTION

The Navajo Nation elders have knowledge on how to collect herbs to heal their people, and bring their teachings to the 21st century, in which we can decode to find solutions from Mother Nature. With these teachings the next generations of new scientist, biologist, researchers, and pharmacists etc., can advance the healing and treatment procedures for the infectious diseases. There is a two way traffic i.e. antibiotics that are working against bacteria and the bacteria that are struggling hard to survive the attack of antibiotics via continuously changing their gene make up i.e. mutations. Abuse of theses antibiotics, unavailability of new antibiotics and multi drug resistant species of bacteria have already resulted in a biggest threat worldwide along with food security and water crises to the public's health. According to WHO, if no significant action is taken by 2050, superbugs will kill more people then cancer and diabetes combined and will result in 300 million premature deaths (1). Current study is an ongoing part of worldwide network of instructors and student researchers tackling the challenge of antibiotic discovery. We are hoping to find some good antibiotic producer strains of bacteria from the soil of deserted dry lands.

Antibiotics have not only saved patient's lives; they have played a pivotal role in achieving major advances in medicine and surgery (2). They have successfully prevented or treated infections that can occur in patients who are receiving chemotherapy treatments; who have chronic diseases such as diabetes, end-stage renal disease, or rheumatoid arthritis; or who have had complex surgeries such as organ transplants, joint replacements, or cardiac surgery (2). Antibiotics have also helped to extend expected life spans by changing the outcome of bacterial infections (3). In 1920, people in the U.S. were expected to live to be only 56.4 years old; now, however, the average U.S. life span is nearly 80 years (2). Antibiotics have had similar beneficial effects worldwide. In developing countries where sanitation is still poor, antibiotics decrease the morbidity and mortality caused by food-borne and other poverty-related infections (4).

These antibiotics have been derived from extracellular metabolites excreted by microorganisms mostly bacteria that are ubiquitous. These bacteria surrounding us are also struggling for survival and hence continuously going through mutations i.e. changing their genetic make up to fight antibiotics. In this scenario, we can never be able to stay ahead of the mutation wave, and that is the reason antibiotics resistance has become one of the biggest threat worldwide along with food security and water crises for the human race.

Although as early as 1945, Sir Alexander Fleming (discoverer of Penicillin) raised the alarm regarding antibiotic overuse when he warned that the "public will demand [the drug and] … then will begin an era … of abuses" The overuse of antibiotics clearly drives the evolution of resistance. As Bonnie Bassler said, "When antibiotics first came out, nobody could have imagined we'd have the resistance problem we face today. We did not give bacteria credit for being able to change and adapt so fast.

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Despite warnings regarding overuse, antibiotics are overprescribed worldwide and the result is here (2,5).

Incorrectly prescribed antibiotics, inappropriate use of antibiotics, over use of antibiotics, prescription of antibiotics without having correct culture and sensitivity results etc. are some factors that contributed to the promotion of superbugs or resistant strains of bacteria. Studies have shown that treatment indication, choice of agent, or duration of antibiotic therapy is incorrect in 30% to 50% of cases (6). Markets are flooded with antibacterial products sold for hygienic or cleaning purposes (hand sanitizers, deodorants etc.), may also contribute to this problem. Since they may limit the development of immunities to environmental antigens in both children and adults (3), consequently, immune-system versatility may be compromised, possibly increasing morbidity and mortality due to infections that would not normally be virulent. According to WHO If no significant action is taken by 2050, superbugs will kill more people than cancer and diabetes combined and will result in 300 million premature deaths (1).

Current Study is influenced by "Tiny Earth", a network of instructors and students focused on crowdsourcing antibiotic discovery from soil. One of the important mission of that network is to address a worldwide health threat—the diminishing supply of effective antibiotics—by tapping into the collective power of many student researchers concurrently tackling the same challenge, living up to its motto "student sourcing antibiotic discovery." Since over two thirds of antibiotics originate from soil bacteria or fungi (7, 8), hence we are hoping to find some good antibiotic producer strains from the soil of Navajo Reservations.

II. MATERIALS AND METHOD

A. Sample collection and preparation:

Soil samples were obtained from soil samples collected using kits (Courtesy Tiny Earth & Thermo Fischer) from different sites in Navajo Reservations near Tuba City, Moenave, Rare metals, and Moencopi Wash during March–July, 2019 (Table #1). Samples were collected from 4-5 cm depth, time of collection, surrounding temperature, temperature of soil, color of soil, type of soil were noted simultaneously. Later we calculated the moisture content in each soil sample using method described by JoVE (10).

Figure 1: Collection of Soil Samples From Tuba City Arizona and surroundings

B. Bacterial isolation:

One gram of each soil sample was re-suspended in 10 ml of Nutrient broth (Carolina Biologicals), homogenized, and serially diluted in sterile nutrient broth using 10^{-1} to 10^{-10} dilutions. Approximately 0.1 ml (100 µl) of each dilution was spread plated (glass beads spread plate method), onto Brain Heart Infusion Agar (Carolina Biologicals) containing 0.1% Cycloheximide solution (Sigma-Aldrich) to inhibit the fungal growth. Plates incubated at 37˚C for 24 -48 hours, CFU/gm of each soil dilution calculated upon incubation and selected colonies were picked for patching (figure 2 & 3) and sub-cultured on Brain Heart Infusion Agar (Carolina Biologicals), EMB Agar (Carolina Biologicals), and McConkey's Agar (Carolina Biologicals) for colonial characteristics, cellular morphology and biochemical identification.

Figure 2: Preparation of Bacterial Culture Media and Serial Dilutions

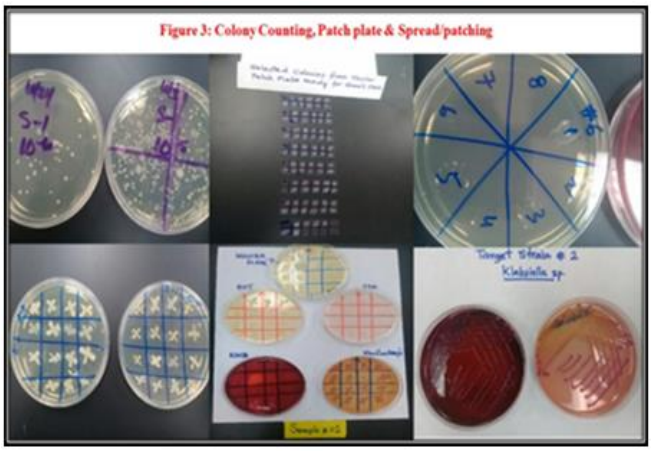
C. Gram Staining:

Gram staining of selected colonies was done using Gram Staining Kit (Carolina Biologicals), and morphological characteristics including shape, size, gram reaction and arrangement of bacteria were noted down.

D. Spot Test:

Only Gram-positive colonies were selected to proceed with, and tested for Catalase, Coagulase, Oxidase production, Nitrocephin test and motility. These gram positive colonies of our soil isolates were then sub cultured on Brain Heart Infusion Agar (Carolina Biologicals) using patch plate method and incubated at 37˚C for 24 hours for further proceedings.

Figure 3: Colony Counting, Patch plate & Spread/patching



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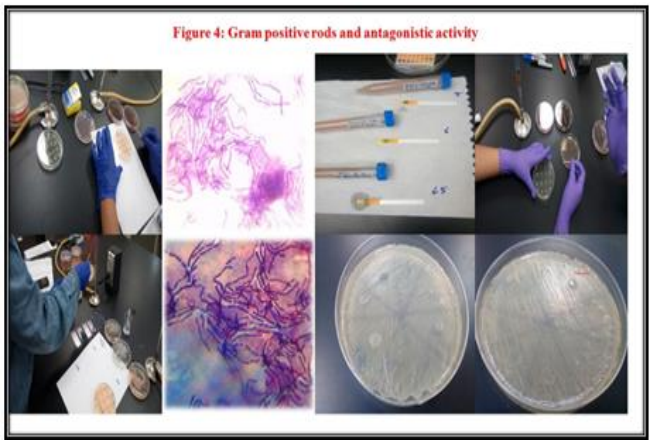
E. Testing against ESBL negative Klebsiella pneumoniae using Spread/patch method:

The patched plates were replicated on the bacterial lawns of extended spectrum beta lactamase (ESBL) negative *Klebsiella pneumoniae* (Carolina Biologicals) seeded on Brain Heart Infusion Agar (Carolina Biologicals) containing 0.1% Cycloheximide solution (Sigma-Aldrich) to look for the presence of any existing antibacterial activity among our soil isolates. Plates were incubated at 37˚C for 48 hours (figure 4).



F. Antibacterial Products or Extracellular metabolites:

The patched colonies were observed for the presence of any clear zone of inhibition surrounding the soil isolates. Presence of a zone of clearance surrounding the soil isolates was considered as indication of positive producer strain and those colonies were selected to proceed further with the extraction of inhibitory compounds or extracellular metabolites.

III. RESULTS

A total of 08 soil samples were processed for isolation of soil bacteria using 10 fold serial dilution. Total of 104 colonies were picked for patching (Table #2 & 3). (16 colonies per plate of Nutrient Agar, BHI Agar, EMB, MacConkey's Agar and BHI Agar with 0.1% Cycloheximide). Those patch plates were replicated on same plates against lawn of *Klebsiella pneumonia* (test strain) using spread/patch method. Out of 104 selected picked colonies, for Master patch plates, only nine (09) gram positive bacterial colonies (rod; spore former), were identified as producer strains. Location of spores were central and they all were catalase positive, oxidase negative and motile.

Table 1: Soil Sample Collection

Soil Sample Data Collection	Soil Sample Number							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Date of Collection	6/13/19	6/11/19	6/11/19	6/11/19	6/11/19	1/28/19	1/28/19	1/30/19
Location	Residents Garden	Reservoir	Moencopi Wash	Moenave	Dodson Pond	Rare Metals	Moenave	Cow-springs
Depth of Collection	4 to 5 cm	4 to 5 cm	4 to 5 cm	4 to 5 cm	4 to 5 cm	4 to 5 cm	4 to 5 cm	4 to 5 cm
Type of Soil	Dry/Soft Sand	Moist Sand	Dry/ Sand	Moist/ Sand	Moist Sand	Dry Sand	Dry Sand	Dry Sand
Temperature of Air	94 degrees	94 degrees	94 degrees	94 degrees	94 degrees	90 degrees	90 degrees	90 degrees
Temperature of Soil	Warm 29 degrees	Cold 23 degrees	Warm 32 degrees	Cold 23 degrees	Cold 19 degrees	Cold 17 degrees	Cold 15 degrees	Cold 17 degrees
Weather condition of Day of Collection	Clear skies	Clear Skies	Clear Skies	Clear Skies	Clear Skies	Clear Breezy	Clear Breezy	Clear Breezy
Sample site Description	Fertile Land	Fertile Land	Dry Wash	Fertile Land	Fertile Land	Dry land	Dry Land	Dry Land
pH of Soil	6.5	7	7	7	6.5	7	6	6.5
Moist content Of Soil	Dry	Moist	Dry/Course	Moist	Moist	Dry	Dry	Dry

These producer strains showed narrow zones of inhibition surrounding their colonies on the lawn of test strain i.e. ESBL negative *Klebsiella pneumoniae* upon 24 hours of incubation at 37˚C. Those zones could be better on 48 hours incubation as spore forming bacteria might need more time to adjust with the environment and start production of antibacterial chemicals.

Table 2: Bacterial Colonies isolated from soil samples

Sample No. & Location	*CFU/**gm of soil in selected dilutions X = 1/dilution factor x number of colonies				
	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
1	UC	37200000	176000000	920000000	8400000000
2	UC	UC	UC	UC	8400000000
3	UC	UC	UC	1890000000	1700000000
4	UC	13200000	56000000	360000000	1200000000
5	UC	26400000	28000000	40000000	400000000
6	110000000000	6000000	40000	200	00
7	40000	200	200	200	200
8	9000000000	70000000	70000000	70000000	500000

*CFU = Colony Forming Unit **gm = Grams

Table 3: Frequency of Antibiotic Producer Bacterial Strain

Culture Media	Number of Patches tested	Isolates Producing antibiotics (number)	Isolates producing antibiotics (%)
Brain Heart Infusion Agar (BHI)	104	0	0
Nutrient Agar	104	0	0
Eosin Methylene Blue Agar (EMB)	104	0	0
MacConkey's Agar	104	0	0
Brain Heart Infusion Agar with 0.1% Cycloheximide solution	104	09	8.6%

IV. DISCUSSION

Although antibiotics is a new innovation, throughout the century's, the traces of tetracycline have been discovered in skeletal remains from ancient Sudanese Nubia dating back to 350-550 CE (12,13), and alongside skeletons in Dakhleh Oasis, Egypt; form the late Roman era (14). However, the "golden era" of antibiotics is in danger now. Centers for Disease Control and Prevention (CDC), Infectious Diseases Society of America, World Economic Forum, and the World Health Organization (WHO) have declared antibiotic resistance to be a "global public health concern" (15,16,17).

At Diné College Tuba City Center, we divided this part of our study into three goals: collection of soil samples from Tuba City and surrounding locations, isolation and identification of soil-derived bacteria (Figures # 1 & 2), and antagonistic activity of the isolated soil strains of bacteria against test strain of Klebsiella pneumoniae. Through this workflow, we identified nine gram positive bacterial strains that showed the antibacterial activity against our test strain of *Klebsiella pneumoniae*. In next part of the study the crude extracellular extract of these 09 strains will be tested against ESKAPE safe relatives (*Enterococcus raffinosus, Staphylococcus epidermidis, Escherichia coli, Acinetobacter baylyi, Pseudomonas putida* and *Enterobacter aerogenes*), and in case of any activity we will purify those extracts and will identify them using 16S ribosomal RNA.

Majority of antibiotic producing organisms are soil inhabitants and among them bacillus species are on the top. These bacteria produce extracellular metabolites that can be bactericidal or bacteriostatic in nature. Also, the production of antibiotics in microorganisms from soil could be affected by many factors including moist content, nitrogen and carbon source or presence of heavy metals like uranium in the surroundings. Temperature variation is another important factor that can affects the activity of microbes in the soil and hence can affect the synthesis of antibiotics too. These antimicrobial substances are synthesized during the idophase stage i.e. the phase of culture growth during which secondary metabolites are produced and yet there is no correlation reported between the bacterial sporulation and antibiotic production ability (9).

Only 09 of our soil isolates showed inhibitory effect against test strains under normal growth conditions. And although the percentage of producer strains was not very promising i.e. only 8.6% (9 out of 104), we believe it could be more if we modify our growth conditions and procedure. It is important to note that all the soil samples we collected were dry soil samples and 90% of the bacterial isolates were gram positive spore former rods. We use the incubation period of 24 hours for all the samples (both stock and diluted samples), these spore former may show good activity if we give them about 24-48 hours to show their response as more than 48 hours can result in overcrowding or mixing of colonies. We collected all our samples from maximum 4-5 cm depth this could also be the reason of not getting some good producer strains as its almost top soil and continuously exposed to sun light that might have inactivated these bacteria. Next, we are willing to collect samples from more deep and areas closer to vegetation where more competition for survival may enhance their antagonistic capabilities.

Another important thing is that out of 09 producer strains we isolated, 03 were from a sample collected from "Rare metals" area that was a Uranium processing facility near Tuba City and have closed in 1990. Soil of that area as the name indicates is rich in rare metals mainly Uranium and we believe that the bacterial strains that we are able to

grow from that soil sample can give us much better results against some other test strains, possibly ESKAPE safe relatives.

V. CONCLUSION

As we have mentioned earlier these are the results from our baseline study of an ongoing work, we are continuously collecting more soil samples from more different areas and places and hoping to expand the study up to molecular level soon. There are numerous possibilities for facilitating the bacterial growth and activity rates in these soil samples. Additionally, the depth of collection sites and close by vegetation are the factors we are also considering to improve our results. We are certainly looking forward to contribute to the continuing efforts against growing burden of anti-microbial resistance through our soil bacteria project.

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