Hunting for New Antibiotics from the Soil Bacteria of Tabuk Natural Reserves

Abstract

Background

Antimicrobial resistance is spreading all over the world and at the same time the rate of discovery of antimicrobials in general and antibiotics in particular is diminished. Historically and currently many antibiotics are discovered and isolated from soil bacteria and other natural sources. In this project our aim is to search for antibiotic producing bacteria from soil samples.

Methods

Soil samples from different locations from King Salaman Bin Abdulaziz Royal Reserve were collected. Soil samples were cultured in Actinomycetes special mediumand incubated at 35°C for 4 days. Isolates were further identified and categorized using VITEK-2 system.Two set of experiments were employed to evaluate the potential of antibiotic production and activity from these isolates against known pathogenic bacteria from both gram-positive and gram-negative Molecular identification of the isolates that exhibited inhibitory activity was performed by PCR

Results

Many samples were identified through VITEK-2 as actinomycetes, others were unidentified and some were known irrelevant samples. Supernatant of the suspension of actinomycetes samples and the other unidentified samples succeeded to inhibit the growth of *Staphylococcus aureus*and *Klebsiella pneumoniae* in the checkerboard experiments.

Conclusion

Some of the isolates were actinomycetes that arepotentially secreting inhibitory substances and some of them were actinomycetes but notproducing inhibitory substances. Further research is recommended to characterize theseinhibitory substances

1. Introduction

The discovery of antibiotics revolutionized modern medicine, offering effective tools to combat bacterial infections. Unfortunately, Antimicrobial resistance (AMR) is moving up to the top of the list of human killers worldwide. Previously it was anticipated that by 2050, AMR will be the leading cause of death amounting to ten million deaths annually(1). In Saudi Arabia, many microorganisms are reported to have developed resistance. And nearly every type of resistance gene can be found in different parts of the country(2). AMR is going to jeopardizes the effectiveness of many medical procedures that rely on antibiotics, such as surgeries, chemotherapy, and organ transplants. Now many microorganisms are being multidrug resistant (MDR), or extensively drug resistant (XDR) or even pan drug resistant (PDR)(3,4)

While the golden era of antibiotic discovery may seem to belong to the past(5), the urgency of addressing antibiotic resistance has reignited interest in the exploration of untapped microbial sources(6). One promising avenue is the isolation of antibiotic-producing bacteria from the diverse ecosystems of the natural environment.

Nature has long been recognized as a vast reservoir of microbial biodiversity, harboring an array of bacteria with the potential to produce novel and potent antibiotics. These bacteria have evolved intricate mechanisms to compete for survival and resources in their ecological niches, and this often involves the production of bioactive compounds with antimicrobial properties(7,8).The isolation process typically entails the collection of environmental samples followed by the cultivation of microorganisms. This step is crucial for obtaining pure bacterial cultures that can be further studied for their antibiotic-producing potential.

Once isolated, the bacterial strains are subjected to screening assays to identify those with antimicrobial activity. These assays often involve challenging the isolated bacteria with target

pathogens to assess their inhibitory effects. Positive results in these screenings indicate the presence of antibiotic compounds and prompt further characterization of the bioactive molecules.

Other methods which do not necessitate culturing of the microorganisms before the isolation of the antibiotics also exist and this has recently led to the discovery of malacidins(9)

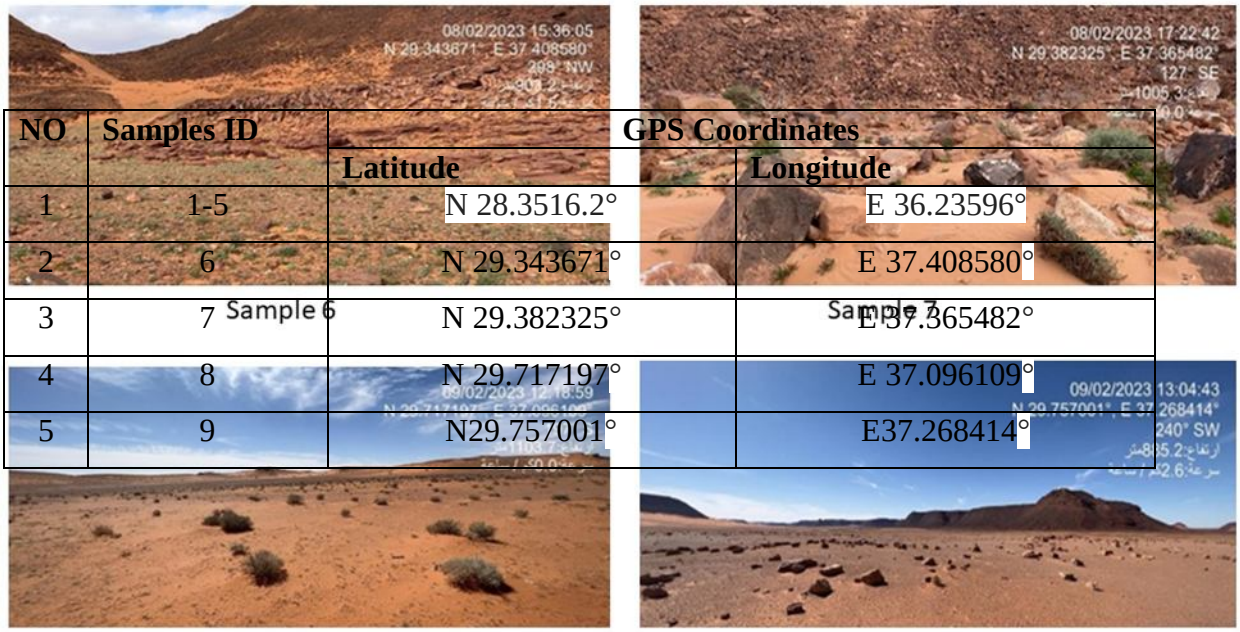
The isolation of antibiotic-producing bacteria from the natural environment not only contributes to the expansion of our antibiotic arsenal but also provides insights into the ecological and evolutionary aspects of microbial communities. Additionally, it holds the promise of discovering antibiotics with novel mechanisms of action, crucial for overcoming the challenges posed by antibiotic-resistant pathogens.

The study's aim is to screen bacteria in their natural environment (Tabuk's nature reserve) for their potential to produce antibiotics.

2. Methods

2.1 Soil sample collection

Nine soil samples were collected in self-seal sterilization pouches from different sites at King Salaman Bin Abdulaziz Royal Reserve (KSBARR) (Table 1 and figure 2).



NO	Samples ID	GPS Coordinates	
		Latitude	Longitude
1	1-5	N 28.3516.2°	E 36.23596°
2	6	N 29.343671°	E 37.408580°
3	7	N 29.382325°	E 37.365482°
4	8	N 29.717197°	E 37.096109°
5	9	N29.757001°	E37.268414°

Figure1: Geographical locations of the samples

Table No 1: GPS coordinates of the geographical locations of the samples

2.2 Bacteria culture and identification

Approximately 1 g of each sample is dissolved in 10 ml sterile water. Samples were then cultured in Actinomycete special agar medium (HIMEDIA, India) and incubated at 35°C for 4 days. Single isolated colonies were sub cultured for further studies. Isolates were further identified based on shape and Gram's reaction. Briefly, thin smear of the bacteria on a clean glass microscope slide was prepared and fixed by passing through flame. Then the primary stain, crystal violet was applied to the smear for 1 minute, and rinsed with water. The mordant, Gram's iodine, was applied to the smear for 1 minute, rinsed with water followed by application of the decolorizer (ethanol) for 15-30 seconds and rinsing with water. Finally, counterstain, safranin, was added to the smear for 1 minute, rinsed with water, and let to air dry. The slides were observed the microscope, shape and reaction to gram were reported. All Gram-negative isolates were excluded from further work. Gram positive isolates were proceeded with for further identification through VITEK-2 system.

Pure, well-isolated colonies from solid culture plates were taken, and emulsified in 3 mL of sterile saline solution until the suspension is homogeneous. The turbidity of the suspension was adjusted to a 0.5 McFarland standard. 1 mL of the adjusted suspension of each sample was added to a VITEK-2 identification tube, and vortexed briefly to mix the suspension. Then the samples were loaded into the Vitek instrument following the manufacturer's instructions(10).

Isolates that were Gram positive bacilli/branching and were either identified as actinomycetes or unidentified by VITEK-2 were taken to the next steps of the research.

2.3 Testing of the antibiotic production potential

Two methods were employed to evaluate the potential of the isolated microorganisms in producing inhibitory substances; perpendicular plate method(11) and checkerboard method(12,13)

2.3.1 Perpendicular streak method

Actinomycete special agar medium was used and each plate was streaked with individual isolates at the center/diameter of the plate and incubated at 30 °C for 7 days(14). Then the activity against pathogenic bacteria was evaluated using both *Staphylococcus aureus* ATCC 25923as a

representative of Gram positive and *Klebsiella pneumonia* human isolate as a representative of Gram negative. A 24 h fresh sub-cultured test bacteria was prepared and streaked perpendicular to the test isolates and incubated at 37 °C for 24 h. Then we evaluated whether there are zones of inhibition near the line of the test isolates

2.3.2 Checkerboard method

Nitroblue tetrazolium (NBT) micro-dilution was used to determine the ability of the isolates to produce inhibitory agents as previously described(15). In brief, 100 µl from well centrifuge (12000 rpm – 2 min) 7 days supernatant broth culture was incubated with 100 µl tested organisms (*Staphylococcus aureus* ATCC 25923 and human isolate *Klebsiella pneumonia*) separately in a microtiter plate and 50 µl of NBT. Compared to positive and negative controls, the plate was examined after 4 hours and overnight incubation at 35 C for the possible development of blue color indicating growth or yellow color indicating inhibition.

2.4 Molecular identification of Actinomycetes:16S rRNA gene amplification and sequencing

Molecular identification was done using 16S rRNA forward primer F243 5'GGATGAGCCCGCGGCCTA3' and reverse primer R513GC 5' CGGCCGCGGCTGCTGGCACGTA3' (16)

First, genomic DNA was isolated from a 2 ml overnight broth culture using bacterial DNA isolation kit (Transiom Genomics, India) following the manufacturer's guide. Second, 25 µl reaction mix was prepared by adding 5 µl of extracted DNA to 12.5 of the 2X PCR master mix(amaROnePCR™, India), 1 µl primers and 5.5 µl nuclease free molecular grades water.

PCR protocol included initial denaturation at 95C for 10 minutes followed by 40X denaturation at 95C for 1 minutes, annealing at 68C for 1 minutes and extension at 72C for 1 minutes with final extension at 72C for 10 minutes. The PCR products was subject to electrophoreses at 80 volts for 40 minutes in 2% Agarose gel with Ethidium bromide. The Gel was examined using Gel documentation system (Gel DocXR, Bio-Rad, USA).

3. Results

3.1 Soil samples bacteria identification

Soil samples cultures obtained were diverse in terms of colors and shapes of colonies (Figure 2).

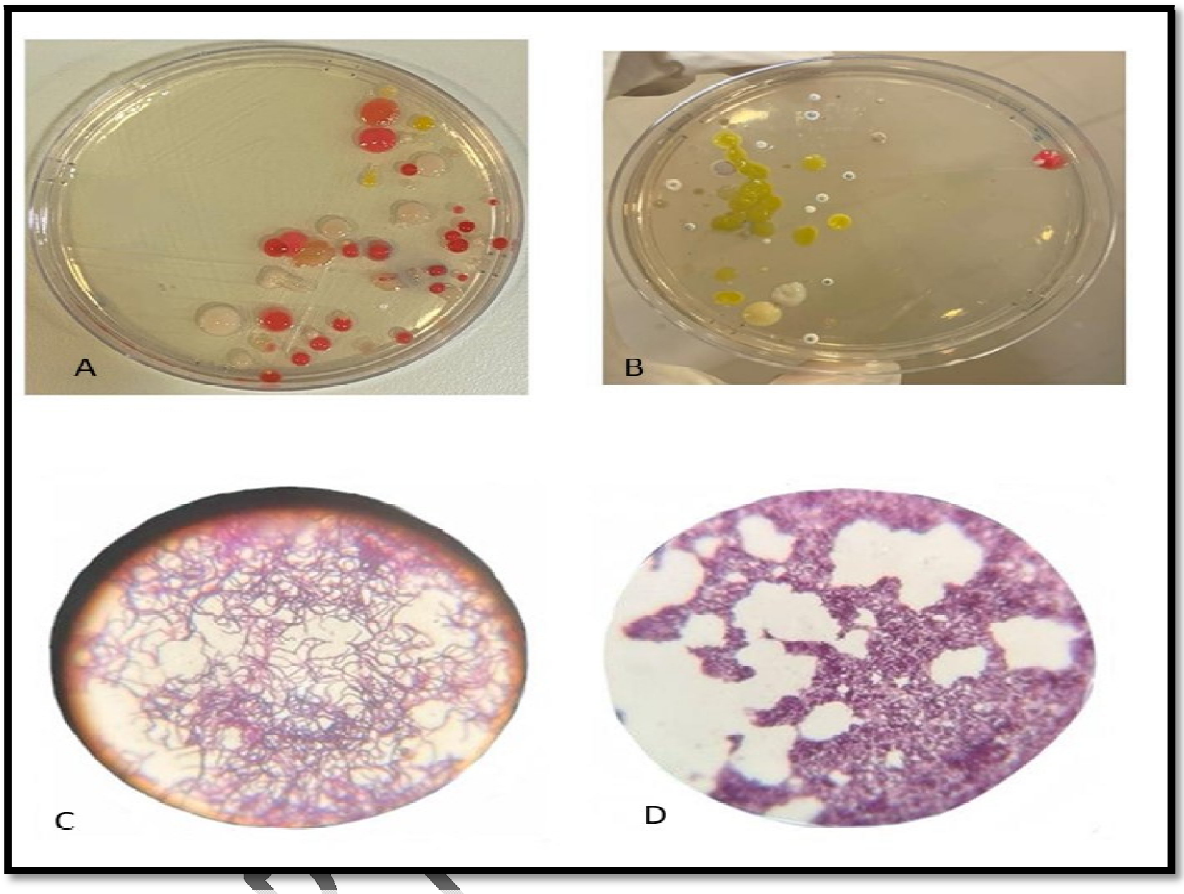


Figure2: Soil samples culture and Gram staining: Figures 2A and 2B are examples of the results of the culture of the soil samples. Figures 2C and 2D are examples of Gram staining results.

Many colonies were not taken into the next steps and have been excluded after they Gram staining steps. Following the identification of all isolates as actinomyces or undefinable by the VITEK-2, as well as the positive Gram staining results and branching shape, the isolates were proceeded with to the stage of antibiotic production potential. (Table No2).

Table No 2: VITEK-2 and Gram staining results of the isolates.

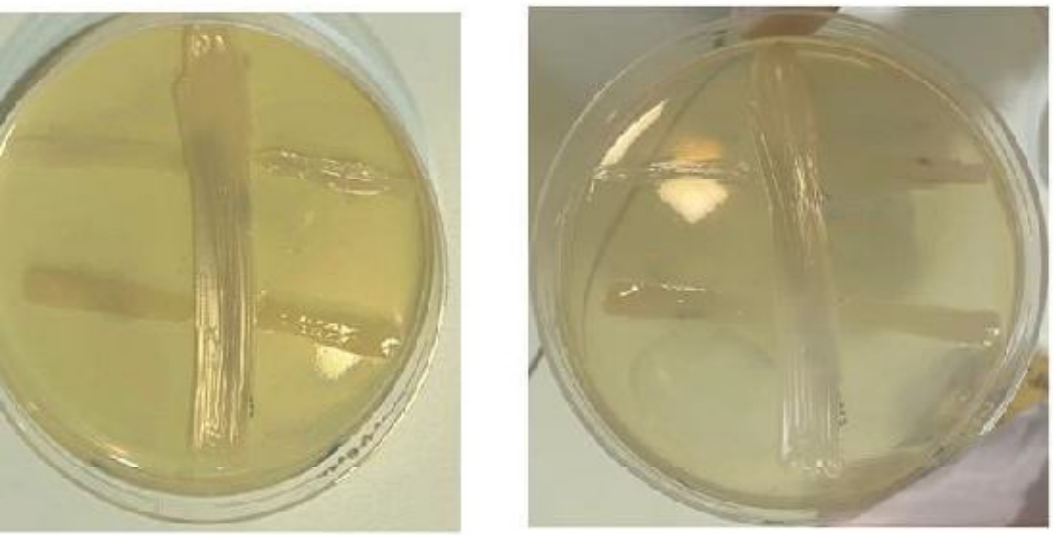
No	Original sample	Isolate code	Vitek-2 system Microbiology Chart Report		Gram stain result
			Isolate Name	Propability	
1	1	100	Unidentified	NA	G+ve short bacilli
		101	Unidentified	NA	G+ve bacilli
		140	*Eggerthiacatenaformis*	99%	Did not proceed further
		104	*Acranobacteriumhaemolyticum*	33%	G+ve bacilli
2	2	022	Unidentified	NA	G+ve bacilli
3	3	301	Unidentified	NA	G+ve bacilli (Branching)
		302	Unidentified	NA	G+ve bacilli
4	4	402	Corynebacterium minutissimum	92%	G+ve bacilli (Branching)
5	5	510	*Actinomyces odontolyticus*	91%	G+ve bacilli (Branching)
		504	Microbacterium spp	88%	Did not proceed further
		540	*Turicellaotitidis*	93%	Did not proceed further
6	8	801	Unidentified	NA	G+ve single bacilli (Branching)
		802	Unidentified	NA	G+ve bacilli
		803	Unidentified	NA	G+ve bacilli (Branching)

Table 3: Antibiotic producing isolates and activity against both Gram positive and negative bacteria based on color change from the checkerboard experiment.

No	Isolate designation	Activity against S.aureus	Activity against Klebsiella Pneumonia
1	100	Present	Present
2	101	Present	Present
3	104	Absent	Absent
4	022	Absent	Absent
5	301	Absent	Absent
6	302	Present	Present
7	402	Present	Present
8	510	Present	Present
9	801	Present	Present
10	802	Absent	Absent
11	803	Absent	Absent

3.2 Assessment of antibiotic production potential

All the isolated did not show any signs of inhibition in the perpendicular experiment results (figure 3). In the second experiment, some samples (100, 101, 302, 402, 510, 801)have exhibited inhibitory activity as witnessed with the color change from yellow to dark blue/black (table 3 and figure4).



Figue3: Perpendicular plate results: samples showing no inhibitory zones noticed perpendicularly to the soil isolate tested.

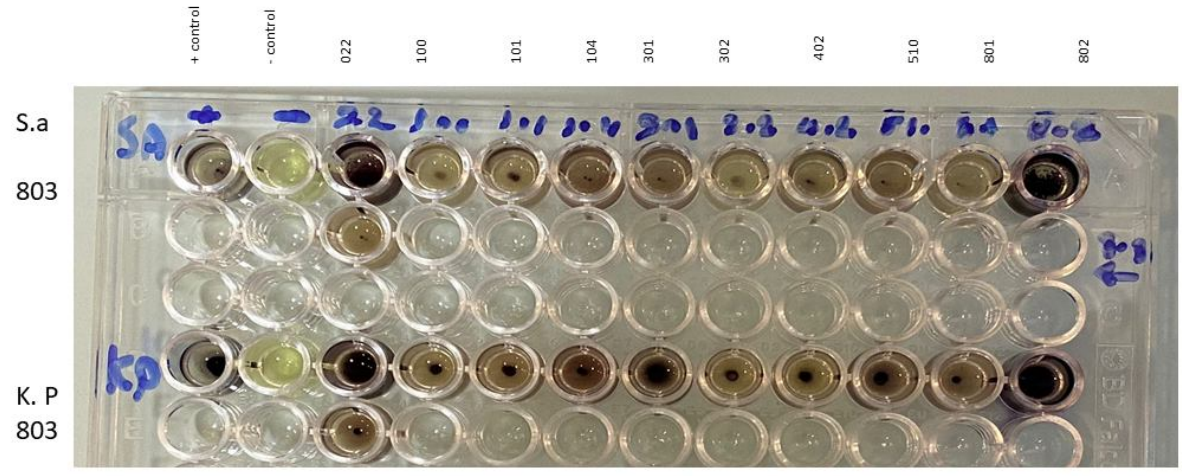


Figure4: Checkerboard experiment results: Positive control means the test bacteria (S.a or K.P) were grown in the media without the addition of the suspension from the test isolate. Negative control meant the well only contain the media and the dye. The other codes correspond to the respective isolates' suspension plus the test organism.

3.3 Molecular identification of the isolates

All the samples that were tested for their potential to produce inhibitory substances, were investigated molecularly through the detection of 16sRNA band to confirm or negate their actinomyces identity. The band with the size 270bp is clear in isolates No 101, 402, and 803. The same band appear in the isolates 801 and 802 but very faint. It was not detected in the other samples.

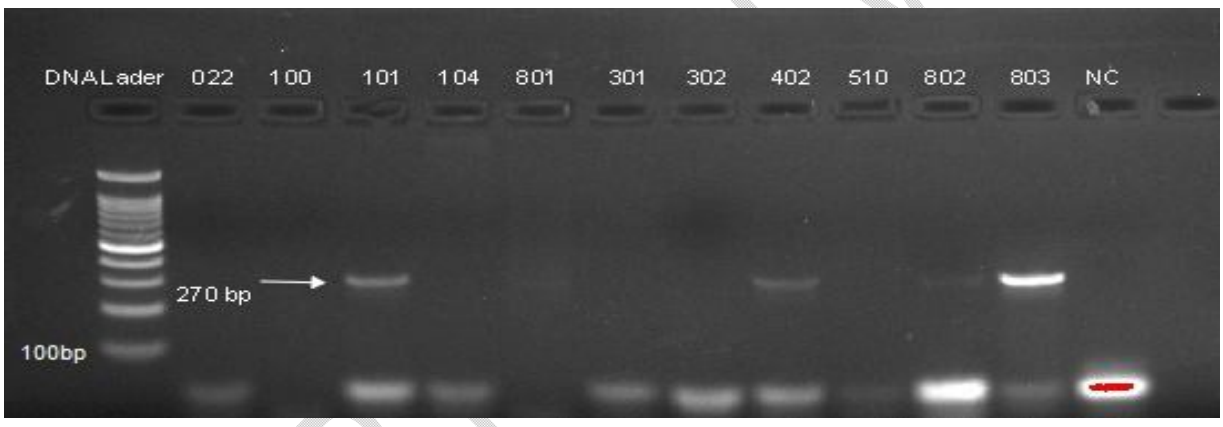


Figure5: Gel image of the bands of the 16sRNA experiments. Band 270 corresponds to actinomyces samples

4. Discussion

Antimicrobial resistance (AMR) is considered one of the top global public health threats, posing a serious and growing danger to human and animal health(17). One of the most important tactics in the fight against AMR is the quest for new classes of antibiotics(18,19).

Screening of microorganisms for antibiotic generating capabilities is part of the search for novel antibiotics. So, in this research we aimed to assess the potential of some soil samples from Tabuk natural reserves for their potential of producing inhibitory substances.Before testing the potential of the isolates we first tried to exclude all gram negative and other non-relevant bacteria and proceeded with those who are likely to be actinomyces based on the gram reaction, branching

shape along with the results from VITEK-2. Since some bacteria turned out to be unidentified from VITEK-2 experiment we decided to proceed with them as they may be previously unknown actinomyces.

When we evaluated the isolates for their potential to produce inhibitory substances, although we did not get positive results from the perpendicular plate method, we were able to detect some inhibitory activity in the checkerboard experiment based on the color change. This is probably due to the inhibitory substances are unable to transverse through the agar media.

One of the ground breaking discovery of antibiotics was the identification of teixobactin(20) which is active against multidrug resistant gram-positive bacteria. Another breakthrough is the discovery of zosurabalpin which is also active against resistant bacteria Carbapenem-resistant *Acinetobacter baumannii* (CRAB)(21). Both of them were from soil bacteria. So, the soil is still rich of promising inhibitory substances. Regarding the actinomycetes we found, there still more work needs to be done. This includes sequencing of the 16sRNA as well as the chemical characterization of the inhibitory substances.

Conclusion

In conclusion, this research isolated potentially antibiotic-producing bacteria from Tabuk soil samples. Further characterization of these isolates is recommended to identify the bacterial species and optimize antibiotic production. Overall, this research demonstrates the presence of antibiotic-producing bacteria in the studied environment and lays the groundwork for further exploration and development of novel antibiotics.

5. References

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