Preliminary Studies on a Novel Antimicrobial Compound Producing Bacterium Discovered in the Rio Grande Valley of Texas

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Abstract

We analyzed different soil samples collected across the Rio Grande Valley (RGV) of Texas for bacteria capable of producing antimicrobial compounds. Of the more than 500 bacterial colonies tested, less than 1 percent gave any indication of the likelihood of producing antimicrobial compounds asdetermined by the diameter of the observed zones of inhibition created when tested on the cultures of safe ESKAPE relatives. One of the soil isolates of interest was further studied with the 16S rRNA gene sequenced and analyzed, and its antimicrobial compound was also extracted using the ethyl acetate and methanol extraction method. The results obtained suggest that the identified bacterium could be a novel specie or at least a sub-specie of the genus *Pseudomonas* and the novel antimicrobial compound discovered possibly broad spectrum in nature considering that it is active against gram-positive and gram-negative bacteria, especially *Staphylococcus epidermidis*, the safe relative of *Staphylococcus aureus*. Furthermore, positive inhibitory results were obtained when the antimicrobial compound was tested on *Bacillus subtilis, Klebsiella pneumoniae, Pseudomonas putida, Enterococcus raffinosus, Erwinia caratovora, Enterobacter aerogenes* and an unknown bacterium from an environmental sample etc. The newly discovered antimicrobial compound has been named **"Anietocin"** and will be the subject of extensive studies in the coming research efforts.

Introduction

The emergence of antimicrobial resistance in pathogenic bacteria has turned into a global phenomenon with the tremendous potential of being the leading cause of death if left unchallenged (Aslam et al., 2018) . For this reason, many researchers across the globe have actively worked and are currently engaged in finding solutions to this problem. A number of scientific articles have been published, showcasing novel discoveries including one by Darabpour et al. (2012)where they described an antibiotic producing *Pseudoalteromonas piscicida* that was effective against most Gram-positive bacteria. Similarly, Pishchany et al. (2018) described the successes of the novel antibiotic Amycomicin on different bacteria including *Staphylococcus aureus*.

Many of the discoveries have made it to patents as novel antibiotics as described by Koulenti et al. (2019). Such novel antibiotics that were described as efficient against multidrug-resistant Gram-positive bacteria include Ceftobiprole, Ceftaroline, Telavancin, Oritavancin, Dalbavancin etc. In addition to antimicrobial compounds derived from bacteria, a few studies have reported the use of other chemical compounds in our fight against multi-drug resistant bacteria, notably Behroozian et al. (2016) reported the successful use of Kisameet clay on ESKAPE pathogens. Similarly, Hijazi et al. (2018) described the antimicrobial property of Gallium compounds on ESKAPE pathogens. Perhaps to be considered an outlier to recent endeavors with novel antimicrobial compounds, is the article by Nakonieczna et al.(2019) describing the latest photoinactivation method used on ESKAPE pathogens.

Members of the ESKAPE pathogens include *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter spp*. (Mulani, et al., 2019). These bacteria are resistant to multiple drugs and are known to be virulent (Mulaniet al., 2019). The mechanisms employed by these ESKAPE pathogens to evade antibiotics include the alteration of the compounds, modification of the binding sites of drugs, intracellular decrease in the accumulation of the drug, loss of porin, the use of efflux pumps etc. (Santajit and Indrawattana, 2016). These mechanisms do not deviate at all from previously established mechanisms of evasion by all bacteria that are sensitive to antibiotics.

In our efforts to contribute to slowing down the depletion of our potent antibiotics and the emergence of superbugs, we discovered a highly effective antimicrobial compound which has been named "Anietocin" and is produced by a possibly novel specie or at least a sub-specie of the genus *Pseudomonas* based on its determined 16S rRNA gene sequence data. This novel *Pseudomonas* spp. grows profusely in liquid and on solid tryptic soy media and creates a significant zone of inhibition when tested on different bacteria including a member of the ESKAPE pathogens and safe ESKAPE relatives.

There are quite a number of reported studies where *Pseudomonas* spp. have been researched for the possibility of producing antimicrobial compounds. For example Raaijmakers et al. (1997) described the production of phanazine-1-carboxylic acid (PCA) and 2,4-diacetlyphloroglucinol (Phl) as it concerns the diseases of wheat while Darabpour et al. (2010) described a *P. aeruginosa* strain PG-01 isolated from the Persian Gulf. The maximum zone of inhibition observed was 33 mm in a culture of Methicillin-resistant *Staphylococcus aureus* (MRSA).

The possible *Pseudomonas* spp. responsible for producing "Anietocin" was isolated from a soil sample, and we hereby present the preliminary results obtained with the raw extracts of this novel antimicrobial compound on safe ESKAPE relatives and Klebsiella pneumoniae.

Materials and Methods

Unless otherwise stated, all methods used were adapted with minor modifications from the Small World Initiative Research Protocols (4th edition) by Broderick and Kurt (2016)

Soil Sample

Soil samples were collected in resealable bags from different locations across the Rio Grande Valley of Texas with particular focus in Hidalgo County. The target soil samples were from the topsoil where organic matter is the most abundant. A soil sample data collection sheet as designed by the Small World Initiative (http://www.smallworldinitiative.org/) was used to document the characteristics of the collected sample which included the general location, GPS coordinates, dates and times collected, sample site descriptors, air temperature in Celsius, humidity, depth, type of soil, soil temperature, pH of soil, soil water

content etc. Soil samples were kept at room temperature and processed within 72 hours of collection.

Broth Culture Preparation

0.1 g of each collected soil sample was aseptically added to 10 ml of sterilized Tryptic Soy Broth (TSB, BD BDL, 9046995, Sparks MD). This was thoroughly mixed using a bench top Vortex machine, and thereafter 0.1 ml of this mix was used to inoculate another 9.9 ml of TSB to bring the final dilution to 10^{-3}. The broth cultures were incubated at 37 °C between 18-24 hours.

Solid Media Plating

10 µl of the 10^{-3} dilution was taken and diluted in 90 µl of freshly prepared TSB broth and a final 100 µl of this dilution was taken and then spread plated on Tryptic Soy Agar plates (TSA, Becton Dickinson BDL, 8346803, Spars MD) prepared with 25 µg/ml of 95% cycloheximide (Acros Organics, A0411477, China) to prevent fungal growth. The plates were incubated at 37 °C for 24-48 hours.

Colony Selection

After 24-48 hours, the incubated plates were observed for the formation of bacterial colonies, with particular attention paid to single colonies with unique characteristics such as size, color, smooth

Antimicrobial Compounds Production Test

The isolated pure colonies were collectively spotted grid style using sterile toothpicks on prepared TSA plates of cultures of safe ESKAPE relatives and *K. pneumoniae*. The plates previously described were prepared using freezer stocks of ESKAPE relatives and *K. pneumoniae*, where single colonies were re-inoculated into fresh broths before being transferred onto TSA plates using the spread plating techniques. These plates were spotted with the suspected antimicrobial compounds producing soil isolates and incubated at 37 °C for 24-48 hours Thereafter they were removed and observed for any noticeable zones of inhibitions.

Antimicrobial Compound Screening

The isolated colonies that showed noticeable zones of inhibition in the antimicrobial compounds production tests previously described were individually spotted on prepared plates of cultures of safe ESKAPE relatives and *K. pneumoniae* and incubated as previously described. The plates were once again screened for evidence of antimicrobial activity by looking for evidence of "zones of inhibition" – a clearing on the bacterial lawn where the isolates were spotted using sterile toothpicks. This procedure is a confirmation of what was done prior in F.

DNA Extraction, Amplification, Gel Electrophoresis and Sequencing

The isolate of interest that demonstrated zones of inhibition against any of the safe ESKAPE relatives and other tested bacteria had its DNA extracted using

the Zymo quick-DNA miniprep plus kit protocols (Zymoresearch, 207601, USA). These isolates were grown in TSB broth overnight at 37 °C before the DNA isolation. The gene sequence of the 16S rRNA were amplified using 16S rRNA forward (AGAGTTTGATCCTGGCTCAG) and 16S rRNA reverse (ACGGCTACCTTGTTACGACTT) sequences primers (integrated DNA Technologies, Inc.) and thereafter, gel electrophoresis was performed to confirm the right size sequence at approximately 1500 base pairs. The amplified gene sequence was cleaned up using the Monarch PCR & DNA Cleanup Kit (New England Biolabs) and sequenced for identification by Eurofins Genomics.

Organic extraction

The antimicrobial producing isolate of interest was grown on solid media and was subjected to organic extraction as described in the Small World Initiative Research Protocols (4th edition) by Broderick and Kurt (2016). A spatula was used to cut 2-3 small pieces of agar (1 inch square) containing the antimicrobial producing culture isolate, which was then transferred into 100 ml glass bottle and placed in -20 °C freezer overnight, this procedure was replicated multiple times to increase the quantity of extract obtained.Thereafter, 15 ml of 99% ethyl acetate (Flint Scientific, 273018, Batavia, IL) and 10 ml of deionized water were added to each bottle and shaken vigorously at room temperature for 20 - 30 minutes. A pipette was then used to transfer the entire liquid contents of the bottles to new bottles, avoiding the agar debris and allowed to sit for as long as needed for the two phases to separate. The separated ethyl acetate layer (top portion) up to 10 ml, was then transferred to new bottles and placed without caps inside the fume hood until all ethyl acetate had evaporated (usually between 96-120 hours). After the entire ethyl acetate had evaporated, leaving behind a white residue hereby referred to as the "the crude extract", the white residue was dissolved by the addition of 80 µl of methanol (VWR, 0000200921, Mississauga ON) in each bottle and the solution kept at 4°C for further use, usually between 12-24 hours after extraction.

Screening for antimicrobial activity

The solution obtained from the organic extraction (white residues dissolved in the methanol) was tested on prepared plates of safe ESKAPE relatives and other bacteria. 30 µl each of the solution (crude extract dissolved in methanol) was dropped on the plates containing the prepared plates, another 30 µl of methanol dropped on a different part of the plate as negative control and in some cases different antibiotics disks (BD BBLTM, Sparks MD) were tested against the solution for comparison. The plates were incubated at 37 °C for 24-48 hours. The zones of inhibition produced by the antimicrobial compound were measured and compared to zones of inhibition produced by the antibiotic disks. These steps were replicated several times and confirmed reproducible.

Infra-red spectroscopy

The antimicrobial compound solution was tested to determine the functional groups in the mixture using the Fourier Transform Infrared Spectrophotometer (Shimadzu). Resolution was set at 4 cm-1 and the Happ-Genzel apodization was utilized for a total of 4 scans.

Results

More than 100 unknown soil bacteria isolates were tested on ESKAPE relatives for the ability to produce antimicrobial compounds. Among them was an isolate with a yellow appearance that created a clear zone of inhibition when tested on *B. subtilis* and *S. epidermidis* and subsequent testing of the isolate of interest on *K. pneumoniae*, *E. raffinosus* and others (not shown) in Figure 1 produced zones of inhibition of varying diameters. The culture of the suspected novel species or at least a sub-specie of *Pseudomonas* was assumed to be producing an antimicrobial compound when spot tested on the 7 listed bacteria in table 1. This spot testing was replicated multiple times and the ability of the bacterium to produce clear zones of inhibition when spotted confirmed its antimicrobial producing ability. It was noted that some inconsistencies were observed when the antimicrobial compound producing bacterium was tested on cultures of *E. coli*, as some of the results were initially positive but came back negative on subsequent spot testing. Cultures of *E. coli* sustained these variations and so were declared non-sensitive to the antimicrobial compound produced by the unknown bacterium.

Table 1 shows the results obtained when testing with the unknown compound on safe ESKAPE relatives and *K. pneumoniae*. *B. subtilis* and *S. epidermidis* showed higher levels of sensitivity to the unknown bacterium, suggesting that perhaps the antimicrobial compound produced could have more effect on Gram-positives species especially the non-sporeforming *S. epidermidis*. The rest of the test cultures showed varying degrees of sensitivity over many replicates, with *E. coli*, appearing to be the least sensitive. The unknown bacterium was used after incubation for 18 – 24 hours for best results. Older cultures of this unknown bacterium showed less ability to inhibit the growth of the test cultures.

Fig 1: Left to right. The unknown bacterium (pointed) on a culture of B. subtilis, the zone of inhibition can be clearly seen on the plates.

Tester Bacterium	Result
1. ***Enterococcus raffinosus***	+
2. ***Escherichia coli***	-
3. ***Erwinia caratovora***	+
4. ***Pseudomonas putida***	+
5. ***Staphylococcus epidermidis***	++
6. ***Enterobacter aerogenes***	+
7. ***Bacillus subtilis***	++

Table 1: Tester bacteria are used to verify the antimicrobial compound produced by the unknown bacterium. - = no zone of inhibition observed, + = zone of inhibition observed, ++ = large zone of inhibition observed

The raw extract from the antimicrobial compound producing bacterium was obtained as described in the materials and methods section and was used on the test cultures to confirm potency. The results obtained showed consistency as earlier observed when the antimicrobial compound was directly used on the test cultures. *S. epidermidis* showed the highest level of sensitivity as can be observed from the zone of inhibition obtained. *E. coli* though, originally declared non-sensitive, appeared this time to show a slight degree of sensitivity, suggesting that perhaps much is left for us to understand on the culturing conditions of this unknown bacterium to produce the maximum and/or most potent form of the antimicrobial compound. For this testing phase, a bacterium obtained from an environmental sample was added to the line-up of test cultures and was sensitive to the crude extract of the antimicrobial compound being tested.

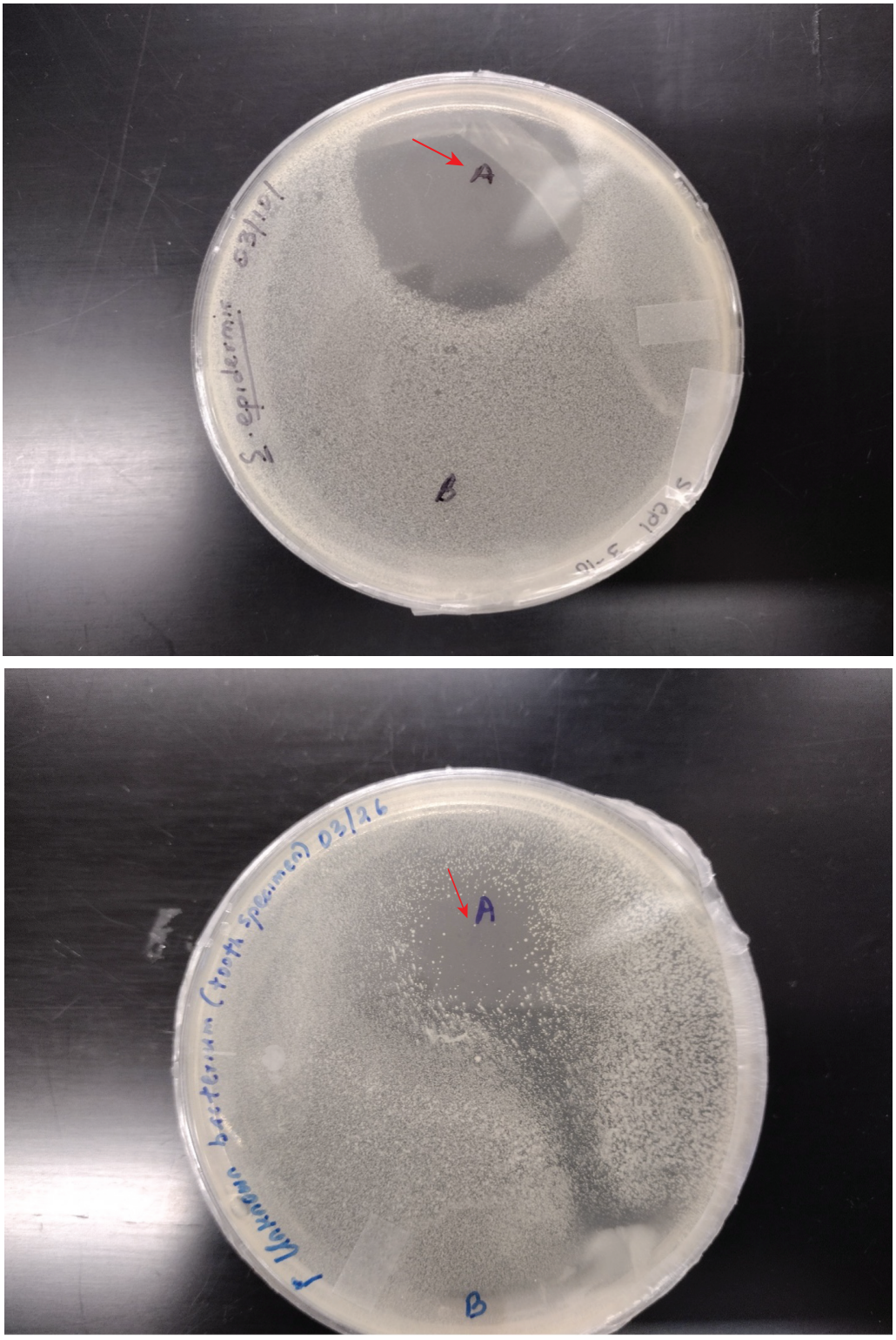

Fig 2: Top to Bottom. The antimicrobial compound zones of inhibition (pointed) on a culture of S. epidermidis, and an unknown environmental sample extract bacterium, Methanol was used as the negative control and represents the alphabet "B" and written as methanol in some of the plates.

To determine this unknown antimicrobial compound's effectiveness when compared with known antibiotics, we set up an experiment with *S. epidermidis* and *K. pneumoniae*, representing gram-positive and gram-negative bacteria respectively. For *S. epidermidis,* Penicillin 10 IU (BD, BDL 9154628, Sparks MD) was used to compare to the unknown antimicrobial compound while 1.25 µg Trimethoprim/ sulfamethoxazole (SXT, BD BDL, 9078838, Sparks MD) was used for the same comparison on *K. pneumoniae*. The choice for these two well-established antibiotics is hinged on their known efficacy when used on the aforementioned bacteria. For example, some strains of *K. pneumoniae* strains are susceptible to SXT as reported by Ballén et al. (2021). Similarly, it is common knowledge that Penicillin is the oldest commercially viable antibiotic in modern medicine. Its mode of action being to interfere with the synthesis of the bacterial cell walls as described by Soares et al. (2012). This novel antimicrobial compound appeared more potent than Penicillin (10 IU) on S. *epidermidis* and SXT (1.25 µg) on K. *pneumoniae* when measured by the observable zones of inhibition produced as can be seen in figure 3 below and this experiment was replicated many times with the same outcomes. Going by the BD BBLTM antimicrobial susceptibility tables, Penicillin (10 IU) with values greater than 29 mm in diameter on *Staphylococcus* species is the top range effectiveness but, we achieved a little more than 500 mm in diameter of zone of inhibition using the novel antimicrobial compound. As far as we know, no research article has ever reported this extremely large number with either crude or refined extract and none of the commercially available antibiotic discs has produced this result on any bacterium. This suggests a unique killing efficiency that may become the new and effective antibiotic against resistant pathogens.

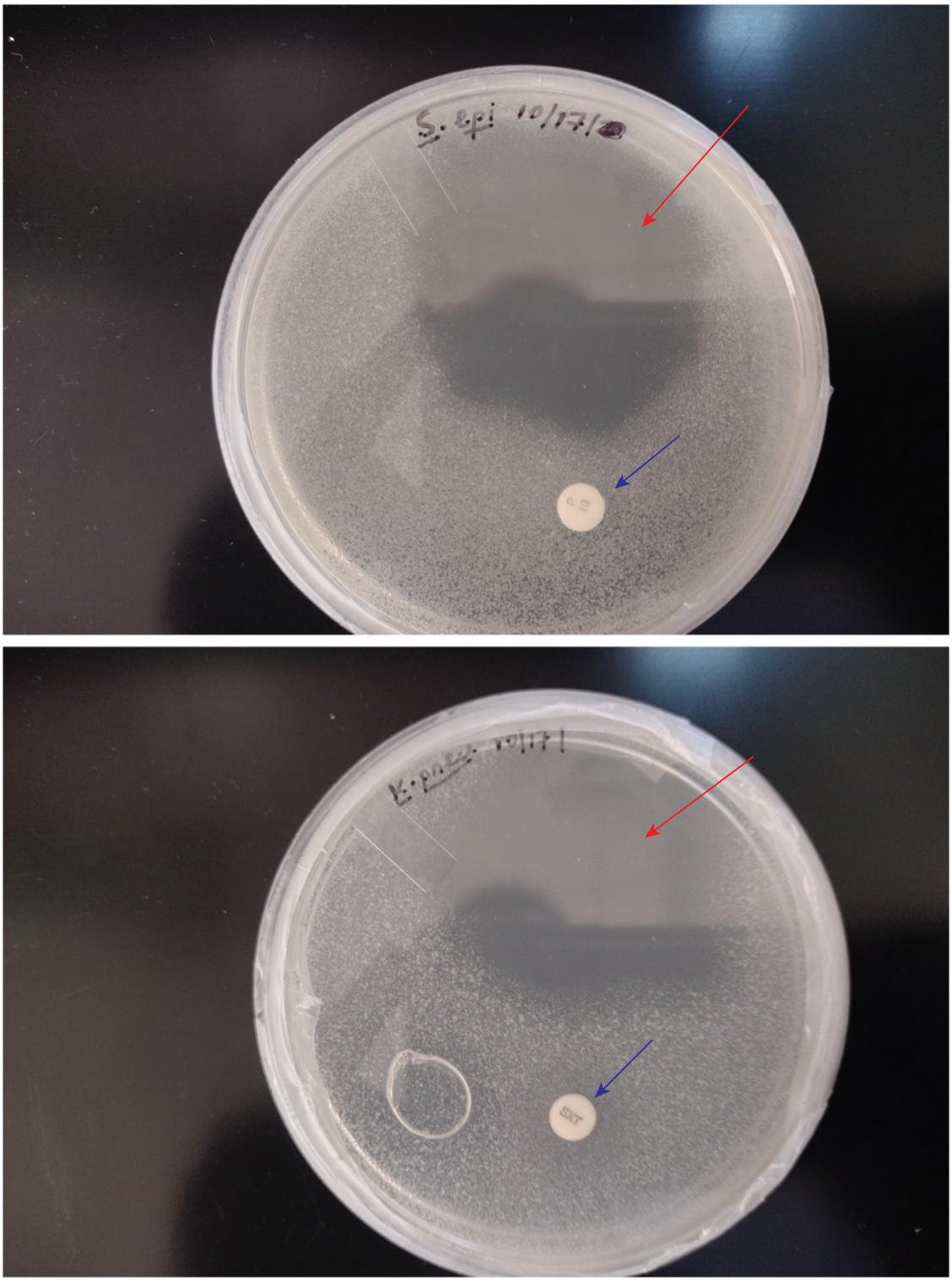

Fig 3: Top to bottom. The unknown antimicrobial compound (red arrow) on S. epidermidis and K. pneumoniae. P-10 and SXT are arrowed in blue. The zone of inhibition produced by the unknown antimicrobial compound on S. epidermidis was slightly above 500 mm in diameter.

The result of 16S rRNA genes sequencing analysis (GenBank accession number ON964909, SeqIDAnieto), suggests the likelihood of a relationship with the genus *Pseudomonas*, Azotobacter, Halopseudomonas, Atopomonas, and Azorhizophilus. The closest matches with a max score and total score of 723 and 86% percent identities suggest a possible closer relationship with *P. aeruginosa* strains DDM 50071, NBRC 12689 and ATCC 10145 (GenBank accession numbers NR_117678.1, NR_113599.1 and NR_114471.1 respectively). Considering the unique nature of this isolate by its ability to produce an antimicrobial compound, previously undescribed as far as we are aware, we hypothesize that this suggested *Pseudomonas* spp. is likely a new addition to the genus or a sub-specie at the minimum. Its antimicrobial compound is equally novel based on the observed preliminary results and therefore referred to as "ANIETOCIN" for the purposes of proper identification.

The results of the infra-red spectroscopy analyses, suggest the possible presence of alkenes (=C-H) and alkanes (C-H) functional groups with peaks seen at 3100-2900 cm-1 regions; however, this can be regarded as inconclusive because the peaks appear weak which does not correlate with the description of known peaks at the same region. This weak appearance could be a function of the crude nature of the extract, with numerous artifacts interfering with the analyses. A critical look at the fingerprint region of the infra-red spectroscopy analyses suggests the likelihood of the presence of methyl or methylene groups (C-H bending) with medium sized peaks at around 1500-1450 cm-1. A very sharp peak at approximately 900 cm-1 suggests the presence of a di- or trisubstituted alkane group.

Discussion

Not much is known about this likely *Pseudomonas* sp. that produces the novel antimicrobial compound Anietocin but the outcomes of the spot testing experiment across a battery of safe ESKAPE relatives and including an ESKAPE pathogen itself suggest that it could well become an effective antimicrobial compound. A much better outcome is seen when tested on Gram-positives than on Gram-negatives, with *S. epidermidis* showing an outstanding level of sensitivity when exposed to Anietocin. As *S. epidermidis* is the safe version of the ESKAPE pathogen S. *aureus*, we are hopeful that this positive result will be replicated when tried on it. The preliminary results obtained show the superiority of Anietocin on *S. epidermidis* by the observed and measured zones of inhibition using only the crude extract when compared to 10U Penicillin. We hypothesize that a cleaned up and pure Anietocin compound will effectively confirm this superiority by giving much wider zones of inhibition with lower minimal inhibitory concentration (MIC) values. Our next focus is to purify the Anietocin antimicrobial compound, determine its chemical structure, minimal inhibitory concentration based on the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines, the full genome analyses of the suspected *Pseudomonas* spp. and other studies that will assist in moving it from laboratory to the pharmacy.

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