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Combating antibiotic resistance: An approach to discover antimicrobial compounds

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Abstract

Objective: This study aims to test a cost-effective approach for discovery of novel molecular compounds with antimicrobial characteristics.

Materials and methods: Thirteen randomly picked objects were swabbed in the area in and around Liverpool. They were further cultivated on BHI-agar plates, and then spread on top of several clinically relevant pathogenic indicator strains including *C. albicans*, *C. auris*, *E. coli*, *M. luteus* and *MRSA*. This system was incubated overnight and then analysed. Colonies which inhibit the growth of indicator strains, preferable more than one, were assessed suitable for further analysis.

Results: From all the cultivated samples**,** no bacterial colonies showed antimicrobial activity against *C. albicans*, *C. auris* and *E. coli*. Multiple colonies demonstrated antimicrobial activity against *M. luteus*, and one clone against *MRSA*.

Conclusion: Based on the result from thirteen samples were four of them demonstrate antimicrobial activity, the pipeline of such an approach to discover new antimicrobial molecules seems promising. It is cost-effective with great potential to detect novel antimicrobial compounds. More resources and time are required to investigate its fully potential.

1. Introduction

Antimicrobial resistance is a well-known increasing problem around the world. Within the lifetime of the current generation, we are at risk of seeing a dramatic increase in treatment failure with antimicrobial agents. This means that the same drugs which have saved uncountable number of lives by now, have the potential not to kill or inhibit bacteria through bacteriostatic or bactericide processes anymore. Earlier easily treated infections (e.g. UTI, strep throat, pericoronitis) and routine procedures (e.g. hip replacement and general surgery for immunosuppressed patients) will be transformed into potentially fatal procedures with higher risk of death if resistance bacteria are the causative agents of such infections. Organ transplantation, treatment of a spectre of autoimmune diseases, chemotherapy and larger surgeries are all examples of procedures dependent on antimicrobial agents that do work (1). Unfortunately, antimicrobial resistance mainly affects the weaker individuals from an immunological perspective, but the healthiest of us might be affected as well. Economists has predicted the antimicrobial resistance to kill 10 000 000 people per year the world over within year 2050 (2) if no action is adopted now to combat the problems. Some people have coined this term for a post-antibiotic era.

Although antimicrobial resistance is an increasing problem, antimicrobial resistance has been a well-known phenomenon since the discovery of antibiotics. Alexander Fleming discovered penicillin in 1928 and before it was introduced as an antimicrobial agent for clinical use, penicillin resistance was identified (3, 4). Penicillinase, an enzyme inhibiting the therapeutic effect of penicillin, was discovered by Flemings colleagues already within 1940 (5). This is the history of all discovered antimicrobial agents by now, which also could describe why microbiome untouched by humans, carry resistant genes (6, 7). All use of antimicrobial agents in clinical settings apply selective pressure towards bacteria (8). This includes both justified and unjustified use in medicine and agriculture (9). Selective pressure promotes the spread of resistant genes between bacteria as a defence mechanism. In addition to the fact that the use of antimicrobial agents has increased sharply since the start of clinical use, the reason why antimicrobial resistance has become a bigger problem now than for forty years ago could partially be explained by drug discovery. Due to several reasons the number of new antimicrobials discovered has decreased, and antimicrobial resistant pathogens increased (10).

In a longer leap, this means that all antimicrobials we have for hand can become ineffective. The situation was the opposite forty years ago.

Antimicrobial drug discovery has been reduced significantly the last decades. Whilst treating chronic diseases require several doses of drugs, infections normally only need treatment for a brief period (11). Newer antibiotics are often saved as emergency-drugs and therefore barely sold. New drugs are also facing resistance problems shortly after, or even before clinical introduction, which leads to minor prescription and a lack of income for the producer (12). Those points, added with the fact that it is both extremely time- and money consuming to develop antimicrobials, has made investing in other fields more interesting for pharmaceutical companies. The current situation of low investing initiatives in new antimicrobials conflicts with the urgent need for new antimicrobial agents with characteristics not affected by already known resistance mechanisms (13).

Technically, antimicrobial resistance can be divided into intrinsic, acquired, and adaptive resistance. Intrinsic resistance means that the microorganism has inherent properties which make them resistant against certain antimicrobial agents, for example penicillinase as mentioned. Acquired resistance is a trait which the microorganism has developed or acquired, either through mutations or horizontal gene transfer (14). On the other hand, adaptive resistance is a newly discovered concept and seems to be transient. In short, it is characterized by gene modulation in response to specific signals from the environment nearby, making the organism resistant if the signal persist (15, 16). There are different mechanisms of antimicrobial resistance as well. In short, bacteria can either lower the concentration of antimicrobials and thus inhibit them or modify their target (17, 18). This can be done with efflux pumps, lowering the permeability of the cell wall, destroying, or modifying the antimicrobial, through altering synthesis to change their target or produce alternative targets. This is illustrated in Figure 1. Spread of intrinsic resistance select resistant strains, while spread of acquired resistance select resistant genetic elements, which means selective pressure is elementary for spread of antimicrobial resistance (15). Horizontal gene transfer is the main route for resistant genes between microorganisms, mainly conducted through transformation, transduction, or conjugation (19). It has also been recognized as elemental for evolution of

bacteria, as a large spectrum of genetic material can be exchanged in the process (20). Transformation involves uptake and incorporation of extracellular DNA, but is exceedingly rare (21). During conjugation, bacteria have direct contact with a conjugation bridge and plasmids are transmitted. Transduction means the transfer of genetic material through bacteriophages.

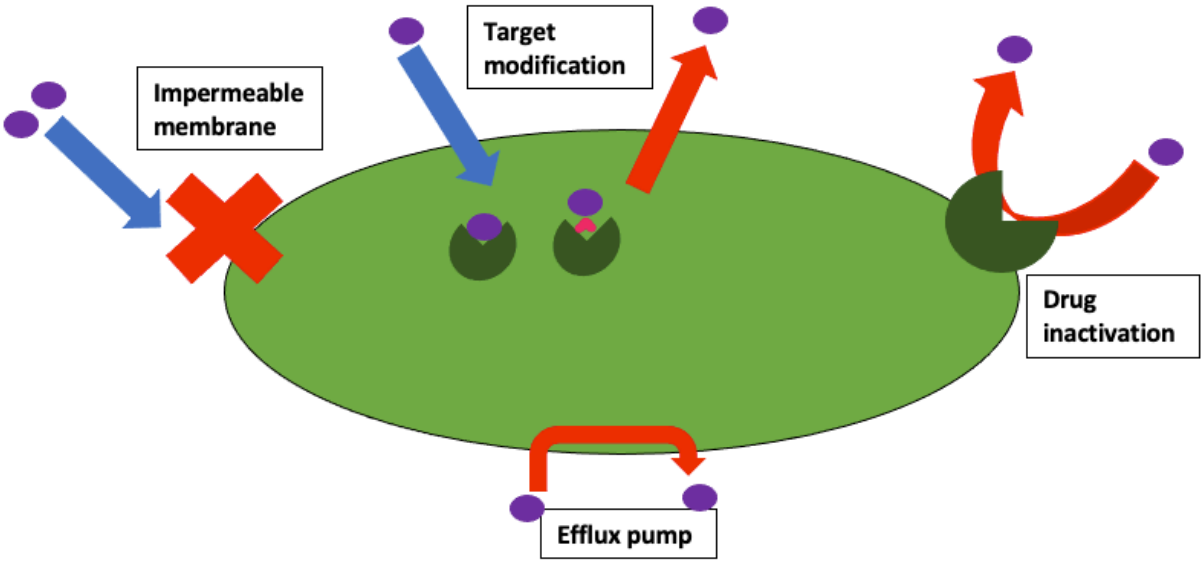

Figure 1: Mechanisms of antimicrobial resistance

It is important to stress that antimicrobial agents are used to treat some oral infections as well as other infections. The location of the oral cavity makes it a potentially dangerous foci of infection with its immediate proximity to essential organs including the brain, trachea, and lungs. It has previously been demonstrated how infections with teeth as origin have led to critical illness and loss of parts of the face (22). The Norwegian guidelines for use of antibiotics for the oral cavity include regimes for acute odontogenic infections, exacerbation of periodontitis and peri-implantitis and prophylactic use for individuals with higher risk of infection (23). Preferred treatment of odontogenic infections is drainage, but antibiotics could be required now and then as the foci of infection often are inaccessible. Apart from antibiotics, other antimicrobial agents are also well used in dentistry. Chlorhexidine is used as a supplement when oral hygiene is not sufficient for the treatment of inflammatory conditions including gingivitis, periodontitis and alveolitis, as well as pre-operatively before oral surgery and in revision treatment in endodontics (24). Another antimicrobial, sodium hypochlorite is

the preferred irrigation agent for root canals (25). In the period from 2010 to 2016 a total of 27 million prescriptions of antibiotics were made in England, Norway, Scotland and Sweden, and dentist accounted for about 2 million of them (26). This means that antimicrobials are important in dentistry, but also that dentists have a role to fight bacterial resistance problem and preserve the effectiveness current available antimicrobials.

Antimicrobial resistance is primarily a global crisis, and tackling it needs multidisciplinary cooperation. Surveillance, to map the extent of the problem, is important. World Health Organization released a report on surveillance in 2014 which showed major differences in how the surveillance work is done around the world (27). European Antimicrobial Resistance Surveillance Network (EARS-Net) even report about inter-laboratory differences in one country (28). Global guidelines for surveillance would benefit all stockholders, without doubt (29). Furthermore, next-generation laboratory methods been highlighted as tools to help combating antimicrobial resistance (30). Utilising next-generation laboratory methods, could leads us to optimize the use of already known antimicrobial agents. The concept of collateral sensitivity has been introduced the last years, as a method where antimicrobial resistance to one agent provokes increased susceptibility to another agent which then could work without resistance issues (31, 32). Despite those measures mentioned here, development of new antimicrobials without known any resistance potential will persist as elemental for continuation of the antibiotic era.

Nearly all infectious agents contain DNA or RNA molecules. This makes sequencing an attractive approach for pathogen detection and analysis (33). Next-generation sequencing (NGS) is a fairly new method of handling larger amounts of genomic materials. The first available NGS technologies came around the turn of the millennium in the form “massively parallel signature sequencing”, a service made available from Lynx Therapeutics Company (now Illumina). The technology was limited and expensive. The next two decades brought along large changes and improvements in the field of metagenomics. The price of sequencing one human genome in was 100.000.000 US dollars in 2001, and 1,000 US dollars in 2020 (34). The current sequencing platforms available are including, but not limited to, iSeq,

MiSeq, MiniSeq, NextSeq, HiSeq, NovaSeq, Ion Torrent, BGISEQ, and Oxford Nanopore Technologies. Excellent descriptions about the technologies are described by Gu *et al* ((33).

NGS has already proven to be an efficient tool in microbiology for analysis of antibiotic resistance and virulence factors. An example of this is in 2018 where they used whole genome sequencing to get insight into phylogeny, antimicrobial resistance, and virulence markers in *Salmonella serovars* (35) . It is hypothesized that as the cost-efficiency of NGS improves, and the data storage and processing problems the large datasets provide are managed, the possibilities of NGS in antimicrobial drug discovery will be an unreplaceable tool.

As a contribution to tackle the antimicrobial resistance crisis we are facing, this project aimed to test a cost-effective approach for discovery of molecular compounds with antimicrobial characteristics. Interesting molecules can then easily be passed on to the next phase towards pharmaceutical development, whilst the other ones are singled out. We hypothesize that this pipeline will be an efficient way of discovering known and de novo antimicrobial traits in randomly selected microorganisms.

2. Methods

Ethical consideration

As this project can be classified as a technical and methodological development work that uses biological material without any personal information being linked to the material, there is no need for any application to the Regional Ethical Committee (REK) and prior approval of the project, according to the current laws and guidelines (36).

Collection of environmental samples

Microbiological samples were obtained from surrounding areas thought to be inhibited by interesting microbials, in and around the city of Liverpool (UK). Seemingly randomly picked objects were swabbed with cotton swabs (Transwab, M40 Compliant, UK) containing Amies^{TM} medium to preserve the swab quality during the transport to the laboratory – illustrated in Figure 2.

Figure 2: Cotton swab stored in Amies^{TM} medium

Cultivation of samples

To cultivate the environmental samples, all swabs were streaked out in 4% Brain-Heart-Infusion (BHI) agar plates and incubated for three days at 37 °C. Brain-Heart-Infusion serves us a medium with growth conditions for a broad range of microbial species, optimal for our purpose. As we work with unknown microbes from the environment, we do not know the optimal temperature or time for incubation. Based on empirical data three days and 37 °C were used. After three days of incubation, 96 of the newly formed colonies were single-handedly picked and placed in 100 ml BHI broth in a 96-well microtiter plate for further handling.

Indicator strains

To discover whether any of the swabbed objects contain any bacterial species that produce molecules with antimicrobial activity, we aimed to grow them in near relation to clinically relevant indicator strains. The selected indicator strains for this project are limited to the fungi *Candida albicans* and *Candida auris*, the gram-positive *Micrococcus luteus* and *methicillin resistant Staphylococcus aureus* (MRSA) and the gram-negative *Escherichia Coli*, all of which are highly relevant microorganisms with known pathogenicity and resistance to antimicrobial treatment in clinical settings (37-41). After two days of incubation of swabbed environmental sample, each indicator strain was streaked on 4% BHI plates and incubated at 37°C overnight, so that all microbes were finish incubated in the third day.

Testing for antimicrobial activity

To test the environmental microbes for antimicrobial activity, we aimed to create a simple system which easily reveal antimicrobial activity. This was done by picking separate colonies from each finally incubated indicator strain and mix them in 1 ml BHI broth. This mix were spread on their individual 100 ml 4% BHI agar plate before drying the plate for 30 minutes, which facilitate the growth of the indicator species at the surface of the BHI. After that, the 96 environmental colonies from the 96-well microtiter plate were picked and transferred to the plate containing dried indicator strains, to facilitate growth of environmental samples on top. This was conducted with a 96-replicator pin (Figure 3) which was ethanol sterilized and set

ablaze prior the picking to prevent contamination, and to dry the metal. A positive control of environmental samples was placed on a 4% BHI agar plate without any indicator strain to ensure that the medium is not the cause for lack of growth. All plates were incubated overnight at 37°C and analyzed the next day.

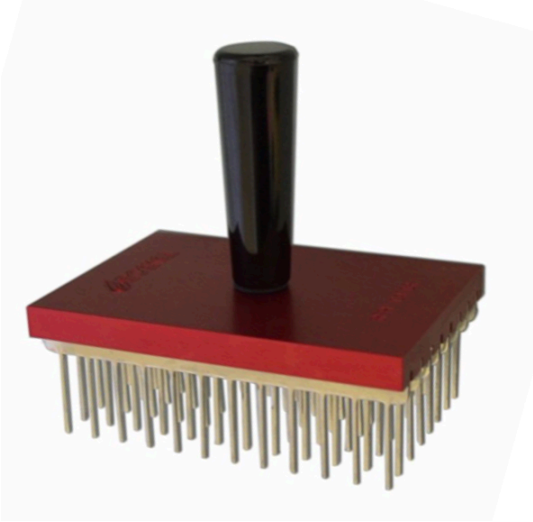

Figure 3: 96-replicator pin (Boekel Scientific)

After incubation overnight of the BHI plates with indicator strains and environmental samples at the top, we did plan to screen every plate for any trace of antimicrobial activity. This was done by eye, looking for any presence of *zone of inhibition* (42) on all plates. This is a qualitative method to evaluate antimicrobial activity. If there are antimicrobial activity in our plates, this will appear as a circle around the environmental colony without any growth of indicator strain. This will help us to further select for clinically relevant characteristics which will be interesting for further characterization and analysis. However, we know from literature that environmental samples will contain species where 50% will show antimicrobial activity against gram-positive bacteria, and 20% against gram-negative bacteria, most of which are known or toxic compounds (43). We have therefore set a cut-off criteria for further analysis, to help us filtrate already known or toxic compounds:

- antimicrobial activity against one gram-positive and one gram-negative bacterium
    - possibly one fungal species in addition
- two fungal species

For further characterization of the potential active compounds, analysis using NGS and bioinformatic analysis would be required. The NGS analysis could help us characterize if there are known or unknown compounds involved. Although NGS has been cheaper the latest years, the resources and capacity available during this master project makes it difficult to purse such analysis. However, we could still test a cost-effective approach for discovery of molecular compounds with antimicrobial characteristics.

3. Results

Collection of environmental samples

When considering areas and objects to be swabbed, with interesting microbial characteristics, we thought of surfaces and objects that are often easily contaminated by humans and during their interaction with the environment. In retrospect it could have been smart to consider areas as to a greater extent addresses the nature as there are many special microbes living out there. Thirteen randomly chosen surfaces (MD113-MD124) were swabbed in and around Liverpool city (UK). Examples of things we did swab include a cup, elevator buttons and the bathroom drain. The origin of each swab is listed in Table 1. After swabbing, all samples were transported to the laboratory.

Table 1: The origin of environmental samples

Swab	Origin
MD114	Coffe cup
MD115	Lift
MD116	Tiles outside
MD117	Sink drain
MD118	Elevator buttons
MD119	TV-remote
MD120	Bathroom tile
MD121	Cab-door
MD122	Sink
MD123	Skin
MD124	Macbook Pro

Cultivation of samples

After three days of incubation at 37 °C in BHI agar, all plates including environmental samples showed growth. This is illustrated in Figure 4A. The numbers of colonies ranged

from two to several hundred. Several types of microbes were represented, including bacteria and fungi. The fact that the number of colonies vary this much, could be attributed to several factors earlier mentioned: growth medium, temperature during incubation and time of incubation. After incubation, we picked 96 randomly colonies and placed them in 100 mL BHI broth each in a 96-well microtiter plate.

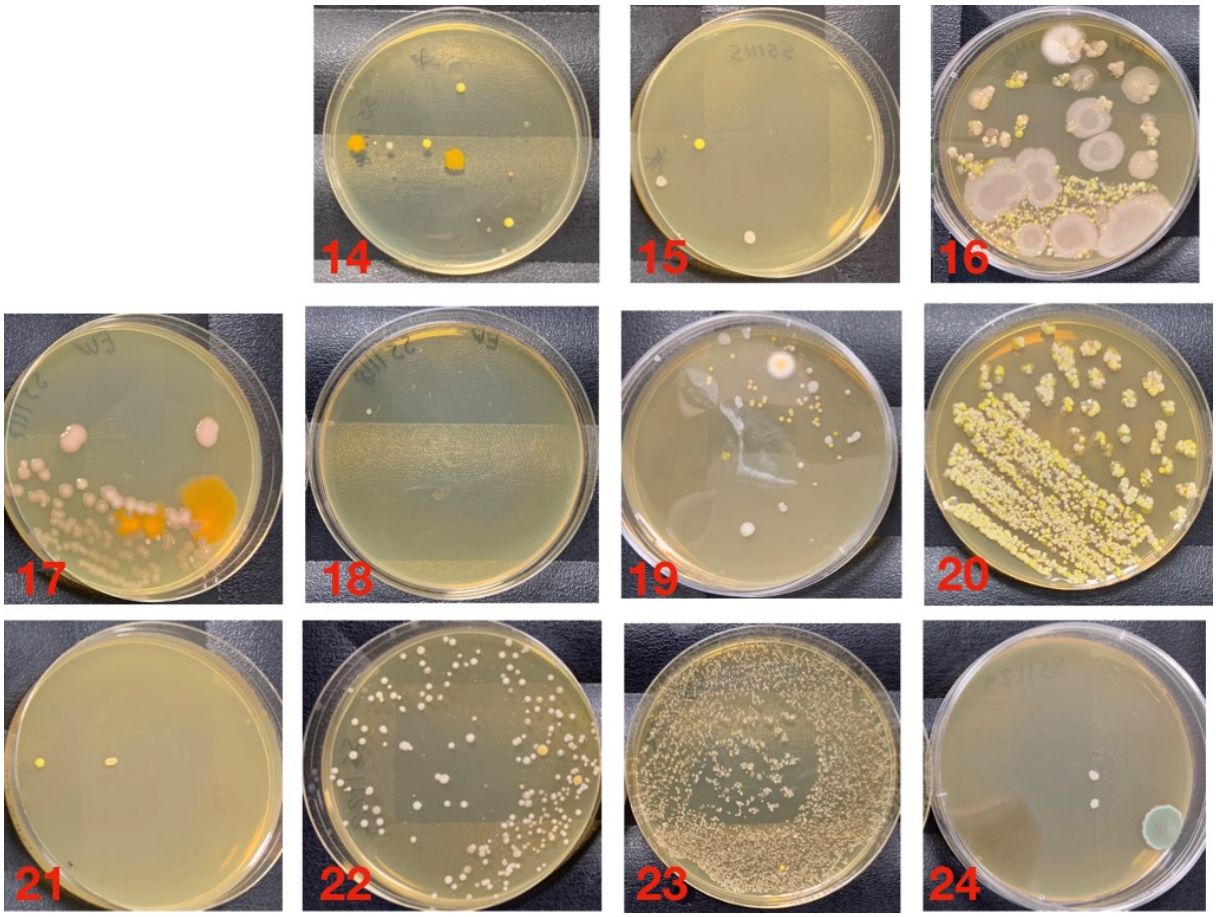

Figure 4A: Colonies on swabbed plates after three days of incubation

Indicator strains

All indicator strains grew as expected, with growth on all plates. These have been isolated from clinical settings, and therefore with known optimal condition for incubation. All indicator strains after incubation are illustrated in Figure 4B.

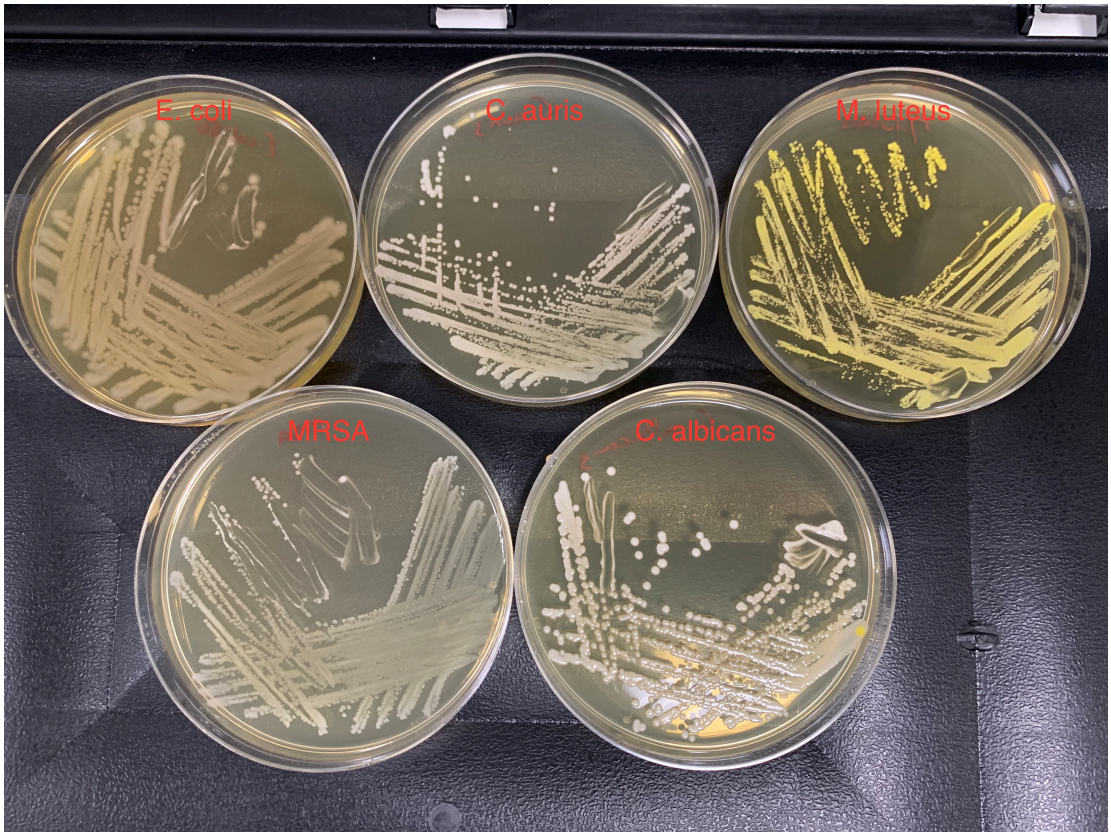

Figure 4B: Indicator strains

Testing for antimicrobial activity

The environmental samples were applied upon the indicator strains as described in the methods section. After incubation of this system overnight there was growth of both indicator strains at the bottom, and environmental samples on top in all plates, shown in figure 4C. Almost all the 96 colonies showed growth on all five plates.

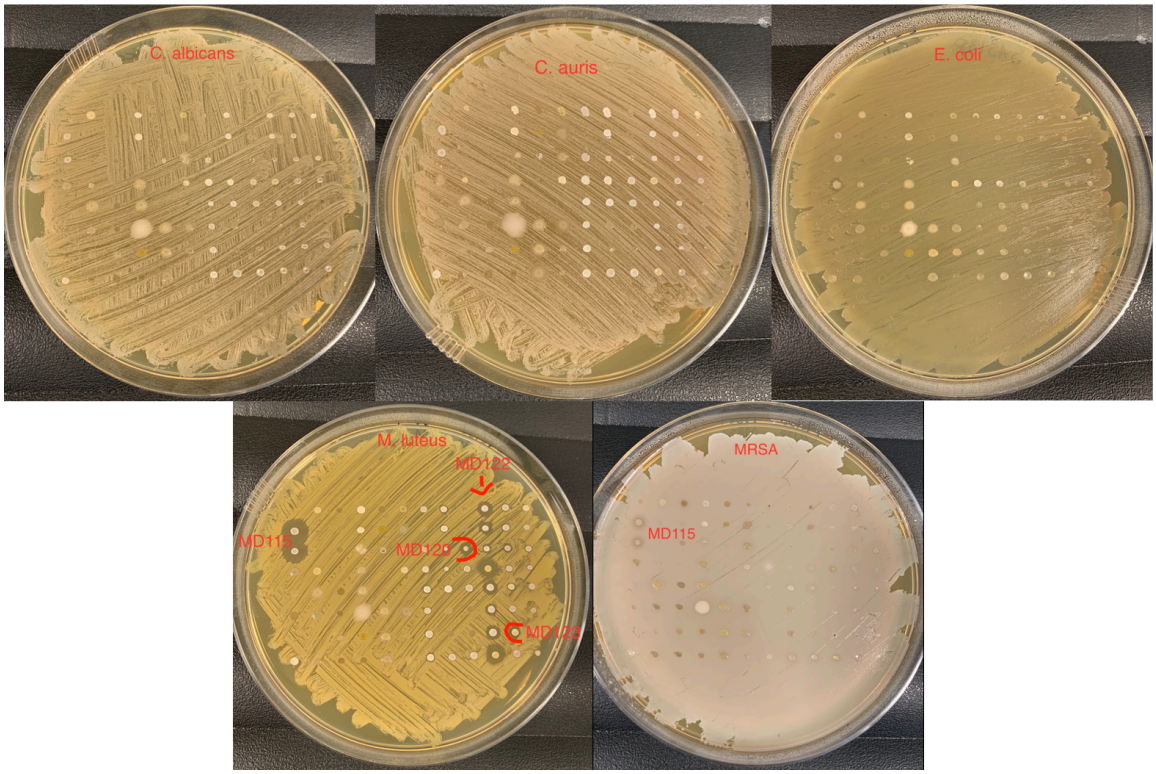

Figure 4C: Indicator strains with environmental samples

The positive control ensuring that the medium is not the cause for lack of growth, showed the same result (Figure 4D). In the first and second rows it appears that there are some colonies without growth. More reasons could describe this finding. It could be colonies which do not survive the transfer from plates to the 96-well microtiter plate, or the lab procedure. It could also be that they need longer time for incubation.

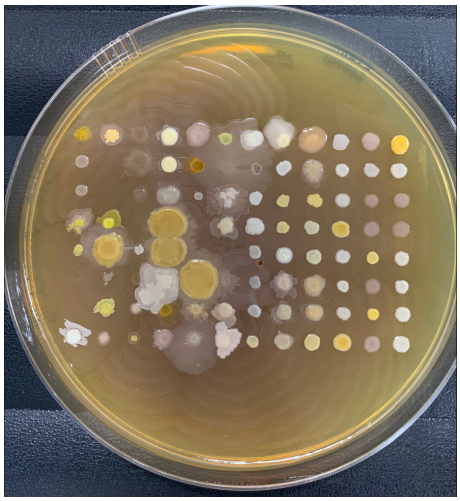

Figure 4D: Positive control

Analysis

Almost all the 96 added colonies showed growth on all five plates. The size varied largely between the colonies. Around 6% of the colonies showed a zone of inhibition towards the indicator strain, with test colony MD115 (lift) showing zone of inhibition towards both *M. luteus* and *MRSA*. On the other hand, MD120 (bathroom tile), MD122 (sink), as well as MD123 (skin) showed a zone of inhibition towards *M. luteus*. These findings are summarized in Table 2. There were as good as no zone of inhibition on the plate containing C. albicans, C. auris, or *E. coli*. Visualization of examples of zone of inhibitions can be found in Figure 4E.

Table 2: Summary of antimicrobial activity

Swab	**Antimicrobial activity against**
MD115 (lift)	*M. luteus*, *MRSA*
MD120 (bathroom tile)	*M. luteus*
MD122 (sink)	*M. luteus*
MD123 (skin)	*M. luteus*

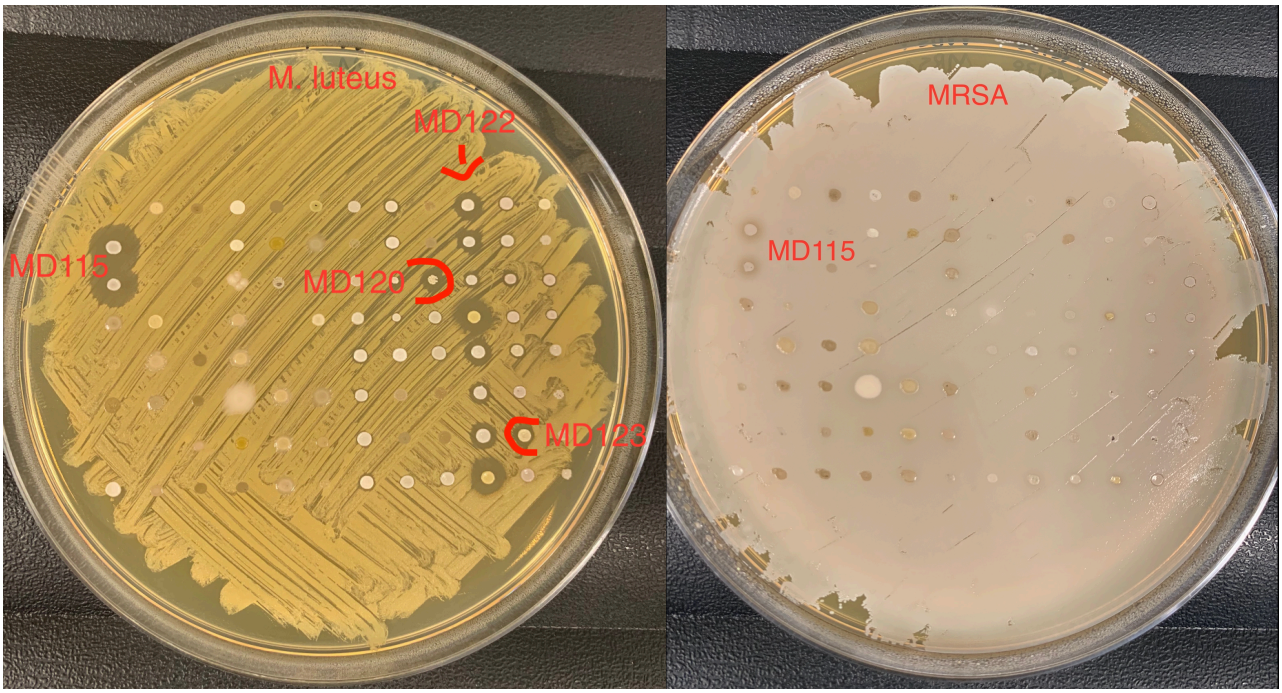

Figure 4E: Antimicrobial activity against M. luteus and MRSA

As this is a master project with limited resources and time, none of the clones were analysed any deeper. In the future, it could be highly relevant to re-run the pipeline and then sequence clones of interest to reveal if there are either novel or known mechanisms behind the antimicrobial activity.

4. Discussion

As knowledge increases in a field of medicine, the drug discovery and drug potency are usually improved. In the field of antimicrobial resistance, this relationship between knowledge and the ability to discover new drugs is paradoxically inverted (44, 45). The more we know about antibiotics, the less we can discover. For the last 50 years the field of microbiology has been studied world-wide, yet there has only been one new antibiotic that has been discovered with traditional methods and made into a drug in clinical practice, the narrow spectrum daptomycin (46). The golden era of antibiotics lasting a short 20 years from the 1940s to the early 1960s brought us our main classes of antibiotics we use today. In the 1960s this drug discovery pathway dried up. The need for a new one is long overdue. Therefore, we must rethink our approach to drug discovery.

One of the strengths of this proposed drug discovery pipeline is the diversity and cost-efficiency. The broad array of microbes gathered for screening in this project is made available by the community-research model and the use of cotton swabs that required no or very miniscule training to use (Figure 2). The model has two great benefits. Firstly, the geographic area covered is vast and almost without restriction. Swabbers can be inspired and contacted through social media, all over the world. Used swabs with microbes are stable and can be sent by mail. Secondly, the gathering of microbes by private personnel is done without cost for the researchers except the cost of the swab kits themselves.

Another advantage of this pipeline is that it includes more than just a research environment; the public could be involved. Public engagement, where the public is involved in research, has become more important with time, and probably it becomes even more important in the upcoming years.

The pipeline focuses on natural compounds produced by the collection of microbes. More specifically compounds harboring antimicrobial properties. Historically, the antibiotics have come to know and use in medical practice are derived from natural sources who produce them organically. An example of this is erythromycin, tetracycline, as well as cephalosporines (47).

The very same study also tells us that more use of bioinformatics whole-genome approaches has been initiated and used systematically the last decade. It will most likely continue to be a key factor in the years to come. Even though around 90% of the antibiotics prescribed today are natural products (48) or synthesized from their natural derivatives. An example of this are the sulfonamide group that was discovered early in the 90's (49). Next generation sequencing has become more cost-efficient the last decade, although the methodology still demands large amount of computer power and knowledge about the specific technology. The presented pipeline to discover new antimicrobial compounds facilitates for this in the way that it filters out all the colonies that does not harbor anti-microbial properties, effectively reducing the data load, or raw data used in a next-generation sequencing analysis (47).

One of the drawbacks of using this pipeline is that it is executed using manual methods. At the laboratory, multiple rounds of swabbing, picking and choosing colonies, not to mention the use of an ethanol sterilized hedgehog instrument. This opens for the possibility of human errors with the potential of contaminating the samples. An upside about this is that the contamination itself is not critical to the pipeline, as it can help produce results if it produces a substance with antimicrobial properties. At the same time, if a there is a find from a contaminated sample, tracking the colony origin will be nearly impossible.

Another drawback is the fact that only one growth medium was used in the study (4% BHI broth and agar) in an attempt to replicate human conditions. Although this growth medium may cover a broad range of microbes, but restricting our study to only one growth medium, i.e. BHI might have resulted in losing out on a somewhat alrge amount of potential finds. In a larger scale study, this problem should be adressed, and the range of growth mediums should be expanded to use. Another way of finding more or different microbal products of interest is to experiment with different lengths and temperatures for incubation. Lastly, the results of using the pipeline are based on finding the zone of inhibtion by researchers' eye. This inhabits the possibility of producing false positives and false negative results that may influence the results. It is also important to note that this test thst depend on researcher's eye for interpertation of result could contains sources of error (42).

The best possible outcome of this pipeline is the discovery of a culture that produces one or more antimicrobial substances and pumps it out towards nearby pathogens. From there it is another task to identify what the substance is, and what sequence of DNA is responsible for its making. This is where a bioinformatical effort would be unreplaceable in the form of a whole genome sequencing (WGS). WGS is a potent tool as we would be able to match our genomic sequences towards all open databases online and check what genes might be encoding for the substance in question, or if it perhaps belongs to a de-novo gene. One of the advantages of using NGS in bacterial genome sequencing for drug discovery is that because of the small genomes that procaryotes have means that many strains can be sequenced per run, making it cost-efficient. The methodology does have some drawbacks, one of them being that short read alignment can result in gaps in coverage owing to repeated sequences (50), as well as the fact that there is currently no way to confirm what de-novo sequence would produce the sequence. A way to get around this would be to use the elimination method to remove the known parts of the sequence and to somehow test the remaining sequence in the laboratory

As mentioned earlier, this project tests the viability of an alternative approach for discovering new antimicrobial compounds. We (the authors of this paper) tested only 13 swabs that we gathered ourselves as an example of how this pipeline will work. The larger scale operation happening simultaneously will fulfill the true potential of this drug-discovery pipeline. It is recorded in literature how an overweight of antibiotics are discovered from microbes in nature. All the swabs from this project were from the city of Liverpool and its infrastructure. This shows the potential for this pipeline, finding antimicrobial substances no matter where the microbes are caught. To develop an antibiotic with one experiment would have been a sensation in all possible ways, but there is no doubt that the study has demonstrated a method to screen for microbes around us which produces antimicrobial compounds in one form or another.

5. Conclusion

This project has tested a pipeline for discovery of novel antimicrobial compounds. Based on the result from thirteen samples, the pipeline seems promising. It is cost-effective, has unlimited potential to detect new antimicrobial compounds and can engage the public. Nevertheless, more research and more resources are needed to investigate the full potential of the method.

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