PhD Chinese Science Council Scholarship project

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Research Proposal

Title

Synergy between novel antimicrobial peptides and conventional antibiotics to eradicate bacterial persisters

Summary

Antimicrobial resistance (AMR) is a major problem raising significant scientific and societal challenges. Not only is genetic resistance a main driver, but bacterial persistence is also crucially contributing to AMR through antimicrobial tolerance development, biofilm derived infections and spore formation. In contrast to the need for genetic mutation as is in antimicrobial resistance development, microbial persistence is based upon the capability of the pathogen to transiently display tolerance to antimicrobial agents through a lowering of their cellular metabolic activity. As conventional antibiotics need active cellular metabolism to be effective, an alternative treatment to eradicate persisters is urgently needed. Antimicrobial peptides (AMPs) have proven to be a promising alternative to antibiotics. Moreover, recent research has provided strong evidence of a positive synergy between AMPs and antibiotics. As synergistic treatments have proven to be effective in the past (e.g. multidrug therapy for tuberculosis), it is feasible to further investigate synergistic treatments in solving the antimicrobial resistance crisis. This study will encompass a microbiological study of medically relevant persisters, their response to promising AMPs, and antibiotics as well as synergistic combinations. Additionally, the research will be focussed on the cellular metabolism of the relevant persisters and how it is affected by the developed treatment protocols. Ultimately, this research will be focussed on the mode of action of the used AMPs and their synergy with antibiotics. Altogether, this study will provide detailed insights into the molecular basis of AMP activity against persister forms of important pathogens, their synergy with antibiotics and will significantly contribute to resolving antimicrobial resistance.

Research field (Research Institute)

Indicate research area(s)

Swammerdam Institute for Life Sciences (Science Faculty)
Amsterdam Infection and Immunity (Academic Medical Centre)

Brief description of research proposal

Introduction

Before the discovery of antibiotics, infectious diseases were the main cause of death. Since the end of World-War II this has changed owing to the advent of antibiotics. However, misuse of antibiotics, as well as their high presence in the environment and the food chain, has led to the emergence of multidrug resistant bacteria [1]. In the present day, antimicrobial resistance (AMR) has reached immense levels to the extent that it is predicted that by 2050 we risk to see more deaths due to AMR than to cancer [2].

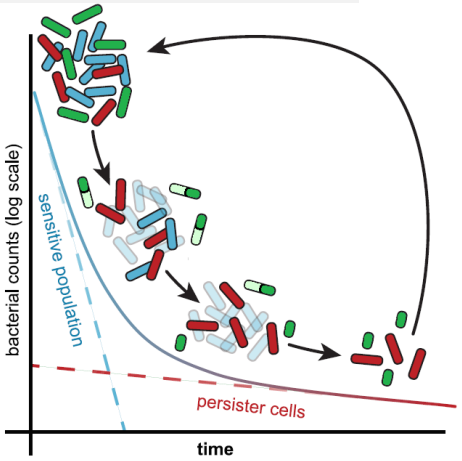

***Figure* 1.** When a lethal dose of a bactericidal antibiotic is added to a population of bacteria it rapidly eradicates the sensitive bulk of the population (blue) (with spore formers represented in Green) until only persister cells (red) and spores (Green) remain. Once the treatment has been completed the original bacterial population can be restored from the remaining persisters.

Genetic mutations have long been thought of as the main driver behind AMR. Nowadays, microbial persisters have also proven to be a crucial contributor to AMR [3]. Microbial persisters are special variants of microbes that have entered a state of dormancy for an extended period of time. This state of dormancy is achieved through a drastic reduction in cellular metabolism and growth rather than through genetic mutations [4]. As antibiotics strongly rely on an active cellular growth and metabolism, they are not active against these persisters [5]. Once the antibiotic treatment is completed the surviving persisters can restore their metabolic activity and reinitiate the infection (*figure* 1). The rate by which these persister form is, however, still largely unknown. Persisters have multiple treatment survival strategies, namely: the development of treatment tolerance (e.g. *Salmonella, E. coli* and *M. tuberculosis*), through biofilm protection (e.g. *P. aeruginosa* and *S. aureus*), by hiding inside host cells (e.g. *Salmonella*) and through spore formation (e.g. *B. cereus* and *C. difficile*) [6]-[9]. A As contemporary antibiotics fail to eradicate these persisters , novel approaches need to be found.

A possible treatment can be the use of antimicrobial peptides (AMPs). AMPs already play an important role in the innate immune system of multicellular organisms where they are capable of eradicating microbes through perturbation of the bacterial cytoplasmic membranes and additionally reacting with intracellular macromolecular components [9]. Recent findings from the department of medical microbiology at the AMC and the department of Molecular biology & microbial food safety at the FNWI have proven two groups of AMPs to be particularly promising; the human platelet thrombocidin derived AMPs and the human cathelicidin LL-37 derived synthetic AMPS. Among the human platelet thrombocidin derived AMPs, TC19 and TC84 were proven to be effective against the model endospore forming bacterium *Bacillus subtilis* [10], [11]. TC19 also showed positive results in eradicating multi-drug resistant *Staphylococcus aureus* [12]. Among the human cathelicidin LL-37 derived synthetic antimicrobial peptides, SAAP-148, SAAP-145 and SAAP-276, were found to be effective against *S. aureus* as well as against a panel of the currently most important multi- and PAN-resistant bacteria [13]–[15]. The effect of the thrombocidin and cathelicidin LL-37 derived AMPs has not yet been tested on separated persister cells and despite the fact that these novel AMPs have promising activity against several tested pathogen populations, any novel type of antimicrobial is in risk of resistance development [13]. Although resistance to AMPs does not readily occur, resistance to AMPs has been described for various pathogens [11]–[13], [16]. Thus, in order to prevent potential resistance development against clinically used AMPs and to prevent further resistance development against the currently used antibiotics a novel treatment strategy has to be found.

One potent treatment strategy, that has proven to be effective in the past, is the use of treatment synergy. For example, a drastic improvement was found when patients suffering from tuberculosis were treated with multiple antibiotics instead of one, bringing the number of patients with resistant tuberculosis down from 70% to almost 0% [17]. Similarly, recent research has shown that combining different AMPs also improves

their effect [18]. Moreover, the effect of LL-37 showed great improvement when tested simultaneously with common antibiotics like teicoplanin and vancomycin [19], [20], [21]. Studying combinational therapy between different AMPs and between AMPs and antibiotics in order to eradicate persisters could therefore be highly promising in resolving AMR.

Research questions / Key objectives

Altogether, this leads to the following research questions/Key objectives:

1. What is the persister development rate under the standardized conditions and which metabolic state characterizes this population?
2. How do antibiotics, AMPs and combinations affect persisters?
3. Does the synergy treatment show similar effects against multidrug resistant microbes whilst not inducing resistance by itself?
4. What is the mode of action of the tested AMPs and synergistic combinations with antibiotics on persister?

Methods/Approach

During this study different persister forms will be investigated, namely:
Antimicrobial tolerant persister cells: *E. coli*
Biofilm related persister: *P. aeruginosa, S. aureus*
Small colony variants: *S. aureus, S. epidermidis*
Spores: *B. cereus*, *C. difficile*

Likewise, different antibiotics and AMPs will be investigated:
Antibiotics: teicoplanin, vancomycin
Thrombocidin derived AMPs: TC19, TC84
Cathelicidin LL37 derived AMPs: SAAP-145, SAAP-148, SAAP-276

For the remainder of the proposal the antimicrobial tolerant cells, biofilm related persisters and small colony variants will be known as **persisters** and the spores as **spores** TC19, TC84, SAAP-145, SAAP-148 and SAAP-276 will be furtherly designated as **AMPs**, teicoplanin and vancomycin will be known as **antibiotics**

What is the persister development rate under the standardized conditions and which metabolic state characterizes this population?
The standardized research condition by which persister cells are formed is through exposing a pre-persister population of the pathogen to 100X the minimal inhibitory and bactericidal concentration (MICs and MBCs) of an antimicrobial compound [22]. The MICs and MBCs for each AMP and antibiotic will, therefore, be determined for the persisters. This can be accomplished through exposing pre-persister populations to different compound concentrations while measuring their growth rates using a continuous recording plate reader. As a control, the growth rates of each pre-persister populations will be determined without any form of treatment. Besides this, the growth processes of the persister populations will be visualized using phase contrast microscopy. The cell conditions will also be determined using a time killing assay were the microbe's ability to form colonies, after exposure to several times the MIC, will be tested. Ultimately, persister populations will be obtained from the pre-persister populations using the standardized (100X MIC/MBC) research condition and be used for further experimentation.

To determine the metabolic state of the obtained persister population, a novel methodology has to be developed, as none is available at the moment. This can only be realized for the metabolically still active persister populations since spores, the "metabolically inactive persisters", do not have a measurable basal metabolism. The novel metabolic measuring methodology can be realized by alternately using an Agilent Seahorse XF (collaboration with the laboratory of Metabolic Genetic Disorders of prof. Ron Wanders and

prof. Hans Waterham. AMC). The Seahorse XF is a state of the art technique that is normally used to measure the cellular metabolism of humane cells. Besides measuring the cellular metabolism, the Agilent Seahorse XF also has the capability to add four different compounds during the measurement after which any metabolic alterations can be observed (*figure* 2). This makes the Seahorse XF exceptionally suitable to test the complex cellular metabolism of metabolically active persisters and the effect that relevant antibiotics, AMPs and combinations have on this metabolic state. The use of the Seahorse method has already been proven to be effective for metabolic measurement in *E. coli* [23]. One important aspect of developing this particular Seahorse XF application is the use of a fixed population size, as population size will contribute to the overall measured metabolic state. This can be realized by incorporating the innovative Cytena cell sorter (Cytena; www.cytena.com), available at the department of Medical Microbiology. The Cytena is capable of sorting cells into identical population sizes for further testing and has ,to the best of our knowledge, never been realized before. The Cytena incorporation will greatly improve quantitative analysis of the differential cell populations. Altogether, the use of plate reader tests, time killing assays, phase contrast microscopy, Cytena cell sorting and the Agilent Seahorse XF will provide the answer to how fast persisters form under the standardized conditions and which metabolic state characterizes this population.

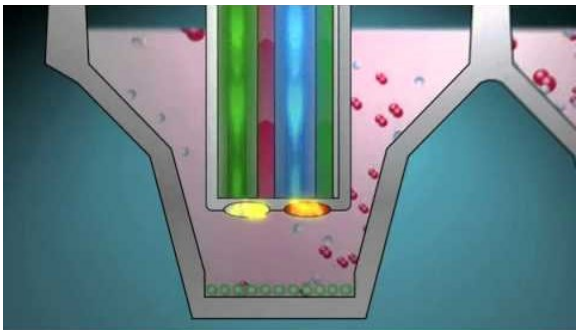

***Figure* 2.** A short movie explaining the workings of the Agilent Seahorse XF. The movie can be activated through clicking on the image while holding down the Ctrl button, or through using this link: https://www.youtube.com/watch?v=5ETh3VBaX3Q&t

What is the effect of the tested antibiotics, AMPs and combinations on persisters?

One major downside of the standardized methodology is the fact that the obtained persister population is undetermined and heterogeneous. To overcome this problem a new method will be developed. In order to effectively separate persister cells from the heterogeneous population obtained under the standardized conditions, the altered metabolism of persister cells will be utilized. Persister formation is controlled by a panel of signalling pathways with (p)ppGpp signalling as an integral part of these pathways [3]. ppGpp levels can be measured using, for instance, the highly selective fluorescent detection methodology composed by Zhang *et al*. [24]. First pre-persister populations of both the metabolically active and inactive persisters will be via the standardized condition (100X MBC/MIC). Subsequently, the surviving cells will be separated from the population using cell death markers and fluorescence-activated cell sorting (FACS). This will sort out the cells sensitive to the compounds. To isolate the persister cells from the remaining unsensitive population, ppGpp labelling and FACS will be used. The FACS will be used to separate the cells regarding their ppGpp label signal. As ppGpp is upregulated in persister cells the cells containing a high ppGpp label signal will be the persister cells. To prove that the obtained persister cells have not acquired any antimicrobial resistance mutations the genome of the persisters cells will be sequenced and compared to the parental strain genome. Using this novel methodology we will be able to effectively separate persister cells from a heterogenous population.

With the persister cells separated from the population, the effect of the relevant antibiotics, AMPs and synergistic combinations can be investigated. To accomplish this, the persister populations will be exposed to different concentrations of the relevant AMPs, antibiotics and combinations. The effect of these compounds will be measures by using plate reader tests, time killing assays, phase contrast microscopy, Cytena cell sorting and the Agilent Seahorse XF. As a benchmark pre-persister populations of the metabolically active and inactive persisters will be tested identically. Altogether, this will provide the data needed to determine the effect of antibiotics, AMPs, and combinational therapy on persisters. Ultimately these experiments will provide the optimal synergistic treatment plan(s) to eradicate persister cells. If the newly developed methodologies prove to be unsuccessful we can still utilize the same standardized methodologies that we try to improve.

Does the synergy treatment show similar effects against multidrug resistant microbes whilst not inducing resistance by itself?

The established optimal treatment plan(s) will subsequently be tested on bacterial strains that are known for their resistance to contemporary treatments. Furthermore, to investigate whether resistance of the survival forms of the used bacteria against the developed treatment can indeed occur, the tested bacteria will be exposed to sub inhibitory concentrations of the synergistic combinations for up to 20 culture passages or more. These experiments will be performed under a broad range of media and culture conditions, with persister cells as well as bacteria in different growth rates including slow growth. This will include experiments under highly controlled growth conditions in chemostat cultures. To mimic the clinical situation, experiments will also be performed in media containing human plasma, serum or blood proteins such as human serum albumin. These results will provide the decisive evidence that the optimal treatment plan(s) are effective and do not select for resistance. In case these studies will yield bacteria which survive the treatment, it will be vital to analyse their response to the antimicrobials using the high-end methodologies described in methods of the first two research questions/key objectives in this section.

What is the mode of action of the tested AMPs and combinations on persister?

To investigate the mode of action of the tested AMPs, antibiotics, and synergistic combinations, the bacteria will be investigated using state of the art super resolution microscopy, among which the recently developed Rescan Confocal Microscopy (RCM) and the high end Structured Illumination Microscopy (SIM), available at Science Park [24],[25]. Both imaging techniques are suitable to image the mode of action in great detail. In order to do so the bacterial cytoplasmic membrane will be labelled with a spectrum of available dyes used for staining membrane processes and composition, all because the cellular membrane is most likely to be affected by the antibiotics and AMPs [26]–[28]. The stained cells will be exposed to labelled antibiotics, AMPs and combinations after which they will be imaged over time. Different labelling of antibiotics and AMPs will allow spatio-temporal resolution of their interaction with the bacterial cell and membrane at near molecular level. This will provide innovative insights into how the antibiotics and antimicrobial compounds precisely affect the bacterial membrane and how the synergistic treatment differs from non-synergistic treatment.

All methodologies are summarized in *Figure* 3 on the page 7

Innovation

This research will provide innovative insights into the molecular basis of AMP activity and their synergy with antibiotics, aiding in resolving the antimicrobial resistance crisis. During this study important novel techniques will be developed to separate persister cells from a cell population and to map the dynamics and the cellular metabolism of a microbial cell population consisting of a specially fixed population size. This study will be the first to apply these state of the art techniques to develop new synergistic treatment strategies in favour of resolving AMR. Moreover, since this study is focussed on treatment development, application and improvement it is readily translatable to the future clinic.

Relevance for science, technology or society

In the AMR field of research it is becoming more apparent that monotherapies continue to fail in resolving infections. This study is aimed towards overcoming this problem by focussing the research on combinational therapy. Moreover, this study is aimed towards proving its mode of action, providing important **scientific insights** into solving AMR and future microbial challenges. When completed the study will also provide a positive stepping stone for future synergy and AMR research. During the study a good amount of time will be spent on developing and optimizing **novel technology** applicable to successfully separate persister cells, create cell populations of a fixed number of cells and to measure the dynamics and metabolic activity of these populations. By using these novel techniques and combining it with well proven measuring methodologies and state of the art techniques like super resolution microscopy to develop new synergistic

treatments we will make a significant contribution to solving the AMR crisis. Considering biofilm and spore related persister formations are currently causing major clinical and **societal challenges**, studies like these are of great importance. Moreover, this study will provide a significant contribution to the 'One Health' concept (http://onehealth.nl). The One Health concept is a global concept for increasing interdisciplinary collaborations and communications in all aspects of health care for humans, animals and the environment. The synergism achieved will advance health care and accelerate biomedical research discoveries, enhancing public health efficacy, expeditiously expanding the scientific knowledge base, and improving medical education and clinical care [29].

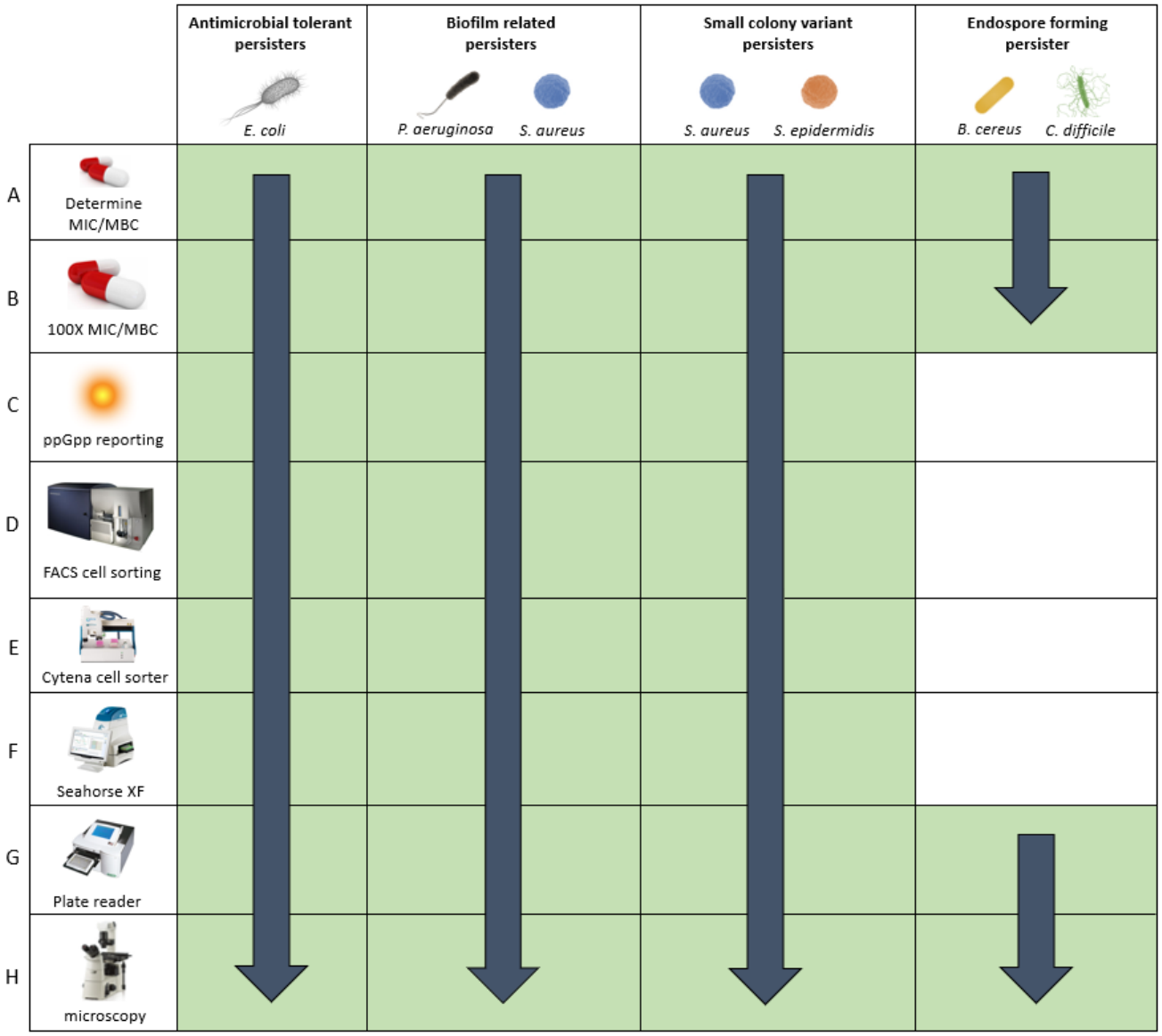

***Figure* 3. Visual summary of the study methodologies.** First, for both the persisters and spores, the MICs and MBCs of the AMPs and antibiotics will be determined (A) after which pre-persister populations will be exposed to 100X the MIC/MBC (B). Of the remainder cells a ppGpp reporter will be applied, spores excluded (C). The persister cells will be isolated using FACS (D). The cellular metabolism of these persisters and how it is affected by the tested antibiotics, AMPs and combinations will be tested using the Seahorse XF (F). The effect of the antibiotics, AMPs and synergistic combinations will be tested for both persister cells and the spores using plate reader tests (G) and phase contrast microscopy (H). Finally, the mode of action will be determined using super resolution microscopy (H).

6f. Literature references

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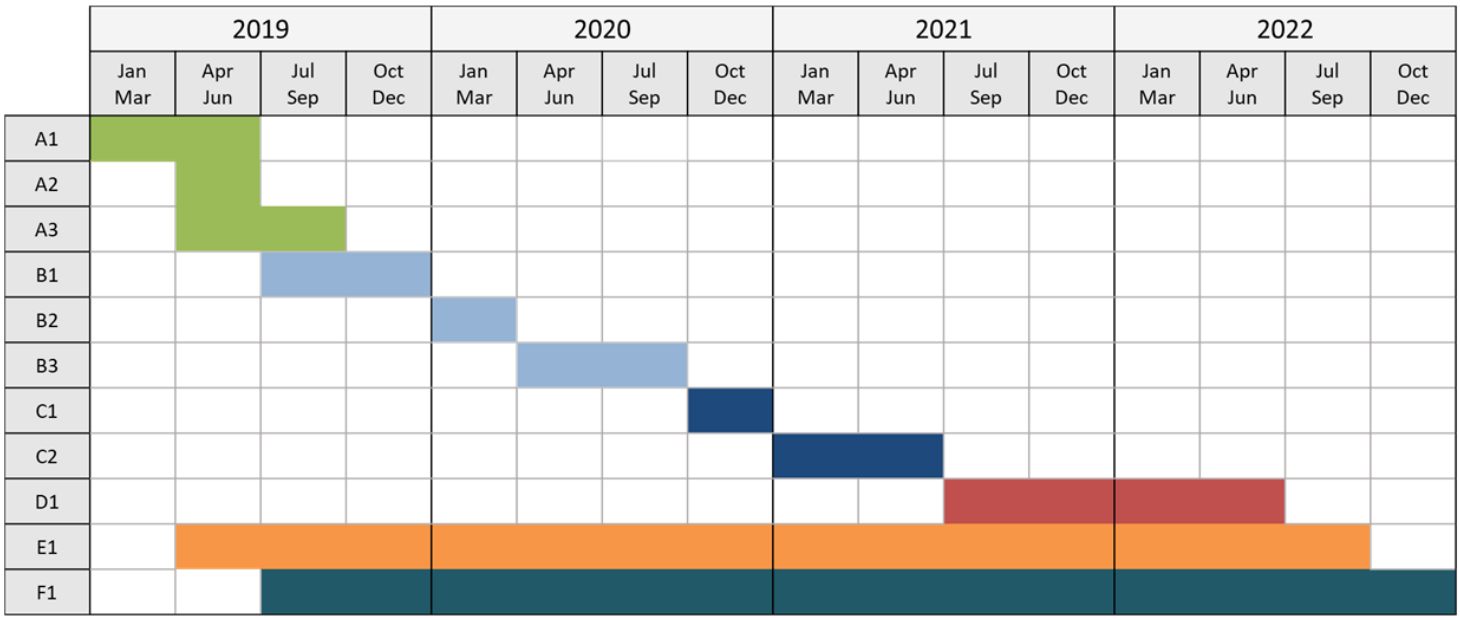
6g. Plan of work (2018-2023)[*]



A1 = Antibiotics, AMPs and synergy MICs, MBCs, Time killing assays and phase contrast microscopy
A2 = Cytena/Seahorse XF combination, adaptation and optimisation
A3 = Determination of pre-persister population dynamics and the effect of the antibiotics, AMPs and combinations.
B1 = Development of persister separation technique
B2 = Persister cell separation and confirmation
B3 = Determination of persister population dynamics and the effect of the antibiotics, AMPs and combinations.
C1 = Effect of synergy treatment on multi-drug resistant bacteria
C2 = Resistance development against synergy treatment
D1 = Mode of action super resolution microscopy
E1 = Data analysis
F1 = Writing