University of Antwerp
LMPH | Laboratory of Microbiology, Parasitology and Hygiene

Faculty of Pharmaceutical, Biomedical and Veterinary Sciences

Department of Pharmaceutical Sciences

Elucidation of the clinical relevance and potential mechanisms of persistence in *Streptococcus pneumoniae*

Opheldering van de klinische relevantie en mogelijke mechanismen van persistentie bij *Streptococcus pneumoniae*

Dissertation submitted in partial fulfilment of the requirements for the degree of doctor of pharmaceutical sciences (University of Antwerp)

To defend by

Nele GEERTS

December 2024

Promotors

Prof. Dr. Paul Cos

Dr. Bram Van den Bergh

Doctoral committee

Prof. Dr. Wim Martinet (chair)

Prof. Dr. Sarah Lebeer

Members of the jury

Prof. Dr. Natalie Lorent

Prof. Dr. Françoise Van Bambeke

Front cover: Nele Geerts

Preface/voorwoord

Dear reader,

Beste lezer,

Before you start reading my thesis, I would like to thank some people who I really appreciated during my PhD.

Eerst en vooral, Paul, bedankt om mij te willen ontvangen op het LMPH en mij de kans te geven om 4 jaar lang aan mijn eigen onderzoek te werken. Bedankt om mij te begeleiden, maar ook om mij de vrijheid te geven om mijn eigen onderzoek te plannen en richting te geven. Natuurlijk ook bedankt om mij de kans te geven om drie fantastische congressen bij te wonen 😊. Bram, ik bewonder jou kennis en nieuwsgierigheid enorm. Ik ben heel dankbaar dat jij mijn promotor was gedurende mijn doctoraat en ik ben ervan overtuigd dat jij mijn onderzoek naar een hoger niveau hebt getild. I would also like to thank Peter, Guy and Alvaro. I would like to thank you for the warm welcome at the LMPH and for your interest into my research.

Dan zou ik graag mijn lieve collega's willen bedanken. Het begon bij jou, Freya. Ik zou jou heel graag willen bedanken om mij te begeleiden in mijn eerste stappen in het onderzoek en mij op weg te helpen met mijn doctoraat. Ik heb veel van jou geleerd en ik heb genoten van onze korte samenwerking. Dan ga ik beginnen bij het bacteriologie team. Tat, jij was een van de eerste mensen die ik ontmoette op het LMPH en die mij op weg hielp bij mijn voorbereiding voor het FWO en bij mijn doctoraat. Ik heb enorm genoten van onze babbels en kon altijd bij jou terecht, bedankt! Laurence, we hebben samen heel wat afgelachen, in het labo en in Hamburg! Ik heb me heel erg geamuseerd met jou en ik hoop echt dat je mijn, ondertussen ons, onderzoek kan verderzetten. Milan, tegen het einde van mijn experimenten was jij het vaakst mijn buur in de lafkast en daar was ik heel blij om! Ik heb ook wat nieuwe muziek geleerd van jou (hopelijk hebben jullie al terug een goedwerkende radio trouwens ;)). Jou enthiousiasme werkt aanstekelijk en heeft mij toch wel geholpen tijdens mijn laatste experimenten. Laurence en Milan, bedankt om mij af en toe eens te laten ventileren bij jullie!! ;) Laure, mijn overbuur op den bureau, bij jou kon ik altijd terecht voor een toffe babbel

over het werk, maar ook over vanalle andere dingen! Ik heb er ook heel erg van genoten om samen te sporten voor of na het werk ☺. Ons avontuur op de Brusselse ring zal ik ook nooit vergeten. Ondanks dat we doornat waren, heb ik toch nog goed gelachen. Linda, het was heel leuk om samen met jou op de bureau te zitten. We hebben goed gelachen samen! Ook bedankt om mij te helpen met mijn doctoraat! I would also like to thank Matthias, Lauren, Natalia and Emine for the nice time we had in the lab.

Dan kom ik bij het virologie team. Eerst en vooral bedankt om mij te verwelkomen op jullie bureau. Lotte, wij zijn samen aan dit avontuur gestart waarvoor ik heel dankbaar ben. Ik kon bij jou altijd terecht voor een goede babbel, over het onderzoek en over mijn persoonlijk leven, maar ook om samen te sporten na het werk. 😊 Kim, ik heb met plezier 3,5 jaar naast jou gezeten op de bureau. We hebben goed gelachen samen, maar ik kon bij jou ook altijd terecht voor statistiek en linux problemen wat mij toch wel hard heeft geholpen! Sofie, jij kon altijd een lach op mijn gezicht brengen met je, soms toch wel chaotische (😉), aanwezigheid. Ik heb ook genoten van onze babbeltjes. Sara, ik ben blij dat ik toch ook een goed half jaar naast jou heb mogen zitten op de bureau. Jou energie werkte aanstekelijk en maakte mij toch altijd nog net iets beter gezind als je aankwam op het werk. 😊 Ik ga jou “in jezelf praten” toch wel missen. Francisco, Noor and Martina, I would like to thank you for the nice conversations we had and to broaden my view on the world.

Het parasitologie team mag ik natuurlijk niet vergeten. Jullie kende ik misschien het minst goed, maar toch kon ik met jullie allemaal goede babbels hebben. Sarah H, jij bent net zoals ik een vroege vogel en dat was ook onze moment om ons hart eens te luchten. Bedankt voor de leuke gesprekken in het myco labo waar jij ook wel eens passeerde! Kayhan, I really appreciated our conversations and we always could have a good laugh together. I really admired your perseverance with the flexiVent (which was necessary to make it work… ;))!

Natascha, bedankt voor jou geduld met al mijn vragen en voor de leuke babbels. Pim, jij bent ook een vroege vogel en dat kon wel eens eindigen in een leuke babbel over verbouwen of planten (hopelijk kan ik ook als ex-collega nog beroep doen op je bloemen-groei kennis ☺). Ook met jou heb ik me goed geamuseerd,

bedankt! An, ik zou je graag willen bedanken met je hulp tijdens mijn doctoraat en voor de babbels aan de lafkast. Daarnaast mag ik Naomi, Margot D., Rik, Margot V. en Mathias zeker niet vergeten. Ik kon bij jullie allemaal terecht met vragen, of gewoon voor een babbeltje, bedank! ☺

Beste collega's, jullie zijn misschien mijn collega's niet meer, maar ik heb er toch wel een paar vrienden aan over gehouden!!

Last but not least zou ik mijn familie willen bedanken. Moeke en vokke, Thijs en Ward, aan jullie mocht ik altijd vertellen over mijn doctoraat (lekker chaotisch als ik ben), ookal verstonden jullie mij niet misschien altijd. Bedankt om naar mij te luisteren en mij te steunen. Dan kom ik bij de laatste persoon die ik graag zou willen bedanken, Jan. Merci. Ik denk niet dat ik hier zonder aan jou begonnen zou zijn. Jij hebt mij het vertrouwen gegeven in mezelf om hieraan te beginnen en om door te zetten, maar ook om nog een extra opleiding te starten. Je hebt altijd geluisterd en kon mijn onderzoek zelfs beter aan anderen uitleggen dan ikzelf ;). We hebben zoveel zotte dingen gedaan tijdens mijn doctoraat. We mochten samen naar Toronto en Kaapstad, we zijn begonnen aan de bouw van ons huis en nog zoveel meer. Jou eeuwige student is jou ook eeuwig dankbaar.

Nele

December 2024

LIST OF ABBREVATIONS

A

AMR	Antimicrobial resistance

B

BA	Blood agar plates
BHI	Brain-Heart Infusion broth

C

CAP	Community-acquired pneumoniae
CbpA	Choline binding protein A
CDC	Centers for Disease Control and Prevention
ChoP	Phosphorylcholine
CLSI	Clinical & Laboratory Standards Institute
CPS	Capsular polysaccharide
cps	Capsular polysaccharide synthesis genes
CSP	Cerebrospinal fluid
CSP-1	Competence-stimulating peptide 1

F

FOB	Functional observational battery

G

GPSCs	Global Pneumococcal Sequence Clusters
GWAS	Genome-wide association studies

H

HGT	Horizontal Gene Transfer
H_2O_2	Hydrogen peroxide

I

IPD	Invasive pneumococcal disease

K

KO	Knockout mutants

L

LytA	Autolysin
LRT	Lower respiratory tract
LB	Luria-Bertani broth

M

MRSA	Methicillin-resistant *Staphylococcus aureus*
MIC	Minimum inhibitory concentration
MHL	Mueller-Hinton broth (cation-adjusted) + 5% lysed horse blood
MLST	Multilocus sequencing typing
MOA	Mechanism-of-action

N

NA	Not available
NanA	Neuraminidase A
NGS	Next-Generation Sequencing
NSAID	Nonsteroidal anti-inflammatory drug

O

OM	Otitis Media

P

PAFR	Platelet activating factor receptor
PavA	Adherence and virulence protein A
PavB	Adherence and virulence protein B
PBS	Phosphate-buffered saline
PBP	Penicillin binding protein
PCV	Pneumococcal conjugate vaccine
PIGR	Polymeric immunoglobulin receptor
Ply	Pneumolysin
PspA	Pneumococcal surface protein A

Q

qPCR	quantitative PCR

S

SNP	Single nucleotide polymorphism
SpxB	Pyruvate oxidase
SREF	Stimulated Raman excited fluorescence

T

TA	Toxin-antitoxin
THY	Todd-Hewitt broth + with 0.5% yeast extract

TSA	Tryptic soy agar

U

URT	Upper respiratory tract

V

VBNC	Viable but nonculturable
VPC	Viable plate count

W

WT	Wild-type
WHO	Word Health Organization

SUMMARY

Infectious diseases exist already for millennia and so does antimicrobial therapy. The past 100 years, major advances were accomplished into the fight against infectious diseases, especially with the discovery of antimicrobial agents. Unfortunately, these discoveries were accompanied with the fast emergence of antimicrobial resistance. Many efforts were made regarding research into antimicrobial resistance, but bacteria can evade antibiotic treatment also in different ways resulting in therapy failure. One way of how bacteria can evade killing by antibiotics, regardless of resistance, is antibiotic persistence. Persister cells are phenotypic variants that exist as a subpopulation within a clonal culture and can transiently switch to a nongrowing state that enables them to survive treatment with a bactericidal drug concentration. Being tolerant to lethal antibiotics, they underly the chronic nature of a variety of infections and even help in acquiring genetic resistance.

Streptococcus pneumoniae is a common colonizer of the mucosal surfaces of the nasopharynx of children, but can also cause life-threating diseases like pneumonia, sepsis and meningitis. *S. pneumoniae* remains a major cause of morbidity and mortality worldwide, despite the availability of a vaccine, and is classified as a priority pathogen by the World Health Organization (WHO) in 2024 for the need of novel antibiotics. Many reports were published about antibiotic persistence in a variety of bacterial species, but for *Streptococcus pneumoniae*, no studies were published. As studies in other bacterial species underscore the importance of persister cells, it is of utmost importance to gain knowledge about *S. pneumoniae* persistence in both acute and chronic pneumococcal infections in order to gain a better understanding of how *S. pneumoniae* evades elimination by antibiotic treatment. We therefore focused on antibiotic persistence in *S. pneumoniae* in this PhD dissertation.

S. pneumoniae has a fastidious and self-limiting nature that results in a limited *in vitro* survival after entering the stationary phase. This self-limitation could explain the lack of reports on antibiotic persistence in *S. pneumoniae.* To study persistence, prolonged antibiotic-induced killing studies are usually employed and therefore any confounding effects of mortality through the self-limiting nature of *S. pneumoniae* is best to

be avoided. We assessed different approaches to circumvent this self-limitation and were able to set-up a long-living *in vitro* model based on a specific liquid growth medium which enabled us to execute prolonged time-killing experiments to study antibiotic persistence. Using these killing studies together with heritability assays, the gold standard assays to determine persistence, we proved the presence of high numbers of genuine persister cells in cultures of a *S. pneumoniae* reference strain.

After we provided the first proof of the presence of persister cells in *S. pneumoniae* cultures, we wanted to investigate potential molecular mechanisms underlying persisters. To study persister mechanisms, we set out experimental evolution experiments with the goal to evolve *S. pneumoniae* cultures to highly persistent populations followed by genetic analysis. Despite sampling a large number of mutations and the ability of *S. pneumoniae* to evolve under experimental conditions, we did not succeed in evolving *S. pneumoniae* strains under our experimental set-up.

Finally, we wondered what the clinical relevance of persisters is for *S. pneumoniae* infections, considering their presence in a *S. pneumoniae* reference strain, but the absence of evolution towards high persistent mutants. We therefore looked for the presence of persistence in a large set of clinical strains (647 strains) and we made the first steps towards the study of *S. pneumoniae* persisters in an *in vivo* mouse model. We explored antibiotic resistance and persistence in our diverse collection with strains expressing different serotypes and originating from different types of infection or from carriage. We found resistance rates of about 8-20 % towards β-lactam antibiotics and no resistant strains were present for the antibiotics moxifloxacin and vancomycin. Persister cells were widely present and highly diverse among our collection of clinical strains, for both antibiotics and for the different types of diseases, which gives an indication of the clinical importance of persistence in *S. pneumoniae* infections. To further explore the clinical relevance of persisters during *S. pneumoniae* infections, we initiated the development of a mouse model to study antibiotic persistence *in vivo*. We studied two models, a lung infection and nasopharyngeal carriage model, with the goal to obtain a chronic *in vivo* model. Finally, we explored *in vivo* persistence using the nasopharyngeal carriage model, but further optimization is required.

We were the first to report the presence of persisters in *S. pneumoniae* cultures, both for a reference strain and a large set of clinical isolates, for different clinically relevant, bactericidal antibiotics (amoxicillin, cefuroxime, moxifloxacin and vancomycin) using our optimized long-living *in vitro* model. Persister cells were widely present and highly diverse among our strain collection. The absence of evolution to a highly persistent phenotype hampered us to investigate the molecular mechanisms behind *S. pneumoniae* persistence. Finally, we made the first steps for the optimization of a long-term *in vivo* model to enable persistence studies in a clinically relevant setting. Altogether, our work advocates for higher interest for persistence in *S. pneumoniae* as a contributing factor for therapy failure and resistance development.

SAMENVATTING

Infectieziekten bestaan al millennia lang, net zoals antimicrobiële therapie. De afgelopen 100 jaar zijn er grote vooruitgangen geboekt in de strijd tegen infectieziekten, vooral met de ontdekking van antimicrobiële middelen. Helaas gingen deze ontdekkingen gepaard met de snelle opkomst van antimicrobiële resistentie. Er zijn veel inspanningen geleverd op het gebied van onderzoek naar antimicrobiële resistentie, maar bacteriën kunnen ook op andere manieren een antibioticumbehandeling omzeilen wat kan resulteren in therapiefalen. Eén manier waarop bacteriën aan afdoding door antibiotica kunnen ontsnappen, ongeacht resistentie, is antibioticum persistentie. Persister cellen zijn fenotypische varianten die als een subpopulatie binnen een klonale cultuur bestaan en tijdelijk kunnen overschakelen naar een niet-groeiende vorm, waardoor ze een behandeling met een bacteriedodende antibioticumconcentratie kunnen overleven. Door hun tolerantie voor antibiotica liggen ze ten grondslag aan de chronische aard van verschillende infecties en dragen ze zelfs bij aan het verwerven van genetische resistentie.

Streptococcus pneumoniae komt frequent voor als commensale bacterie t.h.v. de slijmvliezen van de neuskeelholte bij kinderen, maar kan ook levensbedreigende infecties veroorzaken zoals een longontsteking, sepsis en meningitis. *S. pneumoniae* blijft wereldwijd een belangrijke oorzaak van morbiditeit en mortaliteit, ondanks de beschikbaarheid van een vaccin, en is door de Wereldgezondheidsorganisatie (WHO) in 2024 geclassificeerd als een prioritair pathogeen voor de nood aan nieuwe antibiotica. Er zijn veel rapporten gepubliceerd over antibioticum persistentie bij verschillende bacteriesoorten, maar voor *Streptococcus pneumoniae* zijn er geen studies gepubliceerd. Aangezien studies bij andere bacteriesoorten het belang van persister cellen benadrukken, is het belangrijk om kennis te vergaren over *S. pneumoniae* persistentie bij zowel acute als chronische pneumokokkeninfecties om beter te begrijpen hoe *S. pneumoniae* eliminatie door antibiotica omzeilt. Daarom richtten we ons in dit proefschrift op antibioticum persistentie bij *S. pneumoniae*.

S. pneumoniae is een kieskeurige en zelfbeperkende bacterie van natuur wat resulteert in een beperkte *in vitro* overleving na het bereiken van de stationaire fase. Deze zelfbeperking zou het gebrek aan rapporten

over antibioticum persistentie bij *S. pneumoniae* kunnen verklaren. Om persistentie te bestuderen, worden doorgaans langdurige antibioticum-geïnduceerde afdodingsexperimenten uitgevoerd en moeten de effecten van mortaliteit door de zelfbeperkende aard *van S. pneumoniae* vermeden worden. We hebben verschillende benaderingen beoordeeld om deze zelfbeperking te omzeilen en waren in staat om een langdurig *in vitro* model op te zetten op basis van een specifiek groeimedium waarmee we langdurige afdodingsexperimenten konden uitvoeren om antibioticum persistentie te bestuderen. Met behulp van deze afdodingsstudies gecombineerd met erfelijkheidsexperimenten, de gouden standaardtests om persistentie te bepalen, hebben we de aanwezigheid van hoge aantallen persister cellen aangetoond voor een *S. pneumoniae* referentiestam.

Nadat we het eerste bewijs hadden geleverd voor de aanwezigheid van persister cellen in *S. pneumoniae* culturen wilden we potentiële moleculaire mechanismen achter persistentie onderzoeken. Om persister mechanismen te bestuderen, hebben we experimentele evolutie-experimenten opgezet met als doel *S. pneumoniae* te laten evolueren naar hoog persistente populaties gevolgd door genetische analyse. Ondanks dat we een groot aantal mutaties hebben gesampled en dat *S. pneumoniae* in staat is om te evolueren onder experimentele omstandigheden, zijn we er niet in geslaagd om *S. pneumoniae* te laten evolueren.

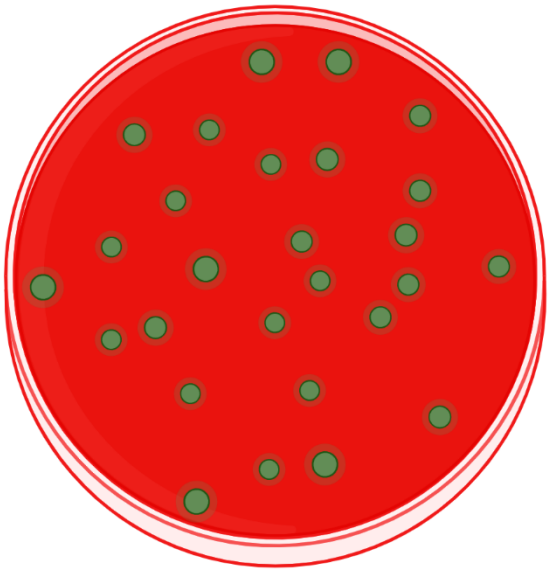
Ten slotte vroegen we ons af wat de klinische relevantie is van persisters bij *S. pneumoniae* infecties, gezien hun aanwezigheid in een *S. pneumoniae* referentiestam, maar het ontbreken van evolutie naar hoog persistente mutanten. We hebben daarom gezocht naar de aanwezigheid van persistentie in een grote set klinische stammen (647 stammen) en we hebben de eerste stappen gezet naar het bestuderen van *S. pneumoniae* persisters in een *in vivo* muismodel. We hebben antibioticumresistentie en persistentie onderzocht in onze diverse collectie met stammen van verschillende serotypes afkomstig van verschillende soorten infecties of uit dragerschap. We vonden resistentiecijfers van ongeveer 8-20% tegen β-lactam antibiotica en er waren geen resistente stammen aanwezig voor de antibiotica moxifloxacine en vancomycine. Persister cellen waren wijdverspreid aanwezig en zeer divers in onze collectie klinische stammen, voor de verschillende antibiotica en voor de verschillende ziektebeelden, wat een indicatie geeft

van het klinische belang van persistentie bij *S. pneumoniae* infecties. Om de klinische relevantie van persisters tijdens *S. pneumoniae* infecties verder te verkennen, hebben we de ontwikkeling van een muismodel opgestart om antibioticum persistentie ook *in vivo* te bestuderen. We hebben twee modellen bestudeerd, een longinfectie- en een dragerschapsmodel in de neuskeelholte, met als doel een chronisch *in vivo* model te bekomen. Ten slotte hebben we *in vivo* persistentie onderzocht met behulp van het dragerschapsmodel, maar verdere optimalisatie is vereist.

We waren de eersten die de aanwezigheid van persisters rapporteerden in *S. pneumoniae*, zowel voor een referentiestam als voor een grote collectie klinische stammen, voor verschillende klinisch relevante bactericide antibiotica (amoxicilline, cefuroxim, moxifloxacine en vancomycine) met behulp van ons geoptimaliseerd *in vitro* model. Persister cellen waren wijdverspreid aanwezig en zeer divers in onze collectie klinische isolaten. Het ontbreken van evolutie naar een hoog persistent fenotype belemmerde ons bij het onderzoeken van de moleculaire mechanismen onderliggend aan *S. pneumoniae* persistentie. Ten slotte hebben we de eerste stappen gezet voor de optimalisatie van een chronisch *in vivo* model om persistentiestudies mogelijk te maken in een klinisch relevante setting. Al met al pleit ons werk voor een hogere interesse in persistentie bij *S. pneumoniae* als bijdragende factor aan therapie falen en resistentieontwikkeling

TABLE OF CONTENTS

1 INTRODUCTION



1.1 Streptococcus pneumoniae

1.1.1 A little bit of history

Streptococcus pneumoniae was discovered in the late 19th century simultaneously by George M. Sternberg (North America) (1) and Louis Pasteur (France) (2,3). Both Sternberg and Pasteur discovered *S. pneumoniae* accidentally by inoculating rabbits with saliva, which led to the unexpected death of these animals through septicemia (1,2). Pasteur already noticed the presence of a surrounding halo, today identified as the polysaccharide capsule, upon microscopic examination of *S. pneumoniae* (2–5). Both researchers experienced difficulties growing and isolating the agent that caused the rabbit's death, as the inoculum only grew in rabbit flesh bouillon after amplification of the inoculum in rabbit's blood (2,3,6). Finally, in 1883 a French physician (Charles Talamon) was the first to succeed in isolating a pure culture of *S. pneumoniae*, or *Micrococcus lanceolatus,* as it was called by William H. Welch in 1892 (3,7). Ever since, *S. pneumoniae* has been recognized as a major pathogen, which was demonstrated by the influenza pandemic during World War I where most deaths were most likely the result of a secondary bacterial pneumonia (8).

Even to date, *S. pneumoniae* is still known as a major pathogen that is fastidious and difficult to grow *in vitro*. It's a Gram-positive, facultative anaerobic bacterium that does not produce catalase (9). *S. pneumoniae* is α-hemolytic and can be identified on blood agar (BA) by the formation of a green halo, which is produced through the oxidation of oxy-hemoglobin to met-hemoglobin (non-oxygen binding) by the production of hydrogen peroxide (H_2O_2) (9,10). A following step in the identification of *S. pneumoniae* is the determination of its serotype (4,11,12). Currently, at least 100 different serotypes are identified (13) that have structurally and serologically different capsular polysaccharides (4). The polysaccharide capsule (CPS) is a major virulence factor that is very important during *S. pneumoniae* pathogenesis by shielding the bacterium from the human host defenses, more specifically from phagocytosis, and is a thus major target for vaccine development (4,11,12). CPS can be produced via two different mechanisms, either synthase-dependent that results in homopolymers that are non-covalently linked to the peptidoglycan layer or Wzy-dependent via UDP-glycosyltransferases that results in a covalently linked capsule of heteropolymers. All *S.*

pneumoniae serotypes, except serotypes 3 and 37, produce their capsule via the Wzy-dependent pathway (14,15). Next to the distinct pathway of capsule production, serotype 3 has a thick capsule with a mucoid morphology and was associated with a higher morbidity than other serotypes (16,17). Unencapsulated *S. pneumoniae* strains on the other hand will more likely only colonize the nasopharynx or cause localized infections, which stresses the importance of the capsule in *S. pneumoniae* virulence (16).

S. pneumoniae is an important, mainly human pathogen causing invasive and non-invasive diseases including meningitis, bacteremia, pneumonia, and acute otitis media. (18,19). Besides, *S. pneumoniae* is a common colonizer of the mucosal surfaces of the nasopharynx of children which also renders it the main reservoir of *S. pneumoniae* (20). *S. pneumoniae* is an opportunistic pathogen which means that it normally does not cause infection in healthy adults. Groups that are at risk for *S. pneumoniae* infection are children, elderly and immunocompromised people (21,22). Risk factors specifically for pneumococcal pneumonia in adults include the presence of chronic pulmonary disease (23,24). Yearly, 2 million people in the United States suffer from pneumococcal infections, resulting in $4 billion in costs (25). It is estimated that 900,000 of these infections are caused by drug-resistant strains (25). Moreover, about 600,000 people died worldwide in 2019 from an *S. pneumoniae* infection related to antibiotic resistance, either directly attributable to the occurrence of a resistant pathogen resulting in therapy failure or associated to a resistant infection without the direct linkage with therapy failure (**Figure 1.1.1**) (26). Therefore, Murray *et al.* (2022) placed *S. pneumoniae* as the 4th most important pathogen on their list and the Centers for Disease Control and Prevention (CDC) considers *S. pneumoniae* a serious threat (25,27).

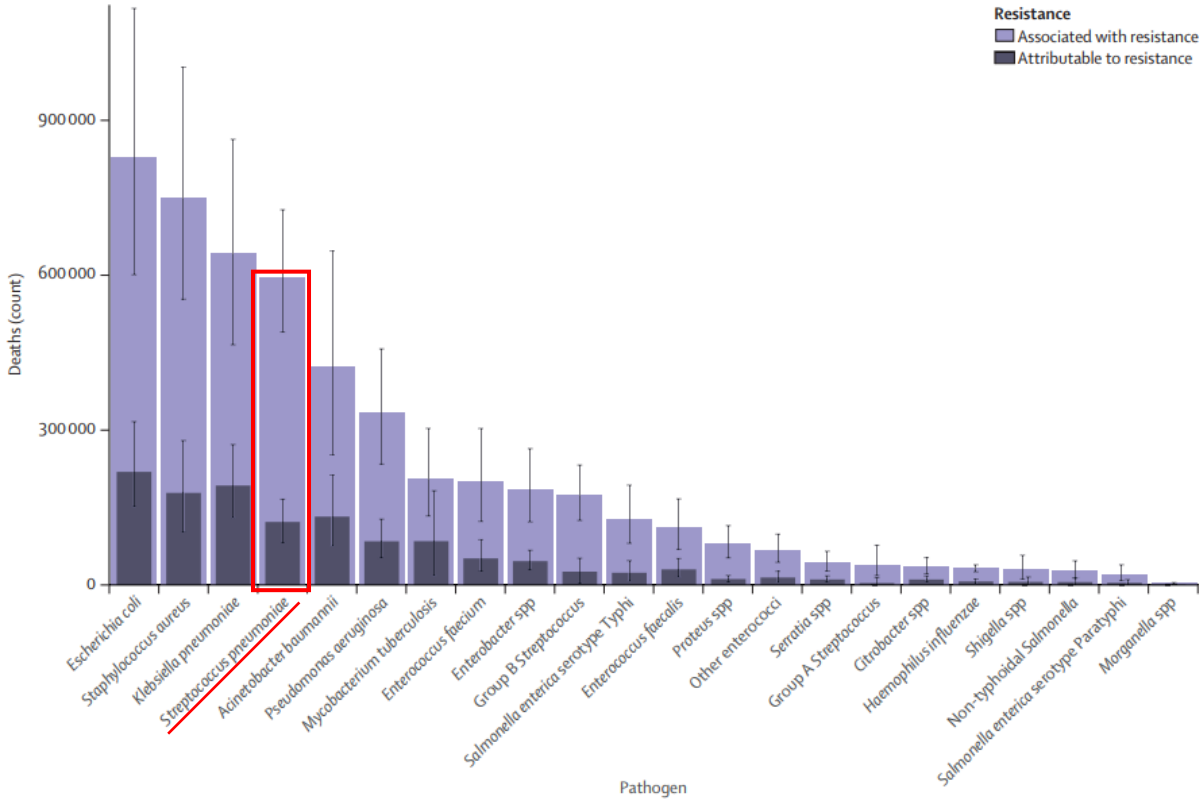


Figure 1.1.1: Global death counts related to antimicrobial resistance for different pathogens in 2019. Globally, about 600,000 people died from an infection from *S. pneumoniae* (highlighted in red), associated or attributed to antimicrobial resistance. Figure is adapted from Murray *et al.* (2022) (26).

1.1.2 The genome of *Streptococcus pneumoniae*

The genome of *S. pneumoniae* is approximately 2 Mb long, encodes for about 2200 coding regions and has an average GC content of 39.6% (28,29). *S. pneumoniae* has a small genome compared to other bacteria, such as *E. coli* with a genome of 4.6 Mb that encodes for about 4500 genes or *P. aeruginosa* with a genome of 6.6 Mb that encodes for about 6200 genes (30–33). The first complete genome of a serotype 4 reference strain, TIGR4, was published in 2001 by Tettelin *et al.* (2001) (34). Since the introduction of next-generation sequencing (NGS) with relatively low costs and a high-throughput set-up, more than 20,000 *S. pneumoniae* genomes were unraveled of which only 14 were closed (28,29).

The pan-genome, also known as the supragenome, is the total repertoire of genes of a phylogenetic clade. The pan-genome can be split into the core genome, the accessory genome and strain-specific genes. The core genome is a set of genes that is shared by all strains in the clade and the accessory genome, also called

the dispensable genome, includes genes that are shared by only a subset of the strains (29,35,36). The pangenome of *S. pneumoniae* is estimated to include at least 3500 genes with a core genome of about 1650 genes (37–39). *S. pneumoniae* has an open pan-genome which means that the pan-genome size increases indefinitely when new genomes are added. Nonetheless, as a newly sequenced *S. pneumoniae* population derives from a common ancestor, the new genomic sequences will provide less new genes (36,38,40). Also *E. coli* and *P. aeruginosa* have open pangenomes (32,33,41,42). Clinical interventions, such as vaccination and antibiotic treatment, are two important examples of drivers of the evolution of the *S. pneumoniae* genome through selective pressure (29,37). The crucial mechanism that *S. pneumoniae* uses to acquire new traits, and thus for its genetic plasticity, is natural transformation (29,37,39). Natural transformation is the ability of bacteria to take up DNA from the environment followed by the incorporation of this DNA into their own genome via homologous recombination (28,29,37,38). Chewapreecha *et al.* (2014) showed that genetic plasticity via natural transformation had a major contribution to the spread of β-lactam resistance within the *S. pneumoniae* population in a refugee camp (43). Different studies have also indicated the important role of natural transformation in serotype replacement after the introduction of serotype-based vaccines (44–46). Another important way for *S. pneumoniae* to acquire new traits is genetic exchange between related species, especially with *S. mitis, S. pseudopneumoniae, S. oralis* and *S. infantis*, that are co-colonizing the upper respiratory tract (URT) (29,37).

Multilocus sequencing typing (MLST) was widely used for the evaluation of evolution of bacterial clones and to characterize strains molecularly (47). The principle of MLST relies on sequencing different housekeeping genes (*aroE, gdh, gki, recP, spi, xpt* and *ddl* for *S. pneumoniae*) across the genome of each clinical isolate in order to classify the isolates into different sequence types (ST) based on the combination of alleles (48,49). MLST was a useful tool to study the population structure of bacteria and to get a look into the evolutionary history (48,49). However, *S. pneumoniae* strains allocated to the same ST do not always share the same serotype, which presumes that MLST may be not able to discriminate closely related strains with a high degree of variability (genetically or phenotypically) (47). Therefore, Crisafulli *et al.* (2012) extended the number of sequenced genomic loci from 7 housekeeping genes to 96 loci within core genes (96-MLST) to

get a better look into the genetic diversity within MLST lineages (47). More recently another approach gained more interest than MLST, Gladstone *et al.* (2019) used whole-genome sequencing of over 20,000 isolates worldwide to look into *S. pneumoniae*'s population structure to cluster them into 621 Global Pneumococcal Sequence Clusters (GPSCs) by genome-wide variation (50). Their goal was to enable investigation of distribution of antibiotic resistance, serotypes and invasiveness across different pneumococcal lineages (50).

1.1.3 The self-limiting nature of *Streptococcus pneumoniae*

S. pneumoniae is a fastidious and fragile bacterium that needs particular nutrient and environmental requirements to grow in the lab (51–53). Together with its self-limiting nature, which is explained more in detail below, it makes *S. pneumoniae* a pathogen that is difficult to culture *in vitro* (51–53). Self-limitation is not restricted to *S. pneumoniae* and is observed for a variety of bacterial species with different functions *in vivo*. Self-limitation has for example a role in biofilm formation for *Staphylococcus aureus* or for sporulation of *Bacillus subtilis* (54–56). *In vitro* self-limitation raises problems in the clinical practice, but also in the context of research. Culture-based methods requiring growth on agar plates are still the golden standard in the routine clinical practice, but recovery of *S. pneumoniae* from respiratory samples can be difficult because of its self-limiting nature and strict nutrient requirements. Therefore, correct preservation and transportation of the samples is important in order to trace *S. pneumoniae* as causative agent. qPCR is an alternative method for detection *of S. pneumoniae* that does not require viable bacteria (57,58). In research, the fastidious and self-limiting nature of *S. pneumoniae* results in a limited survival of *in vitro* cultures and therefore complicate practical experimentation (**Figure 1.1.2**).

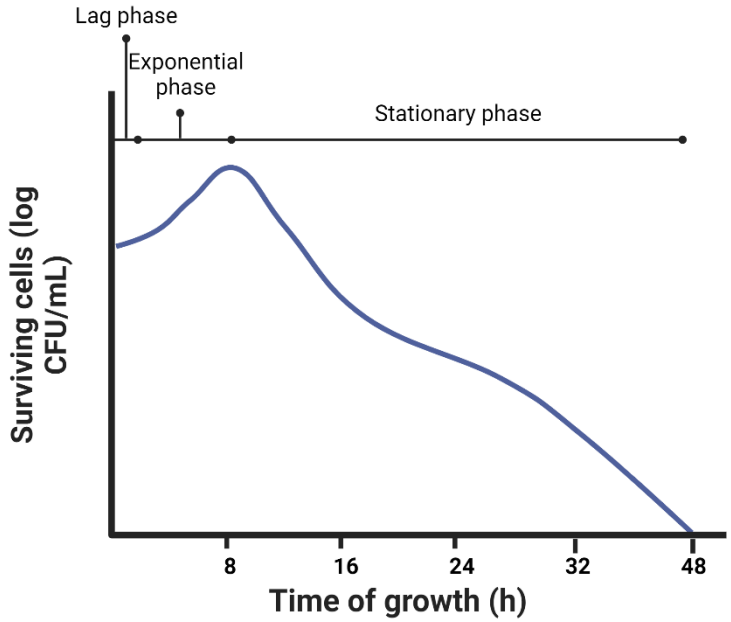


Figure 1.1.2: **The self-limiting *in vitro* nature of *Streptococcus pneumoniae*.** *S. pneumoniae* growth starts with a short lag phase followed by exponential growth to 8 hours. After entering the stationary phase, a fast-killing phase is observed with no survivors after 48h.

Several mechanisms are suggested to contribute to the limited *in vitro* survival of *S. pneumoniae*. The first potential cause is linked to pyruvate oxidase (SpxB), an enzyme of the aerobic metabolism of *S. pneumoniae* (51,52,59). SpxB catalyzes the reaction in which pyruvate is converted to the phosphoryl donor acetyl phosphate with the release of CO_2 and H_2O_2 as by-products. As *S. pneumoniae* lacks the enzyme catalase which has a major role in eliminating H_2O_2 (**Figure 1.1.3**) (51,52,60), the *in vitro* accumulation of H_2O_2 could finally lead to self-killing of *S. pneumoniae* (61). Mutant strains lacking pyruvate oxidase produce 90% less H_2O_2 compared to the wild type which could be a potential strategy to prolong stationary phase survival (51,52,59,61). In the nasopharynx of human, the endogenously produced H_2O_2 does not accumulate, but diffuses into the extracellular milieu of *S. pneumoniae* where it can kill other colonizing bacteria like *H. influenzae* and *S. aureus*, thereby promoting *S. pneumoniae* colonization (52,59,62). Moreover, exposure of epithelial alveolar cells to H_2O_2 induces double-strand breaks in their DNA followed by apoptosis (63). Next to the *in vivo* advantages of the production of H_2O_2 for colonization and invasion, H_2O_2 also contributes to pneumococcal virulence by promoting the release of pneumolysin, a pore-forming toxin that causes damage to lung epithelial cells (52,60). As opposed to the *in vivo* advantages of the production of H_2O_2, the *in vitro*

accumulation is thought to contribute to self-killing of *S. pneumoniae* in high density cultures and mature colonies despite the plethora of detoxification mechanisms against (other than catalase) oxidative stress (52,60,61). Different approaches were described to interfere with SpxB (**Figure 1.1.3)**. First, *S. pneumoniae* is a catalase-negative bacterium, thus catalase supplementation could help in neutralizing the produced H_2O_2 (61). Another suggested way to counteract SpxB is metabolically by growing *S. pneumoniae* under anaerobic conditions, because SpxB needs oxygen for its function (61). Finally, the construction of a *spxB* mutant results in a significant reduction in produced H_2O_2 concentrations and could result in longer stationary phase survival (61,64).

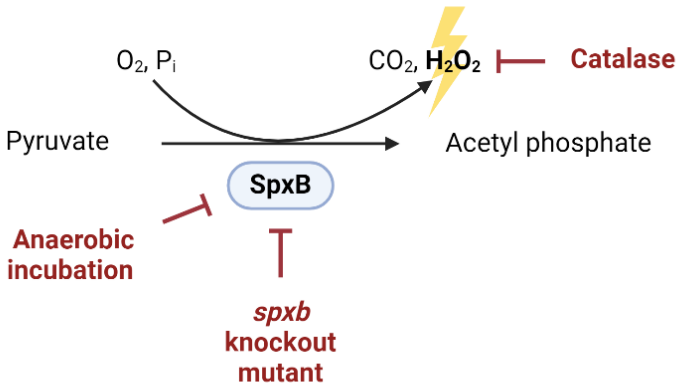


Figure 1.1.3: **Pyruvate oxidase (SpxB) is an enzyme of the aerobic metabolism of *S. pneumoniae* and catalyzes the reaction in which pyruvate is converted to the phosphoryl donor acetyl phosphate with the release of CO_2 and H_2O_2 as by-products** (51,52,59)**.** The function of SpxB can be counteracted by adding catalase to neutralize the produced H_2O_2, by constructing a *spxB* knockout mutant to inhibit the expression of pyruvate oxidase, or by applying anaerobic incubation to inhibit the pneumococcal aerobic metabolism and thus the production of H_2O_2 by pyruvate oxidase (61,64).

Another potential mechanism that could contribute to the self-limiting nature of *S. pneumoniae* is autolysis. LytA, also called N-acetylmuramoyl-l-alanine amidase, is the major autolysin of *S. pneumoniae*. It consists of two domains, an N-terminal amidase domain and a C- terminal choline binding domain (65–68). LytA binds to the cell wall via the phosphocholine residues on the teichoic acids via its choline binding domain (65,69). When attached to the cell wall, LytA induces hydrolysis of the bacterial cell wall by its amidase domain that cleaves the lactyl-amide bond that links the glycan strands with the stem peptides of the peptidoglycan (**Figure 1.1.4**) (65). LytA has a role in pneumococcal growth and division, and contributes to pneumococcal virulence, presumably via its contribution to the release of pneumolysin and inflammatory cell wall

degradation products (65,67,70). LytA is suggested to contribute to cell lysis upon induction of growth arrest, during stationary phase or by antibiotics targeting the cell wall (for example amoxicillin and vancomycin) (65,68). Besides, *S. pneumoniae* can be present in a biofilm in the human nasopharynx where autolysis contributes to, next to natural competence, increasing the genetic diversity of the populations through the release of DNA from lysed cells (55,71). Different research groups, for example Mellroth *et al.* (2012), Martner *et al.* (2009) and Giudicelli *et al.* (1984), indicated already that the function of LytA could be countered by applying an excess of choline chloride in the growth medium to prevent LytA from binding to the choline residues on the cell wall or by constructing a *lytA* knockout mutant (**Figure 1.1.4**) (65,67,69).

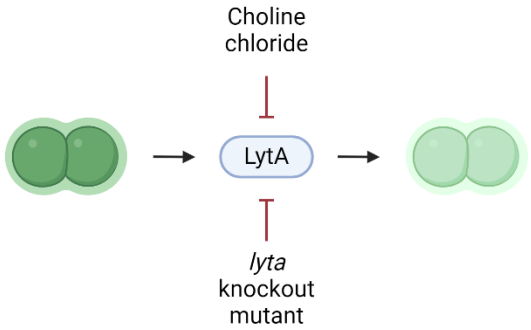


Figure 1.1.4: **LytA, also called N-acetylmuramoyl-l-alanine amidase, is the major autolysin of *S. pneumoniae*.** The function of LytA can be countered by applying an excess of choline chloride in the growth medium or by constructing a *lytA* knockout mutant (65,67,69).

For the research that will be performed in **CHAPTER 4**, it is important that the self-limiting *in vitro* nature of *S. pneumoniae* is avoided. In **CHAPTER 3**, the different strategies that are proposed above to counteract SpxB and LytA will be tested to obtain a long-living *in vitro* model.

1.1.4 Pathogenesis

S. pneumoniae is a mainly airborne pathogen and will transmit from a carrier to a new host via close contact (person-to-person contact or via contaminated surfaces) or via droplet transmission (**Figure 1.1.6**). Transmission is higher in case of a viral infection of the URT, close contact and among young children (20,72,73). Viral infection, for example with influenza, contributes to *S. pneumoniae* transmission by the induction of inflammation in recipients that render them more susceptible to infection and by increasing the nutrient availability resulting in the proliferation of colonizing *S. pneumoniae*. The combination of the increased *S. pneumoniae* density with increased nasal secretions during influenza results in enhanced shedding of *S. pneumoniae* (72). Once *S. pneumoniae* enters a new host, it must colonize the host, as pneumococcal disease is almost always preceded by a period of colonization in the nasopharynx (**Figure 1.1.6**). *S. pneumoniae* is a common colonizer of the URT, with a higher prevalence in children (20-50%) compared to adults (5-20%) (74,75). *S. pneumoniae* exploits several virulence factors to colonize the mucosal surface in the nasopharynx (**Figure 1.1.5**), but also other bacterial species can influence *S. pneumoniae* colonization.

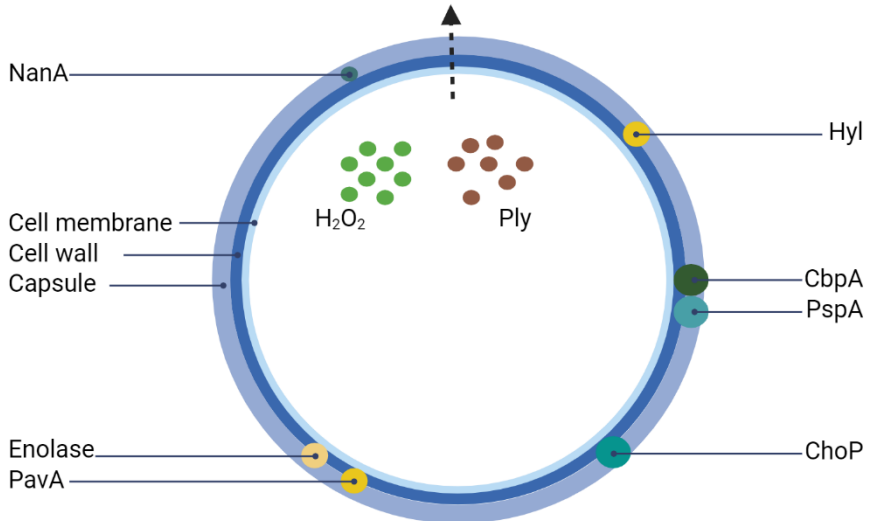


Figure 1.1.5: **Overview of a selection of virulence factors of *Streptococcus pneumoniae*.** *S. pneumoniae* possesses a plethora of virulence factors to colonize the mucosal surface in the nasopharynx and to invade underlying tissues to cause disease. CbpA, choline-binding protein A; ChoP, phosphorylcholine; Hyl, hyaluronate lyase; NanA, neuraminidase A; PavA, pneumococcal adherence and virulence protein A; Ply, pneumolysin; PspA, pneumococcal surface protein A (20,70).

Firstly, *S. pneumoniae* must avoid mucus entrapment to gain access to the nasal epithelium. The CPS surrounding *S. pneumoniae* is negatively charged and will repel the mucopolysaccharides in the nasal mucus facilitating contact with the epithelium (18,20,70,76). Glycosidases (E.g. neuraminidase A, NanA) can reveal receptors at the epithelium for adhesion by releasing sugars from glycoproteins and thereby enhance bacterial adherence, and the degradation of mucus also inhibits mucociliary clearance (18,20,70,76). Pneumococcal adhesins, pneumococcal adherence and virulence proteins A and B (PavA and PavB) and enolase, have an important role in adhesion by binding fibronectin and plasminogen, which are extracellular matrix proteins (18,20,70,76). When present in the nasopharynx, *S. pneumoniae* must evade the host immune response. Interaction with and evasion of the complement system is established by different surface proteins, for example pneumococcal surface protein A (PspA) and choline-binding protein A (CbpA), via blocking complement deposition (18,20,70,76).

Moreover, other bacterial species interfere with *S. pneumoniae* colonization in the URT. Colonizing *S. pneumoniae* is predominantly found in and on the mucus layer overlying the URT epithelium, but the nasopharyngeal microbiota also consists other micro-organisms which can give cooperative or competitive interactions (20). For example, competitive co-colonization of *S. pneumoniae* with *S. aureus* will result in killing of *S. aureus* by *S. pneumoniae* through the production of H_2O_2 (75). *S. pneumoniae* is a common colonizer of the URT of children as a commensal bacterium with a high prevalence (50-70%) between the age of 2-3 years (77). Commonly carried serotypes are 6A, 23F and 19F (78,79). Colonization of the URT typically lasts up to 6 weeks, mostly depending on the serotype, followed by clearance of the bacteria without infection (20,80). Nonetheless, in some cases *S. pneumoniae* penetrates host tissues and causes local or invasive disease exploiting a plethora of virulence factors (**Figure 1.1.5**).

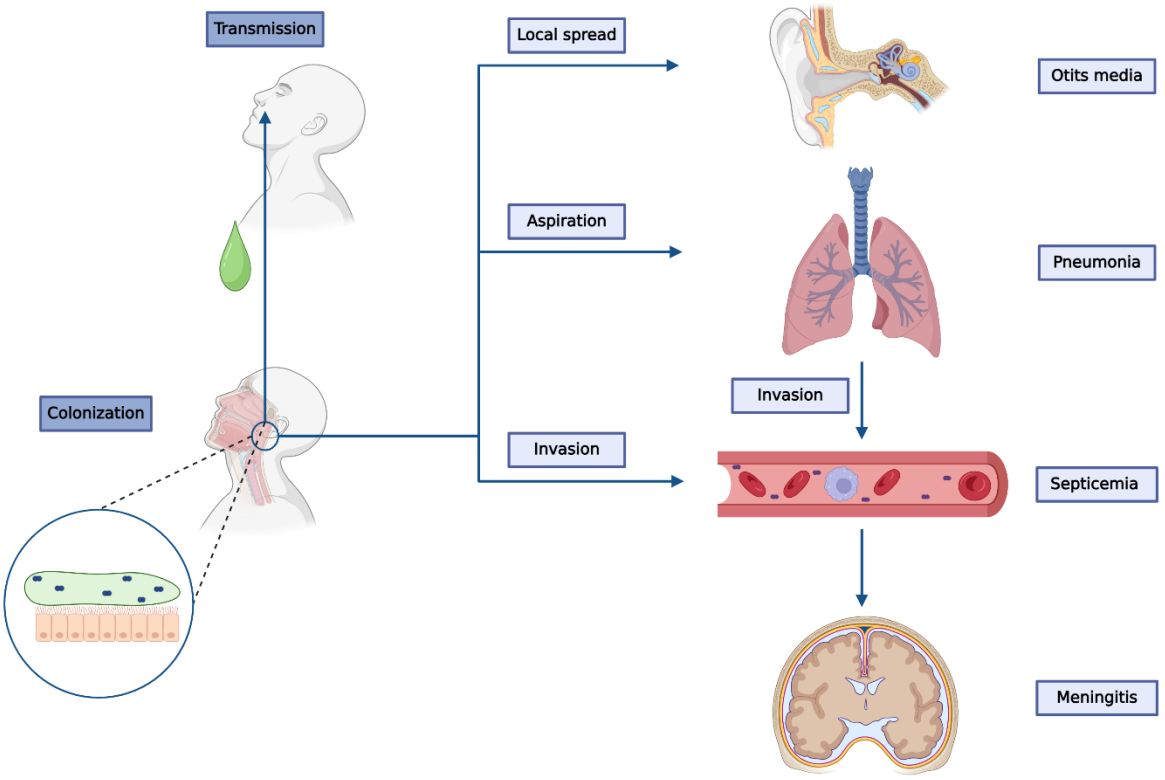


Figure 1.1.6: **Pathogenesis of *Streptococcus pneumoniae*: transmission, colonization and invasion.** *S. pneumoniae* is a common colonizer of the upper respiratory tract (URT), especially in children. Pneumococcal carriage is essential for transmission to a new host (via close contact or droplets) and precedes invasion into other organs, either via local spread (otitis media), aspiration (pneumonia) or by invading the bloodstream (septicemia) or the cerebrospinal fluid (meningitis) (18,20,80).

1.1.4.1 Non-invasive pneumococcal diseases

1.1.4.1.1 Otitis media

Otitis media, or a middle ear infection, is one of the non-invasive diseases caused by *S. pneumoniae*. Patients are typically young children and present with symptoms such as middle ear effusion, irritability, sleep disturbance and tugging at the ear, which points towards ear pain. Potential complications are mastoiditis or meningitis (81,82). Besides, it can reside in biofilms in the middle ear in children, causing recurrent and chronic otitis media. Infection is established via local spread of *S. pneumoniae* to the middle ear, either via ascending the Eustachian tube or by blocking the Eustachian tube resulting in lower oxygen levels and higher dampening on the surface of the middle ear (**Figure 1.1.6**) (80). Neuraminidases (NanA and NanB) play a crucial role during this process by cleaving mucin which reduces the mucosal viscosity and by cleaving sugars

from the host cell surface to gain access to the epithelium and host cell receptors for adhesion (80). Once the middle ear is reached, *S. pneumoniae* will trigger inflammation by the virulence factors pneumolysin and cell wall components which can contribute to hearing loss and cochlear damage (80). Most prevalent serotypes causing otitis media were 3, 6A, 6B, 9V, 14, 19A, 19F and 23F before the introduction of pneumococcal conjugate vaccines (PCV) (83). Due to the temporary switch from PCV13 to PCV10 in Belgium from 2015-2019, serotype 19A, a PCV13-non-PCV10 serotype, is still prevalent in OM next to serotypes 6C, 11A, 15B, 23A and 23B which are not covered by the currently used vaccines (83,84).

1.1.4.1.2 Pneumonia

Pneumonia is characterized by fever, tachycardia and respiratory symptoms, such as cough, chest pain and shortness of breath. Diagnosis of pneumonia is based on physical examination of the patient and laboratory tests followed by confirmation on chest radiography (85). If radiological confirmation cannot be performed, the diagnosis of pneumonia can only be presumed. Pneumonia is established via spread of *S. pneumoniae* to the lower respiratory tract (LRT) (**Figure 1.1.6**). The first barrier *S. pneumoniae* must overcome is the mucociliary escalator that mechanically keeps microorganisms out of the lungs. Again neuraminidases mediate cleavage of mucin in order to enable adherence to the respiratory epithelium and to evade mucociliary clearance (80,86). Surface proteins (ex. PavA and PavB) contribute to attachment via adhering to the extracellular matrix (20,80). Once *S. pneumoniae* is present in the alveoli, an inflammatory process is initiated. Virulence factors that contribute to inflammation and cytotoxicity are the cell wall, pneumolysin and H_2O_2 (80). The cell wall mediates inflammation through activation of the complement pathway and pneumolysin, a pore-forming toxin, mediates cell death through necroptosis. Inflammation at the lungs will result in consolidation by accumulation of erythrocytes and leukocytes in the alveoli (80). *S. pneumoniae* is the most common bacterial cause of community-acquired pneumonia (CAP) (86) and serotypes associated with CAP are 3, 19A, 11A, 11E and 19F (87). Next to pneumonia, *S. pneumoniae* can also cause chronic respiratory diseases, like chronic endobronchial infections (such as protracted bacterial bronchitis, chronic suppurative lung disease and bronchiectasis) in children.

1.1.4.2 Invasive pneumococcal diseases (IPD)

IPD includes sepsis and meningitis and is established after accessing the bloodstream. *S. pneumoniae* is the most important bacterial cause of bacterial meningitis and typical symptoms are neck stiffness, fever and a headache, but also nausea, vomiting and an altered mental status can occur (88,89). Potential neurological complications are hearing loss or memory impairment. Mortality rates are high, depending on the countries health service quality (88). Sepsis is more difficult to diagnose as symptoms are often non-specific, for example fever, tachycardia, confusion, tachypnoea and general malaise (89,90). Also sepsis is associated with a high morbidity and mortality rate, as more than 25-30% of the patients die (90). To cause sepsis or meningitis, *S. pneumoniae* must invade the blood stream by breaching epithelial and endothelial barriers to cause bacteremia (**Figure 1.1.6**). *S. pneumoniae* can access the bloodstream via the URT or LRT (18,20,80). The first pathway involves CbpA that binds to polymeric immunoglobulin receptor (PIGR) and phosphorylcholine (ChoP) that binds to platelet-activating factor receptor (PAFR). Both induce endocytosis at the apical surface of epithelial cells and will result in release of *S. pneumoniae* at the basolateral surface (20,80,91). An alternative pathway is the paracellular route to cross the respiratory epithelial cells, after breakdown of the epithelial barrier. This breakdown is managed via Ply and H_2O_2 which damage the respiratory epithelium combined with hyaluronate lyase and plasmin which degrade the extracellular matrix (20,80). Finally, the bloodstream is reached by crossing the endothelium via CbpA-PIGR and ChoP-PAFR interactions and bacteremia is established (20,80). Once present in the bloodstream, evasion of host defenses is necessary for *S. pneumoniae* to survive acute inflammation. The capsule plays a major role in this by covering underlying bacterial surface structures which inhibits binding of complement, CRP and antibodies resulting in prevention of phagocytosis (20,70,80). Finally, *S. pneumoniae* can also cause meningitis which requires crossing the blood-brain barrier (**Figure 1.1.6**). Invasion from the blood into the cerebrospinal fluid (CSF) is a two-step process. First, *S. pneumoniae* binds the cerebrovascular epithelium via CbpA that recognizes the laminin receptor followed by translocation across the blood-brain barrier via the interaction between ChoP and PAFR (20,80). Again, the virulence of a certain *S. pneumoniae* strain is serotype-dependent, as the serotype defines the ability of *S. pneumoniae* to survive in the bloodstream

(20,70,80). Serotypes 1, 2, 4, 5, 7F, 8, 9, 12F, 14, 16, 18C, and 19A are associated with more invasive disease than serotypes 3, 6A, 6B, 11A, 15B/C, 19, and 23F (22).

S. pneumoniae possesses a plethora of virulence factors which contribute to pneumococcal pathogenesis (70) (**Figure 1.1.5**) and which enable *S. pneumoniae* to cause a wide range of infections, from non-invasive disease to potentially life-threatening infections. *S. pneumoniae* remains a major cause of morbidity and mortality worldwide (18,20,80) and is classified as a priority pathogen (priority 3 – medium) by the World Health Organization (WHO) in 2024 for the need for new antibiotics (92).

1.1.5 Treatment

As previously mentioned, antibiotic resistance is rising for *S. pneumoniae* narrowing the set of antibiotics that can be used to treat pneumococcal infections and therefore the CDC classified *S. pneumoniae* as a serious threat (25).

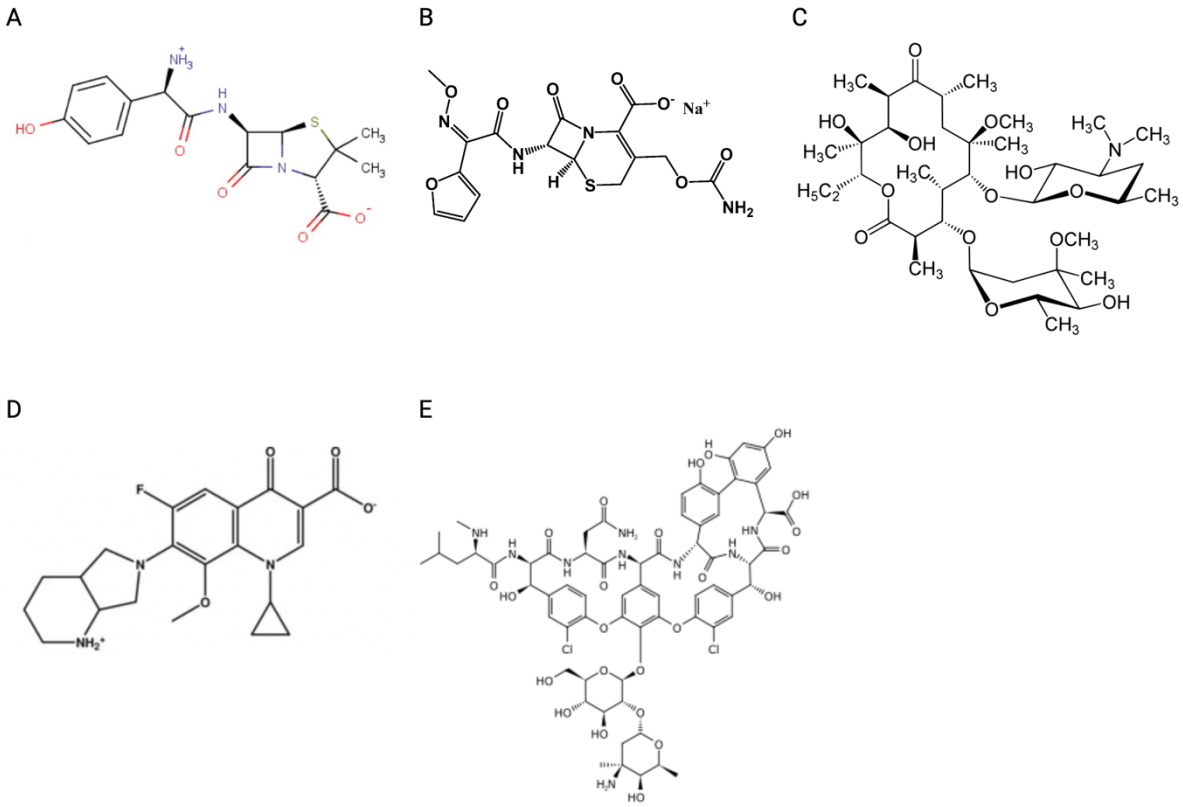


Figure 1.1.7: **Chemical structures of antibiotics used in the treatment of *S. pneumoniae.*** **A.** amoxicillin (penicillin, at physiological pH), **B.** cefuroxime sodium (cephalosporin, at physiological pH), **C.** clarithromycin (macrolide), **D.** moxifloxacin (fluoroquinolone, at physiological pH) and **E.** vancomycin (glycopeptide).

The most important class of antibiotics in treatment of *S. pneumoniae* are the beta-lactam antibiotics. Penicillin was discovered as early as 1928 by Alexander Fleming and the mechanism of action was unraveled by Blumberg and Strominger by 1974 (93,94). The targets of penicillin, transpeptidases or penicillin-binding proteins, catalyze the transpeptidation reaction that results in the final cross-linking of peptidoglycan. Penicillin mimics the structure of the substrate of transpeptidase, the D-Ala-D-Ala terminal of peptidoglycan and will bind transpeptidase covalently resulting in the irreversible inhibition of the enzyme. The resulting weak peptidoglycan formation will disrupt the cell wall's integrity and ultimately cause cell lysis. Beta-lactam antibiotics are therefore classified as bactericidal antibiotics (95,96). Amoxicillin and cefuroxime are two examples (Figure 1.1.7, A and B).

A second group of antibiotics that was frequently administered for *S. pneumoniae* infections are the macrolides. The macrolide structure consists of a 14-, 15- or 16-membered lactone ring with one or multiple sugar moieties (Figure 1.1.7, C). Macrolide antibiotics are bacteriostatic drugs that induce growth arrest via binding to the large ribosomal subunit of the bacterial ribosome resulting in inhibition of protein synthesis (97,98). Macrolide resistance is common among *S. pneumoniae* clinical isolates and are therefore not recommended in first line for treatment of *S. pneumoniae* in Belgium (99). Farrell *et al.* (2008) described an overall rate of macrolide resistance of 37.2% (100,101) while the resistance rate in Belgium in 2017 was estimated at 15.9% (102).

Fluoroquinolones are a third important class of antibiotics in the context of *S. pneumoniae* infections, especially in case of penicillin allergy (99). Fluoroquinolones can only be administered to adult and not to children. Fluoroquinolones inhibit bacterial DNA synthesis by binding DNA gyrase and topoisomerase IV. Fluoroquinolones are classified as bactericidal antibiotics, because the inhibition of DNA replication and transcription results in cell death (103,104). Moxifloxacin is a fourth generation fluoroquinolone with a wider activity against Gram-positive bacteria, including *S. pneumoniae* (Figure 1.1.7, D) (103).

Vancomycin belongs to the glycopeptide antibiotics (**Figure 1.1.7, E**) and also targets the peptidoglycan biosynthesis. Glycopeptide antibiotics do not inhibit the transpeptidase enzyme directly, but block peptidoglycan synthesis through binding the D-Ala-D-Ala sequence followed by a disturbed recognition of this sequence by transpeptidase. The result is again a weak cell wall which leads to bacterial lysis which renders glycopeptides also bactericidal antibiotics (105). Vancomycin is only used as a last resort for *S. pneumoniae* infections, for example in case of penicillin resistance or meningitis (105–108).

Treatment of *S. pneumoniae* depends on the type of infection and does not always include antibiotics (**Figure 1.1.8**). OM and rhinosinusitis are self-limiting infections and treatment is therefore mostly symptomatic, for example by pain release with acetaminophen or a nonsteroidal anti-inflammatory drug (NSAID, ex. ibuprofen), without the use of antibiotics (84). On the other hand, treatment of CAP always includes an antibiotic, but which antibiotic depends on the severity of CAP. Ambulatory patients without comorbidities (subgroup I) are typically treated with a high dose of amoxicillin, as *S. pneumoniae* is the most common bacterial cause of CAP and most *S. pneumoniae* strains are sensitive to amoxicillin in a high dose (84). Subgroup II, ambulant patients with comorbidities, should be treated with amoxicillin combined with clavulanic acid to cover for β-lactamase producing pathogens (ex. *H. influenzae*) (84,109). Subgroup III includes hospitalized patients and subgroup IV are hospitalized patients on the intensive care unit. Patients are respectively treated with amoxicillin combined with clavulanic acid (orally or intravenously) or a beta-lactam (amoxicillin or cefuroxime) antibiotic combined with a macrolide or a fluoroquinolone (84,109). For subgroups I-III, the alternative antibiotic in case of an IgE-mediated penicillin allergy is a fluoroquinolone (moxifloxacin) and the addition of azithromycin (macrolide) is required if no improvement is observed within 48 h after initiation of therapy (84,109). Finally, meningitis and septicemia are always treated with antibiotics. Empirical antibiotic treatment consists of a third generation cephalosporines (ceftriaxone or cefotaxime intravenously). In case of septicemia, these are even combined with clarithromycin (macrolide) or amikacin (aminoglycoside) to also cover for *Enterobacterales* as potential disease-causing pathogen (109).

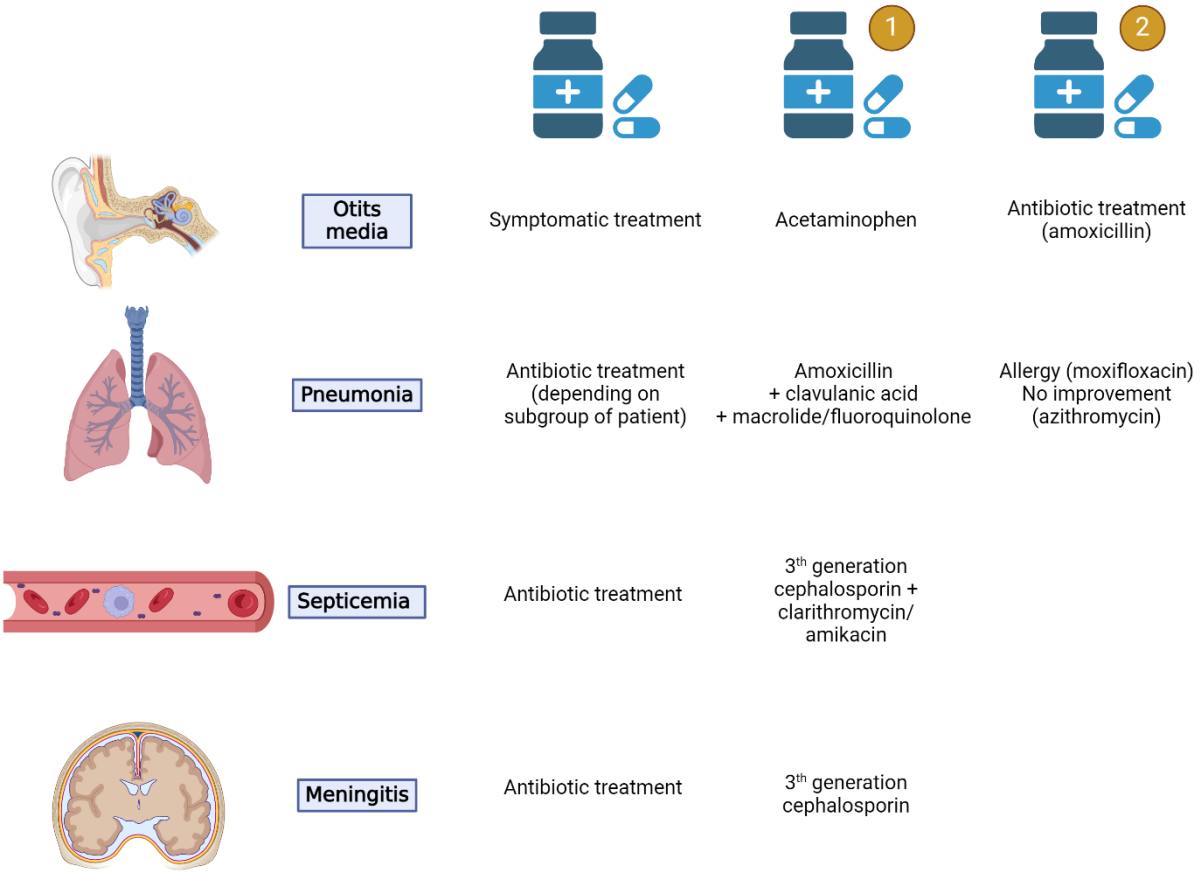


Figure 1.1.8: **Treatment type (symptomatic vs antibiotic) and type of antibiotic for different *S. pneumoniae* infections.** First choice treatment (1) is given followed by alternatives (2) in case of allergy or lack of improvement under first line treatment.

1.1.6 Vaccines and serotype evolution

The first introduction of a pneumococcal vaccine was in 1946, and since 1983, the pneumococcal polysaccharide vaccine PPV23 was used and is still available to date (11). The PPV23 vaccine, Pneumovax®, is a multivalent polysaccharide vaccine that consists purified CPS of 23 *S. pneumoniae* serotypes (**Table 1.1.1**). The vaccine provides good protection in adults and elderly, but lacks efficiency in children and immunocompromised people due to the inability to generate T-cell dependent immunity (110). Besides, it protects poorly against CAP and it does not prevent nasopharyngeal colonization (11). In 2000, the first pneumococcal conjugate vaccine (PCV) was introduced and protected against the 7 most common serotypes (PCV7, Prevnar 7™) (**Table 1.1.1**). Later, more serotypes were included in PCVs, the 13-valent PCV13 (Prevnar 13™) and more recently, the 20-valent PCV20 (Prevnar 20™) (**Table 1.1.1**) (110). Purified CPSs are covalently attached to a highly immunogenic carrier protein in PCVs to enhance T-cell dependent

immune response (11,111). PCVs provide protection against IPD, but also against pneumonia and other mucosal infections. Besides, PCVs reduce carriage of vaccine serotypes, especially in children, which gives herd protection against vaccine-type IPD of unvaccinated elderly (11).

Table 1.1.1 ***S. pneumoniae*** **serotypes included in the different pneumococcal vaccines.**

VACCINE	SEROTYPES INCLUDED
PPV23	1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F, 33F
PCV7	4, 6B, 9V, 14, 18C, 19F and 23F
PCV13	1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F and 23F
PCV15	1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 22F, 23F and 33F
PCV20	1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 18C, 19A, 19F, 22F, 23F and 33F

Belgian guidelines for vaccination of children are summarized in Figure 1.1.9. All infants should receive three doses of a conjugate vaccine (PCV13 or PCV15) at 8 weeks, 16 weeks and 12 months of age (2+1). Premature babies (< 37 weeks) should receive an additional dose at 12 weeks (3+1). For adults, vaccination is recommended for people with an increased risk for *S. pneumoniae* infections (immunocompromised patients, patients with asplenia,…), for people with a comorbidity (chronic diseases) and for all people of 65 years and older. The preferred scheme includes a one-time dose of the conjugate vaccine PCV20 (112).

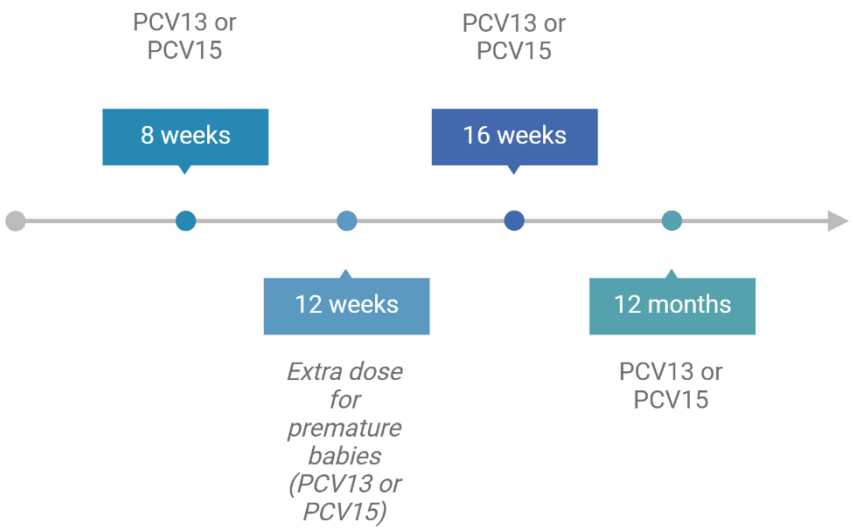


Figure 1.1.9: **Belgian vaccination scheme for children according to the Belgian Superior Health Council** (112).

Currently, all approved vaccines are serotype-based, because binding of a specific antibody to the capsule results in opsonization and rapid clearance of *S. pneumoniae* (4,11,12). The use of serotype-based vaccines leads to a shift in pneumococcal prevalence, also called serotype replacement, and diminishes the protective coverage of the conjugate vaccines (11,113). The so-called vaccine escape recombinants can emerge by capsular switching events that result in the acquirement of capsular polysaccharide synthesis (*cps)* genes from a non-vaccine serotype via natural transformation or they can emerge via minor genetic changes within the *cps* loci, such as deletion, insertion or point mutation, that can change the chemical structure of the CPS (12,37,114–116). For example, Golubchick *et al.* (2012) detected vaccine escape recombinants, namely switching from serotype 4 to 19A, after the introduction of PCV7 (117). The selective pressure by vaccines could also lead to the formation of new serotypes through acquisition of *cps* genes from other commensal streptococci, ex. *S. mitis* (37). The vaccines caused a large reduction in IPD in young children and elderly, despite serotype replacement by non-vaccine serotypes as these serotypes are often less invasive (37).

Nonetheless, serotype-based vaccines have their limitations. About 100 different *S. pneumoniae* serotypes are known today and it's not possible to keep adding serotypes to the vaccines, because it is complex and expensive. Therefore, other antigens have been explored as potential vaccine candidates (110,111).

Proteins antigens, common to all serotypes, can induce a T-cell dependent immune response, can elicit immunological memory and are most likely also immunogenic in infants which renders them suitable vaccine candidates (110,111). Pneumococcal choline-binding proteins are proposed as good antigens to use in a vaccine. Sempere *et al.* (2021) provide an overview of choline-binding proteins that play an important role in *S. pneumoniae* virulence and pathogenesis, for example LytA and PspA. They showed their immunogenicity and their protection against *S. pneumoniae* infection (118). Also Liu *et al.* (2024) described a protein as a vaccine candidate, more specifically a conserved membrane-associated intracellular glycosyltransferase, LafB. They discovered protective immunity in mice via T-helper 17 cells after respiratory immunization with recombinant LafB (119).

S. pneumoniae remains a major pathogen associated with high morbidity and mortality worldwide. Increasing antibiotic resistance and serotype replacement after vaccine introduction contribute to persistence of *S. pneumoniae* infections. The need for novel antimicrobial therapies remains high and therefore we looked into different ways of *S. pneumoniae* to evade antibiotic killing.

1.2 *Streptococcus pneumoniae* and antibiotic escape strategies

1.2.1 Resistance

1.2.1.1 The antibiotic era

Infectious diseases exist already for millennia and so does anti-microbial therapy. More than 2000 years ago, poultices of moldy bread and beer yeast were used to treat wound infections and a list of remedies was written down as early as 1550 BC in the Eber's papyrus (120–122). The first person to describe a natural antibiotic was Bartolomeo Gosio, an Italian medical scientist, who discovered mycophenolic acid as a product from *Pencillium brevi-compactum* in the late 19th century (122). However, it was Paul Ehrlich in 1909 who shaped the concept of chemotherapy with the discovery of salvarsan, the first synthetic antibiotic to treat *Treponema pallidum*, a bacterium causing syphilis (123). Ehrlich set the stage for screening synthetic compounds for their antibacterial activity, which was successfully continued by Domagk, a German physician, resulting in the discovery of sulfa drugs (121,122,124). Sulfonamides were the first antibiotics that were widely used in the clinics and are still in use today (121,122,124). It was Alexander Fleming that discovered penicillin in 1929, accidentally on a contaminated agar plate, which are widespread used antibiotics to date (121,122,124). With these discoveries, the "antibiotic era" was born. Antibiotics have had a great impact on modern medicine and human health by saving countless of lives, but antibiotic discovery was accompanied with the rapid emergence of antimicrobial resistance (AMR) (121,122,124,125). AMR can be measured by the lowest concentration of an antibiotic that inhibits bacterial growth or the minimum inhibitory concentration (MIC). When a bacterium is resistant, the MIC is increased. The MIC can be defined as the MIC_{50} or MIC_{90} indicating the antibiotic concentration needed to inhibit growth of 50% or 90%, respectively, of the bacterial culture. Currently, AMR is one of the biggest challenges in public health. The Centers for Disease Control and Prevention (CDC) estimated that in 2019 the deaths of nearly 5 million people were associated with AMR (25). Future prospectives regarding AMR are bad as described in the famous report of Jim O'Neill. He predicts that by 2050 10 million people will die yearly from AMR infections, which will exceed deaths attributed to cancer (126). The widespread use of antibiotics, the use of antibiotics

as growth promotors in agriculture and the declining development of new antimicrobial agents worsen the antibiotic crisis (127,128).

1.2.1.2 Mechanisms of antibiotic resistance

Bacteria can become resistant towards an antibiotic via different pathways. When a bacterium is intrinsic resistant towards an antibiotic, resistance is chromosome-encoded and the result of inherent bacterial characteristics. On the other hand, acquired resistance confers to resistance through mutations in their chromosome or acquired by horizontal gene transfer (HGT) (129,130). Several mechanisms can render a bacterium resistant. Firstly, the bacterium can keep the intracellular antibiotic concentrations at a low level by lowering the penetration of the antibiotic or via antibiotic efflux. Secondly, the bacterium can modify the target of the antibiotic, genetically or via post-translational modification. Lastly, the bacterium can degrade the antibiotic, for example through hydrolysis (129,130).

1.2.1.3 Antibiotic resistance in *Streptococcus pneumoniae*

AMR in *S. pneumoniae* is also rising as already shown in **Figure 1.1.1**. The CDC classified *S. pneumoniae* as a serious threat, because more than 2 million *S. pneumoniae* infections are counted yearly in the USA of which 30% are caused by resistant strains (25). Also the WHO has included *S. pneumoniae* on the list with antibiotic-resistant priority pathogens for which novel antibiotics are warranted (92). In Belgium, *S. pneumoniae* is considered a pathogen with medium risk (131). The introduction of PCV7 in Belgium has led to a decrease in the circulation of penicillin resistant strains with a penicillin resistance rate of 14.1% in 2022 which was a decrease compared to 2021 (18.3%). Macrolide resistance has been stable for several years with 14.7% in 2022 (131).

S. pneumoniae is naturally competent and exploits a set of mechanisms of HGT and recombination, for example transformation and bacteriophages, which renders *S. pneumoniae* a highly recombinogenic bacterium. B-lactam resistance is mainly mediated via target modification, more specifically via the penicillin binding proteins (PBPs), resulting in a decreased affinity of the antibiotic for the target. The *S. pneumoniae* genome can contain six different PBPs (PBP1a, 1b, 2a, 2x, 2b or 3) whereas PBP1a, 2x and 2b are associated

with β-lactam resistance (108,132,133). Despite the change in PBP, amoxicillin remains efficient when applied in a high dose for strains that display only intermediate resistance (99,108). Macrolide resistance is mainly managed via target modification or via an efflux pump. An erythromycin ribosomal methylase encoded by *erm(B)* methylates the 23S rRNA of the ribosome and prevents binding of the antibiotic to its target. The macrolide efflux pump is encoded by *mefE* or *mefA* (132,133). Due to the widespread presence of macrolide resistance, macrolides are not used as first line antibiotics for non-invasive *S. pneumoniae* diseases in Belgium (99,131). Fluoroquinolone resistance is rare in Belgium, due to the restricted use (84,131). Mutations in the target of fluoroquinolones, topoisomerase IV, encoded by *parC* and *parE*, and DNA gyrase, encoded by *gyrA* and *gyrB*, give raise to resistance via target modification. It is a stepwise process with the accumulation of mutations at the quinolone-resistance-determining regions. Mostly a first mutation in the *parC* gene is followed by a mutation in *gyrA* leading to high-level fluoroquinolone resistance (132,133). Finally, no cases of vancomycin-resistant *S. pneumoniae* were yet reported, but there are several cases of treatment failure due to vancomycin-tolerant *S. pneumoniae* (134).

1.2.2 Tolerance

Antibiotic resistance poses a major threat towards global health. Nonetheless, other mechanisms also contribute to the failure of antibiotic therapy and can worsen the antibiotic crisis. Antibiotic tolerance is the ability of a whole bacterial population to survive antibiotic treatment without an increased MIC through a lowered killing rate (135,136).

Tolerance in *S. pneumoniae* cultures was first described in 1970 (137) and is already extensively studied for the antibiotic vancomycin and in a lesser extent for β-lactam antibiotics (138–142). Limited or no studies were reported for the other antibiotic classes. The mechanism of tolerance relies on the way *S. pneumoniae* is killed after challenge with cell-wall active antibiotics, such as vancomycin and β-lactam antibiotics. β-lactam antibiotics bind PBPs followed by inhibition of the cell wall synthesis and growth arrest. Then, two pathways lead to cell death, a lytic and nonlytic pathway. In the lytic pathway, the bacteria activate their own enzymes, cell wall hydrolyses such as LytA, to achieve bacterial killing via autolysis (138,139,141). In the nonlytic pathway, the inhibition of the transpeptidase results in formation of long peptidoglycan strands followed by precursor depletion and overuse of energy which contributes to cell death (141,143). Tolerance goes along with environmental changes, for example nutrient deprivation or lowering the pH of the growth medium, followed by a decreased synthesis of macromolecules in non- or slow-growing strains (139). Population-wide tolerance is associated with the control regions of the autolytic machinery of *S. pneumoniae* (138–140,142). VncS, a histidine kinase/phosphatase, is a two-component system that contributes to the regulation of triggering autolysis and functional loss of VncS was related to vancomycin tolerance (138). More recently, a four-gene operon (phenotypic tolerance to vancomycin, *ptv*) was induced upon vancomycin treatment and was regulated by a transcriptional repressor, PtvR. The *ptv* operon encodes for, next to PtvR, PtvA, PtvB and PtvC which are associated with the cell membrane and contribution of this operon to antibiotic tolerance is therefore supposed to work via modulation of the *S. pneumoniae* cell membrane (142). The operon is conserved among *S. pneumoniae* strains, but also in closely related *Streptococcus* species, such as *S. mitis* and *S. oralis* (142).

1.2.3 Persistence

A subpopulation of antibiotic-tolerant cells was first described in 1942 by Gladys Hobby, an American microbiologist, during her study of the mode of action of penicillin. She discovered that a fraction of a *Streptococcus* culture was killed at a lower rate and even 1% of the culture survived treatment (144). It was only two years later that these antibiotic-tolerant cells were named 'persister cells' by Joseph Bigger. He also found survivors in cultures of staphylococci after penicillin treatment, which could not be attributed to antibiotic resistance. He described these surviving cells as cells in a dormant, non-dividing state which rendered them insensitive to penicillin (145). It required another forty years before further advances in persistence research were made, for example by Moyed and Bertrand who described high-persistent mutants in *E. coli* cultures (146). From then, persistence research gained interest and in 2019 a Consensus Statement on definitions and guidelines for research on antibiotic persistence was published (135).

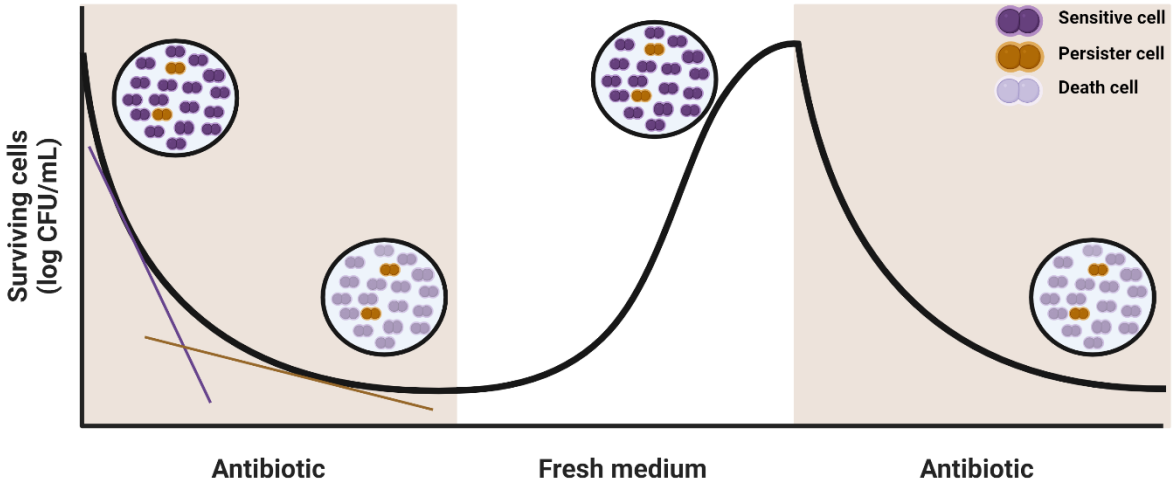


Figure 1.2.1: **The hallmark of antibiotic persistence is the biphasic killing curve.** After initiation of antibiotic treatment, sensitive cells will be rapidly killed (following the purple line) followed by a slower killing of the subpopulation of antibiotic-tolerant persister cells (following the brown line). Persisters are transient, phenotypic variants of the population. When the antibiotic pressure is removed, persisters will reconstitute a new population with similar characteristics (antibiotic susceptibility and persister fraction) to the culture they originated from (147).

Persisters are a subpopulation of cells that can survive treatment with a bactericidal antibiotic, but they cannot grow during treatment (135,147–150). The hallmark of persistence is the biphasic killing curve, which represents different killing rates within a clonal population during antibiotic exposure, i.e. susceptible

cells die fast and persisters die much slower (**Figure 1.2.1**). Antibiotic persistence only weakly depends on the antibiotic concentration and is often present for different antibiotic classes. Persisters are genetically identical to the bulk population, but express another phenotype. This phenotype is only transient. When the antibiotic pressure is removed, persisters will reconstitute a new population with similar characteristics (antibiotic susceptibility and persister fraction) to the culture they originated from (**Figure 1.2.1**) (135,147–150). The persistent phenotype can have different origins. Two types of persistence are frequently described, namely spontaneous and triggered persistence (135,147,148). Spontaneous persistence is the emergence of slow- or non-growing persister cells without any trigger, for example during balanced exponential growth (135,148). These so-called stochastic persisters originate from random variations in gene expression, protein levels or protein stability (147). On the other hand, triggered persistence confers to the generation of persister cells upon a trigger, such as nutrient starvation, exposure to drugs or to the host immune system (135,149). Persistence is often referred to as an evolutionary survival strategy or a bet-hedging strategy (147,148). By switching a part of the population to a different phenotype and introducing heterogeneity in the isogenic population, the population is better prepared to future environmental perturbations, for example antibiotic exposure or starvation, despite the cost of these suboptimal variants under optimal growth conditions (147,148,151).

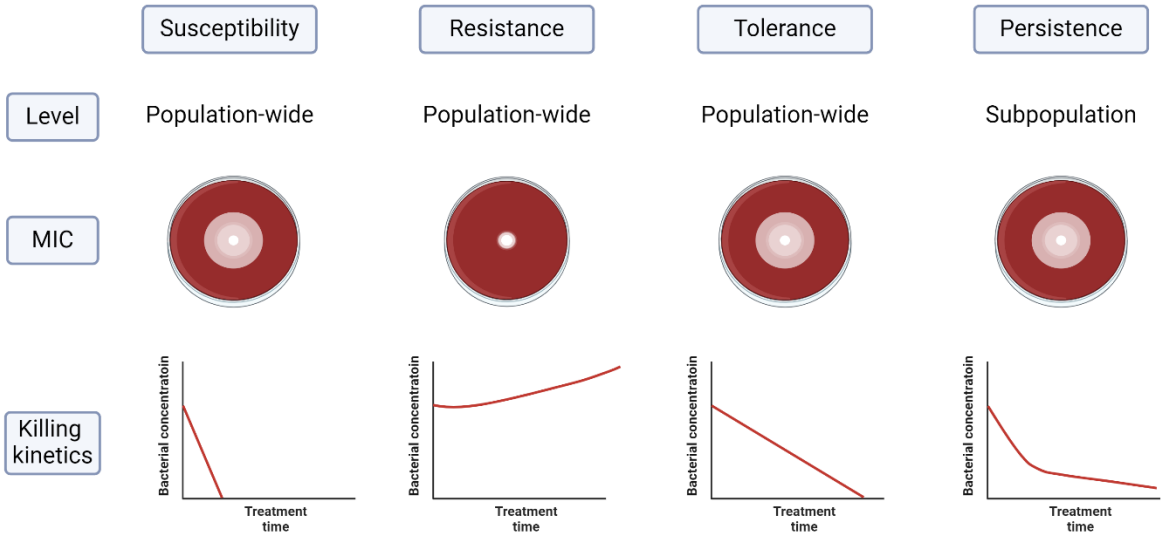


Figure 1.2.2: **Hypothetical representation of different ways of bacteria to evade antibiotic treatment.** The minimum inhibitory concentration (MIC) is presented as the disk diffusion assay, with a wider clear area around the disk depicting a lower MIC and a higher susceptibility towards the antibiotic. Killing kinetics are presented as killing curves (bacterial concentration in function of treatment time) (149,152).

Antibiotic persistence can be distinguished from antibiotic tolerance and resistance (**Figure 1.2.2**). Persistence and tolerance both show a transient, increased survival during antibiotic treatment without an increase in MIC. Tolerance is the ability of the entire population to survive antibiotic treatment better, with an increased duration needed to kill the entire population, whereas for persistence only a subset of the population is antibiotic-tolerant which results in a typical biphasic killing curve (**Figure 1.2.2**) (135,149,152). Resistant bacteria display an increased MIC which results in a higher survival and even the ability to grow during antibiotic treatment. The origin of antibiotic resistance is a genetic mutation, which is in contrast with persistence that confers only a difference towards the original population at the phenotypic level. Resistance is therefore inherited by the daughter population while persistence is not (**Figure 1.2.2**) (135,149,152).

1.2.3.1 Persistence mechanisms

Already for a long time, persister cells were seen as slowly growing or dormant cells with a reduced metabolic activity that can survive antibiotic treatment through target inactivity. Following this statement, antibiotic persisters were often supposed to be multidrug tolerant through the generally dormant state

(147,151,153). Balaban *et al.* (2004) and Correia *et al.* (2006) showed the role of growth arrest in persister formation in *E. coli* already 20 years ago (154). Also in 2024, new studies are executed in order to understand the mechanisms behind dormancy in *E. coli* (155), but also in other pathogens (156,157). A typical pathway to dormancy is a decrease in bacterial energy levels followed by a reduction of the synthesis of DNA, proteins and peptidoglycan which are the targets of fluoroquinolone, aminoglycoside and β-lactam antibiotics, respectively (151). Two examples of biological effectors that influence the energy levels in *E. coli* via pore formation and induce persister formation are toxins TisB and HokB. These toxins are part of a genetic toxin-antitoxin (TA) module encoding for a labile antitoxin (RNA or protein) and a stable toxin. The toxin inhibits an essential cellular function and the antitoxin can inhibit the toxin (by inhibiting either the activity or the synthesis) (147,151). Another way of inducing the persister state is by direct interference with DNA replication and/or transcription. During starvation, the stringent response alarmone (p)ppGpp (guanosine tetra-or pentaphosphate) levels increase and lead to inhibition of DNA replication and transcription, and repression of rRNA and tRNA syntheses (147,148,151). Persister formation can also directly result from blocking translation and thus inhibiting protein synthesis. Common effectors are the toxins YafQ and RelE that target the ribosome and MazF and MqsR that cleave mRNA and prevent formation of new ribosomes. YafQ, RelE, MazF and MqsR also belong to TA modules (147,148,151).

Dormancy remains the most important way in persister formation, but it is not the only explanation for the persistent phenotype (147,151). Both actively growing cultures and non-growing, metabolically active bacteria can give rise to persister cells (158–162). The first mechanism with a more active nature is linked to quinolone antibiotics which cause DNA damage. This damage induces the SOS response and thus active DNA repair which is an intrinsic defense mechanism against antibiotic-induced damage (147,151). Another active way resulting in persisters is by multidrug efflux pumps that keep intracellular antibiotic concentrations low and has been proven important in *E. coli* and *M. smegmatis* persisters (147,151). Antibiotic efflux pumps are also a potential strategy of antibiotic resistance (129,130). In the past, resistance and persistence were seen as different survival strategies of bacteria, however more and more evidence states that resistance and persistence are intertwined at various levels (163).

Taken altogether these findings point out that there is no sole explanation for persistence, but that persistence is a complex phenomenon resulting from the interplay between a great variety of mechanisms, including dormancy and, potentially, active processes. The variety of underlying mechanisms leads also to varying levels of tolerance towards different antibiotics within a persister subpopulation, rather than general multidrug tolerance (147). Many efforts were made regarding persistence and what's behind in a variety of pathogens, but until 2022, no reports were published about persistence, nor persistence mechanisms, in *S. pneumoniae*. Understanding the mechanisms behind persister formation and the contribution of these mechanisms to therapy failure, could help in pointing future efforts towards the most relevant persister mechanisms.

1.2.3.2 Clinical relevance

Persister cells are not only present in bacterial species, but widespread among a variety of organisms, including eukaryotes, which is the first indication of the role of persistence in the wider clinical setting (147,149). A well-studied example is the opportunistic yeast, *Candida*. *C. albicans* persister cells have been detected within biofilms with glucose starvation as an important trigger. Besides, *Candida* persisters manage oxidative stress upon fungicidal drug treatment with for example an upregulation of superoxide dismutases (164). Also for the malaria-causing parasite, *Plasmodium falciparum*, dormant cells were observed after anti-parasite therapy with artemisinin and these cells were linked to therapy failure (165). Finally, eukaryotic cancer cell lines can contain drug-tolerant phenotypic variants (166–169). Different studies indicate the contribution of persisters to therapy failure of different cancers (167,170), but also to the development of drug-resistance cancer cells (166,170).

Next to the fact that persister cells seem to be a universal feature of clonal life forms, persistence is a driver towards the development of antibiotic resistance (147). Clearly, persisters constitute a viable pool of cells that facilitate resistance development by prolonging the presence of viable bacteria during antibiotic treatment (171,172). Nonetheless, various other mechanisms contribute to acquired resistance originating from a persistent subpopulation (173–176). Highly persistent, non-growing strains show an increased

mutation rate when regrown due to spontaneous or antibiotic-induced DNA damage which promotes evolution toward antibiotic resistance (173,177). Moreover, the emergence of resistance is not limited to the drug that gave rise to the persistent subpopulation (171,178). This is illustrated by Swaminath *et al.* (2020) who pointed out that *Mycobacterium smegmatis* persisters following moxifloxacin treatment not only give rise to moxifloxacin resistance, but also to ethambutol (178). Furthermore, Levin-Reisman *et al.* (2019) indicated the role of epistasis, i.e. the interaction between genes contributing to the same phenotype, between antibiotic persistence and resistance mutations (176). Aforementioned studies emphasize the need to combat antibiotic persistence in the fight against antibiotic resistance (136,173).

Finally, the role of persister cells can be demonstrated in the clinical practice. It took a long time before the clinical importance of persistence was acknowledged, because the attention was reserved for antibiotic resistance and the assumption was that the small fraction of persisters would be killed by the host immune system (147,150,179). Mulcahy *et al.* (2010) performed one of the first studies proving the *in vivo* relevance of persisters (180). By following cystic fibrosis patients, who often suffer from chronic respiratory *P. aeruginosa* infections, over an extended period, they detected the presence of high persister mutants during the later stages of infection which could explain the recalcitrant nature of the cystic fibrosis airway infection (180). More recently, Bartell *et al.* (2020) performed a similar study, again proving the link between high persister variants of *P. aeruginosa*, long-term establishment of *P. aeruginosa* in the cystic fibrosis lung environment, and treatment failure (181). High persistent mutants were not only detected for *P. aeruginosa*, but were also present amongst clinical isolates from *C. albicans* (182), uropathogenic *E. coli* (162,183) and *M. tuberculosis* (184). It is clear that persisters contribute to the chronic nature of infections, as high persistent mutants are selected after prolonged antibiotic treatment (180–183). Again, no reports were published studying the clinical relevance of *S. pneumoniae* persistence, which confers a major knowledge gap. The acknowledgement of the clinical importance of persister cells in chronic infections should encourage persistence research to gain insights in how to subvert the matter.

2 THESIS OUTLINE AND AIMS

The past 100 years, major advances were accomplished into the fight against infectious diseases, especially with the discovery of antimicrobial agents. Unfortunately, these discoveries were accompanied with the fast emergence of AMR. Many researchers have investigated AMR, but bacteria can evade antibiotic treatment also in different ways leading to therapy failure. Persistence is an example of how bacteria can evade killing by antibiotics regardless of resistance. Persister cells are phenotypic variants that exist as a subpopulation within a clonal culture. Being tolerant to lethal antibiotics, they underly the chronic nature of a variety of infections and even help in acquiring genetic resistance. The past years, increasing reports were published about antibiotic persistence in many bacterial species, but also in eukaryotic organisms (170,175,180,182,183) and the clinical importance of antibiotic persisters was acknowledged (136,185). Specifically for *S. pneumoniae*, no studies were published about antibiotic persistence (63,186,187) despite the findings of *S. pneumoniae* tolerance after treatment with vancomycin and β-lactam antibiotics (138–142). Antibiotic-tolerant persisters are mostly connected with recurrent and chronic infections, and the role of persisters in acute infections is not clear (147,185). Most infections caused by *S. pneumoniae* have an acute nature. Nonetheless, *S. pneumoniae* is also, albeit to a lesser extent, the causative agent of chronic diseases, like chronic endobronchial infections in children (188–191), and it can reside in biofilms in the middle ear in children, causing recurrent and chronic otitis media (153,192–194). As studies in other bacterial species underscore the importance of persister cells, it is of utmost importance to gain knowledge about *S. pneumoniae* persistence in both acute and chronic pneumococcal infections in order to gain a better understanding of how *S. pneumoniae* evades elimination by antibiotic treatment. We therefore focused on *S. pneumoniae* in this PhD which remains an important human pathogen, being one of the most common causes of community-acquired pneumonia and otitis media.

We set out an optimized model to identify and characterize persistence in *S. pneumoniae.* Furthermore, we studied both resistance and persistence across a diverse collection of *S. pneumoniae* clinical isolates. Finally, we investigated antibiotic persistence in an *in vivo* mouse model. Collectively, this PhD research aimed to get a better understanding of the importance of antibiotic persistence in *S. pneumoniae* infections which

will set stage for characterizing its relevance to clinical outcomes and advocates for increased attention to the phenotype in both fundamental and clinical research (Figure 2.1).

In CHAPTER 1, a general overview is given of the pathogen *S. pneumoniae* with more information about the pathogenesis and current strategies in the fight against this pathogen. Moreover, different mechanisms are explained of how *S. pneumoniae* can evade antibiotic therapy. The goal of this PhD thesis was to study persistence and therefore a robust *in vitro* model needed to be optimized. For this, we had to overcome the self-limiting *in vitro* nature of *S. pneumoniae* in order to study antibiotic persistence over prolonged treatment periods (CHAPTER 3). This model was applied in CHAPTER 4 to make a broad characterization of *S. pneumoniae* persistence using *S. pneumoniae* reference strain D39. Besides, evolution experiments were performed with the intention to elucidate persister mechanisms (CHAPTER 5). In CHAPTER 6, we investigated resistance and persistence levels of a large set of clinical strains (647 strains) against clinically relevant antibiotics. We evaluated the diversity of antibiotic persister levels and the correlations between antibiotic susceptibility and persistence. Clinical strains originated from carriage, non-invasive and invasive *S. pneumoniae* diseases. Additionally, the full genome sequence was determined for 377 strains which gave us a look into the genetic diversity among the clinical isolates. Finally, we made the first steps towards the optimization of a prolonged murine infection model to enable *in vivo* persister studies in CHAPTER 7. We assessed two different murine models, a lung infection and nasopharyngeal carriage model, for their potential to obtain a chronic infection. Subsequently, we chose the nasopharyngeal carriage model to perform an *in vivo* persister experiment. CHAPTER 8 presents a general discussion and the main conclusions of the work performed during the thesis together with future perspectives on persistence research.

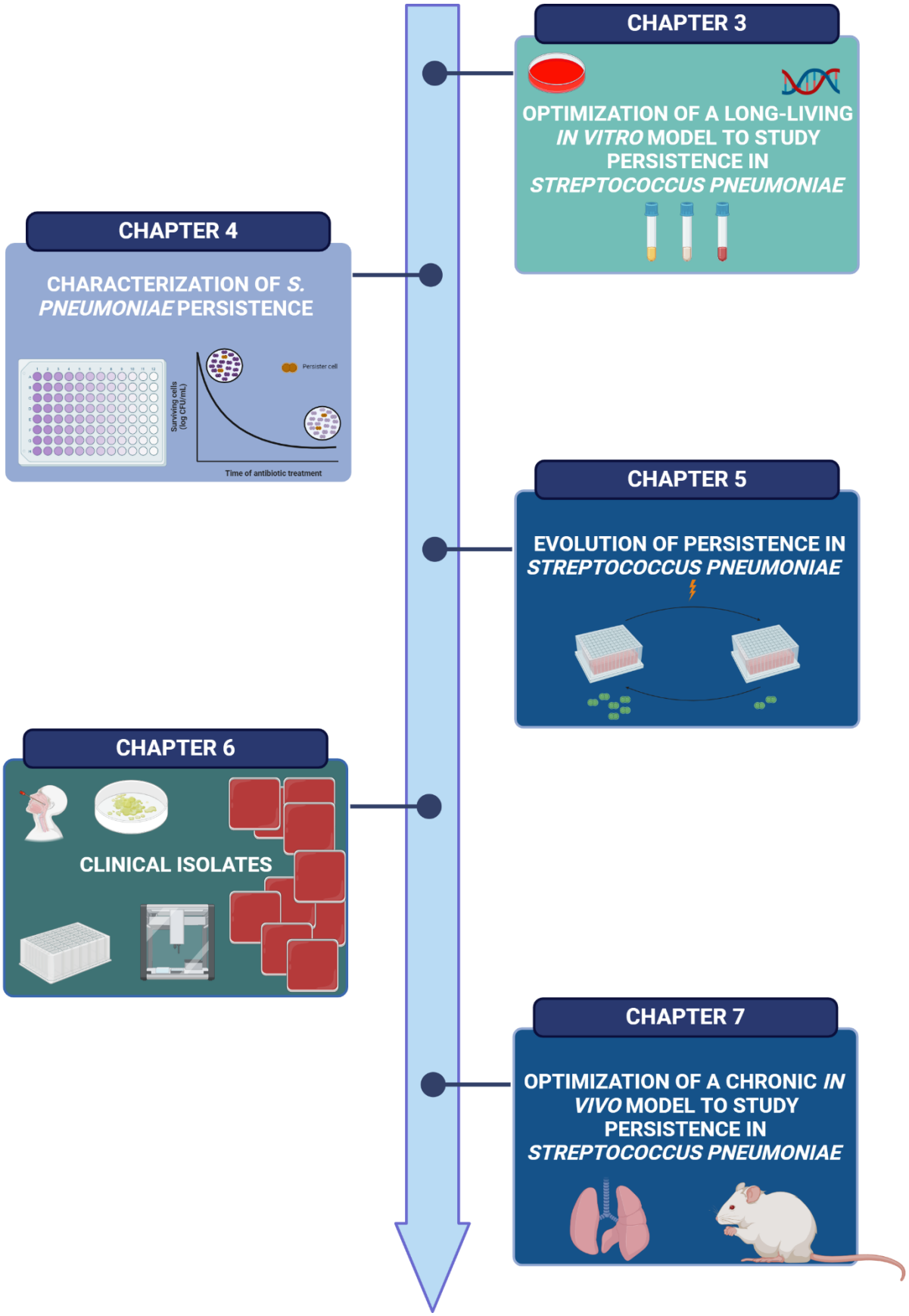
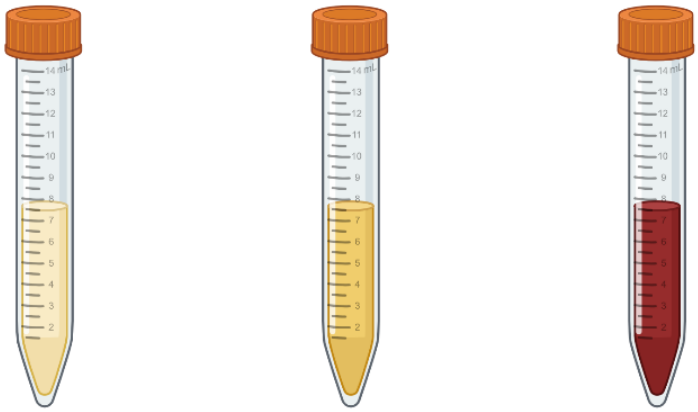


Figure 2.1: Schematic overview of the research chapters.

3 OPTIMIZATION OF A LONG-LIVING *IN VITRO* MODEL TO STUDY PERSISTENCE IN *STREPTOCOCCUS PNEUMONIAE*



"Three different growth media versus one bacterium, how hard can it be?"

Partially published in Nele Geerts, Linda De Vooght, Ioannis Passaris, Peter Delputte, Bram Van den Bergh, Paul Cos., *Antibiotic Tolerance Indicative of Persistence Is Pervasive among Clinical Streptococcus pneumoniae Isolates and Shows Strong Condition Dependence.* Microbiol Spectr. 2022 Dec 21;10(6):e0270122. doi: 10.1128/spectrum.02701-22. Epub 2022 Nov 14. PMID: 36374111; PMCID: PMC9769776.

3.1 Introduction

The presence of antibiotic-tolerant persisters in *S. pneumoniae* has not been investigated to date. In part, the lack of understanding persistence in *S. pneumoniae* stems from the fastidious and self-limiting nature of this bacterium *in vitro*, which hampers studying *S. pneumoniae* cultures during a long term as is needed to study antibiotic persistence, especially in stationary phase (135,195).

Two suggested causes of the fast decrease in survival after entering the stationary phase are the enzymes pyruvate oxidase (SpxB) and autolysin (LytA) (61,65). Pyruvate oxidase is the major producer of H_2O_2 as a by-product of the aerobic metabolism of *S. pneumoniae*, but *S. pneumoniae* lacks the neutralizing enzyme catalase, which could lead to *in vitro* death through an accumulation of H_2O_2 (51,61,64,196). Autolysin, a cell wall-bound amidase that breaks down peptidoglycan, can induce *in vitro* autolysis in stationary-phase cultures (65,67,69,197). We followed two routes targeting the suggested effectors of self-limitation in *S. pneumoniae* in order to prevent *in vitro* death in the absence of antibiotics, thus to avoid the confounding effect of such self-killing during treatment, and to obtain a stable bacterial culture for a prolonged period. First, we added catalase to neutralize the produced H_2O_2, we constructed a *spxB* knockout mutant to inhibit the expression of pyruvate oxidase, or we applied hypoxic incubation (5% CO_2 - 0.1% O_2 - 94.9% N_2) to inhibit the pneumococcal aerobic metabolism and thus the production of H_2O_2 by pyruvate oxidase (61,64). Secondly, we used choline chloride supplementation to prevent the binding of autolysin to the cell wall or we used a *lytA* knockout mutant to inhibit the expression of autolysin (65,67,69,197).

To study persistence, prolonged *in vitro* antibiotic-induced killing studies are usually employed, as shown for other species. Especially when examining antibiotic survival in stationary phase, long-living cultures are needed to allow substantial killing during subsequent treatment. Therefore, any confounding effects of mortality through the self-limiting nature of *S. pneumoniae* is best to be avoided. While previously suggested mechanisms showed only minor improvements, we succeeded in obtaining stable long living *in vitro* cultures by using specific growth conditions. The methodology described here will form the basis for

setting up prolonged antibiotic-induced killing studies (CHAPTER 4, 5 and 6) without confounding the results with the self-limiting nature of *S. pneumoniae*.

3.2 Material and Methods

3.2.1 Bacterial strains and growth conditions

S. pneumoniae strains used are listed in Table 3.2.1. *S. pneumoniae* was cultured statically in Brain-Heart Infusion broth (BHI; Neogen), Todd-Hewitt broth (BD Biosciences) supplemented with 0.5% yeast extract (THY; Gibco), or cation-adjusted Mueller-Hinton broth (Fluka) supplemented with 5% lysed horse blood (MHL; Oxoid) or on blood agar (BA) plates (tryptic soy agar [Neogen] supplemented with 5% defibrinated sheep blood [Oxoid]) at 37°C in 5% CO_2. When specified, catalase (30,000 U/mL; MP Biomedicals) or choline chloride (Sigma-Aldrich) was added or bacteria were grown under hypoxic conditions (5% CO_2 - 0.1% O_2 - 94.9% N_2) in a Whitley H35 Hypoxystation. *Escherichia coli* strain DH5α was cultured shaking in Luria-Bertani broth (Lennox) (LB; Sigma-Aldrich) at 37°C and 175 rpm.

Table 3.2.1: *S. pneumoniae* strains used during this study.

Strain	Serotype	Origin
D39	2	NCTC® 7466
TIGR4	4	ATCC® BAA-334
ATCC49619	19F	ATCC® 49619
R6	2^-	NCTC® 13276
85	14	Cools *et al.* (198)
88	5	Cools *et al.* (198)

3.2.2 Planktonic growth and enumeration of bacteria

Bacteria were grown in different media with or without the supplementation of catalase (500; 1,000 or 2,000 U/mL) or choline chloride (5, 7.5 or 10 mM). At different time points, samples were taken and the bacterial concentration was determined according to the viable plate count (VPC) method. Briefly, a 1:10 serial dilution (10^0 to 10^{-6}) was made in phosphate-buffered saline (PBS) in a 96-well plate. Three drops of

10 µL of a selection of dilutions was plated on BA and incubated for minimum 24 h at 37°C in 5% CO_2 before colonies were counted and suspensions were enumerated.

3.2.3 Long-living *in vitro* culturing

Bacteria from cryopreservation were plated on BA and incubated for 24 to 72 h, followed by subculturing in a tube with MHL for 8 h with a final concentration around $1*10^8$ CFU/mL. Then, bacteria were diluted to $5*10^5$ CFU/mL in fresh MHL and brought into the desired growth state. Stationary-phase bacteria were obtained by overnight growth (16 h). Exponential-phase bacteria were obtained by overnight growth (16 h), dilution to $5*10^5$ CFU/mL in fresh MHL followed by 3 h of growth (**Figure 3.3.5**).

3.2.4 Construction of knockout mutants

(i) Vector construction

The first and last 500-bp regions of the gene (*lytA* or *spxB*) were amplified from *S. pneumoniae* D39 chromosomal DNA by PCR using Q5 high-fidelity DNA polymerase (New England Biolabs). A kanamycin resistance cassette was amplified from pSt-K and a streptomycin resistance cassette from pGMC5-SM-RFP-PFurA-GFP-streptomycin. The PCR primers contained overhang sequences with the antibiotic resistance marker (kanamycin resistance cassette for *lytA* and streptomycin resistance cassette for *spxB*) and the pGEM-T Easy vector (Promega) (**Table 3.5.1**). The first and last 500 bp of the gene and the antibiotic resistance cassette were then introduced into the pGEM-T Easy vector using HiFi DNA assembly (New England Biolabs), resulting in plasmids pLytA and pSpxB containing the target genes disrupted with an antibiotic resistance cassette (**Figure 3.2.1**). The plasmids were used to transform chemocompetent *E. coli* DH5a. The resultant plasmid was verified by PCR and sequencing and used to transform *S. pneumoniae* D39.

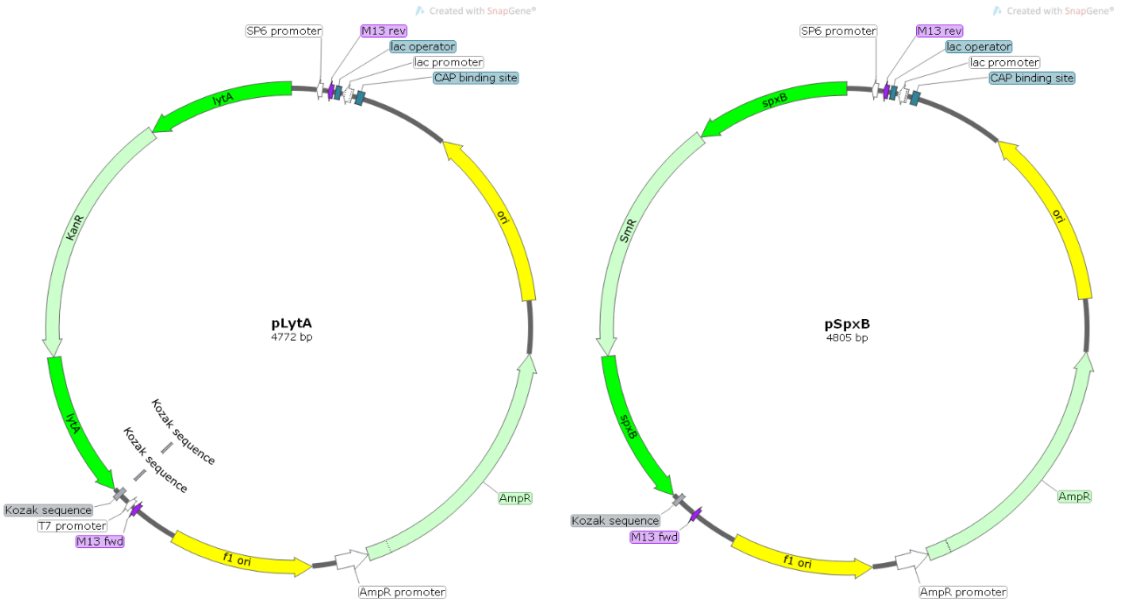


Figure 3.2.1: **Schematic overview of the constructed plasmids to generate knockout mutants of *lytA* (pLytA) and *spxB* (pSpxB) in *S. pneumoniae* D39.** The plasmid contains the first and last 500 bp of the gene (*lytA* or *spxB*) disrupted by an antibiotic resistance marker (kanamycin cassette for *lytA* and streptomycin cassette for *spxB*).

(ii) Transformation

Precompetent *S. pneumoniae* cells were obtained by growing *S. pneumoniae* in THY to $3*10^8$ CFU/mL from a starting concentration of $1*10^6$ CFU/mL. Then, the bacterial suspension was diluted 1:100 in competence medium (THY supplemented with 0.2% bovine serum albumin and 0.01% $CaCl_2$), 10% glycerol (Sigma-Aldrich) was added, and bacteria were stored at -80°C. For transformation, precompetent *S. pneumoniae* were thawed, competence-stimulating peptide 1 (CSP-1) was added (2.5 µg/mL), and natural competence was induced by incubation at 37°C in a water bath. After 20 min, 200 ng of plasmid DNA was added and bacteria were incubated for an additional 60 min at 30°C and transferred to 37°C for 90 min before plating on BA containing 400 µg/mL of kanamycin (*lytA*) (Sigma-Aldrich) or 200 µg/mL of streptomycin (*spxB*) (Sigma-Aldrich). Colonies that had taken up the plasmid via natural competence and subsequently had replaced the functional gene with the kanamycin- or streptomycin-disrupted gene via homologous recombination, could be selected through resistance towards kanamycin or streptomycin. The mutation was confirmed by sequencing and by quantitative PCR (qPCR) (**Figure 3.5.1**).

3.3 Results

3.3.1 *S. pneumoniae* D39 dies fast after entering the stationary phase in the growth media BHI and THY

Before we could study persistence, we needed to obtain long-living cultures to avoid any confounding effects of mortality through the self-limiting nature of *S. pneumoniae.* As a first step, we compared survival over 48 hours between two commonly used growth media, BHI and THY (3,53). As expected, after the regular growth in exponential phase (0-8 h) (53,195), a phase of significant killing was observed when entering the stationary phase (8-16 h). Survival was relatively stable for both growth media between 16-24 h, but at 48 h almost no surviving bacteria were detected in THY and no survivors were detected in BHI (**Figure 3.3.1**).

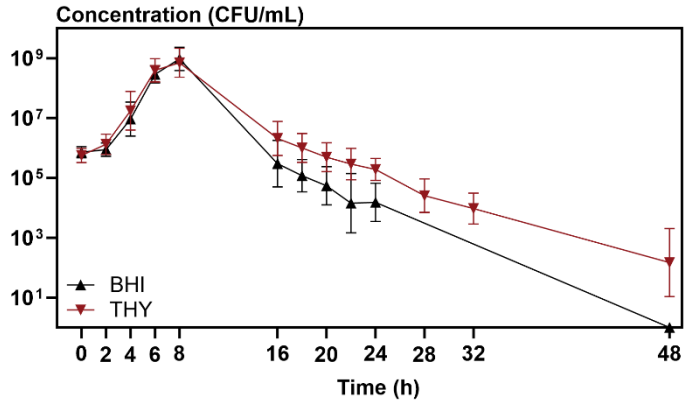


Figure 3.3.1: **No stable stationary phase survival is established when *S. pneumoniae* was grown in BHI or THY.** We compared the planktonic growth curves of *S. pneumoniae* D39 in BHI (Brain-Heart Infusion broth) and THY (Todd-Hewitt broth supplemented with 0.5% Yeast extract). After 8 h of growth, a fast killing phase is observed followed by a relatively stable period between 16 and 24 h. Three biological repeats of the experiment were performed and each value is presented as the mean ± standard deviation (n =3).

3.3.2 Counteracting the enzyme pyruvate oxidase does not improve survival

We used different strategies targeting pyruvate oxidase (SpxB) to prolong survival of *S. pneumoniae* in the growth media BHI and THY (**Figure 3.3.2**). First, we added catalase (1,000 U/mL) to neutralize the produced H_2O_2. The addition of catalase did not prevent the fast killing phase after 8 h of incubation. While a section of the stationary phase was reasonably stable when adding catalase (5- to 20-fold reduction in bacterial

concentration from 16 to 24 h), although comparable to the control, the bacterial concentration was nevertheless reduced up to 1,000-fold before reaching such a stable period (**Figure 3.3.2, A and B**). Similarly to catalase, the *spxB* knockout mutant or hypoxic incubation (5% CO_2 - 0.1% O_2 - 94.9% N_2) did not result in an improved survival (**Figure 3.3.2, C and D**). Together, we were not able to counteract the function of SpxB suggesting that H_2O_2 is not the prime cause of the self-limiting nature of *S. pneumoniae* under our conditions, in the growth media BHI and THY.

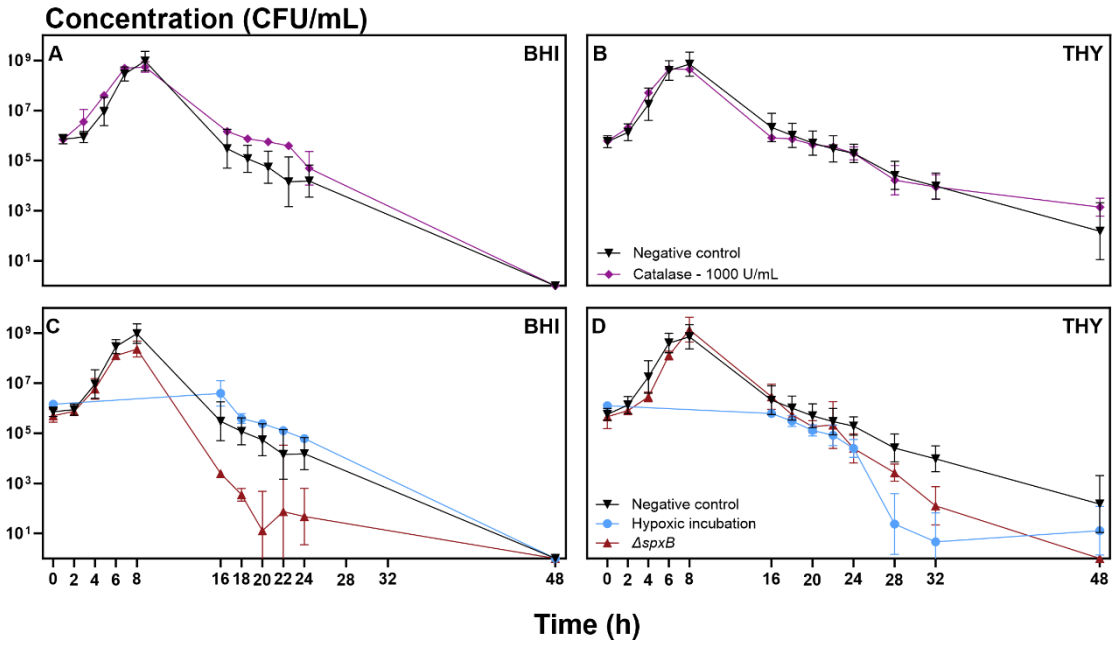


Figure 3.3.2: **The self-limiting *in vitro* nature of *S. pneumoniae* is not counteracted in BHI and THY by different strategies adopted to avoid killing by H_2O_2.** We compared the effect of different strategies to counteract pyruvate oxidase in planktonic growth curves of *S. pneumoniae* D39 in BHI (Brain Heart Infusion broth, **A and C**) and THY (Todd-Hewitt broth supplemented with 0.5% Yeast extract, **B and D**). The strong reduction of viable bacteria after 8 hours of growth could not be avoided, despite the addition of catalase (**A and B**), constructing a *spxB* knockout mutant (**C and D**) or by applying hypoxic incubation (5% CO_2 - 0.1% O_2 - 94.9% N_2, **C and D**) in the media BHI and THY. Two or more biological repeats of the experiment were performed and each value is presented as the mean ± standard deviation (n ≥ 2).

3.3.3 Different strategies adopted to avoid autolysis do not improve survival either

Next, we tried to counteract the effects of the enzyme autolysin to prevent *in vitro* autolysis in stationary-phase cultures (**Figure 3.3.3**) (65,67,69,197). The first applied strategy was choline chloride supplementation (10 mM) to prevent binding of autolysin to the cell wall and thus the cleavage of

peptidoglycan. Secondly, we constructed a *lytA* knockout mutant to inhibit the expression of autolysin. Our observations depended on the used growth medium. We observed significant killing in BHI after *S. pneumoniae* entered the stationary phase, despite the addition of choline chloride or the use of a knockout mutant. In THY, stationary-phase lysis was reduced for the *lytA* knockout mutant with a relatively stable bacterial concentration between 16-22h, although a 1 log reduction in bacterial concentration between 8-16 h was still present. Overall, survival did not improve substantially for any of the tested strategies and therefore, we could not attribute the self-limiting nature of *S. pneumoniae* to LytA.

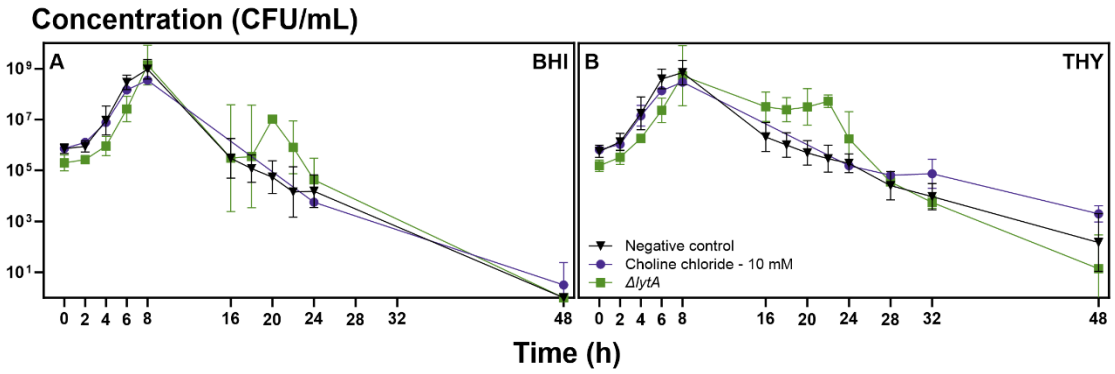


Figure 3.3.3: **The self-limiting *in vitro* nature of *S. pneumoniae* is not counteracted in BHI and THY by different strategies adopted to avoid autolysis.** We compared the effect of different strategies to counteract the effects of autolysin in planktonic growth curves of *S. pneumoniae* D39 in BHI (Brain Heart Infusion broth, **A**) and THY (Todd-Hewitt broth supplemented with 0.5% Yeast extract, **B**). The strong reduction of viable bacteria after 8 hours of growth is still observed despite the adopted strategies. Two or more biological repeats of the experiment were performed and each value is presented as the mean ± standard deviation ($n \geq 2$).

3.3.4 Mueller-Hinton broth supplemented with 5% Lysed horse blood (MHL) abolishes the self-limitation of *S. pneumoniae* until 32 hours of growth

Finally, we tried another growth medium, namely Mueller-Hinton broth supplemented with 5% Lysed horse blood (MHL) as our goal to obtain a stable stationary phase was not reached yet. It is the recommended medium by the Clinical & Laboratory Standards Institute (CLSI) for antibiotic susceptibility testing of *S. pneumoniae,* but its use is less common in research laboratories focusing on other *S. pneumoniae* traits. Surprisingly, when using the less common growth medium MHL, *in vitro* self-limitation was mostly absent during 32 h of incubation. Only after 32 h, a strong death phase occurred with a 10,000- to 1,000,000-fold

reduction in bacterial viability resembling bacterial concentrations after 48 h in the media THY and BHI (**Figure 3.3.4**).

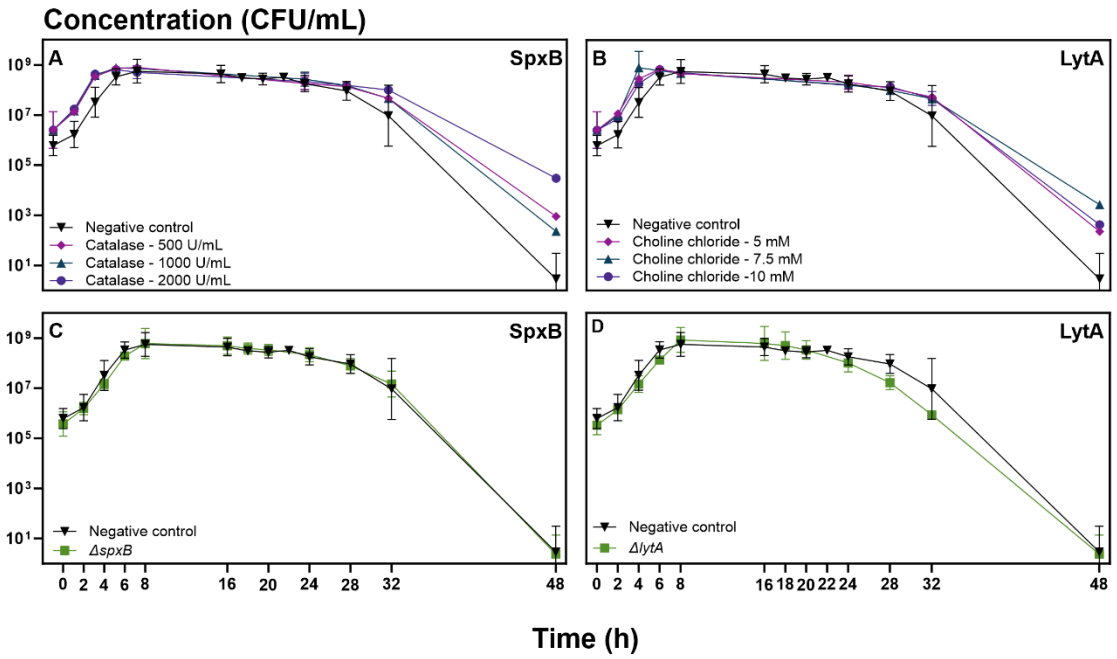


Figure 3.3.4: **MHL abolishes the self-limiting *in vitro* nature of *S. pneumoniae*.** We compared the effect of different strategies to counteract the effects of pyruvate oxidase (**A and C**) or autolysin (**B and D**) in planktonic growth curves of *S. pneumoniae* D39, but none of the strategies could improve survival between 32 and 48 h of growth. Three biological repeats of the experiment were performed, and values are presented as means ± standard deviations (n = 3).

We tried to prolong stable stationary phase survival from 32 to 48 h by adopting different strategies to counteract pyruvate oxidase or autolysin. First, we added different concentrations of catalase (500; 1,000 and 2,000 U/mL), but we did not observe any significant impact on survival by the addition of catalase. Neither the *spxB* knockout mutant or hypoxic incubation (5% CO_2 - 0.1% O_2 - 94.9% N_2) resulted in an improved survival. Secondly, we tried to abolish the effects of LytA by choline chloride supplementation (5, 7.5 and 10 mM) or by the construction of a *lytA* knockout mutant. Again, killing after 32 h of growth could not be avoided. In conclusion, counteracting the suggested effectors of self-limitation in *S. pneumoniae* to prevent *in vitro* death did not lead to prolonged survival under the tested conditions, but when bacteria were grown in MHL, stationary phase survival was stable up to 32 h.

3.3.5 The optimized long-living *in vitro* model

After following *in vitro* survival of *S. pneumoniae* in different culture conditions, we conclude that MHL seems to be the optimal liquid growth medium to obtain a stable long-term bacterial culture. Therefore, we optimized a model based on MHL as growth medium (**Figure 3.3.5**). Briefly, cryopreserved *S. pneumoniae* bacteria were plated on a blood agar plate, followed by inoculation into a tube with MHL. After 8 h of static incubation, the culture was diluted to $5*10^5$ CFU/mL and grown overnight. The overnight culture could be directly used as a stationary-phase culture, diluted 1:10 in fresh MHL to act as a diluted stationary-phase sample or could be diluted to $5*10^5$ CFU/mL in fresh MHL and grown 3 h to obtain an exponential-phase culture.

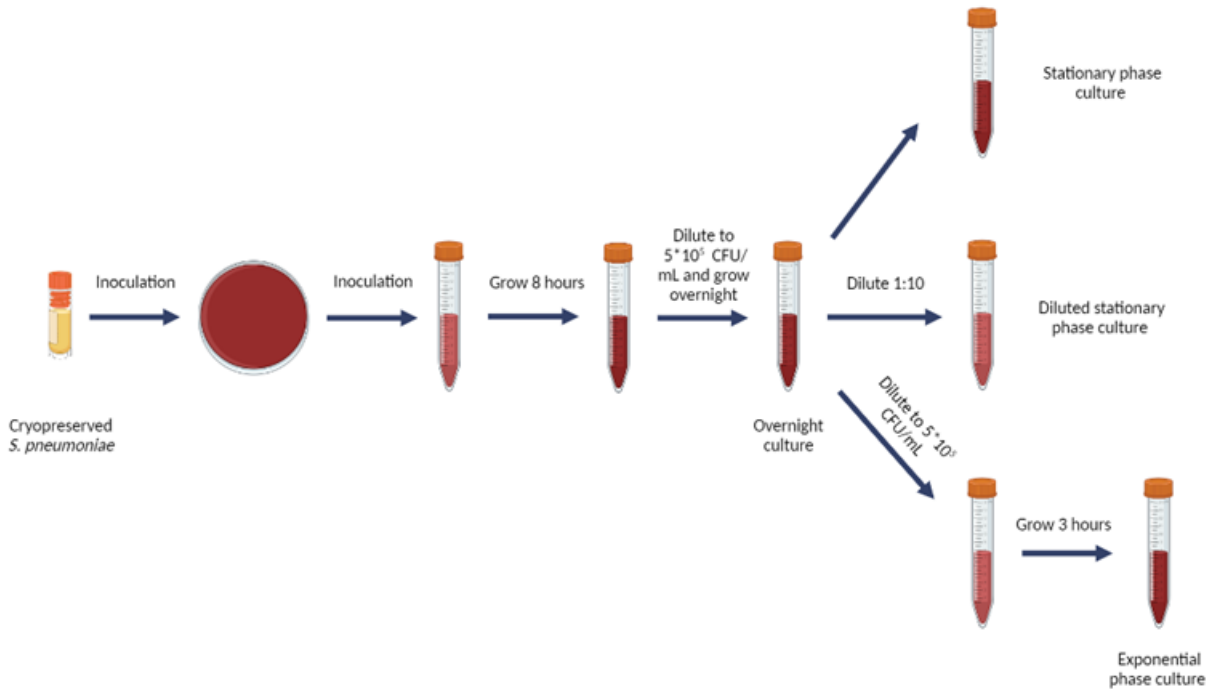


Figure 3.3.5: **Overview of the long-living *in vitro* protocol for stable *S. pneumoniae* bacterial cultures.** Cryopreserved *S. pneumoniae* bacteria are plated on a blood agar plate, followed by inoculation in a tube with MHL. After 8 h of static incubation, the culture is diluted to $5*10^5$ CFU/mL and grown overnight. The overnight culture is either directly used as a stationary-phase culture, diluted 1:10 in fresh MHL to act as a diluted stationary-phase culture, or is diluted to $5*10^5$ CFU/mL in fresh MHL and grown for 3 h to obtain an exponential-phase culture. *MHL, Mueller-Hinton broth (cation-adjusted) + 5% lysed horse blood.*

To validate the model for survival over 24 h and generalize our first results in strain D39, growth curves were obtained for five *S. pneumoniae* strains (**Figure 3.3.6**). These strains are either commonly used lab

strains (TIGR4, ATCC 49619 and R6) or lab strains from previous studies (85 and 88) (**Table 3.2.1**) (48). While these strains show small differences in lag phase, growth rate, and maximal bacterial concentration, overall survival over 24 h is stable among all strains (**Figure 3.3.6**). In conclusion, the optimized long-living model results in a stable bacterial culture until 24 h of growth, which will enable us to execute prolonged time-killing experiments without the self-limiting nature of *S. pneumoniae* as confounding factor.

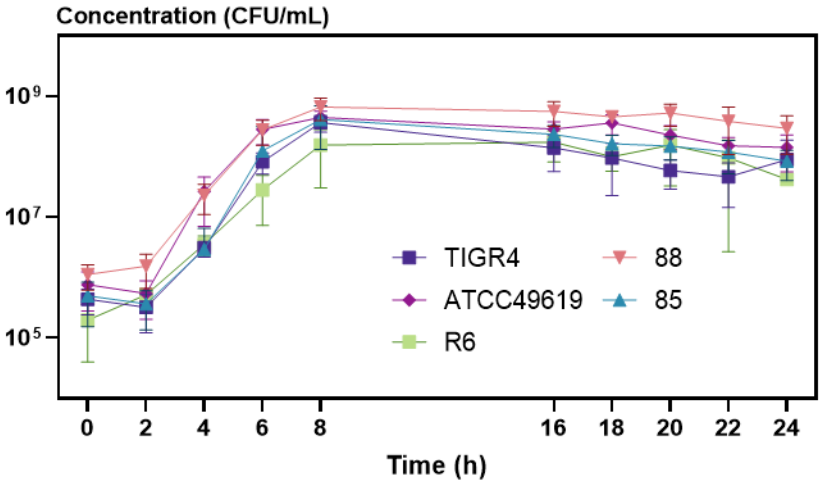


Figure 3.3.6: V**arious *S. pneumoniae* lab strains show robust growth dynamics for up to 24 h of incubation using the optimized long-living *in vitro* model.** Planktonic growth curves of *S. pneumoniae* TIGR4, ATCC 49619, R6, 85 and 88 as a function of time show small differences in lag phase, growth rate, and maximal bacterial concentration, but a stable survival over 24 h. Three biological repeats of the experiment were performed, and values are presented as means ± standard deviations (n = 3).

3.4 Discussion

3.4.1 The disagreement on *in vitro* self-limitation of *S. pneumoniae*

S. pneumoniae is a fastidious, self-limiting organism which makes it difficult to grow and maintain *in vitro* (195). Nonetheless, a high density bacterial culture for a prolonged time can be desirable, for example to obtain an appropriate amount of viable cells to inoculate animals (63,195), in the routine clinical practice to recover *S. pneumoniae* as causative agent (57,58) or, as in this study, to investigate bacterial persistence (199–201). Several causes are suggested that render *S. pneumoniae* a fastidious and self-limiting organism. Firstly, *S. pneumoniae* has a small genome and lacks many genes necessary for aerobic growth, such as genes coding for the enzymes catalase and NADPH peroxidase. *S. pneumoniae* is therefore classified as a facultative anaerobic bacterium (195). In aerobic environments, *S. pneumoniae* produces H_2O_2 up to millimolar concentrations, primarily via the enzyme pyruvate oxidase (SpxB) and to a lesser extent via the enzyme lactate oxidase (LctO) (51,63,64). The lack of the neutralizing enzyme catalase combined with the production of high concentrations of H_2O_2 was suggested as an important cause of *in vitro* death of *S. pneumoniae* via a mechanism reminiscent of apoptosis with externalization of anionic phospholipids, loss of DNA and typical morphological features (61,63,196). Another proposed explanation is the presence of autolysin (LytA) (65,67,69). Autolysin is involved in stationary-phase autolysis and penicillin- and vancomycin induced lysis through the cleavage of peptidoglycan resulting in hydrolysis of the cell wall (65). Next to the lytic pathway leading to cell death, a non-lytic pathway is also involved after antibiotic induced inhibition of transpeptidase via the formation of long peptidoglycan strands followed by precursor and energy depletion (141,143). The spontaneous *in vitro* death of *S. pneumoniae* remains a controversial and unresolved issue throughout history, with different research groups suggesting a variety of causes and solutions (61,64,195). During this study, we therefore investigated different ways to counteract with either SpxB or LytA to evaluate the effect on *in vitro* survival of *S. pneumoniae* to reach stable stationary-phase survival.

3.4.2 Counteracting pyruvate oxidase or autolysin does not impact *in vitro* survival

Regev-Yochay and colleagues performed different studies investigating the role of SpxB in *S. pneumoniae in vitro* survival (61,196). In short, they assessed three different approaches, catalase supplementation, growth under anaerobic conditions and a *spxB* knockout mutant, and all approaches resulted in a significant prolongation of stationary phase survival of *S. pneumoniae* until 32 to 48 hours of incubation (61,196). We assessed the effect of the same approaches on long-term *in vitro* survival in slightly different conditions (different strains and more liquid growth media) (61,196). We could not confirm the results of Regev-Yochay *et al.* (61,196). Altogether, we did not observe an improved survival under any of our test conditions, suggesting that H_2O_2 is not the major cause of the self-limiting nature of *S. pneumoniae*. An explanation could be that *S. pneumoniae* exploits, despite the lack of NADPH peroxidase and catalase, a plethora of protective systems to survive oxidative stress (63). Lisher *et al.* (2017) claim that even SpxB and LctO protect *S. pneumoniae* against exogenous H_2O_2 through their endogenous H_2O_2 production that facilitates the induction of the oxidative stress response of *S. pneumoniae* (51,63). Moreover, the reaction of SpxB results in the generation of ATP which contributes to protection against oxidative stress and the LctO reaction leads to increased pyruvate levels that stimulates the SpxB pathway (63). Also Pericone *et al.* (2003) indicate that pyruvate oxidase is required for survival during exposure to high levels of H_2O_2 and that pyruvate oxidase contributes to H_2O_2 resistance during oxidative stress via an H_2O_2-resistant energy source (62).

Although the role of autolysin during stationary-phase lysis was questioned by Regev-Yochay *et al.* (2007) (61), we investigated two different approaches, choline chloride supplementation and a *lytA* knockout mutant, to counteract the function of this enzyme similar to Mellroth *et al.* (2012) (65,67,69,202). Mellroth *et al.* (2012) observed a stable survival until 16 h of growth, both in presence of a high choline concentration (1% choline chloride) and for the *lytA* knockout mutant (65). Our findings approached the observations of Mellroth *et al.* (2012) when we followed growth of the *lytA* knockout mutant in THY (65), but there was no tested approach sufficient to prevent the fast *in vitro* killing of *S. pneumoniae* in BHI or THY when entering the stationary phase and we could not conclude that LytA has a major role in stationary phase autolysis.

3.4.3 MHL to the rescue

Our final approach was the use of a distinct growth medium, Mueller-Hinton broth supplemented with Lysed horse blood (MHL). Surprisingly, stable stationary phase survival was reached up to 32 h of growth without the need of additional actions. We tested the same strategies targeting SpxB or LytA as we did for the growth media BHI and THY, but also in MHL no substantial improvements were achieved.

Table 3.4.1: **Comparison of the composition of the used liquid growth media**. *BHI, Brain-Heart Infusion broth; THY, Todd-Hewitt broth supplemented with 0.5% yeast extract; MHL, Mueller-Hinton broth supplemented with 5% lysed horse blood.*

	BHI	THY	MHL
NUTRIENT SOURCE	Brain infusions Heart infusions	Heart infusions Yeast extract	Beef extract
ENERGY SOURCE	Glucose	Dextrose	Starch (hydrolysis to dextrose)
OSMOTIC STABILITY/PH	Sodium chloride Disodium phosphate	Sodium chloride Disodium phosphate	Calcium Magnesium

All growth media contain a source of amino acids, nitrogen, vitamins, growth factors and carbon and sugar or starch (after hydrolysis to dextrose during autoclavation) as energy source. Starch will also neutralize toxic substance that could interfere with the effect of the tested antibiotics (**Table 3.4.1**) (203–205). Overall, all growth media are nutrient-rich and the most likely explanation of the superior survival of *S. pneumoniae* in MHL is the presence of lysed horse blood which is a source of catalase with an approximate intracellular concentration of 11 µM (195,206–208). Besides, red blood cells are a source of many other antioxidants, such as glutathione and peroxiredoxin 2 (207). We based our final long-living *in vitro* protocol on the use of MHL (**Figure 3.3.5**) and we used this model in **CHAPTERS 4, 5 and 6**.

Our findings do not clarify the cause of spontaneous *in vitro* death of *S. pneumoniae*, as none of the strategies seemed to significantly impact stationary phase survival, but rather contribute to the uncertainty

of the self-limitation of *S. pneumoniae* (61,64,195). One explanation could be that both SpxB and LytA contribute to the self-limiting nature of *S. pneumoniae* and should be simultaneously inhibited before significant effects on stationary phase survival would be observed. We could either try to interfere with both enzymes at the same time, by the supplementation of choline and catalase at the same time or by the construction of a double knockout mutant. On the other hand, we could inhibit either SpxB or LytA followed by measuring the activity of the enzyme that is not inhibited, for example measuring the activity of LytA during catalase supplementation. To further explore the field, across SpxB and LytA, we could assess *in vitro* survival for a large set of clinical strains followed by genetic analysis to look into genes or mutations associated with increased killing or survival in the stationary phase. We could follow the approach of Lisher *et al.* (2017) who used microarray analysis to compare the difference in gene expression under aerobic and anaerobic conditions (51). We also could include the three liquid growth media described in this chapter during these survival experiments to include an extra variable in the experiments that clearly affects survival. Moreover, to study the influence of lysed horse blood on bacterial survival, we could include a fourth growth medium, Mueller-Hinton broth without supplementation with blood. These suggested strategies to further investigate the self-limiting nature of *S. pneumoniae* were not studied during this PhD, due to time restrictions and as our goal to obtain a long-living model to study persistence was obtained.

In conclusion, the optimized long-living model based on MHL as growth medium results in a stable bacterial culture until 24 h of growth, which enabled us to execute prolonged time-killing experiments in CHAPTER 4 without the self-limiting nature of *S. pneumoniae* as a confounding factor.

3.5 Supplementary data

Table 3.5.1: **Primers used for plasmid construction and validation of the *spxB* and *lytA* knockout mutants.**

Primer	Sequence
Construction of pLytA	
For_lytA_first500	5'-TCCCGTTGAATATGGCTCATCCATTTAGCAAGATATGGATAAGGGTCAAC-3'
Rev_lytA_first500	5'-TATGGTCGACCTGCAGGCGGCCGCGAATTCACTAGTGATTATGGAAATTAATGTGAGTAAATTAAGAACAGATTTGCCTCAAGT -3'
For_kan	5'- ATCCATATCTTGCTAAATGGATGAGCCATATTCAACGGGAAACG -3'
Rev_kan	5'- TTCTCAATATCATGCTTAAATTAGAAAAACTCATCGAGCATCAAATGAAACT -3'
For_lytA_last500	5'-GCATGCTCCCGGCCGCCATGGCGGCCGCGGGAATTCGATTTTATTTTACTGTAATCAAGCCATCTGGCTCTACT- 3'
Rev_lytA_last500	5'- TGCTCGATGAGTTTTTCTAATTTAAGCATGATATTGAGAACGGCTTGAC -3'
Construction of pSpxB	
For_spxB_first500	5'-TATGGTCGACCTGCAGGCGGCCGCGAATTCACTAGTGATTATGACTCAAGGGAAAATTACTGCATCTG -3'
Rev_ spxB_first500	5'- GCGATCACCGCTTCCCTCATGAAGTTTACTGGAATTTCAACAACAGCTGG -3'
For_strep	5'- CGATCTGGATTGTCTTTCTTTTATTTGCCGACTACCTTGGTGATCT -3'
Rev_strep	5'- TTGAAATTCCAGTAAACTTCATGAGGGAAGCGGTGATCGCC -3'
For_ spxB _last500	5'- CCAAGGTAGTCGGCAAATAAAAGAAAGACAATCCAGATCGCCAAG -3'
Rev_ spxB _last500	5'-GCATGCTCCCGGCCGCCATGGCGGCCGCGGGAATTCGATTTTATTTAATTGCGCGTGATTGCAATCCTTCTTCTTCCA -3'
cPCR to check integration	
For_lytA_cPCR	5'- TGCGCTGTTCTGATTTGAAAGA -3'
Rev_lytA_cPCR	5'- AAAGGAGTTTCTGGTTCTGGAT -3'
For_spxB_cPCR	5'-TATGGTCGACCTGCAGGCGGCCGCGAATTCACTAGTGATTATGACTCAAGGGAAAATTACTGCATCTG -3'
Rev_spxB_cPCR	5'-GCATGCTCCCGGCCGCCATGGCGGCCGCGGGAATTCGATTTTATTTAATTGCGCGTGATTGCAATCCTTCTTCTTCCA -3'
qPCR to check expression	
For_lytA_qPCR	5'- CAGATTTGCCTCAAGTCGGC -3'
Rev_lytA_qPCR	5'- ATTCTGGGTCTTTCCGCCAG -3'
For_spxB_qPCR	5'- TCTCCGCTCTTTGCGACAAT -3'
Rev_spxB_qPCR	5'- TGTTGAATGCTCCATCACCCA -3'
For_gdh	5'- GGAGACCTGGCTAAACGCAA -3'
Rev_gdh	5'- GGTCTACGGGCAGTTCCAAT -3'

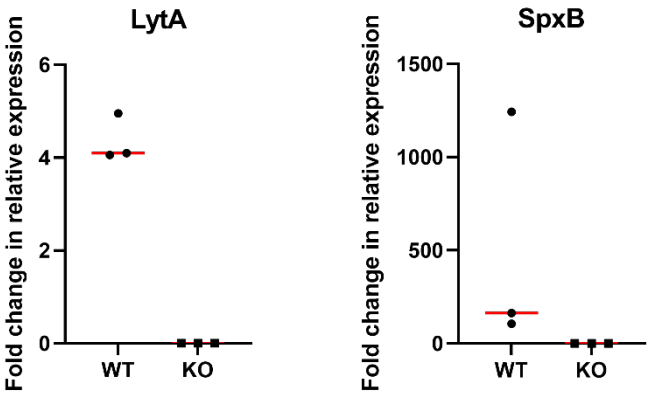
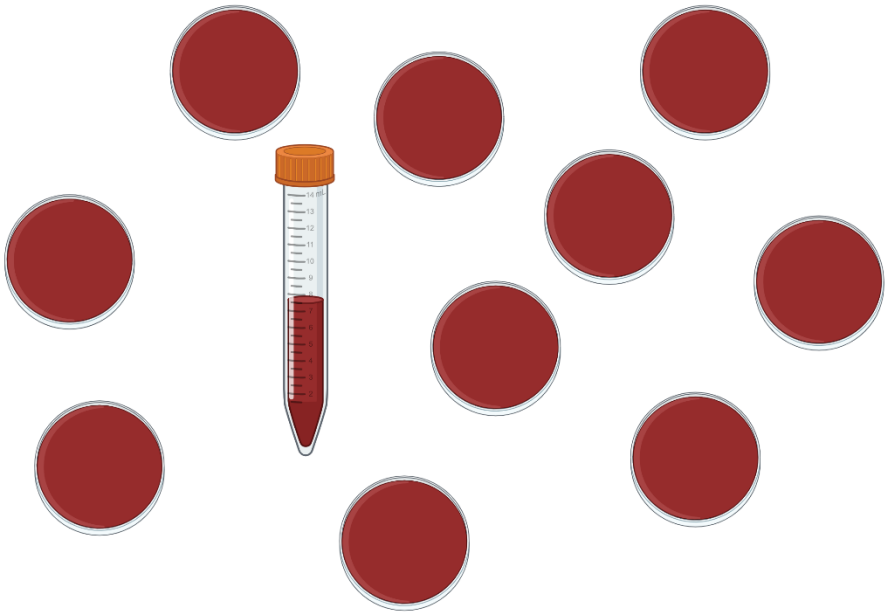


Figure 3.5.1: **The knockout mutants of the enzymes autolysin (LytA) and pyruvate oxidase (SpxB) show no mRNA expression.** The data represent the number of fold-changes in mRNA levels of knockout mutants (KO) and wild-types (WT) relative to the expression of the housekeeping gene *gdh*. The mean is given as the red line with the individual datapoints as dots. Three technical repeats of the experiment were performed (n = 1x3).

4 PERSISTER CELLS ARE PRESENT IN *STREPTOCOCCUS PNEUMONIAE* D39 CULTURES



"For this chapter, I used about 5000 blood agar plates. After this, I stopped counting…"

Adapted from Nele Geerts, Linda De Vooght, Ioannis Passaris, Peter Delputte, Bram Van den Bergh, Paul Cos, *Antibiotic Tolerance Indicative of Persistence Is Pervasive among Clinical Streptococcus pneumoniae Isolates and Shows Strong Condition Dependence.* Microbiol Spectr. 2022 Dec 21;10(6):e0270122. doi: 10.1128/spectrum.02701-22. Epub 2022 Nov 14. PMID: 36374111; PMCID: PMC9769776.

4.1 Introduction

Antibiotic persistent bacteria are a subpopulation of cells that transiently switch to a nongrowing state that enables them to survive treatment with a bactericidal drug concentration. Persisters are phenotypic variants but are genetically identical to the bulk population (135,147,209). As a consequence, antibiotic persisters can transform back into antibiotic-susceptible bacteria and, after the antibiotic pressure is removed, reconstitute a population that displays an antibiotic tolerance identical to that of the starting culture (135,147). Persister cells seem to be a universal feature of clonal life forms. Not only are they identified in many, if not all, bacterial species that have been studied, but also eukaryotic cancer cell lines and fungal populations contain drug-tolerant phenotypic variants (147,164,170). Besides, as persisters are tolerant to lethal antibiotics, they underly the chronic nature of a variety of infections, are linked to therapy failure and even help in acquiring genetic resistance. The presence of antibiotic-tolerant persisters in the clinically important pathogen *S. pneumoniae* has not been investigated to date (137–140,142). Our goal was to determine if persister cells are present in cultures of a *S. pneumoniae* reference strain for different antibiotics and at different growth stages. In part, the lack of understanding persistence in *S. pneumoniae* stems from the self-limiting nature of this bacterium *in vitro* (30). We succeeded in obtaining stable long-living *in vitro* cultures using specific growth conditions, as described in CHAPTER 3, which allowed us to set-up prolonged antibiotic-induced killing studies without confounding the results with the self-limiting nature of *S. pneumoniae*. Using these killing studies together with heritability assays, the gold standard assays to determine persistence (135,147), we proved the presence of high numbers of persister cells in reference strain D39 cultures.

4.2 Material and Methods

4.2.1 Bacterial strains and growth conditions

S. pneumoniae reference strain D39 (serotype 2, NCTC® 7466) was used during this study. ***S. pneumoniae*** was cultured statically in cation-adjusted Mueller-Hinton broth (Fluka) supplemented with 5% lysed horse blood (MHL; Oxoid) or on blood agar (BA) plates (tryptic soy agar [Neogen] supplemented with 5% defibrinated sheep blood [Oxoid]) at 37°C in 5% CO_2.

4.2.2 Long-living *in vitro* culturing and enumeration of bacteria

Bacteria from cryopreservation stocks (BHI + 10 % glycerol) were plated on BA and incubated for 24 to 72 h, followed by subculturing in a static tube with liquid MHL for 8 h with a final, steady concentration of $1*10^8$ CFU/mL, independently of how long they grew on BA. Then, bacteria were diluted to $5*10^5$ CFU/mL in fresh MHL and brought into the desired growth state. Stationary-phase bacteria were obtained by overnight growth (16 h). Diluted stationary-phase bacteria were obtained by overnight growth (16 h) and 1:10 dilution in fresh MHL. Exponential-phase bacteria were obtained by overnight growth (16 h), dilution to $5*10^5$ CFU/mL in fresh MHL and 3 h of growth (**Figure 4.2.1**). The bacterial concentration was determined according to the viable plate count (VPC) method. Briefly, a 1:10 serial dilution (10^0 to 10^{-6}) was made in phosphate-buffered saline (PBS) in a 96-well plate. Three aliquots of 10 µL of a selection of dilutions were plated on BA and incubated for minimum of 48 h before colonies were counted and bacterial concentration (CFU/mL) was calculated.

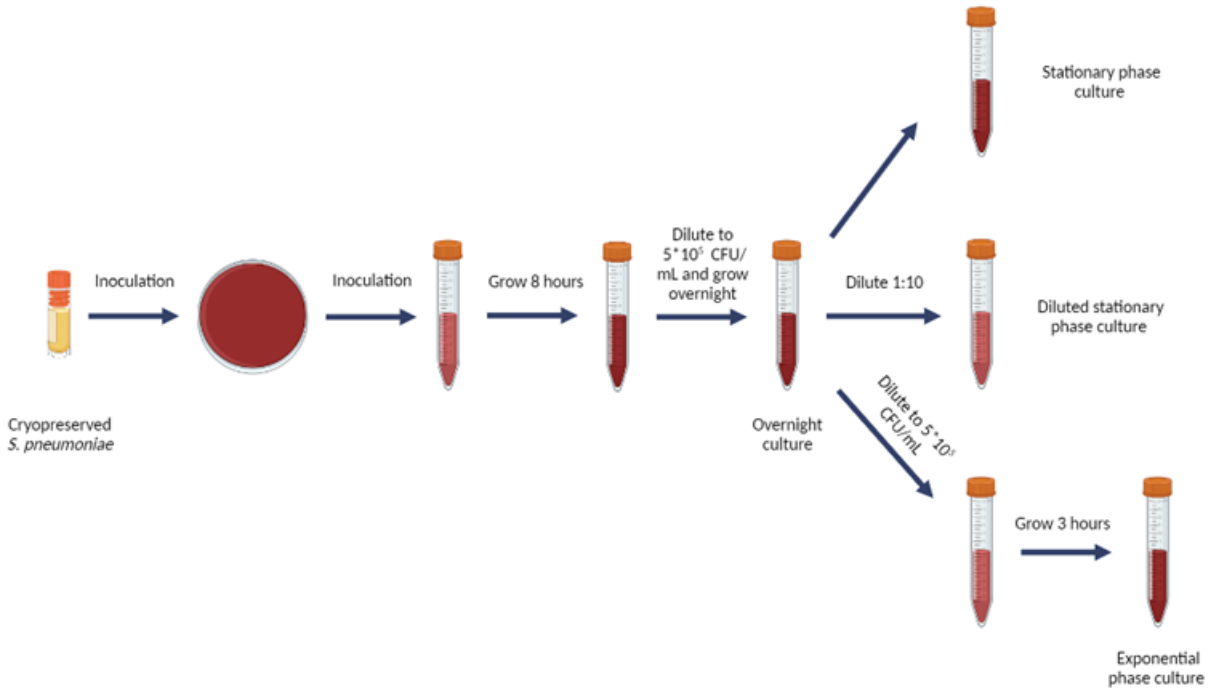


Figure 4.2.1 **Overview of the long-living *in vitro* protocol for stable *S. pneumoniae* bacterial cultures.** Cryopreserved bacteria are plated on a blood agar plate, followed by inoculation in a tube with MHL. After 8 h of static incubation, the culture is diluted to $5*10^5$ CFU/mL and grown overnight. The overnight culture either is directly used as a stationary-phase culture, diluted 1:10 in fresh MHL to act as a diluted stationary-phase culture, or is diluted to $5*10^5$ CFU/mL in fresh MHL and grown for 3 h to obtain an exponential-phase culture.

4.2.3 Antibiotic susceptibility

AMR can be measured by the lowest concentration of an antibiotic that inhibits bacterial growth or the minimum inhibitory concentration (MIC). When a bacterium is resistant, the MIC is increased. Here, we used the MIC_{90} indicating the antibiotic concentration needed to inhibit growth of 90%, of the bacterial culture. The MICs (90 % of growth reduction compared to the positive control) of standard antibiotics were determined using a resazurin assay as described previously (210). Therefore, amoxicillin (Sigma-Aldrich; beta-lactam antibiotic), cefuroxime (Sigma-Aldrich; beta-lactam antibiotic), moxifloxacin (Sigma-Aldrich; fluoroquinolone) and vancomycin (Sigma-Aldrich; glycopeptide) were used. Briefly, a 1:2 serial dilution of the antibiotic was made in triplicates in MHL in a 96-well plate with a final volume of 100 µL. Then, 100 µL of a bacterial suspension was added to each well, except to negative-control wells, to a final concentration of $5*10^5$ CFU/mL in 200 µL. Positive-control wells contained 200 µL of bacterial suspension ($5*10^5$ CFU/mL)

without antibiotics, and negative-control wells contained 200 µL of MHL without antibiotics or bacteria. Plates were incubated at 37°C and 5% CO_2 for 20 h before 20 µL of a 0.005% resazurin solution was added. Plates were further incubated for 90 min and fluorescence was measured (λ_{em} = 590 nm; λ_{ex} = 520 nm) using a spectrophotometer (Promega; Discover).

4.2.4 Dose-dependent and time-dependent kill curves

To obtain dose-dependent kill curves, *S. pneumoniae* cultures were treated for 5 h in the stationary or diluted stationary growth phase with five different antibiotic concentrations (**Table 4.2.1**). After 5 h, bacterial suspensions were centrifuged, resuspended in PBS to wash away antibiotics, and bacterial concentration (CFU/mL) was determined by plating. Colonies were counted after a minimum of 48 h of incubation.

Table 4.2.1: **Antibiotic concentrations (µg/mL) that were used, given as X fold the MIC, for treatment of reference strain D39 to obtain dose-dependent kill curves.**

ANTIBIOTIC	5X	10X	20X	100X	200X
AMOXICILLIN	0.03	0.07	0.14	0.7	1.4
CEFUROXIME	0.14	0.27	0.54	2.7	5.4
MOXIFLOXACIN	1.4	2.9	5.8	29	58
VANCOMYCIN	2.2	4.4	8.8	44	88

To obtain time-kill curves, *S. pneumoniae* was treated in the stationary, diluted stationary or exponential growth state with a fixed antibiotic concentration (100-fold the MIC, **Table 4.2.1**). Bacterial suspensions were incubated up to 24 h. At specified time points, bacterial concentration was determined via plating after centrifugation and resuspension in PBS to wash away the antibiotics. Colonies were counted after a minimum of 48 h of incubation.

4.2.5 Heritability assay

For each condition (growth phase x antibiotic), 5 surviving clones from the initial time-kill experiment were isolated from the blood agar plate from the second killing phase (after 6 h of treatment for the diluted stationary phase and after 18 h of treatment for the exponential phase), regrown in fresh MHL without antibiotics, and stored at -80°C. These bacterial clones were subjected to the same protocol as in the initial time-kill assay. For one of these clones per condition, a time-kill curve was obtained and the MIC value was determined. For the other 4 clones, a fixed time point (6 or 18 h of treatment) was chosen to determine survival levels via plating.

4.2.6 Data analysis and statistics

Student's t test, one-way ANOVA, mixed-effect analysis, or two-way ANOVA was used to compare continuous variables (MIC values and time-kill curves) in GraphPad Prism version 9. A difference between two groups was considered statistically significant when the P value was < 0.05. The R packages *nls.multstart*, *broom*, and *purrr* were used to analyze the time-kill curves mathematically by comparing two models of killing, a uniphasic model with a single killing rate and a biphasic model with two killing rates. The nonlinear fixed-effect model used the $\log_{10}$-transformed fraction of surviving cells. The biphasic model was based on the equation $Log(Y) = log((N-P_0)^{(-kn*t)}+P_0^{(-kp*t)})$ and the monophasic model on $Log(Y) = log((N)^{(-kn*t)})$ with Y survival fraction, t treatment time (in hours), P_0 persister fraction at $t = 0$ and k_n and k_p the killing rate of normal and persister cells (logCFU per hour). Curves were considered biphasic if biphasic fitting was better than uniphasic fitting according to ANOVA (F test), the Akaike information criterion (AIC), the Bayesian information criterion (BIC), and the log likelihood (LogLik). The raw data are available on Zenodo via the following link: https://doi.org/10.5281/zenodo.7147832.

4.3 Results

4.3.1 Persisters are widely present in *S. pneumoniae* reference strain D39 cultures and highly dependent on growth phase and type of antibiotic.

To study persister cells, the concentration of the applied bactericidal antibiotics needs to be well above the MIC to invoke killing of sensitive cells. Reference strain D39 is sensitive to amoxicillin, cefuroxime, moxifloxacin, and vancomycin, i.e. clinically relevant antibiotics of various classes, according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints (**Table 4.3.1**). To evaluate antibiotic-induced killing, an excess of such MICs was thus applied in further experiments (180,211–213). Along with the selection of antibiotics and their concentrations, we tested the effect of different growth phases on treatment of *S. pneumoniae* with the antibiotics. Three growth conditions are frequently used to score persister levels and arguably cover all possible growth conditions: the stationary phase, the diluted stationary phase, and the exponential phase (199–201). The protocol we used to obtain these different growth phases is described in **Figure 4.2.1**.

Table 4.3.1: ***S. pneumoniae* D39 is susceptible to the antibiotics amoxicillin, cefuroxime, moxifloxacin and vancomycin according to the EUCAST breakpoints.** Minimum inhibitory concentration (MIC, µg/mL) of *S. pneumoniae* D39 before and after the initial time-kill curve experiment. Values represent mean ± SD (n = 3). The MIC value was determined for one randomly selected surviving clone of the initial time-kill assay. MIC values before and after the initial time-kill experiment did not significantly differ (Student's T-test), except for the MIC for moxifloxacin in the diluted stationary growth phase that was significantly lower for the repeated experiment (p = 0.001). AB = antibiotic.

MIC (µg/mL)	EUCAST BREAKPOINTS		D39	CLONE AFTER AB TREATMENT IN DILUTED STATIONARY PHASE	CLONE AFTER AB TREATMENT IN EXPONENTIAL PHASE
	Sensitive (µg/mL)	Resistant (µg/mL)			
AMOXICILLIN	≤ 0.5	> 1	0.007 ± 0.002	0,009 ± 0,004	0,010 ± 0,002
CEFUROXIME	≤ 0.25	> 0.25	0.022 ± 0.005	0,031 ± 0,003	0,034 ± 0,018
MOXIFLOXACIN	≤ 0.5	> 0.5	0.233 ± 0.006	**0,071 ± 0,039**	0,282 ± 0,075
VANCOMYCIN	≤ 2	> 2	0.450 ± 0.111	0,426 ± 0,080	0,588 ± 0,155

To evaluate the minimal dose needed to kill sensitive *S. pneumoniae* D39 cells within 5 h, we obtained dose-dependent kill curves by treating *S. pneumoniae* with increasing antibiotic concentrations, i.e., 5-, 10-, 20-, 100-, and 200-fold the MIC. Stationary-phase cultures proved insensitive to any of the antibiotics used, even at the highest dose, but treatment of diluted stationary-phase samples resulted in significant killing of sensitive cells at or above a concentration of 5-fold the MIC (**Figure 4.3.1**). The independence on antibiotic concentration, once a sufficient dose is reached to kill sensitive cells, is a typical hallmark of persistence, while strong correlations would point toward antibiotic resistance as the underlying cause of survival (135). In this case, the independence on antibiotic concentration could be the first indication of the presence of persister cells within *S. pneumoniae* D39 cultures. For the remainder of our work, we chose to apply concentrations of 100-fold the MIC to ensure proper killing of sensitive cells and to level out small differences in threshold concentrations needed to reach a plateau, regarding further experiments in CHAPTER 6 with clinical strains.

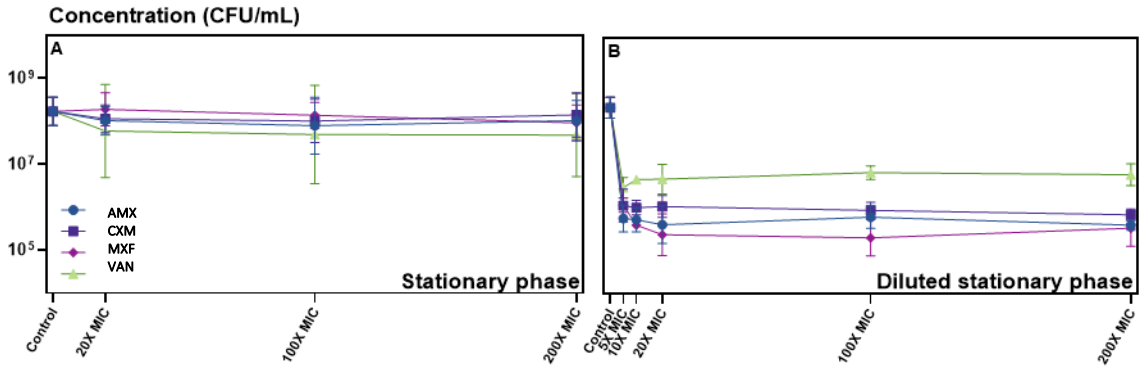


Figure 4.3.1: ***S. pneumoniae* D39 in stationary phase is insensitive to antibiotic treatment, while a dose of 5-fold the MIC is sufficient to kill sensitive cells in a diluted stationary-phase culture.** Dose-dependent kill curves with amoxicillin, cefuroxime, moxifloxacin, and vancomycin of planktonic *S. pneumoniae* D39 are shown in stationary-phase (A) or diluted stationary-phase (B) samples. Antibiotic treatments lasted for 5 h before survivors were enumerated. Applied concentrations were 5-, 10-, 20-, 100-, and 200-fold the MIC (respectively, 0.03, 0.07, 0.14, 0.70, and 1.40 µg/mL for amoxicillin; 0.14, 0.27, 0.54, 2.7, and 5.4 µg/mL for cefuroxime; 1.4, 2.9, 5.8, 29, and 58 µg/mL for moxifloxacin; and 2.2, 4.4, 8.8, 44, and 88 µg/mL for vancomycin). The y axis of panel B corresponds to the y axis of panel A. Three biological repeats of the experiment were performed, and values are presented as means ± standard deviations (n = 3).

4.3.2 *Streptococcus pneumoniae* D39 cultures contain high numbers of persisters.

To investigate the presence of persister cells, we followed survival of cells as a function of time during antibiotic treatment. If the bacterial culture is fully susceptible, the so-called time-kill curves should show a single rate of killing (uniphasic pattern) that describes the population-level tolerance without any subpopulation with increased tolerance (persister cells). However, if a subpopulation of antibiotic-tolerant persister cells is present within the susceptible population, we expect distinctly different killing rates to be apparent in the time-kill curves (biphasic pattern).

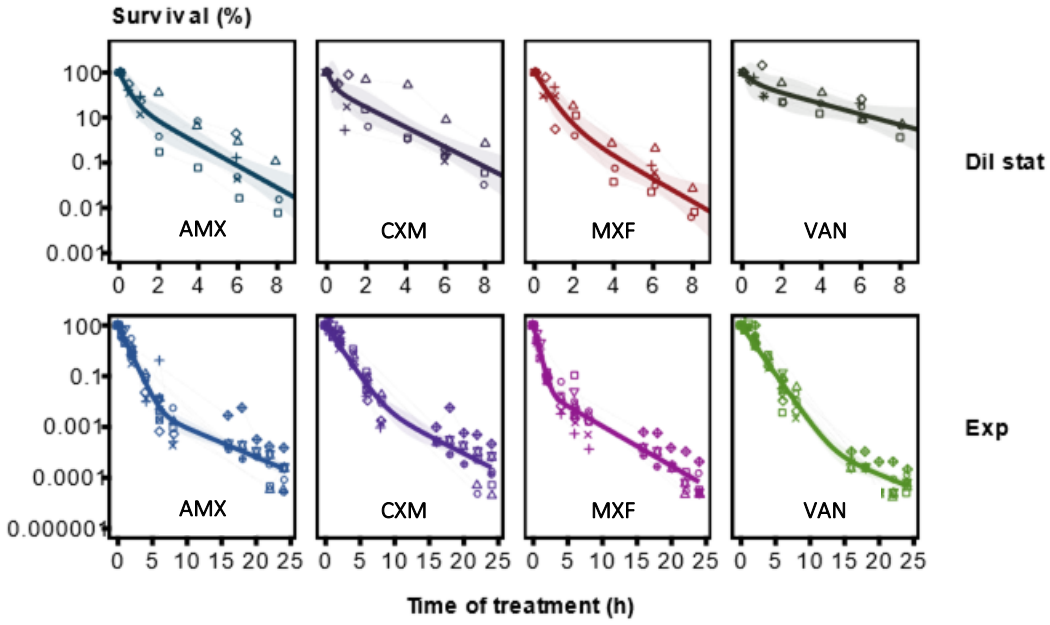


Figure 4.3.2: **Biphasic killing pattern upon antibiotic treatment indicates presence of persister subpopulations.** Fitting of a nonlinear fixed-effect model to $\log_{10}$-transformed survival data upon treatment with amoxicillin, cefuroxime, moxifloxacin, and vancomycin against *S. pneumoniae* D39. Diluted (1:10) stationary-phase (Dil stat) and exponential-phase (Exp) bacteria were treated for 8 or 24 h with the antibiotic (100-fold the MIC; 0.70 µg/mL for amoxicillin, 2.7 µg/mL for cefuroxime, 29 µg/mL for moxifloxacin, and 44 µg/mL for vancomycin). Symbols show the individual repeats (time points connected and in the same shape if coming from the same repeat), and bold lines show the fitted biphasic killing curves ± 95% confidence intervals (shades). Three or more biological repeats of the experiment were performed ($n \geq 3$).

As stationary-phase cultures did not show any killing (**Figure 4.3.1**), we performed time-kill assays on stationary-phase and diluted stationary-phase cultures, but also on exponentially growing samples. Stationary-phase cultures did not show any killing upon antibiotic treatment over an 8 h period (**Figure 4.5.1**). Mathematical analyses of the data showed that the uniphasic killing model is superior to the biphasic

model in describing the data, suggesting the presence of population-wide tolerance rather than the presence of a subpopulation of antibiotic-tolerant persisters. Upon dilution of stationary-phase cultures, antibiotic treatment killed 90 to 99.99% of the cells of strain D39 over an 8 h period, depending on the antibiotic (**Figure 4.3.2**). We observed killing of 90 to 99.99% of the cells after an 8 h treatment of exponentially growing *S. pneumoniae* D39, but when treatment was prolonged to 24 h, antibiotic treatment killed an additional 3 orders of magnitude of the exponentially growing cells (**Figure 4.3.2**). Mathematical analyses of the entire data set, with a global model containing a condition-dependent structure, showed that the biphasic killing model is superior to the uniphasic model in describing the data (analysis of variance [ANOVA; F test], $P = 1.58*10^{-84}$ (**Table 4.5.1**)), which implies that sensitive *S. pneumoniae* D39 populations contain persister cells. When each condition (growth phase x antibiotic) was analyzed separately, the biphasic model was significantly preferred over the uniphasic model for describing the data from all conditions ($P \leq 0.05$), except for data from treatment with cefuroxime and vancomycin in the diluted stationary growth phase. While this might indicate that including a second killing rate does not improve the models for these conditions, P values were close to significance (P = 0.1641 and 0.1074, respectively) and various test statistics (Akaike information criterion [AIC], Bayesian information criterion [BIC], and log likelihood [LogLik]) were either inconclusive or in favor of the biphasic model (**Table 4.5.1**). Overall, we detected relatively high persister levels ranging from 13.74 to 24.31% for amoxicillin and moxifloxacin in the diluted stationary growth phase, compared to lower levels (ranging from 0.02 to 0.5%) in the exponential growth phase. The killing rates of persister cells (0.25 to 0.58 logCFU/h) were comparable between the different conditions and were 3- to 8-fold lower than the killing rates of normal cells (0.89 to 3.78 logCFU/h) (**Table 4.5.2**).

4.3.3 The antibiotic-tolerant *S. pneumoniae* persisters were transient and nonheritable.

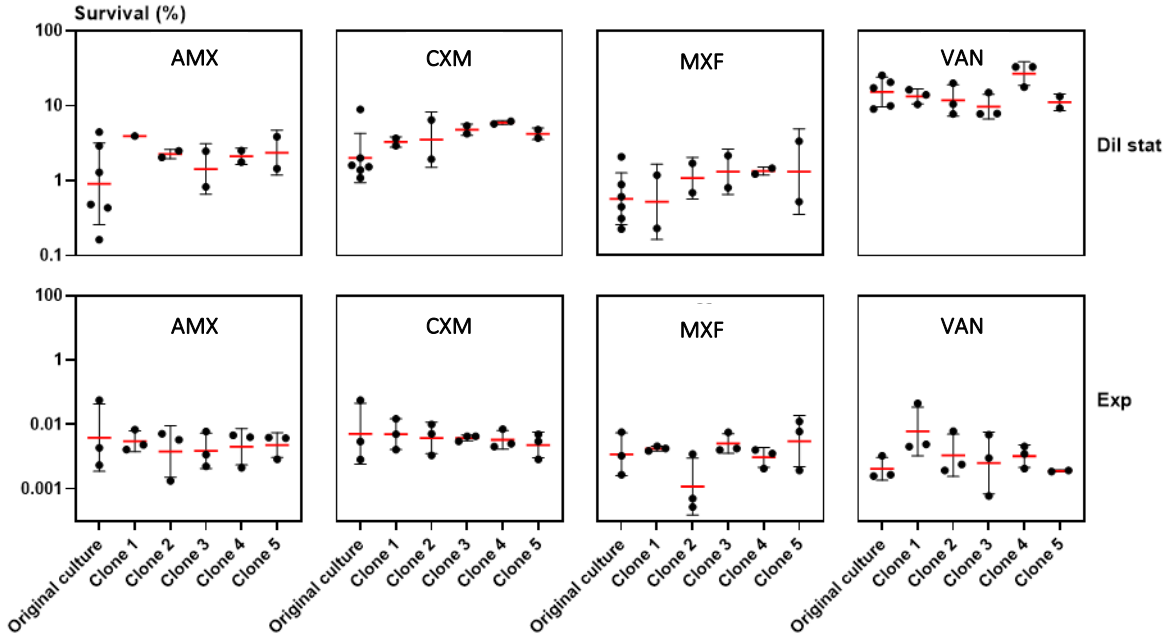


Figure 4.3.3: **The antibiotic tolerance of surviving *S. pneumoniae* cells is transient and nondeterministically inherited by daughter cells.** Antibiotic-tolerant *S. pneumoniae* D39 clones were recovered after 6 (Dil stat) or 18 (Exp) hours of treatment during the initial time-kill assay, regrown without antibiotics, and preserved at -80°C. For these clones arising from potential persister cells, survival was determined after 6 (Dil stat) or 18 (Exp) hours of antibiotic treatment with amoxicillin, cefuroxime, moxifloxacin, and vancomycin in the diluted stationary or the exponential growth phase. Survival of the randomly selected clones was similar to that of the original culture (mixed-effect analysis; clone 1 was excluded from the analysis for amox—Dil stat because we had only one data point). Two or more biological repeats of the experiment were performed, and values are presented as means ± standard deviations ($n \geq 2$).

While biphasic killing patterns are the gold standard to identify persistence, theoretically, such surviving cells could still be the result of emerging resistance or of mutants that display an increased population-wide tolerance, but are initially only present at a low frequency. To confirm the presence of genuine persisters, we performed so-called heritability assays (135). We retested some of the surviving clones of the initial time-kill assay in a subsequent round of antibiotic treatment. If *S. pneumoniae* were resistant, the MIC value would have been increased, and if the persister phenotype was inherited and passed to the entire population of daughter cells, an increased survival would have been observed during the subsequent killing assays. During these subsequent killing assays, we observed a similar survival of randomly selected clones

that survived the initial killing assay (i.e., supposed persisters) (**Figure 4.3.3**), a similar killing dynamic pattern (**Figure 4.3.4),** and MIC values that remained unchanged (**Table 4.3.1**) compared to the original culture. Thus, the surviving cells that we observed were genuine persister cells showing only a transient antibiotic tolerance, as regrown cultures show characteristics similar to those of the culture of origin.

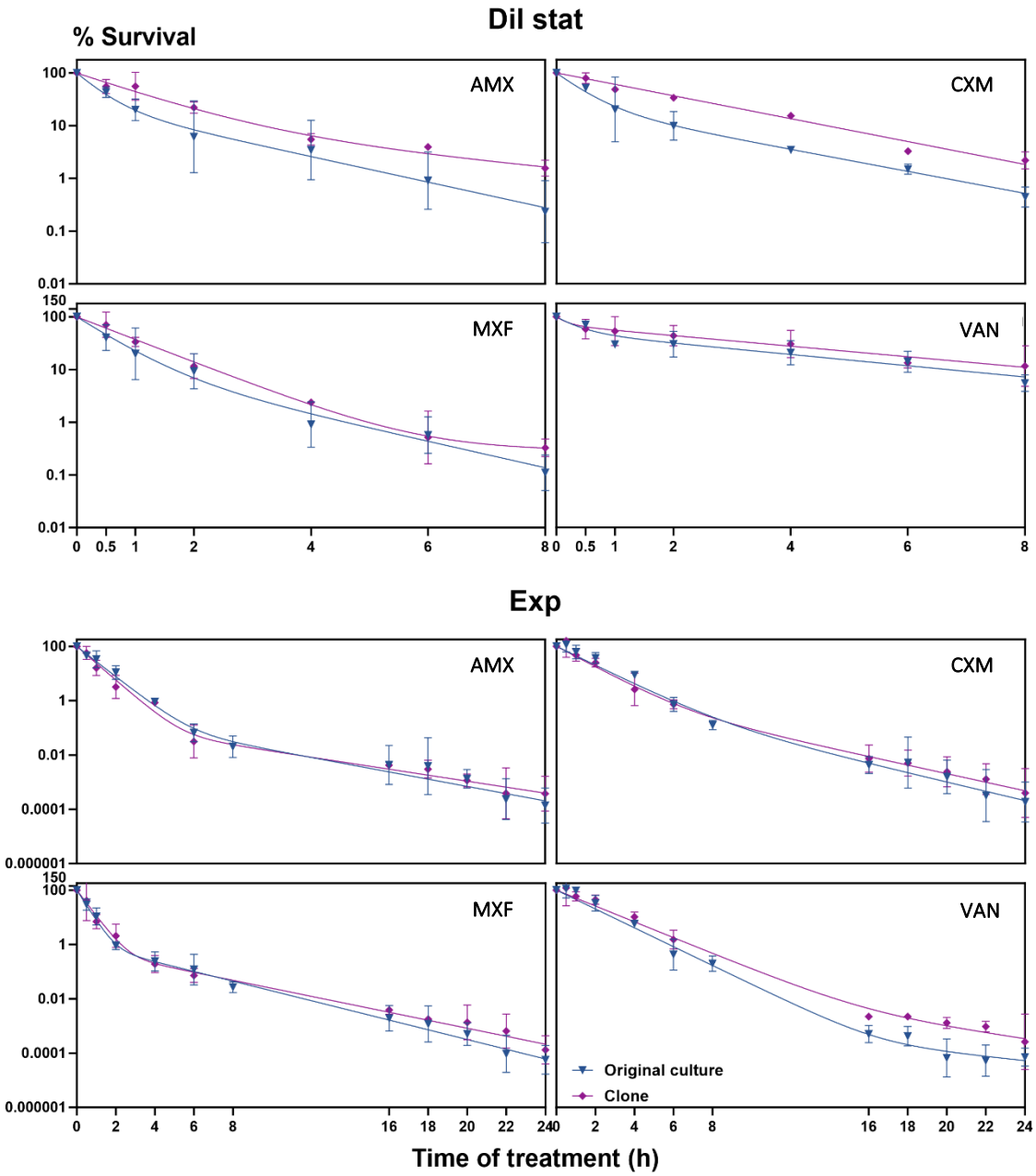


Figure 4.3.4: **The antibiotic tolerance of surviving *S. pneumoniae* cells is transient and non-deterministically inherited by daughter cells.** Fitting of a non-linear fixed-effect model to $\log_{10}$-transformed kill curves of amoxicillin (AMX), cefuroxime (CXM), moxifloxacin (MXF) and vancomycin (VAN) against *S. pneumoniae* D39 planktonic bacteria. AB-tolerant *S. pneumoniae* D39 clones were recovered after 6 (Dil stat, **4 upper panels**) or 18 (Exp, **4 lower panels**) hours of treatment during the initial time-kill assay,

regrown without antibiotic and preserved at -80°C. For one of these clones arising from potential persister cells, survival was determined over 8 or 24 hours of antibiotic treatment with amoxicillin, cefuroxime, moxifloxacin and vancomycin in the diluted stationary or the exponential growth phase. Killing dynamic patterns of the randomly selected clones were similar to the original culture (two-way ANOVA). Three biological repeats of the experiment were performed and each value is presented as the mean ± standard deviation (n = 3).

4.4 Discussion

This study presents a broad characterization of persistence in *S. pneumoniae*. We confirmed the hypothesis that persister cells are present in *S. pneumoniae* cultures by finding strong indications of a biphasic killing pattern, the hallmark of persistence, after treatment with four clinically relevant antibiotics with different modes of action. Additionally, the surviving persisters in *S. pneumoniae* show no inheritable resistance, as tolerance to the antibiotics was not passed from the initial persister cell to the subsequent generation during the heritability assays and the MIC values remained unchanged. After the optimization experiments with strain D39, we assume that persistence is a general trait in *S. pneumoniae* cultures.

Most insights in persistence have been gathered using Gram-negative bacteria, like *E. coli*, *S. typhimurium* and *P. aeruginosa* (147,174,175,214–217). However, different studies already indicated a role for persistence in Gram-positive bacteria as well (147,218–220), and more specifically in various reports on streptococcal species (147,220). Persisters in *S. mutans*, a cariogenic oral bacterium, are tolerant to a wide variety of antibiotics (221), but also to a dental caries defensive agent, dimethylaminododecyl methacrylate (DMADDM) (222,223), and to other antibacterial monomers used in dental medicine (224). As in non-streptococcal bacterial species, toxin-antitoxin systems seem to be involved in the formation of persister cells in *S. mutans*, as well as the quorum-sensing peptide CSP (competence stimulating peptide) (221,225,226). Similarly, antibiotic-tolerant persisters were identified in the zoonotic pathogen *S. suis* by Willenborg *et al.* (2014) (213) and in the opportunistic human pathogen *S. faecalis* as early as 1979 by Soriano *et al.* (1979) (227). Additionally, persistence in *S. pyogenes* was observed by Wood *et al*. (2005) in stationary phase *in vitro* cultures (228) and Martini *et al.* (2021) detected persister cells in *S. pyogenes* biofilms treated with antimicrobials (229). Despite the various reports on persistence in other species of the *Streptococcus* genus, very little was known about antibiotic tolerance, and more specifically about persistence, in the clinically important Gram-positive bacterium *S. pneumoniae* (137,139,140,142).

S. pneumoniae is well-known to cause acute infections while antibiotic tolerance and persistence are mostly connected with recurrent and chronic infections (149,185). Nonetheless, we expected to find persister cells

as persistence is identified in many, if not all, bacterial species that were studied and *S. pneumoniae* causes, to a lesser extent, also recurrent and chronic infections (189,190,193,230). Stationary-phase cultures of *S. pneumoniae* D39 showed minimal killing upon antibiotic treatment and we detected the presence of a uniphasic killing pattern after mathematical analysis. The nearly absent antibiotic-induced killing and the presence of a uniphasic killing pattern point towards the presence of tolerant cells, not only in a subpopulation, but on a population-wide level (135). Typically, population-wide tolerance displays when the growth rate of the entire population is reduced, for example during stationary-phase, and results in a higher survival after treatment with bactericidal antibiotics (149,152). On the other hand, mathematical analyses of the killing data of *S. pneumoniae* D39 indicated a biphasic killing pattern in diluted stationary-phase and exponentially growing cultures, which indicated the presence of persister cells. A major advantage of our approach is that we determined persister fractions and killing rates by mathematical analysis based on kill curves over a prolonged treatment period which enabled us to take into account the killing pattern rather than determine the persister fraction based on a single timepoint. The characteristics of persistence differed between growth phases and antibiotics. Survival levels were vastly higher in diluted stationary phase cultures than for exponentially growing bacteria for all examined strains, as we expected, because persistence is mostly linked to dormancy and bacteria from the diluted stationary phase recently came out the stationary phase and could therefore be less metabolically active (153,209,231). The difference in persistence between antibiotics can be attributed to the different modes of action. Interestingly, treatment of diluted stationary phase cultures with moxifloxacin, a fluoroquinolone that targets the DNA synthesis of bacteria and is less dependent on cell growth than antibiotics that target the bacterial cell wall, resulted in the lowest level of persisters (13,74%) (232–234). If we would determine the persister fraction based on survival fractions after a fixed period of treatment, for example after 8 hours, fractions approximate 0.01-1% as is described for *S. mutans* or *E. coli* stationary phase cultures (200,221). Nonetheless, we used mathematical analysis based on prolonged time-kill curves rather than on survival at a single timepoint and the resulting persister fractions were higher than described in other species.

With this study, we provided proof of the presence of persister cells in *S. pneumoniae*. We detected the presence of a biphasic killing pattern after analyzing antibiotic-induced time-kill assays for diluted stationary-phase and exponentially growing cultures, the hallmark of persistence, and we proved that *S. pneumoniae* persistence is transient and not-heritable. In the next chapter, we wanted to study persistence in *S. pneumoniae* more in depth with the aim to elucidate potential underlying molecular mechanisms.

4.5 Supplementary data

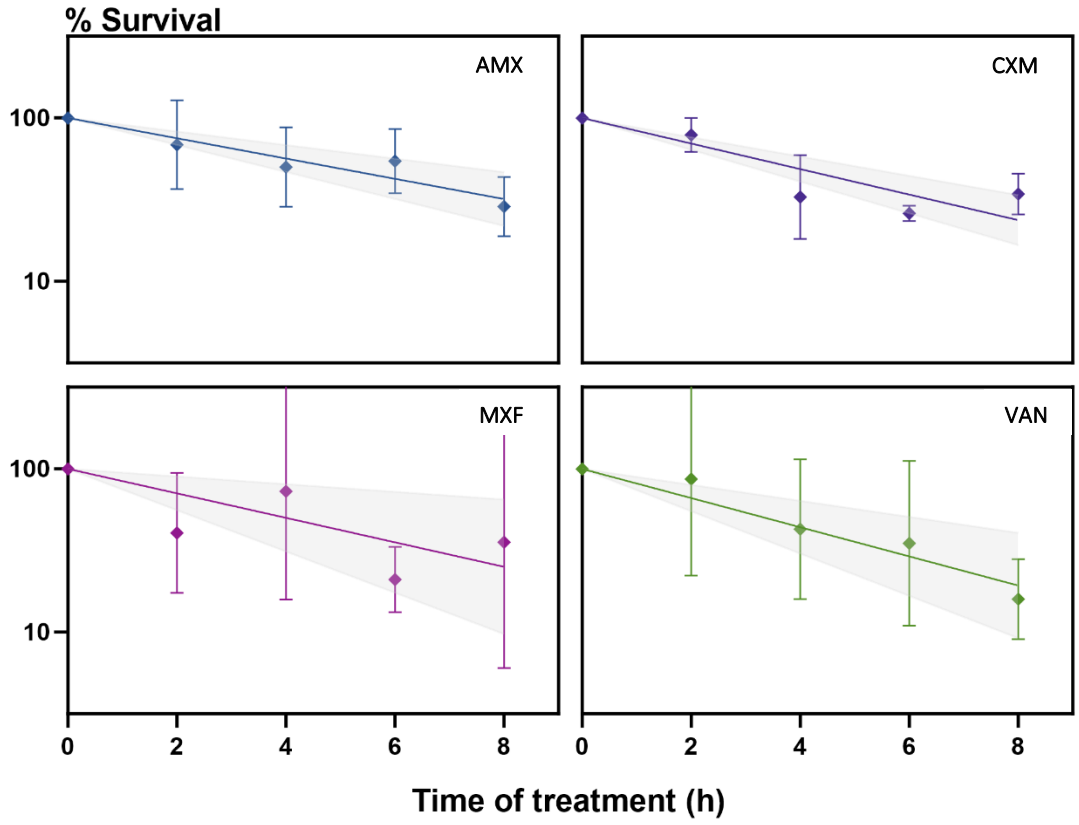


Figure 4.5.1: **Uniphasic killing pattern upon antibiotic treatment indicates absence of persister subpopulations.** Fitting of a nonlinear fixed-effect model to $\log_{10}$-transformed survival data upon treatment with amoxicillin, cefuroxime, moxifloxacin, and vancomycin against *S. pneumoniae* D39. Stationary-phase bacteria were treated for 8 h with the antibiotic (100-fold the MIC; 0.70 µg/mL for amoxicillin, 2.7 µg/mL for cefuroxime, 29 µg/mL for moxifloxacin, and 44 µg/mL for vancomycin). Symbols show the mean ± SD and bold lines show the fitted uniphasic killing curves ± 95% confidence intervals (shades). Three biological repeats of the experiment were performed (n = 3).

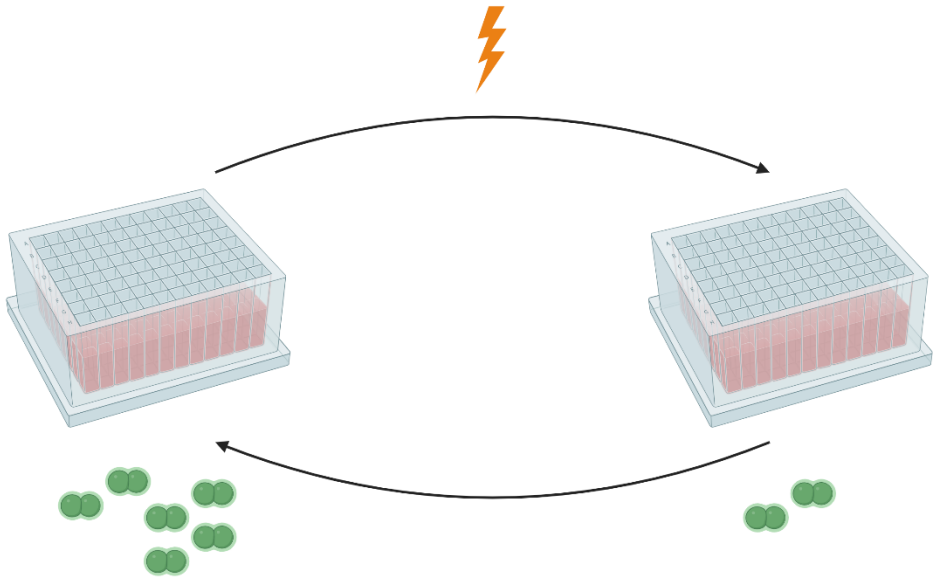
Table 4.5.1: **The biphasic model describes the time-resolved killing data better than the uniphasic model.** Mathematical analyses of the entire dataset, with a global model containing a condition-dependent structure, and of the individual conditions by comparing the fitting of two non-linear fixed-effect models (uniphasic versus biphasic model) to time-kill curves. Probability that the model is correct is determined using the Akaike's information criterion (AIC), the Bayesian information Criterion (BIC) and the Log-Likelihood (LogLik). For the model where the AIC/BIC is lower and the LogLik is higher, the probability is higher that the model is correct. Dil stat, diluted stationary growth phase; Exp, exponential growth phase.

	AIC	**BIC**	**LOGLIK**	AIC	BIC	LOGLIK
GLOBAL MODEL COMPARISON (ACROSS ALL TREATMENTS)						
UNIPHASIC	915.8	951.2	-448.9			
BIPHASIC	481.4	579.8	-215.7			
AMOXICILLIN		**Exp**			**Dil stat**	
UNIPHASIC	204.1	208.5	-100.1	36.3	38.9	-16.2
BIPHASIC	115.7	124.6	-53.9	33.0	38.2	-12.5
CEFUROXIME		**Exp**			**Dil stat**	
UNIPHASIC	146.2	150.7	-71.1	28.9	**31.5**	-12.4
BIPHASIC	104.6	113.5	-48.3	**28.8**	34.0	**-10.4**
MOXIFLOXACIN		**Exp**			**Dil stat**	
UNIPHASIC	219.0	223.4	-107.5	25.0	27.6	-10.5
BIPHASIC	88.8	97.7	-40.4	18.3	23.5	-5.2
VANCOMYCIN		**Exp**			**Dil stat**	
UNIPHASIC	118.4	122.9	-57.2	-7.2	**-4.6**	5.6
BIPHASIC	68.7	77.6	-30.4	**-8.2**	-3.0	**8.1**

Table 4.5.2: **Mathematical analysis of the fitting of a biphasic non-linear fixed-effect model to kill curves of amoxicillin (AMX), cefuroxime (CXM), moxifloxacin (MXF) and vancomycin (VAN) against *S. pneumoniae* D39.** 95% confidence intervals of the parameters are given between brackets. P_0, persister fraction at the start of treatment; K_n , killing rate of normal cells (logCFU/h); K_P, killing rate of persister cells (logCFU/h); Exp, exponential growth phase; Dil stat, diluted stationary growth phase.

ANTIBIOTIC	GROWTH PHASE	P_0	K_N	K_P
AMX	Exp	0.0012 (-0.0098 – 0.0034)	1.3240 (1.1281 – 1.5200)	0.2628 (0.1703 – 0.3553)
	Dil stat	0.2431 (-0.1645 – 0.6507)	2.5802 (-1.3650 – 6.5254)	0.5586 (0.2708 – 0.8463)
CXM	Exp	0.0050 (-0.0117 – 0.0216)	0.9259 (0.7873 – 1.0644)	0.3166 (0.1566 – 0.4765)
	Dil stat	0.4650 (-0.0644 – 0.9944)	3.7800 (-10.5042 – 18.0642)	0.5067 (0.2981 – 0.7153)
MXF	Exp	0.0040 (0.0014 – 0.0065)	2.4595 (2.0477 – 2.8713)	0.3611 (0.3187 – 0.4035)
	Dil stat	0.1374 (-0.1644 – 0.4391)	1.7510 (0.5172 – 2.9848)	0.5756 (0.2320 – 0.9192)
VAN	Exp	0.0002 (-0.0005 – 0.0009)	0.8854 (0.8194 – 0.9513)	0.2510 (0.0962 – 0.4058)
	Dil stat	0.6008 (0.1265 – 1.0750)	2.0722 (-3.5989 – 7.7432)	0.2706 (0.1353 – 0.4059)

5 ABSENCE OF EXPERIMENTAL EVOLUTION OF PERSISTENCE IN *STREPTOCOCCUS PNEUMONIAE*



"Finally, I found a way to reduce the number of agar plates, with deepwell plates!"

Nele Geerts, Linda De Vooght, Bram Van den Bergh, Paul Cos., *Evolution of persistence in Streptococcus pneumoniae,* unpublished

5.1 Introduction

Persisters were first described in 1942 and especially during the last 15 years, intensive research was performed trying to elucidate the molecular mechanisms behind persistence. Many methods are described to study persisters and persistence mechanisms (149). Transcriptomics can help in understanding which transcriptional changes happen in a persister cell and with microfluidics, persisters can be studied at the single-cell level (149). Another way of investigating mechanisms behind persistence is by experimental evolution. Persistence has been shown to be under the direct evolutionary pressure by antibiotic treatment (231,235–240). Evolution experiments showed that persistence can increase rapidly upon frequent antibiotic treatment to extremely high persistence levels (241,242). Moreover, these evolution experiments can be used as a tool to elucidate molecular mechanisms underlying persistence (231). Van den Bergh *et al.* (2016) studied the effect of antibiotic treatment on persistence evolution in *E. coli* (243). They demonstrated that highly tolerant strains develop rapidly *in vitro* after frequent antibiotic exposure, reflecting daily antibiotic treatment in clinic. WGS of these strains revealed single point-mutations that lead to highly tolerant phenotypes, and gave an indication of the genes that contribute to antibiotic persistence (243). Sulaiman *et al.* (2020) used a similar approach for *in vitro* evolution of *E. coli* and applied proteomics on the evolved strains to pinpoint proteins that are involved in persister formation (244).

In CHAPTER 5, we tried to elucidate potential molecular mechanisms behind *S. pneumoniae* persistence by setting-up evolution experiments. Surprisingly, we did not observe evolution towards a high persistent phenotype after frequent antibiotic exposure *in vitro* under the tested experimental conditions.

5.2 Material and Methods

5.2.1 Bacterial strains and growth conditions

S. pneumoniae strains used during this study are listed in Table 5.2.1. *S. pneumoniae* was cultured statically in cation-adjusted Mueller-Hinton broth (Fluka) supplemented with 5% lysed horse blood (MHL; Oxoid) or on blood agar (BA) plates (tryptic soy agar [Neogen] supplemented with 5% defibrinated sheep blood [Oxoid]) at 37°C in 5% CO_2.

Table 5.2.1: *S. pneumoniae* strains used during this study.

Strain	Serotype	Origin	Type
D39	2	NCTC® 7466	Reference strain
TIGR4	4	ATCC® BAA-334	Reference strain
SCI1	19F	Sciensano	Clinical isolate

5.2.2 Long-living *in vitro* culturing and enumeration of bacteria

Bacteria from cryopreservation stocks (BHI + 10 % glycerol) were plated on BA and incubated for 24 to 72h, followed by subculturing statically in a tube with MHL for 8 h with a final concentration of $1*10^8$ CFU/mL. Then, bacteria were diluted to $5*10^5$ CFU/mL in fresh MHL and brought into the desired growth state. Stationary-phase bacteria were obtained by overnight growth (16 h). Diluted stationary-phase bacteria were obtained by overnight growth (16 h) and 1:10 dilution in fresh MHL (Figure 5.2.1). The bacterial concentration was determined according to the viable plate count (VPC) method. Briefly, a 1:10 serial dilution (10^0 to 10^{-6}) was made in phosphate-buffered saline (PBS) in a 96-well plate. Three aliquots of 10 µL of a selection of dilutions were plated on BA and incubated for minimum of 48 h before colonies were counted and bacterial concentration (CFU/mL) was calculated.

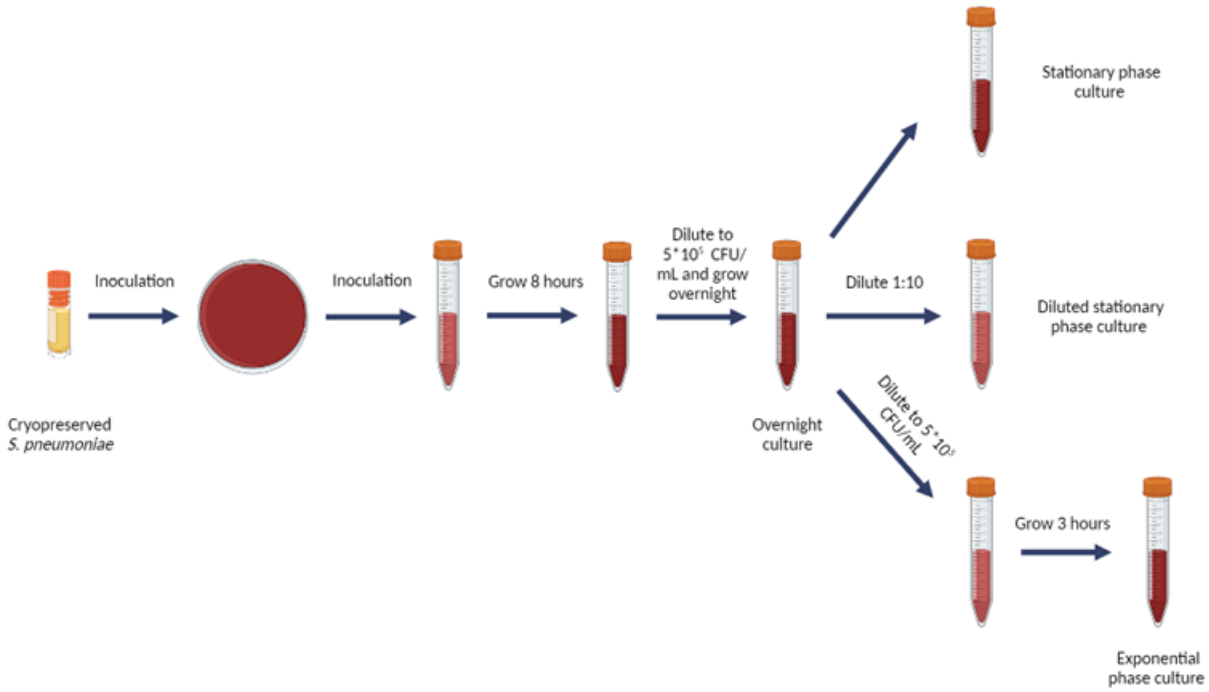


Figure 5.2.1: **Overview of the long-living *in vitro* protocol for stable *S. pneumoniae* bacterial cultures.** Cryopreserved bacteria are plated on a blood agar plate, followed by inoculation in a tube with MHL. After 8 h of static incubation, the culture is diluted to $5*10^5$ CFU/mL and grown overnight. The overnight culture either is directly used as a stationary-phase culture, diluted 1:10 in fresh MHL to act as a diluted stationary-phase culture, or is diluted to $5*10^5$ CFU/mL in fresh MHL and grown for 3 h to obtain an exponential-phase culture.

5.2.3 Evolution experiments

For the assessment of evolution after antibiotic treatment, a 6 h antibiotic treatment was alternated with overnight recovery in fresh medium without antibiotic. Diluted stationary-phase cultures were treated during 6 h with an antibiotic (amoxicillin, cefuroxime, moxifloxacin or vancomycin) at 100X the MIC in either 0.5 or 25 mL in a 96-deepwell plate or in 50 mL Erlenmeyer's, respectively. An overnight stationary phase culture that was not diluted and not treated, was included as a negative control. After 6 h of treatment, bacteria were washed three times in PBS to remove antibiotics followed by resuspension in 0.5 or 25 mL fresh MHL. Bacterial concentration was determined before and after each antibiotic treatment. Bacteria were exposed to 7, 8 or 15 antibiotic treatment cycles.

To follow overnight recovery after antibiotic treatment, 100 µL of the bacterial suspension (after washing away the antibiotic and resuspension in fresh MHL) was transferred to a flat bottom 96-well plate. Bacterial

growth was followed over 20 h (each 30 min for 0-8 h, each 1 h for 8-20 h) by measuring $OD_{600\ nm}$ with a multimode microplate reader (Tecan Spark). Two untreated, negative controls were included, a negative control that was not diluted and one that was diluted to $5*10^5$ CFU/mL after the 6 h period of growth.

5.2.4 Antibiotic susceptibility

MICs of standard antibiotics were determined using the broth microdilution method as described previously (245). The following antibiotics were used: kanamycin (Sigma-Aldrich; aminoglycoside), gentamicin (Gibco; aminoglycoside), tobramycin (LabPro; aminoglycoside) and streptomycin (Sigma-Aldrich; aminoglycoside). Briefly, a 1:2 serial dilution of the antibiotic was made in triplicates in MHL in a 96-well plate with a final volume of 100 µL. Then, 100 µL of a bacterial suspension was added to each well, except to negative-control wells, to a final concentration of $5*10^5$ CFU/mL in 200 µL. Positive-control wells contained 200 µL of bacterial suspension ($5*10^5$ CFU/mL) without antibiotics, and negative-control wells contained 200 µL of MHL without antibiotics or bacteria. Plates were incubated at 37°C and 5% CO_2 for 20 h before the optical density was measured at 600 nm using a spectrophotometer (Promega; Discover).

5.2.5 Dose-dependent and time-dependent kill curves

To obtain dose-dependent kill curves, *S. pneumoniae* cultures were treated for 5 h in the stationary or diluted stationary growth phase with four different antibiotic concentrations (**Table 5.2.2**). After 5 h, bacterial suspensions were centrifuged, resuspended in PBS to wash away antibiotics, and bacterial concentration (CFU/mL) was determined by plating. Colonies were counted after a minimum of 48 h of incubation.

Table 5.2.2: **Antibiotic concentrations (µg/mL), given as X times the MIC, that were used for treatment of reference strain D39 to obtain dose-dependent kill curves for different aminoglycoside antibiotics.**

ANTIBIOTIC	5X	10X	50X	100X
KANAMYCIN	245	490	2450	4900
GENTAMICIN	20	40	200	400
TOBRAMYCIN	70	140	700	1400

STREPTOMYCIN	80	160	800	1600

To obtain time-kill curves, *S. pneumoniae* in the stationary growth state was treated with a fixed antibiotic concentration (100-fold the MIC). Bacterial suspensions were incubated up to 8 h. At specified time points, bacterial concentration was determined via plating after centrifugation and resuspension in PBS to wash away the antibiotics. Colonies were counted after a minimum of 48 h of incubation.

5.2.6 Data analysis and statistics

A simple linear regression model was applied on the survival data (the $\log_{10}$-transformed fraction of surviving cells) in function of round of antibiotic treatment. Overnight recovery was assessed by fitting a logistic growth model with following function $Y = \frac{Ym*Y0}{((Ym-Y0)^{-k*x}+Y0)}$ with Y_0 starting population ($OD_{600\ nm}$); Y_M maximum population ($OD_{600\ nm}$); k rate constant (1/h) and X_{int} X-coordinate of first infliction point. Both analyses were performed in GraphPad Prism version 10. Time-kill curves were analyzed mathematically by comparing two models of killing, a uniphasic model with a single killing rate and a biphasic model with two killing rates. The nonlinear fixed-effect model used the $\log_{10}$-transformed fraction of surviving cells. The biphasic model was based on the equation $Log(Y) = log((N-P_0)^{(-kn*t)}+P_0^{(-kp*t)})$ and the uniphasic model on $Log(Y) = log((N)^{(-kn*t)})$ with *Y* survival fraction, *t* treatment time (in hours), P_0 persister fraction at t = 0 and k_n and k_p the killing rate of normal and persister cells (logCFU per hour). Curves were considered biphasic if biphasic fitting was better than uniphasic fitting according to the Akaike information criterion (AIC).

5.3 Results

Similar as was done before for *E. coli* and *P. aeruginosa* (174,241,244), here, we tried to evolve *S. pneumoniae* persisters *in vitro* by frequent antibiotic treatment as a way to identify potential molecular mechanisms of *S. pneumoniae* persistence. We used different approaches based on Van den Bergh *et al.* (2016) (243).

5.3.1 Experimental *in vitro* evolution of reference strain D39 with clinically relevant antibiotics in small treatment volumes does not lead to highly persistent populations

We treated reference strain D39 with different antibiotics during 7 (cefuroxime) or 15 cycles (negative control, amoxicillin, moxifloxacin or vancomycin) in small volumes (0.5 mL) (**Figure 5.3.1**) resembling the protocol of Van den Bergh and colleagues (241). *S. pneumoniae* D39 became extinct after 10 cycles of antibiotic treatment with vancomycin (**Figure 5.3.1**, Van, depicted with the asterisk), i.e. we did not observe surviving bacteria after the 10^{th} round of antibiotic treatment, and visually, we did not observe an increased survival with increasing antibiotic treatments. Also statistically there was no increased survival after frequent antibiotic exposure, as deduced from a simple linear regression model on the survival data over treatment round. The slope of the fitted line did not significantly differ from zero for all tested antibiotics, which implies that there is no change in survival and thus no evolution towards highly persistent mutants in this experimental setting **(Figure 5.3.1**, **Table 5.5.1**).

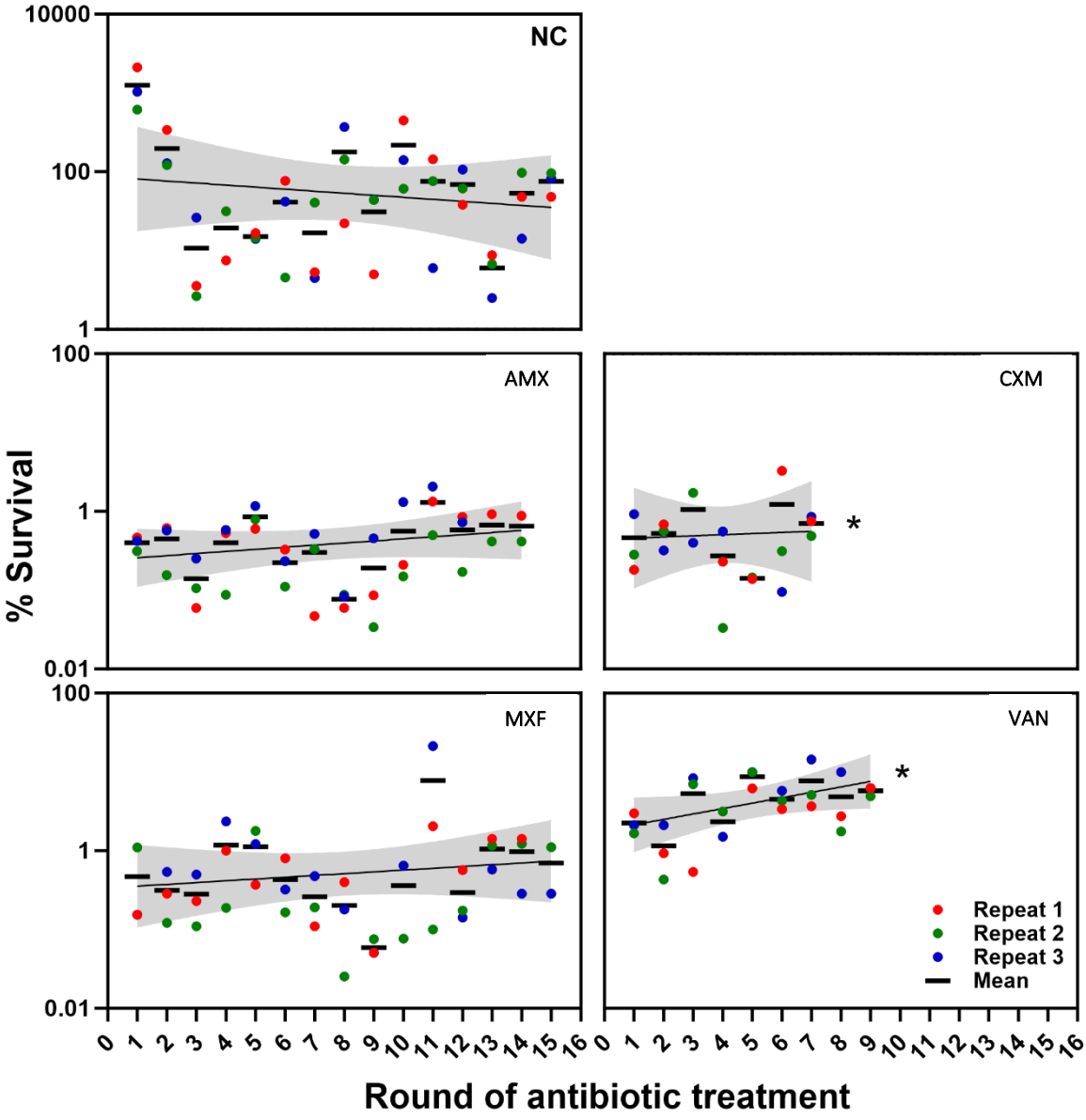


Figure 5.3.1: **Frequent antibiotic exposure of reference strain D39 does not give increased persister fractions under the applied conditions.** 0.5 mL of diluted (1:10) stationary-phase bacteria were treated for 6 h with the antibiotic (100-fold the MIC; 0.70 µg/mL for amoxicillin, 2.7 µg/mL cefuroxime, 29 µg/mL for moxifloxacin, and 44 µg/mL for vancomycin) alternated with overnight recovery in fresh medium. Bacteria were exposed to 7 (cefuroxime, end of cycles depicted with the asterisk (*)) or 15 cycles (negative control, amoxicillin, moxifloxacin and vancomycin) of antibiotic treatment. *S. pneumoniae* D39 became extinct after 10 cycles of antibiotic treatment with vancomycin (depicted with the asterisk (*)). Symbols show the individual repeats (colored according to the repeat) and bold lines shows the mean. The line shows fitting of the means by simple linear regression with the 95% confidence interval (grey shade). Three biological repeats (i.e. 3 different colonies were picked from blood agar) of the experiment were performed (n = 1 x 3).

5.3.2 Antibiotic exposure leads to bacterial killing and not to growth arrest

To ensure that the evolutionary pressure we applied, namely killing through antibiotic treatment, was sufficient to potentiate evolution, we checked if *S. pneumoniae* D39 was properly killed through the antibiotics by following overnight recovery (after washing away the antibiotic and resuspension in fresh medium) (**Figure 5.3.2**). The rationale here is that if antibiotics would only induce growth arrest and not bacterial killing, we would observe a fast increase in bacterial CFU's during overnight recovery after awakening of the surviving bacteria. We included two untreated growth controls to assess recovery of antibiotic-treated cultures: a negative control that was not diluted and one that was diluted to $5*10^5$ CFU/mL after the 6 h period of growth. Undiluted controls showed a fast increase in $OD_{600\,nm}$ as the bacterial density of the starting cultures remained high (+/- $1*10^9$ CFU/mL). Diluted controls ($5*10^5$ CFU/mL) showed a lag phase followed by normal exponential growth and entrance to the stationary phase at 6-8 h of growth. The bacterial density after 6 h of treatment depended on the used antibiotic, ranging from $1*10^4$ CFU/mL for moxifloxacin to $1*10^5$ CFU/mL for amoxicillin and cefuroxime and $1*10^6$ CFU/mL for vancomycin. A logistic growth model was used to fit the data to assess different growth parameters (**Table 5.5.2**). We observed differences in lag phase and maximal bacterial concentration after different treatments. The highest bacterial density was reached for the undiluted control and the lowest after vancomycin treatment, which could explain the extinction of *S. pneumoniae* D39 after 10 cycles of vancomycin treatment (**Table 5.5.2)**. The goal of this recovery experiment was to ensure that the antibiotics were capable of inducing killing, and not only growth arrest on plates, of *S. pneumoniae* D39 cultures. By comparing overnight recovery of treated and untreated controls, we proved that the bacterial density at the start (0 h) was lower for treated cultures than the density of untreated, undiluted cultures, and that treated cultures needed more time to reach the maximum concentration (Y_M) which, along with the low CFU counts after treatment, implies that the antibiotics killed a fraction of the culture (**Figure 5.3.2, Table 5.5.2**).

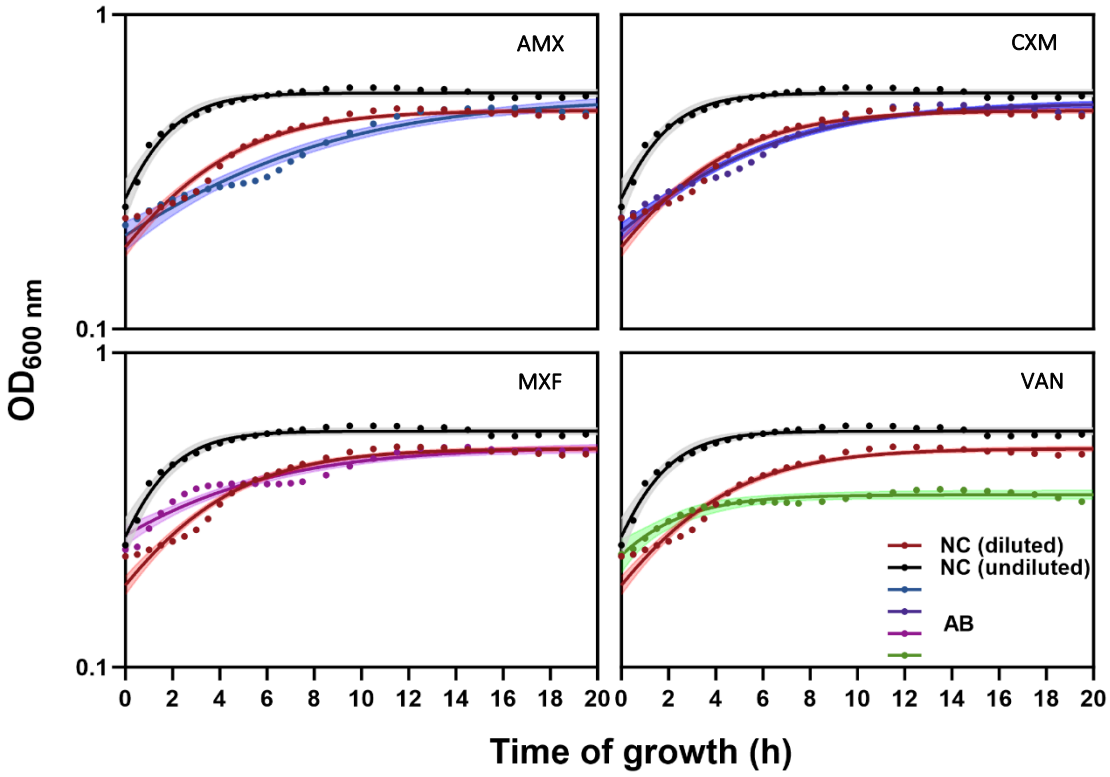


Figure 5.3.2: **Overnight recovery of surviving *S. pneumoniae* D39 after a 6 hours antibiotic treatment**. Growth was measured as $OD_{600\ nm}$ each 30 minutes from 0-8 h and each hour from 8-20 h with a multimode microplate reader (Tecan Spark). Two growth controls were included, a negative control that was not diluted and one that was diluted to $5*10^5$ CFU/mL after the 6 h period of treatment. Symbols show the mean and the lines show the logistic growth model (shade is the 95 % CI) that was used to fit the data. The experiment was performed in triplo (3 technical repeats).

5.3.3 Experimental *in vitro* evolution in large treatment volumes does not lead to highly persistent populations

We increased the population size and thus the number of CFU's that was exposed to the antibiotic by using a higher volume of *S. pneumoniae* D39 cultures to increase the chance for bacteria to mutate. We applied the same protocol as for small treatment volumes, except for the used bacterial culture volume that increased from 0.5 mL to 25 mL. We analyzed the data (survival in function of antibiotic treatment cycle) again by fitting a simple linear regression model to check if survival increased with more antibiotic treatments (**Figure 5.3.3**). The slope of the fitted line ranged from 0.1135 for amoxicillin to 0.2688 for moxifloxacin, which is higher than the slopes from the experiment with small treatment volumes, but still

does not significantly points towards evolution to highly persistent mutants in this experimental setting (Table 5.5.1).

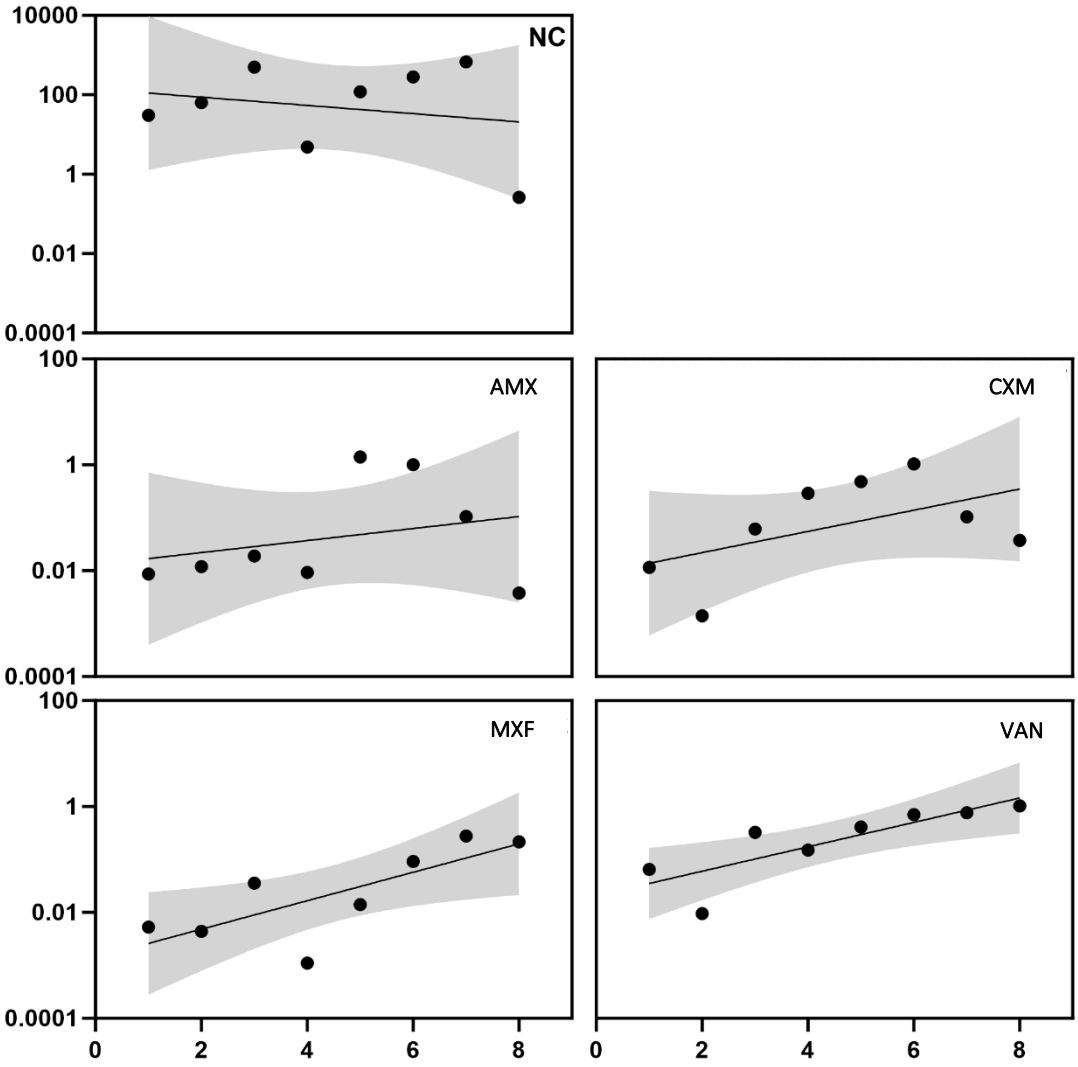


Figure 5.3.3: **Frequent antibiotic exposure of reference strain D39 does not give increased persister fractions under the applied conditions.** 25 mL of diluted (1:10) stationary-phase bacteria were treated for 6 h with the antibiotic (100-fold the MIC; 0.70 µg/mL for amoxicillin, 2.7 µg/mL cefuroxime, 29 µg/mL for moxifloxacin, and 44 µg/mL for vancomycin) alternated with overnight recovery in fresh medium. Bacteria were exposed to 8 cycles of antibiotic treatment. The connection line shows fitting by simple linear regression with the 95% confidence interval (grey shade) (n = 1 x 1).

5.3.4 Experimental *in vitro* evolution of reference strain TIGR4 and clinical strain SCI1 with clinically relevant antibiotics does not lead towards a high-persister phenotype

We were not able to observe evolution of reference strain D39 towards a high-persistent phenotype after treatment with amoxicillin, cefuroxime, moxifloxacin or vancomycin. As this could be a strain-specific limitation, we chose to repeat the initial experiment (repeated antibiotic exposure cycles in a small volume) with two different strains, another reference strain, TIGR4 (**Figure 5.3.4**), and a clinical isolate from carriage, SCI 1 (serotype 19F) (**Figure 5.3.5**). *S. pneumoniae* TIGR4 and SCI1 became extinct, i.e. we did not observe surviving bacteria, after antibiotic treatment with vancomycin, but already at the second round of treatment. Besides, the survival of the negative control seems to decrease with increasing cycles. The negative control was an undiluted overnight culture that was washed, but not diluted, after 6 hours, so the decrease in survival could be attributed to the self-limiting nature of *S. pneumoniae*. After statistical analysis using a simple linear regression model, we could conclude that there is no increase in survival after increasing rounds of treatment, as the slope of the fitted line did not differ from zero for all conditions. This confirms our findings with D39, that, under these experimental conditions, there is no change in survival and thus no evolutionary adaptation towards highly persistent mutants (**Table 5.5.1**).

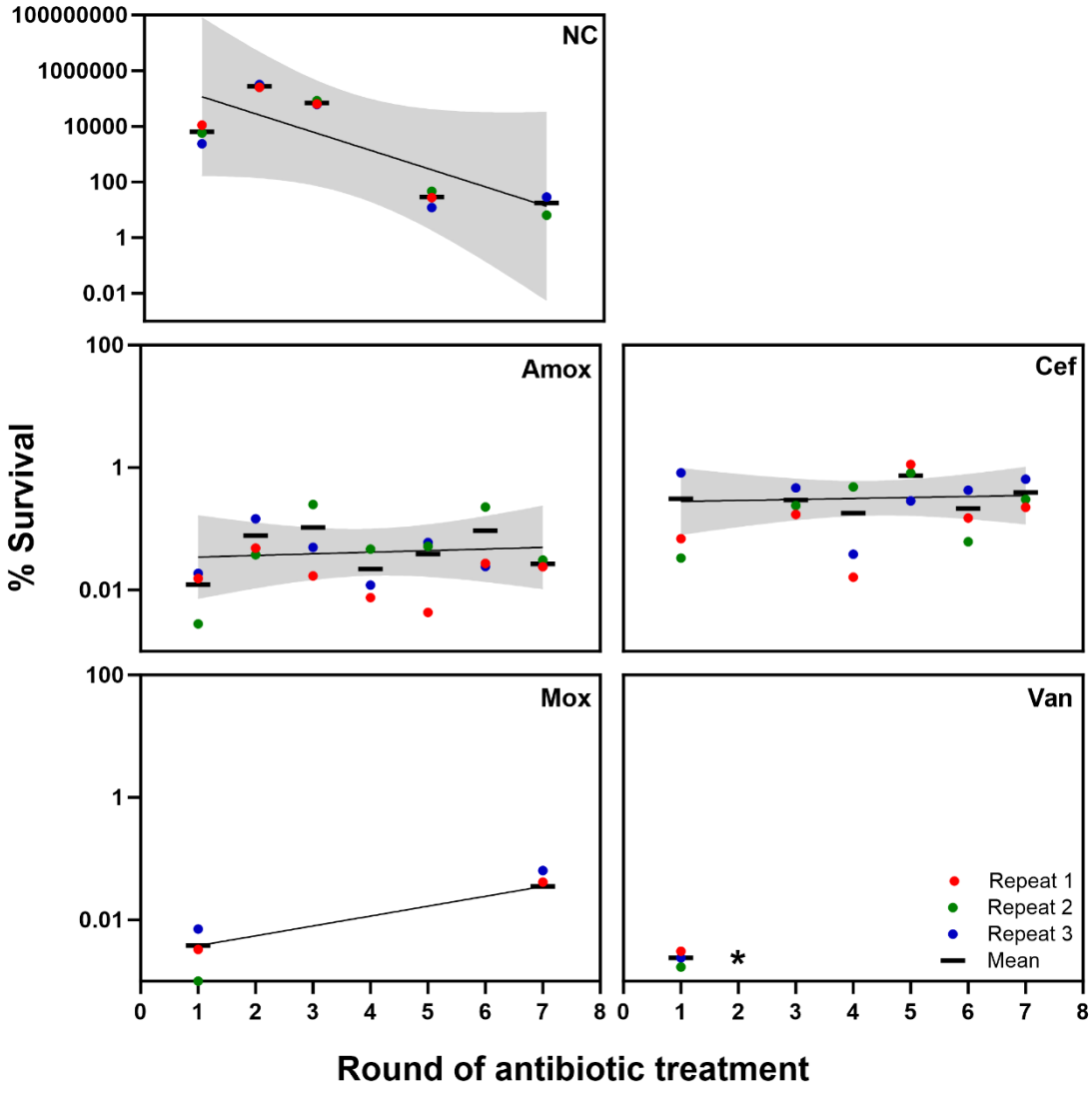


Figure 5.3.4: **Frequent antibiotic exposure of reference strain TIGR4 does not give increased persister fractions under the applied conditions.** 0.5 mL of diluted (1:10) stationary-phase bacteria were treated for 6 h with the antibiotic (100-fold the MIC; 0.70 µg/mL for amoxicillin, 2.7 µg/mL cefuroxime, 29 µg/mL for moxifloxacin, and 44 µg/mL for vancomycin) alternated with overnight recovery in fresh medium. Bacteria were exposed to 7 cycles of antibiotic treatment. *S. pneumoniae* TIGR4 became extinct after 1 cycle of antibiotic treatment with vancomycin (depicted with the asterisk (*****)). Symbols show the individual repeats (colored according to repeat) and bold lines show the mean. The line shows fitting of the means by simple linear regression with the 95% confidence interval (grey shade). Three biological repeats (i.e. 3 different colonies were picked from blood agar) of the experiment were performed (n = 1 x 3).

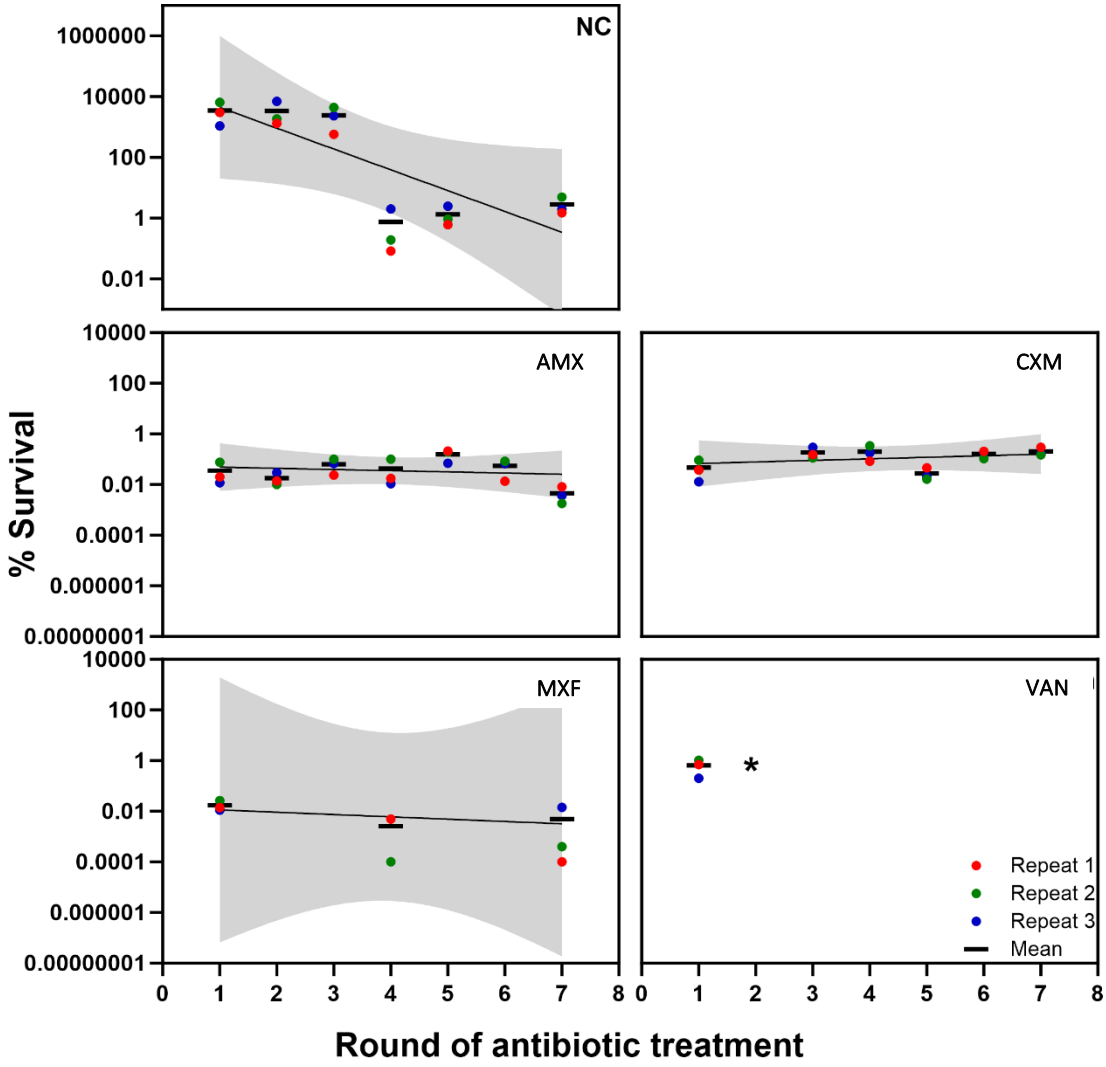


Figure 5.3.5: **Frequent antibiotic exposure of clinical strain SCI1 does not give increased persister fractions under the applied conditions.** 0.5 mL of diluted (1:10) stationary-phase bacteria were treated for 6 h with the antibiotic (100-fold the MIC; 6.25 µg/mL for amoxicillin, 25 µg/mL cefuroxime, 25 µg/mL for moxifloxacin, and 50 µg/mL for vancomycin) alternated with overnight recovery in fresh medium. Bacteria were exposed to 7 cycles of antibiotic treatment. *S. pneumoniae* SCI1 became extinct after 1 cycle of antibiotic treatment with vancomycin (depicted with the asterisk (*****)). Symbols show the individual repeats (colored according to repeat) and bold lines show the mean. The line shows fitting of the means by simple linear regression with the 95% confidence interval (grey shade). Three biological repeats (i.e. 3 different colonies were picked from blood agar) of the experiment were performed (n = 1 x 3).

5.3.5 Experimental *in vitro* evolution of reference strain D39 with aminoglycosides

In our final approach to evolve *S. pneumoniae* persisters *in vitro* by frequent antibiotic treatment, we looked into the use of the aminoglycosides kanamycin, tobramycin, gentamicin and streptomycin, four antibiotics that are not used in the clinic to treat *S. pneumoniae* infections. Gentamicin is more active against Gram-

positive bacteria, especially *Staphylococcus* spp, but *S. pneumoniae* remains relatively resistant (246). Nonetheless, we chose to work with aminoglycosides as these resulted in a very consistent evolution of *E. coli* cultures towards colonies with high persister fractions after two to three treatment cycles (243) and were therefore used despite the lack of clinical relevance for treatment of *S. pneumoniae*. We wanted to look into different strategies, of which aminoglycoside antibiotics was one, to ensure that our observations indicating the absence of evolution were true. Before we could perform evolution experiments with aminoglycosides, the MIC (Table 5.5.3) and survival dynamics of *S. pneumoniae* after treatment with aminoglycosides were determined by setting-up dose-dependent and time-dependent kill curves. First, we investigated survival after 5h of treatment with different doses (ranging from 5X to 100X the MIC) of a set of aminoglycosides (Figure 5.3.6). In contrast to the results with amoxicillin, cefuroxime, moxifloxacin and vancomycin (CHAPTER 4), stationary phase cultures of *S. pneumoniae* were sensitive to a high dose (100X the MIC) of kanamycin and streptomycin and the sensitivity of *S. pneumoniae* in diluted-stationary phase to aminoglycosides was highly dependent on the applied antibiotic concentration, as survival decreased with increasing antibiotic concentrations (Figure 5.3.6). A typical hallmark of persistence is the independence on antibiotic concentration, once a sufficient dose is reached to kill sensitive cells, so the decreased survival with increasing the antibiotic concentration could point towards antibiotic resistance. Another, more convenient, explanation for the concentration-dependence are the extremely high antibiotic concentrations that were used, reaching 400 to 4900 µg/mL at 100X the MIC. These concentrations exceed typical concentrations used in, for example, cytotoxicity screens (here, mostly up to 64 µM is used) which could render the antibiotics rather antiseptics at the highest applied concentrations.

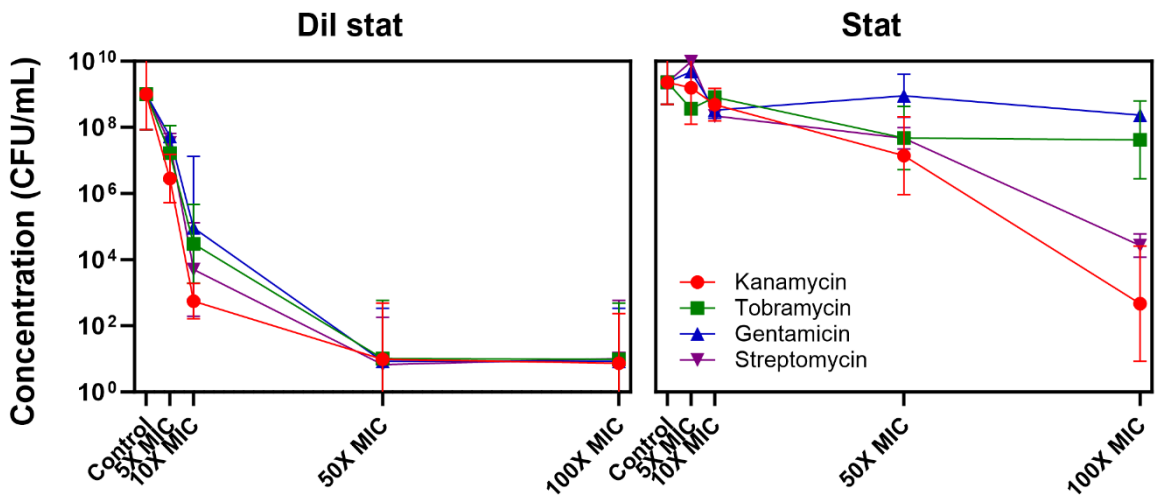


Figure 5.3.6: ***S. pneumoniae* D39 in stationary phase is insensitive to tobramycin and gentamicin, but killing was observed for kanamycin and streptomycin at 100X the MIC. Diluted stationary-phase cultures are highly sensitive to all aminoglycosides with lower survival at higher antibiotic doses.** Dose-dependent kill curves with kanamycin, tobramycin, gentamicin and streptomycin of planktonic *S. pneumoniae* D39 are shown in stationary-phase (Stat) or diluted stationary-phase (Dil stat) samples. Antibiotic treatments lasted for 5 h before survivors were enumerated. Applied concentrations were 5-, 10-, 50-, and 100-fold the MIC (respectively; 245, 490, 2450 and 4900 µg/mL for kanamycin; 20, 40, 200 and 400 µg/mL for gentamicin; 70, 140, 700 and 1400 µg/mL for tobramycin; and 80, 160, 800 and 1600 µg/mL for streptomycin). Three biological repeats of the experiment were performed, and values are presented as means ± standard deviations (n = 3).

Next, we obtained a time-kill curve for the aminoglycoside kanamycin to study survival dynamics. Following analysis of the dose-dependent kill curves, we treated *S. pneumoniae* D39 in the stationary phase with 100X the MIC of kanamycin. We observed a biphasic killing pattern upon treatment with kanamycin, which is the first indication of the presence of persister cells after aminoglycoside treatment, but we did not detect surviving bacteria after 4 h of treatment. We should further optimize the antibiotic concentration to obtain time-kill curves for the different aminoglycosides. Furthermore, to prove the presence of persisters, heritability assays should be performed as described in CHAPTER 4. The following step should be to choose the right conditions to set-up an evolution experiment with aminoglycosides. Due to time constrictions, these last two assays were not performed during this project.

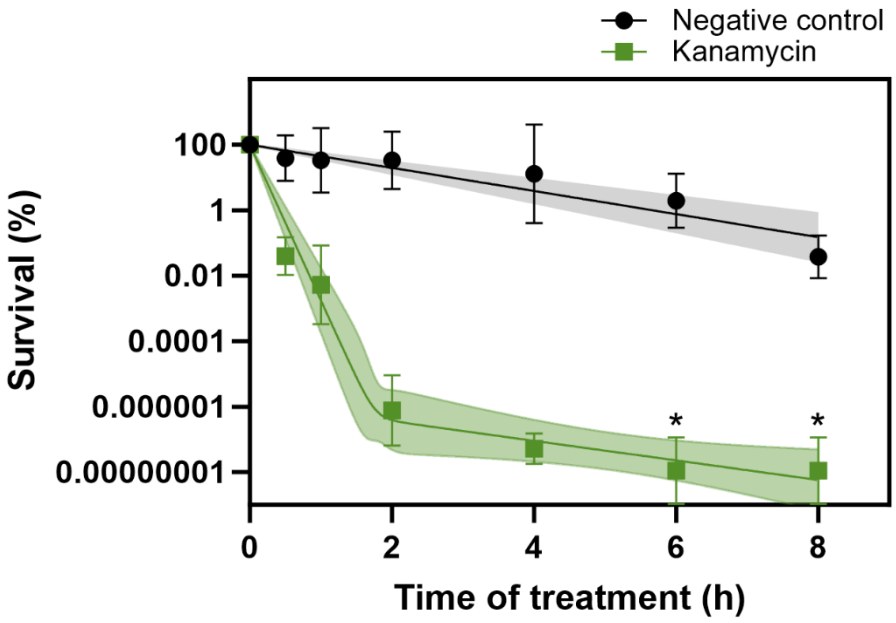


Figure 5.3.7: **Biphasic killing pattern upon antibiotic treatment indicates presence of persister subpopulations, but no surviving bacteria were detected after 4 h of treatment.** Survival data upon treatment with kanamycin against *S. pneumoniae* D39. Stationary-phase bacteria were treated for 8 with the antibiotic (100-fold the MIC; 4.9 mg/mL). Asterisks (*) indicate that no survivors were detected. Three biological repeats of the experiment were performed (n = 3).

5.4 Discussion

We tried to define potential molecular mechanisms of *S. pneumoniae* persistence by setting-up experimental evolution *in vitro* through frequent antibiotic exposure (243). We did not succeed in evolving *S. pneumoniae* strains towards a high-persister phenotype under the tested experimental conditions, unlike other research groups that work on different pathogens (239,243,244,247,248), including on other *Streptococcus* species (249). Fridman *et al.* (2014) used a similar approach to ours and they observed that changes in lag time led to the development of tolerance (248). Also the approach from Van den Bergh *et al.* (2016) and Sulaiman *et al.* (2020) was similar to the setups we attempted and they observed evolved populations that were highly persistent or tolerant, so they used these *in vitro* evolved populations to study potential persister mechanisms via sequencing or via proteomic investigation (243,244). Even the role of evolution was observed *in vivo*, for example for *P. aeruginosa* by Mulcahy *et al.* (2010), who observed the presence of high persister mutants in late *P. aeruginosa* isolates of cystic fibrosis patients after receiving periodic treatments with high doses of antibiotics (180). Similarly, Liu *et al.* (2020) found highly tolerant MRSA isolates in patients with persistent blood infections (250) and Parsons *et al.* (2024) observed isolates with increased persister fractions of *E. coli* causing relapsing bloodstream infections compared to the initial infections (251).

While previous references have used other species to study the evolution of persistence after cyclic antibiotic treatment, experimental evolution was already performed in *S. pneumoniae* to elucidate potential mechanisms in the context of antibiotic tolerance and resistance (252–255). Feng *et al.* (2009) and Carsenti-Dellamonica *et al.* (2005) used experimental evolution *in vitro* to elucidate resistance mechanisms of *S. pneumoniae*, respectively of linezolid and macrolides/linezolid (252,254). More recently, Dao *et al.* (2024) set-up experimental evolution in a murine *in vivo* model to study the role of fluoroquinolone tolerance in evasion of antibiotic-mediated killing (253). More specifically, mice were infected with *S. pneumoniae* TIGR4 for 6 h followed by treatment with levofloxacin. After 12 h of treatment, mice were euthanized to collect *S. pneumoniae* survivors and these survivors were used as inoculum to infect the next group of mice. This was

repeated for 15 passages. Follow-up experiments included time-kill experiments and genetic analysis which revealed the presence of antibiotic-tolerant cells that survived antibiotic treatment better through reduced production of reactive oxygen species (253).

All forementioned studies were successful, as they all led to the discovery of different genes that contribute to either resistance or tolerance (252–254). We tried to evolve *S. pneumoniae* persistence, under different experimental conditions, but these experiments proved not to be successful and we therefore tried to estimate how many mutations were sampled during the evolution experiments. Recently, Jiang *et al.* (2024) discovered that *S. pneumoniae* D39 has a high spontaneous mutation rate (0.02 per genome per cell division) by using mutation-accumulation lines (256). To determine the number of mutations that occurred during our initial experiment, we needed to estimate the number of generations we sampled. When we consider our initial experiment with D39 (15 cycli of treatment) and more specifically treatment with amoxicillin, we sampled about 170 generations during overnight growth ($\sum_n \log_2 \left(\frac{CFU\ at\ the\ end\ of\ the\ cycle}{CFU\ at\ the\ start}\right)$ with n = number of cycli (243)) which confers to about $2*10^{10}$ cell divisions. When we then combine the mutation rate of 0.02 per genome per cell division of *S. pneumoniae* D39 with the number of cell divisions, we can conclude that we sampled about $4*10^{8}$ mutations ((0.02/genome per cell division * $2*10^{10}$ cell divisions) during the 15 cycles of antibiotic treatment with amoxicillin. We sampled a large number of mutations at practically all positions in the *S. pneumoniae* D39 genome, so we can conclude that our study was not limited by the number of sampled mutations. A potential explanation for the absence of evolution could be the presence of cells in the viable but nonculturable (VBNC) state which could impair our evolutionary pressure of cyclic antibiotic treatment. Bacteria in the VBNC state are viable and can remain metabolically active, but are not able to grow on regular growth media (257,258). We used culture-based methods for the detection of survivors of antibiotic treatment, and we could therefore have overlooked the potential presence of VBNC cells. To further investigate the matter, we could use alternative methods that allow detection of VBNC cells via BacLights® Live/Dead assay or via molecular methods (257,258).

Our approach, despite the similarity to other research groups that performed experimental evolution (243,244,248) and the ability of *S. pneumoniae* to evolve *in vitro* (252,254), did not result in evolution of persistence, neither to the identification of potential mechanisms. At this point, we can only conclude that we do not observe evolution of persistence under the tested experimental conditions. This could be attributed to the duration of the period of recovery after antibiotic exposure which is around 18 h. As *S. pneumoniae* is a fastidious and self-limiting bacterium, this period could be too long and provide additional stress upon antibiotic exposure. Moreover, antibiotic exposure is applied to the bacterial population in a diluted stationary-phase in which the cells are most likely in a more dormant/metabolically inactive state. Another approach we could look into is treatment of exponentially growing bacteria which could allow a higher mutation rate during treatment. Further attempts with different experimental set-ups should be tested (such as further exploring aminoglycosides and VBNC cells), but were not performed due to the time limitation of the project. In the next chapters (CHAPTER 6 and CHAPTER 7), we did not focus on unraveling potential underlying mechanisms of *S. pneumoniae* persistence, but on its clinical relevance.

5.5 Supplementary data

Table 5.5.1: **The slope with 95% CI is given after mathematical analysis of the fitting of a simple linear regression model on the survival data of *S. pneumoniae* strain D39 after frequent antibiotic cycling in small (0.5 mL) and large (25 mL) volumes.** 0.5 mL or 25 mL of diluted (1:10) stationary-phase bacteria were treated for 6 h with the antibiotic (100-fold the MIC; 0.70 µg/mL for amoxicillin, 2.7 µg/mL cefuroxime, 29 µg/mL for moxifloxacin, and 44 µg/mL for vancomycin) alternated with overnight recovery in fresh medium. Bacteria were exposed to 7 (cefuroxime) or 15 cycles (negative control, amoxicillin, moxifloxacin and vancomycin) of antibiotic treatment.

	LARGE TREATMENT VOLUME (25 ML)	SMALL TREATMENT VOLUME (0.5 ML)		
	D39		TIGR4	SCI1
NEGATIVE CONTROL	-0.1037 (-0.5619 to 0.3545)	-0.02582 (-0.1052 to 0.05358)	-0.6563 (-1.493 to 0.1800)	-0.6847 (-1.385 to 0.01571)
AMOXICILLIN	0.1135 (-0.2713 to 0.4984)	0.02660 (-0.02011 to 0.07331)	0.02618 (-0.1598 to 0.2122)	-0.04754 (-0.2996 to 0.2045)
CEFUROXIME	0.2003 (-0.1222 to 0.5228)	0.01435 (-0.1599 to 0.1886)	0.01599 (-0.1220 to 0.1540)	0.06129 (-0.1675 to 0.2901)
MOXIFLOXACIN	0.2688 (0.04104 to 0.4965)	0.02262 (-0.03972 to 0.08495)	0.1613 /	-0.09131 (-1.434 to 1.252)
VANCOMYCIN	0.2307 (0.07389 to 0.3875)	0.06868 (-0.001657 to 0.1390)	/	/

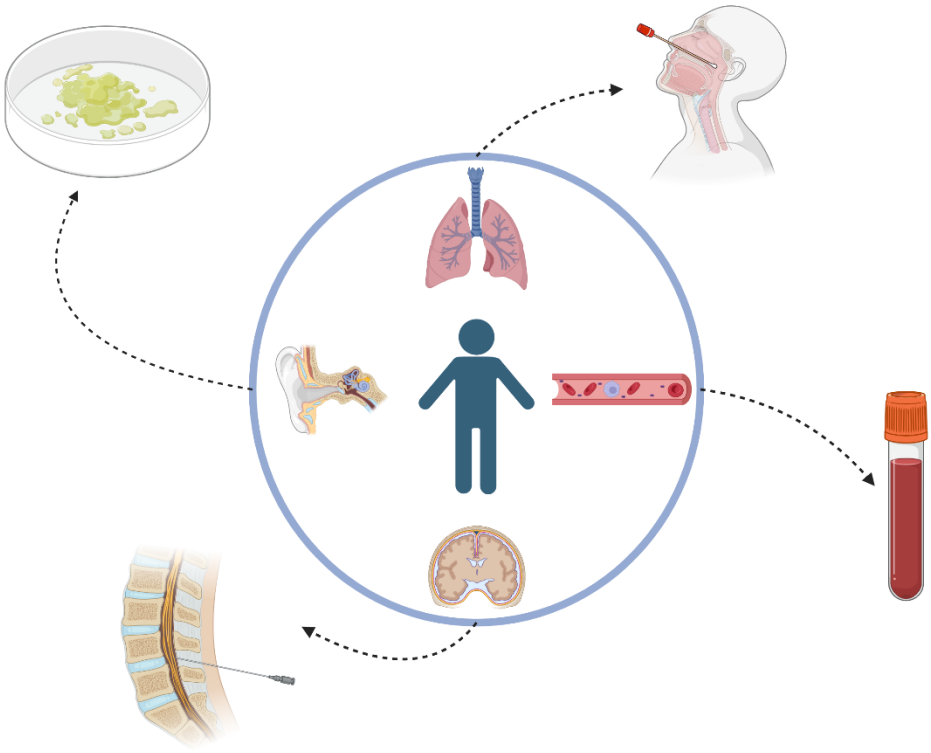
Table 5.5.2: **Mathematical analysis of the fitting of a logistic growth model to growth curves of *S. pneumoniae* D39. Growth** curves ($OD_{600\ nm}$ in function of time) were obtained for the recovery period after antibiotic treatment with amoxicillin (AMX), cefuroxime (CXM), moxifloxacin (MXF) and vancomycin (VAN) or from the untreated negative control (undiluted or diluted to $5*10^5$ CFU/mL). 95% confidence intervals of the parameters are given between brackets. Y_0, starting population ($OD_{600\ nm}$); Y_M, maximum population ($OD_{600\ nm}$); k, rate constant (1/h) and X_{int}, X-coordinate of first infliction point.

PARAMETER	NC (DILUTED)	NC (UNDILUTED)	AMX	CXM	MXF	VAN
Y_M	0.4960 (0.4878 to 0.5045)	0.5635 (0.5500 to 0.5774)	0.5443 (0.5115 to 0.5925)	0.5252 (0.5125 to 0.5394)	0.5024 (0.4841 to 0.5261)	0.3539 (0.3426 to 0.3671)
Y_0	0.1822 (0.1702 to 0.1942)	0.2596 (0.2225 to 0.2959)	0.1982 (0.1786 to 0.2178)	0.2041 (0.1920 to 0.2163)	0.2626 (0.4841 to 0.5261)	0.2271 (0.1969 to 0.2549)
k	0.3360 (0.3063 to 0.3676)	0.6933 (0.5344 to 0.9085)	0.1761 (0.1391 to 0.2155)	0.2343 (0.2094 to 0.2604)	0.2113 (0.1649 to 0.2645)	0.4510 (0.2690 to 0.7368)
X_{INT}	2.976 (2.720 to 3.265)	1.442 (1.101 to 1.871)	5.679 (4.640 to 7.190)	4.268 (3.840 to 4.775)	4.732 (3.780 to 6.064)	2.217 (1.357 to 3.717)

Table 5.5.3: **Minimum inhibitory concentration (MIC, µg/mL) of *S. pneumoniae* D39 against a set of aminoglycosides.** Values represent mean ± SD (n = 3).

	MIC (µg/mL)
KANAMYCIN	49 ± 11.36
GENTAMICIN	14 ± 3.46
TOBRAMYCIN	4 ± 0
STREPTOMYCIN	16 ± 0

6 THE DIVERSITY OF *STREPTOCOCCUS PNEUMONIAE* GENETICS, ANTIBIOTIC RESISTANCE AND PERSISTENCE



"Screen 650 clinical isolates? Why not? Bring the agar plates again!"

Nele Geerts, Laurence Van Moll, Linda De Vooght, Ioannis Passaris, Lize Cuypers, Stefanie Desmet, Bram Van den Bergh and Paul Cos, Genome-wide association study *of Streptococcus pneumoniae* resistance and persistence, unpublished.

6.1 Introduction

Bacteria have developed different strategies to evade antimicrobial therapy. Antimicrobial resistance (AMR) is a well-known mechanism that emerged shortly after the discovery of antimicrobial compounds (121,122,124,125). Resistant bacteria can grow in the presence of antibiotics through the acquisition of genetic changes (129,130). Another escape strategy employed by bacteria is antibiotic persistence. There is no genotypic change, but a phenotypic change that results in a reduced metabolic activity to even dormancy allowing the bacteria to tolerate and survive antibiotic treatment (135).

A lot is known about AMR, AMR mechanisms and the spread of AMR among *S. pneumoniae* (132,133), but nothing was known about *S. pneumoniae* persistence until 2022 (63,186,187). In CHAPTERS 4 and 5, we already made a broad characterization of *S. pneumoniae* persistence, but in this chapter, we wanted to investigate the clinical relevance and the diversity of *S. pneumoniae* resistance and persistence by studying a large set of clinical strains. The inclusion of clinical *S. pneumoniae* strains in studies regarding AMR, more specifically about AMR spread and molecular mechanisms, have already shown their value (259–264). Overall knowledge about antibiotic persistence was gained working with lab strains, but previous studies with clinical strains showed their potential in elucidating its clinical relevance and potential persistence mechanisms (265–267). Hofsteenge *et al.* (2012) and Stewart and Rozen (2013) looked into naturel *E. coli* isolates. They both detected high variation in persister fractions among clinical strains following antibiotic exposure and they both indicated that the persister fraction strongly depends on the antimicrobial compound that was used rather than observing multidrug tolerance (265,266). Similarly, Barth *et al.* (2013) observed a high heterogeneity of persister cell formation among *Acinetobacter baumannii* isolates (267) and for unraveling *P. aeruginosa* persistence, natural strains were used in different set-ups. Longitudinal studies following cystic fibrosis patients showed the presence of highly persistent mutants during the later stages of infection (180,181). On the other hand, Verstraete *et al.* (2023) used a set of environmental and clinical *P. aeruginosa* strains to make a comparison between *in vitro* and *in vivo* persistence levels (215).

Research on clinical strains has proven its value in unraveling the clinical relevance of antibiotic persistence, but also of the underlying mechanisms.

In this chapter, we determined the antibiotic resistance and persistence profiles of our strain collection. To study resistance, we included four clinically relevant, bactericidal antibiotics. Amoxicillin and cefuroxime belong to the β-lactam antibiotics and inhibit cell-wall synthesis through irreversible binding to the transpeptidase or penicillin-binding protein (PBP) (95,96). Resistance is mainly mediated via modification of the PBP (108,132,133). Moxifloxacin is a fluoroquinolone antibiotic that interrupts the DNA synthesis by binding DNA gyrase and topoisomerase IV (103,104) and resistance is caused by mutations in these targets: topoisomerase IV, encoded by *parC* and *parE*, and DNA gyrase, encoded by *gyrA* and *gyrB* (132,133). Vancomycin is a glycopeptide and also inhibits cell-wall synthesis, but via binding the D-Ala-D-Ala sequence and subsequent disruption of the recognition of this sequence by the transpeptidase (105). No cases of vancomycin resistance were reported yet (134). We chose two antibiotics to determine the persistence profiles of our large collection, namely amoxicillin and moxifloxacin. Amoxicillin is the antibiotic of first choice for treatment of *S. pneumoniae* infections and thus widely used (99). Moxifloxacin is an alternative treatment for *S. pneumoniae* infections, for example in case of penicillin allergy, and it has a different mechanism-of-action (MOA) (99). To date, no studies were reported on persistence mechanisms after treatment with β-lactam antibiotics, but two studies described the role of growth arrest and the oxidative stress response in *S. pneumoniae* fluoroquinolone persisters (63,187). Finally, we whole-genome sequenced a part of our strain collection (377 strains) to investigate the genetics of *S. pneumoniae*, such as the population structure and pangenome.

With this study, we demonstrated that both resistance and persistence are highly variable among our collection of clinical strains and highly depend on the used antibiotic. Persisters were widely present among the tested clinical strains with highly diverse persister levels.

6.2 Material and Methods

6.2.1 *Streptococcus pneumoniae* collection

The *S. pneumoniae* strains used in this study are listed in **Appendix A**. *S. pneumoniae* strains were kindly provided by Ioannis Passaris and Pieter-Jan Ceyssens (100 strains, Bacterial Diseases Unit, Sciensano), by Lize Cuypers and Stefanie Desmet (497 strains, National Reference Centre for Invasive Pneumococci, University Hospitals Leuven) and by Françoise Van Bambeke (50 strains, Pharmacologie Cellulaire et Moléculaire, UC Louvain). Bacteria were cultured statically in cation-adjusted Mueller-Hinton broth (Fluka) supplemented with 5% lysed horse blood (MHL; Oxoid), on blood agar (BA) plates (tryptic soy agar (TSA) [Neogen] supplemented with 5% defibrinated sheep blood [Oxoid]) or on TSA plates at 37°C in 5% CO_2.

6.2.2 *De novo* assembly

Reference strain D39 along with 376 clinical strains were whole-genome sequenced. Genomic DNA was extracted with the DNeasy Blood & Tissue kit (Qiagen) according to the manufacturer's instructions. Paired-end DNA libraries were prepared with the xGen™ DNA Library Preparation Kits (IDT). The quality of the DNA samples was assessed before sequencing using a Nanodrop (Thermo Fisher), with a spectrophotometer using the Quant-it 1X dsDNA assay kit (Invitrogen) and gel electrophoresis. Samples were sequenced using the AVITI platform of ElementBio® (VIB, Leuven). The quality of the reads was examined using FastQC and, if necessary, the reads were trimmed and filtered to improve the quality using Trimmomatic with adapter removal, removal of low quality bases at the beginning and end of the reads, a sliding window size of 4 bp with an average quality of 20, and removal of reads shorter than 36 bp. Quality was also assessed after trimming with FastQC. SPAdes was used for the de novo assembly (with the 'careful' option) on the paired-end reads that passed the previous quality controls. The quality of the contigs was assessed with QUAST. Analysis were performed by Joe Ibrahim (BIOMINA) and Dale Annear (BIOMINA).

6.2.3 Genome annotation, pangenome analysis and phylogenetic analysis

The coding sequences of the contigs were predicted with Prokka with the Genbank compliance option. The genome of reference strain D39 (NC_008533) was used for annotation of coding sequences. Roary with default settings was used to investigate core and accessory genes and to construct the pangenome. Core genes were defined as genes identified in at least 95% (≥ 358) of the strains and accessory genes occur in less than 358 strains. The core genes of all sequenced strains were aligned with MAFFT and were used to construct the phylogenetic tree with IQ-Tree with automatic model selection (option 'MF') and ultrafast bootstrapping (UFBoot, 1000 replicates). Resulting phylogenetic trees were visualized with iTOL. Analysis were performed by Joe Ibrahim (BIOMINA) and Dale Annear (BIOMINA).

6.2.4 Long- living *in vitro* culturing

Bacteria from cryopreservation stocks (BHI + 10 % glycerol) were plated on BA and incubated for 24 to 72 h, followed by subculturing statically in a tube with liquid MHL for 8 h with a final concentration of about $1*10^8$ CFU/mL. Then, bacteria were diluted to about $5*10^5$ CFU/mL in fresh MHL and brought into the desired growth state. Diluted stationary-phase bacteria were obtained by overnight growth (16 h) and 1:10 dilution in fresh MHL (Figure 6.2.1).

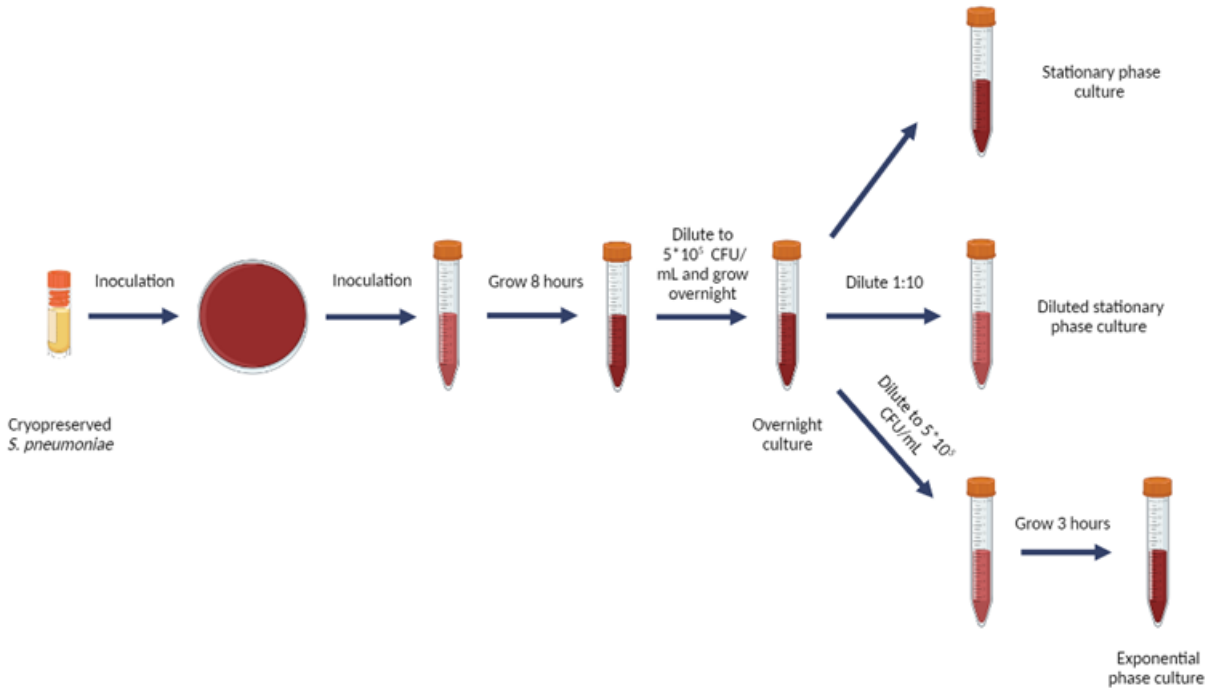


Figure 6.2.1: **Overview of the long-living *in vitro* protocol for stable *S. pneumoniae* bacterial cultures.** Cryopreserved bacteria are plated on a blood agar plate, followed by inoculation in a tube with MHL. After 8 h of static incubation, the culture is diluted to $5*10^5$ CFU/mL and grown overnight. The overnight culture either is directly used as a stationary-phase culture, diluted 1:10 in fresh MHL to act as a diluted stationary-phase culture, or is diluted to $5*10^5$ CFU/mL in fresh MHL and grown for 3 h to obtain an exponential-phase culture.

6.2.5 Antibiotic susceptibility

The minimum inhibitory concentration (MIC), the lowest antibiotic concentration that inhibits bacterial growth, was determined by the agar dilution method according to the EUCAST guidelines for the antibiotics amoxicillin, cefuroxime, moxifloxacin and vancomycin. Bacteria were grown in duplo from BA in 200 µL MHL in 96-deepwell plates (U-bottom) for 6 hours. The concentration of the bacterial suspension was then adjusted to $5*10^6$ CFU/mL before 5 µL was spotted on Mueller-Hinton agar plates supplemented with 5% defibrinated sheep blood containing one of the antibiotics in doubling dilutions. Agar plates were incubated at 37°C and 5% CO_2 for 20 h. The MIC was determined by visual inspection of the plates for growth.

6.2.6 Persistence screening and time-kill curves

Diluted stationary phase cultures were treated with an antibiotic, either amoxicillin or moxifloxacin, at 100X the MIC. The antibiotic concentration was adjusted for each strain based on its MIC and the concentration

of the untreated bacterial suspension was determined before the antibiotic was added. After 6 hours of treatment, bacteria were washed twice with PBS and resuspended in PBS followed by spot plating (7 µL) on TSA plates using an automated system (Biomek 3000, Beckman Coulter). After overnight incubation, a picture was taken of each spot with an inverted Nikon microscope (TiEclipse) at a 4X magnification, using a programmed automated stage. The number of colonies on the pictures was counted with the retrained model of Cellpose (268). Survival fractions were calculated by the ratio of the number of bacteria surviving antibiotic treatment and the number of bacteria before treatment. For time-kill curves, some strains were treated during 8 h and samples were taken at 30 min, 1, 2, 4, 6 and 8 h post treatment. CFU determination was performed similarly to the screening.

6.2.7 Data analysis and statistics

GraphPad Prism version 10 was used to analyze the time-kill curves mathematically. The nonlinear fixed-effect model used the $\log_{10}$-transformed fraction of surviving cells. The biphasic model was based on the equation $Log(Y) = \log((N-P_0)^{(-kn*t)}+P_0^{(-kp*t)})$ with Y survival fraction, t treatment time (in hours), P_0 persister fraction at t = 0 and k_n and k_p the killing of normal and persister cells (logCFU per hour). Correlation analyses were performed via Pearson correlation in GraphPad Prism version 10. The correlation was considered statistically significant when the P value was < 0.05. For the correlation analysis, MIC values, normalized MIC values (**Table 6.5.1**) or $\log_{10}$-transformed survival data were used.

6.3 Results

6.3.1 A look into our *Streptococcus pneumoniae* collection

During this study 648 *S. pneumoniae* strains were collected. The collection consisted of 1 reference strain, D39, and 647 clinical isolates. All strains were isolated from human specimens in Belgium, as *S. pneumoniae* is exclusively found in human. The strains were divided into four groups: strains from carriage, from non-invasive and invasive *S. pneumoniae* disease (IPD) and strains causing a surinfection (secondary infection superimposed on an initial infection). For 7 strains, no data on the type of infection was available (not available, NA) (**Figure 6.3.1**). Most strains were isolated from patients with invasive *S. pneumoniae* disease (497 of 648 strains). The strains isolated from patients with invasive diseases were collected from 2018-2022. Strains isolated from carriage and non-invasive diseases were isolated between 2020-2023 (Sciensano) or between 2006-2009 (UC Louvain). The first *S. pneumoniae* vaccine, PCV7, was implemented in the vaccine schedule of children in 2007 and PCV13 was introduced in 2011 (269), so almost all strains were collected after the introduction of the pneumococcal vaccine.

Figure 6.3.1 **Type of infection caused by the strains in our collection**. Numbers depict the number of isolates included in our dataset for the different types of infection. NA, not available.

Next, we listed the serotype distribution among the collection (**Figure 6.3.2**). Serotypes 6A, 19F and 23F were commonly found in asymptomatic carriage in earlier studies (78,79), but as we looked into our dataset, isolates from carriers were mostly from serotypes 11A, 19F, 23B and 35B which can be explained by the introduction of PCV7 (covering for 19F and 23F) in 2007 and of PCV13 (covering for 6A, 19F and 23F) in 2011. Serotypes 1, 2, 4, 5, 7F, 8, 9, 12F, 14, 16, 18C, and 19A are associated with more invasive disease than serotypes 3, 6A, 6B, 11A, 15B/C, 19, and 23F (22). The most prevalent serotypes in our dataset causing non-invasive disease were serotypes 3, 19A and 6C and the most prevalent serotypes causing IPD were 3, 19A, 23B, 6C and 9N, which depicts an overlay in serotypes between the two types of disease. The conjugate vaccine PCV13 is widely used in Belgium since 2011 and covers for serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F and 23F. Despite the widespread vaccination of children in Belgium with PCV13, we still observe a high burden of serotypes 3, 4 and 19A in our dataset. The collection of clinical strains is summarized in **Appendix A**.

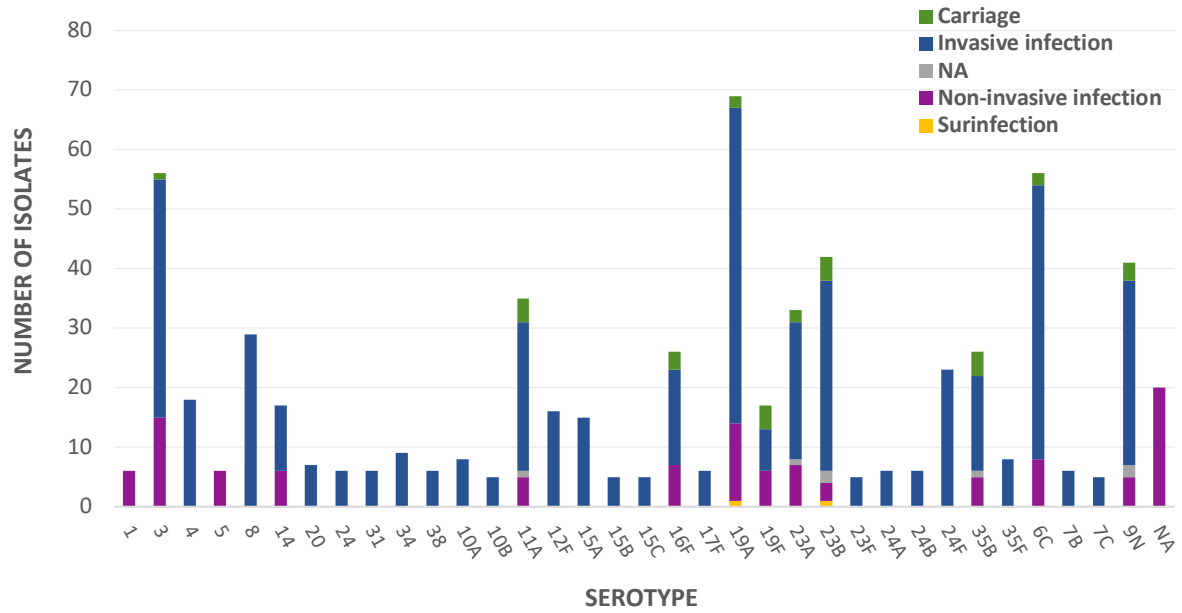


Figure 6.3.2: **Serotype distribution of the strains included in the collection.** Number of strains is given per serotype and colors indicate the type of infection the strains were isolated from.

Finally, we analyzed the genomes of our *S. pneumoniae* collection (**Figure 6.3.3**). As a start, we whole-genome sequenced a subset of the collection, de novo assembled the short reads into contigs and

annotated them (377 strains). Next, we performed a pangenome analysis on the draft assemblies of the set of strains (**Figure 6.3.3, A**). We identified a total of 10,761 genes of which 1,543 genes were conserved among 95% of the isolates and constitute the core genome. The remaining 9,218 genes that are present in less than 95% of the strains form the accessory genome. Moreover, plotting the number of genes in function of the number of sequenced genomes shows an increasing number of genes with more sequenced genomes which indicates that *S. pneumoniae* has an open pangenome (**Figure 6.3.3, B**). An important note here is the potential presence of fragmented genes that are not correctly annotated via Prokka, for example *lytA* is annotated as lytA_1, lytA_2, lytA_3, lytA_4 and lytA_5. Probably, these different gene fragments would together represent the full gene. Due to the presence of fragmented gene annotations, we get an overestimation of the number of genes in the pangenome of *S. pneumoniae* and a false indication of how 'open' the pangenome of *S. pneumoniae* really is. To further investigate the pangenome, we should study the presence of fragmented genes more in depth. Finally, we used the core genome to construct a phylogenetic tree (**Figure 6.3.3, C**). We displayed the origin of the strain (reference strain, isolated from non-invasive or invasive infection) and the serotype on the tree. Strains from the same serotype seem to be close to each other and show local clustering, but some serotypes (e.g. 19A and 23B) can be found all over the tree similarly to strains from different types of infection. To study the epidemiology and population structure more in depth, clades should be detected and MLST analysis should be performed. These analysis were not performed during this thesis due to time limitations.

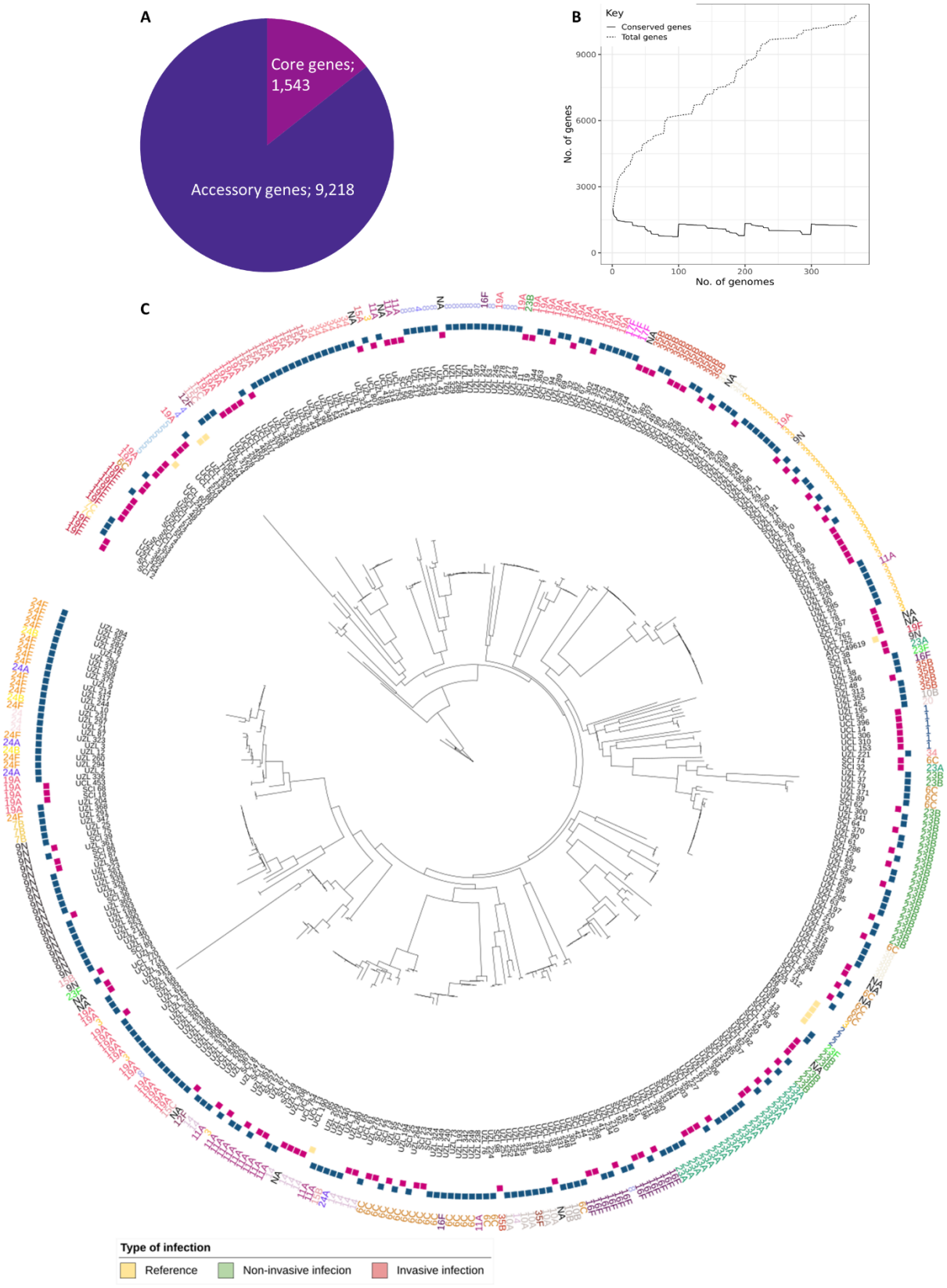


Figure 6.3.3: **The genomics of *S. pneumoniae*.** (A) The pangenome of *S. pneumoniae* with 1,543 core genes (present in 95% of the strains) and 9,218 accessory genes (present in less than 95% of the strains). (B) Pangenome plot of the gene repertoire of *S. pneumoniae*. (C) Phylogenetic tree annotated with origin and serotype. The inner circle represents the origin of the strain (infection type) and the outer circle represents the serotype with the same serotype displayed in the same color.

6.3.2 Antibiotic resistance is diverse among our *S. pneumoniae* collection depending on the type of antibiotic

We characterized the entire collection of strains for resistance against amoxicillin, cefuroxime, moxifloxacin and vancomycin. Depending on their MIC, strains were considered susceptible or resistant towards an antibiotic according to their EUCAST breakpoints in 2024 (**Table 6.3.1**). When strains had a MIC above the clinical breakpoint for resistance, strains were considered resistant.

Table 6.3.1 **EUCAST breakpoints to define whether a *S. pneumoniae* strain is susceptible or resistant towards an antibiotic for 4 antibiotics.**

MIC	EUCAST BREAKPOINTS	
	Sensitive (µg/mL)	Resistant (µg/mL)
AMOXICILLIN	≤ 0.5	> 1
CEFUROXIME	≤ 0.25	> 0.25
MOXIFLOXACIN	≤ 0.5	> 0.5
VANCOMYCIN	≤ 2	> 2

When we analyzed the resistance profile of the strains for the different antibiotics (**Figure 6.3.4)**, we observed a resistance rate of about 8% of the strains towards amoxicillin and about 20% towards cefuroxime which is in line with general numbers of penicillin resistance in Belgium for invasive strains with 18.3% in 2021 and 14.1% in 2022 (131). Our collection does not contain resistant strains towards moxifloxacin or vancomycin which is also consistent with resistance rates both in Belgium and globally (131,134). Resistance rates differed among the different types of infection. About 13-14% of the isolates from carriage and non-invasive diseases were resistant towards amoxicillin, but only about 6.5% of the isolates from invasive diseases. For cefuroxime, the resistance rate was lowest for the carriage group (17%) followed by invasive isolates (19.5%) and non-invasive isolates (26%).

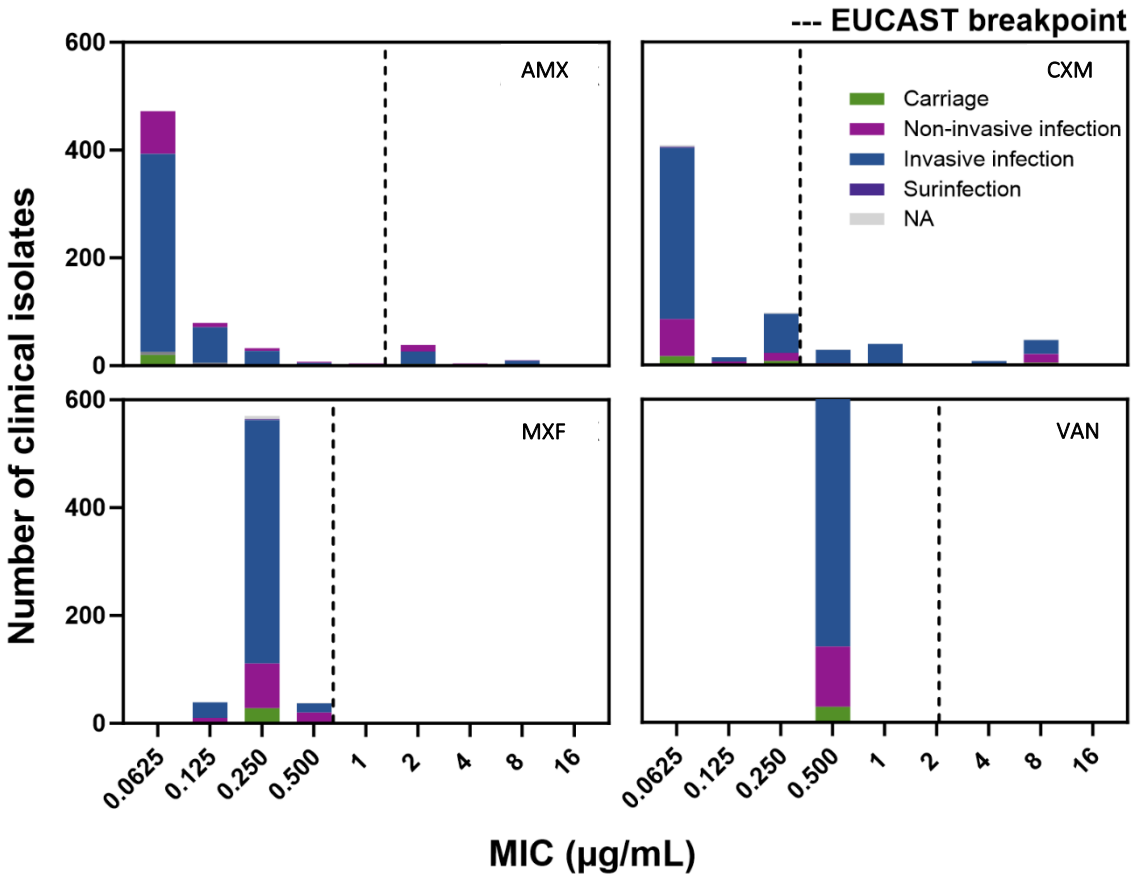


Figure 6.3.4: **Minimal inhibitory concentration (MIC) of 647 *S. pneumoniae* clinical strains, from carriage, invasive and non-invasive infections, to four clinically relevant antibiotics: amoxicillin (AMX), cefuroxime (CXM), moxifloxacin (MXF) and vancomycin (VAN).** About 8% of the strains was resistant towards amoxicillin and about 20% towards cefuroxime. All strains were susceptible to moxifloxacin and vancomycin according to their EUCAST breakpoints (dotted lines).

The following step was to search for correlations between the MIC values of the different antibiotics (**Figure 6.3.5**). We therefore used normalized MIC values according to the distance of the MIC value to the EUCAST breakpoints. Strains with a MIC value that corresponded to the breakpoint were assigned a score of 0. A score of +1 or -1 was given to strains with a MIC one twofold dilution above or below the breakpoint, respectively. Resistant strains therefore received positive values and sensitive strains were assigned 0 or a negative value. MIC values further away from the breakpoint increased or decreased with 1 per twofold dilution (**Table 6.5.1**). We observed a strong positive and significant correlation between the normalized MICs of amoxicillin and cefuroxime (Pearson, $R = 0.8775$, $R^2 = 0.77$, $p < 0.001$) (**Figure 6.3.5**). Both antibiotics are β-lactam antibiotics and have the same MOA, which indicates that a mutual resistance mechanism will

likely explain resistance towards both antibiotics. We observed a weak negative, but significant correlation between MICs for moxifloxacin and amoxicillin (Pearson, R = -0.1268, R^2 = 0.016, p = 0,0013) or moxifloxacin and cefuroxime (Pearson, R = -0,1029, R^2 = 0.01060, p = 0,0089) (**Figure 6.3.5**). A potential explanation is the absence of resistant strains against moxifloxacin while some of these strains were resistant against amoxicillin and/or cefuroxime. As the MIC value for vancomycin was 0.5 µg/mL for all strains, no correlation between vancomycin and the other antibiotics was observed. We also plotted the data on the phylogenetic tree which visually shows the positive correlation between the MICs for amoxicillin and cefuroxime, and the negative correlation between the β-lactam antibiotics and moxifloxacin (**Figure 6.5.1**).

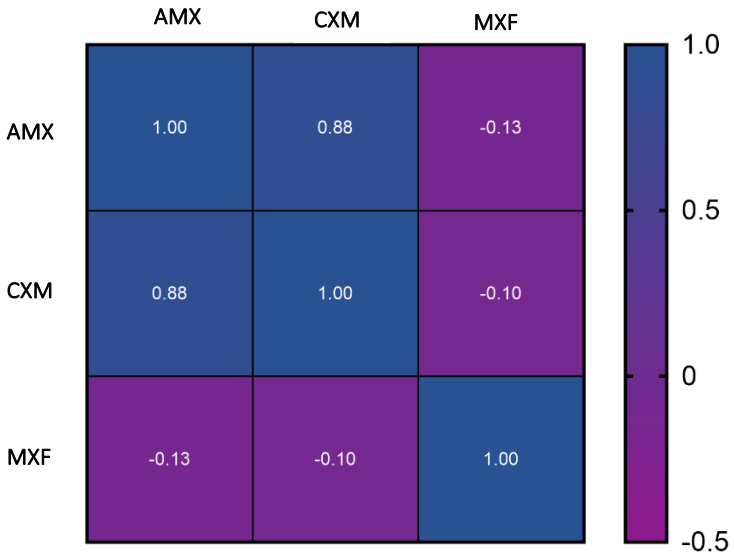


Figure 6.3.5: **Correlation plot of the normalized MICs of 3 different antibiotics.** The correlation between amoxicillin and cefuroxime is positive (Pearson, R=0.877, p < 0.001) which can be related to the common mechanism-of-action (MOA). The correlation between amoxicillin/cefuroxime and moxifloxacin is negative (Pearson, R = -0.1268 and p = 0,0013, R = -0,1029 and p = 0,0089, respectively) which could be explained to the distinct MOAs of the antibiotics.

6.3.3 Persister cells are both prevalent and highly variable among *S. pneumoniae* clinical isolates

The next step was to determine the persistence profile of the entire collection of *S. pneumoniae* clinical isolates. We set-up a screening model based on the killing dynamics of *S. pneumoniae* reference strain D39 (**CHAPTER 4**). Treatment of D39 at a 100X the MIC of amoxicillin, cefuroxime, moxifloxacin and vancomycin,

in both the diluted stationary and exponential growth state, resulted in a biphasic killing pattern. After 4 hours of treatment of diluted stationary-phase cultures, D39 reached a slower killing phase indicating the presence of persister cells for all antibiotics. To prove that the clinical strains displayed similar killing dynamics, kill curves were determined for a part of the collection (31 strains, **Figure 6.3.7**). Our collection contained too many strains to obtain a full killing curve for each strain and we therefore opted for a screening model (**Figure 6.3.6**). More specifically, we looked into persister levels after treatment at a 100X the MIC of amoxicillin or moxifloxacin, two antibiotics with a different MOA, in the diluted stationary phase. We determined survival after 6 hours of treatment, to ensure we reached the second, slower killing phase of persister cells. The applied antibiotic concentration was adjusted for each strain based on its MIC to rule out potential confounding effects of AMR. As we did not obtain a kill curve for each isolate, we could not determine the real persister level using mathematical analysis and we therefore used the term survival level/fraction instead of persister level/fraction.

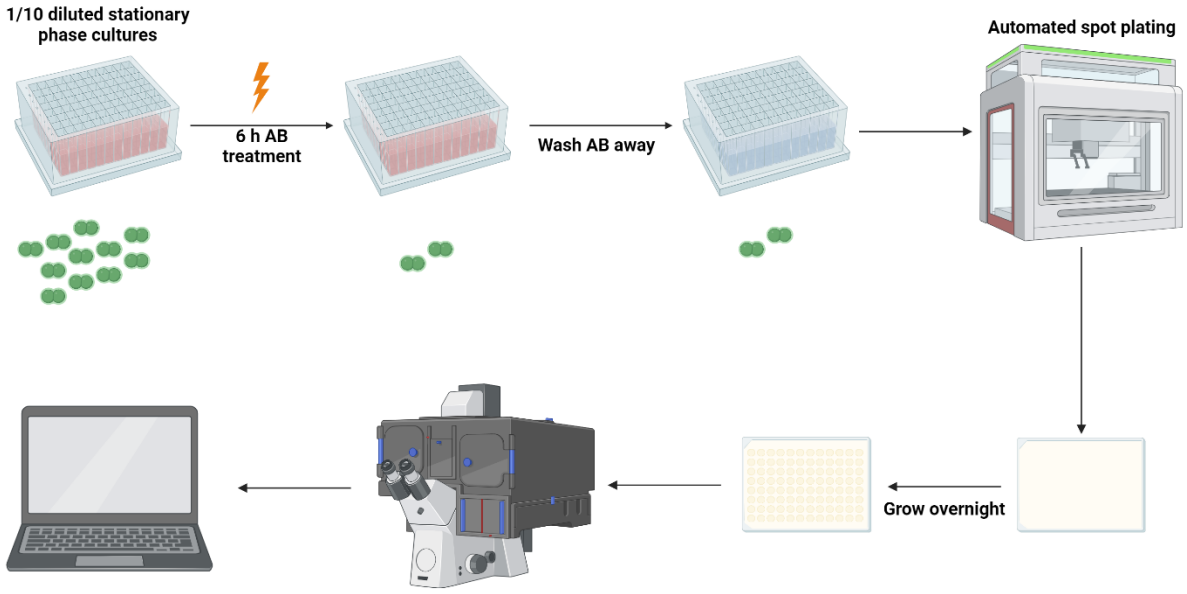


Figure 6.3.6: **Screening model of *S. pneumoniae* clinical isolates for persistence.** Diluted stationary phase cultures were treated during 6 hours with amoxicillin or moxifloxacin at 100X the MIC followed by washing away the antibiotics with PBS. Spot plating was performed automatically on TSA plates using an automated system (Biomek 3000, Beckman Coulter). After overnight incubation, a picture was taken of each spot with an inverted Nikon microscope (TiEclipse) at a 4X magnification, using a programmed automated stage. The number of colonies on the pictures was counted with the retrained model of Cellpose (46).

The survival fraction was determined by the number of cells before and after treatment according to following formula.

$$survival\ fraction = \frac{number\ of\ cells\ after\ treatment}{number\ of\ cells\ before\ treatment}$$

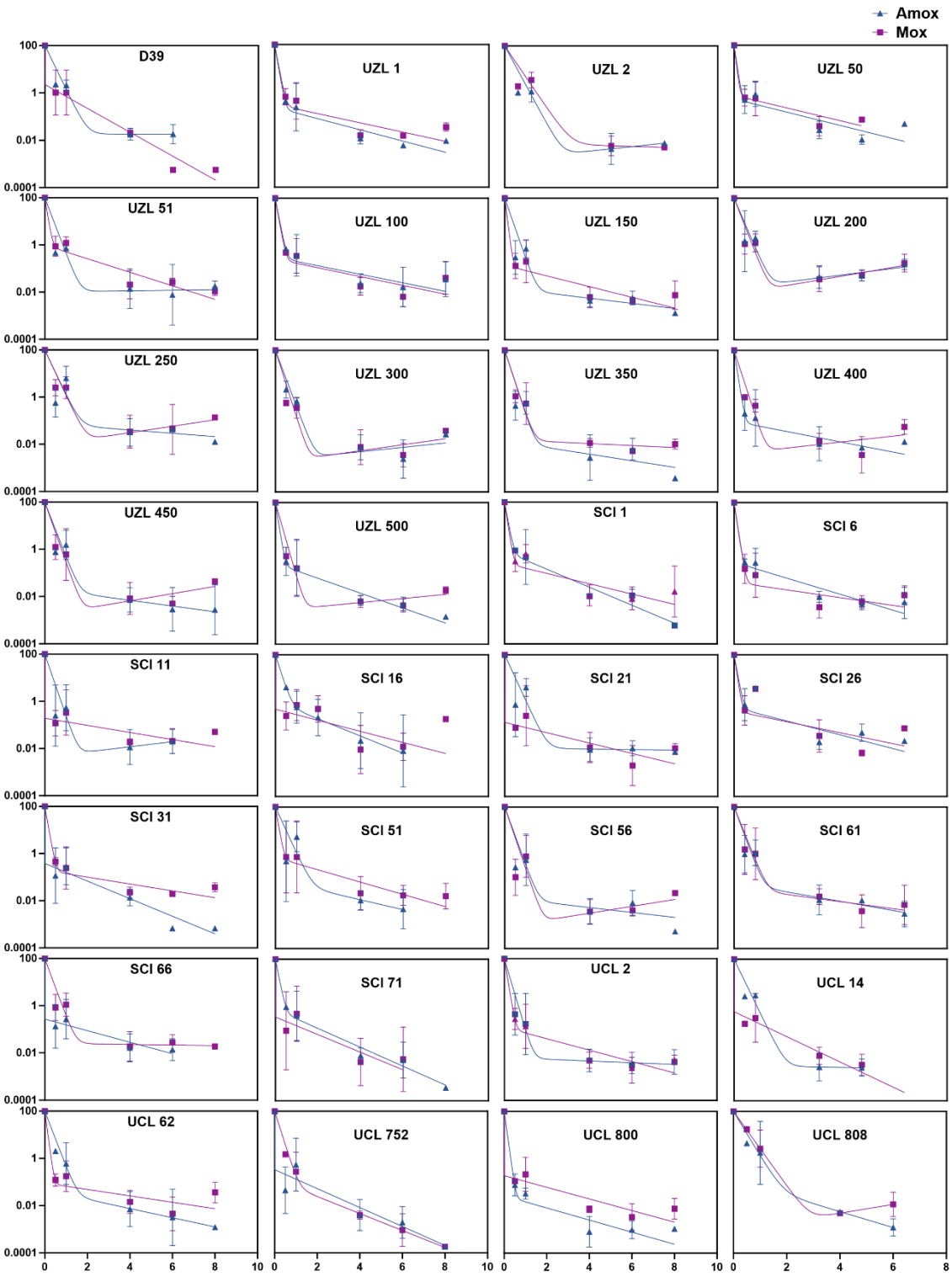


Figure 6.3.7: **Biphasic killing pattern upon antibiotic treatment indicates the presence of persister subpopulations for different *S. pneumoniae* clinical isolates.** Fitting of a nonlinear fixed-effect model to $\log_{10}$-transformed survival data upon treatment with amoxicillin or moxifloxacin. Diluted (1:10) stationary-phase bacteria were treated for 8 h with the antibiotic at 100-fold the MIC. Bold lines show the fitted biphasic killing curves. Three biological repeats of the experiments were performed (n = 3).

We observed a high diversity in survival fractions among clinical isolates after exposure to amoxicillin, ranging from a 3 log to a 7 log reduction in bacterial concentration, or even the full sterilization of a culture with no surviving cells after 6 hours of treatment of 7 strains (8 log reduction or more) (**Figure 6.3.8**). The median survival fraction after amoxicillin treatment was $2.8*10^{-5}$.

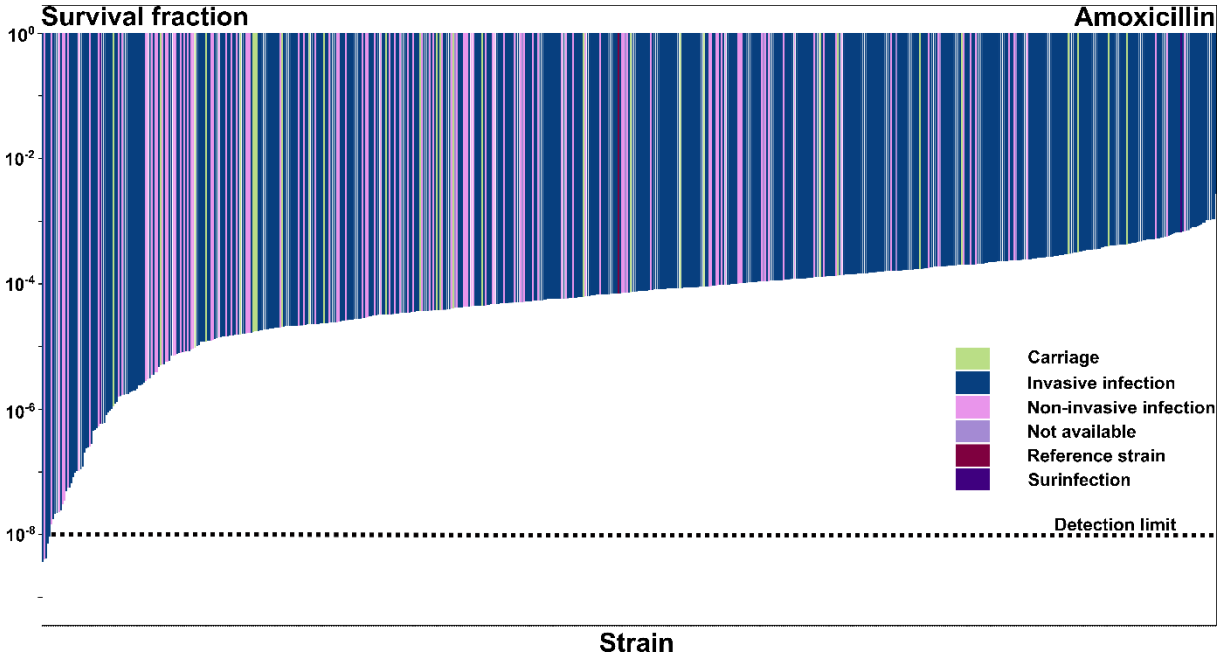


Figure 6.3.8: **The amoxicillin persistence profile.** The mean survival fraction of each strain is presented with a bar after a 6 hour treatment in the diluted stationary phase with amoxicillin at 100X the MIC. The color of the bar represents the origin of the strain and the detection limit is given by the dotted line.

Similar results were observed for moxifloxacin. Again, survival fractions ranged from a 3 log to a 7 log reduction and for 14 of the *S. pneumoniae* cultures, we did not detect survivors after 6 hours of treatment (8 log reduction or more) (**Figure 6.3.9**). The median survival fraction after moxifloxacin treatment was $4.9*10^{-6}$ and thus lower than the median survival fraction after amoxicillin treatment. This corresponds to our results that we obtained with reference strain D39 (**CHAPTER 4**) where we observed a persister fraction after treatment with moxifloxacin of 13.74% compared to a higher fraction after amoxicillin treatment of 24.31%. A potential explanation is the difference in MOA of the antibiotics.

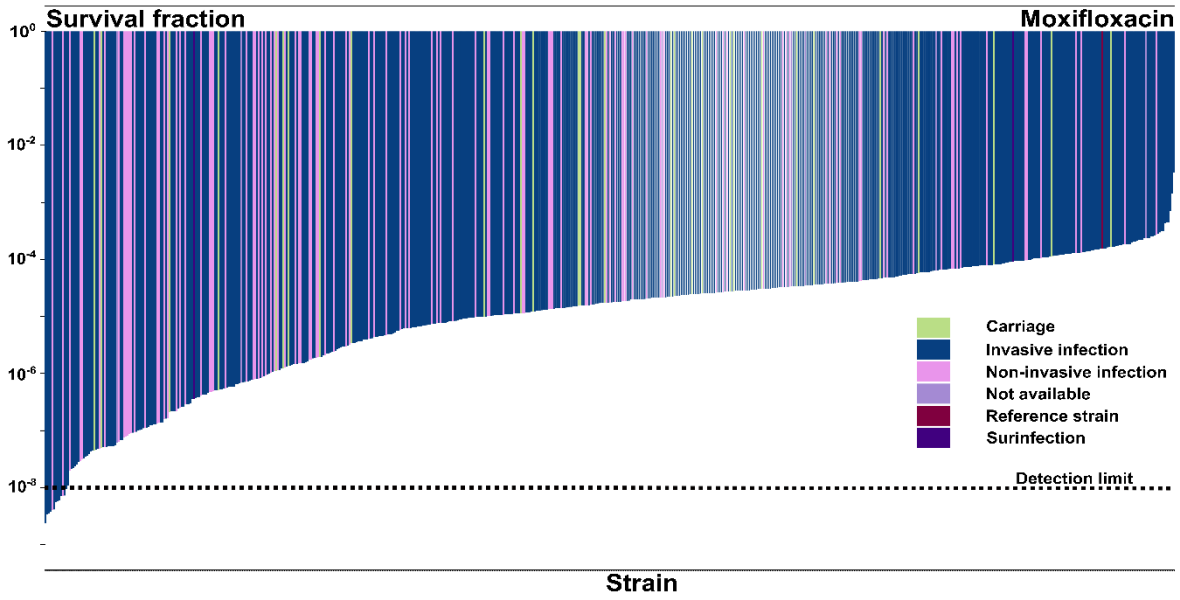


Figure 6.3.9: **The moxifloxacin persistence profile.** The mean survival fraction of each strain presented with a bar after a 6 hour treatment in the diluted stationary phase with moxifloxacin at 100X the MIC. The color of the bar represents the origin of the strain and the detection limit is given by the dotted line.

Given the strong variation between strains, we wondered whether some strains show antibiotic survival specific to one condition or whether survival levels of these strains can be correlated between different conditions. To further investigate the difference in survival levels between amoxicillin and moxifloxacin, we therefore performed a correlation analysis on the persister fractions after treatment (**Figure 6.3.10**). We observed a significant positive correlation (Pearson, $R^2 = 0.1187$, $p < 0.001$) between the survival fraction after lethal exposure of both antibiotics, which could indicate multidrug tolerance or a similar mechanism contributing to amoxicillin/moxifloxacin persistence. Nonetheless, this positive correlation indicates that only about 12% of the variation of the phenotype following amoxicillin treatment explains the variation observed in the phenotype after moxifloxacin treatment which could indicate that also non-related mechanisms contribute to amoxicillin and moxifloxacin persistence. We also plotted the data on the phylogenetic tree which visually shows the small positive correlation between the persister levels for amoxicillin and moxifloxacin (**Figure 6.5.2**). For a long time, dormancy was proposed as the mechanism behind antibiotic persistence and persisters were supposed to be multidrug tolerant (147,151,153). We also studied correlations between growth, i.e. survival after 6 h of growth without treatment compared to the

start (0 h), and persisters by comparing survival fractions of treated and untreated cells after 6 hours to investigate the potential role of overall dormancy in the persister phenotype (**Figure 6.3.10**). Surprisingly, we observed a small, but significant positive correlation between increased growth and increased survival (Pearson, R^2 from 0.04452 to 0.1151, $P < 0.0001$), which does not substantiate the common belief that slow growth induces persister formation. A lot of studies underscore the role of growth arrest and metabolic inactivity in survival of antibiotic therapy (154–157,270), but dormancy is not the sole explanation for the persister phenotype (147,151). Moreover, both non-growing, metabolically active and actively growing bacteria can give rise to persister cells (158–162). The variety of underlying mechanisms can also lead to varying levels of tolerance towards different antibiotics within a persister subpopulation, rather than general multidrug tolerance (147).

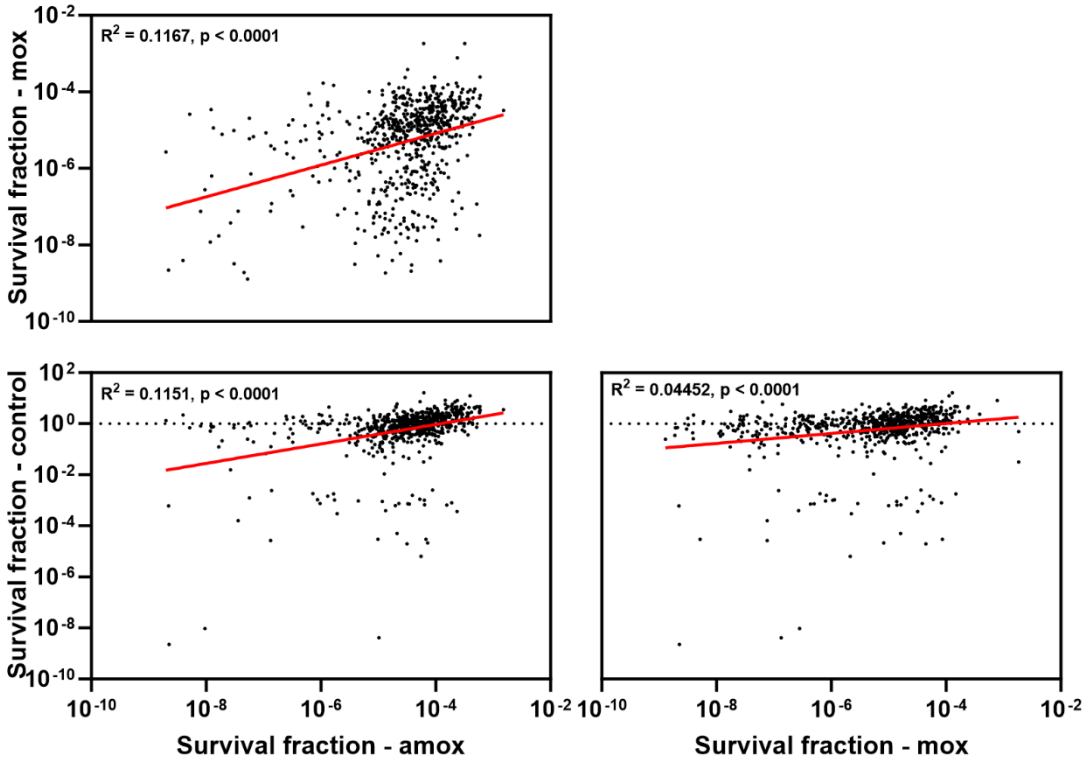


Figure 6.3.10 **Correlation analysis of survival fractions between amoxicillin and moxifloxacin show strong correlations, but also a significant correlation was observed between antibiotic treatment and growth.** Pearson correlation coefficient (R^2) is given for each correlation.

Finally, we were wondering if antibiotic sensitivity (MIC) was associated with higher survival levels. We used the MIC and survival fraction to investigate the potential correlation (**Figure 6.3.11**). For none of the antibiotics, a significant correlation was observed (Pearson, $P > 0.05$) which indicates that higher survival levels are not related to a higher MIC or resistance. To further study the correlation between antibiotic sensitivity and antibiotic persistence, categories should be made based on the type of infection (invasive vs non-invasive) the clinical strain cause. This analysis was not performed due to time limitations.

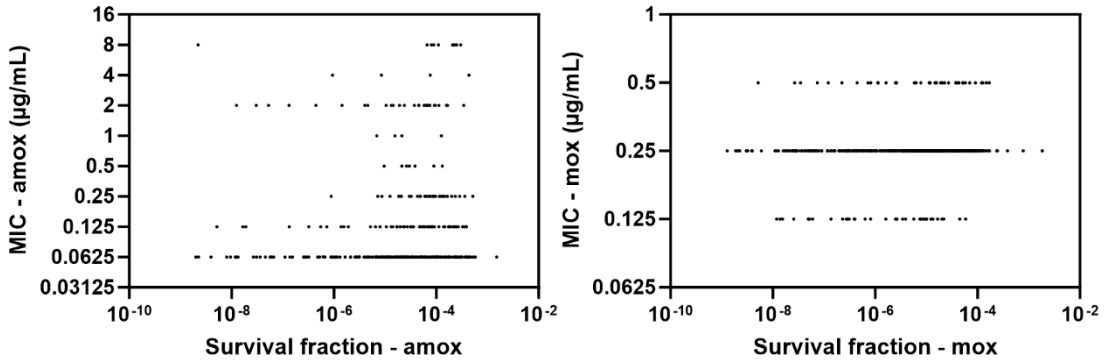


Figure 6.3.11: **Antibiotic resistance and antibiotic persistence are not associated for amoxicillin and moxifloxacin.** The correlation between the MIC values and the survival fraction after a 6 hours treatment at 100X the MIC was determined. No significant correlation was observed for amoxicillin (Pearson, $R^2 = 0.0004$, $P = 0.6341$) or moxifloxacin (Pearson, $R^2 = 0.005$, $P = 0.0733$).

6.4 Discussion

In this study, we used a large collection of clinical *S. pneumoniae* isolates to explore antibiotic resistance and persistence in *S. pneumoniae*. Our collection is diverse with strains expressing different serotypes and originating from different types of infection (surinfection, invasive or non-invasive infections) or from carriage, which is an important requisite for infection. This collection can give us new insights in how *S. pneumoniae* can evade antibiotic exposure via a clinically relevant approach.

6.4.1 The antibiotic resistance profile

We observed high resistance rates among the clinical strains towards the β-lactam antibiotics (amoxicillin and cefuroxime), but none of the strains was resistant towards the fluoroquinolone (moxifloxacin) or the glycopeptide antibiotic (vancomycin). Penicillin-resistant *S. pneumoniae* is since 2017 included on the list of priority pathogens for antibiotic research of the WHO. B-lactam resistance is worldwide widespread, but resistance rates differ between countries (133). Amoxicillin resistance was reported to be as high as 35.7% in 2020 in Spain (271), whereas the amoxicillin resistance rate was only 0.7% in Argentina among IPD isolates in children from 2006-2019 (272). A multicenter study in China from 2010-2015 reported a resistance rate of less than 3% for amoxicillin in contrast to 60% for cefuroxime (273). In Belgium, the reported penicillin resistance rate was 18.3% in 2021 and 14.1% in 2022 (131) which is comparable to our findings of a resistance rate of about 8% for amoxicillin and 20% for cefuroxime. Fluoroquinolone resistance was reported to remain low worldwide, despite the increased use for treatment of *S. pneumoniae* infections (132,133). No moxifloxacin resistant strains were present in our collection of strains, which is consistent with a multicenter study conducted in China from 2010-2015 (273) and with a study in Argentina from 2006-2019 (272) who also did not observe moxifloxacin resistance. The use of vancomycin in treatment of *S. pneumoniae* infections is limited, as vancomycin is only indicated as last resort in case of penicillin allergy, for meningitis or when first- and second-line therapies are failing (105–108). No cases of vancomycin were reported to date, which corresponds to our findings of no vancomycin resistance among our strain

collection (133,134). Nonetheless, cases of treatment failure were reported due to vancomycin-tolerant *S. pneumoniae* (138–142).

We compared the resistance profile of amoxicillin, cefuroxime and moxifloxacin. Vancomycin was excluded from this analysis, because all strains were susceptible towards vancomycin and had the same MIC (0.5 µg/mL). We observed a positive correlation between the normalized MICs of amoxicillin and cefuroxime, but a negative correlation between amoxicillin/cefuroxime and moxifloxacin. The positive correlation can be attributed to the same MOA, interference with the cell wall synthesis, and thus similar molecular mechanisms of resistance for both β-lactam antibiotics, for example via alterations of the PBP (132–134,139). Antibiotic-susceptibility in the clinic is based on the oxacillin disk-diffusion test that is used for the detection of penicillin resistance and for the prediction of the susceptibility for both amoxicillin (penicillin) and cefuroxime (cephalosporin) (274). The negative correlation between the β-lactam antibiotics and moxifloxacin, a fluoroquinolone targeting the bacterial DNA synthesis, can again be explained by the MOA, which differs between both antibiotic classes.

6.4.2 Antibiotic persistence is both prevalent and highly variable among *S. pneumoniae* clinical isolates

This is the first study that employs a large collection of clinical strains to investigate antibiotic persistence in *Streptococcus pneumoniae*. Previous studies on antibiotic persistence in clinical or natural isolates were often limited in the number of strains. Stewart and Rozen (2011), for example, used 24 clinical *E. coli* strains to investigate genetic variation in antibiotic persistence (266) and Goneau *et al.* (2014) studied the underlying mechanism of persistence in *E. coli* by 10 clinical strains (162). Other research was rather limited in diversity of the set of clinical strains, because strains were collected during a longitudinal study (180–182). On the other hand, persistence was studied in *Mycobacterium tuberculosis* on a set of about 50 clinical isolates (184) and for a set of 375 clinical staphylococcal isolates (275).

We observed a high frequency of persisters in our collection with a high variability in survival levels. Survivors were detected for almost all strains, ranging from 0.1% to 0.00001% surviving cells for both

amoxicillin and moxifloxacin. Hofsteenge *et al.* (2013) and Stewart and Rosen (2011) detected comparable variations in survival after antibiotic treatment of a set of natural and environmental *E. coli* isolates, respectively (265,266). Similarly, Barth *et al.* (2013) observed a high heterogeneity of persister cell formation among *A. baumannii* isolates (267). The median survival fraction after amoxicillin treatment ($2.8*10^{-5}$) was higher compared to moxifloxacin ($4.9*10^{-6}$). These observations are in concordance with our results with reference strain D39 (CHAPTER 4), but also with other research groups investigating persistence in clinical strains (162,265,266,275). The diversity in survival levels upon antibiotic treatment could be related to the different MOA of amoxicillin and moxifloxacin and is the first indication of distinct mechanisms underlying amoxicillin and moxifloxacin persistence (162,266). Despite the difference in median survival level, we observed a significant positive correlation (Pearson, $R^2 = 0.1187$, $p < 0.001$) between the survival levels after lethal amoxicillin and moxifloxacin exposure. This could point towards an overall metabolically inactive state or to a common mechanism contributing to amoxicillin and moxifloxacin persistence. Previous findings for natural bacterial strains were contradictory. Hofsteenge *et al.* (2013) and Stewart and Rosen (2011) did not observe positive correlations between different antibiotics for *E. coli* isolates (265,266) and Barth *et al.* (2013) reported the absence of correlations between persister levels for different antibiotics in *A. baumannii* isolates (267). On the other hand, research investigating *P. aeruginosa* or *M. tuberculosis* isolates reported positive correlations between persister levels after aminoglycoside and fluoroquinolone treatment (174,184). Finally, we looked for associations between growth and antibiotic survival, and surprisingly, we saw a slight positive correlation between how fast the strains grow and how well they survive antibiotic treatment, especially after treatment with amoxicillin. These correlations imply that if the bacteria grew faster, they survived antibiotic treatment better. This was unexpected, as persistence is linked to dormancy of bacterial cells and we expected that if cells were less actively dividing and less metabolically active, they would survive antibiotic treatment better. However, different studies state that global metabolic dormancy is not solely responsible for tolerance (158,159,276–278). For example, Stapels *et al.* (2018) and Peyrusson *et al.* (2020) demonstrated the presence of nondividing but metabolically active *Salmonella* and *Staphylococcus aureus* persisters, respectively, during intracellular infections (160,161), and

Goneau *et al.* (2014) stated that antibiotic tolerance is caused more likely by selective target inactivation than by global metabolic dormancy in uropathogens (162). Finally, we compared MIC values with survival fractions for potential associations, but no significant correlation was observed, neither for amoxicillin or moxifloxacin (Pearson, $P > 0.05$) which could indicate that higher survival levels were not related to a higher MIC or resistance.

We found persister cells in a wide range of *S. pneumoniae* clinical isolates, mostly originating from acute infections. Despite the absence of evolution in an experimental set-up towards a high persister mutant, the widespread presence of persisters in *S. pneumoniae* strains indicates that they are relevant for *S. pneumoniae* infections, both in carriage, non-invasive and invasive diseases. To further explore the clinical relevance of persisters during *S. pneumoniae* infections, we initiated the development of a mouse model to study antibiotic persistence *in vivo* in CHAPTER 7.

6.5 Supplementary data

Table 6.5.1: **Normalized MIC scores of amoxicillin, cefuroxime, moxifloxacin and vancomycin.**

MIC (µg/mL)	0.0625	0.125	0.250	0.500	1	2	4	8	16
AMX	-4	-3	-2	-1	**0**	1	2	3	4
CXM	-2	-1	**0**	1	2	3	4	5	6
MXF	-3	-2	-1	**0**	1	2	3	4	5
VAN	-5	-4	-3	-2	-1	**0**	1	2	3

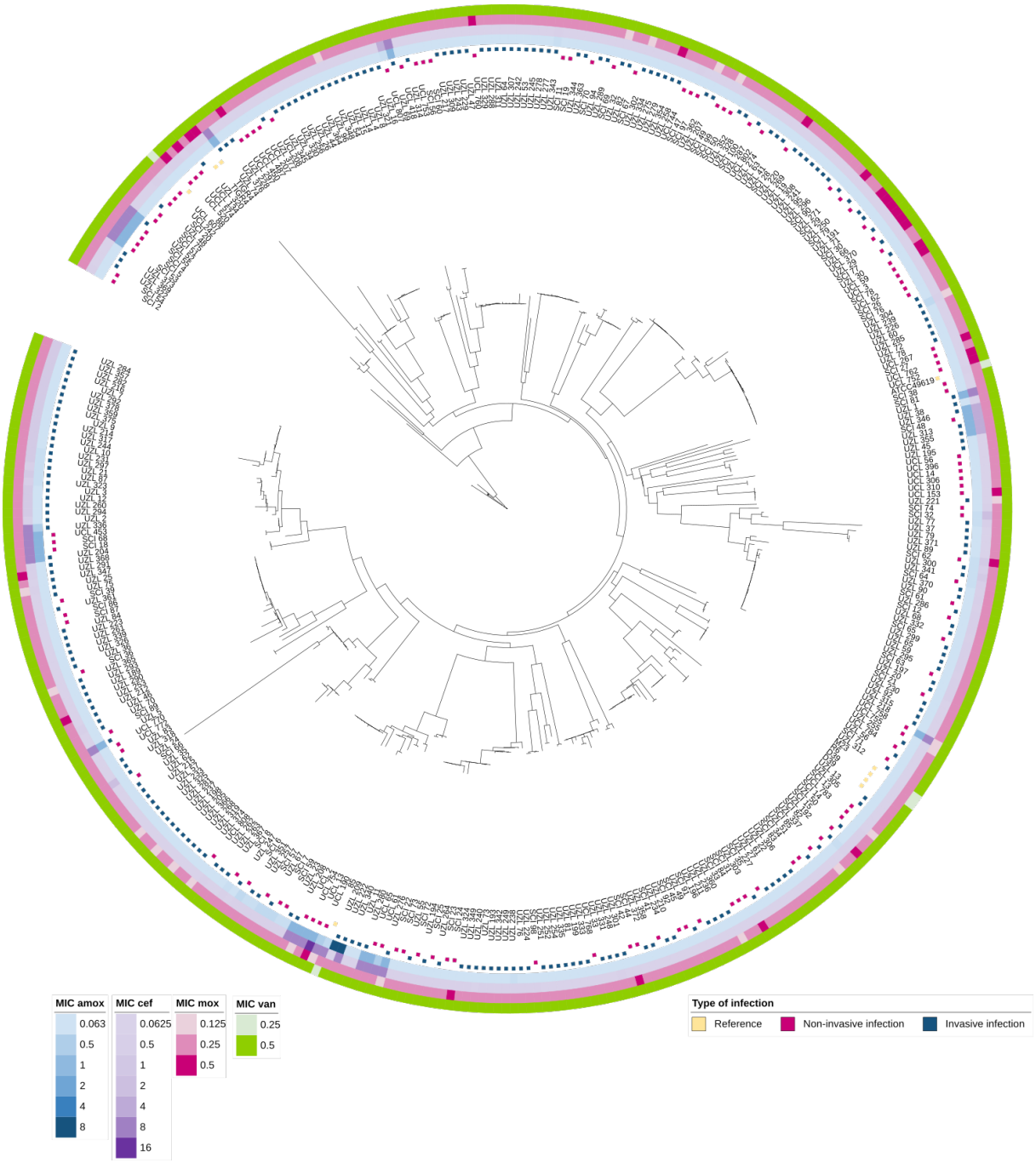


Figure 6.5.1: **Phylogenetic tree annotated with origin and MIC towards amoxicillin (amox), cefuroxime (cef), moxifloxacin (mox) and vancomycin (van).** The inner circle represents the origin of the strain (infection type) and the outer circle represents the MIC towards the four different antibiotics.

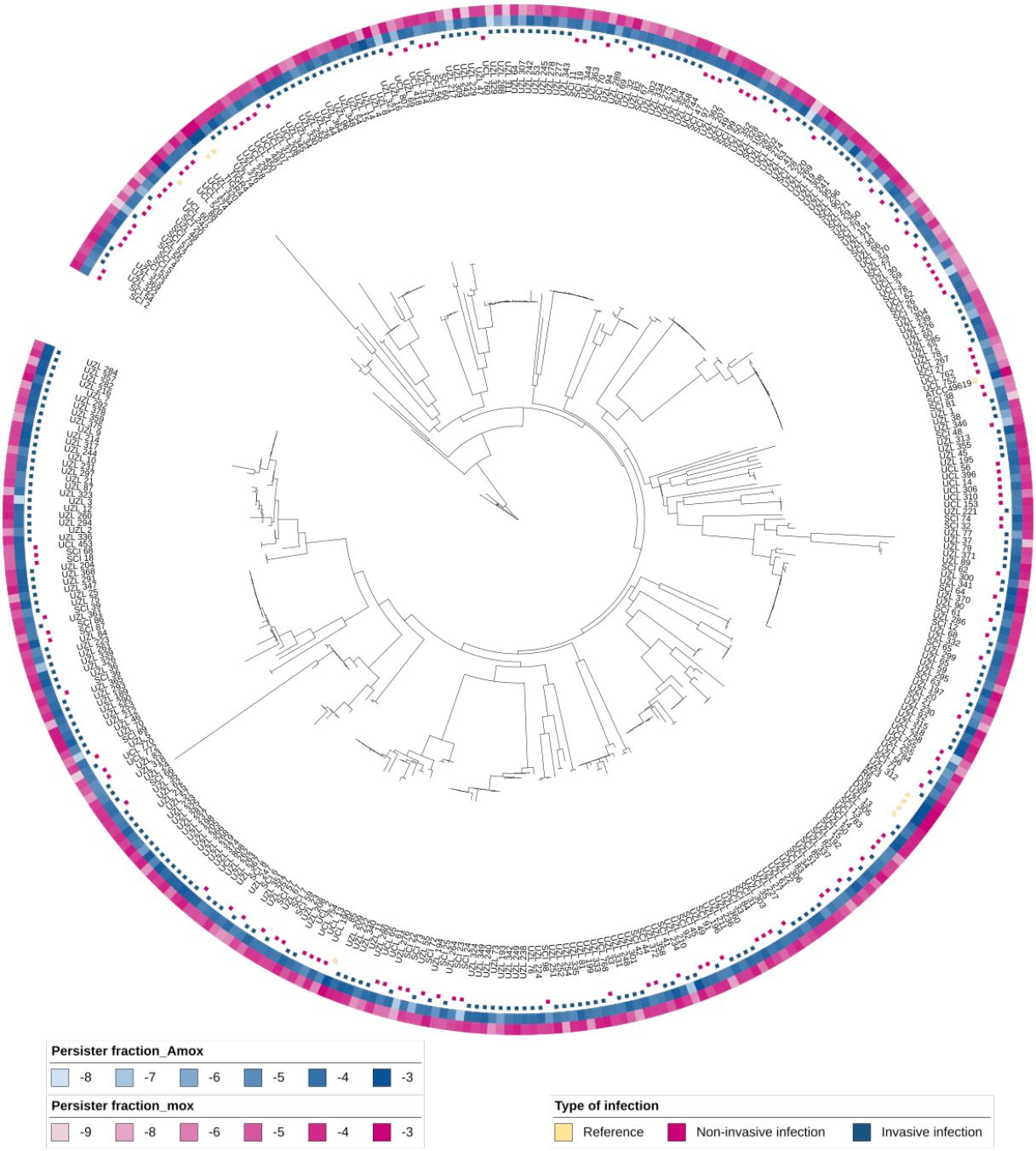


Figure 6.5.2: **Phylogenetic tree annotated with origin and persister levels towards amoxicillin (amox) and moxifloxacin (mox).** The inner circle represents the origin of the strain (infection type) and the outer circle represents the persister levels towards the two different antibiotics.

7 EXPLORATION OF A CHRONIC *IN VIVO* MODEL TO STUDY INFECTION AND PERSISTENCE IN *STREPTOCOCCUS PNEUMONIAE*

Nele Geerts, An Matheeussen, Linda De Vooght, Bram Van den Bergh and Paul Cos., *Optimization of a chronic in vivo model to study Persistence in Streptococcus pneumoniae,* unpublished

7.1 Introduction

In recent years, persistence research gained increased interest which resulted in many reports on the clinical relevance of persistence (147,181). However, persister cells are hard to study, due to the small persister fractions and the transiency of the phenotype. Also, demonstrating the causality between persisters and therapy failure remains challenging (185). Therefore, *in vivo* studies are needed next to *in vitro* experiments to support the hypothesis that persisters contribute to therapy failure and to the chronic nature of infections (147,185). At present, only a limited number of studies have been reported to translate *in vitro* findings into an *in vivo* model (279,280). Helaine *et al.* (2014) detected *Salmonella* persisters in a nonreplicating state within minutes after infection of macrophages. Among these, they found phenotypic heterogeneity with some cells resuming growth intracellularly and others remaining in a nonreplicating state (281). Moreover, different murine models were described to study *in vivo* persistence of *Salmonella* (280,282,283). To study *E. coli* persistence, a model of chronic murine urinary tract infection exists and was already employed to study the role of metabolites in the eradication of persisters (284). Also *in vivo* persistence studies involving clinical strains were performed. Verstraete *et al.* (2023) explored *in vivo* persistence in a murine lung infection model and they observed strong correlations between *in vitro* and *in vivo* survival of natural *P. aeruginosa* isolates after antibiotic challenge (215). Finally, potential persistence targets were studied by Dhar and McKinney (2010) for *M. tuberculosis* through a screening in antibiotic-treated mice (285). More specifically, they infected mice with *M. tuberculosis* mutants followed by isoniazid treatment which allowed them to screen for mutants that affected *in vivo* persistence and to identify potential persistence targets (285). These studies, employing a variety of bacterial species, show the opportunities of *in vivo* persistence models to unravel persistence mechanisms in a complex environment, which could guide future efforts in fighting persistence towards these relevant mechanisms. Furthermore, *in vivo* persistence studies could be useful to elucidate the clinical relevance of persister cells and to investigate ways for eradication of persister cells, namely anti-persister therapies.

In Chapter 4 and 6, we showed the presence of antibiotic-tolerant persisters *in vitro* in a variety of *S. pneumoniae* strains, including reference strains and clinical isolates, suggesting persistence is a general trait in *S. pneumoniae* cultures. Currently, an *in vivo* model to study persistence in *S. pneumoniae* is lacking. Our aim was to develop an *in vivo* mouse model to validate, similarly to Verstraete *et al.* (2023) (215), if persister levels obtained via *in vitro* assays are reflected in a more complex environment such as the mouse.

7.2 Material and Methods

7.2.1 Bacterial strains and culture conditions

S. pneumoniae reference strain D39 (serotype 2, NCTC® 7466) was used during this study. *S. pneumoniae* was cultured statically in cation-adjusted Mueller-Hinton broth (Fluka) supplemented with 5% lysed horse blood (MHL; Oxoid) or on blood agar (BA) plates (tryptic soy agar [Neogen] supplemented with 5% defibrinated sheep blood [Oxoid]) at 37°C in 5% CO_2. Either stationary-phase (overnight culture) or exponentially growing (after 6 h of growth in fresh medium) bacteria were used to prepare the inoculum after centrifugation and resuspension in PBS.

7.2.2 Mice

All animal experiments were authorized and approved by the Ethical committee of the University of Antwerp (approval numbers 2022-77 and 2023-73). Female BALB/c mice from 8-12 weeks old and female SWISS mice from 6-7 weeks were managed in accordance to the guidelines provided by the European Directive for Laboratory Animal Care (Directive 2010/63/EU of the European Parliament). The welfare of the mice was monitored daily during the experiments using a functional observational battery (FOB) scoring system **(Figure 7.5.1)**.

7.2.3 Pneumonia mouse model

BALB/c mice from 8-12 weeks old were briefly sedated with isoflurane (Halocarbon) and were then held in supine position to infect them intranasally or intratracheally. For intranasal infection, the bacterial suspension was pipetted onto the outside of each nostril. For intratracheal infection, the bacterial suspension was pipetted above the vocal cords. Mice were held in supine position for one minute afterwards. For dose optimization, different doses (ranging from $4*10^4$ – $1*10^8$ CFU/mL) and volumes (30 or 50 µL) of the inoculum were tested. After infection, mice had unlimited access to food and water. Mice were monitored daily by observation and body weight. Mice were sacrificed at 24, 48, 72, 96 h or 7 days p.i. by injection with sodium pentobarbital (200 mg/kg, Kela). The left lung lobe, the left lateral lobe of the liver

and the spleen were extracted and homogenized using a TissueRuptor (Qiagen) in 1 mL PBS. CFU counts were performed by serial dilution of the organ homogenates followed by plating on BA to determine the number of colonized bacteria per organ. The bacterial burden was expressed in gram of organ.

7.2.4 Nasopharyngeal carriage mouse model

SWISS mice from 6-7 weeks were briefly sedated with isoflurane (Halocarbon), held in supine position and then intranasally infected. For intranasal infection, 5 µL the bacterial suspension was pipetted onto the outside of each nostril (10 µL in total) and mice were held in supine position for one minute afterwards. The small volume will ensure that the bacteria remain mainly in the nasopharynx and are not deposited into the lungs. For dose optimization, different doses (ranging from $7*10^4$ – $2*10^6$ CFU/mL) were tested. After infection, mice had unlimited access to food and water. Mice were monitored daily by observation and body weight. Mice were sacrificed at 24, 72, 96 h or 7 days p.i. by injection with sodium pentobarbital (200 mg/kg, Kela). The nasopharynx, left lung lobe, the left lateral lobe of the liver and the spleen were extracted and homogenized using a TissueRuptor (Qiagen) in 1 mL PBS. CFU counts were performed by serial dilution of the organ homogenates followed by plating on BA to determine the number of colonized bacteria per organ. The bacterial burden was expressed in gram of organ.

7.2.5 FlexiVent analysis

We measured the lung parameters of a selection of mice with the FlexiVent™ FX equipment (SCIREQ, Canada), i.e. a system that measures the mechanics of the murine respiratory system (286). After applying a volume-driven perturbation to the subject's airway, parameters such as pressure, volume and flow are measured by the equipment and analyzed with the flexiVent software (286). This perturbation can be a single frequency oscillation waveform matching the breathing rate and tidal volume of a normal mouse (='snapshot'), or a combination of perturbations at varying frequencies ('prime perturbation') (286). For the respiratory measurements, mice were euthanized with pentobarbital (200 mg/kg, Kela), intubated with a 19 G blunt tip canula and connected to the FlexiVent™ ventilator. Various lung function parameters were characterized, including elastance (E_{rs}), an index for airway stiffness, the total airway resistance (R_{rs}), which

includes resistance from the conducting and peripheral tract, lung tissue and chest walls and compliance (C_{rs}), the ease with which the respiratory system can be extended (286,287).

7.2.6 *In vivo* killing assay

After 3 days of infection to induce nasopharyngeal carriage, one group of mice received a single dose of amoxicillin at 100X the MIC (10 µL of a 6.25 µg/mL stock solution) via the intranasal route. An infected, non-treated control group was included that received 10 µL of PBS. Mice were sacrificed at 4 or 24 h post treatment by injection with sodium pentobarbital (200 mg/kg, Kela). The nasopharynx, left lung lobe, the left lateral lobe of the liver and the spleen were extracted and homogenized using a TissueRuptor (Qiagen) in 1 mL PBS. CFU counts were performed by serial dilution of the organ homogenates followed by plating on BA to determine the number of colonized bacteria per organ. The bacterial burden was expressed in gram of organ.

7.2.7 Data analysis and statistics

Wilcoxon signed rank tests and Mann-Whitney U tests were used to compare continuous variables (non-treated vs treated). FlexiVent data were analyzed with a one-way ANOVA. All analyses were performed in GraphPad Prism version 10 and a difference between two groups was considered statistically significant when the P value was < 0.05.

7.3 Results

Before we could assess *in vivo* persistence levels, we had to optimize an infection model. For this, we choose to work with an animal model, as animal studies remain an essential tool to study infectious diseases (288). The mouse is the most important host to study *S. pneumoniae* infections, more specifically for pneumonia (288). The mouse has several advantages over other, larger animals such as the rat or chinchilla. Mice are small and thus easier to handle and house. Due to the widespread use of *S. pneumoniae* mouse models, extensive literature concerning infective dose, inoculation route, sedation and choice of mice strain is available as a basis for the optimization and implementation of a novel animal model of infection (288). Since antibiotic-tolerant persisters are mostly connected with recurrent and chronic infections, we chose to optimize a chronic *in vivo* model to adequately study pneumococcal persistence.

7.3.1 The pneumonia mouse model has an acute nature

Several chronic *S. pneumoniae in vivo* models have been reported. Yang *et al.* (2018) and Murrah *et al.* (2015) described a chronic *S. pneumoniae* otitis media (OM) model with infection over 7 days in chinchilla (34,289). However, the housekeeping of chinchillas requires large housing and large restraining devices compared to mice (290). Next to a chronic OM model, a chronic pulmonary infection model exists in mice (291,292). This model has proven to contain bacteria up to 28 days after infection in BALB/c mice (291). BALB/c mice are the most common laboratory animals used in experimental bacterial research and female BALB/c mice were used for *in vivo* persister studies with *P. aeruginosa* (215). Since the highest mortality rate of *S. pneumoniae* is recorded via lung infections (19), we tried to obtain a chronic lung infection model in mice with reference strain D39 with the intend to finally assess *in vivo* persistence levels of *S. pneumoniae*. We considered the infection as chronic from 3 days post infection, based on other research groups reporting acute and chronic pneumococcal infections (185,291,293–295).

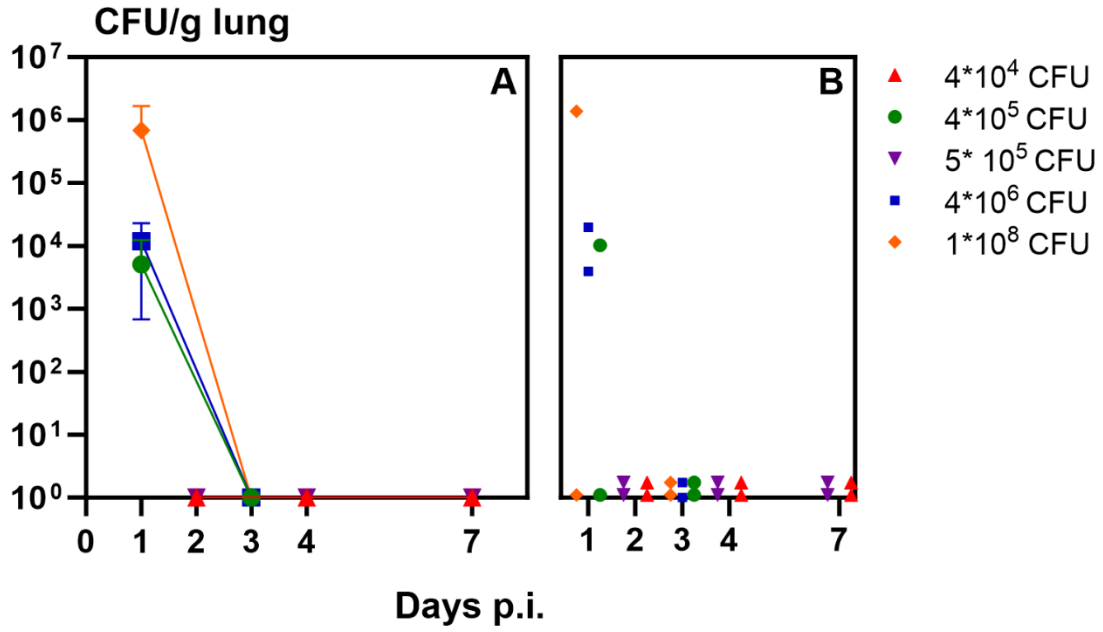


Figure 7.3.1: ***S. pneumoniae* D39 is rapidly cleared from the murine lungs after infection.** Female BALB/c mice were infected intranasally with different infection doses of *S. pneumoniae* D39 in 30 µL ($4*10^4$, $4*10^5$, $5*10^5$ and $4*10^6$ CFU/mouse) or 50 µL of PBS ($1*10^8$ CFU/mouse). Bacterial load of the left lung lobe is given as CFU/g lung. **A.** Each point represents the mean of 2 mice ± SD. **B.** Each point represents an individual mouse. Overlying data points were nudged to improve visibility.

Different parameters were tested, ranging from infection dose to route of infection, in order to obtain a chronic *in vivo* lung infection. Firstly, we set-up a dose-response experiment in which we tested different doses of the *S. pneumoniae* reference strain D39, ranging from $4*10^4$ to $1*10^8$ CFU/mouse. We started with a low dose, $4*10^4$ - $1*10^5$ CFU/mouse, similar to Briestenská *et al.* (2021). Murine survival is also impacted by the volume of the inoculum, as was observed by Haste *et al.* (2014). A higher dose volume was correlated with a higher lethality while keeping the number of CFU's constant (291). We therefore chose to start with a dose volume of 30 µL via intranasal infection. We followed survival and bacterial load, by plating and CFU determination, in the lungs over 7 days (2, 4 and 7 days p.i.) (**Figure 7.3.1**). After infection with $4*10^4$ CFU/mouse in 30 µL, bacteria were rapidly cleared after 24 h. Subsequently, we tested higher inocula and different days p.i. (1 and 3 days p.i.), to $4*10^6$ CFU/mouse in 30 µL or even $1*10^8$ CFU/mouse in 50 µL. The bacterial load at 24 h post infection increased with increasing infection doses, but nonetheless bacteria were cleared from the lungs thereafter and no chronic lung infection was established (**Figure 7.3.1**). Mice showed signs of a mild respiratory infection up to 2 days p.i.. To analyze murine lung function, we used the

flexiVent to assess different lung parameters after infection of mice with the different infection doses ($4*10^4$ – $1*10^8$ CFU/mL) at 1, 2, 3, 4 or 7 days p.i.: total airway resistance, elastance and compliance (emka Technologies/SCIREQ), (**Figure 7.3.2, right panels**). We did not observe differences between the infected groups and the PBS control, for none of the tested infection doses and independently of the day post infection, which confirms the data of the bacterial load in the lungs and the limited symptoms the mice showed.

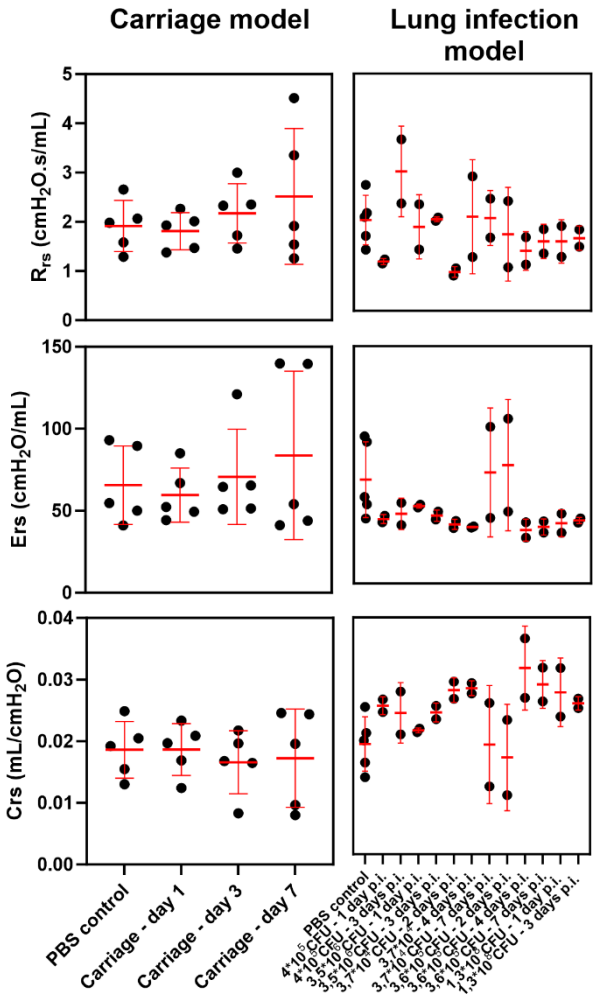


Figure 7.3.2: ***S. pneumoniae*** **D39 infection has no effect on the murine lung function.** The total airway resistance (R_{rs}), total airway elastance (E_{rs}) and total airway compliance (C_{rs}) were measured by flexiVent analysis after induction of nasopharyngeal carriage (left panels) or respiratory infection (right panels). Infection with *S. pneumoniae* did not affect any of the tested parameters for none of the conditions. Data were analyzed using a one-way ANOVA and $p > 0.05$ for all conditions.

Secondly, we tried to change the route of infection, from intranasal to intratracheal infection (**Figure 7.3.3**). Intranasal infection, trough aspiration, mimics natural transmission and the method is fast and easy to perform. Intratracheal infection on the other hand is a more complex technique, which ensures a high delivery of the bacteria to the lungs with a rapid induction of pneumonia without intermediate pathogenic steps (288). We observed *S. pneumoniae* D39 in the lungs of the mice 3 days after intratracheal infection, but with an approximate reduction in bacterial load of 3 log compared to 1 day after infection (**Figure 7.3.3**). Mice showed signs of a mild respiratory infection up to 2 days p.i. after intranasal infection. After intratracheal infection, mice seemed sicker according to the FOB scoring system (**Figure 7.5.1**), especially at day 1 p.i. infection with signs of pneumonia (ruffed fur, tachypnea and overall poor appearance). The overall condition improved from day 2 p.i., which is consistent to the bacterial load found in the lungs. We also assessed the lung function to look for effects of the different routes of infection on the lung parameters, but no differences were detected (**Figure 7.3.4**).

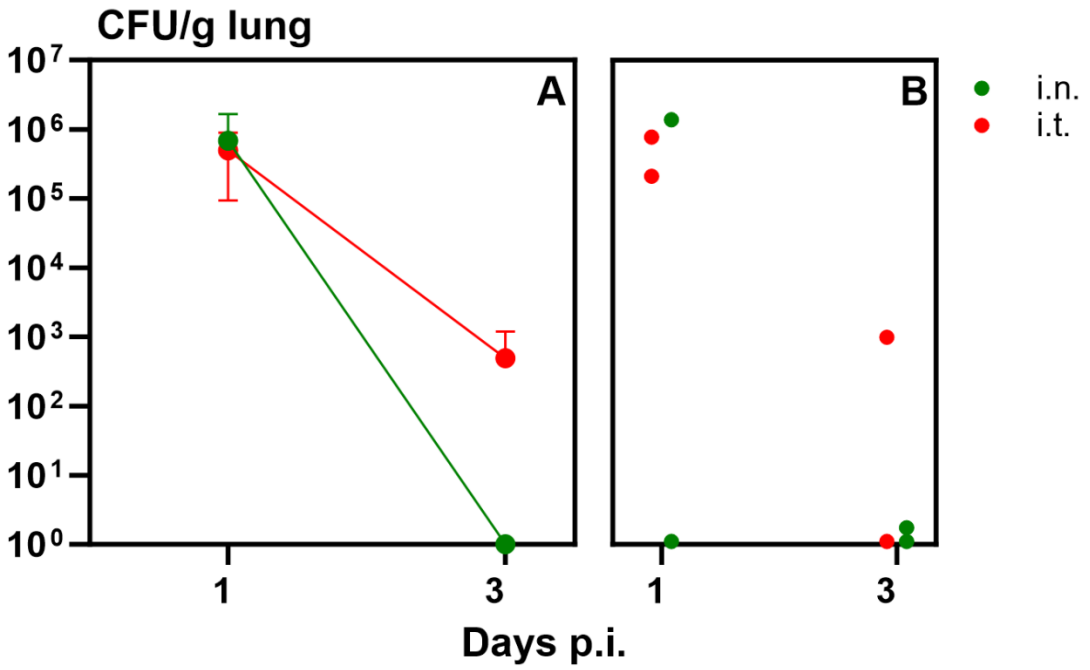


Figure 7.3.3 ***S. pneumoniae* D39 is rapidly cleared from the murine lungs after infection via the intranasal route (i.n.), but persists longer in the lungs after intratracheal infection (i.t.).** Female BALB/c mice were infected with different infection doses of *S. pneumoniae* D39 in 50 µL, intranasally ($1*10^8$ CFU/mouse) or intratracheally ($1*10^6$ CFU/mouse). Bacterial load of the left lung

lobe is given as CFU/g lung. **A.** Each point represents the mean of 2 mice ± SD. **B.** Each point represents an individual mouse. Overlying data points were nudged to improve visibility.

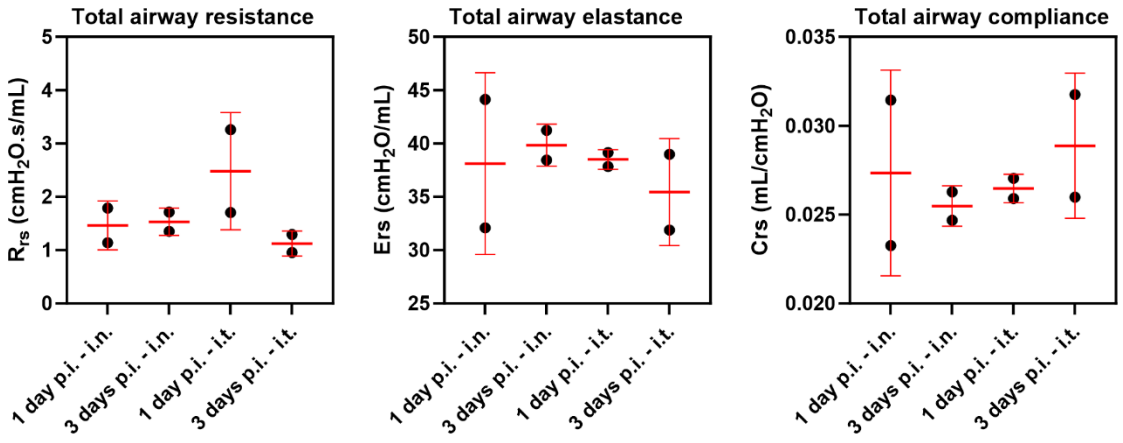


Figure 7.3.4: **Route of *S. pneumoniae* D39 infection has no effect on the murine lung function.** The total airway resistance, total airway elastance and total airway compliance were measured by flexiVent analysis after respiratory infection. Different routes of infection (intranasally, i.n., vs intratracheally, i.t.) with *S. pneumoniae* D39 did not affect any of the tested parameters for none of the conditions. Data were analyzed using a one-way ANOVA and $p > 0.05$ for all conditions.

The next step would be treatment of the mice with a high dose of the antibiotic amoxicillin to check for surviving bacteria, more specifically persisters. As we already observed a 3 log reduction after 3 days of infection without antibiotic treatment, we decided to discontinue the pneumonia model and switched to another chronic mouse model.

7.3.2 The carriage mouse model tends to the chronic presence of *S. pneumoniae* in the murine nasopharynx

Next to the chronic OM model and the chronic pulmonary infection model (291,292), a chronic nasopharyngeal carriage model exists for *S. pneumoniae* in mice (296–298). Because of the common presence of *S. pneumoniae* in the nasopharynx and the link between carriage and disease (299), a chronic carriage model in mice was validated for assessing *in vivo* persistence levels of *S. pneumoniae*.

A murine nasopharyngeal carriage model for *S. pneumoniae* was already used before by different research groups (296–298,300,301). The main principle of nasopharyngeal carriage induction is the use of a small

dose volume (i.e. 10 µL) delivered to the mice via intranasal infection. The small volume will ensure that the bacteria remain mainly in the nasopharynx and are not deposited into the lungs (296–298,300,301). Both inbred mice, such as BALB/c and C57BL/6 mice (297,298,300), and outbred mice, such as MF1 and SWISS mice, (297,298,301) can be used. The model is suited for long-term studies, as *S. pneumoniae* can be found in the nasopharynx up to 21-28 days post infection (296,297,301).

To validate the model in-house, we tested a limited number of conditions. We chose SWISS mice, outbred mice that are less expensive than inbred mice, but suitable for the nasopharyngeal carriage model. We infected female SWISS mice intranasally with different doses of reference strain D39 ($1*10^5$ and $1*10^6$ CFU/mouse) in 10 µL of PBS. Again, we followed survival and bacterial load, by plating and CFU determination, in the nasopharynx over 7 days (**Figure 7.3.5**). Mice did not show any symptoms of an acute respiratory infection, according to the FOB scoring system (**Figure 7.5.1**), which could point towards the presence of *S. pneumoniae* D39 in the nasopharynx as an asymptomatic colonizer and not as a pathogen further invading into the lungs. The bacterial load was slightly higher after infection with $1*10^6$ CFU/mouse than with $1*10^5$ CFU/mouse and the load increased from 1 to 3 days post infection for both infection doses, but 7 days post infection, *S. pneumoniae* D39 was completely cleared from the nasopharynx. Again, we assessed the murine lung function (**Figure 7.3.2, left panels**). We did not observe differences between the infected groups and the PBS control, for none of the infection doses and independently of the day post infection, which confirms the data of the bacterial load in the lungs and the absence of respiratory symptoms of the mice.

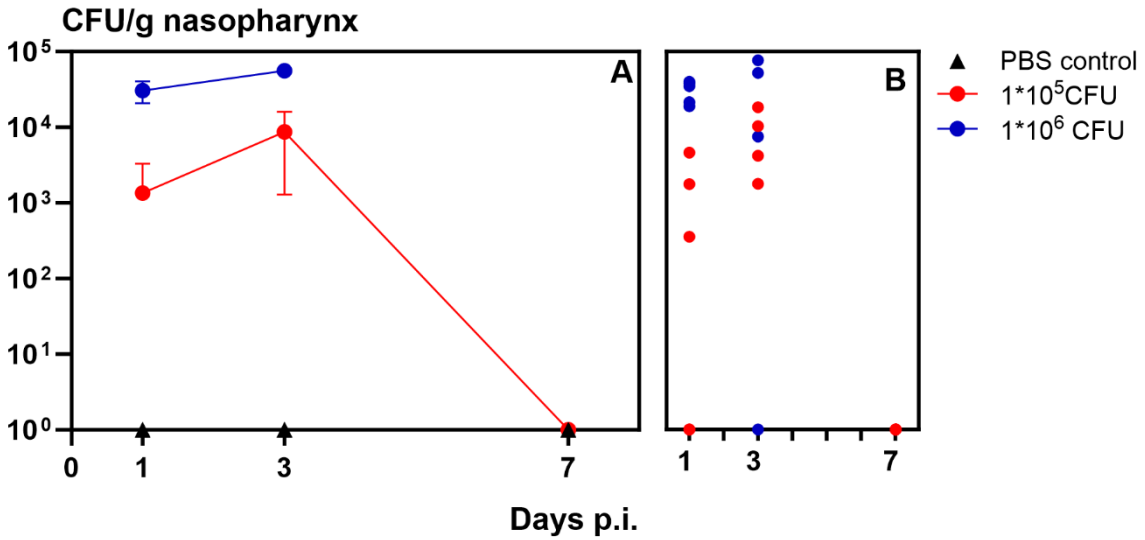


Figure 7.3.5 **Bacterial load in the nasopharynx is stable upon 3 days of infection with *S. pneumoniae* D39.** Female SWISS mice were infected intranasally with different infection doses of *S. pneumoniae* D39 in 10 µL ($1*10^5$ and $1*10^6$ CFU/mouse). Bacterial load of the nasopharynx is given as CFU/g nasopharynx. **A.** Each point represents the mean of 5 mice ± SD. **B.** Each point represents an individual mouse.

We decided to consider the infection as chronic from 3 days post infection, based on other research groups reporting on acute and chronic pneumococcal infections (185,291,293–295). In the next step, we tended to characterize bacterial survival, i.e. persistence, in the nasopharynx after treatment with a high dose of an antibiotic. We chose to treat the mice at 3 days post infection, the chronic stage of carriage, with a dose of $1*10^6$ CFU/mouse, as this resulted in the highest bacterial load in the nasopharynx that was stable until at least 3 days post infection.

7.3.3 *In vivo* killing points towards the presence of antibiotic-tolerant cells

We treated the mice with amoxicillin, a commonly used antibiotic to treat *S. pneumoniae* infections, at 100X the MIC which corresponds to our *in vitro* experiments, but which also should ensure proper killing of antibiotic-sensitive cells (99), at least *in vitro*. We also opted for the same infection route for infection and treatment, namely intranasally, to ensure direct delivery of the antibiotic to the nasopharyngeal tissue and thus to ensure direct contact between the bacteria and amoxicillin. The experimental set-up is given in Figure 7.3.6.

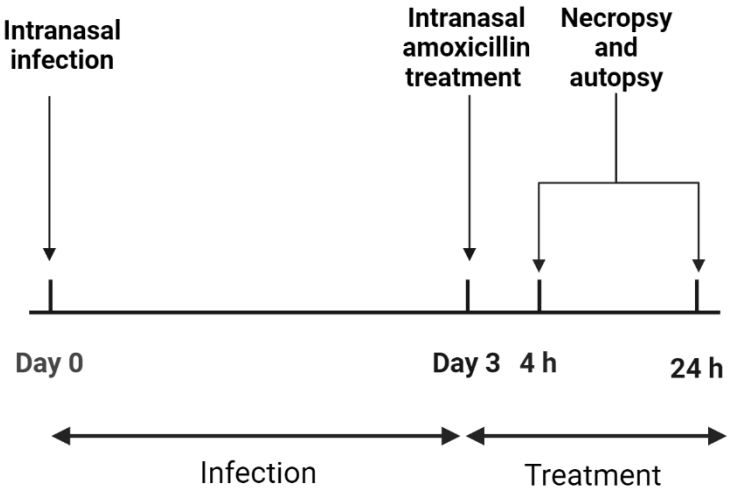


Figure 7.3.6: **Experimental set-up to induce nasopharyngeal carriage with *S. pneumoniae* D39 followed by antibiotic treatment to determine survival of antibiotic-tolerant bacteria in mice.**

The bacterial burden in the nasopharynx was determined at 4 and 24 h post treatment (Figure 7.3.7). We included a control group that was not treated with amoxicillin, but with 10 µL of PBS to follow bacterial burden in the nasopharynx and to ensure *S. pneumoniae* was killed through the antibiotic and not cleared by the immune system of the mice. No significant decrease in CFU was detected for the non-treated control group from 4 to 24 h (Wilcoxon signed rank test; $p = 0.25$) which indicates stable survival of *S. pneumoniae* during the period of treatment and killing in the treated group could be attributed to the antibiotic. The bacterial load did not decrease significantly upon treatment with amoxicillin at 100X the MIC compared to the non-treated control group (Mann-Whitney U; $p = 0.1143$ at 4h, $p > 0.9999$ at 24 h post treatment) and remained stable from 4 to 24 h post treatment (Wilcoxon signed rank test; $p > 0.9999$) (Figure 7.3.7). We observed surviving *S. pneumoniae* bacteria in the *in vivo* nasopharyngeal carriage model after treatment with a high dose of amoxicillin, which gives the first indication of the presence of antibiotic-tolerant cells. To differentiate between population-wide tolerance or tolerance limited to a subpopulation (i.e. persistence), we should include more animals and more timepoints to evaluate the killing pattern (uniphasic vs biphasic killing). We did not perform flexiVent analysis for this experiment.

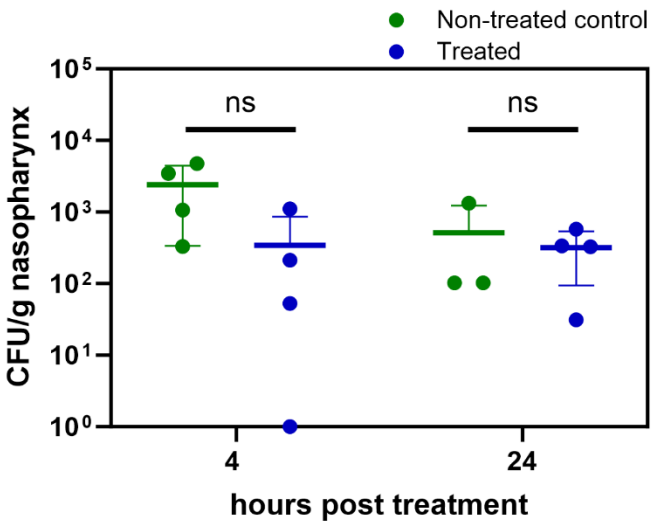


Figure 7.3.7: **Bacterial load in the nasopharynx remained high despite antibiotic treatment, which could indicate the presence of antibiotic-tolerant cells.** Female SWISS mice were infected intranasally with $1*10^6$ CFU/mouse of *S. pneumoniae* D39 in 10 µL. After 3 days of infection, a group mice was treated with 100X the MIC of amoxicillin (6.25 µg/mL) in 10 µL via the intranasal route for 4 or 24 h. A control group treated with 10 µL PBS after 3 days of infection was included. Each point represents the mean of 4 mice ± SD. Bacterial load of the nasopharynx is given as CFU/g nasopharynx. No significant decrease in bacterial CFU in the nasopharynx was observed upon treatment with amoxicillin at 100X the MIC compared to the non-treated control group (Mann-Whitney U; $p = 0.1143$ at 4h, $p > 0.9999$ at 24 h post treatment).

7.4 Discussion

Persistence research gained interest over the past 15 years (147,181). Especially, a large number of *in vitro* studies were executed for different pathogens (bacteria and yeast) (164,174,175,214,302) and even for cancer cells (170). Despite the increasing interests in persister research, limited *in vivo* studies have been reported (147) and none are reported for *S. pneumoniae*. To fill this knowledge gap, we intended to optimize a chronic murine model to enable *S. pneumoniae* persister studies *in vivo*. We studied two models, a lung infection and nasopharyngeal carriage model, to obtain a chronic *in vivo* model. Finally, we explored *in vivo* persistence using the nasopharyngeal carriage model, but further optimization is required.

7.4.1 Optimization of a chronic infection model

Persister cells are connected to recurrent and chronic infections (147). *P. aeruginosa*, for example, is a common cause of chronic infection in cystic fibrosis patients. Isolates sampled during a later stage of the infection were exposed to long-term antibiotic treatment and showed increased persister levels (180,181). Another study on *P. aeruginosa* describes the correlation between *in vitro* and *in vivo* persister levels of natural *P. aeruginosa* strains which underscores the clinical relevance of persisters (215). Besides, different *in vivo* studies were reported on *Salmonella* persistence, both in macrophages and in mice, to emphasize the clinical relevance, but also to study the underlying mechanisms of persistence (280–283). Finally, for clinical isolates of *Candida albicans*, uropathogenic *E. coli* and *M. tuberculosis*, highly persistent mutants were found in patients under antibiotic treatment (162,182–184). These studies suggest the link between long-term infection and antibiotic persistence emphasizing the clinical relevance of persister cells (147). Since antibiotic-tolerant persisters are mostly connected with recurrent and chronic infections, we chose to optimize a chronic *in vivo* model to adequately study *S. pneumoniae* persistence.

Our first attempt involved a lung infection in BALB/c mice, since a lung infection is one of the most important infections caused by *S. pneumoniae* (19). Two studies reported the presence of *S. pneumoniae* in the murine lungs up till 6 or even 28 days post infection (291,292). For our optimization experiments, we started from the optimal parameters that were used in these studies. We started with a low dose ($1*10^4$ – $1*10^5$

CFU/mouse) of *S. pneumoniae* D39 in a small inoculum volume (30 µL), as higher volumes were associated with higher lethality (291). BALB/c mice were chosen as these are widely used in experimental bacterial research, female BALB/c mice have shown as successful hosts for *in vivo* persister studies with *P. aeruginosa* (215) and Briestenská *et al.* (2021) presented them suited to acquire long-term survival of *S. pneumoniae* in the lungs using a *S. pneumoniae* reference strain (A66.1) (292). Nonetheless, we did not observe surviving *S. pneumoniae,* reference strain D39*,* in the lungs of BALB/c mice for more than 24 h (intranasal infection) or 72 h (intratracheal infection). Different studies reported the susceptibility of inbred mice to infection with *S. pneumoniae* (303–306). Their conclusion was that different types of mice markedly differed in their response to *S. pneumoniae* infection. BALB/c mice were considered highly resistant through their ability to maintain the burden of *S. pneumoniae* low in the lungs via a fast influx of neutrophils and mast cells which triggers the release of TNF-α (303–306). The high resistance of BALB/c mice against *S. pneumoniae* infections likely explains why *S. pneumoniae* was rapidly cleared from the lungs and no chronic infection could be established.

We changed our approach to the optimization of a chronic carriage model in the murine nasopharynx. This model was already optimized and used by different research groups (296–298,301,307). Hansol *et al.* (2022) used the model to study the impact of carbohydrate availability on *S. pneumoniae* physiology and virulence during colonization (300). On the other hand, Bricio-Moreno *et al.* (2020) and Jacques *et al.* (2020) applied the model to study a particular *S. pneumoniae* serotype, serotype 1 (296,297). Additionally, colonization of the host is under most circumstances a pre-requisite for *S. pneumoniae* disease which renders colonization an important step in *S. pneumoniae* pathogenesis (20). We started with the in-house validation of the model described by Shears *et al.* (2020) (298). They established asymptomatic nasopharyngeal carriage up till 10 days post infection, without dissemination to the lungs, by intranasally infecting CD1 (SWISS mice) mice with $1*10^5$ CFU/mouse (strain D39) in 10 µL (298). We applied the same conditions, but with an additional infection dose of $1*10^6$ CFU/mouse. We observed a steady bacterial load in the nasopharynx, even with a small increase in CFU's, from day 1 to 3, but no bacteria were recovered after 7 days p.i. which is contradictory to the findings of other research groups (296–298,301,307). Ogunniyi

*et al. (*2007) found *S. pneumoniae* D39 up to 7 days p.i. in the nasopharynx, but they started from a higher infection dose (2-3*10^7 CFU/mouse) (307). Similarly, we could test a higher inoculum to obtain prolonged carriage in the nasopharynx. The type of *S. pneumoniae* strain could also influence the number of CFUs that colonize the nasopharynx. *S. pneumoniae* D39 has been widely used in mouse models to study *S. pneumoniae* pathogenesis and to test vaccine antigens (288), but *S. pneumoniae* strain TIGR4 is better at colonizing the murine nasopharynx than strain 6A-10 followed by D39 (300,308). Changing the type of *S. pneumoniae* strain could be a useful approach to obtain a prolonged presence of bacteria in the nasopharynx. Another variable that could explain the limited recovery of bacteria from the nasopharynx is the complex extraction of the nasopharynx from the mouse. It is a small tissue that needs delicate handling and this could be a source of variation between laboratories. A potential solution for this is a change in experimental set-up. A nasal wash could be used to determine CFUs in the nasopharynx instead of extraction of nasopharyngeal tissue. It is an easy operation: 10 µL of PBS has to be pipetted in the nasal cavity of a sedated mouse followed by extraction of the liquid (which may be around 2 µL) and enumeration of bacteria via plating this liquid (309). This procedure would also allow us to use the same animals throughout the experiment, instead of killing the mice at each timepoint. We should repeat the experiment with more mice to validate the other approaches. Within the limited timeframe of the study, the nasopharyngeal carriage model was not further optimized, but we proceeded to the *in vivo* killing assay with the current set-up.

7.4.2 *In vivo* persistence assay

We performed a single experiment, with 4 mice per group, to assess survival of *S. pneumoniae* D39 in the nasopharynx after antibiotic challenge (**Figure 7.3.7**). We observed no significant difference in number of CFUs between non-treated controls and mice treated intranasally with amoxicillin at 100X the MIC, which could be the first indication of the presence of *S. pneumoniae* antibiotic-tolerant cells *in vivo*. Besides, the number of CFUs was stable during the period of treatment, which could point towards a biphasic killing pattern which is an indicator of the presence of persister cells. To really prove that the killing pattern is

biphasic and that the recovered bacteria are persister cells, additional experiments should be performed. First, the concentration of amoxicillin at the nasopharynx should be determined to ensure that the antibiotic concentration remains well above the MIC at the nasopharynx during treatment which could be executed via LC/MS-MS (310) or via the agar well diffusion method (311). Briefly, an agar plate is inoculated by spreading the microbial inoculum over the entire surface. In the agar, a hole is punched and the nasopharyngeal homogenate is introduced in the well (a control solution of amoxicillin is introduced in another well) followed by incubation. After incubation, the diameter of inhibition can be measured and compared between the nasopharyngeal homogenate and the standard amoxicillin solution (311). To further validate the presence of persister cells, more timepoints should be included to follow bacterial survival after antibiotic treatment which will allow the detection of a biphasic killing pattern, if present (215). The number of mice could be reduced by using nasal washes instead of extraction of the nasopharynx for CFU determination (309). A nasal wash is an easy procedure compared to the extraction of the nasopharynx and it would allow to set-up a longitudinal study in which the same mice could be used throughout the experiment instead of killing the mice at each timepoint (309). A potential disadvantage of a nasal wash is the limited disruption of the nasal surface followed by a lower recovery of bacteria from the nasopharynx (312).

The lung infection model in BALB/c mice was not suited to prolong infection until considered chronic. Therefore, we switched to a nasopharyngeal carriage model which has the potential to be optimized to a chronic model with the presence of *S. pneumoniae* over a prolonged period. A first antibiotic challenge experiment indicated the potential of the model to detect antibiotic-tolerant survivors in the murine nasopharynx after treatment with a high dose of amoxicillin. We took the first steps of optimizing a chronic murine infection model to check for *in vivo* persistence, but further optimization is required.

7.5 Supplementary data

Figure 7.5.1 **Functional observational battery (FOB) scoring sheet.** Humane endpoints are applied when mice score > 9 on the FOB analysis, or when an acute weight loss of >20% occurs.

I. Pick up cage, don't touch animals

1. Body position
   0: (S): Sitting or normally standing (walking, exploring)
   0: (R, rearing): Standing on hind limbs
   1: (hunchback): Back is rounded, even when walking
   2: (apathic): sitting or lying (not asleep) but without interest in surroundings
   3: (lying): Lying on side
   4: (flattened): Animal is spread out with abdomen pressed to floor
   5: (catalepsy): Animal is in a cataleptic-like state, must maintain an unnatural posture

2. Respiration
   0: Normal
   1: Tachypnea or bradypnea
   2: Weak breathing (breathing very little)

3. Fur condition
   0: Normal
   1: Slightly raised/rough hair (localized)
   2: Overall bad fur-condition

4. Overall condition
   0: normal appearance
   1: poor appearance
   2: very poor appearance

5. Occurrence of stereotype behaviour or abnormal behaviour? (no=0, yes=1)
   Stereotype: turning around, squeaking, shaking head, and other repetitive behaviour. Abnormal: e.g., squirming, running backwards, labored movement, disregarding stereotypical activity.

II. Touch/Pick up animal

6. Palpebral closure:
   0: Normal (eyes are open)
   1: Slightly sagging/half shut (eyelids slightly lowered)
   2: Shut (eyelids are closed)

7. Involuntary movement/nerve symptoms:
   0: None/normal
   1: Head tremor (when picked up)
   2. Tilted head or circular pacing
   3. Head tremor (spontaneous) or extended contraction of limbs (rigid)
   4. Generalized tremors or sudden jumping (all limbs off the floor)
   5. Seizures/convulsions

8. Handling/state of activity:
   0: Normal resistance (none-slight)
   1: Overly excited/aroused
   2: Apathic, can be picked up easily without resistance or attempts to escape

8 CONCLUSION AND FUTURE PERSPECTIVES

Infectious diseases exist already for millennia and so does antimicrobial therapy. In the early 20th century, antibiotics as we know today were discovered. Antibiotics have had a great impact on modern medicine and human health by saving countless lives, but antibiotic discovery was accompanied with the rapid emergence of antimicrobial resistance (AMR). Today, AMR makes the control of bacterial infections challenging (121,122,124,125). Nonetheless, AMR is not the only way for bacteria to evade antibiotic therapy and another mechanism, of which the impact was underestimated for a long time, is the ability of transient antibiotic-tolerant persister cells to survive antibiotic treatment thereby contributing to the antibiotic crisis and therapy failure.

The aim of this thesis was to elucidate the importance and potential underlying mechanisms of antibiotic persistence for the clinically important bacterium *Streptococcus pneumoniae,* a major human pathogen causing a high morbidity and mortality worldwide with increasing AMR which classifies *S. pneumoniae* as a serious public health threat according to the CDC (25). Surprisingly, no studies were reported on antibiotic persistence for this bacterium before 2022. We employed the golden standard assays in persister research to make a broad characterization *of S. pneumoniae* persistence using a *S. pneumoniae* reference strain followed by a more in-depth characterization via experimental evolution. In addition to a reference strain, we also generalized our findings and studied a large set of clinical strains to determine the clinical relevance of *S. pneumoniae* persistence. Finally, we used mouse studies to further prove the clinical relevance.

8.1 A first look into *Streptococcus pneumoniae* persistence

S. pneumoniae is a fastidious and fragile bacterium that needs strict nutrient requirements, which led to the difficult isolation and characterization of *S. pneumoniae* in the late 19th century (2,3,6,51–53). Even today, the fragile nature of *S. pneumoniae* results in a limited *in vitro* survival which hampered us to set-up prolonged antibiotic-induced killing assays. We assessed different approaches targeting SpxB or LytA, including the generation of knockout mutants, but there was no tested approach sufficient to prevent the fast *in vitro* killing of *S. pneumoniae* in BHI or THY when entering the stationary phase or to prolong survival in MHL. We could not conclude that either SpxB or LytA alone has a major role in stationary phase autolysis.

Finally, we succeeded in setting-up a model to study antibiotic persistence by implementing Mueller-Hinton broth supplemented with 5% Lysed horse blood (MHL) which is an approach that can be easily applied for screening purposes, in contradiction to the generation of knockout mutants (CHAPTER 3).

Persister cells seem to be a universal feature of clonal life forms, as they have been identified for almost every, if not all, bacterial species that were studied and furthermore also in eukaryotic cancer cells and fungal populations (147,164,170). Before we could investigate potential persistence mechanisms and the clinical relevance of *S. pneumoniae* persistence, we had to prove that *S. pneumoniae* cultures contained persister cells. We assessed antibiotic persistence for reference strain D39 following the guidelines of Balaban *et al.* (2019) (135). Using killing studies together with heritability assays, the gold standard assays to determine persistence (4, 18), we proved the presence of high numbers of genuine persister cells in reference strain D39 cultures. Balaban *et al.* (2019) presented the definitions and guidelines for research on antibiotic persistence following the workshop on 'Bacterial Persistence and Antimicrobial Therapy' in 2018 in which they describe persisters as a subpopulation of cells with an increased minimum duration of killing (MDK), but not necessarily cells that are not killed anymore (135), as we observed for strain D39. We used exponentially growing, diluted stationary phase and stationary phase bacteria to obtain antibiotic induced time-kill curves. Starvation is a common trigger of tolerance and persistence, as we observed after treatment of stationary-phase cultures. To really study drug-induced persistence, steady-state growth should be applied to avoid stationary-phase induced persistence (135). To obtain diluted stationary phase cultures, we diluted the overnight culture 1:10 in fresh medium followed by immediate antibiotic exposure. Here, it is difficult to distinguish whether increased survival is the result of spontaneous or drug-induced persistence. We could further study the actual trigger by either apply persistence studies at the single-cell level or by studying the underlying mechanism (135).

8.2 Unraveling the mechanisms behind *Streptococcus pneumoniae* persistence – lessons learned and future strategies

We tried to unravel the mechanisms behind *S. pneumoniae* persistence. We therefore set-up experimental evolution experiments, which did not result in the discovery of potential persistence mechanisms.

Bacteria can adapt easily to stressful environments, for example to antibiotic exposure. One way to evade antibiotics is via the presence of a persister subpopulation within the bacterial culture which allows survival of antibiotic exposure (313). Bacteria can become increasingly tolerant through genetic changes, directly or by influencing the expression of other tolerance genes, and persistence has been shown to be under the direct evolutionary pressure by antibiotic treatment (231,235–240). The adaptability of bacteria to antibiotics can be investigated through experimental evolution, as evolution experiments showed that *in vitro* persistence can increase rapidly upon frequent antibiotic treatment to extremely high persistence levels (241,242). We did not succeed in evolving *S. pneumoniae* strains towards a high-persister phenotype under the tested experimental conditions, despite sampling a large number of mutations and the ability of *S. pneumoniae* to evolve under experimental conditions, for example in the context of antibiotic resistance (252,254), and even *in vivo* in the context of antibiotic tolerance (253). We could further investigate experimental evolution by checking more conditions or we could also draw other conclusions from the absence of *S. pneumoniae* evolution towards a high-persistent phenotype. Antibiotic-tolerant persisters are mostly connected with recurrent and chronic infections, and the role of persisters in acute infections is not clear (147,185). Moreover, *S. pneumoniae* typically causes infections with an acute nature. Our observations could point towards the limited contribution of persisters in evading antibiotic exposure by *S. pneumoniae*.

Our research reached out to experimental evolution to explore molecular mechanisms of persistence, but many other tools were exploited by other research groups. We investigated bacterial persisters by analyzing the bacterial genome. Another way to find determinants of persister formation is via the bacterial transcriptome (149). The transcriptome is the set of all RNA transcripts, coding and non-coding, on a specific moment in time that can be studied via transcriptomics (314). Two major techniques are microarrays and

RNA seq. Microarrays consist of probes, short nucleotide oligomers, to which fluorescently labelled transcripts can bind. Transcript abundance can be detected through the fluorescence intensity at each location. A disadvantage of this technique is the need of prior knowledge about the studied organisms (314), but it has proven successful for the identification of the persister transcriptome of *M. tuberculosis* which pointed towards potential mechanisms involved in persister formation including downregulation of genes involved in growth and energy and upregulation of TA modules (315). Nowadays, RNA seq gained more interest and is replacing microarrays (314). RNA seq, or RNA sequencing, is a next-generation sequencing technology that does not require previous knowledge about the genome of interest. RNA seq is useful for the identification of genes, but it also allows to read counts and quantify the gene expression level (314). RNA seq revealed pathways involving persistence for different bacteria, such as *Salmonella enterica*, *Listeria monocytogenes*, *Staphylococcus aureus* and *Streptococcus mutans* (161,226,237,316,317). The approaches we applied to determine persister mechanisms involved *S. pneumoniae* populations, but current techniques enable the study of persisters at the single-cell level (318–320). Ma *et al.* (2023) published a method called 'BacDrop' which enabled single-cell RNA seq via a droplet-based technology and they applied this to study persistence in *Klebsiella pneumoniae* clinical isolates. This technique allowed the detection of transcriptionally distinct subpopulations upon antibiotic treatment, whereas bulk RNA seq cannot detect different cellular states within a bacterial population (319,321). Another principle to study persisters at the single-cell level is based on fluorescence. Fluorescent reporter plasmids allow the determination of the transcription levels of genes associated with persistence. Fluorescent reporters enabled Stapels *et al.* (2018) to detect the presence of metabolically active *Salmonella* persister during macrophage infection which indicates that the persister state does not rely solely on dormancy (160). Moreover, fluorescent-activated cell sorting (FACS) enables cell sorting according to the type of cell and the fluorescent characteristics of the cell. These sorted cells can be used in different follow-up experiments, for example in biological assays or for sequencing (320). Flow cytometry has proven useful in the quantification of *S. pneumoniae* in the context of antimicrobial susceptibility testing (322) or pneumococcal agglutination following antibody binding (323), but was not used to date to study persistence. Henry and Brynhildsen (2016) developed a Persister-FACSeq

that combined antibiotic tolerance assays and next-generation sequencing (NGS) with FACS for bacteria that can harbor a fluorescent reporter. More specifically, FACS is parallelized with a fluorescent reporter library. By introducing a positive control and NGS, gene expression distribution of normal cells and persisters can be determined in the untreated and treated samples (319,324). They demonstrated that this assay can rapidly quantify persister physiology and its heterogeneity (324). An additional way for visualizing persister cells and characterize persister morphology is via microfluidics coupled to time-lapse microscopy. One way is to grow bacteria in grooves which results in the formation of linear colonies. This tool allows the detection of cells that have the potential to persist (149,319,320). Balaban *et al.* (2004) demonstrated the presence of preexisting heterogeneity withing *E. coli* populations using microfluidic devices which allowed the detection of persister cells with reduced growth rates (154). Next to sequencing techniques, fluorescence and microfluidics, spectrometry is a useful approach to study persistence. Raman spectroscopy allows single-cell identification via the distinction between spectral fingerprints for different subpopulations and this technology provides detailed molecular analysis via single-cell metabolomics. Moreover, it is non-destructive and therefore allows downstream analysis (319,320). For example, Wang *et al.* (2022) identified *E. coli* persisters and assessed their metabolic activity using this technique (320). Xiong *et al.* (2019) even showed that Raman spectroscopy can be combined with fluorescence into stimulated Raman excited fluorescence (SREF) to obtain sensitive detection of single molecules together with more information about the chemical specificity (325).

All aforementioned techniques have proven successful in the identification of molecular mechanisms behind antibiotic persistence, yet focus on species other than *S. pneumoniae*. Hernandez-Morfa *et al.* (2022) published a report on the underlying mechanism of fluoroquinolone persistence in *S. pneumoniae*. The mechanism relies on overcoming oxidative stress upon fluoroquinolone treatment via the pneumococcal response to oxidative stress involving pyruvate oxidase (SpxB) and ROS-detoxifying enzymes, such as thiol peroxidase and superoxide dismutase (63,187). Another potential mechanism of *S. pneumoniae* persistence related to fluoroquinolones could be the need for DNA repair machinery (for example the SOS response, RecA and RecB) following antibiotic treatment as persister cells can experience DNA damage equally to their

non-persistent counterparts as shown for *E. coli* persisters (234,326). More active pathways could also explain persistence in *S. pneumoniae*, as we observed a slight positive correlation between how fast the strains grow and how well they survive antibiotic treatment. An example of such mechanism is the expression of drug efflux pumps to lower intracellular antibiotic concentrations, as was shown to be the cause of fluoroquinolone persistence of *A. baumannii* (177). These potential underlying mechanisms of *S. pneumoniae* persistence could be further exploited in the future to extend knowledge on *S. pneumoniae* persistence mechanisms as insights in persistence mechanisms could finally lead to new ways of therapeutic intervention.

8.3 Unraveling the clinical relevance of *Streptococcus pneumoniae* persistence: a look into clinical strains

We obtained prove that *S. pneumoniae* persisters were present in reference strain D39 cultures, but one could wonder what the clinical relevance of these persisters is for *S. pneumoniae* infections, also considering the absence of evolution towards high persistent mutants. We therefore looked for the presence of persistence in a large set of clinical strains and we made the first steps towards the study of *S. pneumoniae* persisters in an *in vivo* mouse model.

Persister cells were widely present among our collection of clinical strains, for both antibiotics and for the different types of diseases, which indicates the clinical importance of persistence in *S. pneumoniae* infections. This panel of clinical strains could be extended and used in future research regarding *S. pneumoniae* in other research fields than we applied it for. At present, we have extended our strain collection with about 300 strains from Serbia, but data collection still needs to be performed. *In vivo* studies are another way to prove the clinical relevance of persisters in therapy failure. Moreover, *in vivo* studies are useful to study potential persistence mechanisms and to evaluate future anti-persister therapies. As an *in vivo* model to study *S. pneumoniae* persistence was still lacking, we took the first steps of optimizing a chronic murine infection model to assess *in vivo* persistence, but further optimization is required to prove that these survivors are genuine tolerant or persistent cells. *In vivo* persistence was already extensively studied for the pathogen *Salmonella* indicating the contribution of persisters in the recalcitrant nature of *Salmonella* infections using murine infection models (327–332). Furthermore, Verstraete *et al.* (2023) indicated the clinical relevance of *P. aeruginosa* persisters by correlating *in vitro* and *in vivo* persistence of natural *P. aeruginosa* isolates using mice (215). Next to applying *in vivo* models to study the clinical relevance of persisters, it can be exploited to study persistence evolution and potential persister mechanisms. For example, Dao *et al.* (2024) presented the utility of a murine model to study *S. pneumoniae* tolerance towards fluoroquinolones through *in vivo* evolution (253). Also for other pathogens, *in vivo* models have proven useful for the study of persister determinants (279,281,285). When potential persister

determinants are identified, anti-persister therapies can be explored (284,285,333–335). An optimized *in vivo* persistence model for *S. pneumoniae* could create opportunities to study persistence in a clinically relevant setting which renders further optimization of the model warranted.

We found persister cells in a wide range of *S. pneumoniae* clinical isolates, mostly originating from acute infections. Despite the absence of evolution in an experimental set-up towards a high persister mutant, the widespread presence of persisters in *S. pneumoniae* strains could indicate that persisters are important for *S. pneumoniae* infections, both in carriage, non-invasive and invasive diseases. Moreover, the antibiotic challenge study in our *in vivo* nasopharyngeal model gave the first indication of the potential of *S. pneumoniae* to form persisters in a clinically relevant setting. The exact role of persisters in *S. pneumoniae* therapy failure and resistance development should be further explored by extending our strain collection with strains from more disease types and from different countries next to the further optimization of the *in vivo* persistence model.

8.4 Future perspectives on antibiotic persistence

The importance of persister cells was neglected for a long time due to their small fraction, but to date, the role of persister cells in therapy failure and their contribution to the development of AMR is widely acknowledged (147,148,150,153,185,336). Moreover, high persister mutants were present after long-term infections with frequent antibiotic treatment indicating the potential of bacteria to evolve to high persistent mutants *in vivo* (180–182). The relevance of persisters is widely recognized especially in immunocompromised patients and for biofilm-related and chronic infections such as cystic fibrosis pneumonia, tuberculosis and candidiasis (185). Our research also emphasized the clinical relevance of persister cells as they were widely present among a large set of *S. pneumoniae* clinical isolates.

In the clinic, persisters are not considered in either diagnostics or for antibacterial therapy. As persisters are highly relevant, as stated above, there is an urgent need for the implementation of strategies to detect and fight persisters in the clinical setting. The diagnosis of a bacterial infection is focused on the identification of the causative agent followed by the determination of its susceptibility towards antibiotics. The standard assay to assess antibiotic susceptibility is the MIC assay, which determines at which antibiotic concentration a bacterium stops growing. The susceptibility is then defined according to the EUCAST breakpoint for a certain organism and antibiotic (337). Nonetheless, MIC values are not always a reliable predictor for treatment outcome, as the MICs of antibiotic-tolerant cells assume that they are susceptible towards an antibiotic, but the presence of tolerant cells can lead to therapy failure. Implementing diagnostics to enable the detection of antibiotic-tolerant cells should therefore be included in the clinical practice. The minimum duration of killing (MDK) was proposed as a parameter as it is prolonged for persistence, but it is a time-consuming approach that could take a long time before the results are known (152,336,338,339). Another culture-based approach is the Tolerance Disk Test (TD test). Briefly, bacteria are inoculated on an agar plate on which an antibiotic disk is placed, similar to the Kirby-Bauer disk diffusion test. After overnight incubation, the antibiotic disk is replaced with a disk containing nutrients to stimulate resuscitation of the surviving, tolerant cells. Bacteria that grow in the initial zone of inhibition are considered tolerant or persistent

(340,341). It is again a labor-intensive test that requires manual handling and there is a lack of external triggers to induce persister formation, such as the host environment, which limits the accuracy of the test (341). Also molecular techniques are available in the clinical lab and could therefore be deployed for the detection of persistence, either via sequencing a part of the bacterial genome to look for specific biomarkers linked to antibiotic persistence, or via sequencing the entire genome. In contrast to the culture-based techniques, this approach requires knowledge on the genomic biomarkers related to antibiotic persistence (336,339,342). With the improving techniques to detect persister cells, the implementation of a persister screen should be considered in the clinical environment.

When a patient is diagnosed with a bacterial infection, empirical antibiotic treatment will be initiated. Adjustment of this therapy can be considered when the causative agent and its susceptibility profile become available. Within this process, the causative agent is assumed to be killed following, seemingly appropriate, antibiotic treatment. However, due to the potential presence of persister cells, this scenario is not always realistic (336). It is thus important to, next to considering persisters in clinical diagnostics, also take into account anti-persister therapies when antibiotic treatment is initiated (**Figure 8.4.1**).

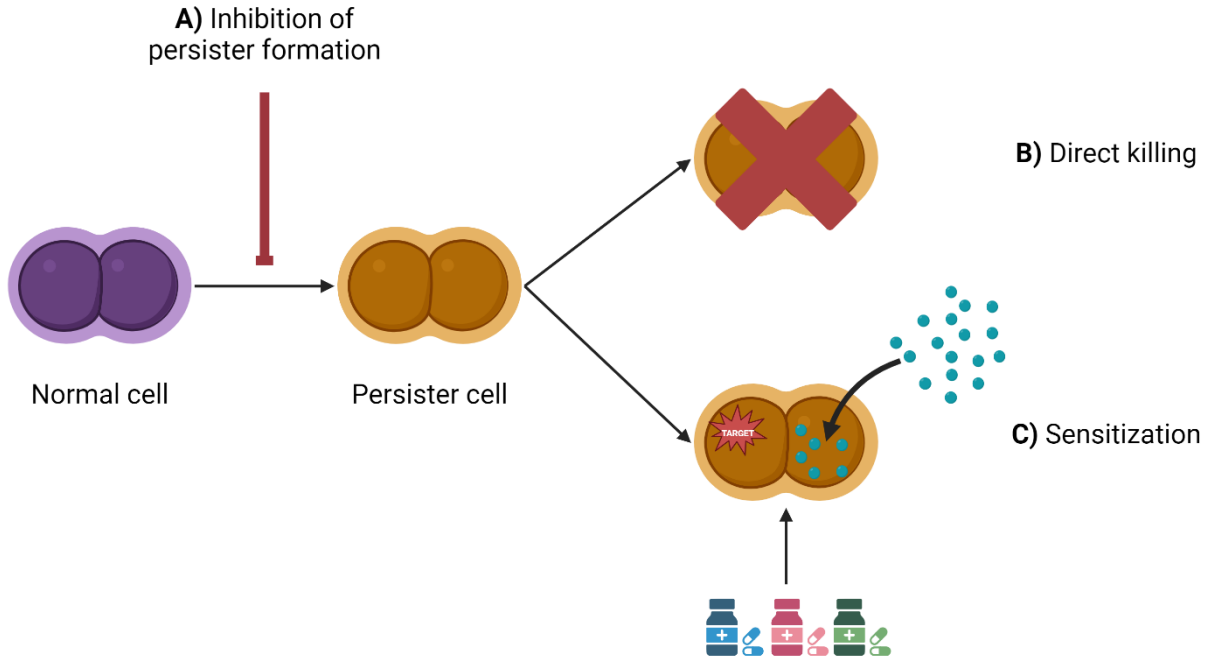


Figure 8.4.1: **Overview of the main strategies to eradicate persister cells**. **A)** Inhibition of persister formation, **B)** direct killing of persister cells by targeting non-active processes and **C)** sensitization of persister cells for conventional antibiotics via stimulation of

the antibiotic target, via stimulation of antibiotic influx or via combinational therapy of different conventional antibiotics (11,29,65,67,78).

Theoretically, there are three main ways of eradicating persisters, A) inhibition of persister formation, B) direct killing of persister cells and C) sensitization (147,148,185,336,343). The first strategy is the prevention of persister formation, which could prevent infections from becoming chronic (**Figure 8.4.1, A**). For this, knowledge on the mechanisms behind antibiotic persistence is warranted to interfere with these processes. An example of this includes the inhibition of the quorum sensing (QS) regulator, that normally controls persister formation, through benzamide in *P. aeruginosa* (344). For direct killing of persister cells, anti-persister compounds should target an alternative, non-active process (**Figure 8.4.1, B**). An example of such compounds are the antimicrobial peptides (AMPs) that will disrupt the integrity of the bacterial membrane which results in direct killing of non-growing bacteria (148,345). The third strategy involves sensitization of persister cells for conventional antibiotics through resuscitation and thereby activating the antibiotic target or stimulating antibiotic influx (**Figure 8.4.1, C**). Sensitization of persister cells is also possible through combinational therapy of different conventional antibiotics (**Figure 8.4.1, C**) (147,148,185,336,343). The metabolism of *E. coli* persisters can be re-activated by the addition of chemical compound C10 which renders them sensitive again against conventional antibiotics (346). The antibiotic influx can be increased via metabolites such as glucose and mannitol and re-sensitize *E. coli*, *P. aeruginosa* and *S. aureus* persister cells (284,347). Persister subpopulations are often tolerant towards only one or a small group of antibiotics. Combination of conventional antibiotics with different modes of action could therefore kill metabolically-inactive persister cells through synergism (343). An example of this is the combination of colistin, which induces membrane damage and enhances uptake of other antibiotics, with gentamicin or ofloxacin for treatment of uropathogenic *E. coli* (348). Anti-persister therapies could be a promising approach in the fight against infectious diseases by decreasing therapy failure and by reducing the chance that bacteria become resistant.

8.5 Final conclusion

We were the first to report the presence of persisters in *S. pneumoniae* cultures for different clinically relevant, bactericidal antibiotics (amoxicillin, cefuroxime, moxifloxacin and vancomycin) using our optimized long-living *in vitro* model. We detected high persister levels for *S. pneumoniae* reference strain D39 varying according to the growth phase and the antibiotic. Experimental evolution did not lead to the formation of a highly persistent phenotype which hampered us to investigate the molecular mechanisms behind *S. pneumoniae* persistence. Furthermore, we employed a large set of *S. pneumoniae* clinical isolates to prove the clinical relevance of *S. pneumoniae* persistence as persister cells were widely present in our strain collection. Finally, we made the first steps for the optimization of a long-term *in vivo* model to enable persistence studies in a clinically relevant setting. Altogether, our work advocates for higher interest for persistence in *S. pneumoniae* as a contributing factor for therapy failure and resistance development.

APPENDIX

Overview of the characteristics of the *S. pneumoniae* clinical strains

Table A: **Overview of the *S. pneumoniae* isolates (serotype), their origin (type of infection and clinical diagnosis) together with their antibiotic persistence and resistance profile and whether they were whole-genome sequenced or not.** MIC, minimum inhibitory concentration; PF, persister fraction; NA, not available; CAP, community-acquired pneumoniae; COPD, chronic obstructive pulmonary disease.

STRAIN	SEROTYPE	INFECTION TYPE	CLINICAL DIAGNOSIS	MIC (µG/ML)				LOG_{10} (PF)		WGS
				AMX	CXM	MXF	VAN	AMX	MXF	
SCI 1	19F	Carriage	/	0.0625	0.25	0.25	0.5	-4.75	-4.78	Yes
SCI 2	19F	Non-invasive	Aspiration pneumonia	0.0625	0.25	0.25	0.5	-4.70	-4.28	Yes
SCI 3	19F	Non-invasive	Otitis media	2	8	0.25	0.5	-4.18	-5.13	Yes
SCI 4	19F	Non-invasive	CAP	2	8	0.25	0.5	-3.95	-5.34	Yes
SCI 5	19F	Non-invasive	Aspiration pneumonia	2	8	0.25	0.5	-4.84	-5.23	Yes
SCI 6	11A	Carriage	/	0.5	0.25	0.25	0.5	-5.02	-6.94	Yes
SCI 7	11A	Non-invasive	CAP	0.5	0.25	0.25	0.5	-4.52	-5.13	Yes
SCI 8	11A	Non-invasive	CAP	0.25	0.25	0.25	0.5	-4.68	-6.52	Yes
SCI 9	11A	NA	NA	8	8	0.25	0.5	-4.05	-6.42	Yes
SCI 10	11A	Carriage	/	8	8	0.25	0.5	-3.66	-4.35	Yes
SCI 11	23B	Carriage	/	0.125	0.0625	0.25	0.5	-4.33	-4.82	Yes
SCI 12	23B	NA	NA	0.125	0.5	0.25	0.5	-4.22	-4.72	Yes
SCI 13	23B	Surinfection	Covid pneumonia	0.0625	0.0625	0.25	0.5	-3.44	-4.30	Yes
SCI 14	23B	Carriage	/	0.125	0.25	0.25	0.5	-5.28	-5.20	Yes
SCI 15	23B	NA	NA	0.125	0.25	0.5	0.5	-4.64	-3.89	Yes
SCI 16	19A	Non-invasive	Conjunctivitis	0.0625	0.0625	0.25	0.5	-4.25	-6.22	Yes

SCI 17	19A	Non-invasive	COPD exacerbation	1	8	0.25	0.5	-4.67	-6.08	Yes
SCI 18	19A	Non-invasive	Ventilator associated pneumonia	2	8	0.25	0.5	-4.21	-6.02	Yes
SCI 19	19A	Surinfection	Covid pneumonia	0.125	0.0625	0.25	0.5	-6.49	-6.71	Yes
SCI 20	19A	Non-invasive	Otitis media	0.0625	0.0625	0.25	0.5	-5.40	-7.96	Yes
SCI 21	6C	Non-invasive	CAP	0.0625	0.0625	0.25	0.5	-5.05	-7.80	Yes
SCI 22	6C	Non-invasive	COPD exacerbation	0.125	0.25	0.25	0.5	-7.78	-7.76	Yes
SCI 23	6C	Non-invasive	CAP	0.125	0.25	0.25	0.5	-5.10	-5.38	Yes
SCI 24	6C	Non-invasive	CAP	0.125	0.25	0.5	0.5	-7.72	-5.11	Yes
SCI 25	6C	Non-invasive	CAP	0.125	0.25	0.25	0.5	-5.72	-5.65	Yes
SCI 26	3	Non-invasive	COPD exacerbation	0.0625	0.0625	0.25	0.5	-4.49	-6.30	Yes
SCI 27	3	Non-invasive	Otitis media	0.0625	0.0625	0.25	0.5	-4.80	-7.35	Yes
SCI 28	3	Non-invasive	COPD exacerbation	0.0625	0.0625	0.5	0.5	-5.78	-3.83	Yes
SCI 29	3	Non-invasive	CAP	0.0625	0.0625	0.5	0.5	-7.25	-4.69	Yes
SCI 30	3	Non-invasive	NA	0.0625	0.0625	0.5	0.5	-6.06	-5.94	Yes
SCI 31	23A	Non-invasive	Sinusitis	0.0625	0.0625	0.5	0.5	-4.41	-6.09	Yes
SCI 32	23A	Non-invasive	COPD exacerbation	0.25	2	0.25	0.5	-4.22	-4.67	Yes
SCI 33	23A	NA	NA	0.0625	0.0625	0.25	0.5	-3.89	-4.46	Yes
SCI 34	23A	Non-invasive	CAP	0.0625	0.0625	0.25	0.5	-5.05	-5.79	Yes
SCI 35	23A	Non-invasive	?	0.0625	0.0625	0.25	0.5	-4.64	-4.42	Yes
SCI 36	9N	Non-invasive	CAP	0.0625	0.0625	0.25	0.5	-4.56	-4.58	Yes
SCI 37	9N	NA	NA	0.0625	0.0625	0.25	0.5	-3.98	-4.74	Yes
SCI 38	9N	Non-invasive	COPD exacerbation	0.0625	0.0625	0.25	0.5	-6.53	-6.58	Yes
SCI 39	9N	NA	NA	0.0625	0.0625	0.25	0.5	-4.55	-4.93	Yes

SCI 40	9N	Non-invasive	?	0.0625	0.0625	0.25	0.5	-4.74	-6.40	Yes
SCI 41	16F	Non-invasive	COPD exacerbation	0.0625	0.0625	0.25	0.5	-4.30	-4.99	Yes
SCI 42	16F	Non-invasive	COPD exacerbation	0.0625	0.0625	0.5	0.5	-5.05	-4.92	Yes
SCI 43	16F	Non-invasive	COPD exacerbation	0.0625	0.0625	0.5	0.5	-4.62	-7.46	Yes
SCI 44	16F	Non-invasive	Sinusitis	0.0625	0.0625	0.25	0.5	-4.75	-4.95	Yes
SCI 45	16F	Carriage	/	0.0625	0.0625	0.25	0.5	-4.39	-4.60	Yes
SCI 46	35B	Carriage	/	0.0625	0.0625	0.25	0.5	-4.89	-7.57	Yes
SCI 47	35B	NA	NA	0.0625	0.25	0.25	0.5	-4.40	-7.47	Yes
SCI 48	35B	Non-invasive	CAP	2	4	0.25	0.5	-7.52	-5.01	Yes
SCI 49	35B	Non-invasive	Convulsive encephalopathy	0.0625	0.0625	0.25	0.5	-4.36	-4.75	Yes
SCI 50	35B	Non-invasive	COPD exacerbation	0.0625	0.0625	0.25	0.5	-5.34	-6.58	Yes
SCI 51	19F	Non-invasive	Otitis Media (OM)	0.063	0.125	0.25	0.5	-4.55	-5.99	Yes
SCI 52	19F	Carriage	/	0.063	0.125	0.25	0.5	-5.02	-5.07	Yes
SCI 53	19F	Carriage	/	2.000	8	0.25	0.5	-4.13	-4.73	Yes
SCI 54	19F	Non-invasive	Otitis Media (OM)	0.063	0.125	0.25	0.5	-5.50	-4.84	Yes
SCI 55	19F	Carriage	/	2.000	8	0.25	0.5	-4.15	-6.17	Yes
SCI 56	11A	Carriage	/	0.063	0.0625	0.25	0.5	-5.18	-5.08	Yes
SCI 57	11A	Non-invasive	Otitis Media (OM)	0.500	0.25	0.25	0.5	-4.58	-4.81	Yes
SCI 58	11A	Non-invasive	Otitis Media (OM)	8.000	8	0.25	0.5	-8.65	-8.65	Yes
SCI 59	11A	Carriage	/	0.250	0.25	0.25	0.5	-4.69	-5.17	Yes
SCI 60	11A	Non-invasive	Sinusitis (SIN)	0.063	0.0625	0.25	0.5	-4.96	-4.58	Yes
SCI 61	23B	Carriage	/	0.063	0.25	0.25	0.5	-5.06	-4.71	Yes
SCI 62	23B	Non-invasive	Otitis Media (OM)	0.250	1	0.5	0.5	-3.87	-4.42	Yes
SCI 63	23B	Non-invasive	Otitis Media (OM)	0.063	0.5	0.25	0.5	-4.79	-4.69	Yes
SCI 64	23B	Carriage	/	0.250	0.5	0.25	0.5	-3.78	-4.21	Yes
SCI 65	23B	Non-invasive	Sinusitis (SIN)	0.063	0.5	0.25	0.5	-3.99	-4.65	Yes
SCI 66	19A	Non-invasive	Sinusitis (SIN)	0.063	0.0625	0.25	0.5	-4.28	-4.74	Yes
SCI 67	19A	Carriage	/	0.063	0.0625	0.25	0.5	-4.65	-5.73	Yes

SCI 68	19A	Carriage	/	2.000	8	0.25	0.5	-4.02	-6.12	Yes
SCI 69	19A	Non-invasive	Sinusitis (SIN)	0.063	0.0625	0.25	0.5	-5.16	-4.00	Yes
SCI 70	19A	Non-invasive	Otitis Media (OM)	0.063	0.0625	0.25	0.5	-4.01	-7.37	Yes
SCI 71	6C	Carriage	/	0.063	0.0625	0.25	0.5	-5.03	-4.82	Yes
SCI 72	6C	Non-invasive	Sinusitis (SIN)	0.125	0.25	0.25	0.5	-4.57	-5.47	Yes
SCI 73	6C	Non-invasive	Sinusitis (SIN)	0.125	0.25	0.25	0.5	-4.63	-5.02	Yes
SCI 74	6C	Carriage	/	0.063	0.25	0.25	0.5	-3.63	-4.50	Yes
SCI 75	6C	Non-invasive	Otitis Media (OM)	0.063	0.125	0.25	0.5	-4.69	-7.55	Yes
SCI 76	3	Non-invasive	Otitis Media (OM)	0.063	0.0625	0.25	0.5	-4.06	-7.32	Yes
SCI 77	3	Non-invasive	Sinusitis (SIN)	0.063	0.0625	0.25	0.5	-4.55	-7.38	Yes
SCI 78	3	Non-invasive	Otitis Media (OM)	0.063	0.0625	0.25	0.5	-4.30	-7.22	Yes
SCI 79	3	Carriage	/	0.063	0.0625	0.25	0.5	-3.96	-4.86	Yes
SCI 80	3	Non-invasive	Sinusitis (SIN)	0.063	0.0625	0.25	0.5	-4.75	-7.06	Yes
SCI 81	23A	Non-invasive	Otitis Media (OM)	0.063	0.0625	0.25	0.5	-4.58	-6.80	Yes
SCI 82	23A	Carriage	/	0.063	0.0625	0.25	0.5	-4.68	-4.90	Yes
SCI 83	23A	Non-invasive	Sinusitis (SIN)	0.063	0.0625	0.25	0.5	-4.62	-5.21	Yes
SCI 84	23A	Carriage	/	0.063	0.0625	0.25	0.5	-4.68	-5.26	Yes
SCI 85	23A	Non-invasive	Otitis Media (OM)	0.063	0.0625	0.25	0.5	-4.28	-5.00	Yes
SCI 86	9N	Carriage	/	0.063	0.0625	0.25	0.5	-4.95	-6.55	Yes
SCI 87	9N	Non-invasive	Otitis Media (OM)	0.063	0.0625	0.25	0.5	-3.50	-4.42	Yes
SCI 88	9N	Non-invasive	Sinusitis (SIN)	0.063	0.0625	0.25	0.5	-4.92	-4.93	Yes
SCI 89	9N	Carriage	/	0.063	0.0625	0.25	0.5	-4.47	-5.02	Yes
SCI 90	9N	Carriage	/	0.063	0.0625	0.25	0.5	-6.21	-4.04	Yes
SCI 91	16F	Carriage	/	0.063	0.0625	0.25	0.5	-4.91	-7.61	Yes
SCI 92	16F	Non-invasive	Otitis Media (OM)	0.063	0.0625	0.25	0.5	-4.22	-5.49	Yes
SCI 93	16F	Non-invasive	Otitis Media (OM)	0.063	0.0625	0.25	0.5	-3.42	-4.74	Yes
SCI 94	16F	Carriage	/	0.063	0.25	0.25	0.5	-3.76	-4.91	Yes
SCI 95	16F	Non-invasive	Otitis Media (OM)	0.250	0.0625	0.25	0.5	-3.54	-7.04	Yes
SCI 96	35B	Carriage	/	0.063	0.0625	0.25	0.5	-5.56	-5.98	Yes
SCI 97	35B	Non-invasive	Otitis Media (OM)	0.063	0.0625	0.25	0.5	-4.27	-4.44	Yes
SCI 98	35B	Non-invasive	Sinusitis (SIN)	0.125	0.25	0.25	0.5	-5.81	-5.06	Yes
SCI 99	35B	Carriage	/	0.063	0.0625	0.125	0.5	-4.31	-4.87	Yes

SCI 100	35B	Carriage	/	0.063	0.0625	0.25	0.5	-4.61	-6.22	Yes
UCL 2	14	Non-invasive	CAP	2	8	0.125	0.5	-4.72	-4.78	Yes
UCL 3	3	Non-invasive	CAP	0.0625	0.0625	0.5	0.5	-6.86	-6.92	Yes
UCL 13	14	Non-invasive	CAP	2	16	0.5	0.5	-4.13	-4.42	Yes
UCL 14	1	Non-invasive	CAP	0.0625	0.0625	0.25	0.5	-5.31	-4.96	Yes
UCL 59	3	Non-invasive	CAP	0.0625	0.0625	0.5	0.5	-5.67	-5.59	Yes
UCL 61	14	Non-invasive	CAP	1	8	0.25	0.5	-4.81	-6.37	Yes
UCL 62	3	Non-invasive	CAP	0.0625	0.0625	0.25	0.5	-8.10	-7.12	Yes
UCL 65	14	Non-invasive	CAP	2	8	0.25	0.5	-3.96	-4.83	Yes
UCL 134	5	Non-invasive	CAP	0.0625	0.125	0.5	0.5	-4.62	-4.15	Yes
UCL 153	1	Non-invasive	CAP	0.0625	0.0625	0.5	0.5	-4.90	-4.36	Yes
UCL 156	1	Non-invasive	CAP	0.0625	0.0625	0.25	0.5	-3.89	-4.28	Yes
UCL 190	14	Non-invasive	CAP	1	8	0.125	0.5	-5.16	-6.86	Yes
UCL 196	14	Non-invasive	CAP	2	8	0.25	0.5	-4.63	-5.27	Yes
UCL 207	19A	Non-invasive	CAP	0.0625	0.0625	0.25	0.5	-4.69	-5.26	Yes
UCL 208	19A	Non-invasive	CAP	0.0625	0.0625	0.25	0.5	-4.43	-6.16	Yes
UCL 242	5	Non-invasive	CAP	0.0625	0.25	0.25	0.5	-4.26	-5.06	Yes
UCL 267	3	Non-invasive	CAP	0.0625	0.0625	0.5	0.5	-6.04	-6.36	Yes
UCL 297	19A	Non-invasive	CAP	0.0625	0.0625	0.5	0.5	-4.86	-4.64	Yes
UCL 306	1	Non-invasive	CAP	0.0625	0.0625	0.25	0.5	-4.16	-4.67	Yes
UCL 310	1	Non-invasive	CAP	0.0625	0.0625	0.25	0.5	-4.87	-6.57	Yes
UCL 370	3	Non-invasive	CAP	0.0625	0.0625	0.5	0.5	-4.40	-7.13	Yes
UCL 396	1	Non-invasive	CAP	0.0625	0.0625	0.25	0.5	-4.29	-5.38	Yes
UCL 418	3	Non-invasive	CAP	0.0625	0.0625	0.25	0.5	-4.20	-5.98	Yes
UCL 440	5	Non-invasive	CAP	0.0625	0.25	0.25	0.5	-5.51	-6.32	Yes
UCL 450	19A	Non-invasive	CAP	0.0625	0.0625	0.25	0.5	-5.35	-6.10	Yes
UCL 453	19A	Non-invasive	CAP	2	8	0.25	0.5	-3.95	-6.02	Yes
UCL 487	19A	Non-invasive	CAP	0.0625	0.0625	0.25	0.5	-4.25	-5.25	Yes
UCL 520	5	Non-invasive	CAP	0.0625	0.25	0.5	0.5	-4.65	-4.83	Yes
UCL 574	5	Non-invasive	CAP	0.0625	0.25	0.25	0.5	-4.46	-6.36	Yes
UCL 607	5	Non-invasive	CAP	0.0625	0.0625	0.25	0.5	-5.12	-5.75	Yes
UCL 744	/	Non-invasive	AECB	0.0625	0.125	0.25	0.5	-4.53	-5.20	Yes

UCL 752	/	Non-invasive	AECB	0.0625	0.0625	0.5	0.5	-4.49	-6.65	Yes
UCL 753	/	Non-invasive	AECB	0.0625	0.0625	0.25	0.5	-5.68	-4.51	Yes
UCL 754	/	Non-invasive	AECB	2	8	0.25	0.5	-5.40	-6.25	Yes
UCL 755	/	Non-invasive	AECB	0.0625	0.0625	0.125	0.5	-4.88	-7.31	Yes
UCL 758	/	Non-invasive	AECB	2	8	0.125	0.5	-7.90	-6.20	Yes
UCL 760	/	Non-invasive	AECB	0.0625	0.0625	0.5	0.5	-4.61	-5.20	Yes
UCL 761	/	Non-invasive	AECB	0.0625	0.0625	0.25	0.5	-4.76	-8.41	Yes
UCL 762	/	Non-invasive	AECB	0.0625	0.0625	0.5	0.5	-5.36	-7.58	Yes
UCL 768	/	Non-invasive	AECB	0.0625	0.0625	0.25	0.5	-4.73	-5.13	Yes
UCL 770	/	Non-invasive	AECB	0.0625	0.0625	0.25	0.5	-4.93	-5.40	Yes
UCL 772	/	Non-invasive	AECB	0.0625	0.0625	0.25	0.5	-5.55	-5.88	Yes
UCL 783	/	Non-invasive	AECB	0.0625	0.0625	0.125	0.5	-5.08	-7.29	Yes
UCL 784	/	Non-invasive	AECB	0.0625	0.0625	0.25	0.5	-5.30	-5.63	Yes
UCL 785	/	Non-invasive	AECB	0.0625	0.0625	0.125	0.5	-4.41	-4.23	Yes
UCL 799	/	Non-invasive	AECB	4	8	0.125	0.5	-4.12	-5.06	Yes
UCL 800	/	Non-invasive	AECB	4	8	0.125	0.5	-5.07	-4.99	Yes
UCL 802	/	Non-invasive	AECB	0.125	0.0625	0.25	0.5	-4.50	-5.01	Yes
UCL 807	/	Non-invasive	AECB	0.25	4	0.25	0.5	-4.15	-4.14	Yes
UCL 808	/	Non-invasive	AECB	0.0625	0.0625	0.125	0.5	-3.89	-5.47	Yes
UZL 1	23F	Invasive	pneumonia	2	8	0.25	0.5	-4.43	-8.68	Yes
UZL 2	24A	Invasive	pneumonia with bacteraemia	0.0625	0.5	0.25	0.5	-5.04	-7.15	Yes
UZL 3	24A	Invasive	meningitis	0.125	1	0.25	0.5	-8.29	-4.58	Yes
UZL 4	34	Invasive	sepsis	0.0625	0.0625	0.25	0.5	-3.88	-5.45	Yes
UZL 5	23F	Invasive	sepsis	2	8	0.25	0.5	-4.88	-8.73	Yes
UZL 6	24A	Invasive	sepsis	0.25	1	0.25	0.5	-3.29	-6.93	Yes
UZL 7	24B	Invasive	bacteraemia	0.125	1	0.25	0.5	-4.48	-3.95	Yes
UZL 8	34	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-3.24	-7.75	Yes
UZL 9	24A	Invasive	pneumonia with bacteraemia	0.125	1	0.25	0.5	-4.31	-7.61	Yes
UZL 10	24B	Invasive	pneumonia with bacteraemia	0.125	1	0.25	0.5	-4.98	-6.93	Yes

UZL 11	24A	Invasive	bacteraemia	0.125	0.0625	0.25	0.5	-4.88	-7.17	Yes
UZL 12	24B	Invasive	pneumonia	0.125	1	0.25	0.5	-4.09	-4.44	Yes
UZL 13	23F	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.74	-4.27	Yes
UZL 14	34	Invasive	pneumonia	0.0625	0.0625	0.25	0.5	-4.93	-4.08	Yes
UZL 15	34	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-4.75	-4.01	Yes
UZL 16	34	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-7.57	-7.42	Yes
UZL 17	23F	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.93	-4.52	Yes
UZL 18	4	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-7.91	-4.46	Yes
UZL 19	3	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.01	-5.05	Yes
UZL 20	23B	Invasive	meningitis	0.0625	0.5	0.25	0.5	-5.49	-6.07	Yes
UZL 21	24	Invasive	bacteraemia	0.25	0.5	0.25	0.5	-5.15	-5.24	Yes
UZL 22	23A	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-4.03	-4.74	Yes
UZL 23	11A	Invasive	bacteraemia	0.25	0.25	0.25	0.5	-3.90	-4.31	Yes
UZL 24	3	Invasive	pneumonia with bacteraemia	0.125	2	0.25	0.5	-4.45	-4.83	Yes
UZL 25	7B	Invasive	pneumonia with bacteraemia	0.0625	0.25	0.125	0.5	-3.73	-4.66	Yes
UZL 26	15A	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-5.59	-7.05	Yes
UZL 27	23A	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-5.06	-5.24	Yes
UZL 28	4	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.99	-6.88	Yes
UZL 29	3	Invasive	meningitis	0.0625	0.0625	0.5	0.5	-4.70	-4.72	Yes
UZL 30	3	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.86	-6.71	Yes
UZL 31	3	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.56	-5.38	No
UZL 32	19A	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.125	0.5	-4.75	-7.22	Yes
UZL 33	10B	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-4.82	-4.86	Yes

UZL 34	15A	Invasive	sepsis	0.0625	0.0625	0.25	0.5	-3.96	-5.19	Yes
UZL 35	19A	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.91	-4.81	Yes
UZL 36	9N	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-4.31	-5.67	Yes
UZL 37	23B	Invasive	meningitis	0.0625	0.0625	0.25	0.5	-4.42	-8.52	Yes
UZL 38	16F	Invasive	pneumonia	0.0625	0.0625	0.25	0.5	-4.90	-5.11	Yes
UZL 39	4	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.31	-4.88	Yes
UZL 40	4	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.5	0.5	-7.87	-4.94	Yes
UZL 41	3	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-4.45	-5.64	Yes
UZL 42	6C	Invasive	bacteraemia	0.125	0.25	0.125	0.5	-4.18	-6.55	No
UZL 43	19A	Invasive	meningitis	0.0625	0.0625	0.25	0.5	-7.17	-5.17	No
UZL 44	19A	Invasive	meningitis	0.0625	0.0625	0.25	0.5	-4.93	-4.73	Yes
UZL 45	10B	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.50	-4.35	Yes
UZL 46	9N	Invasive	pneumonia with bacteraemia	0.125	0.5	0.25	0.5	-5.10	-5.38	Yes
UZL 47	8	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.07	-5.16	Yes
UZL 48	19A	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-6.96	-5.07	Yes
UZL 49	16F	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.93	-4.52	Yes
UZL 50	23B	Invasive	bacteraemia	0.125	0.25	0.25	0.5	-4.76	-5.19	Yes
UZL 51	23A	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-5.09	-7.43	Yes
UZL 52	19F	Invasive	pneumonia with bacteraemia	2	8	0.25	0.5	-7.28	-8.89	Yes
UZL 53	8	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-4.99	-5.36	Yes
UZL 54	15A	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-5.10	-5.86	Yes
UZL 55	6C	Invasive	bacteraemia	0.125	0.25	0.25	0.5	-6.87	-5.41	Yes
UZL 56	6C	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.14	-5.09	Yes

UZL 57	19A	Invasive	sepsis	0.0625	0.0625	0.25	0.5	-4.58	-5.20	Yes
UZL 58	4	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.72	-4.85	Yes
UZL 59	23B	Invasive	meningitis	0.0625	0.5	0.25	0.5	-5.16	-6.93	Yes
UZL 60	3	Invasive	pneumonia	0.0625	0.0625	0.25	0.5	-6.81	-5.49	Yes
UZL 61	23A	Invasive	meningitis	0.0625	0.125	0.25	0.5	-4.10	-4.77	Yes
UZL 62	19A	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-4.73	-5.09	Yes
UZL 63	31	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-8.65	-8.65	Yes
UZL 64	8	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.47	-4.90	Yes
UZL 65	23B	Invasive	pneumonia with bacteraemia	0.0625	0.5	0.25	0.5	-4.51	-4.72	Yes
UZL 66	3	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.5	0.5	-4.91	-5.24	Yes
UZL 67	11A	Invasive	pneumonia	0.5	0.25	0.25	0.5	-4.67	-4.79	Yes
UZL 68	23B	Invasive	bacteraemia	0.0625	0.5	0.25	0.5	-5.25	-5.30	Yes
UZL 69	15A	Invasive	pneumonia with bacteraemia	2	8	0.25	0.5	-6.88	-7.12	Yes
UZL 70	15B	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-8.02	-6.56	Yes
UZL 71	3	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.5	0.5	-5.01	-8.29	Yes
UZL 72	3	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.26	-5.67	Yes
UZL 73	6C	Invasive	pneumonia with bacteraemia	0.125	0.25	0.25	0.5	-4.98	-7.25	Yes
UZL 74	12F	Invasive	pneumonia with bacteraemia	2	8	0.25	0.5	-4.98	-4.89	Yes
UZL 75	9N	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-5.36	-5.71	Yes
UZL 76	6C	Invasive	pneumonia with bacteraemia	0.125	0.25	0.25	0.5	-4.29	-4.81	Yes
UZL 77	23B	Invasive	meningitis	0.25	0.5	0.25	0.5	-6.05	-4.81	Yes
UZL 78	3	Invasive	meningitis	0.0625	0.0625	0.25	0.5	-4.30	-5.37	Yes
UZL 79	6C	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-4.20	-5.16	Yes

UZL 80	31	Invasive	bacteraemia	0.0625	0.0625	0.5	0.5	-5.12	-5.60	No
UZL 81	35F	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.55	-7.30	Yes
UZL 82	19A	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-4.33	-6.44	Yes
UZL 83	19A	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-5.38	-7.17	Yes
UZL 84	9N	Invasive	meningitis	0.0625	0.0625	0.25	0.5	-5.19	-5.33	Yes
UZL 85	19A	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.92	-5.40	Yes
UZL 86	23B	Invasive	pneumonia with bacteraemia	0.0625	0.5	0.25	0.5	-4.70	-6.84	Yes
UZL 87	24	Invasive	bacteraemia	0.25	1	0.25	0.5	-5.07	-4.96	Yes
UZL 88	6C	Invasive	pneumonia with bacteraemia	0.25	0.25	0.25	0.5	-4.49	-6.50	Yes
UZL 89	6C	Invasive	pneumonia with bacteraemia	0.125	0.0625	0.25	0.5	-4.17	-4.07	Yes
UZL 90	23B	Invasive	bacteraemia	0.0625	0.5	0.25	0.5	-4.56	-5.08	Yes
UZL 91	14	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-3.84	-4.41	Yes
UZL 92	38	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-4.26	-4.87	Yes
UZL 93	6C	Invasive	meningitis	0.125	0.25	0.25	0.5	-5.09	-6.63	Yes
UZL 94	19A	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-6.49	-4.73	Yes
UZL 95	3	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-6.56	-4.87	Yes
UZL 96	15A	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-5.01	-5.62	Yes
UZL 97	35B	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-3.50	-2.74	No
UZL 98	3	Invasive	meningitis	0.0625	0.0625	0.25	0.5	-4.21	-2.74	No
UZL 99	24	Invasive	pneumonia with bacteraemia	0.25	1	0.25	0.5	-4.20	-5.46	No
UZL 100	6C	Invasive	bacteraemia	0.125	0.25	0.25	0.5	-4.14	-5.11	No
UZL 101	6C	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-4.18	-4.03	No

UZL 102	35F	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.14	-5.34	No
UZL 103	23B	Invasive	meningitis	0.0625	0.25	0.25	0.5	-3.89	-4.31	No
UZL 104	15A	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.66	-5.01	No
UZL 105	19A	Invasive	pneumonia	0.0625	0.0625	0.25	0.5	-3.77	-4.86	No
UZL 106	11A	Invasive	bacteraemia	8	8	0.125	0.5	-3.96	-7.86	No
UZL 107	15A	Invasive	meningitis	0.0625	0.0625	0.25	0.5	-3.85	-4.24	No
UZL 108	16F	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-3.97	-4.51	No
UZL 109	11A	Invasive	pneumonia with bacteraemia	8	8	0.25	0.5	-3.52	-4.42	No
UZL 110	11A	Invasive	pneumonia with bacteraemia	2	8	0.25	0.5	-4.34	-5.52	No
UZL 111	19A	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-4.04	-4.75	No
UZL 112	19A	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.06	-7.53	No
UZL 113	12F	Invasive	sepsis	0.0625	0.25	0.25	0.5	-5.06	-5.46	No
UZL 114	35B	Invasive	bacteraemia	0.125	0.25	0.25	0.5	-6.26	-5.25	No
UZL 115	19A	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.71	-5.00	No
UZL 116	19F	Invasive	pneumonia with bacteraemia	2	8	0.25	0.5	-4.20	-4.79	No
UZL 117	24A	Invasive	pneumonia with bacteraemia	0.0625	1	0.25	0.5	-4.42	-5.34	No
UZL 118	4	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-5.97	-4.61	No
UZL 119	3	Invasive	pneumonia with bacteraemia	0.0625	0.125	0.25	0.5	-4.56	-3.89	No
UZL 120	7C	Invasive	meningitis	0.0625	0.0625	0.25	0.5	-4.83	-7.67	No
UZL 121	6C	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-4.98	-7.28	No
UZL 122	35F	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-4.03	-5.43	No
UZL 123	23B	Invasive	pneumonia with bacteraemia	0.0625	0.5	0.25	0.5	-4.92	-4.96	No

UZL 124	8	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.93	-4.86	No
UZL 125	3	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.5	0.5	-4.43	-4.80	No
UZL 126	6C	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-4.57	-4.31	No
UZL 127	8	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.32	-5.51	No
UZL 128	12F	Invasive	pneumonia with bacteraemia	0.0625	0.25	0.25	0.5	-4.88	-6.60	No
UZL 129	19A	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.45	-5.77	No
UZL 130	14	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-6.59	-5.62	No
UZL 131	34	Invasive	bacteraemia	0.0625	0.5	0.25	0.5	-4.50	-4.28	No
UZL 132	8	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.53	-4.49	No
UZL 133	35B	Invasive	pneumonia with bacteraemia	0.125	0.25	0.25	0.5	-5.19	-5.26	No
UZL 134	23A	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-4.32	-4.96	No
UZL 135	19A	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.43	-4.98	No
UZL 136	17F	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-8.70	-5.57	No
UZL 137	23B	Invasive	pneumonia with bacteraemia	0.25	1	0.25	0.5	-3.63	-4.69	No
UZL 138	12F	Invasive	meningitis	0.0625	0.25	0.25	0.5	-4.75	-5.22	No
UZL 139	23A	Invasive	pneumonia with bacteraemia	0.0625	0.25	0.25	0.5	-4.75	-4.73	No
UZL 140	3	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-5.88	-4.28	No
UZL 141	3	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.5	0.5	-3.89	-5.79	No
UZL 142	3	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.5	0.5	-5.96	-3.77	No

UZL 143	3	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.5	0.5	-4.06	-3.92	No
UZL 144	3	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.61	-8.22	No
UZL 145	9N	Invasive	meningitis	0.0625	0.0625	0.25	0.5	-4.16	-4.86	No
UZL 146	20	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-3.55	-4.02	No
UZL 147	3	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-3.92	-8.42	No
UZL 148	17F	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.62	-4.75	No
UZL 149	9N	Invasive	meningitis	0.0625	0.0625	0.25	0.5	-3.84	-4.66	No
UZL 150	10B	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.23	-6.69	No
UZL 151	7B	Invasive	pneumonia	0.0625	0.25	0.5	0.5	-4.06	-4.29	No
UZL 152	3	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.5	0.5	-7.22	-6.14	No
UZL 153	23B	Invasive	pneumonia with bacteraemia	0.0625	0.5	0.25	0.5	-2.82	-4.48	No
UZL 154	6C	Invasive	pneumonia with bacteraemia	0.125	0.25	0.25	0.5	-4.22	-5.82	No
UZL 155	3	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.82	-3.79	No
UZL 156	9N	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-4.32	-4.77	No
UZL 157	19A	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.20	-7.54	No
UZL 158	19A	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-5.24	-7.63	No
UZL 159	23B	Invasive	bacteraemia	0.0625	0.5	0.25	0.5	-4.05	-4.36	No
UZL 160	24	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.125	0.5	-4.48	-7.81	No
UZL 161	9N	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.32	-5.37	No
UZL 162	11A	Invasive	bacteraemia	2	8	0.25	0.5	-4.42	-5.29	No

UZL 163	11A	Invasive	pneumonia with bacteraemia	8	8	0.25	0.5	-3.65	-4.69	No
UZL 164	14	Invasive	pneumonia with bacteraemia	2	8	0.25	0.5	-4.24	-5.81	No
UZL 165	20	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-4.62	-5.03	No
UZL 166	16F	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.09	-4.38	No
UZL 167	8	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-5.01	-4.82	No
UZL 168	14	Invasive	pneumonia with bacteraemia	2	8	0.25	0.5	-3.78	-6.23	No
UZL 169	9N	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-3.71	-4.02	No
UZL 170	24	Invasive	pneumonia with bacteraemia	0.125	1	0.25	0.5	-3.45	-4.19	No
UZL 171	9N	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-4.08	-6.38	No
UZL 172	8	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.36	-6.44	No
UZL 173	11A	Invasive	meningitis	0.25	0.25	0.25	0.5	-3.94	-4.93	No
UZL 174	16F	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-3.87	-4.61	No
UZL 175	23B	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.125	0.5	-4.89	-5.13	No
UZL 176	3	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.125	0.5	-7.93	-7.93	No
UZL 177	6C	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.125	0.5	-4.39	-6.51	No
UZL 178	23F	Invasive	pneumonia with bacteraemia	4	16	0.25	0.5	-3.36	-4.79	No
UZL 179	6C	Invasive	pneumonia with bacteraemia	0.125	0.25	0.25	0.5	-3.95	-4.93	No
UZL 180	23A	Invasive	bacteraemia	0.0625	0.0625	0.125	0.5	-3.93	-4.91	No
UZL 181	3	Invasive	meningitis	0.0625	0.0625	0.25	0.5	-4.42	-4.57	No
UZL 182	8	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-4.69	-7.93	No
UZL 183	35F	Invasive	meningitis	0.0625	0.0625	0.25	0.5	-4.11	-4.55	No

UZL 184	8	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-5.34	-6.17	No
UZL 185	23A	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-5.76	-6.00	No
UZL 186	20	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-3.98	-4.82	No
UZL 187	6C	Invasive	bacteraemia	0.0625	0.0625	0.125	0.5	-4.59	-6.41	No
UZL 188	6C	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-3.94	-5.43	No
UZL 189	9N	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-4.34	-7.89	Yes
UZL 191	3	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.5	0.5	-4.50	-5.95	Yes
UZL 192	23A	Invasive	bacteraemia	0.25	0.25	0.25	0.5	-4.05	-6.27	Yes
UZL 193	6C	Invasive	pneumonia with bacteraemia	0.125	0.25	0.25	0.5	-6.14	-6.20	Yes
UZL 194	6C	Invasive	meningitis	0.125	0.25	0.25	0.5	-4.49	-4.03	Yes
UZL 195	20	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.06	-4.85	Yes
UZL 196	16F	Invasive	meningitis	0.0625	0.0625	0.25	0.5	-4.16	-4.88	Yes
UZL 197	23B	Invasive	bacteraemia	0.0625	0.5	0.25	0.5	-3.98	-4.17	Yes
UZL 198	19A	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.125	0.5	-3.55	-4.78	Yes
UZL 199	10A	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-4.89	-4.34	Yes
UZL 200	23B	Invasive	bacteraemia	0.0625	0.5	0.25	0.5	-3.93	-4.54	Yes
UZL 201	9N	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-3.59	-4.19	Yes
UZL 203	15B	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-3.81	-4.85	No
UZL 204	19A	Invasive	pneumonia with bacteraemia	2	8	0.25	0.5	-3.80	-5.01	Yes
UZL 205	19A	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-4.18	-5.10	Yes
UZL 206	4	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-4.10	-4.37	Yes
UZL 207	35B	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-3.89	-5.08	Yes

UZL 208	11A	Invasive	pneumonia with bacteraemia	2	8	0.25	0.5	-4.34	-5.60	Yes
UZL 209	11A	Invasive	pneumonia with bacteraemia	8	> 16	0.25	0.5	-4.18	-4.25	Yes
UZL 210	16F	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-3.22	-4.09	Yes
UZL 211	23A	Invasive	meningitis	0.0625	0.0625	0.25	0.5	-4.81	-4.95	Yes
UZL 212	9N	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-3.72	-4.08	Yes
UZL 213	23B	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.125	0.5	-4.02	-4.78	Yes
UZL 214	24F	Invasive	meningitis	0.125	1	0.25	0.5	-3.57	-5.15	Yes
UZL 215	19A	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-3.82	-4.52	Yes
UZL 216	24F	Invasive	bacteraemia	0.125	1	0.25	0.5	-3.44	-4.69	Yes
UZL 217	8	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-5.08	-6.55	Yes
UZL 218	38	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-3.25	-4.40	Yes
UZL 219	4	Invasive	meningitis	0.0625	0.0625	0.25	0.5	-4.35	-4.46	Yes
UZL 220	3	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.68	-4.39	Yes
UZL 221	34	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.125	0.5	-4.89	-4.70	Yes
UZL 222	11A	Invasive	bacteraemia	8	8	0.25	0.5	-3.68	-4.42	Yes
UZL 223	9N	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-4.76	-4.89	Yes
UZL 224	6C	Invasive	pneumonia with bacteraemia	0.125	0.25	0.25	0.5	-4.08	-5.27	Yes
UZL 225	24B	Invasive	pneumonia with bacteraemia	0.125	1	0.125	0.5	-5.71	-7.21	Yes
UZL 226	3	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-5.94	-5.69	Yes
UZL 227	23A	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-4.51	-5.65	Yes
UZL 228	15C	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.30	-4.66	Yes

UZL 229	8	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-5.41	-8.50	Yes
UZL 230	38	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-3.35	-4.15	Yes
UZL 231	24F	Invasive	pneumonia with bacteraemia	0.25	1	0.25	0.5	-4.37	-5.29	Yes
UZL 232	19A	Invasive	pneumonia with bacteraemia	0.25	0.5	0.125	0.5	-3.84	-5.02	Yes
UZL 233	3	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.93	-3.97	Yes
UZL 234	8	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-7.44	-7.12	Yes
UZL 235	8	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.40	-6.41	Yes
UZL 236	23A	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.15	-4.25	Yes
UZL 237	8	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-5.08	-7.25	Yes
UZL 238	11A	Invasive	bacteraemia	0.25	0.25	0.25	0.5	-4.31	-5.02	Yes
UZL 239	11A	Invasive	pneumonia with bacteraemia	0.5	0.25	0.125	0.5	-4.04	-5.60	Yes
UZL 240	16F	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-3.86	-4.88	Yes
UZL 241	19A	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-3.33	-4.60	Yes
UZL 242	16F	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-3.98	-6.50	Yes
UZL 243	4	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.28	-4.23	Yes
UZL 244	24F	Invasive	bacteraemia	0.125	1	0.25	0.5	-3.64	-5.12	Yes
UZL 245	19A	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-3.91	-6.10	Yes
UZL 246	6C	Invasive	bacteraemia	0.125	0.25	0.25	0.5	-4.50	-5.14	Yes
UZL 247	3	Invasive	meningitis	0.0625	0.0625	0.25	0.5	-4.14	-7.26	Yes

UZL 248	6C	Invasive	pneumonia with bacteraemia	0.125	0.25	0.25	0.5	-4.50	-7.55	Yes
UZL 249	6C	Invasive	pneumonia with bacteraemia	0.125	0.25	0.25	0.5	-4.45	-4.18	Yes
UZL 250	3	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-4.54	-4.34	Yes
UZL 251	10A	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.60	-4.13	Yes
UZL 252	14	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-4.94	-7.72	Yes
UZL 253	9N	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.5	0.5	-4.27	-4.04	Yes
UZL 254	10A	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-5.18	-4.57	Yes
UZL 255	6C	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-5.89	-4.36	Yes
UZL 256	15A	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.45	-6.68	Yes
UZL 257	11A	Invasive	pneumonia with bacteraemia	0.25	0.25	0.25	0.5	-4.02	-6.34	Yes
UZL 258	17F	Invasive	meningitis	0.0625	0.0625	0.25	0.5	-4.38	-4.62	Yes
UZL 259	9N	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-6.48	-5.72	No
UZL 260	24F	Invasive	pneumonia with bacteraemia	0.125	1	0.25	0.5	-4.32	-4.24	Yes
UZL 261	9N	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-7.25	-5.22	Yes
UZL 262	12F	Invasive	pneumonia with bacteraemia	0.0625	0.25	0.25	0.5	-4.94	-5.46	Yes
UZL 263	14	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.69	-5.56	Yes
UZL 264	6C	Invasive	pneumonia with bacteraemia	0.125	0.25	0.25	0.5	-4.12	-5.84	Yes
UZL 265	4	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.21	-4.46	Yes
UZL 266	4	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-4.63	-4.92	Yes

UZL 267	35B	Invasive	bacteraemia	2	4	0.25	0.5	-4.26	-4.89	No
UZL 268	19A	Invasive	pneumonia with bacteraemia	0.125	0.0625	0.125	0.5	-3.96	-6.56	Yes
UZL 269	3	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-6.61	-5.32	Yes
UZL 270	4	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-3.96	-4.39	Yes
UZL 271	3	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.72	-4.14	Yes
UZL 272	4	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.47	-4.81	Yes
UZL 273	12F	Invasive	pneumonia with bacteraemia	0.0625	0.25	0.25	0.5	-3.81	-5.09	Yes
UZL 274	31	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-3.70	-4.62	Yes
UZL 275	19A	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.78	-6.26	Yes
UZL 276	14	Invasive	pneumonia with bacteraemia	2	8	0.125	0.5	-4.17	-6.40	Yes
UZL 277	8	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-6.02	-5.54	Yes
UZL 278	8	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-4.61	-4.98	Yes
UZL 279	17F	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.14	-5.90	Yes
UZL 280	14	Invasive	bacteraemia	2	8	0.25	0.5	-4.24	-6.53	Yes
UZL 281	19A	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-3.56	-4.28	Yes
UZL 282	24F	Invasive	pneumonia with bacteraemia	0.125	1	0.25	0.5	-3.64	-7.59	Yes
UZL 283	3	Invasive	meningitis	0.0625	0.0625	0.25	0.5	-4.96	-4.59	No
UZL 284	24F	Invasive	pneumonia with bacteraemia	0.125	1	0.25	0.5	-3.45	-5.66	Yes
UZL 285	3	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-3.81	-5.88	Yes

UZL 286	23B	Invasive	pneumonia with bacteraemia	0.0625	0.5	0.25	0.5	-3.89	-4.80	Yes
UZL 287	31	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.33	-4.76	Yes
UZL 288	8	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-6.28	-4.88	Yes
UZL 289	19A	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.25	-5.97	Yes
UZL 290	9N	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.69	-4.18	Yes
UZL 291	7B	Invasive	pneumonia with bacteraemia	0.0625	0.25	0.5	0.5	-3.66	-4.24	Yes
UZL 292	24F	Invasive	pneumonia with bacteraemia	0.125	1	0.25	0.5	-3.66	-7.20	Yes
UZL 293	9N	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-3.98	-4.61	Yes
UZL 294	24F	Invasive	pneumonia with bacteraemia	0.0625	1	0.25	0.5	-4.23	-5.02	Yes
UZL 295	23B	Invasive	bacteraemia	0.0625	0.25	0.25	0.5	-3.64	-3.77	Yes
UZL 296	3	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-4.91	-5.60	Yes
UZL 297	24	Invasive	bacteraemia	0.25	1	0.25	0.5	-4.12	-5.41	Yes
UZL 298	3	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-4.12	-4.15	No
UZL 299	23B	Invasive	bacteraemia	0.0625	0.5	0.25	0.5	-4.42	-4.83	Yes
UZL 300	23B	Invasive	meningitis	0.25	0.5	0.25	0.5	-4.53	-6.39	Yes
UZL 301	16F	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-4.11	-4.87	Yes
UZL 302	19A	Invasive	bacteraemia	0.0625	0.0625	0.125	0.5	-4.48	-7.59	Yes
UZL 303	23A	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.67	-5.00	Yes
UZL 304	3	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.37	-7.16	Yes
UZL 305	23B	Invasive	pneumonia	0.0625	0.0625	0.25	0.5	-3.56	-4.33	Yes
UZL 306	23A	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-5.55	-4.78	Yes
UZL 307	8	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-6.32	-7.53	Yes

UZL 308	3	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-6.18	-4.35	Yes
UZL 309	8	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-7.34	-8.72	Yes
UZL 310	12F	Invasive	pneumonia with bacteraemia	0.0625	0.25	0.25	0.5	-4.82	-5.69	Yes
UZL 311	8	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-7.52	-8.49	Yes
UZL 312	6C	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-3.79	-3.81	Yes
UZL 313	35B	Invasive	pneumonia with bacteraemia	2	4	0.25	0.5	-3.47	-4.40	Yes
UZL 314	11A	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.33	-4.70	Yes
UZL 315	38	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-3.34	-4.00	Yes
UZL 316	12F	Invasive	pneumonia with bacteraemia	0.0625	0.25	0.25	0.5	-4.52	-5.27	Yes
UZL 317	24F	Invasive	pneumonia with bacteraemia	0.125	1	0.25	0.5	-3.41	-5.12	Yes
UZL 318	19A	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-5.11	-5.04	Yes
UZL 319	19A	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-3.51	-4.66	Yes
UZL 320	19A	Invasive	bacteraemia	0.0625	0.25	0.25	0.5	-4.19	-5.91	Yes
UZL 321	34	Invasive	meningitis	0.0625	0.0625	0.25	0.5	-4.49	-3.42	Yes
UZL 322	19F	Invasive	bacteraemia	0.0625	0.125	0.25	0.5	-5.17	-5.57	Yes
UZL 323	24F	Invasive	pneumonia with bacteraemia	0.25	0.5	0.25	0.5	-4.32	-7.56	Yes
UZL 324	6C	Invasive	pneumonia with bacteraemia	0.125	0.25	0.25	0.5	-4.07	-4.66	Yes
UZL 325	19A	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.125	0.5	-5.99	-5.11	Yes
UZL 326	9N	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-3.65	-4.31	Yes

UZL 327	24F	Invasive	pneumonia with bacteraemia	0.0625	0.5	0.25	0.5	-3.65	-5.15	Yes
UZL 328	35B	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-4.35	-5.28	Yes
UZL 329	8	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-8.40	-8.40	Yes
UZL 330	15A	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-4.09	-4.43	Yes
UZL 331	10B	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-3.97	-5.91	Yes
UZL 332	23B	Invasive	bacteraemia	0.0625	0.25	0.25	0.5	-4.11	-4.47	Yes
UZL 333	10A	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-4.57	-4.11	Yes
UZL 334	19A	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.50	-6.00	Yes
UZL 335	10A	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-3.91	-4.38	Yes
UZL 336	19A	Invasive	bacteraemia	1	8	0.25	0.5	-3.90	-4.70	Yes
UZL 337	23A	Invasive	bacteraemia	0.0625	0.0625	0.125	0.5	-3.61	-5.79	Yes
UZL 338	12F	Invasive	sepsis	0.0625	0.25	0.25	0.5	-4.15	-5.27	Yes
UZL 339	9N	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-3.91	-4.76	Yes
UZL 340	15B	Invasive	meningitis	0.0625	0.0625	0.25	0.5	-3.79	-4.75	Yes
UZL 341	23B	Invasive	pneumonia with bacteraemia	0.25	1	0.25	0.5	-3.44	-4.00	Yes
UZL 342	6C	Invasive	bacteraemia	0.125	0.25	0.25	0.5	-4.78	-4.58	Yes
UZL 343	19A	Invasive	pneumonia	0.25	0.25	0.25	0.5	-4.50	-4.85	Yes
UZL 344	19A	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.35	-5.34	Yes
UZL 345	15A	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-3.72	-4.22	Yes
UZL 346	35B	Invasive	pneumonia with bacteraemia	2	4	0.25	0.5	-6.35	-5.29	Yes
UZL 347	7B	Invasive	pneumonia with bacteraemia	0.0625	0.125	0.25	0.5	-4.52	-6.09	Yes
UZL 348	15A	Invasive	meningitis	0.0625	0.0625	0.125	0.5	-4.33	-6.09	Yes
UZL 349	6C	Invasive	bacteraemia	0.125	0.25	0.25	0.5	-4.08	-5.22	Yes
UZL 350	4	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-6.02	-5.22	Yes

UZL 351	11A	Invasive	pneumonia with bacteraemia	0.25	0.25	0.25	0.5	-4.90	-5.24	Yes
UZL 352	35B	Invasive	pneumonia	0.0625	0.0625	0.25	0.5	-4.61	-7.71	Yes
UZL 354	17F	Invasive	meningitis	0.0625	0.0625	0.5	0.5	-4.26	-4.57	Yes
UZL 355	35B	Invasive	bacteraemia	2	4	0.25	0.5	-5.34	-4.66	Yes
UZL 356	24F	Invasive	bacteraemia	0.125	1	0.125	0.5	-4.96	-5.41	No
UZL 357	24F	Invasive	pneumonia with bacteraemia	0.0625	1	0.25	0.5	-3.38	-4.68	Yes
UZL 358	16F	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-3.58	-4.13	Yes
UZL 359	24F	Invasive	meningitis	0.125	1	0.25	0.5	-3.72	-5.18	Yes
UZL 360	23A	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.125	0.5	-4.35	-6.64	Yes
UZL 361	9N	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.85	-4.72	Yes
UZL 362	35B	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.125	0.5	-4.66	-5.58	Yes
UZL 363	19A	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.57	-5.06	Yes
UZL 364	15A	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.15	-4.15	Yes
UZL 365	9N	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.125	0.5	-4.24	-5.24	Yes
UZL 366	19A	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-3.83	-4.36	Yes
UZL 367	14	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.32	-4.53	Yes
UZL 368	24F	Invasive	pneumonia with bacteraemia	0.125	0.5	0.25	0.5	-3.76	-5.16	Yes
UZL 369	7C	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-4.56	-5.03	Yes
UZL 370	23B	Invasive	pneumonia with bacteraemia	0.0625	0.25	0.25	0.5	-3.32	-3.92	Yes
UZL 371	6C	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-3.82	-5.44	Yes

UZL 372	16F	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-4.34	-5.70	Yes
UZL 373	7C	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-4.01	-4.17	Yes
UZL 374	15C	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-3.73	-4.20	Yes
UZL 375	24F	Invasive	bacteraemia	0.125	1	0.25	0.5	-4.04	-6.67	Yes
UZL 376	15C	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-3.55	-4.84	Yes
UZL 377	19A	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.84	-5.44	Yes
UZL 378	24F	Invasive	bacteraemia	0.125	1	0.25	0.5	-3.85	-5.47	Yes
UZL 379	15C	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-3.71	-6.85	No
UZL 380	16F	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.59	-6.80	No
UZL 381	6C	Invasive	bacteraemia	0.125	0.25	0.25	0.5	-4.24	-5.18	No
UZL 382	8	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-3.54	-4.91	No
UZL 383	16F	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-3.84	-4.48	No
UZL 384	12F	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-4.63	-5.28	No
UZL 385	19A	Invasive	pneumonia	0.0625	0.0625	0.25	0.5	-4.73	-4.00	No
UZL 386	38	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-3.98	-4.05	No
UZL 387	6C	Invasive	bacteraemia	0.125	0.25	0.25	0.5	-4.23	-4.66	No
UZL 388	11A	Invasive	bacteraemia	0.25	0.25	0.25	0.5	-4.73	-4.61	No
UZL 389	8	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.63	-4.95	No
UZL 390	20	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-4.33	-4.72	No
UZL 391	6C	Invasive	bacteraemia	0.125	0.25	0.25	0.5	-4.16	-6.05	No
UZL 392	35B	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.39	-7.53	No
UZL 393	11A	Invasive	pneumonia with bacteraemia	0.5	0.25	0.25	0.5	-3.88	-4.81	No
UZL 394	6C	Invasive	pneumonia with bacteraemia	0.125	0.25	0.25	0.5	-3.83	-4.81	No
UZL 395	10B	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-3.71	-4.37	No

UZL 396	24F	Invasive	bacteraemia	0.0625	1	0.25	0.5	-3.83	-5.47	No
UZL 397	24F	Invasive	pneumonia with bacteraemia	0.125	1	0.25	0.5	-3.61	-4.93	No
UZL 398	17F	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-4.43	-4.65	No
UZL 399	7C	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-3.91	-4.05	No
UZL 400	31	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.35	-4.61	No
UZL 401	19A	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-3.63	-5.34	No
UZL 402	10A	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-4.09	-4.10	No
UZL 403	23B	Invasive	bacteraemia	0.25	0.25	0.25	0.5	-4.07	-3.85	No
UZL 404	14	Invasive	pneumonia with bacteraemia	2	8	0.25	0.5	-4.04	-3.92	No
UZL 405	9N	Invasive	bacteraemia	2	8	0.25	0.5	-3.96	-4.58	No
UZL 406	9N	Invasive	bacteraemia	0.125	0.0625	0.25	0.5	-3.75	-4.73	No
UZL 407	12F	Invasive	meningitis	0.0625	0.0625	0.25	0.5	-4.41	-4.92	No
UZL 408	6C	Invasive	bacteraemia	0.125	0.25	0.25	0.5	-3.96	-4.12	No
UZL 409	12F	Invasive	pneumonia with bacteraemia	0.0625	0.25	0.25	0.5	-4.84	-6.49	No
UZL 410	34	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-3.95	-5.06	No
UZL 411	23B	Invasive	meningitis	0.125	0.25	0.25	0.5	-4.70	-4.48	No
UZL 412	35F	Invasive	sepsis	0.0625	0.0625	0.25	0.5	-4.57	-4.16	No
UZL 413	16F	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-3.90	-4.96	No
UZL 414	12F	Invasive	bacteraemia	0.0625	0.25	0.25	0.5	-4.88	-6.36	No
UZL 415	23B	Invasive	bacteraemia	0.25	1	0.25	0.5	-4.20	-4.87	No
UZL 416	19F	Invasive	pneumonia with bacteraemia	2	8	0.25	0.5	-5.84	-6.31	No
UZL 417	11A	Invasive	pneumonia with bacteraemia	0.5	0.25	0.25	0.5	-4.41	-5.72	No
UZL 418	10A	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-3.62	-3.86	No
UZL 419	11A	Invasive	bacteraemia	0.25	0.25	0.5	0.5	-3.70	-4.28	No

UZL 420	19A	Invasive	meningitis	0.0625	0.0625	0.25	0.5	-4.00	-4.75	No
UZL 421	15B	Invasive	meningitis	0.0625	0.0625	0.25	0.5	-3.87	-3.89	No
UZL 422	11A	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.02	-3.62	No
UZL 423	35B	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.37	-4.90	No
UZL 424	9N	Invasive	bacteraemia	4	8	0.25	0.5	-6.03	-5.05	No
UZL 425	9N	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-3.40	-3.95	No
UZL 426	23A	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-3.42	-4.21	No
UZL 427	8	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-3.95	-4.67	No
UZL 428	4	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-3.63	-4.49	No
UZL 429	11A	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-3.24	-4.17	No
UZL 430	35F	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.96	-6.10	No
UZL 431	24F	Invasive	meningitis	0.125	1	0.25	0.5	-4.31	-4.73	No
UZL 432	9N	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-3.74	-4.13	No
UZL 433	24F	Invasive	bacteraemia	0.125	1	0.25	0.5	-3.59	-4.44	No
UZL 434	19A	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-4.33	-4.22	No
UZL 435	6C	Invasive	pneumonia with bacteraemia	0.125	0.25	0.125	0.5	-4.43	-4.35	No
UZL 436	23A	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.36	-6.49	No
UZL 437	10A	Invasive	meningitis	0.0625	0.0625	0.25	0.5	-4.37	-6.08	No
UZL 438	19F	Invasive	pneumonia with bacteraemia	0.0625	0.125	0.25	0.5	-4.23	-4.43	No
UZL 439	6C	Invasive	pneumonia with bacteraemia	0.125	0.25	0.25	0.5	-4.49	-4.58	No
UZL 440	12F	Invasive	pneumonia with bacteraemia	0.0625	0.25	0.25	0.5	-3.99	-5.24	No

UZL 441	15A	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-3.56	-4.36	No
UZL 442	35F	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.05	-4.48	No
UZL 443	19A	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.06	-4.44	No
UZL 444	7B	Invasive	pneumonia with bacteraemia	0.0625	0.125	0.25	0.5	-4.10	-4.19	No
UZL 445	23A	Invasive	pneumonia with bacteraemia	0.0625	0.125	0.25	0.5	-4.20	-5.21	No
UZL 446	12F	Invasive	bacteraemia	0.0625	0.25	0.25	0.5	-4.75	-6.43	No
UZL 447	11A	Invasive	bacteraemia	8	8	0.125	0.5	-4.10	-5.01	No
UZL 448	15C	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-3.90	-5.11	No
UZL 449	11A	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-3.67	-4.96	No
UZL 450	31	Invasive	pneumonia with bacteraemia	0.0625	0.5	0.25	0.5	-4.83	-4.78	No
UZL 451	23A	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.24	-4.55	No
UZL 452	8	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.72	-4.83	No
UZL 453	19A	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-3.86	-6.12	No
UZL 454	16F	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.17	-4.43	No
UZL 455	6C	Invasive	bacteraemia	0.125	0.25	0.25	0.5	-4.85	-5.38	No
UZL 456	8	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.11	-6.80	No
UZL 457	6C	Invasive	pneumonia with bacteraemia	0.125	0.25	0.125	0.5	-5.86	-4.71	No
UZL 458	19A	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-3.23	-3.61	No
UZL 459	23A	Invasive	meningitis	0.0625	0.0625	0.25	0.5	-4.54	-4.16	No
UZL 460	8	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-4.02	-3.85	No

UZL 461	19A	Invasive	bacteraemia	> 8	> 16	0.25	0.5	-3.53	-3.93	No
UZL 462	35F	Invasive	bacteraemia					-4.90	-5.26	No
UZL 463	4	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.00	-4.66	No
UZL 464	6C	Invasive	pneumonia with bacteraemia	0.25	0.25	0.25	0.5	-4.07	-4.53	No
UZL 465	38	Invasive	meningitis	0.0625	0.0625	0.25	0.5	-3.28	-4.37	No
UZL 466	9N	Invasive	meningitis	0.0625	0.0625	0.25	0.5	-4.09	-5.20	No
UZL 467	23B	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-4.04	-3.99	No
UZL 468	4	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.38	-6.08	No
UZL 469	14	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.58	-4.11	No
UZL 470	24B	Invasive	bacteraemia	0.125	1	0.25	0.5	-4.27	-4.08	No
UZL 471	35B	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.54	-3.63	No
UZL 472	23B	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-4.14	-4.96	No
UZL 473	35B	Invasive	bacteraemia	0.125	0.25	0.25	0.5	-4.69	-4.09	No
UZL 474	23B	Invasive	pneumonia with bacteraemia	0.0625	0.5	0.25	0.5	-3.88	-4.50	No
UZL 475	20	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-3.95	-4.27	No
UZL 476	8	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.09	-4.73	No
UZL 477	7B	Invasive	pneumonia with bacteraemia	0.0625	0.25	0.5	0.5	-3.88	-4.38	No
UZL 478	19A	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.50	-5.15	No
UZL 479	19A	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-3.36	-4.93	No
UZL 480	9N	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-3.87	-4.80	No
UZL 481	20	Invasive	meningitis	0.0625	0.0625	0.25	0.5	-4.37	-4.62	No
UZL 482	15A	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-3.99	-4.40	No

UZL 483	6C	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.68	-4.93	No
UZL 484	19F	Invasive	pneumonia with bacteraemia	2	8	0.25	0.5	-4.18	-4.97	No
UZL 485	23A	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.61	-6.79	No
UZL 486	4	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-4.54	-4.65	No
UZL 487	24F	Invasive	bacteraemia	0.125	1	0.25	0.5	-3.76	-5.43	No
UZL 488	6C	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-5.36	-5.21	No
UZL 489	11A	Invasive	pneumonia with bacteraemia	8	8	0.25	0.5	-3.60	-6.63	No
UZL 490	9N	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-4.20	-3.89	No
UZL 491	19F	Invasive	pneumonia with bacteraemia	0.0625	0.125	0.25	0.5			No
UZL 492	3	Invasive	bacteraemia	0.0625	0.0625	0.25	0.5	-3.94	-4.22	No
UZL 493	12F	Invasive	pneumonia with bacteraemia	0.0625	0.25	0.25	0.5	-3.52	-4.29	No
UZL 494	24B	Invasive	pneumonia with bacteraemia	0.125	1	0.25	0.5	-3.49	-4.70	No
UZL 495	15A	Invasive	pneumonia with bacteraemia	0.0625	1	0.25	0.5	-3.62	-3.11	No
UZL 496	7C	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.27	-4.62	No
UZL 497	35B	Invasive	pneumonia with bacteraemia	2	4	0.25	0.5	-3.80	-4.78	No
UZL 498	19A	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.81	-5.32	No
UZL 499	6C	Invasive	pneumonia with bacteraemia	0.125	0.25	0.25	0.5	-4.06	-4.37	No
UZL 500	15B	Invasive	pneumonia with bacteraemia	0.0625	0.0625	0.25	0.5	-4.19	-5.28	No

BIBLIOGRAPY

1. Sternberg GM. Special Report to the National Board of Health– Experimental Investigation Relating to the Etiology of the Malarial Fevers. Annu Rep Natl Board Heal [Internet]. 1881;65–91. Available from: https://hdl.handle.net/2027/iau.31858045161712

2. Pasteur L. Bulletin de l'Académie Nationale de Médecin. Paris: Elsevier Masson; 1881.

3. Sanchez-Rosario Y, Johnson MDL. Media Matters, Examining Historical and Modern Streptococcus pneumoniae Growth Media and the Experiments They Affect. Front Cell Infect Microbiol [Internet]. 2021 Mar 23 [cited 2024 Feb 28];11. Available from: https://pubmed.ncbi.nlm.nih.gov/33834003/

4. Paton JC, Trappetti C. Streptococcus pneumoniae Capsular Polysaccharide. Microbiol Spectr [Internet]. 2006 [cited 2019 Oct 20];7(2). Available from: http://www.ncbi.nlm.nih.gov/pubmed/30977464

5. Austrian R. Pneumococcus: the first one hundred years. Rev Infect Dis [Internet]. 1981 [cited 2024 Apr 9];3(2):183–9. Available from: https://pubmed.ncbi.nlm.nih.gov/7020040/

6. Sternberg GM. A Text-book of bacteriology. William Wood and Company; 1896.

7. Talamon, C. H., Broca, A. A. D. T., Proust, R. A. D. T., Feindel, E. A. D. T. A, Texte S. Bulletins de la Socié té anatomique de Paris. Paris: V. Masson and G. Steinheil; 1883.

8. Morens DM, Taubenberger JK, Fauci AS. Predominant role of bacterial pneumonia as a cause of death in pandemic influenza: implications for pandemic influenza preparedness. J Infect Dis [Internet]. 2008 Oct 1 [cited 2024 Apr 9];198(7):962–70. Available from: https://pubmed.ncbi.nlm.nih.gov/18710327/

9. Bridy-Pappas AE, Margolis MB, Center KJ, Isaacman DJ. *Streptococcus pneumoniae* : Description of the Pathogen, Disease Epidemiology, Treatment, and Prevention. Pharmacotherapy [Internet]. 2005 Aug 2 [cited 2019 Oct 10];25(9):1193–212. Available from:

http://www.ncbi.nlm.nih.gov/pubmed/16164394

10. McDevitt E, Khan F, Scasny A, et al. Hydrogen Peroxide Production by Streptococcus pneumoniae Results in Alpha-hemolysis by Oxidation of Oxy-hemoglobin to Met-hemoglobin. mSphere [Internet]. 2020 Dec 23 [cited 2024 Apr 9];5(6). Available from: https://pubmed.ncbi.nlm.nih.gov/33298575/

11. Geno KA, Gilbert GL, Song JY, et al. Pneumococcal Capsules and Their Types: Past, Present, and Future. Clin Microbiol Rev [Internet]. 2015 Jul [cited 2019 Oct 26];28(3):871–99. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26085553

12. Gao S, Jin W, Quan Y, et al. Bacterial capsules: Occurrence, mechanism, and function. NPJ biofilms microbiomes [Internet]. 2024 Dec 1 [cited 2024 Apr 9];10(1). Available from: https://pubmed.ncbi.nlm.nih.gov/38480745/

13. Ganaie F, Saad JS, McGee L, et al. A New Pneumococcal Capsule Type, 10D, is the 100th Serotype and Has a Large cps Fragment from an Oral Streptococcus. MBio [Internet]. 2020 [cited 2024 Apr 9];11(3). Available from: https://pubmed.ncbi.nlm.nih.gov/32430472/

14. Larson TR, Yother J. Streptococcus pneumoniae capsular polysaccharide is linked to peptidoglycan via a direct glycosidic bond to β-D- N-acetylglucosamine. Proc Natl Acad Sci U S A [Internet]. 2017 May 30 [cited 2024 Jun 26];114(22):5695–700. Available from: https://pubmed.ncbi.nlm.nih.gov/28495967/

15. Wen Z, Zhang JR. Bacterial Capsules. Mol Med Microbiol. 2015 Jan 1;33–53.

16. Luck JN, Tettelin H, Orihuela CJ. Sugar-Coated Killer: Serotype 3 Pneumococcal Disease. Front Cell Infect Microbiol [Internet]. 2020 Dec 23 [cited 2024 Jun 26];10. Available from: https://pubmed.ncbi.nlm.nih.gov/33425786/

17. Fuji N, Pichichero M, Kaur R. Pathogenesis of Streptococcus pneumoniae serotype 3 during natural colonization and infections among children and its IgG correlate of protection in a mouse model.

Vaccine [Internet]. 2022 Oct 19 [cited 2024 Jun 26];40(44):6412–21. Available from: https://pubmed.ncbi.nlm.nih.gov/36192274/

18. Subramanian K, Henriques-Normark B, Normark S. Emerging concepts in the pathogenesis of the *Streptococcus pneumoniae*: From nasopharyngeal colonizer to intracellular pathogen. Cell Microbiol [Internet]. 2019 Nov 1 [cited 2022 Jul 8];21(11). Available from: https://pubmed.ncbi.nlm.nih.gov/31251447/

19. Troeger C, Blacker B, Khalil IA, et al. Estimates of the global, regional, and national morbidity, mortality, and aetiologies of lower respiratory infections in 195 countries, 1990-2016: a systematic analysis for the Global Burden of Disease Study 2016. Lancet Infect Dis [Internet]. 2018 Nov 1 [cited 2022 Jan 13];18(11):1191–210. Available from: https://pubmed.ncbi.nlm.nih.gov/30243584/

20. Weiser JN, Ferreira DM, Paton JC. Streptococcus pneumoniae: transmission, colonization and invasion. Nat Rev Microbiol [Internet]. 2018 Jun 29 [cited 2019 Oct 7];16(6):355–67. Available from: http://www.ncbi.nlm.nih.gov/pubmed/29599457

21. Örtqvist Å, Hedlund J, Kalin M. Streptococcus pneumoniae: epidemiology, risk factors, and clinical features. Semin Respir Crit Care Med [Internet]. 2005 Dec [cited 2024 Apr 10];26(6):563–74. Available from: https://pubmed.ncbi.nlm.nih.gov/16388428/

22. Oligbu G, Fry NK, Ladhani SN. The Pneumococcus and Its Critical Role in Public Health. Methods Mol Biol [Internet]. 2019 [cited 2024 Apr 22];1968:205–13. Available from: https://pubmed.ncbi.nlm.nih.gov/30929217/

23. Ochoa-Gondar O, Torras-Vives V, de Diego-Cabanes C, et al. Incidence and risk factors of pneumococcal pneumonia in adults: a population-based study. BMC Pulm Med [Internet]. 2023 Dec 1 [cited 2024 Apr 10];23(1). Available from: https://pubmed.ncbi.nlm.nih.gov/37291502/

24. Sohail I, Ghosh S, Mukundan S, Zelewski S, Khan MN. Role of Inflammatory Risk Factors in the Pathogenesis of Streptococcus pneumoniae. Front Immunol [Internet]. 2018 Oct 2 [cited 2024 Apr

10];9(OCT). Available from: https://pubmed.ncbi.nlm.nih.gov/30333833/

25. Centers for Disease Control U. Antibiotic Resistance Threats in the United States, 2019 [Internet]. 2019 Dec [cited 2022 Feb 17]. Available from: http://dx.doi.org/10.15620/cdc:82532.

26. Murray CJ, Ikuta KS, Sharara F, et al. Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. Lancet (London, England) [Internet]. 2022 Feb 12 [cited 2024 Apr 10];399(10325):629–55. Available from: https://pubmed.ncbi.nlm.nih.gov/35065702/

27. Murray CJ, Ikuta KS, Sharara F, et al. Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. Lancet. 2022 Feb 12;399(10325):629–55.

28. Santoro F, Iannelli F, Pozzi G. Genomics and Genetics of Streptococcus pneumoniae. Microbiol Spectr [Internet]. 2019 May 31 [cited 2024 Apr 10];7(3). Available from: https://pubmed.ncbi.nlm.nih.gov/31111814/

29. Hiller NL, Sá-Leão R. Puzzling Over the Pneumococcal Pangenome. Front Microbiol [Internet]. 2018 Mar 29 [cited 2024 Apr 10];9. Available from: https://pubmed.ncbi.nlm.nih.gov/30425695/

30. Shen CH. The genome. Diagnostic Mol Biol. 2023 Jan 1;127–56.

31. Ochman H, Caro-Quintero A. Genome Size and Structure, Bacterial. Encycl Evol Biol. 2016 Jan 1;179–85.

32. Ozer EA, Allen JP, Hauser AR. Characterization of the core and accessory genomes of Pseudomonas aeruginosa using bioinformatic tools Spine and AGEnt. BMC Genomics [Internet]. 2014 Aug 29 [cited 2024 Jun 27];15(1). Available from: https://pubmed.ncbi.nlm.nih.gov/25168460/

33. Park SC, Lee K, Kim YO, Won S, Chun J. Large-Scale Genomics Reveals the Genetic Characteristics of Seven Species and Importance of Phylogenetic Distance for Estimating Pan-Genome Size. Front Microbiol [Internet]. 2019 [cited 2024 Jun 27];10(APR). Available from: https://pubmed.ncbi.nlm.nih.gov/31068915/

34. Tettelin H, Nelson KE, Paulsen IT, et al. Complete genome sequence of a virulent isolate of Streptococcus pneumoniae. Science [Internet]. 2001 Jul 20 [cited 2024 Apr 10];293(5529):498–506. Available from: https://pubmed.ncbi.nlm.nih.gov/11463916/

35. Vernikos G, Medini D, Riley DR, Tettelin H. Ten years of pan-genome analyses. Curr Opin Microbiol [Internet]. 2015 Feb 1 [cited 2024 Apr 10];23:148–54. Available from: https://pubmed.ncbi.nlm.nih.gov/25483351/

36. Bosi E, Fani R, Fondi M. Defining orthologs and pangenome size metrics. Methods Mol Biol [Internet]. 2015 [cited 2024 Apr 10];1231:191–202. Available from: https://pubmed.ncbi.nlm.nih.gov/25343867/

37. Straume D, Stamsås GA, Håvarstein LS. Natural transformation and genome evolution in Streptococcus pneumoniae. Infect Genet Evol [Internet]. 2015 Jul 1 [cited 2024 Apr 10];33:371–80. Available from: https://pubmed.ncbi.nlm.nih.gov/25445643/

38. Donati C, Hiller NL, Tettelin H, et al. Structure and dynamics of the pan-genome of Streptococcus pneumoniae and closely related species. Genome Biol [Internet]. 2010 Oct 29 [cited 2024 Apr 10];11(10). Available from: https://pubmed.ncbi.nlm.nih.gov/21034474/

39. Chaguza C, Cornick JE, Everett DB. Mechanisms and impact of genetic recombination in the evolution of Streptococcus pneumoniae. Comput Struct Biotechnol J [Internet]. 2015 [cited 2024 Apr 18];13:241–7. Available from: https://pubmed.ncbi.nlm.nih.gov/25904996/

40. Muzzi A, Donati C. Population genetics and evolution of the pan-genome of Streptococcus pneumoniae. Int J Med Microbiol [Internet]. 2011 Dec [cited 2024 Apr 10];301(8):619–22. Available from: https://pubmed.ncbi.nlm.nih.gov/22000739/

41. Hall RJ, Whelan FJ, Cummins EA, Connor C, McNally A, McInerney JO. Gene-gene relationships in an Escherichia coli accessory genome are linked to function and mobility. Microb genomics [Internet]. 2021 [cited 2024 Jun 27];7(9). Available from:

https://pubmed.ncbi.nlm.nih.gov/34499026/

42. Rasko DA, Rosovitz MJ, Myers GSA, et al. The pangenome structure of Escherichia coli: comparative genomic analysis of E. coli commensal and pathogenic isolates. J Bacteriol [Internet]. 2008 Oct [cited 2024 Jun 27];190(20):6881–93. Available from: https://pubmed.ncbi.nlm.nih.gov/18676672/

43. Chewapreecha C, Harris SR, Croucher NJ, et al. Dense genomic sampling identifies highways of pneumococcal recombination. Nat Genet [Internet]. 2014 [cited 2024 Apr 10];46(3):305–9. Available from: https://pubmed.ncbi.nlm.nih.gov/24509479/

44. Dagan R, Klugman KP. Impact of conjugate pneumococcal vaccines on antibiotic resistance. Lancet Infect Dis [Internet]. 2008 Dec [cited 2024 Apr 10];8(12):785–95. Available from: https://pubmed.ncbi.nlm.nih.gov/19022193/

45. Mendes RE, Costello AJ, Jacobs MR, Biek D, Critchley IA, Jones RN. Serotype distribution and antimicrobial susceptibility of USA Streptococcus pneumoniae isolates collected prior to and post introduction of 13-valent pneumococcal conjugate vaccine. Diagn Microbiol Infect Dis [Internet]. 2014 [cited 2024 Apr 10];80(1):19–25. Available from: https://pubmed.ncbi.nlm.nih.gov/24974272/

46. Weinberger DM, Malley R, Lipsitch M. Serotype replacement in disease after pneumococcal vaccination. Lancet (London, England) [Internet]. 2011 [cited 2024 Apr 10];378(9807):1962–73. Available from: https://pubmed.ncbi.nlm.nih.gov/21492929/

47. Crisafulli G, Guidotti S, Muzzi A, et al. An extended multi-locus molecular typing schema for Streptococcus pneumoniae demonstrates that a limited number of capsular switch events is responsible for serotype heterogeneity of closely related strains from different countries. Infect Genet Evol [Internet]. 2013 Jan [cited 2024 Apr 25];13(1):151–61. Available from: https://pubmed.ncbi.nlm.nih.gov/23022733/

48. Robinson DA, Thomas JC, Hanage WP. Population Structure of Pathogenic Bacteria. Genet Evol

Infect Dis. 2011 Jan 1;43–57.

49. Achtman M. Population structure of pathogenic bacteria revisited. Int J Med Microbiol [Internet]. 2004 [cited 2024 Apr 25];294(2–3):67–73. Available from: https://pubmed.ncbi.nlm.nih.gov/15493816/

50. Gladstone RA, Lo SW, Lees JA, et al. International genomic definition of pneumococcal lineages, to contextualise disease, antibiotic resistance and vaccine impact. EBioMedicine [Internet]. 2019 May 1 [cited 2024 Apr 25];43:338–46. Available from: https://pubmed.ncbi.nlm.nih.gov/31003929/

51. Lisher JP, Tsui HCT, Ramos-Montañez S, et al. Biological and Chemical Adaptation to Endogenous Hydrogen Peroxide Production in *Streptococcus pneumoniae* D39. mSphere [Internet]. 2017 Feb 22 [cited 2022 Mar 7];2(1). Available from: https://pubmed.ncbi.nlm.nih.gov/28070562/

52. Mraheil MA, Toque HA, La Pietra L, et al. Dual Role of Hydrogen Peroxide as an Oxidant in Pneumococcal Pneumonia. Antioxid Redox Signal [Internet]. 2021 Apr 20 [cited 2024 Feb 14];34(12):962–78. Available from: https://pubmed.ncbi.nlm.nih.gov/32283950/

53. Suárez N, Texeira E. Optimal Conditions for *Streptococcus pneumoniae* Culture: In Solid and Liquid Media. Methods Mol Biol [Internet]. 2019 [cited 2022 Jul 4];1968:3–10. Available from: https://pubmed.ncbi.nlm.nih.gov/30929201/

54. Lewis K. Programmed death in bacteria. Microbiol Mol Biol Rev [Internet]. 2000 Sep [cited 2024 Aug 26];64(3):503–14. Available from: https://pubmed.ncbi.nlm.nih.gov/10974124/

55. Popp PF, Mascher T. Coordinated Cell Death in Isogenic Bacterial Populations: Sacrificing Some for the Benefit of Many? J Mol Biol [Internet]. 2019 Nov 22 [cited 2024 Aug 26];431(23):4656–69. Available from: https://pubmed.ncbi.nlm.nih.gov/31029705/

56. Lee H, Lee DG. Programmed Cell Death in Bacterial Community: Mechanisms of Action, Causes and Consequences. J Microbiol Biotechnol [Internet]. 2019 Jul 1 [cited 2024 Aug 26];29(7):1014–21. Available from: https://pubmed.ncbi.nlm.nih.gov/31216790/

57. Ikken Y, Charof R, Elouennass M, Sekhsokh Y. The novel biphasic medium for transport, culture and conservation at an ambient temperature of Neisseria meningitidis, Streptococcus pneumoniae and Haemophilus influenzae. World J Microbiol Biotechnol [Internet]. 2021 Nov 1 [cited 2024 Feb 14];37(11). Available from: https://pubmed.ncbi.nlm.nih.gov/34586515/

58. Blaschke AJ. Interpreting assays for the detection of Streptococcus pneumoniae. Clin Infect Dis [Internet]. 2011 May 1 [cited 2024 Feb 15];52 Suppl 4(Suppl 4). Available from: https://pubmed.ncbi.nlm.nih.gov/21460292/

59. Wu X, Gordon O, Jiang W, et al. Interaction between Streptococcus pneumoniae and Staphylococcus aureus Generates ·OH Radicals That Rapidly Kill Staphylococcus aureus Strains. J Bacteriol [Internet]. 2019 [cited 2024 Feb 14];201(21). Available from: https://pubmed.ncbi.nlm.nih.gov/31405914/

60. Yesilkaya H, Andisi VF, Andrew PW, Bijlsma JJE. Streptococcus pneumoniae and reactive oxygen species: an unusual approach to living with radicals. Trends Microbiol [Internet]. 2013 Apr [cited 2024 Feb 14];21(4):187–95. Available from: https://pubmed.ncbi.nlm.nih.gov/23415028/

61. Regev-Yochay G, Trzcinski K, Thompson CM, Lipsitch M, Malley R. SpxB is a suicide gene of *Streptococcus pneumoniae* and confers a selective advantage in an *in vivo* competitive colonization model. J Bacteriol [Internet]. 2007 Sep [cited 2021 Dec 16];189(18):6532–9. Available from: https://pubmed.ncbi.nlm.nih.gov/17631628/

62. Pericone CD, Park S, Imlay JA, Weiser JN. Factors contributing to hydrogen peroxide resistance in Streptococcus pneumoniae include pyruvate oxidase (SpxB) and avoidance of the toxic effects of the fenton reaction. J Bacteriol [Internet]. 2003 Dec [cited 2024 Apr 5];185(23):6815–25. Available from: https://pubmed.ncbi.nlm.nih.gov/14617646/

63. Hernandez-Morfa M, Olivero NB, Zappia VE, et al. The oxidative stress response of Streptococcus pneumoniae: its contribution to both extracellular and intracellular survival. Front Microbiol

[Internet]. 2023 [cited 2024 Apr 5];14. Available from: https://pubmed.ncbi.nlm.nih.gov/37789846/

64. Bryant JC, Dabbs RC, Oswalt KL, et al. Pyruvate oxidase of *Streptococcus pneumoniae* contributes to pneumolysin release. BMC Microbiol [Internet]. 2016 Nov 9 [cited 2022 Jun 7];16(1):1–12. Available from: https://pubmed.ncbi.nlm.nih.gov/27829373/

65. Mellroth P, Daniels R, Eberhardt A, et al. LytA, Major Autolysin of *Streptococcus pneumoniae*, Requires Access to Nascent Peptidoglycan. 2012;

66. Mellroth P, Sandalova T, Kikhney A, et al. Structural and functional insights into peptidoglycan access for the lytic amidase LytA of Streptococcus pneumoniae. MBio [Internet]. 2014 Feb 11 [cited 2024 Feb 15];5(1). Available from: https://pubmed.ncbi.nlm.nih.gov/24520066/

67. Martner A, Skovbjerg S, Paton JC, Wold AE. *Streptococcus pneumoniae* autolysis prevents phagocytosis and production of phagocyte-activating cytokines. Infect Immun [Internet]. 2009 Sep [cited 2022 Jan 21];77(9):3826–37. Available from: https://pubmed.ncbi.nlm.nih.gov/19528220/

68. Ji H, Zhou Y, Zhang L, et al. Function analysis of choline binding domains (CBDs) of LytA, LytC and CbpD in biofilm formation of Streptococcus pneumoniae. Microb Pathog [Internet]. 2023 Jan 1 [cited 2024 Feb 15];174. Available from: https://pubmed.ncbi.nlm.nih.gov/36521655/

69. Giudicelli S, Tomasz A. Attachment of Pneumococcal Autolysin to Wall Teichoic Acids, an Essential Step in Enzymatic Wall Degradation. J Bacteriol. 1984;158(3):1188–90.

70. Kadioglu A, Weiser JN, Paton JC, Andrew PW. The role of Streptococcus pneumoniae virulence factors in host respiratory colonization and disease. Nat Rev Microbiol [Internet]. 2008 Apr [cited 2019 Oct 20];6(4):288–301. Available from: http://www.ncbi.nlm.nih.gov/pubmed/18340341

71. Eldholm V, Johnsborg O, Haugen K, Ohnstad HS, Havastein LS. Fratricide in Streptococcus pneumoniae: contributions and role of the cell wall hydrolases CbpD, LytA and LytC. Microbiology [Internet]. 2009 [cited 2024 Aug 26];155(Pt 7):2223–34. Available from:

https://pubmed.ncbi.nlm.nih.gov/19389766/

72. Zafar MA, Wang Y, Hamaguchi S, Weiser JN. Host-to-Host Transmission of Streptococcus pneumoniae Is Driven by Its Inflammatory Toxin, Pneumolysin. Cell Host Microbe [Internet]. 2017 Jan 11 [cited 2024 Apr 11];21(1):73–83. Available from: https://pubmed.ncbi.nlm.nih.gov/28081446/

73. Morimura A, Hamaguchi S, Akeda Y, Tomono K. Mechanisms Underlying Pneumococcal Transmission and Factors Influencing Host-Pneumococcus Interaction: A Review. Front Cell Infect Microbiol [Internet]. 2021 Apr 28 [cited 2024 Apr 11];11. Available from: https://pubmed.ncbi.nlm.nih.gov/33996623/

74. Brooks LRK, Mias GI. Streptococcus pneumoniae's Virulence and Host Immunity: Aging, Diagnostics, and Prevention. Front Immunol [Internet]. 2018 Jun 22 [cited 2024 Jun 26];9(JUN). Available from: https://pubmed.ncbi.nlm.nih.gov/29988379/

75. Shak JR, Vidal JE, Klugman KP. Influence of bacterial interactions on pneumococcal colonization of the nasopharynx. Trends Microbiol [Internet]. 2013 Mar [cited 2024 Apr 11];21(3):129–35. Available from: https://pubmed.ncbi.nlm.nih.gov/23273566/

76. Soni J, Sinha S, Pandey R. Understanding bacterial pathogenicity: a closer look at the journey of harmful microbes. Front Microbiol [Internet]. 2024 [cited 2024 Apr 11];15. Available from: https://pubmed.ncbi.nlm.nih.gov/38444801/

77. Bogaert D, De Groot R, Hermans PWM. Streptococcus pneumoniae colonisation: The key to pneumococcal disease. Lancet Infect Dis [Internet]. 2004 Mar [cited 2024 Apr 11];4(3):144–54. Available from: https://pubmed.ncbi.nlm.nih.gov/14998500/

78. Chaguza C, Andam CP, Harris SR, et al. Recombination in Streptococcus pneumoniae Lineages Increase with Carriage Duration and Size of the Polysaccharide Capsule. MBio [Internet]. 2016 Sep 1 [cited 2024 Apr 30];7(5). Available from: https://pubmed.ncbi.nlm.nih.gov/27677790/

79. Ritchie ND, Mitchell TJ, Evans TJ. What is different about serotype 1 pneumococci? Future Microbiol [Internet]. 2012 Jan [cited 2024 Apr 30];7(1):33–46. Available from: https://pubmed.ncbi.nlm.nih.gov/22191445/

80. Loughran AJ, Orihuela CJ, Tuomanen EI. Streptococcus pneumoniae: Invasion and Inflammation. Microbiol Spectr [Internet]. 2019 Apr 12 [cited 2024 Apr 10];7(2). Available from: https://pubmed.ncbi.nlm.nih.gov/30873934/

81. Paul CR, Moreno MA. Acute Otitis Media. JAMA Pediatr [Internet]. 2020 Mar 1 [cited 2024 Apr 22];174(3):308. Available from: https://pubmed.ncbi.nlm.nih.gov/31985755/

82. Venekamp RP, Schilder AGM, Van Den Heuvel M, Hay AD. Acute otitis media in children. BMJ [Internet]. 2020 Nov 18 [cited 2024 Apr 22];371. Available from: https://pubmed.ncbi.nlm.nih.gov/33208317/

83. Ekinci E, Desmet S, Van Heirstraeten L, et al. Streptococcus pneumoniae Serotypes Carried by Young Children and Their Association With Acute Otitis Media During the Period 2016-2019. Front Pediatr [Internet]. 2021 Jul 5 [cited 2024 Apr 23];9. Available from: https://pubmed.ncbi.nlm.nih.gov/34291017/

84. BAPCOC. Belgische gids voor anti-infectieuze behandeling in de ambulante praktijk. 2022;

85. Mandell LA. Community-acquired pneumonia: An overview. Postgrad Med [Internet]. 2015 Jan 1 [cited 2024 Apr 23];127(6):607–15. Available from: https://pubmed.ncbi.nlm.nih.gov/26224210/

86. Steel HC, Cockeran R, Anderson R, Feldman C. Overview of community-acquired pneumonia and the role of inflammatory mechanisms in the immunopathogenesis of severe pneumococcal disease. Mediators Inflamm [Internet]. 2013 [cited 2024 Apr 23];2013. Available from: https://pubmed.ncbi.nlm.nih.gov/24453422/

87. Lansbury L, Lim B, McKeever TM, Lawrence H, Lim WS. Non-invasive pneumococcal pneumonia due to vaccine serotypes: A systematic review and meta-analysis. EClinicalMedicine [Internet]. 2022

Feb 1 [cited 2024 Apr 24];44. Available from: https://pubmed.ncbi.nlm.nih.gov/35112072/

88. Hasbun R. Progress and Challenges in Bacterial Meningitis: A Review. JAMA [Internet]. 2022 Dec 6 [cited 2024 Apr 24];328(21):2147–54. Available from: https://pubmed.ncbi.nlm.nih.gov/36472590/

89. Center for Disease Control and Prevention. Pneumococcal Disease | Clinical | Features | CDC [Internet]. 2017 [cited 2019 Oct 10]. Available from: https://www.cdc.gov/pneumococcal/clinicians/clinical-features.html

90. Cecconi M, Evans L, Levy M, Rhodes A. Sepsis and septic shock. Lancet (London, England) [Internet]. 2018 Jul 7 [cited 2024 Apr 24];392(10141):75–87. Available from: https://pubmed.ncbi.nlm.nih.gov/29937192/

91. Engholm DH, Kilian M, Goodsell DS, Andersen ES, Kjærgaard RS. A visual review of the human pathogen Streptococcus pneumoniae. FEMS Microbiol Rev [Internet]. 2017 Nov 1 [cited 2019 Oct 7];41(6):854–79. Available from: https://academic.oup.com/femsre/article/41/6/854/4345647

92. World Health Organization. WHO bacterial priority pathogens list, 2024: Bacterial pathogens of public health importance to guide research, development and strategies to prevent and control antimicrobial resistance. 2024.

93. Abraham EP. Fleming's discovery. Rev Infect Dis. 1980;2(1):140.

94. Blumberg PM, Strominger JL. Interaction of penicillin with the bacterial cell: penicillin-binding proteins and penicillin-sensitive enzymes. Bacteriol Rev [Internet]. 1974 Sep [cited 2024 Apr 24];38(3):291–335. Available from: https://pubmed.ncbi.nlm.nih.gov/4608953/

95. Worthington RJ, Melander C. Overcoming resistance to β-lactam antibiotics. J Org Chem [Internet]. 2013 May 3 [cited 2024 Apr 24];78(9):4207–13. Available from: https://pubmed.ncbi.nlm.nih.gov/23530949/

96. Lima LM, Silva BNM da, Barbosa G, Barreiro EJ. β-lactam antibiotics: An overview from a medicinal chemistry perspective. Eur J Med Chem [Internet]. 2020 Dec 15 [cited 2024 Apr 24];208. Available from: https://pubmed.ncbi.nlm.nih.gov/33002736/

97. Gaynor M, Mankin A. Macrolide antibiotics: binding site, mechanism of action, resistance. Curr Top Med Chem [Internet]. 2003 Mar 25 [cited 2024 Apr 24];3(9):949–60. Available from: https://pubmed.ncbi.nlm.nih.gov/12678831/

98. Vázquez-Laslop N, Mankin AS. How Macrolide Antibiotics Work. Trends Biochem Sci [Internet]. 2018 Sep 1 [cited 2024 Apr 24];43(9):668–84. Available from: https://pubmed.ncbi.nlm.nih.gov/30054232/

99. De Sutter A, Piessens V, Poelman P, Van Roy K. BCFI | Methodologie van de update van de gids 2019/2021 [Internet]. 2021 [cited 2022 Feb 25]. Available from: https://www.bcfi.be/nl/chapters/12?matches=BAPCOC&frag=8901316

100. Farrell DJ, Couturier C, Hryniewicz W. Distribution and antibacterial susceptibility of macrolide resistance genotypes in Streptococcus pneumoniae: PROTEKT Year 5 (2003-2004). Int J Antimicrob Agents [Internet]. 2008 Mar [cited 2024 Apr 24];31(3):245–9. Available from: https://pubmed.ncbi.nlm.nih.gov/18178388/

101. Schroeder MR, Stephens DS. Macrolide Resistance in Streptococcus pneumoniae. Front Cell Infect Microbiol [Internet]. 2016 [cited 2019 Oct 20];6:98. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27709102

102. European centre for disease prevention and control. Surveillance of antimicrobial resistance in Europe 2017 [Internet]. 2017 [cited 2019 Oct 9]. Available from: https://www.ecdc.europa.eu/sites/default/files/documents/EARS-Net-report-2017-update-jan-2019.pdf

103. Ezelarab HAA, Abbas SH, Hassan HA, Abuo-Rahma GEDA. Recent updates of fluoroquinolones as

antibacterial agents. Arch Pharm (Weinheim) [Internet]. 2018 Sep 1 [cited 2024 Apr 24];351(9). Available from: https://pubmed.ncbi.nlm.nih.gov/30048015/

104. Jia Y, Zhao L. The antibacterial activity of fluoroquinolone derivatives: An update (2018-2021). Eur J Med Chem [Internet]. 2021 Nov 15 [cited 2024 Apr 24];224. Available from: https://pubmed.ncbi.nlm.nih.gov/34365130/

105. Flint AJ, Davis AP. Vancomycin mimicry: towards new supramolecular antibiotics. Org Biomol Chem [Internet]. 2022 Sep 27 [cited 2024 Apr 24];20(39):7694–712. Available from: https://pubmed.ncbi.nlm.nih.gov/36165239/

106. Levine DP. Vancomycin: a history. Clin Infect Dis [Internet]. 2006 Jan 1 [cited 2024 Apr 24];42 Suppl 1(SUPPL. 1). Available from: https://pubmed.ncbi.nlm.nih.gov/16323120/

107. Rodriguez CA, Atkinson R, Bitar W, et al. Tolerance to vancomycin in pneumococci: detection with a molecular marker and assessment of clinical impact. J Infect Dis [Internet]. 2004 Oct 15 [cited 2022 Feb 25];190(8):1481–7. Available from: https://pubmed.ncbi.nlm.nih.gov/15378442/

108. Johnson CN, Wilde S, Tuomanen E, Rosch JW. Convergent impact of vaccination and antibiotic pressures on pneumococcal populations. Cell Chem Biol [Internet]. 2024 Feb 15 [cited 2024 Apr 12];31(2):195–206. Available from: https://pubmed.ncbi.nlm.nih.gov/38052216/

109. Antibioticatherapiebeleidsgroep UZ Leuven. Antibioticagids UZ Leuven [Internet]. [cited 2024 Apr 12]. Available from: https://www.antibioticagids.be/pages

110. Duke JA, Avci FY. Emerging vaccine strategies against the incessant pneumococcal disease. NPJ vaccines [Internet]. 2023 Dec 1 [cited 2024 Apr 25];8(1). Available from: https://pubmed.ncbi.nlm.nih.gov/37591986/

111. Briles DE, Paton JC, Mukerji R, Swiatlo E, Crain MJ. Pneumococcal Vaccines. Fischetti VA, Novick RP, Ferretti JJ, Portnoy DA, Braunstein M, Rood JI, editors. Microbiol Spectr [Internet]. 2019 Dec 19 [cited 2024 Apr 25];7(6). Available from: https://pubmed.ncbi.nlm.nih.gov/31858954/

112. Hoge Gezondheidsraad. Vaccinatie van kinderen en adolescenten tegen pneumokokken [Internet]. Brussel; 2023. Available from: https://www.health.belgium.be/sites/default/files/uploads/fields/fpshealth_theme_file/20230117_hgr-9746_vaccinatie_tegen_pneumokokken_vweb.pdf

113. Weinberger DM, Malley R, Lipsitch M. Serotype replacement in disease after pneumococcal vaccination. Lancet (London, England) [Internet]. 2011 [cited 2024 Apr 25];378(9807):1962–73. Available from: https://pubmed.ncbi.nlm.nih.gov/21492929/

114. Croucher NJ, Finkelstein JA, Pelton SI, et al. Population genomics of post-vaccine changes in pneumococcal epidemiology. Nat Genet [Internet]. 2013 Jun [cited 2024 Apr 18];45(6):656–63. Available from: https://pubmed.ncbi.nlm.nih.gov/23644493/

115. Sari RF, Fadilah F, Maladan Y, Sarassari R, Safari D. A narrative review of genomic characteristics, serotype, immunogenicity, and vaccine development of Streptococcus pneumoniae capsular polysaccharide. Clin Exp Vaccine Res [Internet]. 2024 [cited 2024 Jun 26];13(2):91. Available from: https://pubmed.ncbi.nlm.nih.gov/38752009/

116. Wyres KL, Lambertsen LM, Croucher NJ, et al. Pneumococcal capsular switching: a historical perspective. J Infect Dis [Internet]. 2013 Feb 1 [cited 2024 Jun 26];207(3):439–49. Available from: https://pubmed.ncbi.nlm.nih.gov/23175765/

117. Golubchik T, Brueggemann AB, Street T, et al. Pneumococcal genome sequencing tracks a vaccine escape variant formed through a multi-fragment recombination event. Nat Genet [Internet]. 2012 Mar [cited 2024 Apr 25];44(3):352–5. Available from: https://pubmed.ncbi.nlm.nih.gov/22286217/

118. Sempere J, Llamosí M, Menéndez IDR, Ruiz BL, Domenech M, González-Camacho FN. Pneumococcal Choline-Binding Proteins Involved in Virulence as Vaccine Candidates. Vaccines [Internet]. 2021 Feb 1 [cited 2024 Apr 25];9(2):1–20. Available from: https://pubmed.ncbi.nlm.nih.gov/33672701/

119. Liu X, Van Maele L, Matarazzo L, et al. A conserved antigen induces respiratory Th17-mediated

broad serotype protection against pneumococcal superinfection. Cell Host Microbe [Internet]. 2024 Mar 13 [cited 2024 Apr 25];32(3):304-314.e8. Available from: https://pubmed.ncbi.nlm.nih.gov/38417443/

120. Haas LF. Papyrus of Ebers and Smith. J Neurol Neurosurg Psychiatry [Internet]. 1999 [cited 2024 May 2];67(5):578. Available from: https://pubmed.ncbi.nlm.nih.gov/10519860/

121. Hutchings M, Truman A, Wilkinson B. Antibiotics: past, present and future. Curr Opin Microbiol [Internet]. 2019 Oct 1 [cited 2024 May 2];51:72–80. Available from: https://pubmed.ncbi.nlm.nih.gov/31733401/

122. Mohr KI. History of Antibiotics Research. Curr Top Microbiol Immunol [Internet]. 2016 Dec 1 [cited 2024 May 2];398:237–72. Available from: https://pubmed.ncbi.nlm.nih.gov/27738915/

123. Ehrlich P BA. Über das salzsaure 3,3’-Diamino-4,4’-dioxyarsenobenzol und seine nächsten Verwandten.Über das salzsaure 3,3’-Diamino-4,4’-dioxyarsenobenzol und seine nächsten Verwandten. Berichte der Dtsch Chem Gesellschaft [Internet]. 1912;(45):756–66. Available from: https://www.pei.de/DE/institut/paul-ehrlich/publikationen-von-paul-ehrlich/publikationen-von-paul-ehrlich-node.html

124. Aminov RI. A brief history of the antibiotic era: lessons learned and challenges for the future. Front Microbiol [Internet]. 2010 [cited 2024 May 2];1(DEC). Available from: https://pubmed.ncbi.nlm.nih.gov/21687759/

125. J D, D D. Origins and evolution of antibiotic resistance. Microbiol Mol Biol Rev [Internet]. 2010 [cited 2024 May 2];74(3):9–16. Available from: https://pubmed.ncbi.nlm.nih.gov/20805405/

126. Jim O. TACKLING DRUG-RESISTANT INFECTIONS GLOBALLY: FINAL REPORT AND RECOMMENDATIONS [Internet]. 2016. Available from: https://amr-review.org/sites/default/files/160525_Final paper_with cover.pdf

127. Prescott JF. The resistance tsunami, antimicrobial stewardship, and the golden age of microbiology.

Vet Microbiol [Internet]. 2014 Jul 16 [cited 2024 May 2];171(3–4):273–8. Available from: https://pubmed.ncbi.nlm.nih.gov/24646601/

128. Conly JM, Johnston BL. Where are all the new antibiotics? The new antibiotic paradox. Can J Infect Dis Med Microbiol = J Can des Mal Infect la Microbiol medicale [Internet]. 2005 [cited 2024 May 2];16(3):159–60. Available from: https://pubmed.ncbi.nlm.nih.gov/18159536/

129. Blair JMA, Webber MA, Baylay AJ, Ogbolu DO, Piddock LJV. Molecular mechanisms of antibiotic resistance. Nat Rev Microbiol [Internet]. 2015 Jan 11 [cited 2024 May 3];13(1):42–51. Available from: https://pubmed.ncbi.nlm.nih.gov/25435309/

130. Peterson E, Kaur P. Antibiotic Resistance Mechanisms in Bacteria: Relationships Between Resistance Determinants of Antibiotic Producers, Environmental Bacteria, and Clinical Pathogens. Front Microbiol [Internet]. 2018 Nov 30 [cited 2024 May 3];9(NOV). Available from: https://pubmed.ncbi.nlm.nih.gov/30555448/

131. Environment HFCS. BELMAP 2023 - One Health Report on the Antimicrobial use and Resistance in Belgium [Internet]. 2023. Available from: https://www.health.belgium.be/en/belmap-2023-report

132. Gergova R, Boyanov V, Muhtarova A, Alexandrova A. A Review of the Impact of Streptococcal Infections and Antimicrobial Resistance on Human Health. Antibiot (Basel, Switzerland) [Internet]. 2024 Apr 15 [cited 2024 May 3];13(4):360. Available from: https://pubmed.ncbi.nlm.nih.gov/38667036/

133. Li L, Ma J, Yu Z, Li M, Zhang W, Sun H. Epidemiological characteristics and antibiotic resistance mechanisms of Streptococcus pneumoniae: An updated review. Microbiol Res [Internet]. 2023 Jan 1 [cited 2024 Apr 12];266. Available from: https://pubmed.ncbi.nlm.nih.gov/36244081/

134. Cillóniz C, Garcia-Vidal C, Ceccato A, Torres A. Antimicrobial Resistance Among Streptococcus pneumoniae. Antimicrob Resist 21st Century [Internet]. 2018 [cited 2024 May 3];13. Available from: /pmc/articles/PMC7122384/

135. Balaban NQ, Helaine S, Lewis K, et al. Definitions and guidelines for research on antibiotic persistence. Nat Rev Microbiol [Internet]. 2019 Jul 1 [cited 2022 Feb 16];17(7):441–8. Available from: https://pubmed.ncbi.nlm.nih.gov/30980069/

136. Windels EM, Michiels JE, van den Bergh B, Fauvart M, Michiels J. Antibiotics: Combatting Tolerance To Stop Resistance. MBio [Internet]. 2019 Sep 1 [cited 2022 Feb 17];10(5). Available from: https://pubmed.ncbi.nlm.nih.gov/31506315/

137. Tomasz A, Albino A, Zanati E. Multiple antibiotic resistance in a bacterium with suppressed autolytic system. Nature [Internet]. 1970 [cited 2022 Feb 17];227(5254):138–40. Available from: https://pubmed.ncbi.nlm.nih.gov/4393335/

138. Novak R, Henriques B, Charpentier E, Normark S, Tuomanen E. Emergence of vancomycin tolerance in *Streptococcus pneumoniae*. Nature [Internet]. 1999 Jun 10 [cited 2022 Jul 4];399(6736):590–3. Available from: https://pubmed.ncbi.nlm.nih.gov/10376600/

139. Charpentier E, Tuomanen E. Mechanisms of antibiotic resistance and tolerance in *Streptococcus pneumoniae*. Microbes Infect [Internet]. 2000 [cited 2022 Feb 17];2(15):1855–64. Available from: https://pubmed.ncbi.nlm.nih.gov/11165930/

140. Normark BH, Normark S. Antibiotic tolerance in pneumococci. Clin Microbiol Infect [Internet]. 2002 Oct 1 [cited 2022 Feb 17];8(10):613–22. Available from: https://pubmed.ncbi.nlm.nih.gov/12390279/

141. Dörr T. Understanding tolerance to cell wall-active antibiotics. Ann N Y Acad Sci [Internet]. 2021 [cited 2024 May 3];1496(1):35–58. Available from: https://pubmed.ncbi.nlm.nih.gov/33274447/

142. Liu X, Li JW, Feng Z, Luo Y, Veening JW, Zhang JR. Transcriptional Repressor PtvR Regulates Phenotypic Tolerance to Vancomycin in *Streptococcus pneumoniae*. J Bacteriol [Internet]. 2017 Jul 1 [cited 2022 Feb 17];199(14). Available from: https://pubmed.ncbi.nlm.nih.gov/28484041/

143. Cho H, Uehara T, Bernhardt TG. Beta-lactam antibiotics induce a lethal malfunctioning of the

bacterial cell wall synthesis machinery. Cell [Internet]. 2014 Dec 4 [cited 2024 May 3];159(6):1300–11. Available from: https://pubmed.ncbi.nlm.nih.gov/25480295/

144. Hobby GL, Meyer K, Chaffee E. Observations on the Mechanism of Action of Penicillin.∗. https://doi.org/103181/00379727-50-13773 [Internet]. 1942 Jun 1 [cited 2024 May 6];50(2):281–5. Available from: https://journals.sagepub.com/doi/abs/10.3181/00379727-50-13773

145. Bigger JW. TREATMENT OF STAPHYLOCOCCAL INFECTIONS WITH PENICILLIN BY INTERMITTENT STERILISATION. Lancet [Internet]. 1944 Oct 14 [cited 2024 May 6];244(6320):497–500. Available from: https://app.dimensions.ai/details/publication/pub.1022929986

146. Moyed HS, Bertrand KP. hipA, a newly recognized gene of Escherichia coli K-12 that affects frequency of persistence after inhibition of murein synthesis. J Bacteriol [Internet]. 1983 [cited 2024 May 6];155(2):768–75. Available from: https://pubmed.ncbi.nlm.nih.gov/6348026/

147. Van den Bergh B, Fauvart M, Michiels J. Formation, physiology, ecology, evolution and clinical importance of bacterial persisters. FEMS Microbiol Rev [Internet]. 2017 May 1 [cited 2022 Feb 17];41(3):219–51. Available from: https://pubmed.ncbi.nlm.nih.gov/28333307/

148. Urbaniec J, Xu Y, Hu Y, Hingley-Wilson S, McFadden J. Phenotypic heterogeneity in persisters: a novel “hunker” theory of persistence. FEMS Microbiol Rev [Internet]. 2022 Jan 18 [cited 2022 Feb 18];46(1). Available from: https://pubmed.ncbi.nlm.nih.gov/34355746/

149. Gollan B, Grabe G, Michaux C, Helaine S. Bacterial Persisters and Infection: Past, Present, and Progressing. Annu Rev Microbiol [Internet]. 2019 Sep 8 [cited 2020 Jul 6];73(1):359–85. Available from: https://pubmed.ncbi.nlm.nih.gov/31500532/

150. Jung SH, Ryu CM, Kim JS. Bacterial persistence: Fundamentals and clinical importance. J Microbiol [Internet]. 2019 Oct 1 [cited 2022 Mar 10];57(10):829–35. Available from: https://pubmed.ncbi.nlm.nih.gov/31463787/

151. Wilmaerts D, Windels EM, Verstraeten N, Michiels J. General Mechanisms Leading to Persister

Formation and Awakening. Trends Genet. 2019 Jun 1;35(6):401–11.

152. Brauner A, Fridman O, Gefen O, Balaban NQ. Distinguishing between resistance, tolerance and persistence to antibiotic treatment. Nat Rev Microbiol [Internet]. 2016 May 1 [cited 2024 May 6];14(5):320–30. Available from: https://pubmed.ncbi.nlm.nih.gov/27080241/

153. Lewis K. Persister cells. Annu Rev Microbiol [Internet]. 2010 Oct 13 [cited 2022 Feb 17];64:357–72. Available from: https://pubmed.ncbi.nlm.nih.gov/20528688/

154. Balaban NQ, Merrin J, Chait R, Kowalik L, Leibler S. Bacterial persistence as a phenotypic switch. Science [Internet]. 2004 Sep 10 [cited 2024 May 6];305(5690):1622–5. Available from: https://pubmed.ncbi.nlm.nih.gov/15308767/

155. Li Y, Chen X, Zhang W, et al. The metabolic slowdown caused by the deletion of pspA accelerates protein aggregation during stationary phase facilitating antibiotic persistence. Antimicrob Agents Chemother [Internet]. 2024 Feb 1 [cited 2024 May 6];68(2). Available from: https://pubmed.ncbi.nlm.nih.gov/38169282/

156. Sturm A, Sun P, Avila-Pacheco J, et al. Genetic factors affecting storage and utilization of lipids during dormancy in Mycobacterium tuberculosis. MBio [Internet]. 2024 Feb 1 [cited 2024 May 6];15(2). Available from: https://pubmed.ncbi.nlm.nih.gov/38236034/

157. Kim H, Oh S, Song S. Lactobacillus Persisters Formation and Resuscitation. J Microbiol Biotechnol [Internet]. 2024 Jan 29 [cited 2024 May 6];34(4). Available from: https://pubmed.ncbi.nlm.nih.gov/38326923/

158. Orman MA, Brynildsen MP. Dormancy is not necessary or sufficient for bacterial persistence. Antimicrob Agents Chemother [Internet]. 2013 Jul [cited 2022 Jun 3];57(7):3230–9. Available from: https://pubmed.ncbi.nlm.nih.gov/23629720/

159. Hill PWS, Moldoveanu AL, Sargen M, et al. The vulnerable versatility of *Salmonella* antibiotic persisters during infection. Cell Host Microbe [Internet]. 2021 Dec 8 [cited 2022 Jul 8];29(12):1757-

1773.e10. Available from: https://pubmed.ncbi.nlm.nih.gov/34731646/

160. Stapels DAC, Hill PWS, Westermann AJ, et al. *Salmonella* persisters undermine host immune defenses during antibiotic treatment. Science [Internet]. 2018 Dec 7 [cited 2022 Jul 8];362(6419):1156–60. Available from: https://pubmed.ncbi.nlm.nih.gov/30523110/

161. Peyrusson F, Varet H, Nguyen TK, et al. Intracellular *Staphylococcus aureus* persisters upon antibiotic exposure. Nat Commun [Internet]. 2020 Dec 1 [cited 2022 Jul 8];11(1). Available from: https://pubmed.ncbi.nlm.nih.gov/32366839/

162. Goneau LW, Yeoh NS, MacDonald KW, et al. Selective target inactivation rather than global metabolic dormancy causes antibiotic tolerance in uropathogens. Antimicrob Agents Chemother [Internet]. 2014 [cited 2022 Jun 3];58(4):2089–97. Available from: https://pubmed.ncbi.nlm.nih.gov/24449771/

163. Vogwill T, Comfort AC, Furió V, MacLean RC. Persistence and resistance as complementary bacterial adaptations to antibiotics. J Evol Biol [Internet]. 2016 Jun 1 [cited 2024 Jun 26];29(6):1223–33. Available from: https://pubmed.ncbi.nlm.nih.gov/26999656/

164. Wuyts J, Van Dijck P, Holtappels M. Fungal persister cells: The basis for recalcitrant infections? PLoS Pathog [Internet]. 2018 Oct 1 [cited 2022 Jul 8];14(10). Available from: https://pubmed.ncbi.nlm.nih.gov/30335865/

165. Codd A, Teuscher F, Kyle DE, Cheng Q, Gatton ML. Artemisinin-induced parasite dormancy: a plausible mechanism for treatment failure. Malar J [Internet]. 2011 [cited 2024 May 7];10. Available from: https://pubmed.ncbi.nlm.nih.gov/21385409/

166. Ramirez M, Rajaram S, Steininger RJ, et al. Diverse drug-resistance mechanisms can emerge from drug-tolerant cancer persister cells. Nat Commun [Internet]. 2016 Feb 19 [cited 2022 Feb 17];7. Available from: https://pubmed.ncbi.nlm.nih.gov/26891683/

167. Shen S, Vagner S, Robert C. Persistent Cancer Cells: The Deadly Survivors. Cell [Internet]. 2020 Nov

12 [cited 2024 May 7];183(4):860–74. Available from: https://pubmed.ncbi.nlm.nih.gov/33186528/

168. Pearl Mizrahi S, Gefen O, Simon I, Balaban NQ. Persistence to anti-cancer treatments in the stationary to proliferating transition. Cell Cycle [Internet]. 2016 Dec 16 [cited 2024 May 7];15(24):3442–53. Available from: https://pubmed.ncbi.nlm.nih.gov/27801609/

169. Haven B, Heilig E, Donham C, et al. Registered report: A chromatin-mediated reversible drug-tolerant state in cancer cell subpopulations. Elife [Internet]. 2016 Feb 23 [cited 2024 May 7];5(FEBRUARY2016). Available from: https://pubmed.ncbi.nlm.nih.gov/26905833/

170. Oliver L, Lalier L, Salaud C, Heymann D, Cartron PF, Vallette FM. Drug resistance in glioblastoma: are persisters the key to therapy? Cancer drug Resist (Alhambra, Calif) [Internet]. 2020 [cited 2022 Jul 8];3(3):287–301. Available from: https://pubmed.ncbi.nlm.nih.gov/35582442/

171. Sebastian J, Swaminath S, Nair RR, Jakkala K, Pradhan A, Ajitkumar P. *De Novo* Emergence of Genetically Resistant Mutants of *Mycobacterium tuberculosis* from the Persistence Phase Cells Formed against Antituberculosis Drugs *In Vitro*. Antimicrob Agents Chemother [Internet]. 2017 Feb 1 [cited 2022 Apr 7];61(2). Available from: https://pubmed.ncbi.nlm.nih.gov/27895008/

172. Levin-Reisman I, Ronin I, Gefen O, Braniss I, Shoresh N, Balaban NQ. Antibiotic tolerance facilitates the evolution of resistance. Science [Internet]. 2017 Feb 24 [cited 2022 Mar 9];355(6327):826–30. Available from: https://pubmed.ncbi.nlm.nih.gov/28183996/

173. Windels EM, Michiels JE, Fauvart M, Wenseleers T, Bram Van Den Bergh, Michiels J. Bacterial persistence promotes the evolution of antibiotic resistance by increasing survival and mutation rates. ISME J [Internet]. 2019 [cited 2020 Jul 6];13:1239–51. Available from: https://doi.org/10.1038/s41396-019-0344-9

174. Santi I, Manfredi P, Maffei E, Egli A, Jenal U. Evolution of Antibiotic Tolerance Shapes Resistance Development in Chronic *Pseudomonas aeruginosa* Infections. MBio [Internet]. 2021 [cited 2022 Jun

29];12(1):1–17. Available from: https://pubmed.ncbi.nlm.nih.gov/33563834/

175. Bakkeren E, Huisman JS, Fattinger SA, et al. *Salmonella* persisters promote the spread of antibiotic resistance plasmids in the gut. Nature [Internet]. 2019 Sep 12 [cited 2022 Jun 29];573(7773):276–80. Available from: https://pubmed.ncbi.nlm.nih.gov/31485077/

176. Levin-Reisman I, Brauner A, Ronin I, Balaban NQ. Epistasis between antibiotic tolerance, persistence, and resistance mutations. Proc Natl Acad Sci U S A [Internet]. 2019 Jul 7 [cited 2022 Jul 1];116(29):14734–9. Available from: /pmc/articles/PMC6642377/

177. Huo W, Busch LM, Hernandez-Bird J, et al. Immunosuppression broadens evolutionary pathways to drug resistance and treatment failure during *Acinetobacter baumannii* pneumonia in mice. Nat Microbiol [Internet]. 2022 Jun [cited 2022 Jul 8];7(6):796–809. Available from: https://pubmed.ncbi.nlm.nih.gov/35618774/

178. Swaminath S, Paul A, Pradhan A, Sebastian J, Nair RR, Ajitkumar P. Mycobacterium smegmatis moxifloxacin persister cells produce high levels of hydroxyl radical, generating genetic resisters selectable not only with moxifloxacin, but also with ethambutol and isoniazid. Microbiology [Internet]. 2020 [cited 2024 May 7];166(2):180–98. Available from: https://pubmed.ncbi.nlm.nih.gov/31746727/

179. Balaban NQ. Persistence: mechanisms for triggering and enhancing phenotypic variability. Curr Opin Genet Dev [Internet]. 2011 Dec [cited 2024 May 7];21(6):768–75. Available from: https://pubmed.ncbi.nlm.nih.gov/22051606/

180. Mulcahy LR, Burns JL, Lory S, Lewis K. Emergence of *Pseudomonas aeruginosa* strains producing high levels of persister cells in patients with cystic fibrosis. J Bacteriol [Internet]. 2010 Dec [cited 2022 Feb 24];192(23):6191–9. Available from: https://pubmed.ncbi.nlm.nih.gov/20935098/

181. Bartell JA, Cameron DR, Mojsoska B, et al. Bacterial persisters in long-term infection: Emergence and fitness in a complex host environment. PLoS Pathog [Internet]. 2020 Dec 14 [cited 2022 Jun

29];16(12). Available from: https://pubmed.ncbi.nlm.nih.gov/33315938/

182. LaFleur MD, Qi Q, Lewis K. Patients with long-term oral carriage harbor high-persister mutants of Candida albicans. Antimicrob Agents Chemother [Internet]. 2010 Jan [cited 2024 Apr 30];54(1):39–44. Available from: https://pubmed.ncbi.nlm.nih.gov/19841146/

183. Schumacher MA, Balani P, Min J, et al. HipBA-promoter structures reveal the basis of heritable multidrug tolerance. Nature [Internet]. 2015 Aug 6 [cited 2022 Mar 9];524(7563):59–66. Available from: https://pubmed.ncbi.nlm.nih.gov/26222023/

184. Torrey HL, Keren I, Via LE, Lee JS, Lewis K. High Persister Mutants in Mycobacterium tuberculosis. PLoS One [Internet]. 2016 May 1 [cited 2024 May 7];11(5). Available from: https://pubmed.ncbi.nlm.nih.gov/27176494/

185. Fauvart M, de Groote VN, Michiels J. Role of persister cells in chronic infections: clinical relevance and perspectives on anti-persister therapies. J Med Microbiol [Internet]. 2011 Jun [cited 2022 Feb 18];60(Pt 6):699–709. Available from: https://pubmed.ncbi.nlm.nih.gov/21459912/

186. Geerts N, De Vooght L, Passaris I, Delputte P, Van den Bergh B, Cos P. Antibiotic Tolerance Indicative of Persistence Is Pervasive among Clinical Streptococcus pneumoniae Isolates and Shows Strong Condition Dependence. Microbiol Spectr [Internet]. 2022 Dec 21 [cited 2023 Nov 14];10(6). Available from: https://pubmed.ncbi.nlm.nih.gov/36374111/

187. Hernandez-Morfa M, Reinoso-Vizcaíno NM, Olivero NB, et al. Host Cell Oxidative Stress Promotes Intracellular Fluoroquinolone Persisters of Streptococcus pneumoniae. Microbiol Spectr [Internet]. 2022 Dec 21 [cited 2024 May 7];10(6). Available from: https://pubmed.ncbi.nlm.nih.gov/36445159/

188. Verhagen LM, de Groot R. Recurrent, protracted and persistent lower respiratory tract infection: A neglected clinical entity. J Infect [Internet]. 2015 Jun 1 [cited 2020 Jul 23];71(S1):S106–11. Available from: https://pubmed.ncbi.nlm.nih.gov/25917807/

189. Hare KM, Leach AJ, Smith-Vaughan HC, Chang AB, Grimwood K. *Streptococcus pneumoniae* and chronic endobronchial infections in childhood. Pediatr Pulmonol [Internet]. 2017 Dec 1 [cited 2022 Feb 17];52(12):1532–45. Available from: https://pubmed.ncbi.nlm.nih.gov/28922566/

190. Inchingolo R, Pierandrei C, Montemurro G, Smargiassi A, Lohmeyer FM, Rizzi A. Antimicrobial Resistance in Common Respiratory Pathogens of Chronic Bronchiectasis Patients: A Literature Review. Antibiot (Basel, Switzerland) [Internet]. 2021 [cited 2022 Jul 8];10(3). Available from: https://pubmed.ncbi.nlm.nih.gov/33804631/

191. Welp AL, Bomberger JM. Bacterial Community Interactions During Chronic Respiratory Disease. Front Cell Infect Microbiol [Internet]. 2020 May 14 [cited 2024 Apr 11];10. Available from: https://pubmed.ncbi.nlm.nih.gov/32477966/

192. Hoa M, Tomovic S, Nistico L, et al. Identification of adenoid biofilms with middle ear pathogens in otitis-prone children utilizing SEM and FISH. Int J Pediatr Otorhinolaryngol [Internet]. 2009 Sep [cited 2022 Apr 22];73(9):1242–8. Available from: https://pubmed.ncbi.nlm.nih.gov/19525016/

193. Hall-Stoodley L, Hu FZ, Gieseke A, et al. Direct detection of bacterial biofilms on the middle-ear mucosa of children with chronic otitis media. JAMA [Internet]. 2006 Jul 12 [cited 2022 Feb 18];296(2):202–11. Available from: https://pubmed.ncbi.nlm.nih.gov/16835426/

194. Nistico L, Kreft R, Gieseke A, et al. Adenoid reservoir for pathogenic biofilm bacteria. J Clin Microbiol [Internet]. 2011 Apr [cited 2022 Apr 22];49(4):1411–20. Available from: https://pubmed.ncbi.nlm.nih.gov/21307211/

195. Restrepo A V., Salazar BE, Agudelo M, Rodriguez CA, Zuluaga AF, Vesga O. Optimization of culture conditions to obtain maximal growth of penicillin-resistant *Streptococcus pneumoniae*. BMC Microbiol [Internet]. 2005 Jun 2 [cited 2022 Jul 4];5. Available from: https://pubmed.ncbi.nlm.nih.gov/15932633/

196. Regev-Yochay G, Trzciński K, Thompson CM, Malley R, Lipsitch M. Interference between

Streptococcus pneumoniae and *Staphylococcus aureus*: *In vitro* hydrogen peroxide-mediated killing by *Streptococcus pneumoniae*. J Bacteriol [Internet]. 2006 Jul [cited 2020 Jul 13];188(13):4996–5001. Available from: https://pubmed.ncbi.nlm.nih.gov/16788209/

197. Herta T, Bhattacharyya A, Bollensdorf C, et al. DNA-release by *Streptococcus pneumoniae* autolysin LytA induced Krueppel-like factor 4 expression in macrophages. Sci Rep [Internet]. 2018 Dec 1 [cited 2022 Jun 7];8(1). Available from: https://pubmed.ncbi.nlm.nih.gov/29636524/

198. Cools F, Torfs E, Vanhoutte B, et al. Streptococcus pneumoniae galU gene mutation has a direct effect on biofilm growth, adherence and phagocytosis *in vitro* and pathogenicity *in vivo*. Pathog Dis [Internet]. 2018 Oct 1 [cited 2022 Jan 5];76(7). Available from: https://pubmed.ncbi.nlm.nih.gov/30215741/

199. Luidalepp H, Jõers A, Kaldalu N, Tenson T. Age of inoculum strongly influences persister frequency and can mask effects of mutations implicated in altered persistence. J Bacteriol [Internet]. 2011 Jul [cited 2022 Mar 10];193(14):3598–605. Available from: https://pubmed.ncbi.nlm.nih.gov/21602347/

200. Keren I, Kaldalu N, Spoering A, Wang Y, Lewis K. Persister cells and tolerance to antimicrobials. FEMS Microbiol Lett [Internet]. 2004 Jan 15 [cited 2022 Mar 10];230(1):13–8. Available from: https://pubmed.ncbi.nlm.nih.gov/14734160/

201. Grant SS, Hung DT. Persistent bacterial infections, antibiotic tolerance, and the oxidative stress response. Virulence [Internet]. 2013 [cited 2022 Apr 11];4(4):273–83. Available from: https://pubmed.ncbi.nlm.nih.gov/23563389/

202. Howard L V., Gooder H. Specificity of the autolysin of Streptococcus (Diplococcus) pneumoniae. J Bacteriol [Internet]. 1974 [cited 2024 Apr 8];117(2):796–804. Available from: https://pubmed.ncbi.nlm.nih.gov/4149515/

203. Sigma-Aldrich. T1438 Todd Hewitt Broth [Internet]. [cited 2024 May 31]. Available from:

https://www.sigmaaldrich.com/deepweb/assets/sigmaaldrich/product/documents/119/730/T1438dat.pdf

204. Sigma-Aldrich. Mueller Hinton Broth 2, Cation-Adjusted (M-H 2 Broth; Mueller Hinton II Broth). 2018.

205. Neogen. Technical Specification Sheet Brain Heart Infusion Broth (NCM0016).

206. Orrico F, Möller MN, Cassina A, Denicola A, Thomson L. Kinetic and stoichiometric constraints determine the pathway of H2O2 consumption by red blood cells. Free Radic Biol Med [Internet]. 2018 Jun 1 [cited 2024 Jun 17];121:231–9. Available from: https://pubmed.ncbi.nlm.nih.gov/29753074/

207. Möller MN, Orrico F, Villar SF, et al. Oxidants and Antioxidants in the Redox Biochemistry of Human Red Blood Cells. ACS omega [Internet]. 2022 [cited 2024 Jun 17];8(1). Available from: https://pubmed.ncbi.nlm.nih.gov/36643550/

208. Gaetani G, Galiano S, Canepa L, Ferraris A, Kirkman H. Catalase and Glutathione Peroxidase Are Equally Active in Detoxification of Hydrogen Peroxide in Human Erythrocytes. Blood. 1989 Jan 1;73(1):334–9.

209. Jung SH, Ryu CM, Kim JS. Bacterial persistence: Fundamentals and clinical importance. J Microbiol [Internet]. 2019 Oct 1 [cited 2022 Apr 11];57(10):829–35. Available from: https://pubmed.ncbi.nlm.nih.gov/31463787/

210. Cools F, Torfs E, Vanhoutte B, et al. *Streptococcus pneumoniae galU* gene mutation has a direct effect on biofilm growth, adherence and phagocytosis *in vitro* and pathogenicity *in vivo*. Pathog Dis [Internet]. 2018 [cited 2019 Nov 1];76(7). Available from: http://www.ncbi.nlm.nih.gov/pubmed/30215741

211. Barrett TC, Mok WWK, Murawski AM, Brynildsen MP. Enhanced antibiotic resistance development from fluoroquinolone persisters after a single exposure to antibiotic. Nat Commun [Internet]. 2019

Dec 1 [cited 2022 Jun 3];10(1). Available from: https://pubmed.ncbi.nlm.nih.gov/30862812/

212. Nierman WC, Yu Y, Losada L. The In vitro Antibiotic Tolerant Persister Population in *Burkholderia pseudomallei* is Altered by Environmental Factors. Front Microbiol [Internet]. 2015 [cited 2022 Sep 14];6(DEC). Available from: https://pubmed.ncbi.nlm.nih.gov/26696964/

213. Willenborg J, Willms D, Bertram R, Goethe R, Valentin-Weigand P. Characterization of multi-drug tolerant persister cells in *Streptococcus suis*. BMC Microbiol. 2014 May;14:120.

214. Kawano A, Yamasaki R, Sakakura T, et al. Reactive Oxygen Species Penetrate Persister Cell Membranes of *Escherichia coli* for Effective Cell Killing. Front Cell Infect Microbiol [Internet]. 2020 Sep 18 [cited 2022 Jun 29];10. Available from: https://pubmed.ncbi.nlm.nih.gov/33042869/

215. Verstraete L, Aizawa J, Govaerts M, et al. In Vitro Persistence Level Reflects In Vivo Antibiotic Survival of Natural Pseudomonas aeruginosa Isolates in a Murine Lung Infection Model. Microbiol Spectr [Internet]. 2023 Jun 15 [cited 2024 Apr 8];11(3). Available from: https://pubmed.ncbi.nlm.nih.gov/37140371/

216. Ronneau S, Michaux C, Giorgio RT, Helaine S. Intoxication of antibiotic persisters by host RNS inactivates their efflux machinery during infection. PLoS Pathog [Internet]. 2024 Feb 1 [cited 2024 Apr 8];20(2). Available from: https://pubmed.ncbi.nlm.nih.gov/38421944/

217. Maffei E, Fino C, Harms A. Antibiotic Tolerance and Persistence Studied Throughout Bacterial Growth Phases. Methods Mol Biol [Internet]. 2021 [cited 2024 Apr 8];2357:23–40. Available from: https://pubmed.ncbi.nlm.nih.gov/34590249/

218. Ma D, Mandell JB, Donegan NP, et al. The Toxin-Antitoxin MazEF Drives *Staphylococcus aureus* Biofilm Formation, Antibiotic Tolerance, and Chronic Infection. MBio [Internet]. 2019 Nov 1 [cited 2022 Jul 4];10(6). Available from: https://pubmed.ncbi.nlm.nih.gov/31772059/

219. Savijoki K, Miettinen I, Nyman TA, et al. Growth Mode and Physiological State of Cells Prior to Biofilm Formation Affect Immune Evasion and Persistence of *Staphylococcus aureus*.

Microorganisms [Internet]. 2020 Jan 1 [cited 2022 Jul 15];8(1). Available from: https://pubmed.ncbi.nlm.nih.gov/31940921/

220. Suppiger S, Astasov-Frauenhoffer M, Schweizer I, Waltimo T, Kulik EM. Tolerance and Persister Formation in Oral Streptococci. Antibiot (Basel, Switzerland) [Internet]. 2020 Apr 1 [cited 2022 Jul 4];9(4). Available from: https://pubmed.ncbi.nlm.nih.gov/32276310/

221. Leung V, Lévesque CM. A stress-inducible quorum-sensing peptide mediates the formation of persister cells with noninherited multidrug tolerance. J Bacteriol [Internet]. 2012 May [cited 2022 Apr 8];194(9):2265–74. Available from: https://pubmed.ncbi.nlm.nih.gov/22366415/

222. Jiang YL, Qiu W, Zhou XD, et al. Quaternary ammonium-induced multidrug tolerant *Streptococcus mutans* persisters elevate cariogenic virulence *in vitro*. Int J Oral Sci. 2017 Dec;9(12):e7–e7.

223. Lu J, Cheng L, Huang Y, et al. Resumptive Streptococcus mutans Persisters Induced From Dimethylaminododecyl Methacrylate Elevated the Cariogenic Virulence by Up-Regulating the Quorum-Sensing and VicRK Pathway Genes. Front Microbiol [Internet]. 2020 Jan 21 [cited 2024 Apr 8];10. Available from: https://pubmed.ncbi.nlm.nih.gov/32038546/

224. Wang S, Zhou C, Ren B, et al. Formation of persisters in *Streptococcus mutans* biofilms induced by antibacterial dental monomer. J Mater Sci Mater Med. 2017 Nov;28(11):178.

225. Dufour D, Mankovskaia A, Chan Y, Motavaze K, Gong SG, Lévesque CM. A tripartite toxin-antitoxin module induced by quorum sensing is associated with the persistence phenotype in *Streptococcus mutans*. Mol Oral Microbiol. 2018 Dec;33(6):420–9.

226. Dufour D, Li H, Gong SG, Lévesque CM. Transcriptome Analysis of Streptococcus mutans Quorum Sensing-Mediated Persisters Reveals an Enrichment in Genes Related to Stress Defense Mechanisms. Genes (Basel) [Internet]. 2023 Oct 1 [cited 2024 Apr 8];14(10). Available from: https://pubmed.ncbi.nlm.nih.gov/37895236/

227. Soriano F, Greenwood D. Action and interaction of penicillin and gentamicin on enterococci. J Clin

Pathol. 1979 Nov;32(11):1174–9.

228. Wood DN, Chaussee MA, Chaussee MS, Buttaro BA. Persistence of *Streptococcus pyogenes* in stationary-phase cultures. J Bacteriol. 2005 May;187(10):3319–28.

229. Martini CL, Coronado AZ, Melo MCN, et al. Cellular Growth Arrest and Efflux Pumps Are Associated With Antibiotic Persisters in *Streptococcus pyogenes* Induced in Biofilm-Like Environments. Front Microbiol [Internet]. 2021 Sep 21 [cited 2022 Apr 12];12. Available from: https://pubmed.ncbi.nlm.nih.gov/34621249/

230. Thornton RB, Rigby PJ, Wiertsema SP, et al. Multi-species bacterial biofilm and intracellular infection in otitis media. BMC Pediatr [Internet]. 2011 Oct 24 [cited 2022 Feb 18];11. Available from: https://pubmed.ncbi.nlm.nih.gov/22018357/

231. Verstraete L, Van den Bergh B, Verstraeten N, Michiels J. Ecology and evolution of antibiotic persistence. Trends Microbiol [Internet]. 2022 May 1 [cited 2022 Jun 29];30(5):466–79. Available from: https://pubmed.ncbi.nlm.nih.gov/34753652/

232. Lewis K. Multidrug tolerance of biofilms and persister cells. Curr Top Microbiol Immunol [Internet]. 2008 [cited 2022 Feb 25];322:107–31. Available from: https://pubmed.ncbi.nlm.nih.gov/18453274/

233. Pham TDM, Ziora ZM, Blaskovich MAT. Quinolone antibiotics. Medchemcomm [Internet]. 2019 [cited 2022 Feb 25];10(10):1719–39. Available from: https://pubmed.ncbi.nlm.nih.gov/31803393/

234. Mok WWK, Brynildsen MP. Timing of DNA damage responses impacts persistence to fluoroquinolones. Proc Natl Acad Sci U S A [Internet]. 2018 Jul 3 [cited 2022 Jul 8];115(27):E6301–9. Available from: https://pubmed.ncbi.nlm.nih.gov/29915065/

235. Bakkeren E, Diard M, Hardt WD. Evolutionary causes and consequences of bacterial antibiotic persistence. Nat Rev Microbiol [Internet]. 2020 Sep 1 [cited 2024 May 14];18(9):479–90. Available from: https://pubmed.ncbi.nlm.nih.gov/32461608/

236. Windels EM, Cool L, Persy E, et al. Antibiotic dose and nutrient availability differentially drive the evolution of antibiotic resistance and persistence. ISME J [Internet]. 2024 May 1 [cited 2024 May 14]; Available from: https://pubmed.ncbi.nlm.nih.gov/38691440/

237. Ghoshal M, Bechtel TD, Gibbons JG, McLandsborough L. Transcriptomic analysis using RNA sequencing and phenotypic analysis of Salmonella enterica after acid exposure for different time durations using adaptive laboratory evolution. Front Microbiol [Internet]. 2024 [cited 2024 May 15];15. Available from: https://pubmed.ncbi.nlm.nih.gov/38476938/

238. Maeda T, Furusawa C. Laboratory Evolution of Antimicrobial Resistance in Bacteria to Develop Rational Treatment Strategies. Antibiot (Basel, Switzerland) [Internet]. 2024 Jan 1 [cited 2024 Apr 18];13(1). Available from: https://pubmed.ncbi.nlm.nih.gov/38247653/

239. Michiels JE, Van Den Bergh B, Verstraeten N, Fauvart M, Michiels J. *In Vitro* Emergence of High Persistence upon Periodic Aminoglycoside Challenge in the ESKAPE Pathogens. Antimicrob Agents Chemother [Internet]. 2016 Aug 1 [cited 2022 Jul 4];60(8):4630–7. Available from: https://pubmed.ncbi.nlm.nih.gov/27185802/

240. Mechler L, Herbig A, Paprotka K, Fraunholz M, Nieselt K, Bertram R. A novel point mutation promotes growth phase-dependent daptomycin tolerance in Staphylococcus aureus. Antimicrob Agents Chemother [Internet]. 2015 Sep 1 [cited 2024 May 15];59(9):5366–76. Available from: https://pubmed.ncbi.nlm.nih.gov/26100694/

241. Van Den Bergh B, Michiels JE, Wenseleers T, et al. Frequency of antibiotic application drives rapid evolutionary adaptation of *Escherichia coli* persistence. Nat Microbiol [Internet]. 2016 Mar 7 [cited 2022 Feb 17];1(5). Available from: https://pubmed.ncbi.nlm.nih.gov/27572640/

242. Brauner A, Balaban NQ. Quantitative biology of survival under antibiotic treatments. Curr Opin Microbiol [Internet]. 2021 Dec 1 [cited 2024 May 14];64:139–45. Available from: https://pubmed.ncbi.nlm.nih.gov/34715469/

243. Van Den Bergh B, Michiels JE, Wenseleers T, et al. Frequency of antibiotic application drives rapid evolutionary adaptation of Escherichia coli persistence. Nat Microbiol [Internet]. 2016 Mar 7 [cited 2020 Aug 14];1(5):1–7. Available from: https://www.nature.com/articles/nmicrobiol201620

244. Sulaiman JE, Lam H. Proteomic Investigation of Tolerant Escherichia coli Populations from Cyclic Antibiotic Treatment. J Proteome Res [Internet]. 2020 Feb 7 [cited 2024 Apr 9];19(2):900–13. Available from: https://pubmed.ncbi.nlm.nih.gov/31920087/

245. Wiegand I, Hilpert K, Hancock REW. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nat Protoc [Internet]. 2008 Feb [cited 2024 Aug 27];3(2):163–75. Available from: https://pubmed.ncbi.nlm.nih.gov/18274517/

246. Groman RP. Aminoglycosides. Small Anim Crit Care Med. 2009 Jan 1;830–4.

247. Sulaiman JE, Wu L, Lam H. Mutation in the Two-Component System Regulator YycH Leads to Daptomycin Tolerance in Methicillin-Resistant Staphylococcus aureus upon Evolution with a Population Bottleneck. Microbiol Spectr [Internet]. 2022 Aug 31 [cited 2024 Apr 18];10(4). Available from: https://pubmed.ncbi.nlm.nih.gov/35913149/

248. Fridman O, Goldberg A, Ronin I, Shoresh N, Balaban NQ. Optimization of lag time underlies antibiotic tolerance in evolved bacterial populations. Nature [Internet]. 2014 Sep 18 [cited 2024 Apr 17];513(7518):418–21. Available from: https://pubmed.ncbi.nlm.nih.gov/25043002/

249. Fernander MC, Parsons PK, Khaled B, Bradley A, Graves JL, Thomas MD. Adaptation to simulated microgravity in Streptococcus mutans. NPJ microgravity [Internet]. 2022 Dec 1 [cited 2024 Aug 28];8(1). Available from: https://pubmed.ncbi.nlm.nih.gov/35654802/

250. Liu J, Gefen O, Ronin I, Bar-Meir M, Balaban NQ. Effect of tolerance on the evolution of antibiotic resistance under drug combinations. Science [Internet]. 2020 Jan 10 [cited 2024 Apr 17];367(6474):200–4. Available from: https://pubmed.ncbi.nlm.nih.gov/31919223/

251. Parsons JB, Sidders AE, Velez AZ, et al. In-patient evolution of a high-persister Escherichia coli strain

with reduced in vivo antibiotic susceptibility. Proc Natl Acad Sci U S A [Internet]. 2024 [cited 2024 May 15];121(3). Available from: https://pubmed.ncbi.nlm.nih.gov/38190524/

252. Feng J, Lupien A, Gingras H, et al. Genome sequencing of linezolid-resistant Streptococcus pneumoniae mutants reveals novel mechanisms of resistance. Genome Res [Internet]. 2009 Jul [cited 2024 Apr 17];19(7):1214–23. Available from: https://pubmed.ncbi.nlm.nih.gov/19351617/

253. Dao TH, Echlin H, McKnight A, et al. Streptococcus pneumoniae favors tolerance via metabolic adaptation over resistance to circumvent fluoroquinolones. MBio [Internet]. 2024 Feb 1 [cited 2024 Apr 17];15(2). Available from: https://pubmed.ncbi.nlm.nih.gov/38193698/

254. Carsenti-Dellamonica H, Galimand M, Vandenbos F, et al. In vitro selection of mutants of Streptococcus pneumoniae resistant to macrolides and linezolid: relationship with susceptibility to penicillin G or macrolides. J Antimicrob Chemother [Internet]. 2005 Oct [cited 2024 Apr 17];56(4):633–42. Available from: https://pubmed.ncbi.nlm.nih.gov/16135525/

255. Liakopoulos A, Aulin LBS, Buffoni M, Fragkiskou E, Coen van Hasselt JG, Rozen DE. Allele-specific collateral and fitness effects determine the dynamics of fluoroquinolone resistance evolution. Proc Natl Acad Sci U S A [Internet]. 2022 May 3 [cited 2024 Apr 17];119(18). Available from: https://pubmed.ncbi.nlm.nih.gov/35476512/

256. Jiang W, Lin T, Pan J, et al. Spontaneous mutations and mutational responses to penicillin treatment in the bacterial pathogen Streptococcus pneumoniae D39. Mar Life Sci Technol 2024 [Internet]. 2024 Apr 16 [cited 2024 Apr 18];1–14. Available from: https://link.springer.com/article/10.1007/s42995-024-00220-6

257. Liu J, Yang L, Kjellerup BV, Xu Z. Viable but nonculturable (VBNC) state, an underestimated and controversial microbial survival strategy. Trends Microbiol [Internet]. 2023 Oct 1 [cited 2024 Aug 28];31(10):1013–23. Available from: https://pubmed.ncbi.nlm.nih.gov/37225640/

258. Oliver JD. Recent findings on the viable but nonculturable state in pathogenic bacteria. FEMS

Microbiol Rev [Internet]. 2010 [cited 2024 Aug 28];34(4):415–25. Available from: https://pubmed.ncbi.nlm.nih.gov/20059548/

259. Beheshti M, Jabalameli F, Feizabadi MM, Hahsemi FB, Beigverdi R, Emaneini M. Molecular characterization, antibiotic resistance pattern and capsular types of invasive Streptococcus pneumoniae isolated from clinical samples in Tehran, Iran. BMC Microbiol [Internet]. 2020 Jun 16 [cited 2024 May 16];20(1). Available from: https://pubmed.ncbi.nlm.nih.gov/32546124/

260. Manyahi J, Moyo SJ, Langeland N, Blomberg B. Genetic determinants of macrolide and tetracycline resistance in penicillin non-susceptible Streptococcus pneumoniae isolates from people living with HIV in Dar es Salaam, Tanzania. Ann Clin Microbiol Antimicrob [Internet]. 2023 Dec 1 [cited 2024 May 16];22(1). Available from: https://pubmed.ncbi.nlm.nih.gov/36803640/

261. Schmitz FJ, Perdikouli M, Beeck A, Verhoef J, Fluit AC. Molecular surveillance of macrolide, tetracycline and quinolone resistance mechanisms in 1191 clinical European Streptococcus pneumoniae isolates. Int J Antimicrob Agents [Internet]. 2001 [cited 2024 May 16];18(5):433–6. Available from: https://pubmed.ncbi.nlm.nih.gov/11711257/

262. Broskey J, Coleman K, Gwynn MN, et al. Efflux and target mutations as quinolone resistance mechanisms in clinical isolates of Streptococcus pneumoniae. J Antimicrob Chemother [Internet]. 2000 [cited 2024 May 16];45 Suppl 1(4 SUPPL. S1):95–9. Available from: https://pubmed.ncbi.nlm.nih.gov/10824039/

263. Peela MSR, Sistla S, Tamilarasu K, Krishnamurthy S, Adhisivam B. Antimicrobial resistance in clinical isolates of streptococcus pneumoniae: Mechanisms and association with serotype patterns. J Clin Diagnostic Res. 2018 Nov 1;12(11).

264. Ktari S, Ben Ayed N, Ben Rbeh I, et al. Antibiotic resistance pattern, capsular types, and molecular characterization of invasive isolates of Streptococcus pneumoniae in the south of Tunisia from 2012 to 2018. BMC Microbiol [Internet]. 2023 Dec 1 [cited 2024 May 16];23(1). Available from:

https://pubmed.ncbi.nlm.nih.gov/36739390/

265. Hofsteenge N, Van Nimwegen E, Silander OK. Quantitative analysis of persister fractions suggests different mechanisms of formation among environmental isolates of *E. coli*. BMC Microbiol [Internet]. 2013 [cited 2022 Jul 6];13(1). Available from: https://pubmed.ncbi.nlm.nih.gov/23379956/

266. Stewart B, Rozen DE. Genetic variation for antibiotic persistence in *Escherichia coli*. Evolution [Internet]. 2012 Mar [cited 2022 Jul 6];66(3):933–9. Available from: https://pubmed.ncbi.nlm.nih.gov/22380453/

267. Barth VC, Rodrigues BA, Bonatto GD, et al. Heterogeneous persister cells formation in *Acinetobacter baumannii*. PLoS One [Internet]. 2013 Dec 31 [cited 2022 Jul 6];8(12). Available from: https://pubmed.ncbi.nlm.nih.gov/24391945/

268. Stringer C, Wang T, Michaelos M, Pachitariu M. Cellpose: a generalist algorithm for cellular segmentation. Nat Methods [Internet]. 2021 Jan 1 [cited 2024 Jun 24];18(1):100–6. Available from: https://pubmed.ncbi.nlm.nih.gov/33318659/

269. Wouters I, Desmet S, Heirstraeten L Van, et al. How nasopharyngeal pneumococcal carriage evolved during and after a PCV13-to-PCV10 vaccination programme switch in Belgium, 2016 to 2018. Euro Surveill [Internet]. 2020 Feb 6 [cited 2024 May 17];25(5). Available from: https://pubmed.ncbi.nlm.nih.gov/32046817/

270. Correia FF, D'Onofrio A, Rejtar T, et al. Kinase activity of overexpressed HipA is required for growth arrest and multidrug tolerance in Escherichia coli. J Bacteriol [Internet]. 2006 Dec [cited 2024 May 6];188(24):8360–7. Available from: https://pubmed.ncbi.nlm.nih.gov/17041039/

271. Sempere J, González-Camacho F, Domenech M, et al. A national longitudinal study evaluating the activity of cefditoren and other antibiotics against non-susceptible Streptococcus pneumoniae strains during the period 2004-20 in Spain. J Antimicrob Chemother [Internet]. 2022 Apr 1 [cited

2024 May 24];77(4):1045–51. Available from: https://pubmed.ncbi.nlm.nih.gov/35045160/

272. Zintgraff J, Gagetti P, Napoli D, et al. Invasive Streptococcus pneumoniae isolates from pediatric population in Argentina for the period 2006-2019. Temporal progression of serotypes distribution and antibiotic resistance. Vaccine [Internet]. 2022 Jan 24 [cited 2024 May 24];40(3):459–70. Available from: https://pubmed.ncbi.nlm.nih.gov/34920903/

273. Zhou M, Wang Z, Zhang L, et al. Serotype Distribution, Antimicrobial Susceptibility, Multilocus Sequencing Type and Virulence of Invasive Streptococcus pneumoniae in China: A Six-Year Multicenter Study. Front Microbiol [Internet]. 2022 Jan 13 [cited 2024 May 24];12. Available from: https://pubmed.ncbi.nlm.nih.gov/35095809/

274. von Specht M, García Gabarrot G, Mollerach M, et al. Resistance to β-lactams in Streptococcus pneumoniae. Rev Argent Microbiol. 2021 Jul 1;53(3):266–71.

275. Manandhar S, Singh A, Varma A, Pandey S, Shrivastava N. High level of persister frequency in clinical staphylococcal isolates. BMC Microbiol [Internet]. 2022 Dec 1 [cited 2024 May 23];22(1). Available from: https://pubmed.ncbi.nlm.nih.gov/35448965/

276. Kaldalu N, Hauryliuk V, Tenson T. Persisters-as elusive as ever. Appl Microbiol Biotechnol [Internet]. 2016 Aug 1 [cited 2022 Jun 3];100(15):6545–53. Available from: https://pubmed.ncbi.nlm.nih.gov/27262568/

277. Conlon BP, Rowe SE, Gandt AB, et al. Persister formation in *Staphylococcus aureus* is associated with ATP depletion. Nat Microbiol [Internet]. 2016 Apr 18 [cited 2022 Jul 8];1(5). Available from: https://pubmed.ncbi.nlm.nih.gov/27398229/

278. Shan Y, Gandt AB, Rowe SE, Deisinger JP, Conlon BP, Lewis K. ATP-Dependent Persister Formation in *Escherichia coli*. MBio [Internet]. 2017 Jan 1 [cited 2022 Jul 8];8(1). Available from: https://pubmed.ncbi.nlm.nih.gov/28174313/

279. Nguyen D, Joshi-Datar A, Lepine F, et al. Active starvation responses mediate antibiotic tolerance

in biofilms and nutrient-limited bacteria. Science [Internet]. 2011 Nov 18 [cited 2024 Apr 29];334(6058):982–6. Available from: https://pubmed.ncbi.nlm.nih.gov/22096200/

280. Claudi B, Spröte P, Chirkova A, et al. Phenotypic variation of Salmonella in host tissues delays eradication by antimicrobial chemotherapy. Cell [Internet]. 2014 Aug 14 [cited 2024 Apr 29];158(4):722–33. Available from: https://pubmed.ncbi.nlm.nih.gov/25126781/

281. Helaine S, Cheverton AM, Watson KG, Faure LM, Matthews SA, Holden DW. Internalization of Salmonella by macrophages induces formation of nonreplicating persisters. Science [Internet]. 2014 [cited 2024 Apr 29];343(6167):204–8. Available from: https://pubmed.ncbi.nlm.nih.gov/24408438/

282. Newson JP, Gaissmaier MS, McHugh SC, Hardt WD. Studying antibiotic persistence in vivo using the model organism Salmonella Typhimurium. Curr Opin Microbiol [Internet]. 2022 Dec 1 [cited 2024 May 15];70. Available from: https://pubmed.ncbi.nlm.nih.gov/36335713/

283. Rossi O, Dybowski R, Maskell DJ, Grant AJ, Restif O, Mastroeni P. Within-host spatiotemporal dynamics of systemic Salmonella infection during and after antimicrobial treatment. J Antimicrob Chemother [Internet]. 2017 Dec 1 [cited 2024 May 15];72(12):3390–7. Available from: https://pubmed.ncbi.nlm.nih.gov/28962012/

284. Allison KR, Brynildsen MP, Collins JJ. Metabolite-enabled eradication of bacterial persisters by aminoglycosides. Nature [Internet]. 2011 May 12 [cited 2024 May 15];473(7346):216–20. Available from: https://pubmed.ncbi.nlm.nih.gov/21562562/

285. Dhar N, McKinney JD. Mycobacterium tuberculosis persistence mutants identified by screening in isoniazid-treated mice. Proc Natl Acad Sci U S A [Internet]. 2010 Jul 6 [cited 2024 May 15];107(27):12275–80. Available from: https://pubmed.ncbi.nlm.nih.gov/20566858/

286. McGovern TK, Robichaud A, Fereydoonzad L, Schuessler TF, Martin JG. Evaluation of respiratory system mechanics in mice using the forced oscillation technique. J Vis Exp [Internet]. 2013 [cited

2024 May 15];(75). Available from: https://pubmed.ncbi.nlm.nih.gov/23711876/

287. Mabille D, Dirkx L, Thys S, et al. Impact of pulmonary African trypanosomes on the immunology and function of the lung. Nat Commun [Internet]. 2022 Dec 1 [cited 2024 May 15];13(1). Available from: https://pubmed.ncbi.nlm.nih.gov/36400767/

288. Chiavolini D, Pozzi G, Ricci S. Animal models of Streptococcus pneumoniae disease [Internet]. Vol. 21, Clinical Microbiology Reviews. Clin Microbiol Rev; 2008 [cited 2020 Jul 27]. p. 666–85. Available from: https://pubmed.ncbi.nlm.nih.gov/18854486/

289. Murrah KA, Pang B, Richardson S, et al. Nonencapsulated Streptococcus pneumoniae causes otitis media during single-species infection and during polymicrobial infection with nontypeable Haemophilus influenzae. Pathog Dis [Internet]. 2015 Jul 1 [cited 2022 Sep 13];73(5). Available from: https://pubmed.ncbi.nlm.nih.gov/26014114/

290. Suckow M, Stevens K, Wilson R. The Laboratory Rabbit, Guinea Pig, Hamster, and Other Rodents. Lab Rabbit Guinea Pig, Hamster, Other Rodents. 2012;

291. Haste L, Hulland K, Bolton S, Yesilkaya H, McKechnie K, Andrew PW. Development and characterization of a long-term murine model of Streptococcus pneumoniae infection of the lower airways. Infect Immun [Internet]. 2014 [cited 2020 Jul 27];82(8):3289–98. Available from: https://pubmed.ncbi.nlm.nih.gov/24866797/

292. Briestenská K, Mikušová M, Tomčíková K, Kostolanský F, Varečková E. Quantification of bacteria by in vivo bioluminescence imaging in comparison with standard spread plate method and reverse transcription quantitative PCR (RT-qPCR). Arch Microbiol [Internet]. 2021 Sep 1 [cited 2022 Sep 13];203(7):4737–42. Available from: https://pubmed.ncbi.nlm.nih.gov/34184097/

293. Borsa N, Di Pasquale M, Restrepo MI. Animal Models of Pneumococcal pneumonia. Int J Mol Sci [Internet]. 2019 Sep 1 [cited 2024 Apr 29];20(17). Available from: https://pubmed.ncbi.nlm.nih.gov/31466400/

294. Cripps AW, Kyd JM. Comparison of mucosal and parenteral immunisation in two animal models of pneumococcal infection: otitis media and acute pneumonia. Vaccine [Internet]. 2007 Mar 22 [cited 2024 Apr 29];25(13):2471–7. Available from: https://pubmed.ncbi.nlm.nih.gov/17110001/

295. Tasaka S, Richer SE, Mizgerd JP, Doerschuk CM. Very late antigen-4 in CD18-independent neutrophil emigration during acute bacterial pneumonia in mice. Am J Respir Crit Care Med [Internet]. 2002 Jul 1 [cited 2024 Apr 29];166(1):53–60. Available from: https://pubmed.ncbi.nlm.nih.gov/12091171/

296. Bricio-Moreno L, Chaguza C, Yahya R, et al. Lower Density and Shorter Duration of Nasopharyngeal Carriage by Pneumococcal Serotype 1 (ST217) May Explain Its Increased Invasiveness over Other Serotypes. MBio [Internet]. 2020 Nov 1 [cited 2024 Apr 29];11(6):1–15. Available from: https://pubmed.ncbi.nlm.nih.gov/33293378/

297. Jacques LC, Panagiotou S, Baltazar M, et al. Increased pathogenicity of pneumococcal serotype 1 is driven by rapid autolysis and release of pneumolysin. Nat Commun [Internet]. 2020 Dec 1 [cited 2024 Apr 29];11(1). Available from: https://pubmed.ncbi.nlm.nih.gov/32312961/

298. Shears RK, Jacques LC, Naylor G, et al. Exposure to diesel exhaust particles increases susceptibility to invasive pneumococcal disease. J Allergy Clin Immunol [Internet]. 2020 Apr 1 [cited 2024 Apr 29];145(4):1272-1284.e6. Available from: https://pubmed.ncbi.nlm.nih.gov/31983527/

299. Simell B, Auranen K, Käyhty H, Goldblatt D, Dagan R, O'Brien KL. The fundamental link between pneumococcal carriage and disease. Expert Rev Vaccines [Internet]. 2012 Jul [cited 2024 Apr 29];11(7):841–55. Available from: https://pubmed.ncbi.nlm.nih.gov/22913260/

300. Im H, Kruckow KL, Mello AD, et al. Anatomical Site-Specific Carbohydrate Availability Impacts Streptococcus pneumoniae Virulence and Fitness during Colonization and Disease. Infect Immun [Internet]. 2022 Jan 1 [cited 2024 Apr 30];90(1). Available from: https://pubmed.ncbi.nlm.nih.gov/34748366/

301. Richards L, Ferreira DM, Miyaji EN, Andrew PW, Kadioglu A. The immunising effect of pneumococcal nasopharyngeal colonisation; protection against future colonisation and fatal invasive disease. Immunobiology [Internet]. 2010 Apr [cited 2024 Apr 30];215(4):251–63. Available from: https://pubmed.ncbi.nlm.nih.gov/20071053/

302. Joshi H, Kandari D, Bhatnagar R. Insights into the molecular determinants involved in *Mycobacterium tuberculosis* persistence and their therapeutic implications. Virulence [Internet]. 2021 [cited 2022 Jun 29];12(1):2721–49. Available from: https://pubmed.ncbi.nlm.nih.gov/34637683/

303. Kerr AR, Irvine JJ, Search JJ, et al. Role of inflammatory mediators in resistance and susceptibility to pneumococcal infection. Infect Immun [Internet]. 2002 [cited 2024 Apr 30];70(3):1547–57. Available from: https://pubmed.ncbi.nlm.nih.gov/11854243/

304. Gingles NA, Alexander JE, Kadioglu A, et al. Role of genetic resistance in invasive pneumococcal infection: identification and study of susceptibility and resistance in inbred mouse strains. Infect Immun [Internet]. 2001 [cited 2024 Apr 30];69(1):426–34. Available from: https://pubmed.ncbi.nlm.nih.gov/11119534/

305. Kadioglu A, Andrew PW. Susceptibility and resistance to pneumococcal disease in mice. Brief Funct Genomic Proteomic [Internet]. 2005 Nov [cited 2024 Apr 30];4(3):241–7. Available from: https://pubmed.ncbi.nlm.nih.gov/16420749/

306. Preston JA, Beagley KW, Gibson PG, Hansbro PM. Genetic background affects susceptibility in nonfatal pneumococcal bronchopneumonia. Eur Respir J [Internet]. 2004 Feb [cited 2024 Apr 30];23(2):224–31. Available from: https://pubmed.ncbi.nlm.nih.gov/14979496/

307. Ogunniyi AD, LeMessurier KS, Graham RMA, et al. Contributions of pneumolysin, pneumococcal surface protein A (PspA), and PspC to pathogenicity of Streptococcus pneumoniae D39 in a mouse model. Infect Immun [Internet]. 2007 Apr [cited 2024 Apr 30];75(4):1843–51. Available from:

https://pubmed.ncbi.nlm.nih.gov/17261599/

308. D'Mello A, Riegler AN, Martínez E, et al. An in vivo atlas of host–pathogen transcriptomes during Streptococcus pneumoniae colonization and disease. Proc Natl Acad Sci U S A [Internet]. 2020 Dec 26 [cited 2024 Apr 30];117(52):33507–18. Available from: https://pubmed.ncbi.nlm.nih.gov/33318198/

309. Lane JR, Tata M, Yasmin R, Im H, Briles DE, Orihuela CJ. PspA-mediated aggregation protects Streptococcus pneumoniae against desiccation on fomites. MBio [Internet]. 2023 Dec 1 [cited 2024 Apr 30];14(6). Available from: https://pubmed.ncbi.nlm.nih.gov/37982608/

310. Hamamoto K, Mizuno Y. LC-MS/MS measurement of ampicillin residue in swine tissues at 5 days after in-feed administration. J Vet Med Sci [Internet]. 2015 Dec 1 [cited 2024 Jun 18];77(11):1527–9. Available from: https://pubmed.ncbi.nlm.nih.gov/26084485/

311. Balouiri M, Sadiki M, Ibnsouda SK. Methods for in vitro evaluating antimicrobial activity: A review. J Pharm Anal [Internet]. 2016 Apr 1 [cited 2024 Apr 30];6(2):71–9. Available from: https://pubmed.ncbi.nlm.nih.gov/29403965/

312. Schenck LP, McGrath JJC, Lamarche D, Stämpfli MR, Bowdish DME, Surette MG. Nasal Tissue Extraction Is Essential for Characterization of the Murine Upper Respiratory Tract Microbiota. mSphere [Internet]. 2020 Dec 23 [cited 2024 May 15];5(6). Available from: https://pubmed.ncbi.nlm.nih.gov/33328347/

313. Windels EM, Van Den Bergh B, Michiels J. Bacteria under antibiotic attack: Different strategies for evolutionary adaptation. PLoS Pathog [Internet]. 2020 May 1 [cited 2024 May 29];16(5). Available from: https://pubmed.ncbi.nlm.nih.gov/32379814/

314. Lowe R, Shirley N, Bleackley M, Dolan S, Shafee T. Transcriptomics technologies. PLoS Comput Biol [Internet]. 2017 May 1 [cited 2024 May 29];13(5). Available from: https://pubmed.ncbi.nlm.nih.gov/28545146/

315. Keren I, Minami S, Rubin E, Lewis K. Characterization and transcriptome analysis of Mycobacterium tuberculosis persisters. MBio [Internet]. 2011 [cited 2024 May 29];2(3). Available from: https://pubmed.ncbi.nlm.nih.gov/21673191/

316. Wu S, Yu PL, Wheeler D, Flint S. Transcriptomic study on persistence and survival of Listeria monocytogenes following lethal treatment with nisin. J Glob Antimicrob Resist [Internet]. 2018 Dec 1 [cited 2024 May 29];15:25–31. Available from: https://pubmed.ncbi.nlm.nih.gov/29933119/

317. Mattiello SP, Barth VC, Scaria J, Ferreira CAS, Oliveira SD. Fluoroquinolone and beta-lactam antimicrobials induce different transcriptome profiles in Salmonella enterica persister cells. Sci Rep [Internet]. 2023 Dec 1 [cited 2024 May 29];13(1). Available from: https://pubmed.ncbi.nlm.nih.gov/37907566/

318. Alnahhas RN, Dunlop MJ. Advances in linking single-cell bacterial stress response to population-level survival. Curr Opin Biotechnol [Internet]. 2023 Feb 1 [cited 2024 May 29];79. Available from: https://pubmed.ncbi.nlm.nih.gov/36641904/

319. Pan X, Liu W, Du Q, Zhang H, Han D. Recent Advances in Bacterial Persistence Mechanisms. Int J Mol Sci [Internet]. 2023 Sep 1 [cited 2024 May 29];24(18). Available from: https://pubmed.ncbi.nlm.nih.gov/37762613/

320. Hare PJ, Lagree TJ, Byrd BA, Demarco AM, Mok WWK. Single-Cell Technologies to Study Phenotypic Heterogeneity and Bacterial Persisters. Microorganisms [Internet]. 2021 Nov 1 [cited 2024 May 29];9(11). Available from: https://pubmed.ncbi.nlm.nih.gov/34835403/

321. Ma P, Amemiya HM, He LL, et al. Bacterial droplet-based single-cell RNA-seq reveals antibiotic-associated heterogeneous cellular states. Cell [Internet]. 2023 Feb 16 [cited 2024 May 29];186(4):877-891.e14. Available from: https://pubmed.ncbi.nlm.nih.gov/36708705/

322. Sawada T, Katayama M, Takatani S, Ohiro Y. Early detection of drug-resistant Streptococcus pneumoniae and Haemophilus influenzae by quantitative flow cytometry. Sci Rep [Internet]. 2021

Dec 1 [cited 2024 Jun 25];11(1). Available from: https://pubmed.ncbi.nlm.nih.gov/33536509/

323. Habets MN, Van Selm S, Van Der Gaast De Jongh CE, Diavatopoulos DA, De Jonge MI. A novel flow cytometry-based assay for the quantification of antibody-dependent pneumococcal agglutination. PLoS One [Internet]. 2017 Mar 1 [cited 2024 Jun 25];12(3). Available from: https://pubmed.ncbi.nlm.nih.gov/28288168/

324. Henry TC, Brynildsen MP. Development of Persister-FACSeq: a method to massively parallelize quantification of persister physiology and its heterogeneity. Sci Rep [Internet]. 2016 Jul 6 [cited 2024 May 30];6(1). Available from: https://pubmed.ncbi.nlm.nih.gov/27142337/

325. Xiong H, Shi L, Wei L, et al. Stimulated Raman Excited Fluorescence Spectroscopy and Imaging. Nat Photonics [Internet]. 2019 Jun 1 [cited 2024 Jun 25];13(6):412–7. Available from: https://pubmed.ncbi.nlm.nih.gov/32607124/

326. Völzing KG, Brynildsen MP. Stationary-Phase Persisters to Ofloxacin Sustain DNA Damage and Require Repair Systems Only during Recovery. MBio [Internet]. 2015 [cited 2024 Jun 19];6(5). Available from: https://pubmed.ncbi.nlm.nih.gov/26330511/

327. Maskell DJ, Horrnaeche CE. Relapse following cessation of antibiotic therapy for mouse typhoid in resistant and susceptible mice infected with salmonellae of differing virulence. J Infect Dis [Internet]. 1985 Nov 1 [cited 2024 May 31];152(5):1044–9. Available from: https://pubmed.ncbi.nlm.nih.gov/3930625/

328. Easmon CSF, Blowers A. Ciprofloxacin treatment of systemic salmonella infection in sensitive and resistance mice. J Antimicrob Chemother [Internet]. 1985 Nov [cited 2024 May 31];16(5):615–9. Available from: https://pubmed.ncbi.nlm.nih.gov/2934362/

329. Easmon CSF, Crane JP, Blowers A. Effect of ciprofloxacin on intracellular organisms: in-vitro and in-vivo studies. J Antimicrob Chemother [Internet]. 1986 [cited 2024 May 31];18 Suppl D(SUPPL. D):43–8. Available from: https://pubmed.ncbi.nlm.nih.gov/3542948/

330. Fu KP, Hilliard J, Isaacson D, Tobia AJ, Rosenthale ME, Mcguire JL. In-vivo evaluation of ofloxacin in Salmonella typhimurium infection in mice. J Antimicrob Chemother [Internet]. 1990 Feb [cited 2024 May 31];25(2):263–8. Available from: https://pubmed.ncbi.nlm.nih.gov/2184159/

331. Griffin AJ, Li LX, Voedisch S, Pabst O, McSorley SJ. Dissemination of persistent intestinal bacteria via the mesenteric lymph nodes causes typhoid relapse. Infect Immun [Internet]. 2011 Apr [cited 2024 May 31];79(4):1479–88. Available from: https://pubmed.ncbi.nlm.nih.gov/21263018/

332. Endt K, Maier L, Käppeli R, et al. Peroral ciprofloxacin therapy impairs the generation of a protective immune response in a mouse model for Salmonella enterica serovar Typhimurium diarrhea, while parenteral ceftriaxone therapy does not. Antimicrob Agents Chemother [Internet]. 2012 May [cited 2024 May 31];56(5):2295–304. Available from: https://pubmed.ncbi.nlm.nih.gov/22354292/

333. Lebeaux D, Chauhan A, Létoffé S, et al. pH-mediated potentiation of aminoglycosides kills bacterial persisters and eradicates in vivo biofilms. J Infect Dis [Internet]. 2014 Nov 1 [cited 2024 May 31];210(9):1357–66. Available from: https://pubmed.ncbi.nlm.nih.gov/24837402/

334. Defraine V, Schuermans J, Grymonprez B, et al. Efficacy of Artilysin Art-175 against Resistant and Persistent Acinetobacter baumannii. Antimicrob Agents Chemother [Internet]. 2016 Jun 1 [cited 2024 May 31];60(6):3480–8. Available from: https://pubmed.ncbi.nlm.nih.gov/27021321/

335. RubenMorones-Ramirez J, Winkler JA, Spina CS, Collins JJ. Silver enhances antibiotic activity against gram-negative bacteria. Sci Transl Med [Internet]. 2013 Jun 19 [cited 2024 May 31];5(190). Available from: https://pubmed.ncbi.nlm.nih.gov/23785037/

336. Dewachter L, Fauvart M, Michiels J. Bacterial Heterogeneity and Antibiotic Survival: Understanding and Combatting Persistence and Heteroresistance. Vol. 76, Molecular Cell. Cell Press; 2019. p. 255–67.

337. Kowalska-Krochmal B, Dudek-Wicher R. The Minimum Inhibitory Concentration of Antibiotics: Methods, Interpretation, Clinical Relevance. Pathog (Basel, Switzerland) [Internet]. 2021 Feb 1

[cited 2024 May 30];10(2):1–21. Available from: https://pubmed.ncbi.nlm.nih.gov/33557078/

338. Brauner A, Shoresh N, Fridman O, Balaban NQ. An Experimental Framework for Quantifying Bacterial Tolerance. Biophys J [Internet]. 2017 Jun 20 [cited 2024 May 30];112(12):2664–71. Available from: https://pubmed.ncbi.nlm.nih.gov/28636922/

339. Van den Bergh B, Michiels JE, Fauvart M, Michiels J. Should we develop screens for multi-drug antibiotic tolerance? Expert Rev Anti Infect Ther [Internet]. 2016 Jul 2 [cited 2024 May 30];14(7):613–6. Available from: https://pubmed.ncbi.nlm.nih.gov/27227426/

340. Gefen O, Chekol B, Strahilevitz J, Balaban NQ. TDtest: easy detection of bacterial tolerance and persistence in clinical isolates by a modified disk-diffusion assay. Sci Rep [Internet]. 2017 Feb 1 [cited 2024 May 30];7. Available from: https://pubmed.ncbi.nlm.nih.gov/28145464/

341. Kotková H, Cabrnochová M, Lichá I, et al. Evaluation of TD test for analysis of persistence or tolerance in clinical isolates of Staphylococcus aureus. J Microbiol Methods [Internet]. 2019 Dec 1 [cited 2024 May 30];167. Available from: https://pubmed.ncbi.nlm.nih.gov/31472196/

342. Andersson DI, Balaban NQ, Baquero F, et al. Antibiotic resistance: turning evolutionary principles into clinical reality. FEMS Microbiol Rev [Internet]. 2020 [cited 2024 May 30];44(2). Available from: https://pubmed.ncbi.nlm.nih.gov/31981358/

343. Defraine V, Fauvart M, Michiels J. Fighting bacterial persistence: Current and emerging anti-persister strategies and therapeutics [Internet]. Vol. 38, Drug Resistance Updates. Churchill Livingstone; 2018 [cited 2020 Jul 15]. p. 12–26. Available from: https://pubmed.ncbi.nlm.nih.gov/29857815/

344. Starkey M, Lepine F, Maura D, et al. Identification of anti-virulence compounds that disrupt quorum-sensing regulated acute and persistent pathogenicity. PLoS Pathog [Internet]. 2014 Aug 21 [cited 2024 May 31];10(8). Available from: https://pubmed.ncbi.nlm.nih.gov/25144274/

345. Chen X, Zhang M, Zhou C, Kallenbach NR, Ren D. Control of bacterial persister cells by Trp/Arg-

containing antimicrobial peptides. Appl Environ Microbiol [Internet]. 2011 Jul [cited 2022 Feb 24];77(14):4878–85. Available from: https://pubmed.ncbi.nlm.nih.gov/21622798/

346. Kim JS, Heo P, Yang TJ, et al. Selective killing of bacterial persisters by a single chemical compound without affecting normal antibiotic-sensitive cells. Antimicrob Agents Chemother [Internet]. 2011 Nov [cited 2024 May 30];55(11):5380–3. Available from: https://pubmed.ncbi.nlm.nih.gov/21844322/

347. Barraud N, Buson A, Jarolimek W, Rice SA. Mannitol enhances antibiotic sensitivity of persister bacteria in Pseudomonas aeruginosa biofilms. PLoS One [Internet]. 2013 Dec 13 [cited 2024 May 30];8(12). Available from: https://pubmed.ncbi.nlm.nih.gov/24349568/

348. Cui P, Niu H, Shi W, et al. Disruption of Membrane by Colistin Kills Uropathogenic Escherichia coli Persisters and Enhances Killing of Other Antibiotics. Antimicrob Agents Chemother [Internet]. 2016 Nov 1 [cited 2024 May 30];60(11):6867–71. Available from: https://pubmed.ncbi.nlm.nih.gov/27600051/

CURRICULUM VITAE

PERSONAL INFORMATION

Name: Nele Geerts
Date of birth: 24/10/1997
Address: Kerkstraat 32, 2980 Zoersel
Tel: 0474/06.16.31
Email: nele.geerts@hotmail.com
LinkedIn: www.linkedin.com/in/nele-geerts-a70a2b20b

EDUCATION

University of Antwerp, Nov 2020 – Oct 2024

- PhD in Pharmaceutical Sciences: Microbiology
- FWO fellowship fundamental research
- Skill set

  Time management, writing protocols, independent research, efficient literature searches…

University of Antwerp, 2022 – 2028

- Advanced master of Laboratory Medicine

University of Antwerp, 2018 – 2020

- Master in Pharmaceutical Sciences: Drug Development – Pharmacist

  Degree with great distinction
- Master's thesis "*In vitro* and *in vivo* evaluation of *in silico* predicted streptococcal UDPG:PP inhibitors"
- Granted with the price 'Nedeljkovitch' 2020

University of Antwerp, 2015 – 2018

- Bachelor in Pharmaceutical Sciences

  Degree with great distinction.

- Granted with the price for the most distinguished student of the bachelor pharmaceutical sciences of 2018

Sint-Jan Berchmanscollege, Westmalle, 2009 – 2015

- Science and Mathematics

  Degree with distinction.

LIST OF SCIENTIFIC PUBLICATIONS

Geerts N, De Vooght L, Passaris I, Delputte P, Van den Bergh B, Cos P. Antibiotic Tolerance Indicative of Persistence Is Pervasive among Clinical *Streptococcus pneumoniae* Isolates and Shows Strong Condition Dependence. Microbiol Spectr. 2022 Dec 21;10(6):e0270122. doi: 10.1128/spectrum.02701-22. Epub 2022 Nov 14. PMID: 36374111; PMCID: PMC9769776.

Cools F, Triki D, Geerts N, Delputte P, Fourches D, and Cos P. *In vitro* and *in vivo* evaluation of *in silico* predicted pneumococcal UDPG:PP inhibitors. Front Microbiol. Doi: 10.3389/fmicb.2020.01596

CONTRIBUTIONS TO SCIENTIFIC MEETINGS AND CONFERENCES

Oral presentations

Nele Geerts, Linda De Vooght, Ioannis Passaris, Peter Delputte, Bram Van den Bergh, Paul Cos (2022). Antibiotic-tolerant persisters are relevant in *Streptococcus pneumoniae*. Presentation at the International Symposium on Pneumococci and Pneumococcal Diseases (ISPPD-12), 21/06/2022, Toronto, Canada.

Nele Geerts, Linda De Vooght, Ioannis Passaris, Peter Delputte, Bram Van den Bergh, Paul Cos (2023). Antibiotic tolerance indicative of persistence is pervasive among clinical *Streptococcus pneumoniae* isolates and shows strong condition dependence. Presentation at the Departmental Research Day of Pharmaceutical Sciences, 10/05/2023, Antwerp, Belgium

Nele Geerts, Linda De Vooght, Ioannis Passaris, Peter Delputte, Bram Van den Bergh, Paul Cos (2023). *S. pneumoniae's* survival symphony: a first step towards deciphering the clinical relevance. Presentation at the FWO Research Community "Biology and Ecology of Bacterial & Fungal Human Biofilms": 6th Workshop on Bacterial and Fungal Biofilms, 22/09/2023, Leuven, Belgium.

Poster presentations

Nele Geerts, Linda De Vooght, Ioannis Passaris, Peter Delputte, Bram Van den Bergh, Paul Cos (2023). Antibiotic-tolerant persisters are pervasive in clinical *Streptococcus pneumoniae* isolates. Poster presentation at FEMS2023, 9-13/07/2023, Hamburg, Germany.

Nele Geerts, Linda De Vooght, Ioannis Passaris, Lize Cuypers, Stefanie Desmet, Peter Delputte, Bram Van den Bergh, Paul Cos (2024). Antibiotic-tolerant persisters are pervasive in clinical *Streptococcus pneumoniae* isolates. Poster presentation at the International Symposium on Pneumococci and Pneumococcal Diseases (ISPPD-13), 17-20/03/2024, Cape Town, South-Africa.

SCIENTIFIC GRANT

FEMS Congress Attendance Grant. 10th FEMS Congress of European Microbiologists. 09/07/2023-13/07/2023, Hamburg, Germany