Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Medicine and Health Sciences
Department of Clinical and Molecular Medicine

Synnøve Nordsteien Rigstad

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Supervisor: Jan Egil Afset
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Abstract

Relaxed strategies in infection control and high antimicrobial use in some countries give rise to resistant bacteria, which may easily spread in the modern interconnected world. BSIs are among the top seven causes of death in North America and Europe. In Norway, and the world, there are increased incidence rates of bloodstream infections (BSI). The rise in BSI rates combined with the ever-increasing rate of antimicrobial resistance, composes a challenge for health care facilities to give accurate, and timely treatment to even more patients. Optimised treatment can be given when all laboratory tests have been run, gram staining, organism ID and AST. Most ASTs used today are culture based, meaning they rely on the ability of the bacteria to grow. EUCAST methods for AST vary from 18 ± 2 hours to 4 hours incubation time. If one could detect antimicrobial susceptibility faster, optimised treatment could be given sooner thus improving clinical outcomes.

This project's aim is to phenotypically determine the susceptibility profile of *E. coli* isolated from BSI to gentamicin through a Real-Time PCR-method. It will evaluate a boiling lysis method for fast DNA preparation and optimise the incubation period of *E. coli* in Mueller-Hinton broth to distinguish resistant and susceptible strains.

A Real-Time PCR assay was established, and it measured the relative change in the 16S rRNA gene. Fourteen strains of *E. coli* were individually incubated in Mueller-Hinton broth with and without gentamicin. Most growth experiments lasted 2.5-5 hours. The boiling lysis method was optimised and used when preparing DNA for the Real-Time PCR. Boiling lysis was also compared to a commercial automatic DNA extraction method, EZ1. Turbidity was measured for growth curves spectrophotometrically at 600 nm (OD600).

The Real-Time PCR established for the 16S rRNA gene was based on St. Olavs hospital's Real-Time PCR for 16S rRNA gene detection from direct material. To determine if strains were susceptible or resistant it took 4 hours of incubation if using boiling lysis and Real-Time PCR. Using only OD600 measurements, it took 1.5 hours of incubation to distinguish susceptible and resistant strains. The automated DNA extraction method, EZ1, had lower run time, hands-on work, and more stable results than boiling lysis.

An increase in the 16S rRNA gene in the gentamicin broth would indicate growth, meaning the bacteria was resistant. Detecting bacterial growth using Real-Time PCR seemed unreliable due to the weak relationship between growth and Cq-values, a 10-fold increase in DNA template is needed for a 3.32 decrease in Cq-value. Depending on available resources, automated DNA extraction, EZ1, seems to be the better choice for bacterial DNA preparation. Boiling lysis produced less consistent results and took longer to perform than EZ1. OD600 measurements seems a better option for detecting bacterial growth because of the direct correlation between cell density and turbidity.

Only a small selection of *E. coli* stains were tested. It would be advantageous to test both a larger group of bacteria and antimicrobials in different combinations. An interesting thought to explore would be the possibility of broth microdilutions directly from blood culture samples with a shortened incubation time.

Sammendrag

Slappe strategier for infeksjonskontroll og høyt forbruk av antimikrobielle stoffer i noen land gir god grobunn for resistente bakterier, og med en svært sammenkoblet verden kan mikrober spre seg fort. Blodstrømsinfeksjoner (BSI) er en av de syv største årsakene til død i Nord-Amerika og Europa, og i Norge, som resten av verden, er det en økning i tilfeller av BSI. Denne økningen kombinert med økningen i antimikrobiell resistens, gir utfordringer for mange helseinstitusjoner i å gi effektiv og tidlig behandling til flere pasienter. Optimalisert behandling kan gis når alle laboratorier tester er kjørt, som gramfarging, organisme ID og antimikrobiell følsomhets testing (AFT). De fleste AFTer brukt nå bygger på kulturer, som betyr at de baserer seg på bakteriens evne til å vokse. EUCAST metoder for AFT varierer fra 18 ± 2 timer til 4 timers inkubasjon. On man kunne detektere antimikrobiell følsomhet raskere, kan optimalisert behandling gis tidligere og de kliniske utfallene bedres.

Dette prosjektets mål er å fenotypisk avgjøre følsomhetsprofiler for *E. coli* isolater fra BSI mot gentamicin vha. en Real-Time PCR metode. Prosjektet vil også evaluere en kokelyseringsmetode for rask DNA preparasjon, og optimere inkubasjonstiden for *E. coli* i Muellr-Hinton buljong for å differensiere følsomme og resistente stammer.

En Real-Time PCR ble etablert som målte relativ forandring ï 16S rRNA genet. Fjorten stammer *E. coli* ble ble individuelt inkubert i Mueller-Hinton buljong med og uten gentamicin. De fleste vekstforsøk varte i 2.5-5 timer. Kokelyseringsmetoden ble optimalisert og ble brukt som DAN preparasjon før Real-Time PCRen. Kokelyseringen ble også sammenlignet med en kommersiell automatisk DNA ekstraksjonsmetode, EZ1. Turbiditet ble målt for vekstkurvene spektrofotometrisk på 600 nm (OD600).

Real-Time PCRen etablert for 16S rRNA genet var basert på St. Olavs hospitals Real-Time PCR for 16S rRNA gen deteksjon fra direkte materiale. Å avgjøre om stammer var resistente eller følsomme tok 4 timer med inkubering ved å bruke kokelysering og Real-Time PCR. Med kun OD600 målinger tok det 1.5 time med inkubering for å differensiere resistente og følsomme stammer. Den automatiske DNA ekstraksjonen, EZ1, hadde et lavere EZ1 lavere tidsbruk, «hands-on» arbeid og mer stabile resultater enn kokelysering.

En økning i 16S rRNA genet i gentamicin-buljongen ville bety vekst, som indikerer at bakterien er resistent. Å detektere bakteriell vekst vha. Real-Time PCR virket for upålitelig pga. det svake forholdet mellom vekst og Cq-verdier, en 10-folds økning i DNA templat er nødvendig for en 3,32 reduksjon i Cq-verdi. Avhengig av ressurser, virker den automatiske DNA ekstraksjonen som et bedre valg for bakteriell DNA preparasjon. Kokelyseringen produserte mindre konsise resultater og brukte lenger tid enn EZ1. OD600 målinger virket som et mye bedre valg for deteksjon av bakteriell vekst pga. det direkte forholdet mellom celletetthet og turbiditet.

Bare en liten gruppe stammer *E. coli* ble testet. Det ville være interessant teste både flere grupper av bakterier og antimikrobielle stoffer i forskjellige kombinasjoner. Et interessant perspektiv å utforske ville være muligheten for mikrobuljongfortynninger med forkortet inkubasjonstid med prøver direkte fra blodkultur.

Acknowledgements

And my heart aches, in hopeless pain

Exhausted with repinings vain,

That I shall greet them ne’er again

— Emily Brontë, Best Poems of the Brontë Sisters

Contents

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Equations

Abbreviations

AAC – Aminoglycoside N-acetyltransferases

AME – Aminoglycoside-modifying enzymes

ANT –Aminoglycoside O-nucleotidyltransferases

APH - Aminoglycoside O-phosphotransferases

AST – Antimicrobial susceptibility testing

BC – Blood culture

BLBLI – β-lactam/β-lactamase inhibitor

bp – base pair

Kbp – kilo (1000) bp

BSI – Blood stream infection

CFU/mL – Colony forming unites per milli litre

CI – Confidence interval

Cq – Cycle of quantification

DNA – Deoxyribonucleic acid

dsDNA – double stand DNA

ssDNA – single stand DNA

dNTP – deoxynucleotide triphosphates

E. coli *– Escherichia coli*

ECDC - European Centre for Disease Control

EUCAST – The European Committee on Antimicrobial Susceptibility Testing

ESBL – Extended spectrum β-lactamase

GE – Growth experiment

LOD – Limit of detection

MH – Mueller-Hinton broth

MIC – Minimum inhibition concentration

NordicAST – Nordic Committee on Antimicrobial Susceptibility Testing

OD – Optimal density

ON – Over-night

oriC – Origin of replication in bacterial chromosomes

PBP3 – Penicillin-binding protein 3

PCR – Polymerase chain reaction

dPCR – digital PCR

Real-Time PCR – Real-Time PCR

RT-PCR – Reverse-transcription PCR

RAST – Rapid antimicrobial susceptibility testing

RMT - 16S rRNA methyltransferases

RNA – Ribonucleic acid

mRNA – messenger RNA

tRNA – transport RNA

rpm – revolutions per minute

S. aureus – *Staphylococcus aureus*

S-I-R – Susceptible, Intermediate, Resistant

UTI – Urinary tract infection

WHO – World Health Organization

1 Introduction

The world is becoming increasingly interconnected, and this has been happening for decades. Through international travel and trade, more people than ever are scaling the world [1, 2]. They bring with them all what that entails, including potential pathogenic organisms [3, 4]. The COVID-19 pandemic has displayed on a global scale the ability of a pathogen to encompass the earth and its population in a relative short time. COVID-19 was a novel pathogen, known pathogens might acquire novel resistance mechanisms from far-off places, and be disguised as a well-known infection. This transfer of resistance can lead to a rise in multi-drug-resistant bacteria [5]. For local health care this leads to a unique issue, the standard action of treatment might not work in every case, complicating both diagnosis and treatment [5]. A solution to the issue is testing, especially that of organism identity and resistance mechanisms. Diagnostic techniques should be accurate and fast, both for the health of the patient and the efficiency of the hospital.

1.1 Blood stream infections (BSI)

Blood stream infections (BSI) are among the top seven causes of death in North America and Europe [6], and a leading cause of death in immunocompromised and severely sick patients [7]. Modern medicine has created even more immunocompromised patients through treatments impairing the immune system, thus putting more people at risk for infections by opportunistic pathogens [8]. Norway, as the rest of the world, has experienced an increased incidence rate of BSI [7]. Mortality among patients with BSI in Norway is quite steady, with only a small increase related to antibiotic resistance [9]. The rise in BSI rates combined with the ever-increasing rate of antibiotic resistance, composes a challenge for health care facilities to give accurate and effective treatment to even more patients.

Bacteraemia can be defined as viable bacteria found in the blood stream [5, 10]. Asymptomatic bacteraemia in healthy people can be transient without displays of symptoms. In other cases bacteraemia can develop into BSI and other severe diseases like sepsis, septic shock or multiple organ dysfunction syndrome (MODS) [5, 10]. Sepsis and BSI have in some cases been used interchangeably, though their definitions vary. Sepsis is defined by The Third International Consensus Definition for Sepsis and Septic Shock as "*life-threatening organ dysfunction caused by a dysregulated host response to infection*" [11]. Whilst BSI is defined by Huerta and Rice as "*a pathogenic organism in the bloodstream that causes disease*" [12]. It is therefore the hosts immune response to the initial bacteraemia that determines the severity of the infection. Especially sepsis and septic shock are related to prolonged hospital stays, higher costs and increased mortality and morbidity [10, 13]. Of the most clinically important bacteria in bacteraemia, *Escherichia coli* tops the statistics for gram-negative bacteria, whilst *Staphylococcus aureus* is the most common gram-positive organism [5].

Treatment of BSI, and especially sepsis, needs to happen as quickly as possible, preferably within 6 hours to reduce morbidity and mortality [10, 13, 14]. The gold

standard for diagnosing BSI is blood culture (BC) [10, 13, 14], meaning large blood samples are placed in optimal conditions to cultivate any potential organisms in the blood. This method is time consuming, taking 1-6 days to determine growth by bacteria, identify the bacteria and determine any antimicrobial resistance [10, 13]. Until a gram-stain can be completed the patient will receive empirical treatment with broad-spectrum antimicrobials for both gram-positive and gram-negative microorganisms [13]. This includes extended-generation cephalosporins or beta-lactamase inhibitors [5]. In Norway, standard empiric treatment for BSI includes an aminoglycoside, like gentamicin or tobramycin, in combination with benzylpenicillin [15]. When the gram profile is known, the antimicrobial treatment might be altered to match the pathogen. The optimised antimicrobial treatment will be given after the Susceptible-Intermediate-Resistant (S-I-R) profile is known. The S-I-R profile is uncovered by methods like agar disk diffusion, diffusion strips or broth microdilution.

1.2 Antimicrobial agents

The first recorded use of antibiotic-like substances dates back 2,500 years. Both the Chinese and the Egyptians discovered some medical benefit from mould derived from bean curd or bread [16]. But it was not until the 19th century one had the ability to study these elusive substances and the term antibiotics was coined [16]. Here, antimicrobial/antimicrobial agent is used as a blanket term for antimicrobial agents made both by microorganisms and synthetically made by humans. Antibiotic will be used when referring the metabolic end products or intermediate products, made by different microorganisms to protect themselves against other microorganisms [16].

The first antibiotic derived from microorganisms used for therapeutic application was in 1889 by Emmerich. The substance was called pyocyanase derived from a culture of *Bacillus pyocyaneus* and was probably a mixture of two antibiotics [16]. But it was not until 1928, with the discovery of penicillin, that the potential of antibiotics were unlocked. [16]. Penicillin was discovered by Alexander Fleming [16-18], as an isolate from *Penicillium notatum* [16]. It was later, in 1941, made commercially available by Ernst Chain and Sir Howard W. Florey as they manufactured the antimicrobial with assistance from the U.S. government [16]. The discovery of antimicrobials has had a great impact on the world and human life [16]. It is one of the most influential medical discoveries of the 20th century, furthering public health and increasing life expectancy though control of infectious diseases [16, 17]. More than 100 antimicrobials have been discovered since Fleming's discovery, and almost 90% are derived from microorganisms, while the rest are either wholly or partially synthetic [16].

Antimicrobials can be organised in several different ways, based on chemical structure or mechanism of action [19]. Here they are organised into five groups based on their chemical structure; beta lactams (e.g. penicillin, carbapenem), tetracyclines (e.g. doxycycline), macrolides (e.g. erythromycin), aminoglycosides (e.g. gentamicin, streptomycin) and others (e.g. chloramphenicol, vancomycin, fusidic acid) [18-20]. They have several different mechanisms of action, through interference with cell wall synthesis, DNA or RNA synthesis, protein synthesis, or through other mechanisms [21, 22]. They can also generally be labelled as either bacteriostatic or bactericidal [19], meaning inhibiting bacterial growth or killing the bacteria. Another classification is broad or narrow spectrum. Broad spectrum antibiotics target both gram-negative and -positive

bacteria, whilst narrow spectrum antimicrobials are more specialised and work only on a few or one bacterial species [17-19].

1.2.1 Aminoglycosides – Gentamicin

Aminoglycosides are one of the main groups of antimicrobials, first discovered in 1944 by Waksman and colleagues [16, 23-25]. Streptomycin was the first discovery, with gentamicin being discovered in 1963 by Marvin Weinstein's group at Schering Plough [16]. Aminoglycosides are small, polycationic molecules, with similarities to carbohydrates due to their six-membered aminocyclitol ring [16] (*Figure 1.1*). Aminoglycosides are natural or semisynthetic antibiotics derived from different species from the phylum *Actinobacteria* [16, 24, 26]. *Actinobacteria* includes the class *Actinomycetes*, which is a large class of gram-positive filamentous bacteria [16, 26]. Most aminoglycosides, 70-80%, are derived from *Streptomyces* species in the class *Actinomycetes*. Gentamicin was though the first antimicrobial to be derived from another species, namely *Micromonospora purpurea* and/or *M. echinospora,* also in the *Actinomycetes class*. *Micromonospora* is the second largest group of culturable *Actinomycetes* in soil and a great source of antibiotics [16].

Aminoglycosides are bactericidal and have a concentration-dependent killing mechanism, meaning a higher concentration of antibiotic corelates to a higher rate of antimicrobial death [15, 23, 24]. They are broad spectrum antimicrobial and work best in aerobic/facultative anaerobic bacteria [23, 24], and are potent against both *E. coli* and *S. aureus* [24]. It can be used in combination with other antibiotics, or as a solo treatment, both empirically or specifically [24].

Gentamicin is a complex of different agents built around a central aminocyclitol ring, specifically a deoxystreptamine ring [16, 24, 27] (*Figure 1.1*). Complexes C1 and C2 are the most potent [28, 29], and are what is most likely found in commercially available gentamicin [16]. Gentamicin’s mechanism of action is inhibition of protein synthesis [24, 29]. Gentamicin enters the bacterial cell through an oxygen-dependent active transport, thus making it ineffective in anaerobic bacteria living in oxygen deplete environments [23, 24]. Inside the bacterial cell, gentamicin binds to the A-site on 30S subunits of ribosomes. The A-site is partially made of 16S rRNA and is the entry site for tRNA in protein synthesis [23, 24]. The binding of gentamicin into the A-site disrupts translation by misreading codons, which leads to truncated and non-functional proteins [23, 24]. It is not completely understood how these non-functional proteins can be bactericidal [23], here, two hypothesises will be presented. First hypothesis; the incorporation of these non-functional and truncated proteins into the cell wall can make it weak and more permeable, thus even more gentamicin can enter the cell [23, 24]. This speeds up the mistranslation and ends in death for the cell [24]. Second hypothesis; if proteins involved in essential functions, like oxidation-reduction, get truncated and non-functional, reactive oxygen species might accumulate, leading to cell death [23].

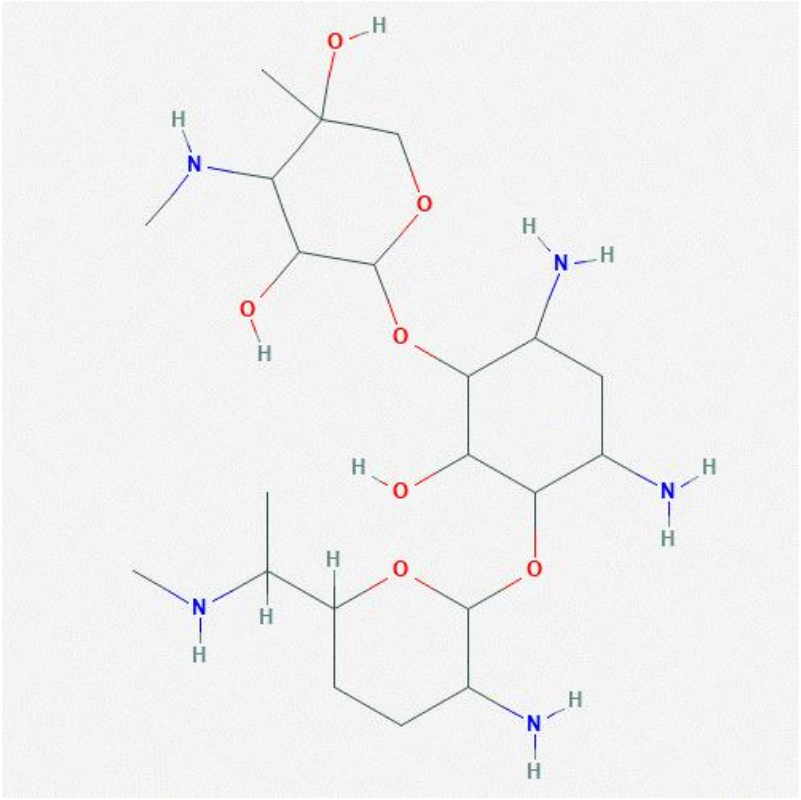

Figure 1.1 Gentamicin's molecular structure [30], with the deoxystreptamine ring in the middle, a version of an aminocyclitol ring.

1.2.2 Antimicrobial resistance

Antimicrobial resistance was already seen and cautioned against by Fleming himself in 1945, only a year after the use of penicillin got widespread [31, 32]. Now it is an increasing problem, putting modern medicine at risk. It is a threat to global health and longevity, predicted to cause 10 million deaths by 2050 [3, 4, 18, 33]. Many modern medical procedures, such as most surgeries and immune compromising procedures, cannot be safely performed without the use of antibiotics [3]. The environment is a key player in antimicrobial resistance as a reservoir of resistance genes. Antimicrobial resistance can be especially rampant in the intersection between environment and agriculture. Antimicrobials are used prophylactically in animals whilst they are in close contact with the environment's large reservoir of resistance genes, a perfect match to generate resistant bacteria [4]. These resistant bacteria can be transferred to humans through direct contact of through food products [4].

Resistance to antimicrobial agents is a survival mechanism on the part of the bacteria. It protects its prolonged survival by resisting the antimicrobial agent's mode of action. Most antimicrobial agents are derived from other microorganisms, therefore bacteria would be exposed to them in the wild. Antimicrobial resistance would be a valuable survival tool, most likely first developed by the antimicrobial producers themselves [24]. Resistance can be introduced to bacteria through mutations or plasmids [18]. Genes can be transferred between species or within species by horizontal gene transfer [31]. The mechanisms involved in horizontal gene transference are transduction; bacteriophages spreading genetic information, conjugation; transference of genes from bacteria to bacteria, or transformation; uptake of genetic material from the environment. Plasmids

can be transferred from one bacterium to another by conjugation, and is a type of resistance often seen in hospital settings [18, 31]. Genes may code for proteins that specifically target the antimicrobial agent, or modifications in the antimicrobial target can make the antimicrobial ineffective. Environments with prevalence of antimicrobial agents cause a selective pressure for bacteria with antimicrobial resistance mutations or genes to survive [18].

There are several mechanisms responsible for resistance to aminoglycosides. It can be efflux of the antimicrobial, modifications of the target through genetic mutation or modifications to the antimicrobial agent itself, often several mechanisms are involved at once [24]. Modifications to the antimicrobial agent can happen through aminoglycoside-modifying enzymes (AMEs), commonly found on plasmids including other AMEs or β-lactamases [24]. AMEs can be divided into three groups, aminoglycoside N-acetyltransferases (AACs), aminoglycoside O-nucleotidyltransferases (ANTs), and aminoglycoside O-phosphotransferases (APHs). These enzymes modify the aminoglycoside thereby decreasing the affinity of the aminoglycoside to its target, leading to decreased potency [24]. To combat this, chemically modified antimicrobials have been developed, like isepamicin derived from gentamicin [25]. Target site modification can happen through 16S rRNA methyltransferases (RMTs) [4, 24]. These enzymes methylate either position N7 of nucleotide G^{1405}, or position N1 of nucleotide A^{1408}, making the bacteria resistant to either 4,6-di-substituted aminoglycosides (including gentamicin) or 4,6- and 4,5-di-substituted aminoglycosides [24]. Genes that code for these RMTs are e.g. *armA [4], rmtB1, rmtB2, rmtC* and *rmtD*, all found on plasmids [24]. Efflux of the antimicrobial agent in *E. coli* is mediated by a homologous transporter called AcrD [24]. AMEs are commonly found in combination with other resistance elements like carbapenemases and extended spectrum β-lactamases (ESBLs) [4, 24], creating multi-resistant bacteria.

Estimations of antimicrobial consumption between 2000 to 2010 calculated an increase of 35 %, where Russia, India, China, Brazil, and South Africa were responsible for 76% of the increase [34]. Norway has a moderate consumption of antimicrobials compared to other countries, even though the use has increased by 30% in the last decades [17]. Penicillin is the most widely used antibiotic in Norway [18], with gentamicin as part of standard treatment of BSI [15]. In Europe there is a clear north-to-south gradient, where the southern regions have a larger percentage of resistance compared to northern regions. This gradient is probably reflective of differences in infection control and antimicrobial use between countries, with inappropriate antimicrobial use being one of the factors perpetuating this phenomenon [4]. An example from 2011 is the prevalence of multi-resistance isolates, which ranged from about 1% in Estonia, Iceland and Sweden, to more than 10% in Romania, Slovakia and Cyprus [4]. *E. coli* in Europe can be seen mostly resistance to these antibiotics: β-lactams, quinolones, and aminoglycosides [4]. In 2015, the European Centre for Disease Control (ECDC) estimated the total deaths caused by antimicrobial resistance to be approx. 33,000 per year [35]. In the same period the USA and China estimated the same for their countries, and came to a conclusion of 100,000 and 80,000 deaths respectively [32]. The data available in Africa does suggest the region experiences the same increasing trend in drug resistance as the rest of the world [36]. Globally, *E. coli* is reported to have a high resistance to third-generation cephalosporins. In 2014 worldwide reports showed 0-82% and 0-98% of isolates were resistant to cephalosporins and fluoroquinolones respectively [36].

Aminoglycosides are still valuable options for many gram-negative infections, despite increased rates of resistance [16, 23, 24]. It is regarded as a globally important antimicrobial [4, 29] and is on the WHO's model list of essential medicines, a list of the medicines needed for a basic health-care system [37]. To keep the current antimicrobial resistance stable, cooperation between countries is key. Resistant bacteria know no borders, threatening all countries regardless of monitoring and healthcare systems [32].

1.3 Antimicrobial susceptibility testing (AST)

Antimicrobial susceptibility tests (AST) are designed to uncover antimicrobial traits in microorganisms. These can test for phenotypic or genotypic resistant traits through culture based or molecular methods. Rapid and accurate AST are important to stop the possible spread and selection towards resistant pathogens [7]. Testing also guides the antimicrobial treatment [38], leading to a better outcome for the patient [39]. AST is currently performed by disc diffusion, agar gradient diffusion or broth microdilutions. Disc diffusion is the most widespread routine method, broth microdilution is the gold standard [34, 40, 41]. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) has developed breakpoints for these tests, where the zone diameter from disk diffusion are calibrated to match minimum inhibitory concentrations (MIC) values from broth microdilutions [42]. To use the EUCAST breakpoints are used in a lab, they should calibrate their methods to methods used by EUCAST [43]. EUCAST has also developed a rapid antimicrobial susceptibility test (RAST) using disc diffusion for a select few bacteria, including *E. coli* and *S. aureus*. This is used only for blood cultures (BC) [44].

The principle behind disk diffusion methods is the agar's ability to diffuse the antimicrobial out beyond the disks, with a decreasing antimicrobial concentration outward from the disk. Resistant bacteria can tolerate a higher concentration of antimicrobial, thus growing closer to the disk. Susceptible or intermediate bacteria can not tolerate such high concentrations and grow further away from the disk. The zone created around the disk were bacteria can not grow is referred to as the inhibition zone (*Figure 1.2*). These diameters are standardised by organisations such as EUCAST and NordicAST. Both organisations publish documents with updated MIC-values and inhibition zone diameters used in comparison to results in diagnostic labs. MIC values and zone diameters can determine the S-I-R profile of an organisms, meaning if it's susceptible (S), intermediate (I) or resistant (R) to the antimicrobial agent tested.

The EUCAST guidelines for disk diffusion stated the use of Mueller-Hinton (MH) agar as standard growth media, with horse blood and β-NAD added for fastidious organisms. After inoculum is spread and antimicrobial disks are placed, incubation is 18 ± 2 hours [45]. A similar method to disk diffusion, RAST, was developed by EUCAST to be able to test directly from positive blood culture bottles. Undiluted BC is spread and antimicrobial disks placed, whilst incubation here is only 4, 6 or 8 hours depending on the organisms growth [46]. Agar diffusion gradient relies on the same principle as the two methods above, agar and diffusion of antimicrobial agents. It doesn't use disks, but rather plastic strips laced with an antimicrobial gradient. The bacteria grow beside the strip at low concentration and diverge from the strip at higher concentrations (*Figure 1.2*). The method and how to read the results are described in *3.2.1 Minimum Inhibitory Concentration testing*.

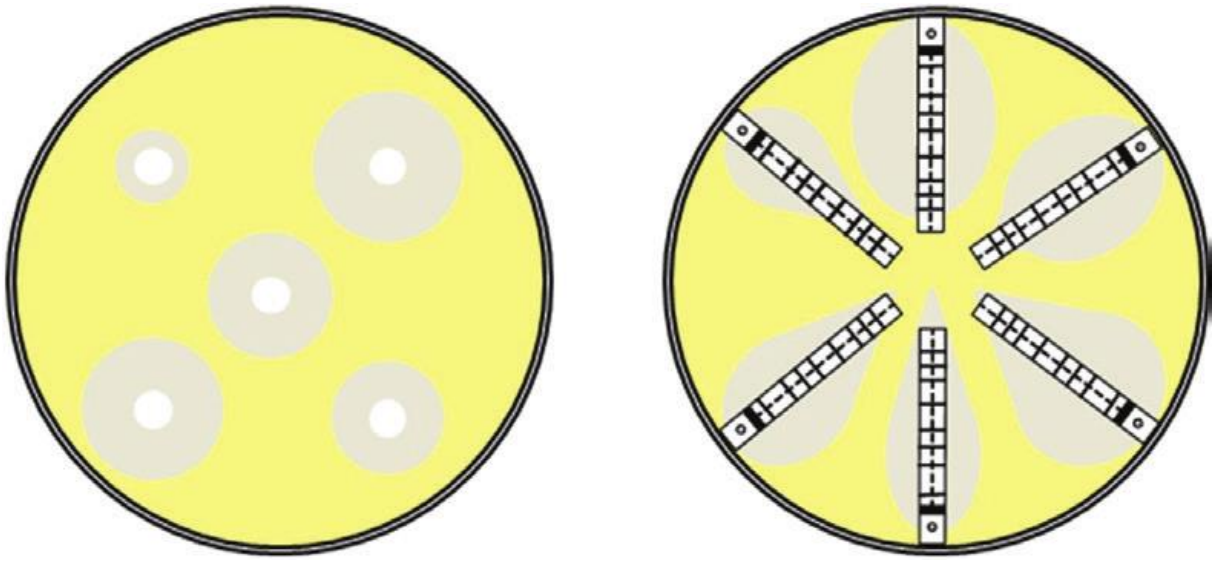

Figure 1.2 Disk diffusion and agar diffusion gradient. The illustrations to the left shows disk diffusion. White circles are the antimicrobial disks, grey zones are the inhibition zones, and yellow indicates bacterial growth. To the right is an illustration of an agar diffusion gradient. Yellow indicates bacterial growth with the antimicrobial strips in a star formation. The grey fields are the inhibition zones [47].

Broth microdilution is the reference method for AST [41, 48]. It uses another principle compared to the methods above to determine MIC-values. A bacterial solution is inoculated in a two-fold dilution series of an antimicrobial using cation-adjusted MH broth in volumes ≤ 500 mL [34, 49]. The final bacterial concentration should be $5x10^5$ CFU/mL [48]. In a full-range MIC-test five to twelve concentrations are used, whilst in a breakpoint analysis, one to three are tested [48, 50]. After an incubation period of 18 ± 2 hours the plate would be read. The lowest concentration with no bacterial growth represented the MIC-value [39] (*Figure 1.3*).

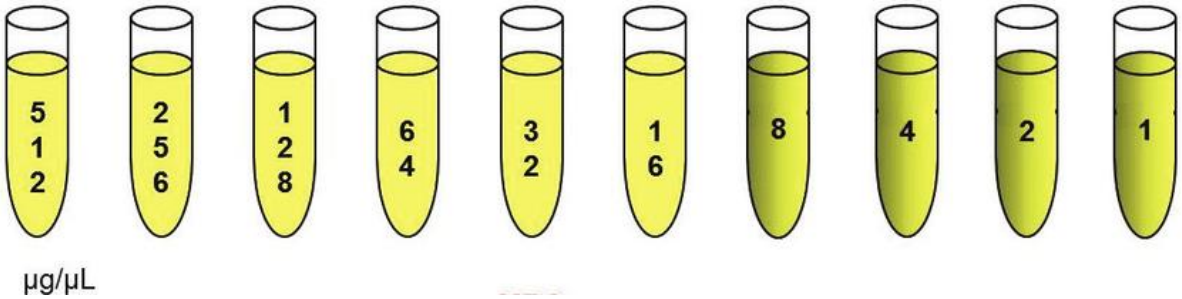

Figure 1.3 The illustration shows a broth microdilution with 10 dilutions, from 1 to 512 µg/µL. Clear yellow indicates no bacterial growth, whilst darker yellow indicates growth [47].

Positive BCs can be run with RAST and have an S-I-R profile ready in 4-8 hours for select microorganisms. In addition to RAST, gram-stain and organism ID must be assessed for the S-I-R profile to be useful. The other methods need a fresh preculture to make the inoculum for the final test, this typically takes over night from a positive blood culture.

Time is the common denominator for all these methods. Identification of eventual resistance traits of the bacteria in question should happen as fast as possible, as there is evidence for increased morbidity and mortality after delayed treatment with the appropriate antibiotic [5]. They all rely on monocultures, and in most cases a preculture to enhance the bacterial load.

There are also commercial methods being developed for RAST from BC, like the dRAST method from Quanta Matrix (Republic of Korea). The dRAST uses positive BC samples directly in a form of broth microdilution monitored by microscopic timelapse imaging. MIC values and phenotypic resistance can be available in as little as 4 hours, but information about organism ID and gram-type must be known beforehand (Quanta Matrix's homepage https://www.quantamatrix.com). A similar product on the market is the ASTar by Q-linea (Sweden). It also uses positive BC samples, the ID can be input at any time and is only needed to calculate the results of the assay. The bacteria are isolated and inoculum using media for both normal and fastidious bacteria is made. The inoculum is then placed in the AST cartridge to incubate, whilst monitored by an optical detection system. MIC values are available after approx. 6 hours (Q-lineas homepage https://www.qlinea.com/). Both these methods support several samples running at the same time as well as random samples initiation.

There are also some PCR-assays like SepsiTest (Molzym, Germany), SeptiFast (Roche Molecular System, Switzerland), and PLEX-ID (Abbot Molecular, USA) [14] on the commercial market. They all test for genotypic resistance and organism ID directly from blood samples using either a multiplex or a broad range PCR, sometimes combined with other techniques like sequencing or electrophoresis [14].

1.4 *Escherichia coli*

Escherichia coli is the most common cause of BSI in humans and the most researched bacterium available. *E. coli* is a species of the *Enterobacteriaceae* family, under the gamma subdivision of the phylum *Proteobacteria* [4]. It is a gram negative, non-spore forming, rod bacteria, 1 – 4 µm x 0.5-1 µm in size [51, 52]. It has one chromosome located in the cytoplasm, containing approx. 5000 genes [52, 53], and it can have several plasmids with additional genes. Its natural habitat is the colon of warm-blooded animals, like humans, but it may survive in a range of other habitats like water, soil, and food [4, 54, 55]. Normally a harmless commensal bacterium, there are pathogenic strains responsible for disease [4, 55]. They can be divided into two main groups, intestinal pathogenic *E. coli* (IPEC) and extraintestinal pathogenic *E. coli* (ExPEC) [38, 55, 56]. They can be further categorised based on certain pathogenic traits [4]. ExPEC groups included meningitis-associated *E. coli* (MNEC), uropathogenic *E. coli* (UPEC) and septicaemia-associated *E. coli* (SEPEC) [55, 56]. ExPEC is now viewed as one whole group, where strains contain at least two of the following virulence factors: *papA* and/or *papC, sfa/foc, afa/draBC, kpsM II* and *iutA*. This change came about after isolates assigned to one ExPEC group were observed causing infections in other anatomical sites [38].

An example of ExPEC's course of disease is colonization of the bowel by a pathogenic strain, which by itself does not cause an immediate threat. Disease may occur if the strain ends up somewhere it shouldn't be, such as the urethra. If *E. coli* moves from the intestine to the urethra an environmental selection for the best adaptations takes place.

If the bacteria then get a foothold it may lead to urinary tract infection (UTI). Untreated UTI may develop into pyelonephritis [51], leading to damage of the proximal tubules. This gives the bacteria access to the bloodstream, which may lead to bacteraemia [56], BSI and sepsis [38]. *E. coli's* ability to enter the blood stream through the mucosal cells in the intestine [38] makes it able to enter the blood stream directly from the intestine as well.

In neonates with Gram-negative meningitis, ExPEC is the most common cause [4, 51], and is often preceded by high-level bacteraemia [38]. The fatality rate is approaching 40%, whilst up to 50% of survivors experience severe neurological defects [38, 56]. It appears as if early-onset sepsis in infants with *E. coli* is increasing, whilst the incidences caused by Gram-positive organisms are decreasing [56].

1.4.1 Replication and growth of *E. coli*

E. coli is a fast-growing bacterium in optimal conditions, with a generation time as short as 20 min [52]. The parameters temperature, oxygen, pH, osmotic pressure and access to nutrients are parameters must be regulated for optimal growth [57]. *E. coli* reproduces through binary fission [52] (*Figure 1.2*). Division starts when the bacterial cell attains a critical mass/volume, and the first step is replication. Replication of the chromosome starts at every copy of oriC when the initiation potential is reached [58] (*Figure 1.2 1*). The old strands of the chromosomes are methylated, whilst the new strands are not. Methylation of the new strands takes minutes, except for the oriC region, where it takes 30-40% of the cell cycle, meaning another replication cannot take place immediately [58]. Partitioning of the cell commences immediately after replication, with the two sister chromosomes are relocated to each end of the cell [58] (*Figure 1.2 2*).

The rod-shaped bacterium divides in the middle with the help of FtsZ proteins [58, 59]. The placement of the FtsZ proteins is decided by the DNA concentration, either the middle, or the end with lower concentration [60]. The Min system blocks division at the cells poles, so the only option for FtsZ proteins would be midcell [60] (*Figure 1.2 3*). FtsZ form a Z ring at the point of division on the inside of the cell membrane. The Z ring's circumference decreases as the septum is formed, with the ring disappearing when division is complete [58] (*Figure 1.2 4 and 5*). The FtsZ ring itself does not exude enough force to pull in the cell wall and membrane. The force thought to be responsible for creating the septum is rather cell wall synthesis [59]. Other proteins are also required for division, such as FtsA, FtsQ, FtsI(PBP3), FtsW, and FtsE, but FtsZ is arguably the most important component for division [58-60]. FtsZ is also widely conserved between species. It is present in almost all eubacteria, most archaea, and is found in the organelles of many eukaryotes [60]. Growth is continual except during chromosome replication, partition of sister chromosome and cell division [58].

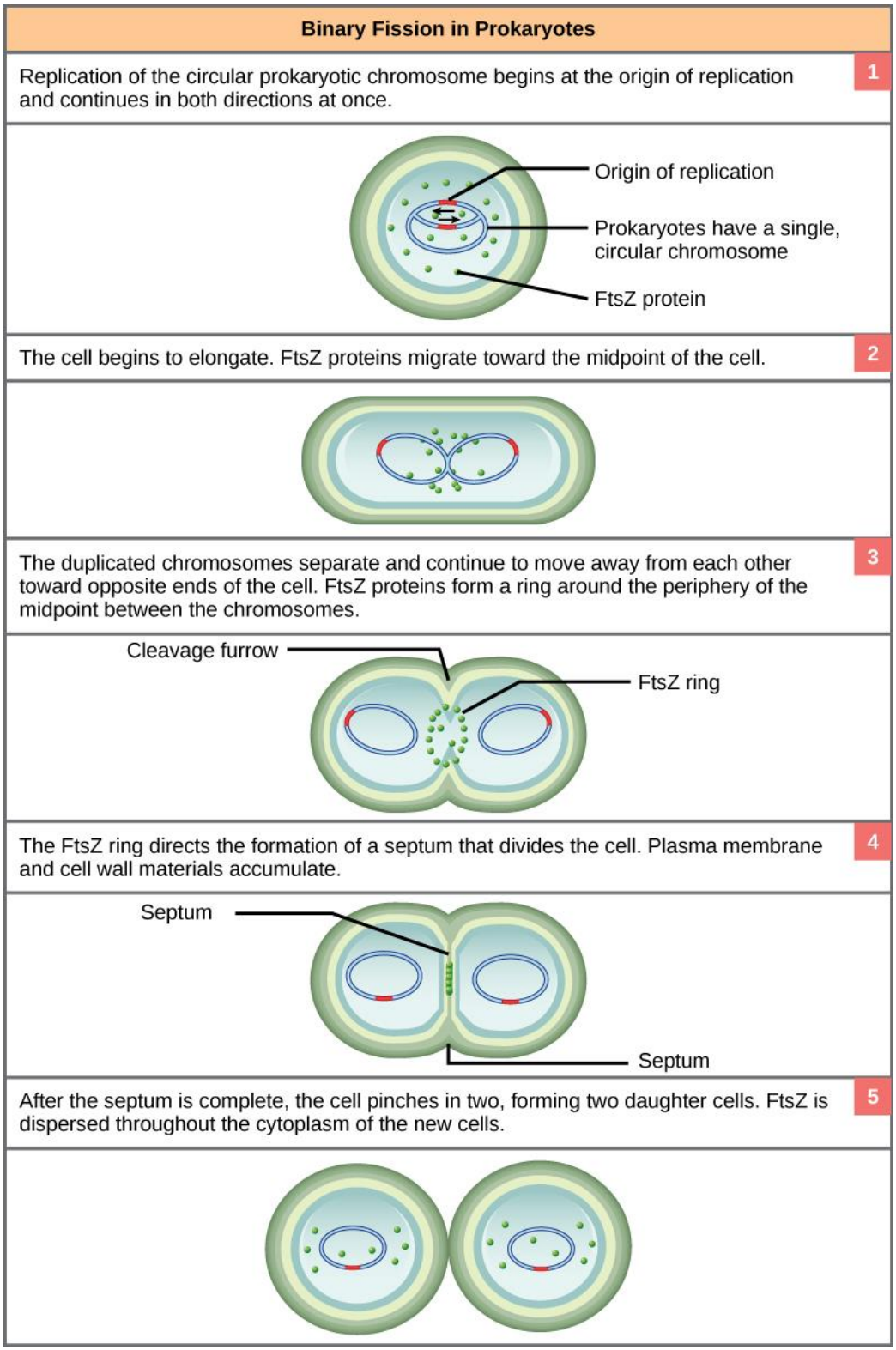

Figure 1.4 Displaying binary fission, the method of which bacteria divide. 1. When the cell reaches a critical mass, replication of the chromosome starts at the oriC (origin of replication). 2. When the sister chromosomes start to separate, FtsZ proteins start to accumulate midcell. 3. The sister chromosomes separate and are transported to each end of the cell. FtsZ proteins form the Z ring on the inside of the cell membrane midcell, in between the chromosomes. A cleavage furrow forms as a precursor of the septum. 4. Cell wall synthesis in cooperation with the Z ring divides the cell in the middle. 5. When division is complete two daughter cells are formed, each with a chromosome and FtsZ. [61]

1.4.1.1 Growth curves

Growth curves can be made to visualise the bacterial growth. These graphs plot time and growth measurements, such as optical density (OD) or colony forming unites pr. millilitre (CFU/ml), against each other to make a visual representation of the amount and speed of growth [52, 57, 62, 63]. In microbiology, growth is often measured by spectrophotometer at 600 nm (OD600) [64], as it has been shown to correlate well with the number of bacteria in suspension. McFarland is also used in microbiology to determine the number of cells in a solution as it has a direct relationship to CFU/ml [65, 66]. An increased turbidity reflects an increase in cell density. One should note that OD and McFarland measurements do not distinguish between live and dead cells [63], and neither is a definite measure of the number of cells in a solution. Plate spreading is a more direct count, although more time and labour intensive, it does distinguish between live and dead cells [52]. It is important to understand growth curves, to be able to interpret changes to them, such as antimicrobial addition [63].

A growth curve in a closed system can be split into four stages, lag, exponential, stationary and death phase [62, 63] (*Figure 1.3*). Lag phase is a rearrangement phase where the bacteria often need to adapt to a new milieu or condition [52]. Changing conditions are not optimal, therefore growth will be slow to none in this phase [62]. When the bacteria have adjusted, the growth rate picks up and the exponential phase begins [52]. In this phase, the growth rate is exponential with bacteria dividing freely [62]. Nutrients and oxygen are readily available, and the pH and osmotic pressure are not changed if the temperature is stable. As the resources in the closed system deplete, the growth will slow to the stationary phase [52, 62]. The population is no longer rising, but there are a sustained number of cells as some start to die but replication still occurs. Nutrients become sparse, pH, osmotic pressure changes occur and waste products build up as cells start to die, making the conditions no longer optimal [52]. This leads to the last phase, the death phase. As resources are spent, growth declines and the rate of cell death increases [62]. The dead cells decompose somewhat, returning some resources back into the system but not enough to increase growth.

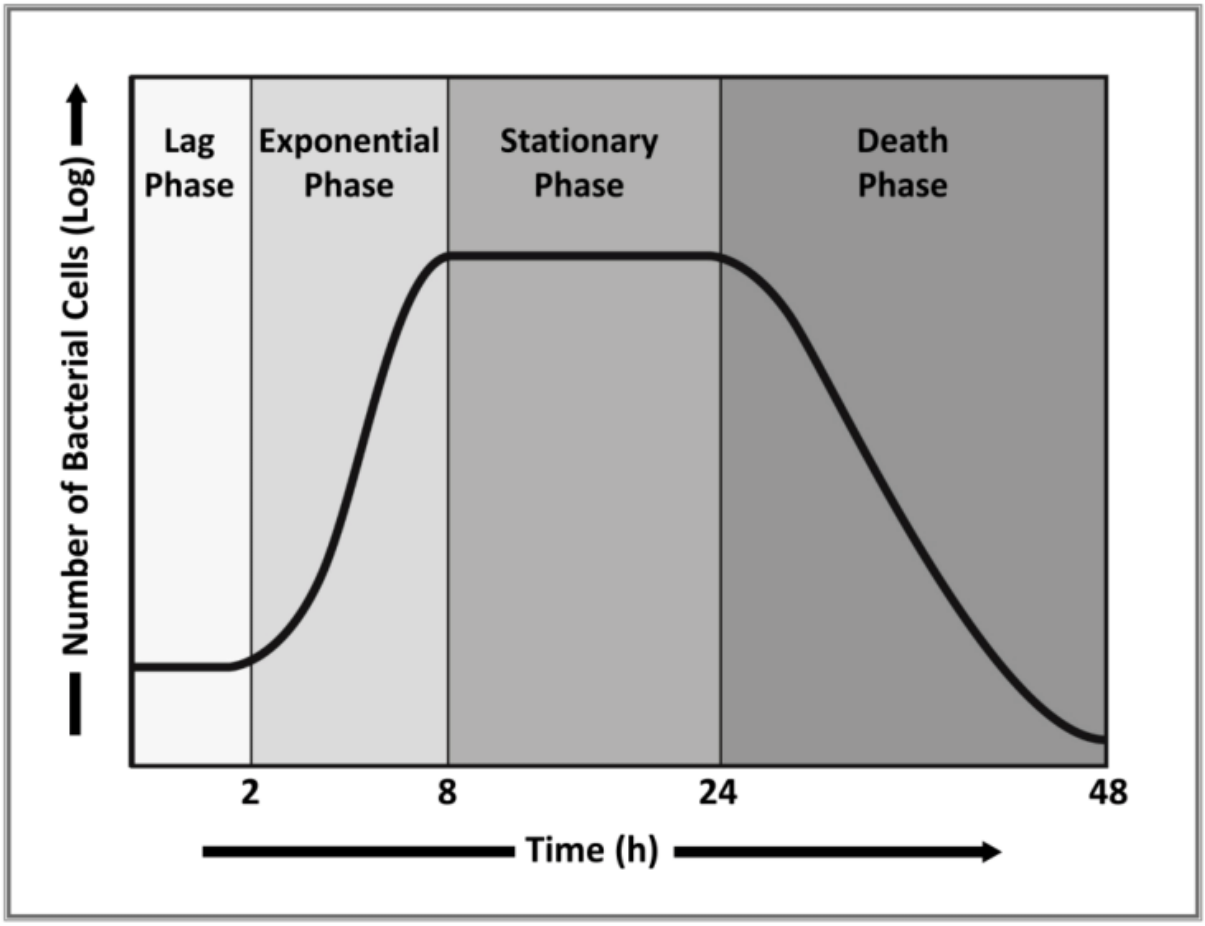

Figure 1.5 An example of a growth curve , showing the four phases; lag, exponential, stationary and death. The number of cells is plotted against time [67].

1.4.2 Genome

E. coli possesses one haploid circular chromosome, highly condensed and organised into a nucleoid (a nucleus-like form lacking a nuclear membrane) [68]. By weight the nucleoid consists of 80% genomic DNA, 10% RNA and 10% protein [68]. The circular genome is organised into supercoils, half of which are free plectonemic supercoils (imagine twisting a hair tie), the other half a mix of forms like the toroidal form (similar to a curly telephone wire) and bound plectonemic supercoils (like above only bound by proteins) [68]. There is another element to the genome, plasmids. Plasmids are extrachromosomal rings of DNA, they are self-replicating and spread through horizontal gene transfer [69, 70]. Some plasmids may be linked to specific phenotypes, such as antibiotic resistance, whilst others have no apparent function [69].

The size of the genome varies between isolates, but an approximate size is around 5,000 kilo base pairs (Kbp), harbouring approximately 5000 genes [53]. *E. coli* shares its core genome with related genera like *Citrobacter* and *Salmonella* [53]. The core genome is conserved, with interspersed variable regions for accessory genes called genomic islands [53]. These genomic islands encode lifestyle-specific functions, with related functions often clustered in the same genomic island [53]. The genomic islands are acquired through horizontal gene transfer, and have a different C+G content then the rest of the genome [53]. There exists a genomic gene pool in nature where natural selective forces

modify and influence the available genes [69]. A calculation has set the gene pool to contain more than 13,000 genes, which has an enormous impact on *E. coli's* diversity and ability to cause disease [71].

1.4.2.1 16S rRNA gene

16S rRNA genes are found in all bacteria [72-74], and together with proteins the 16S rRNA makes up the 30S subunit of ribosomes. The 16S rRNA gene is built up of nine variable regions flanked by ten conserved regions [74-76] (*Figure 1.4*). For years, this gene has been used for taxonomic assignment and phylogenetic trees [77]. The 16S rRNA gene accumulates mutations at a constant slow rate, where the variable regions are different between different bacteria [72, 74, 75]. Based on the variable regions sequences it is possible to distinguish one bacteria species from another [75]. The conserved regions are conserved between species, making it ideal for universal primer design, and it is now the most used gene marker for genotypic bacterial community profiling [72, 74, 75]. Regions V1, V2 and V6 have the greatest diversity between species according to Coenye and Vandamme [78], whilst Chakravorty, Helb et al. site V2, V3 and V6 as best to distinguish species [75]. Other rRNA genes that can serve the same function for bacterial detection are 5S and 23S, along with the intergenic space between 23S and 16S [72]. The 16S rRNA gene is still widely used because of its presence in at least one copy in every genome, the conserved regions enable easy identification through PCR and information about bacterial family, genus and species if sequenced [77].

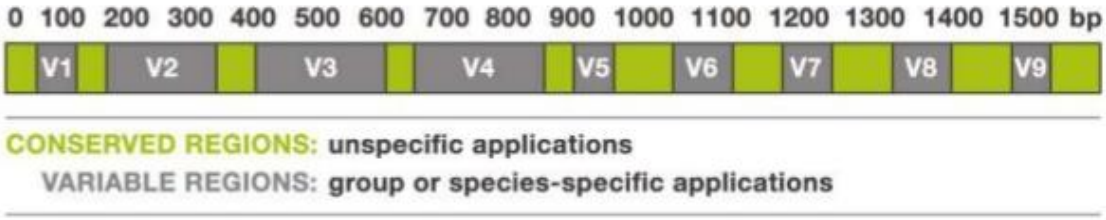

Figure 1.6 The different regions of the 16S rRNA gene. Gray represents the nine variable regions, flanked by ten green conserved regions [79].

The distribution of copy numbers of the 16S rRNA gene in bacteria is not identical in all species. Some taxa have up to 15 gene copies, whilst others make do with one or two [73]. It seems the copy numbers in species are the same, although some variation have been seen between strains [73]. When bacteria have several copy numbers of the 16S rRNA gene they are not necessarily identical, with the number of variants estimated to be 2.5 times greater than the number of bacterial species [73]. Calculations of the relative amount of bacteria in complex populations become unreliable based on 16S rRNA sequencing, because of the difference in copy number and in sequence [73].

1.4.3 Genotypic/phenotypic detection

Detection and typing of bacteria have been practised for decades by phenotypic tests. These rely on the bacteria's different expressed traits to differentiate them, known as phenotypic detection. Example of phenotypic tests are AST disk diffusion and immunological serotyping [72, 80, 81]. These tests can be time consuming and very

hands on. Another form of detection is genotypic. This relies on the presence of DNA or mRNA from the bacteria, and by detection of specific genes or sequences, strains and groups can be differentiated. This can be done by PCR, alternatively with an additional sequencing step or other methods like pulse-field gel electrophoresis (PFGE) or whole genome sequencing [80].

Some genotypic test results can easily be quantified and standardised between different bacteria, and are generally more specific than phenotypic markers [82]. It is also fast, and easy to perform [81]. Genotypic determination, especially related to resistance, still has some limitations. The presence of a gene does not mean the expression of that trait. A bacterium might contain a resistance gene but not express it [81, 83]. An explanation of this might be that environmental factors affect gene expression, leading to a down regulation and a decrease in the phenotypic expression [83], or mutations causing the products loss of intended effect. In regards to antibiotic resistance this means genes can be present and dormant until an antibiotic is detected in the environment, leading to an upregulation of the gene when needed, or when upregulated the product does not work. The genotypic tests are faster and may give a preliminary hint, but as the presence of resistance genes not automatically means expression, genotypic results should be validated with biochemical tests. [84].

1.4.4 Antimicrobial resistance of *E. coli*

In Norway, most *E. coli* are susceptible to regular antimicrobials, such as tetracyclines, sulphonamides, trimethoprim, chloramphenicol, ampicillin, cephalosporins, mecillinam, quinolones and nitrofurantoin. They tend to be intrinsically resistant to therapeutic levels of penicillin G and V [4, 51], but research indicates resistance exists for all major groups of antibiotics [31]. In Norway the percentage of *E. coli* isolates resistant to gentamicin, or ciprofloxacin, reported in 2019 to be 5.8 %, 11.3 % respectively, with ESBL producing isolates at 7.1% [85]. Bacteria resistant to three or more antibiotic classes are deemed multi-drug resistant (as long as the resistant isn't intrinsic to the species) and is on the rise world-wide [4]. An example is the ExPEC isolate ST131, resistant to both aminoglycosides and fluoroquinolones, with ESBL [31, 38]. For *E. coli* strains that harbour no resistance genes or mutations, treatment options are penicillins, cephalosporins, fluoroquinolones, β-lactam/β-lactamase inhibitor combinations (BLBLI) and aminoglycosides [31]. In Europe *E. coli* is mostly seen resistance to these antibiotics: β-lactams, quinolones, and aminoglycosides [4]. Globally, *E. coli* is reported to have a high resistance to third-generation cephalosporins. From 0 to 82 % of isolates were reported resistant to cephalosporins worldwide in 2014. In the same period resistance to fluoroquinolones were reported in 0-98 % of isolates [36].

1.5 Polymerase chain reaction

From its birth in 1985, polymerase chain reaction (PCR) has become one the most fundamental tools in the molecular biology lab, earning its inventor, Kary B. Mullis, the Nobel prize in Chemistry in 1993 [86-88]. Today the technique is widely used, from forensics, and environmental biology to research, and medical diagnostics. The technique amplifies DNA or RNA through temperature cycles, primers, and polymerases. Through the years it has been improved and fine-tuned, now including several different iterations, like Reverse-transcription PCR (RT-PCR), real time PCR, and digital PCR (dPCR). This project will revolve around Real-Time PCR for bacterial detection.

The basic components of a PCR are DNA/RNA template, polymerase, a primer pair, Mg^{2+}, deoxynucleotide triphosphates (dNTP) and a temperature cycle. The temperature cycles initiate three stages: denaturation, annealing and extension. In stage one DNA denatures into single strand DNA (ssDNA) at approx. 95°C. In stage two primers anneal to the ssDNA at approx. 50-65 °C depending on the primers. In stage three polymerase extends the 3'-end of the primers at around 70-75°C. This happens in cycles, from 20-40 times, each cycle doubling the amount of target DNA [89]. This means small amounts of DNA/RNA can be copied exponentially, into large amounts, which could be analysed in different ways, or just detected through a Real-Time PCR. To make a PCR Real-Time the inclusion of a dye or fluorophore is needed. This makes the amplification products visible whilst the reaction is running.

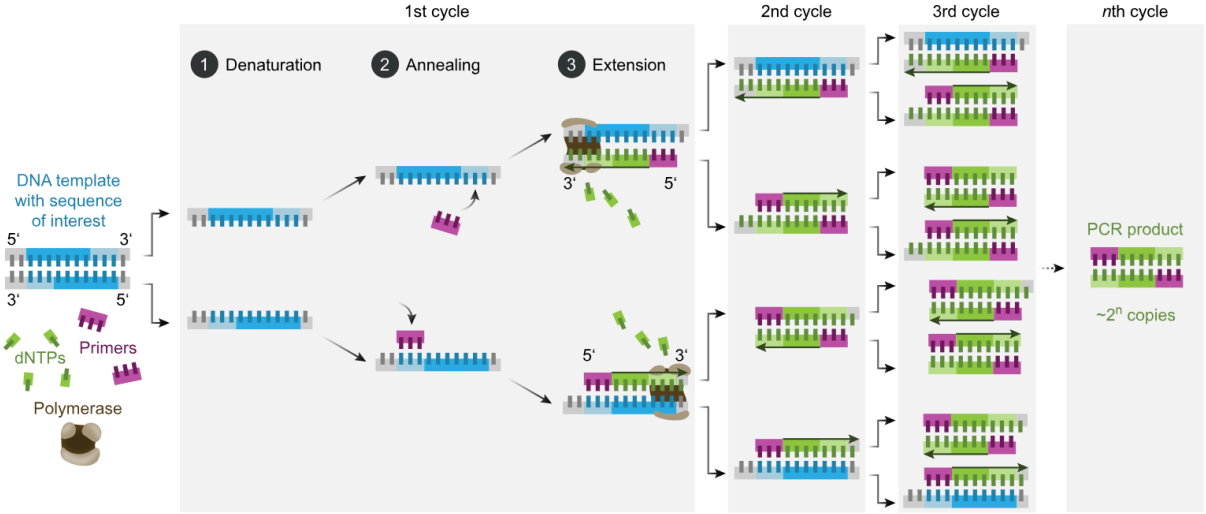

Figure 1.7 A visual depiction of PCR. The starting components are DNA template, deoxynucleotide triphosphates (dNTPs), primers and polymerase. Through several thermal cycles, normally 20-35, the region of interest is copied exponentially. A cycle consists of tree stages, denaturing, annealing and extension. The first step denatures the DNA, the second anneals the primers to the ssDNA, and the third extends upon the primers by way of the polymerase [89, 90].

1.5.1 Primers

Primers are short oligonucleotides, approx. 20 base pairs (bp) in length. They are often used in pairs, where one corresponds to the 3' end of the coding stand, and the other to the 3' end of the template strand. They are integral to PCR as without them the polymerase would have nowhere to start amplification. To design primers, one has to know the sequence of the site, meaning primer sites can't be unknow, but the sequence between a pair can be unknown. PCR is therefore not ideal for entirely novel sequences.

Primer design will not be discussed in depth as it was not part of this project, but some important things to consider are: length of primers, placement of primers which determines product length, melting temperature, GC content, 3'end stability, and amplification efficiency [91]. Some other factors to consider is what one wants to detect, DNA, RNA, or both, and for what the amplification product is to be used for [91].

1.5.2 SYBR Green

In Real-Time PCR, amplification of template is detected by fluorescent techniques after each cycle, visualising the reaction progress continuously. Hydrolysis probes such as TaqMan are specific and will only fluoresce when its specified target has undergone amplification. SYBR Green is a non-specific dye, adhering to any double-stranded DNA (dsDNA) (*Figure 1.6 A and C*) [92]. When unbound, SYBR Green will not fluoresce. When bound to DNA it will absorb blue light (λmax= 497 nm), and emit green light (λmax= 520 nm) (*Figure 1.6 B*) [91]. In Real-Time PCR the SYBR Green fluorescence signal is measured in the extension phase for each cycle [91]. SYBR Green being a non-specific dye, will bind to the minor groove of all dsDNA with no regard to its source [91]. Primer dimers and other non-specific products are components SYBR Green will bind to that might skew the results. It can seem as if there is more target amplification than there actually is. There is therefore a need for optimisation of the assay to ensure minimal production of non-specific amplification products [82].

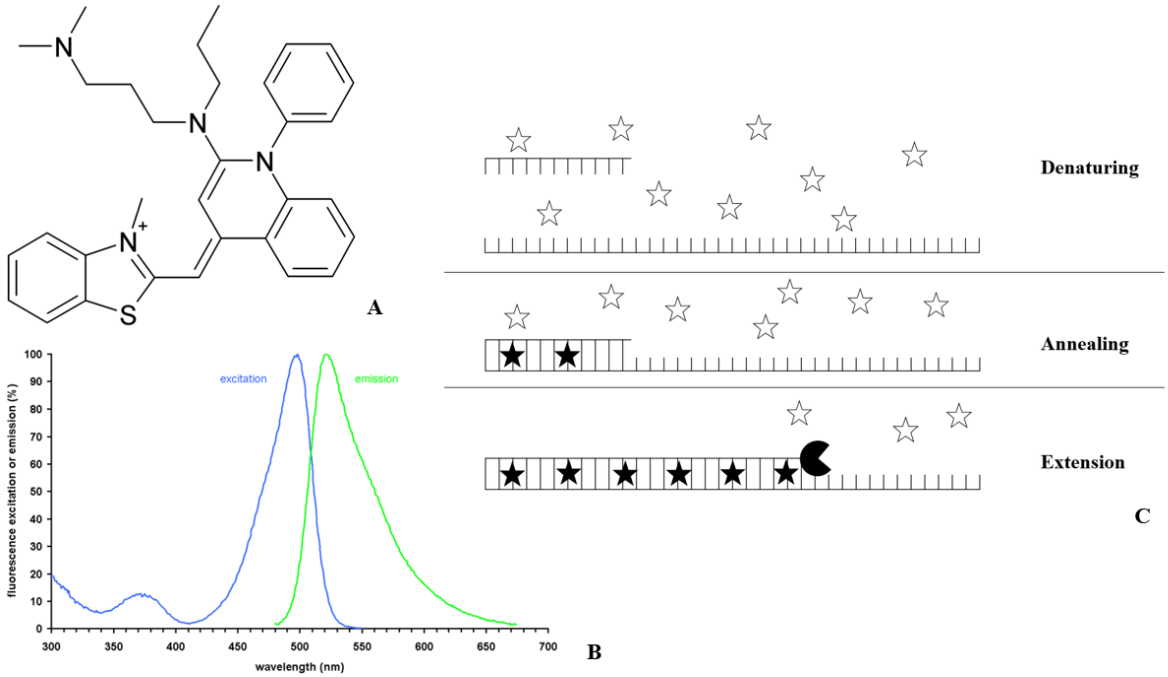

Figure 1.8 SYBR Green information. A. Chemical structure of SYBR Green I [93]. B. Emission and fluorescent excitation spectra for SYBR Green I [94]. It absorbs blue light (λmax= 497 nm) and emits green light (λmax= 520 nm). C. Simplified drawing of SYBR Greens attachment to DNA. Blank stars = inactive SYBR Green, black stars = active SYBR Green. ¾ circle = polymerase. Short ticked line = primer. Long ticked line = amplification target. SYBR Green is free in solution and inactive when the DNA is denatured, in the annealing face it starts to bind if a minor groove forms at the newly attached primer. As the polymerase synthesises new DNA in extension face, SYBR Green attaches to the minor groove, and is now able to be excited and fluoresce.

As the concentration of dsDNA increases with each cycle, the fluorescent signal from the dye increases proportionally. The reaction is exponential, each cycle doubling the number of amplification products. The increase in fluorescent signals is displayed as an amplification plot (*Figure 1.7*). The horizontal green line indicates the threshold, separating noise from fluorescence from synthesised amplification product. Lines under the threshold are the baseline, where fluorescence from synthesised amplification product can't be distinguished from noise. The point where a plotline crosses the

threshold indicates its Cq-value. Cq-value is the number of cycles it takes for a sample to be amplified above the background noise.

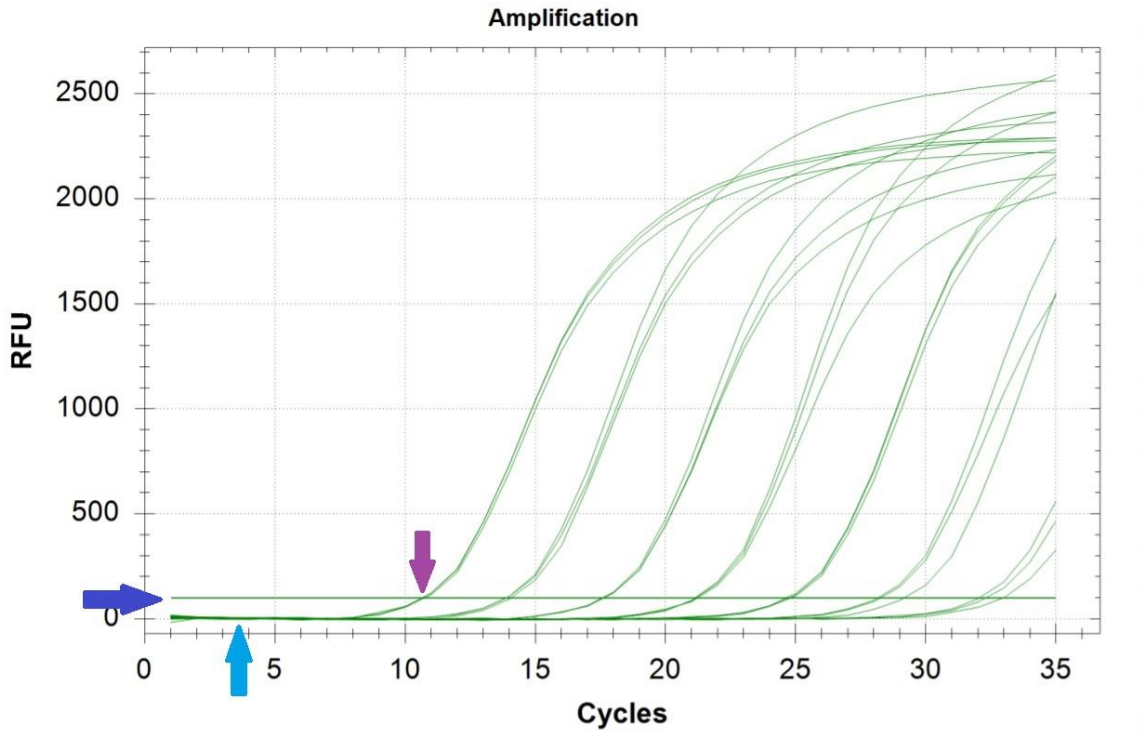

Figure 1.9 Amplification plot showing baseline (light blue arrow), threshold (dark blue arrow), and Cq (purple arrow) for the first plot (Own data).

Compared to other methods like probes, SYBR Green is usually the cheaper option [91]. SYBR green can also be used with different sets of primers since it is not specific to one sequence unlike hydrolysis probes. Despite its popularity SYBR Green I is not the most stable dye, contributing to dye-dependent PCR inhibition.

1.5.3 Melt curve analysis

Melt curve analysis can be done as an additional step in a Real-time PCR, as an alternative to gel electrophoresis for product analysis [95]. The amplification products are put through a range of temperatures, including the melting temperature of the product, whilst the fluorescence is continuously monitored [96]. The product's GC contents, length and sequence determine their melting points. This can be used to separate amplification products by their melting temperature, with less difference than 2 °C [95]. Non-specific products and amplicon can be differentiated based on these results [82, 91, 95, 96]. Negative controls are added to compare is there are contaminants in any of the reagents [82].

1.5.4 Real-Time PCR in diagnostics now

Real-Time PCR has a place in the molecular microbiology lab at the present. It can detect and characterise viral, bacterial, fungal, and protozoan nucleic acids [82, 84]. With the increasing amount of available data, it could be possible to develop a PCR for every

microorganism. It is also fast and has a high-throughput, with detection of nucleic acid sequences from different matrices [84]. The technique is especially useful for viruses, as they are notoriously hard to grow in many cases, and Real-Time PCR eliminates the need for culturing with viruses. It is also very advantageous to be able to quickly determine if an infection is viral or not, which can lead to better targeted therapy [82]. In bacteriology Real-Time PCR has been used to detect bacteria, type strains and isolates, look for the presence of specific genes and alleles, like resistance genes and toxin production [82, 84]. They can in many cases be faster than the standard method of culture and biochemical testing, leading to an early recognition and treatment [82]. For public health, early identification for optimised treatment is of tremendous value [82].

1.5.5 Broadrange 16S rRNA gene PCR

The 16S rRNA gene being universally present and conserved in all bacteria, has made it a popular universal target for bacterial PCRs. The conserved regions have made it possible to design universal primers that will detect every bacterium, whilst sequencing of the variable regions has the power to differentiate between a large amount of bacterial species/families [75]. Due to the universal design, 16S PCR assays are sensitive to contamination [72]. Especially when working with low concentration samples, small amounts of contamination might overshadow the sample due to PCR core ability, nuclei acid amplification. A universal 16S PCR would pick up any contamination by other bacterial DNA, simply because it is designed to work with as many bacterial species as possible. Contamination has led to false-positive results, and thus to a more complicated clinical interpretation, with little benefit for the patient. It is therefore important to limit the opportunities where contamination might happen [72]. There have been examples of contamination by bacterial DNA found in commercially available BC bottles, even though they are considered sterile [72]. To reduce the chances of contamination, sample preparation should be as simple as possible. Reagents should be of high quality and might be pre-treated with UV-radiation or filtering to remove any possible contamination. Results from these assays should be interpreted in a relevant biological setting to determine if they can be clinically plausible.

1.5.6 MIQE guidelines

Earlier there was not much of an agreement on what should be included when writing papers about PCR. The MIQE guidelines were therefore made to serve as a guide for anyone writing such papers. They state that information about the following must be included: experimental design, sample, DNA/RNA extraction, reverse transcription if used, PCR target information, PCR primers, PCR protocol, PCR validation and data analysis [91, 97].

2 Aim of study

The aim of this project was to evaluate the use of a Real-Time PCR-method for molecular characterization of phenotypic susceptibility of *E. coli* isolates from bloodstream infections to the aminoglycoside gentamicin, a part of the standard sepsis regiment in Norway.

According to our hypothesis, this method has the ability to be faster than the standard method of antimicrobial susceptibility testing (AST) in use now, like disc diffusion and microbroth dilutions.

Specific objectives:

- Establish a Real-Time PCR protocol for the detection of the 16S rRNA gene
- Evaluate a fast boiling lysis method for *E. coli* cultured in Mueller-Hinton (MH) broth
- Establish a method for optimal incubation of *E. coli* in MH broth to determine resistance or susceptibility to gentamicin by Real-Time PCR analysis

This could serve as a starting point for St. Olavs hospital to further evaluate if the methods developed here have a place in diagnosing antibiotic susceptibility in blood cultures.

3 Materials and Methods

Here the methods used in this project are presented. From an overview of the literature searches, information about the bacterial strains used, how growth experiments and DNA preparations were performed and the optimising and use of a Real-Time PCR assay.

3.1 Literature search

Scientific literature for this project was found through a series of searches in Google, Google scholar and PubMed. Search for primers was done using the search terms *'16S primer qPCR E. coli'*, *'16S primer qPCR universal'*, and *'16S primer qPCR efficiency'*. Primers were located both in the primary articles and their references. Some literature was already collected and available from previous projects and supervisor's collections. Searches for other topics were done by snowball literature search; starting with articles from the project description, relevant references were chosen from their bibliographies, then relevant references were chosen from their bibliographies and so forth. A couple of systematic literature searches were performed in the later stages of the project. The following query is an example of a systematic PubMed search: *((((((("Escherichia coli"[Mesh]) OR ("Escherichia coli")) OR ("E. coli")) AND ((((("Microbial Sensitivity Tests"[Mesh]) OR ("Microbial Sensitivity Tests*")) OR ("antibiotic Susceptibility Tests*")) OR ("antimicrobial Susceptibility Tests*"))) AND (("optical density") OR ("OD"))) AND ((("Clinical Laboratory Techniques"[Mesh]) OR ("Clinical Laboratory Techniques*")) OR ("Clinical Laboratory Test*"))*.

3.2 Bacterial strains

E. coli was selected as the model bacteria for this study because of it being the most common cause of BSI, and the ease of obtaining BSI strains from the St. Olavs hospital laboratory. Since *E. coli* is the most commonly used model organism this also means there is an immense amount of research data available. A total of fourteen strains of *E. coli* were used in this study, thirteen of whom were isolated from patients with BSI. The thirteen strains were made available from the Department of medical microbiology at St. Olavs hospital with no other information than their BSI-origin and antibiotic susceptibility profile. Strains were selected based on their susceptibility to gentamicin, ether susceptible or resistant. One of these, denoted Res in this thesis, was used as a control for gentamicin resistance. The additional twelve strains were denoted A to L. The fourteenth strain, denoted Sus in this thesis, CCUG 17620 (same as ATCC 25922), was retrieved from NTNU's *E. coli* strain collection, and was used as a control for gentamicin susceptibility.

3.2.1 Minimum Inhibitory Concentration testing

To determine the level of gentamicin susceptibility of the *E. coli* strains in this study their MIC-values were measured using agar diffusion gradient method. The method was performed in cooperation with an experienced staff member at Department of medical

microbiology, St. Olavs hospital. Fresh blood agar plates (Oxoid blood agar base CM0271 with ox blood) of all strains were prepared and the next day suspensions of bacteria in sterile saline were made and adjusted to a concentration of 0.5 McFarland with additional saline or bacteria. The suspensions were spread on MH agar plates with cotton swabs on a rotating plate inoculator. MIC-test strips with gentamicin (Liofilchem, Roseto degli Abruzzi, Italy) were placed one on each plate. The agar plates were incubated at 35 °C over-night, for approx. 18 hours, before they were read. Values were read as geometric sequence of two, example: 2, 4, 8, 16, 32..., and if values were in between the higher number was read. Cut of values set for gentamicin by NordicAST were at 2 mg/L [98]. All culture media was delivered by the Department of medical microbiology, St. Olavs hospital.

3.3 Growth experiments

Analysis of growth curves for all the *E. coli* strains was done to determine the minimal time it would take for there to be a large enough difference between susceptible and resistant strains in growth. Again, speed is important, so the time it takes to determine the result should be as short as possible.

As the experiments related to determining antibiotic susceptibility it was decided to follow the standard method for susceptibility testing recommended by EUCAST [99], as closely as possible. MH broth was used as the standard growth medium. The broth was supplied to the Department of medical microbiology, and they made the final product. Supplier for the initial part of the study was Gibco (Thermo Fisher Scientific, Waltham, USA). For the final part supplier was changed to Merck (Darmstadt, Germany). Some experimentation regarding volume of broth and size of growth containers were done. Depending on the amount of bacterial suspension needed for each experiment, 25 to 150 ml of MH broth in one 50 to 200 ml conical flask was chosen for optimal growth conditions.

3.3.1 Measurements for growth curves

To make growth curves, turbidity was measured at set time intervals, where increasing turbidity reflects an increase in cell density. Turbidity was measured by a densitometer and a spectrophotometer. McFarland was measured by the densitometer Den-1 (BioSan, Riga, Latvia), the turbidity of the sample was measured by 565 nm light through a glass tube containing the sample. To measure optical density a UV-1700 spectrometer (Shimadzu, Kyoto, Japan) was used with a 70 µl micro cuvette (Brand, Frankfurt, Germany). It measured the optical density (OD) at 600 nm, displaying the results as OD values at OD600 [100]. A test of the 70 µl micro cuvettes was done as initial measurements were not consistent. A large number of blank measurements, approx. 170, were done with MH broth, ranging from 70 µl to 600 µl all at once (the 70 µl cuvettes were large enough to accommodate larger volumes). 200 µl seemed to give more precise measurements and was utilised throughout the project.

The starting concentration of the growth flasks was set to 0.1 OD, as it was close to the concentration of a positive BC [101]. Samples for OD600 and boiling lysis were taken for every experiment. McFarland was deemed redundant for the last growth experiment but was measured for every former experiment (*Table 3.1*). In one experiment clear MH broth was included to check it the colour of the broth changed throughout the incubation period. A total of eight experiments were done, were six were analysed by Real-Time PCR (*Table 3.1*).

Determining the lag and exponential phase was done by visual analysis of the graphs. The steepest part of the graph was identified visually and established as the exponential phase. The length of the exponential phase was determined to be the length of the steepest part in time. Using a ruler, the length of the steepest part of the graph was set. The lag phase was established as the amount of time preceding the exponential phase.

Table 3.1 Here the eight different growth experiments are shown , along with what measurements and analyses were performed for each experiment.

Growth experiment	**I** (Failure)	**II**	**III**	**IV**	**V**	**VI**	**VII**	**VIII**
PCR		X	X	X	X	X	X	
McFarland		X	X	X	X	X	X	
OD600	X	X	X	X	X	X	X	X
Boiling lysis		X	X	X	X	X	X	
EZ1							X	

3.3.2 General method for growth experiments

The chosen E. coli strain was inoculated on a blood agar plate either from frozen form or from an older plate. The new plate was incubated over night at 37 °C. The next day an overnight (ON) culture was made by picking one distinct colony from the plate and inoculating it in MH broth, 25-150 ml, in a conical flask. The ON culture was incubated at 37 °C at 200 revolutions per minute (rpm) over-night, for 18-24 hours. The following day growth flasks were made: the required amount of MH broth was pipetted into conical flask and put in a heating cabinet for 30 min. A bacterial suspension of 25-150 ml was made from the heated MH broth and the ON culture. It was made in a 50-200 ml conical flask by adding 0.5 – 4 ml ON culture to the heated MH broth. The concentration would be tweaked with bacterial suspension or MH broth to reach a concentration of 0.1 OD ± 0.01 OD when necessary. If the strain was to be grown in gentamicin the MH broth would contain 2 mg/L. This bacterial suspension would be split into other conical flask depending on the number of parallels. The flasks were then incubated at 37 °C at 200 rpm.

Samples were taken every 30 min, starting at 0 min (before incubation), for 3 hours, then every hour. Growth experiments ranged from 2.5 – 8 hours. Samples taken were 2,0 ml for McFarland measurement, 200 µl for spectrophotometric measurement, 200 µl for EZ1 and 1 ml for boiling lysis. When cultures were put back in incubation, boiling lysis (*3.4.2 Boiling lysis*) was performed immediately (*Figure 3.1*). Processed boiling lysis samples were kept refrigerated until Real-Time PCR analysis.

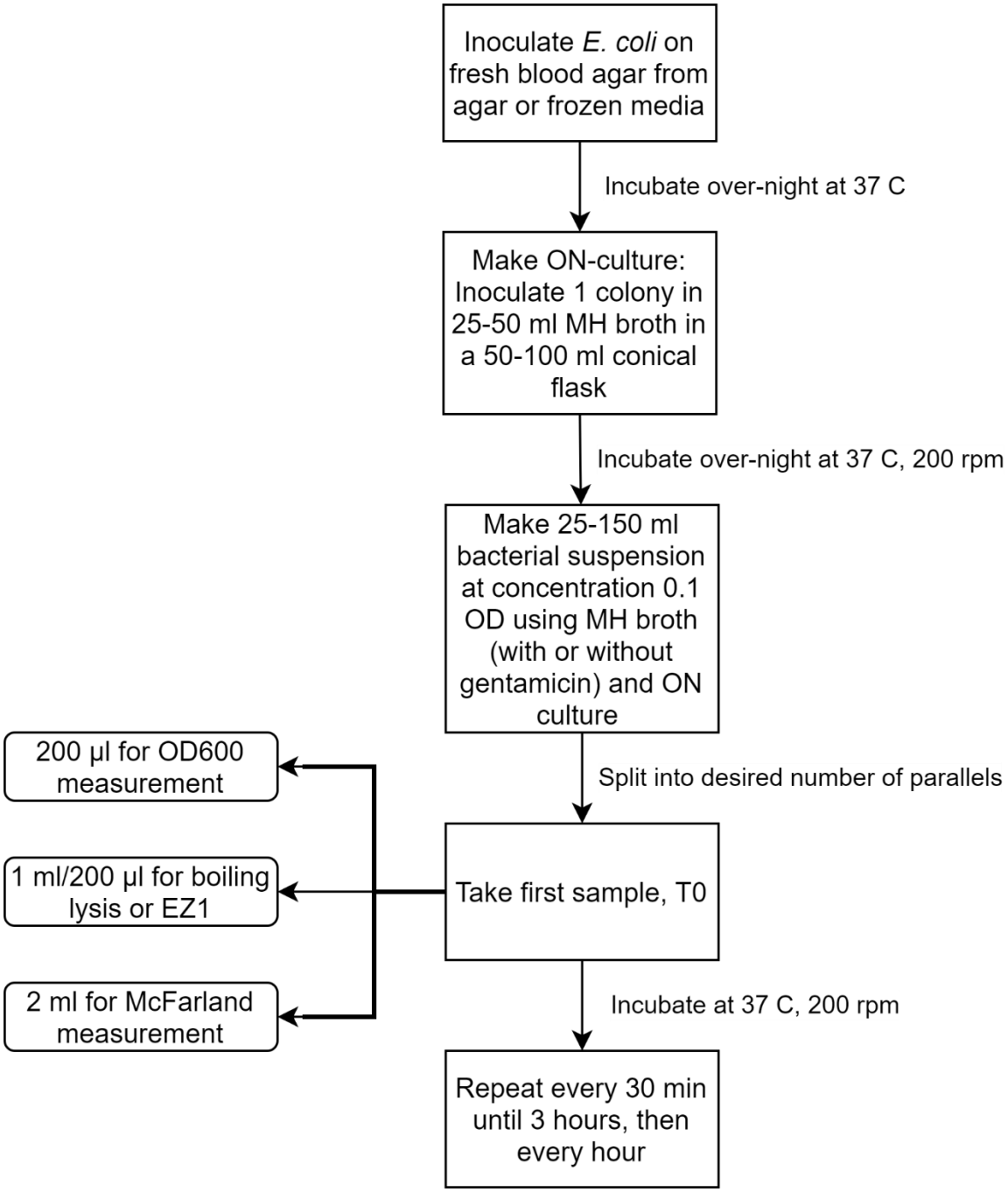

Figure 3.1 A flowchart representation of the workflow in growth experiments.

3.4 Preparation of bacterial DNA

DNA extraction is needed before a PCR to expose the DNA and remove cell debris that could inhibit PCR. This could be performed in a number of ways, but in this study one facet is speed. To achieve this, the time it takes to extract DNA needs to be as quick as possible. Less hands-on time is better for reducing the time from sample preparation to result. Since lysis by boiling is a very fast way of lysing bacteria, it was decided to test this method to see if that could be a viable option for this project. In addition, DNA extraction was done by two commercial methods, one manual and one automated method.

No DNA concentration was measured, no quality of the DNA was measured.

3.4.1 Manual DNA extraction

DNeasy Blood and Tissue kit (Qiagen, Hilden Germany) was used to obtain bacterial DNA for Real-Time PCR optimisation because it is a fast and reliable method for DNA extraction. The principle behind this method is based on the ability of DNA to bind to the silica-based membrane in the mini spin column in the presence of chaotropic salt. First, proteinase K is added, this lyses the cells/tissue, then the mixture is placed into the mini spin column. When centrifuged, the DNA will selectively bind to the membrane whilst cell debris and other contaminants are washed out. Both DNA and the silica membrane are negatively charged, but the presence of the chaotropic salts builds a "bridge" between them, decreasing the forces repelling them from each other [102, 103]. Extra wash steps ensure that remaining contaminants and other enzyme inhibitors are removed. When the chaotropic salts are removed/diluted by water or buffer, the DNA releases from the silica membrane and is eluted [104].

Figure 3.2 The DNeasy workflow. Round arrows represent centrifugations.

Protocol: a fresh colony of *E. coli* was picked and suspended in 180 µl buffer ATL in a 1.5 ml tube (*Figure 3.2)*. 20 µl Proteinase K was added and mixed thoroughly by vortex. The mixture was incubated for 30 min at 56 °C on a thermal heating block, (Thermomixer comfort, Eppendorf, Hamburg, Germany), with shaking, 300 rpm. After a 15 sec vortex, 200 µl Buffer AL was added to the sample before

vortexing again. Immediately, 200 µl of ethanol (96 – 100 %) was added and the mix was vortexed. A DNeasy Mini spin column was placed into a 2 ml collection tube before the mix was pipetted into the Mini spin column. The tube was centrifugated for 1 min at 8000 rpm in a MiniSpin® plus instrument (Eppendorf, Hamburg, Germany), and the flow-through and collection column was discarded. The Mini spin column was placed into a new collection tube, and 500 µl Buffer AW1 was added before a second centrifugation for 1 min at 8000 rpm was done. Flow-through and collection tube was discarded. Again, the Mini spin column was placed in a new collection tube. 500 µl AW2 was added, and the tube was centrifuged for 3 min at 14,000 rpm to dry the membrane. Flow-through and collection tube was discarded, being careful not to let the membrane touch the flow-through. The Mini spin column was placed into a new 1.5 µl microcentrifuge tube, and 200 µl Buffer AE was pipetted directly onto the membrane. After a 1 min incubation in room temperature, a final centrifugation of 8000 rpm for 1 min was completed, and the DNA was eluted into the microcentrifuge tube and refrigerated until needed.

3.4.2 Boiling lysis

Boiling lysis was tested as it's the fastest available method for preparation of bacterial DNA for PCR amplification. It is cheap and easy to preform, and has already been used to obtain DNA for PCR [105]. High temperatures near the boiling point of water can lyse cells, making them release their contents into the environment. Cell debris and DNA can be separated through centrifugation, before the DNA can be used in other methods, like PCR [106, 107].

3.4.2.1 Initial testing of boiling lysis

Two protocols were tested against each other for speed, ease of use and amplification efficiency. Both were run in Real-Time PCR, then one was chosen as a starting point for further optimisation. Protocol 1: 3-4 colonies were dispersed in 200 µl molecular grade water in a 1.5 ml Eppendorf tube with a pricked lid. It was boiled on a thermal block for 15 min at 95 °C with 300 rpm shaking. After a 2 min centrifugation step at 13,200 rpm, the supernatant was pipetted into a new tube and put on ice awaiting further use. Protocol 2: as with Protocol 1, 3-4 colonies were dispersed in 200 µl molecular grade water in a 1.5 ml Eppendorf tube with a pricked lid. It was boiled on a thermal block for 15 at 99 °C, without shaking. A 5 min centrifugation step was done before pipetting the supernatant into a new tube. The tube was put back on the thermal block for another 10 min at 99 °C, before being put on ice (*Table 3.2*). Both protocols were run on an amplification efficiency analysis PCR (*3.5.4 Amplification efficiency analysis*). Based on ease of use and speed, protocol 1 was chosen for further optimisation.

Table 3.2 The two boiling lysis protocols tested in this study. Protocol 1 included 95°C boiling for 15 min on 300 rpm, with the supernatant being put on ice. Protocol 2 was 99 °C for 15 min without shaking, then boiling the supernatant a second time for 10 min before putting on ice.

Protocol 1	Protocol 2
The lid of an Eppendorf tube was pricked to let steam escape	The lid of an Eppendorf tube was pricked to let steam escape
Using the aforementioned tube, 3-4 colonies of *E. coli* were dispersed in 200 µl MGW	Using the aforementioned tube, 3-4 colonies of *E. coli* were dispersed in 200 µl MGW

The tube was put on a heating block for 15 min at *95 °C with 300 rpm shaking*	The tube was put on a heating block for 15 min at *99 °C without shaking*
The tube was centrifuged at 13,200 rpm for *2 minutes* at 21 °C	The tube was centrifuged at 13,200 rpm for *5 minutes* at 21 °C
Supernatant was pipetted into a new tube	Supernatant was pipetted into a new tube
-	The supernatant was put *back on the heating block for 10 min at 99 °C without shaking*
Tube with supernatant was put on ice until further use	Tube with supernatant was put on ice until further use

To standardise the sample preparation when optimising protocol 1, two sample preparations were used depending on the amount bacterial suspension needed for the tests. Sample preparation one: a fresh culture from the Sus strain was inoculated in 2.5 ml molecular grade water. 200 µl were distributed to 12 Eppendorf tubes. This number of tubes was consistent with the number of tubes needed when utilising sample preparation one. Sample preparation two: 1 ml ON culture (*3.3 Growth experiments*) was distributed to the desired amount of tubes. Sample volume was increased from 200 µl to 1 ml, to ensure enough material after lysis.

A Real-Time PCR with melt curve analysis (*3.5.1 Real-Time PCR*) was run for each optimisation test, with all samples in duplicates.

Protocol 1 was chosen as a starting point for further testing based on speed and ease of use. Protocol 1's variables were to be optimised (*Table 3.3* and *Figure 3.3*), and the first was temperature for the boiling step. Both 95 °C and 99 °C were tested. Sample prep. one was used as described above. Six tubes were run as described for Protocol 1 in *Table 3.2* (with the altered samples prep.), and six tubes were run identically, but at 99 °C. The chosen temperature was 95 °C after testing.

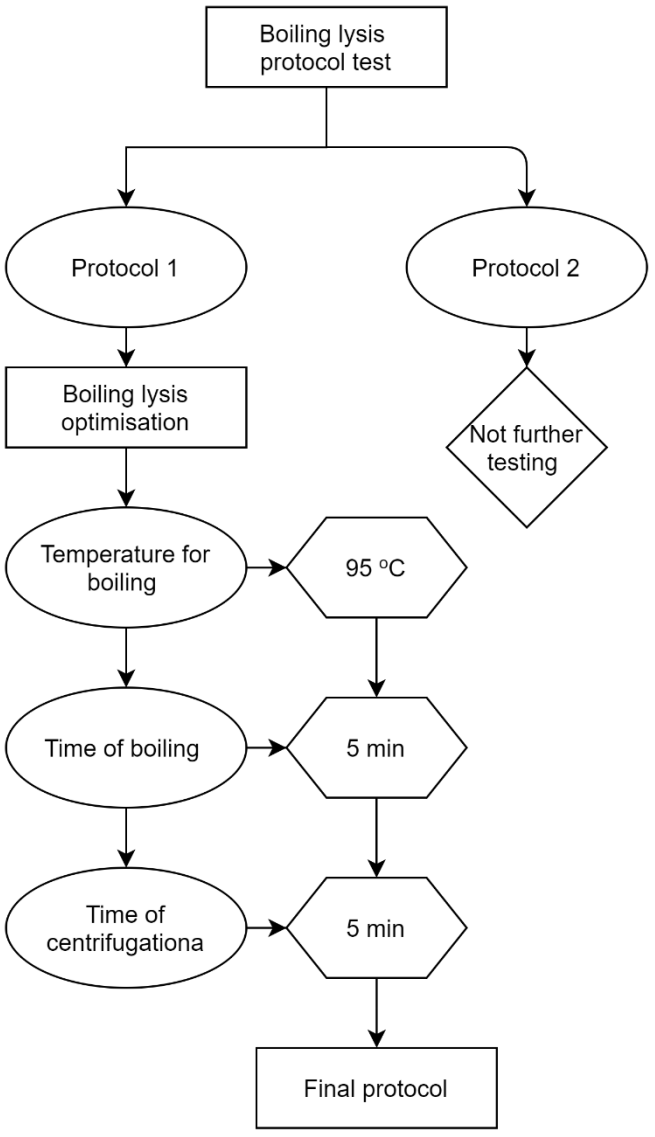

Figure 3.3 A flowchart of the different steps taken to optimise the boiling lysis method.

Table 3.3 The variables tested for optimising protocol 1. Temperatures for boiling, time for boiling and time for centrifugation were optimised with several parameters. Two temperatures were tested, five times for boiling and 7 times for centrifugation were tested. Parameters marked in cursive were the final choices.

Variables in protocol 1	**Parameters tested**						
Temperatures for boiling	*95 °C*	99 °C					
Duration of boiling	1 min	2 min	3 min	*5 min*	10 min		
Duration of centrifugation	30 sec	1 min	1,5 min	2 min	3 min	*5 min*	10 min

The next variable to test was the duration of boiling. Five times were tested, 1, 2, 3, 5 and 10 minutes. Two tests were done, each using one of the sample prep. methods, following protocol 1 in *Table 3.2*. Three samples were run per boiling time. The optimal time for boiling was set to 5 min after testing.

Centrifugation duration was optimised. Centrifugation rotation was set to the centrifuges max setting, 13,200 rpm, and was not subject to optimisation. There had been some issues with the results from PCR analyses that might be attributed to the amount of broth in the lysate. The solution was to centrifugate samples before lysis and resuspend the pellet in an equal amount of molecular grade water. It was decided to make the protocol easy to follow so the centrifugation steps were to be identical. Thus, two centrifugation steps were optimised. The times 30 sec, 1, 1.5, 2, 3, 5 and 10 min were tested. Protocol 1 (*Table 3.2*) was followed with the additional centrifugation step for broth removal. Sample prep. two was followed, with three samples per centrifugation time. The optimal duration of centrifugation was set to 5 min.

3.4.2.2 Final boiling lysis protocol

After having optimised boiling temperature, boiling duration and centrifugation duration, the final protocol for boiling lysis was as follows; 1 ml of bacterial suspension was transferred to a 1.5 ml Eppendorf tube with a hole pricked in the lid. It was centrifuged for 5 min at 13,200 rpm at 21 °C in a Centrifuge 5415 (Eppendorf, Hamburg, Germany). Supernatant was removed and replaced by an equal amount molecular grade water, and mixed either by pipette or vortex. Then the mix was heated on a thermal block (Eppendorf, Hamburg, Germany) at 95 °C for 5 min with 300 rpm shaking. Another centrifugation identical to the one prior was done before pipetting the supernatant into a new 1.5 ml Eppendorf tube and refrigerated. Final protocol can also be seen in *Table 3.4*.

Table 3.4 The final boiling lysis protocol included an initial centrifugation step to remove and replace the MH broth, boiling at 95 °C for 5 min with 300 rpm shaking, and refrigerating the supernatant until further use.

Final boiling lysis protocol
The lid of an Eppendorf tube was pricked to let steam escape
1 ml samples were collected in the Eppendorf tubes with pricked lids
Centrifugation at 13,200 rpm for 5 minutes

Removal of supernatant, replaced by equal amount molecular grade water and vortexed
Tubes were put on heating block for 5 min at 95 °C with 300 rpm shaking
The tube was centrifuged at 13,200 rpm for 5 minutes at 21 °C
Supernatant was pipetted into a new tube
Tube with supernatant was refrigerated until further use

3.4.3 Automated DNA extraction

Evaluation of another DNA extraction method was done to compare to boiling lysis. EZ1® DNA Tissue kit (Qiagen, Hilden Germany) was chosen as it is an automated method available at St. Olavs hospital. This method relies on magnetic silica particles binding DNA in the presence of chaotropic salt, as described in *3.4.1 Manual DNA extraction, DNeasy (Figure 3.4)*. Using a magnet, silica particles with DNA are separated from the lysate, then washed. The DNA is then eluted in elution buffer [108].

The protocol used is as follows: 200 µl samples of bacterial broth was collected and centrifuged for 5 min at 13,200 rpm. This step was identical to that of the boiling lysis centrifugation. Supernatant was removed and replaced by 200 µl Buffer G2. When all samples were collected and prepared, they were taken to the EZ1 instrument. Elution volume was set to 200 µl, and reagent cartridges were loaded. Open elution tubes were placed in the EZ1 rack's first row. Tip holders and filter-tips were loaded into the second row, whilst the samples with open caps were placed in the fourth row. The instrument was initiated. After extraction, all samples were run in duplicates on Real-Time PCR with melt curve analysis.

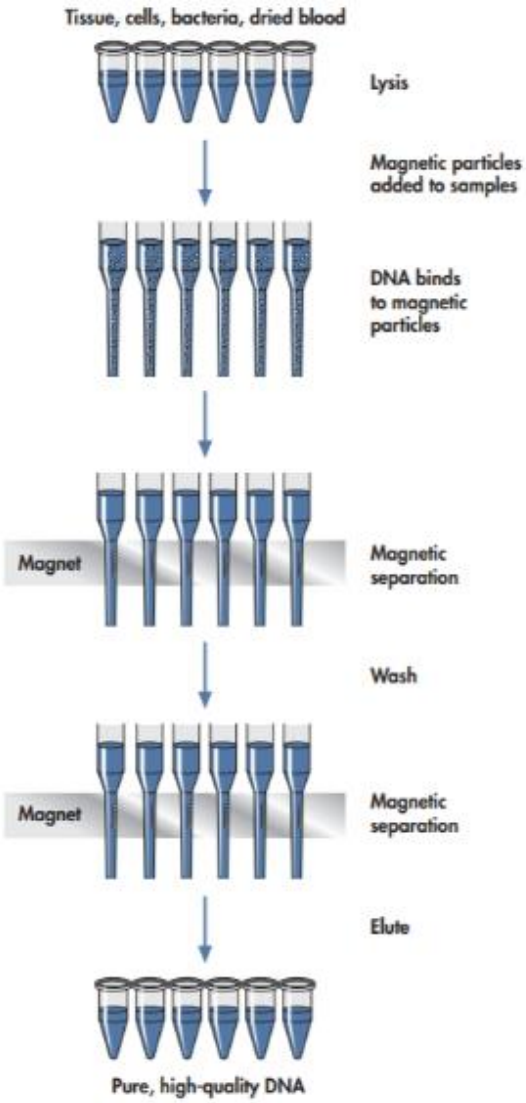

Figure 3.4 Automated workflow of EZ1 DNA Tissue kit.

3.5 Primers and Real-time PCR

Primers are an integral part of any PCR. The goal in this study was to use the relative amount of amplification product of the 16S rRNA gene as a measure of growth, and thereby determine the strain's sensitivity to gentamicin. To do that the primer pair needed to be complimentary to the nucleotide sequence in question. To make the protocol as useful as possible, universal primer pairs for the 16S rRNA gene nucleotide sequence conserved in both Gram-positive and Gram-negative bacteria were chosen.

A reference genome (*Escherichia coli* str. K-12 substr. MG1655) for *E. coli* was selected from NCBI and uploaded to Geneious Prime, a sequence analysis tool (Biomatters, Ltd., Auckland, New Zealand). To identify the 16S gene the whole genome was aligned with the 16S rRNA gene nucleotide sequence (GenBank: J01859.1). Conserved and variable regions on the gene were marked according to Chakravorty S. et al. [75]. Primer pairs targeting the 16S rRNA gene identified by a broad literature search was pasted into the program (*Table 3.5 and Table 3.6*), eight in total including the primer pair used by St. Olavs hospital for direct material. The PCR product lengths, amplified region, and nucleotide sequence were compared. The primer pairs were also BLASTED in NCBI's Primer design tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) using the following parameters: 16S rRNA gene (GenBank: J01859.1) in FASTA format as PCR template, exclusion of uncultured/environmental sample sequences, with all other parameters as default. Product lengths, primer-dimers and complimentary human genome sequences were assessed. One of the primer pairs had other letters than the standard A, T, C, G annotations, and needed to be altered to be run in BLAST. Following "*Nomenclature for incompletely specified bases in nucleic acid sequences: recommendations 1984*" [109], the alternative letters were replaced by A, T, C or G based on *E. coli's* 16S rRNA gene (GenBank: J01859.1).

Based on the results of the Geneious Prime and Primer-BLAST analyses, six primer pairs including the Hospital primer pair, were chosen to further characterise (*Table 3.5* and *Table 3.6*). These primer pairs were analysed in Oligo 7 (Molecular Biology Insights, Inc, Colorado Springs, USA), a primer analysis program, for GC content, self-complementarity, hairpin structures and internal stability. Based on this analysis two primer pairs, Horz and Muyzer (*Table 3.5*), were chosen for further testing. The Muyzer primer pair was found in an article by G. Muyzer et al. [110]. The Horz primer pair was found in an article by H. P. Horz, et al. [111]. The Horz primer pair was designed for the V7 region, and Muyzer for the V3 region. The Hospital primer pair, designed for the V3 to V5 region, was included as a reference for a primer pair used in diagnostics. The Hospital primer pair had the same forward primer as the Muyzer primer pair, whilst the reverse hospital primer was a modified version from an article by Schabereiter-Gurtner et al. [112].

Table 3.5 The different primer pairs chosen after Geneious Primer and Oligo analysis. Their sequence, length of product and provider are displayed.

Primer pair	Reference	Sequence	Length of product (bp)	GC%	Provider
Hospital	[110, 112]	**341F** – CCT ACG GGA GGC AGC AG **927R** – CCC GTC AAT TCA TTT GAG TTT	587	F – 70.6 R – 38.1	Department of medical microbiology, St. Olavs hospital, Trondheim
Horz	[111]	**F** – GTG STG CAY GGY TGT CGT CA	147	F – 70.6 R – 67.7	Thermo Fisher Scientific, Waltham, USA

		R – ACG TCR TCC MCA CCT TCC TC *Modified for Primer-BLAST* ***F** – GTG CTG CAT GGC TGT CGT CA* ***R** – ACG TCA TCC CCA CCT TCC TC*			
Muyzer	[110]	**F** – CCT ACG GGA GGC AGC AG **R** – ATT ACC GCG GCT GCT GG	194	F – 70.6 R – 67.7	Thermo Fisher Scientific, Waltham, USA

Table 3.6 Table of primer pairs that were tried out in Geneious Prime but not tested any further.

Reference	Sequence	Length of product (bp)
Chakravorty, Helb et al. [75]	**F** – CCA GAC TCC TAC GGG AGG CAG **R** – CGT ATT ACC GCG GCT GCT G	203
Chakravorty, Helb et al. [75]	**F** – TCG ATG CAA CGC GAA GAA **R** – ACA TTT CAC AAC ACG AGC TGA CGA	57
Suzuki, Taylor et al. [113]	**F** – AGA GTT TGA TCM TGG CTC AG **R** –	-
Clifford, Milillo et al. [114]	**F** – ACT CCT ACG GGA GGC AGC AGT **R** – TAT TAC CGC GGC TGC TGG C	180
Lee, Bae et al. [115]	**F** – AGA AGC TTG CTC TTT GCT GA **R** – CTT TGG TCT TGC GAC GTT AT	120

Horz and Muyzer primer pairs were ordered from Thermo Fisher Scientific, whilst the Hospital primer pair was acquired from the Department of medical microbiology, St. Olavs hospital and was received pre-diluted, ready to use. Freeze-dried primers were diluted to 100 µM stock solutions according to specifications from manufacturer. Working solutions of 6, 8 and 10 µM were made, as primer concentrations of 300, 400 and 500

nM would be tested in PCR optimisation experiments. There was no effort to optimize forward or revers primers individually, as primer pairs in the same reaction would be run with the same concentration. The Hospital primer pair was run according to specification in the procedure from Department of medical microbiology, St. Olavs Hospital, of 300 nM in the Real-Time PCR.

3.5.1 Real-Time PCR

The 16S rRNA Real-Time PCR protocol used in this study was acquired from the Department of medical microbiology at St. Olavs hospital, and was in use for direct material (*Appendix 1.*). The PCR reaction mix contained 10 µl PerfeCTa SYBR Green FastMix (Quantabio, Beverly, MA, USA), 2 µl of 300 nM Forward primer, 2 µl of 300 nM Reverse primer, 1 µl molecular grade water, and 5 µl template DNA. Two to four negative controls were added to each run depending on the number of samples. Instead of Template DNA the negative controls contained 5 µl PerfeCTa SYBR Green FastMix (*Table 3.7*). A three-step PCR with melting curve and SYBR Green detection was used (*Figure 3.5)* and run on a CFX Connect Real-Time PCR Detection System (BIO-RAD, Hercules, CA, USA). Initial denaturing at 95 °C for 3 min, then denaturing at 95 °C for 10 sec, annealing at 58 °C for 15 sec, and extension at 72 °C for 10 sec. This was repeated a total of 35 times, with detection of SYBR Green in extension phase. The melt curve analysis went from 50 to 95 °C, in 0.5 °C steps lasting 5 sec each.

Table 3.7 Amounts and concentrations for reagents in Real-Time PCR.

Reagent	**Amount (µl)**	**Initial concentration**
PerfeCTa SYBR Green FastMix	10	-
Forward primer	2	6 µM
Reverse primer	2	6 µM
Molecular grade water	1	-
Template DNA	5	Undiluted
Total	*20*	

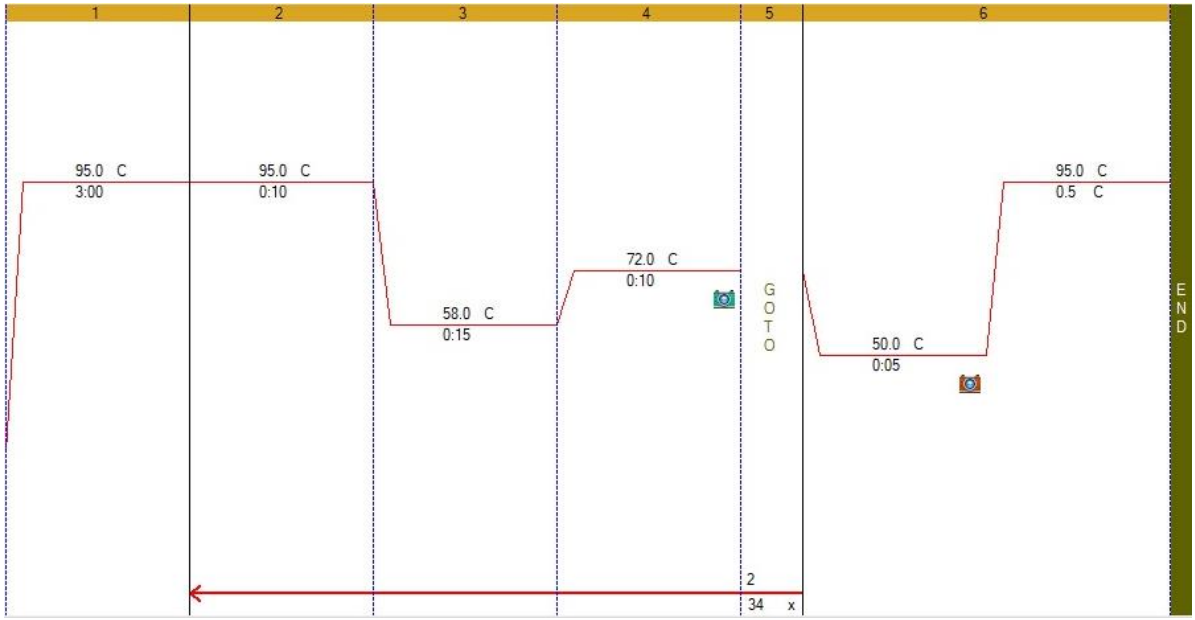

Figure 3.5 Three-step Real-Time PCR with a melt curve analysis. Initial denaturing at 95 °C for 3 min, then denaturing at 95 °C for 10 sec, annealing at 58 °C for 15 sec, and extension at 72 °C for 10 sec. This was repeated a total of 35 times, with detection of SYBR Green in extension. The melt curve analysis went from 50 to 95 °C, in 0.5 °C steps lasting 5 sec each.

3.5.2 Gel electrophoresis

Gel electrophoresis can be used to separate PCR products. It does so by applying an electric field to an agarose gel loaded with PCR samples. Since DNA is negatively charged, it will wander towards the positive electrode. Larger molecules will move slower, and smaller ones will move faster due to the gel's agarose matrix. Ladders with known sizes can be added to determine the size of the PCR products [116].

Gel electrophoresis was performed as a quality control step, to confirm the presence of amplification products and test for non-specific PCR products such as primer dimers. E-gel with SYBR Safe (Thermo Fisher Scientific, Waltham, USA) was loaded with 10 µl undiluted sample, both samples and negative controls, with one or two ladders (10 Kb Plus, Thermo Fisher Scientific, Waltham, USA) in each gel. The edge lanes of the first run seemed to give smudged results, so the edge lanes were avoided in all following runs (*Appendix* 2.). The gel was run for 30 min. Fluorescence bands were captured by image analysis using Gel Doc (BIO-RAD, Hercules, CA, USA). This was performed for the initial Real-Time PCR test run, the temperature gradient analysis and two of the amplification efficiency analyses.

3.5.3 Temperature gradient analysis

To optimise the annealing temperatures for all primer pairs, a temperature gradient analysis was performed. The calculated theoretical temperatures can differ from the optimal practical temperatures, thus optimising this ensures a more specific reaction. Too high a temperature might lead to less efficient amplification of the target, whilst too low a temperature might lead to amplification of nonspecific products [117]. To find the optimal temperature many real-time PCR instruments have a gradient feature. In this experiment CFX's gradient feature was used. Each row holds a different temperature

programmed by the user, usually the theoretical annealing temperature represented in the middle. The optimal temperature should result in the lowest Cq-value with specific amplification and without nonspecific amplification [117].

Using undiluted DNA eluate from strain Sus, extracted by DNeasy Blood and tissue kit (*3.4.1 Manual DNA extraction, DNeasy*) a temperature gradient analysis was performed for all primer pairs. Samples were run in one parallel, with a negative control for each temperature and primer. The temperature gradient ran from 65.0 °C in row A to 55.0 °C in row H. Due their difference in temperature being under 1 °C from rows A and H, rows B and G were unused, see *Table 3.8* for all temperatures.

Table 3.8 Overview of the wells and temperatures used in the temperature gradient. Wells A and H differ from B and G under 1 °C, so B and G were not used.

Row	Temperature °C
A	65.0
B	*64.5*
C	63.3
D	61.4
E	59.0
F	57.0
G	*55.7*
H	55.0

3.5.4 Amplification efficiency analysis

Amplification efficiency should be calculated when evaluating a PCR to determine how good it is at effectively amplifying its target. Efficiency can be defined as a quantitative measure of the increase in amplicons per cycle [118]. In this study, efficiency of the Real-Time PCR was calculated using the gold standard; standard curve analysis [119]. After making and analysing the ten-fold serial diluted samples, PCR Cq-values can be plotted against the dilution factor in a 10 semi-logarithmic graph [120], then fitting a regression line to the plots. A slope of -3.32 in equivalent to 100% efficiency, while a steeper slope indicates lower efficiency and a shallower slope indicates higher efficiency [120, 121]. The slope is used in calculating the efficiency using the equation below:

I. $E = 10^{1/slope} - 1$ [122]

E is the theoretical efficiency and *slope* is the slope from the regression line made from the dilution factors and Cq-values.

DNA extracted from strain Sus was used in the efficiency analyses, the same as in *3.5.3 Temperature gradient*. Using the template eluate, six ten-fold dilutions were made, 1:1 - $1:10^5$. In the first analysis, a predilution of $1:10^2$ for the first sample was done, this was later changed to an undiluted first sample. PCR amplification was run with the three primer pairs, Horz, Muyzer and Hospital, each in triplicate. Two negative controls were included for each primer pair, to rule out contamination. Real-time PCR protocol was performed as described in *3.5.1 Real-Time PCR*. Four efficiency analyses were completed, three only to measure amplification efficiency and one as part of a boiling protocol test. After Analysis 2 a gel electrophoresis was run for confirmation of the expected amplification product in sample wells and the lack of products in negative controls.

Calculations were performed in Excel using a template received from a supervisor (*Appendix* 3.). The excel sheet uses the recorded Cq-values, dependent Y values, and plots them against the dilutions, independent X values. The slope of the regression line is then used to calculate the amplification efficiency in percent using the following equation in excel:

II. =(OPPHØYD.I(10;-1/L7)-1)*100

3.6 Statistics

Calculations of error bars in various graphs were done with 95% confidence interval (CI) in Graph Pad when possible. Calculation of CI in tables was done by arranging the Cq-values in an excel sheet, calculating mean and standard deviation using excel formulas (formula for standard deviation is shown in *Formula III*), and plotting these values including number of parallels into an online statistics calculator, https://www.socscistatistics.com/confidenceinterval/default2.aspx, which used the formula shown under, IV.

III. =STDAV.S

IV. $\mu = M \pm t(S_M)$ [123]

M is the sample mean, *t* is the t-statistic determined by the confidence level and S_M is standard error.

4 Results

The results are presented in four parts: an account of the growth curves with their lag and log phases, a presentation and comparison of bacterial DNA preparations boiling lysis and EZ1, the results of Real-Time PCR with optimisation steps, and a description of predictions of S-I-R profiles using these results.

4.1 Growth experiments

The aim was to establish how long it would take to differentiate resistant strains from susceptible strains of *E. coli* by growth in MH broth with and without gentamicin. A total of eight growth experiments (GE) were performed. Of the eight, the first failed, two experiments were run with the Res strain, four were run with the Sus strain, four were run with gentamicin in the broth, and three with regular MH broth (*Table 4.1* and *Table 4.1*).

When testing the MH broth for colour changes none were observed (*Figure 4.1*).

Growth experiments started out at five hours duration. After completing two growth experiments at five hours, both lag and exponential phase appeared before the three hour mark (*Table 4.1* and *Figure 4.1*). It was then deemed sufficient with 2.5-3.0 hours growth experiments.

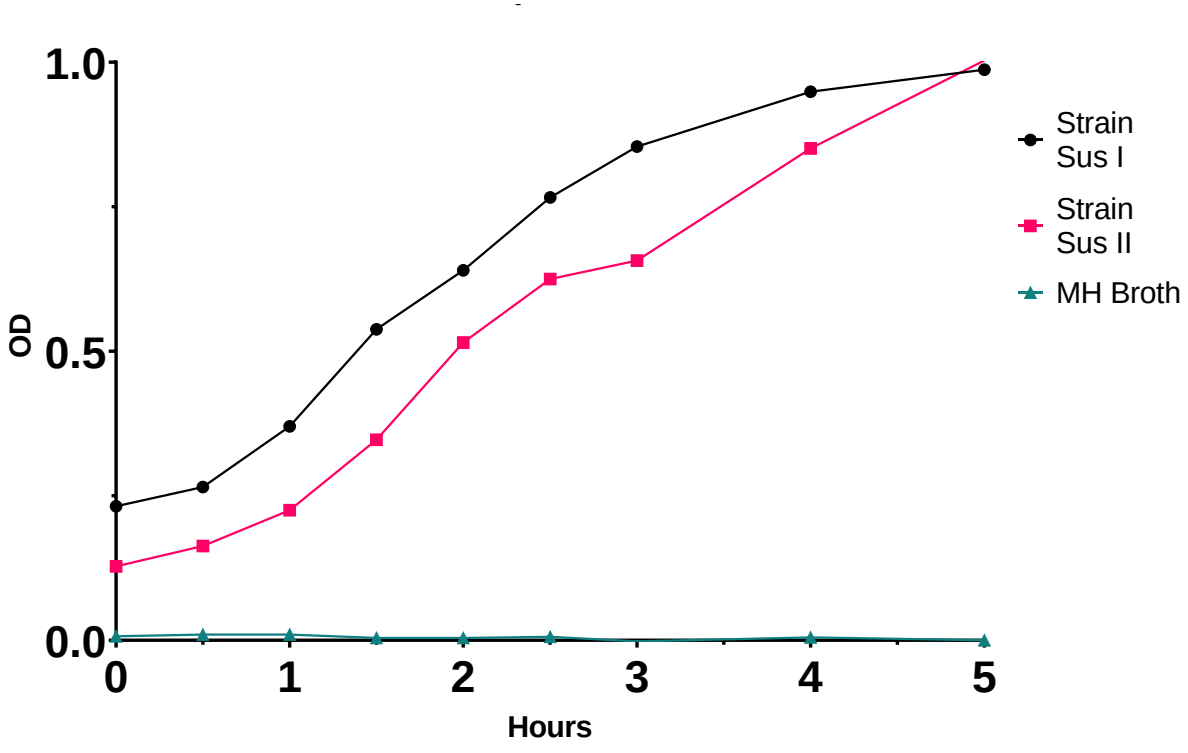

Figure 4.1 This graph shows the growth curve of two samples of the Sus strain (black and red) and one sample containing only Mueller-Hinton broth (MH) (blue) derived from

OD measurements. Strain Sus I (black) had a starting concentration of approx. 0.2 OD, whilst strain Sus II had a starting concentration of approx. 0.1 OD. The plain MH was included to look for any colour changes in in the broth when incubated. Lag and exponential phases are in both samples present before 2.5 hours.

4.1.1 Lag phase and exponential phase

To reduce the time of results, the lag phase should be as short as possible. To facilitate this the MH broth was heated to lessen the shock the bacteria could experience when changing milieu. Estimations of lag phase for all strains were between 1 and 1.5 hours when grown in MH broth without gentamicin. The exponential phase lasted approx. 0.5 hour for all samples (*Table 4.1* and *Table 4.2*). These were estimations made by eye assisted by a ruler based on OD measurements (*Figure 4.1* and *Figure 4.2*).

Table 4.1 Lag and exponential phases length in hours for Sus and Res strains in the different growth experiments (GE). These measurements were estimated from OD600 values. Where there are two numbers, there are two parallels or samples of the same strain. (g) annotates experiments run with gentamicin in the Mueller-Hinton broth. *Lag* is the lag phase and *Exp.* is the exponential phase.

Strain	**GE II**		**GE III**		**GE IV**		**GE V (g)**		**GE VI (g)**		**GE VII (g)**	
	Lag	*Exp.*	*Lag*	*Exp.*	*Lag*	*Exp.*	*Lag*	*Exp.*	*Lag*	*Exp.*	*Lag*	*Exp.*
Sus			1 1.5	0.5 0.5	1 1.5	1 0.5	1	0.5			1.5	0.5
Res	1	0.5							1.5	0.5		

Table 4.2 Lag and exponential phases in hours for growth experiment (GE) VIII run with strains A - L. These measurements were estimated from OD600 values.

Strain	**GE VIII (run with gentamicin)**	
	Lag phase	Exponential phase
A	1.5	0.5
B	1.5	0.5
C	1	0.5
D	1	0.5
E	1	0.5
F	1.5	0.5
G	1.5	0.5
H	1.5	0.5
I	1.5	0.5
J	1.5	0.5
K	1.5	0.5
L	1.5	0.5

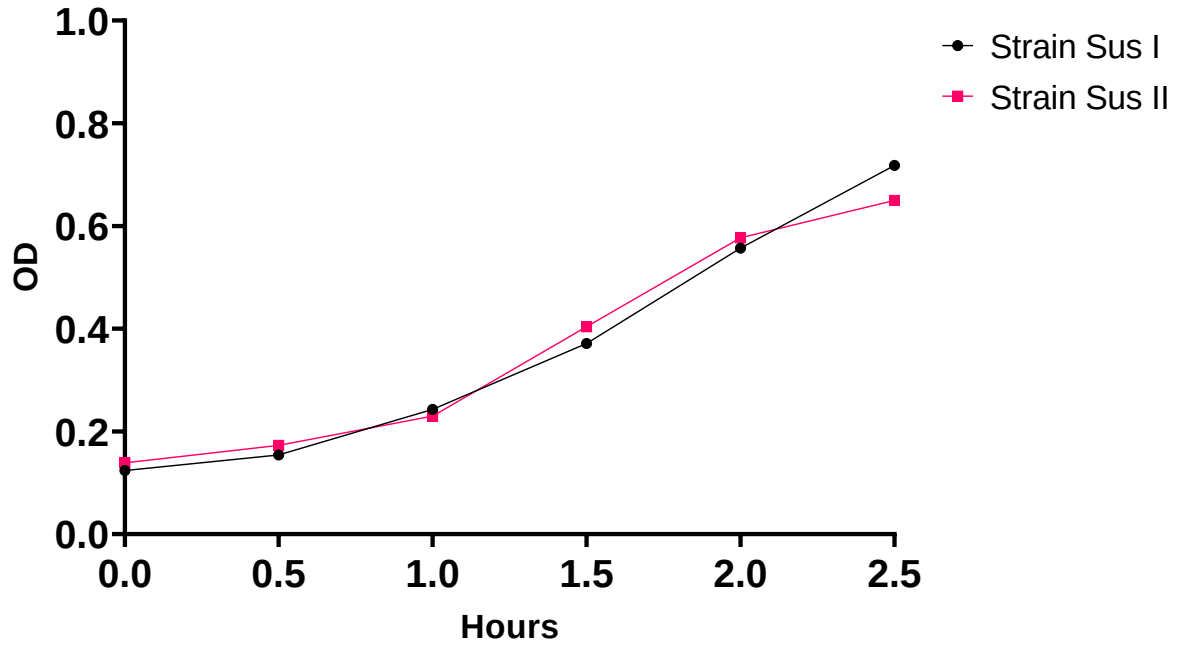

Figure 4.2 This graph shows a 2.5 hour growth curve of two parallels of Strain Sus. Strain Sus was in two parallels, I and II, both having a start concentration of approx. 0.1 OD600. The lag phase seamed to last for 1 (black, Sus I) and 1.5 hours (red, Sus II) for the two parallels. The exponential phase lasted 1 hours for Sus I (black), and 0.5 hours for Sus II (red). These measurements were made by eye and a ruler.

4.2 Preparation of bacterial DNA

Three methods were used, DNeasy, boiling lysis and EZ1. DNeasy was only used to obtain DNA for the optimisation steps in PCR. It was never reviewed as a viable option for DNA preparation in this project and will be discussed no further. Boiling lysis was optimised and compared to EZ1, and both were evaluated as options for DNA preparation for this project.

4.2.1 Boiling lysis

The final optimised method gave results in the Real-Time PCR assay. As the method was run several times some discrepancies were seen, but most resolved after removing the MH broth before running Real-Time PCR as described in *3.5.1 Real-Time PCR*. Running nine samples took approx. 20-25 min. There was a good amount of hands-on work, but incredibly cheap as material needed for this method usually will be available in a lab as basic equipment.

Two boiling lysis protocols were tested, Protocol 1 and Protocol 2. Two primer pairs, Muyzer and Hospital, were tested with several 10-fold dilutions using two different boiling lysis protocols. Only the results regarding the boiling lysis protocols are shown here. Results regarding primer pairs are shown under *4.3 Test of and optimisation of PCR*. Protocol 2 has the overall lowest Cq-values for both primer pairs. With primer pair Muyzer the greatest difference is in dilution $1{:}10^3$, with a difference of 1.59 Cq in favour of Protocol 2. The lowest difference is in dilution $1{:}10^5$ with 0.71 Cq, also in favour of Protocol 2. With primer pair Hospital it's the same, protocol 2 is lower overall, with the

highest difference being dilution $1:10^2$ with 1.22 Cq and the lowest in dilution $1:10^5$ with 0.01 Cq (***Feil! Fant ikke referansekilden.****Table 4.3*).

Table 4.3 Mean PCR Cq-values from analysis of 10-fold dilutions of samples prepared with two different boiling lysis methods. Two different PCR primer pairs were used, but only results from primer pair Hospital is shown. Differences in Cq-values between the two protocols were calculated. 95% Confidence Intervals (CI) are shown in brackets.

Dilution	Protocol 1	Difference between protocols	Protocol 2
1	12.24 (10.86-13.62)	0,59	11.65 (10.68-12.62)
1:10	15.92 (14.29-17.55)	1,22	14.70 (13.84-15.56)
$1:10^2$	19.35 (17.82-20.88)	1,19	18.16 (17.45-18.87)
$1:10^3$	22.64 (21.36-23.92)	1,13	21.51 (21.16-21.86)
$1:10^4$	24.83 (24.19-25.47)	0,57	24.26 (23.89-24.63)
$1:10^5$	25.30 (25.02-25.58)	0,01	25.29 (25.14-25.44)

Testing of the boiling temperature and duration was performed several times. The results from two runs are included here in *Table 4.4* and *Table 4.5.* Several different times of boiling were tested alongside the temperatures, from 1 – 15 min. Usually, the longer the samples boiled the lower the Cq-value, except for 15 min. For 99 °C the Cq-value went up 0.50 compared to 5 min. In the first temperature check (*Table 4.4*), Cq- values ranged from 7.02 to 7.54, not a large difference. 95% confidence intervals (CI 95%) overlapped at 15 minutes, and overlapped by 0.01 C values at 5 minutes. Based on that test no definite decision was made. The boiling lysis time check (*Table 4.5*) showed a larger range of Cq-values, from 16.04 for 1 min at 95 °C, to 10.16 for 5 min at 99 °C. For every time that was tested, Cq-values for 99 °C were lower. CI 95% overlapped at 1, 2, and 3 min, but were separate at 5 min. 95 °C at 5 min was the chosen temperature after these tests.

Table 4.4 95 °C and 99 °C were tested for 5 and 15 min in boiling lysis in three parallels. **Mean Cq-values ranged from 7.02 for 5 min at 99°C, to 7.54 for 5 min at 95°C. Confidence interval was calculated at 95% and are shown in brackets.**

	95 °C	99 °C	Difference between 95 and 99 °C
5 min	7.54 (7.12-7.96)*	7.02 (6.92-7.12)*	0.52
15 min	7.35 (7.14-7.56)	7.52 (7.44-7.61)	-0.17

** almost no overlap between 95% confidence intervals*

Table 4.5 95 °C and 99 C° were tested for boiling lysis, here for 1, 2, 3 and 5 minutes boiling. Cq-values ranged from 16.04 for 1 min at 95 C°, to 10.16 for 5 min at 99 C°. Confidence interval was calculated at 95% and are shown in brackets.

	95 °C	**99 °C**	**Difference between 95 and 99 °C**
1 min	16.04 (15.40-16.68)	14.71 (13.89-15.53)	1.33
2 min	14.20 (12.21-16.20)	11.87 (10.52-13.22)	2.33
3 min	12.84 (11.21-14.47)	10.83 (9.66-12.00)	2.04
5 min	12.70 (12.31-13.10)*	10.16 (9.92-10.40)*	2.54

** no overlap between 95% confidence intervals*

Duration of centrifugation of the samples was tested against duration of boiling. Samples were boiled for 3, 5 or 10 min, and centrifuged for 1, 3, 5 or 10 min. 5 minutes boiling had the lowest Cq-values all over, whilst boiling for 10 min has the highest overall values. Mean Cq-values from the boiling and centrifugation test varied from 14.71 – 16.80 (*Table 4.6*). Centrifugation times didn't seem to have a great impact on the Cq-values. Although centrifugation for 1 min gave slightly higher Cq for 3 and 5 min boiling. 5 minutes was the chosen time both for boiling and centrifugation.

Table 4.6 Shows mean of the Cq-values after centrifugation and boiling test. Samples were run in three parallels for the boiling lysis, and two parallels for Real-Time PCR. Samples were boiled for 3, 5 or 10 min, and centrifuged for 1, 3, 5 or 10 min. 5 minutes boiling had the lowest Cq-values all over, whilst boiling for 10 min had the highest all over values. Centrifugation for 1 min gave slightly higher Cq for 3 and 5 min boiling.

		Boiling (min)		
		3	*5*	*10*
Centrifugation (min)	*1*	16.20 (16.02-16.38)	15.11 (14.95-15.27)	16.06 (15.91-16.21)
	3	15.60 (15.29-15.91)	14.86 (14.51-15.21)	16.74 (16.48-17.00)
	5	15.13 (15.01-15.25)	14.71 (14.29-15.13)	16.40 (15.95-16.71)
	10	15.40 (15.17-15.62)	14.89 (14.76-15.02)	16.80 (16.62-16.96)

A run of the finalised boiling lysis protocol compared to EZ1 was done. Boiling lysis results are presented below (*Table 4.7*) and a comparison to EZ1 results are performed in chapter *4.2.3 Boiling lysis and EZ1 compared*. When interpreting Cq-values the thing to keep in mind is that Cq-values are inverse with template DNA concentration – higher Cq-values mean lower concentrations of template DNA. The samples grown without gentamicin are clearly dropping in Cq value from 17.87 to 12.54, although both 2 and 2.5 hours means are lower than 3 hours. Of the samples grown with gentamicin there is an increase in Cq value from 16.32 to 17.69. Here as well the Cq value at 3 hours is not the value farthest away from 0 hours, here that would be 1.5 hour at 18.48 Cq. For confidence intervals from samples run without gentamicin to not overlap with samples run with gentamicin it would take one hour according to this boiling lysis run.

Table 4.7 The table displays the mean Cq of a growth experiment using strain Sus and boiling lysis. In the growth experiment two parallels were run without gentamicin, and two were run with. Samples were run in parallel in a Real-Time PCR assay. Confidence interval was calculated and are shown in brackets behind the mean.

	Boiling lysis	
Incubation time (hours)	*Without gentamicin*	*With gentamicin*
0	17.87 (17.46-18.28)	16.32 (15.72-16.92)
0.5	18.22 (13.97-22.47)	17.22 (15.61-18.83)
1	15.00 (13.51-16.49) *	16.90 (16.55-17.25) *
1.5	13.66 (12.55-14.77) *	18.48 (17.61-19.35) *
2	12.20 (11.69-12.71) *	17.56 (17.10-18.09) *
2.5	11.75 (11.49-12.01) *	18.28 (17.53-19.03) *
3	12.54 (12.02-13.06) *	17.69 (17.07-18.31) *

** There was no overlap in the confidence interval when comparing Cq-values from samples grown with or without gentamicin.*

4.2.2 Automated DNA extraction, EZ1

This method gave the best results of the three methods for DNA preparation. A relatively fast and with minimal hands-on work method. It took 17 minutes to run 14 samples, not including the setup. There were though some problems acquiring kits, and it's probably more expensive compared to boiling lysis.

When looking at a susceptible strain grown in gentamicin one would expect no growth thus Cq-values to be unchanged or rise. This does not happen in EZ1 samples. There is a slight drop in Cq-value from 0 to 3 hours for the Sus strain grown in gentamicin (*Table 4.8*). The drop is not large, but there is no rise as expected. Grown without gentamicin the drop in Cq value is large, from 13.90 to 9.39 Cq. After one and a half hours the confidence intervals from samples grown without and with gentamicin are separated.

Table 4.8 The table displays the mean Cq of a growth experiment using strain Sus and EZ1 as DNA preparation method. In the growth experiment two parallels were run without gentamicin, and two were run with. Samples were run in parallel in a Real-Time PCR assay. Confidence interval was calculated and are shown in brackets behind the mean. After 1.5 hours the confidence interval of samples growth without gentamicin no longer overlap with the confidence interval of samples growth in gentamicin.

	DNA extraction EZ1	
Incubation time (hrs)	Without gentamicin	With gentamicin
0	13.90 (13.62-14.18)	13.97 (13.46-14.48)
0.5	13.99 (13.77-14.21)	13.74 (13.61-13.87)
1	14.21 (13.74-14.68)	13.36 (13.11-13.61)
1.5	11.88 (11.53-12.23) *	13.46 (13.31-13.61) *

2	10.65 (10.31-10.99) *	13.01 (12.79-13.23) *
2.5	9.61 (9.59-9.63) *	12.87 (12.61-13.13) *
3	9.39 (8.52-10.26) *	12.64 (12.50-12.78) *

** There was no overlap in the confidence interval when comparing Cq-values from samples grown with or without gentamicin.*

4.2.3 Boiling lysis and EZ1 compared

The two DNA preparation methods, boiling lysis and EZ1, were tested against each other using the same parallels grown with and without gentamicin for 3 hours. The samples were then run on a Real-Time PCR in parallels.

When comparing the two methods, EZ1 stands out with the lowest and most consistent Cq-values. Samples run without gentamicin show growth when the Cq-values drop, and both methods show this drop (*Figure 4.3* and *Table 4.9*). The difference is EZ1's much lower Cq-value at the start and its lower error bars, indicating more consistent results. When strain Sus is grown in gentamicin no growth is expected, thus no change in Cq-values are expected. In the samples grown with gentamicin EZ1 attains the lowest Cq-values as well, but it also has a downward slope, indicating bacterial growth (*Figure 4.4*). Boiling lysis has a higher start Cq and rises slowly, indicating no growth. EZ1 has a more consistent line compared to boiling lysis' line, which fluctuated.

Table 4.9 This table compares the means of DNA preparations EZ1 and boiling lysis from the same growth experiment using strain Sus. In the growth experiment two parallels were run, as well as with or without gentamicin. 200 µl samples taken for EZ1 and 1ml samples taken for boiling lysis. Samples were run in parallel in a Real-Time PCR assay. Confidence interval was calculated and are shown in brackets behind the mean.

Incubation time (hours)	EZ1 (without gentamicin)	Boiling lysis (without gentamicin)
0	13.90 (13.62-14.18)	17.87 (17.46-18.28)
0.5	13.99 (13.77-14.21)	18.22 (13.97-22.47)
1	14.21 (13.74-14.68)	15.00 (13.51-16.49)
1.5	11.88 (11.53-12.23)	13.66 (12.55-14.77)
2	10.65 (10.31-10.99)	12.20 (11.69-12.71)
2.5	9.61 (9.59-9.63)	11.75 (11.49-12.01)
3	9.39 (8.52-10.26)	12.54 (12.02-13.06)

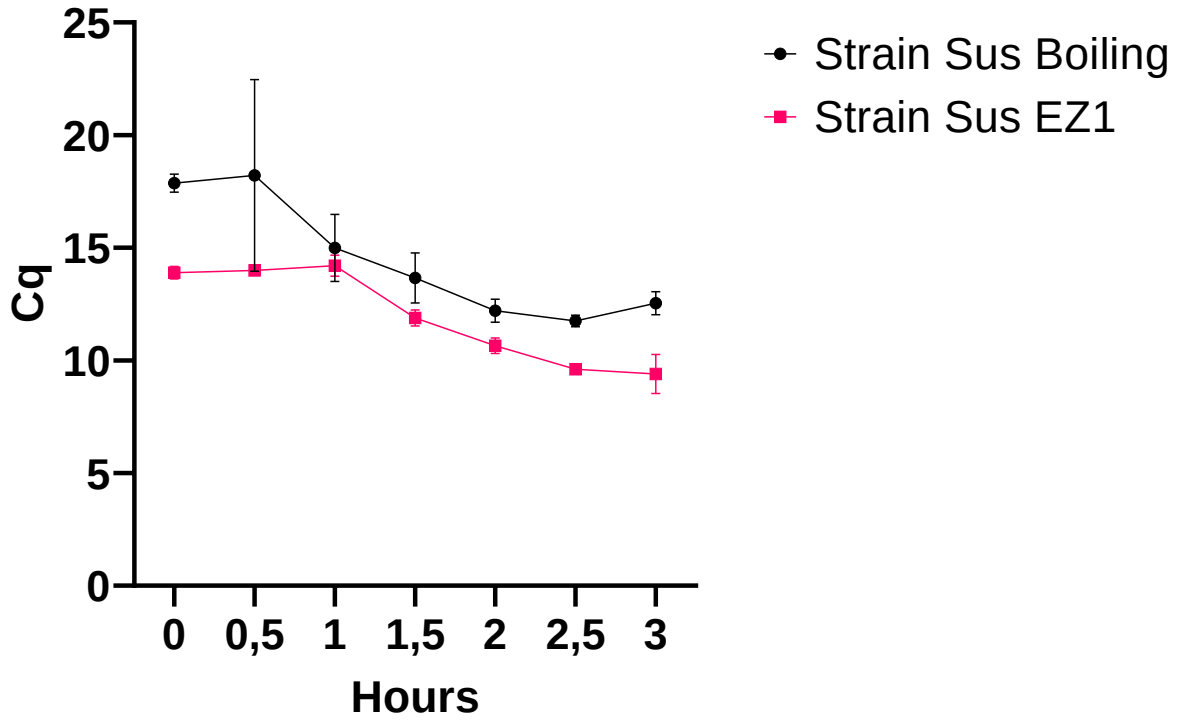

Figure 4.3 This graph shows the Cq-values of strain Sus grown without gentamicin then treated with two different DNA preparations methods , boiling lysis (black) and EZ1 (red). Two parallels were run in the Real-Time PCR. EZ1 clearly has the lowest Cq-values throughout, still the shapes are similar between the two methods. Error bars were calculated using 95% CI.

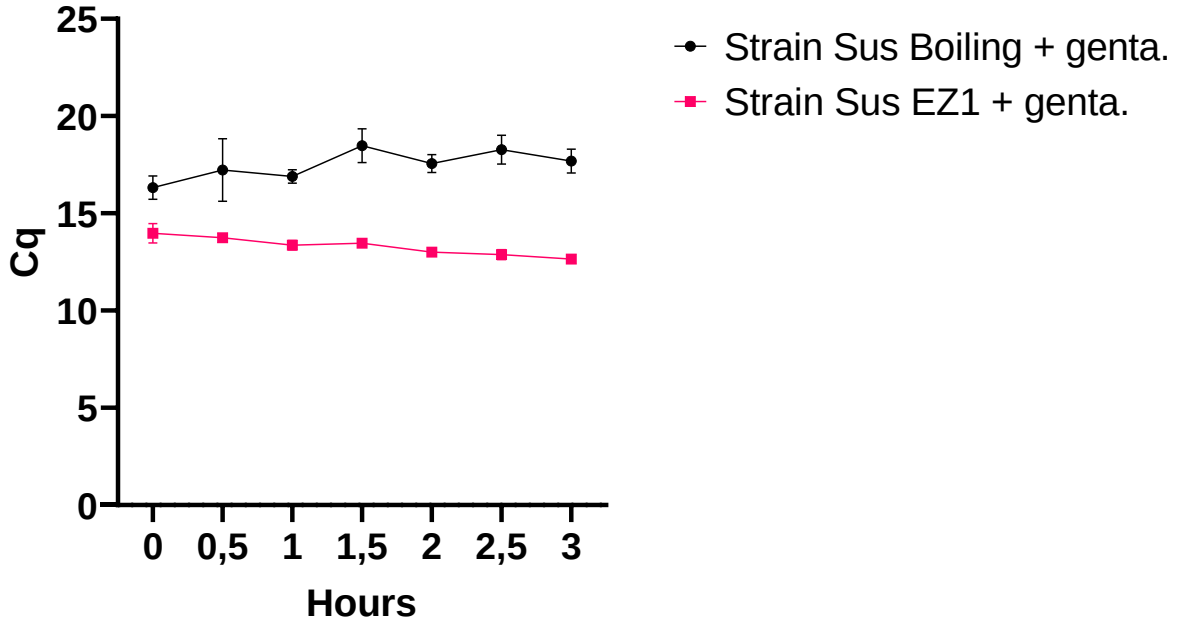

Figure 4.4 The graph shows the Cq-values of strain Sus grown with gentamicin then treated with two different DNA preparations methods , boiling lysis (black) and EZ1 (red). Two parallels were run in the Real-Time PCR. EZ1 has the lowest Cq-values and is more stable than the boiling lysis numbers. Error bars were calculated using 95% CI.

4.3 Primers and Real-Time PCR

A visual representation from Geneious Prime of all eight primers tested in this study and their alignment on the 16S rRNA gene is included below (*Figure 4.5*Figure 4.5 Alignment in Geneious Prime of the eight primer pairs initially chosen to the 16S rRNA gene. Dark green represents the forward primers, and light green represents the reverse primers. Gray arrows under the sequence represents the variable regions, from V1 to V9.). The Hospital primer pair had the longest amplification product, stretching from 340 to 926 bp. This meant it covered three variable regions, V3, V4 and V5. Horz was the primer pair positioned closest to the 3' end on the 16S rRNA gene, amplifying the V7 region, spanning bp 1047 – 1193 (GenBank: J01859.1). Primer pair Muyzer spanned the V3 region, having the same forward primer as the Hospital primer pair, from 340 to 533 bp (GenBank: J01859.1).

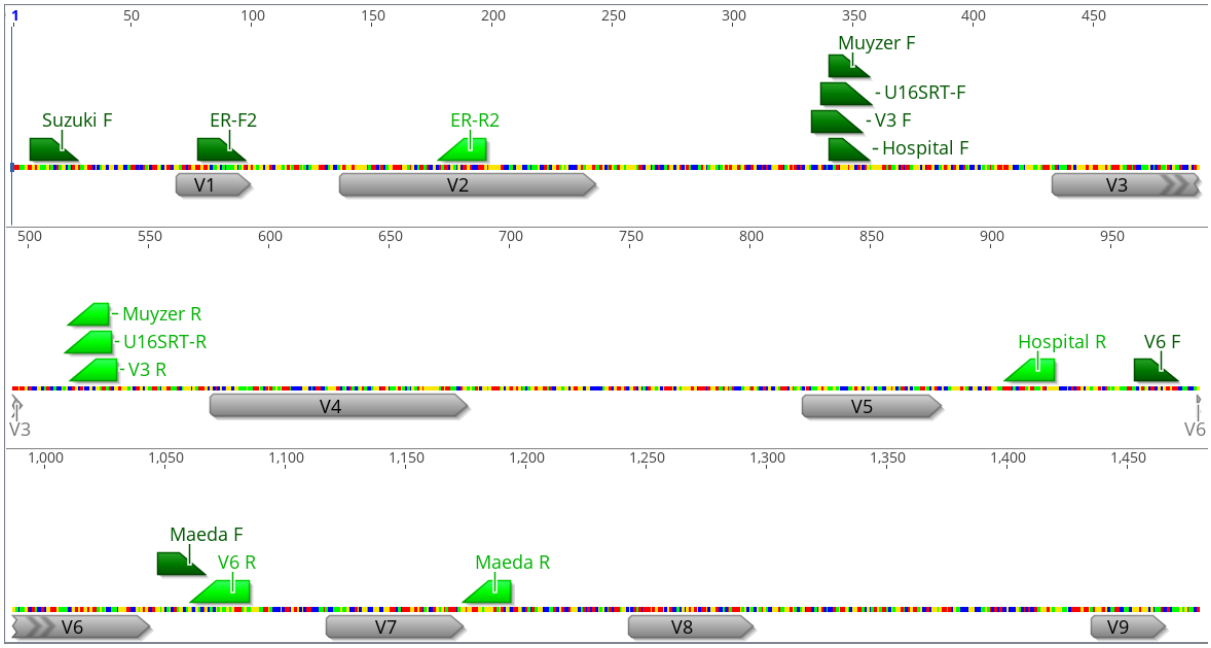

Figure 4.5 Alignment in Geneious Prime of the eight primer pairs initially chosen to the 16S rRNA gene. Dark green represents the forward primers, and light green represents the reverse primers. Gray arrows under the sequence represents the variable regions, from V1 to V9.

Binding complementary to human genomic DNA was confirmed for all three primer pairs but in varying degree. The Hospital primer pair had two alternate bindings, homo sapiens zinc finger BED-type containing 1, HECT domain E3 ubiquitin ligase 4 and aryl hydrocarbon receptor nuclear translocator. With 1598 bp, 534 bp and 1582-1588 bp amplification products, respectively. Horz had the least potential for binding complementary to human genomic DNA with only two possibilities, calmodulin binding transcription activator 1 at 2517 bp and a non-selective sodium leak channel at 757 bp. Muyzer had many possible bindings complementary to human genomic DNA, including gap junction protein gamma 2 (1126 bp), zink finger BED-type containing 1 (1598 bp) and HECT domain E3 ubiquitin protein ligase 4 (534 bp).

4.3.1 Real-Time PCR

For the first Real-Time PCR a primer optimisation was performed alongside a confirmation of the amplification products (*Figure 4.6, Figure 4.7* and *Table 4.10*). Concentrations of 300, 400 and 500 nM were run, with template DNA from both the Sus and Res strains. Samples had Cq-values between 9.28 and 11.34. The difference between concentration of one primer was at maximum 0.77 Cq, and at minimum 0.24 Cq. As there were no major differences in Cq between the concentrations, it was decided to use 300 nM. Negative control ranged from 29.19 - 32.38 in Cq-value. Total time for this assay was approx. 1 hour and 35 min.

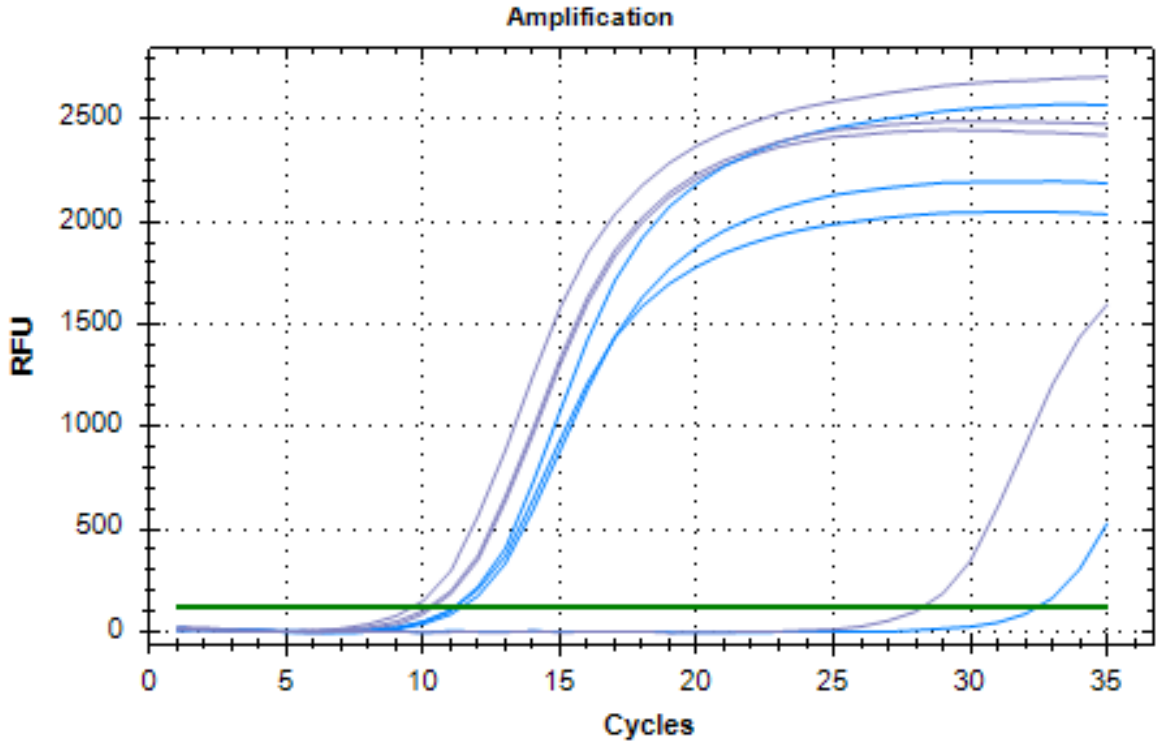

Figure 4.6 This shows the amplification curves of the first Real-Time PCR with primer pair Horz , using both strain Sus (blue) and Res (purple). The run was to optimise the primer concentration, with 300, 400 and 500 nM final concentration. One negative controlwas included per strain.

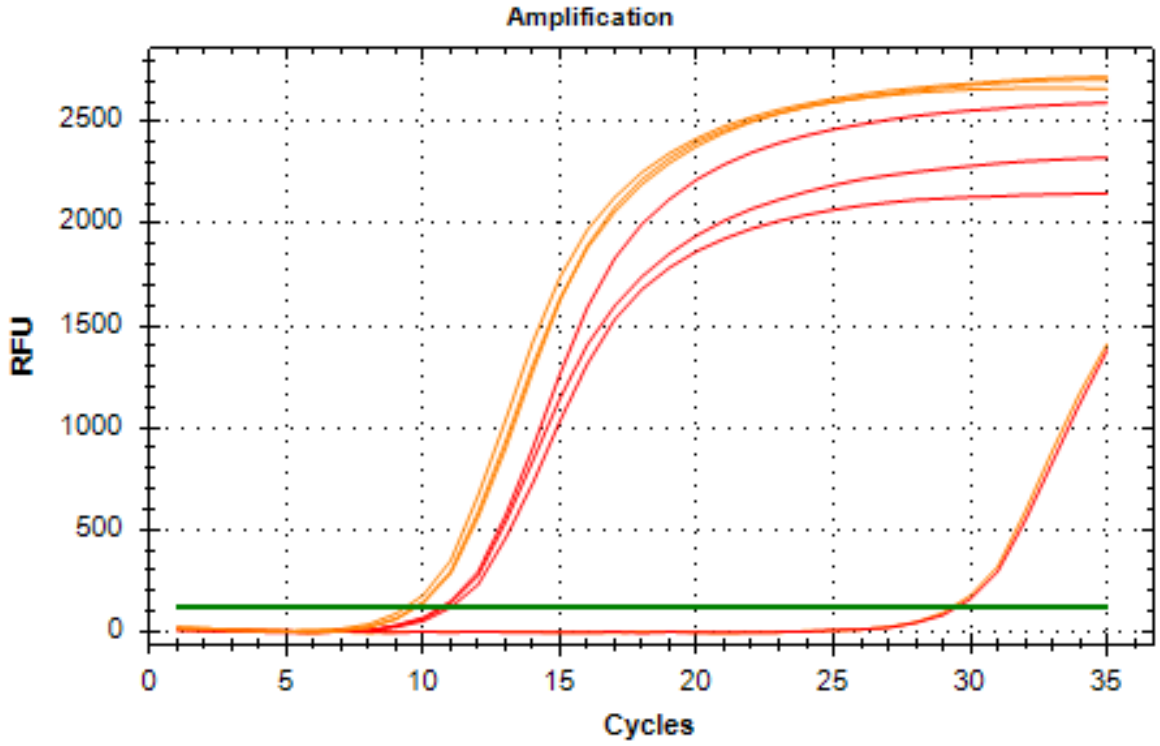

Figure 4.7 This shows the amplification curves of the first Real-Time PCR with primer pair Horz , using both strain Sus (red) and Res (orange). The run was to optimise the primer concentration, with 300, 400 and 500 nM final concentration. One negative controlwas included per strain.

Table 4.10 Table of Cq-values from the first Real-Time PCR performed , were optimisation of primer concentrations were done as well. 300, 400 and 500 nM concentrations were tested for the Horz and Muyzer primer pairs. Template DNA from both the Sus and Res stains were used. The difference between concentration for one primer pair was at maximum 0.77 Cq, and at minimum 0.24 Cq.

Concentration (nM)	**Horz**		**Muyzer**	
	Sus strain	*Res strain*	*Sus strain*	*Res strain*
300	11.34	10.29	10.93	9.67
400	11.11	10.14	10.57	9.59
500	11.10	9.52	10.68	9.28
Negative control	32.38	28.19	29.42	29.26

4.3.2 Identification of non-specific amplification products

Testing for non-specific amplification products were done by including a melt curve analysis in the Real-Time PCR and running gel electrophoresis.

4.3.2.1 Melt peak analysis

Melt peaks usually showed the samples as clear peaks at approx. 84 °C. Negative controls were also peaking at approx. 84 °C but they were not as pointed and were a lot lower. Two examples are shown below from the first primer optimisation (*Figure 4.8* and *Figure 4.9*). Other melt peaks were similar to these.

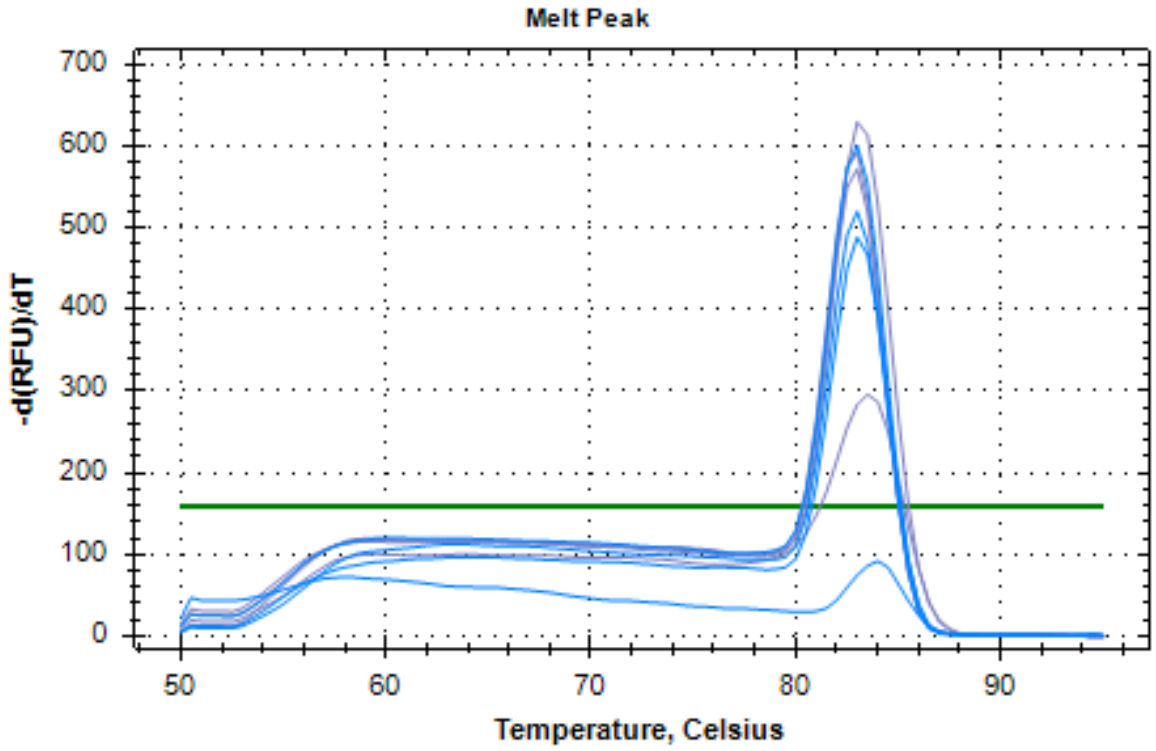

Figure 4.8 Melt peak from the first Real-Time PCR primer concentration optimisation with primer pair Muyzer. 300, 400 and 500 nM concentrations were tested with both the Sus (blue) and Res (purple) strains. Two negative samples were included.

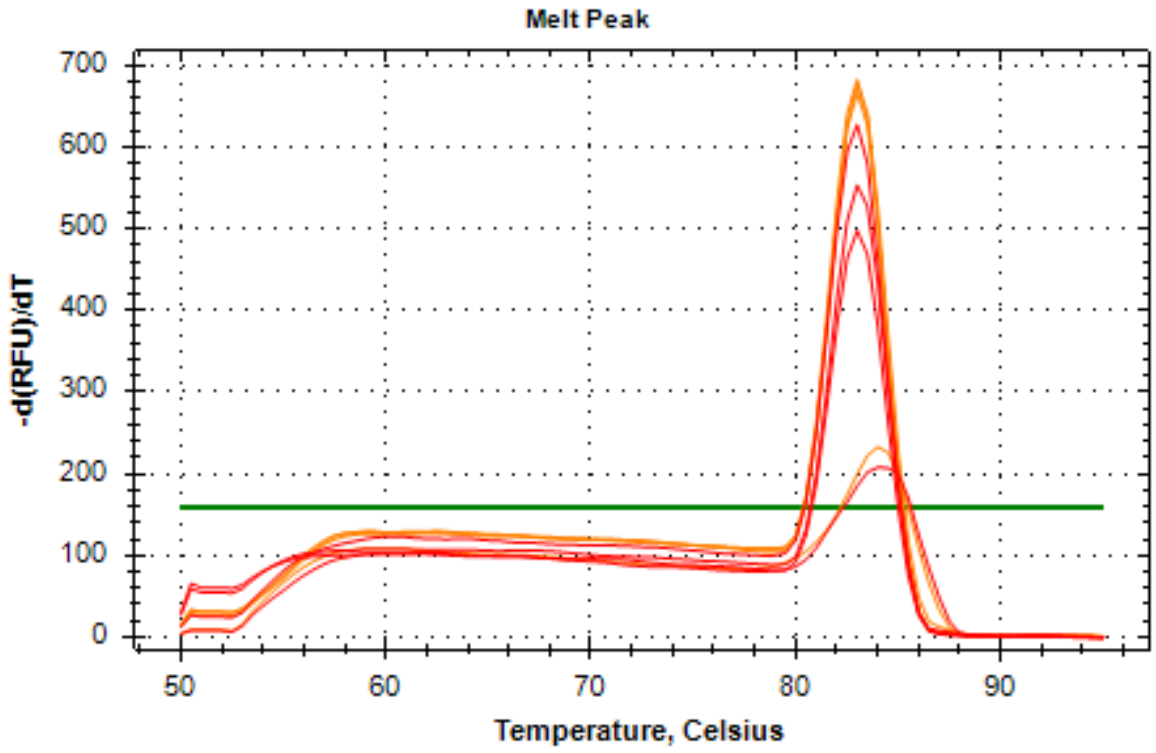

Figure 4.9 Melt peak from the first Real-Time PCR primer concentration optimisation with primer pair Horz. 300, 400 and 500 nM concentrations were tested with both the Sus (red) and Res (orange) strains. Two negative samples were included.

4.3.2.2 Gel electrophoresis

Agarose gel electrophoresis was run as a quality control step to confirm target amplification product and ensure absence of non-specific amplification products. The gels run for the temperature gradient analysis are shown below as an example (*Figure 4.10* and *Figure 4.11*). Samples of amplification product from primer pairs Horz, Muyzer and Hospital were analysed alongside amplification products from negative controls from all three primer pairs. Negative controls from primer pair Horz showed no visible bands. Product from primer pair Muyzer had one faint band on negative control 6 just above 200 bp. Both primer pair Horz and Muyzer had a strong visible band right above 200 bp for their normal samples. For primer pair Muyzer it fits well with its predicted amplification product length of 194 bp. The amplification product from primer pair Horz being over 200 bp doesn't match its predicted amplification product length of 147 bp. Product from the Hospital primer pair sample had a visible band just under 600 bp, which was consistent with its amplification product length of 587 bp. The negative control for the Hospital primer pair showed faint, smudged bands under 100 bp, an indication of possible primer dimers.

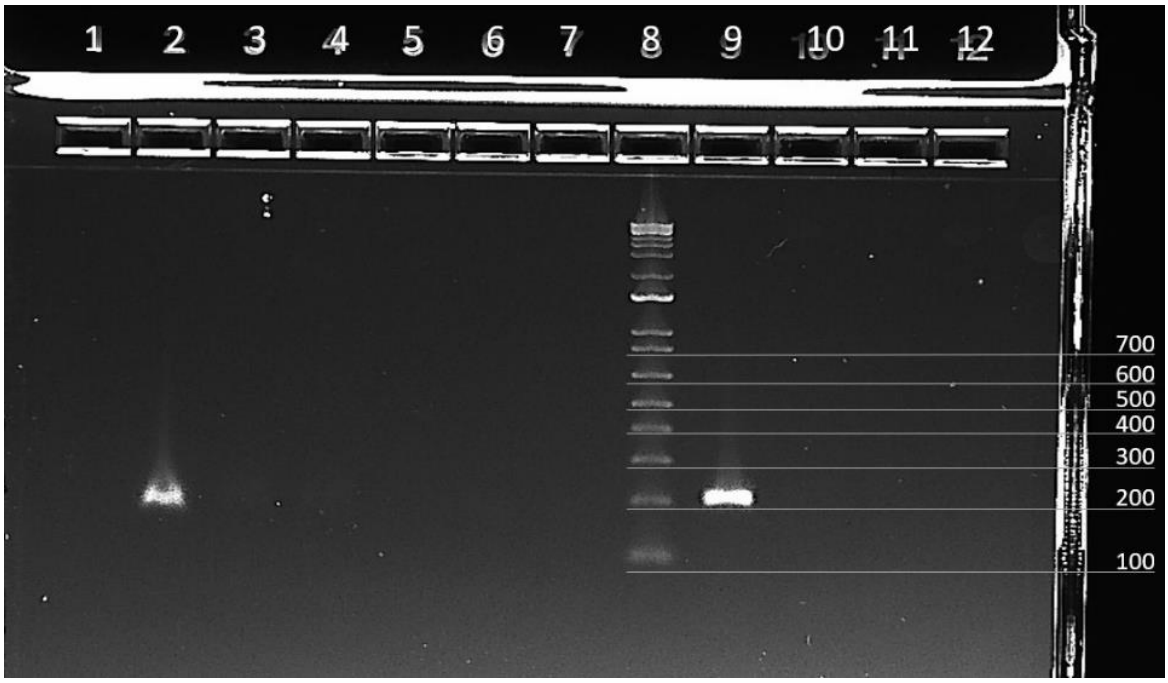

Figure 4.10 Gel electrophoresis image from temperature gradient. Lane 1 is empty. Lane 2 contains amplification product from primer pair Horz sample 2 (63.3 °C). Lanes 3-7 contains amplification product from primer pair Horz negative controls 2 – 6 (63.3 – 55.0 °C). Lane 8 is the 10 Kb ladder. Lane 9 is amplification product from primer pair Muyzer sample 2 (63.3 °C). Lanes 10 and 11 contain amplification product from primer pair Muyzer negative controls 2 and 3 (63.3 and 61.4 °C), whilst lane 12 is empty.

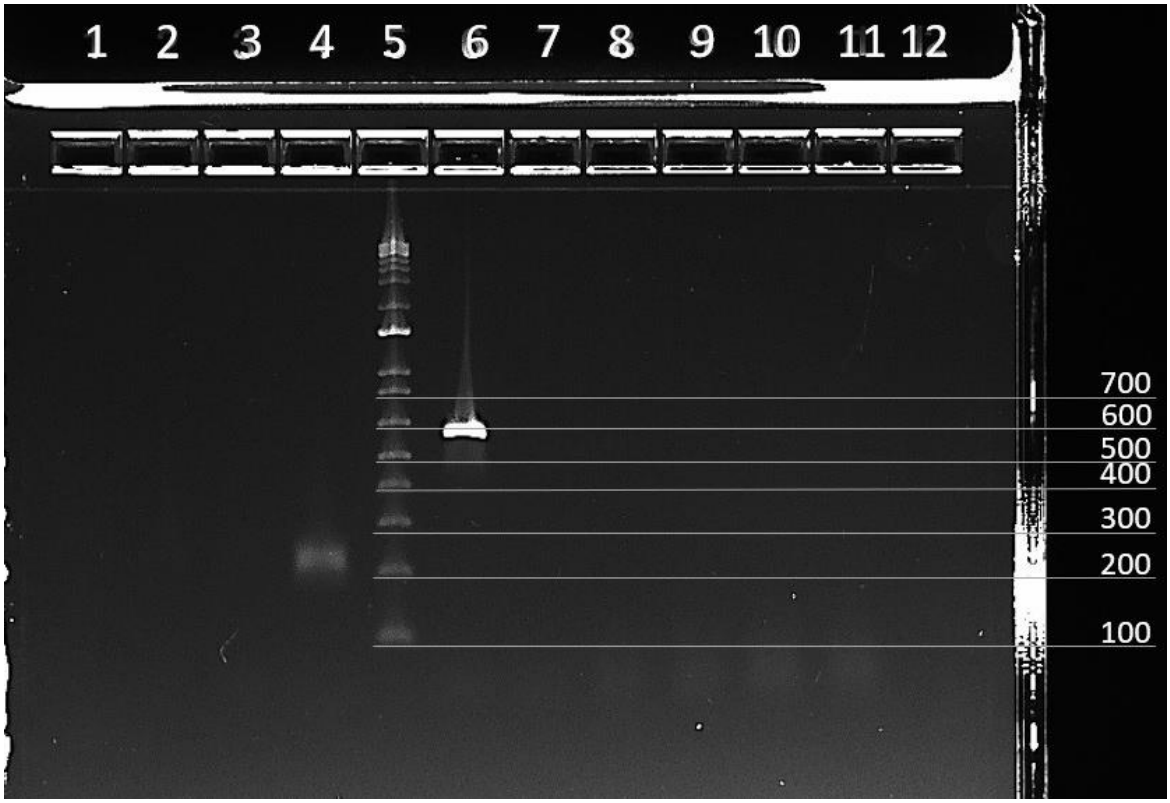

Figure 4.11 Gel electrophoresis image from temperature gradient. Lane 1 is empty. Lanes 2-4 contain amplification product from primer Muyzer negative controls 4 – 6 (59 - 55°C). Lane 5 is the 10 Kb ladder. Lane 6 contains amplification product from primer Hospital sample 2 (63.3 °C). Lanes 7-11 contain amplification product from primer Hospital negative controls 2 – 6 (63.3 – 55.0 °C).

4.3.3 Temperature gradient analysis

The temperature gradient was performed with undiluted bacterial DNA extracted by DNeasy. After Real-Time PCR, all three primer pairs, Horz, Hospital, and Muyzer, were relatively similar in Cq-values (*Figure 4.12*). The Cq-values obtained from primer pairs Horz and Muyzer were almost identical, whilst the Cq-values from primer pair Hospital were slightly lower until the last temperature where the Cq value was the same as that of primer pairs Horz and Muyzer. There were significant differences in the Cq-values between the negative controls and the samples Cq-values. All negative controls, except one, were over 30, and all samples were between 15 and 18 Cq. There were no Cq-values at 65 °C from any of the negative controls.

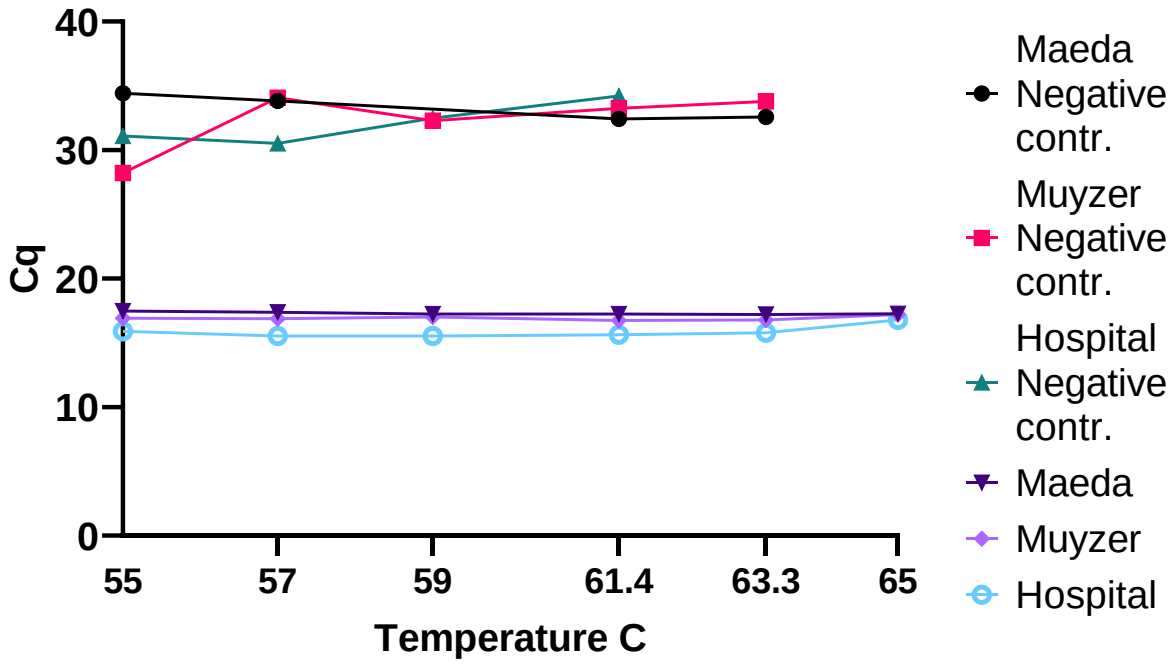

Figure 4.12 This graph displays the temperature gradient for primer pairs Horz (dark purple inverted triangle), Muyzer (purple square on point) and Hospital (blue hollow circle) with their respective negative controls (black circle, red square, and green triangle). Temperature ranged from 55 – 65 °C. There were no available Cq results for 65 °C for any of the negative controls, and no results for 63.3 °C for primer pair Hospitals negative control.

4.3.4 Amplification efficiency analysis

To measure the primers efficiency at amplifying the desired region, an efficiency analysis was performed. It was done four different times, three independent efficiency analyses using DNA extracted by DNeasy (*3.4.1 Manual DNA extraction, DNeasy*) and one efficiency analysis as part of boiling lysis protocol test. All in all, the Hospital primer pair was tested four times, Muyzer primer pair three times and primer pair Horz two times. No preliminary dilution was made for the rest of the analyses, the first sample was undiluted. This resulted in straighter regression lines. Primer pairs Horz and Hospital were run in the analysis, and their efficiencies were calculated to be 108.2% and 96.7%

respectively (*Figure 4.13 A* and *B*). The third analysis included only primer pair Muyzer and resulted in an efficiency of 90.4% (*Figure 4.13 C*). The fourth analysis tested boiling protocol 1 and 2, and used primer pairs Muyzer and Hospital. The efficiency of primer pair Muyzers decreased to 89% for Protocol 1 and 88 % for Protocol 2. The regression line of primer pair Hospital was not straight, this was corrected by removing the two most diluted values (*Figure 4.14*). The efficiency of primer pair Hospital was 133.0% for protocol 1 and 123.6% for protocol 2 before correction. The corrected efficiency was calculated at 94.4% and 100.9% for protocol 1 and 2 respectively

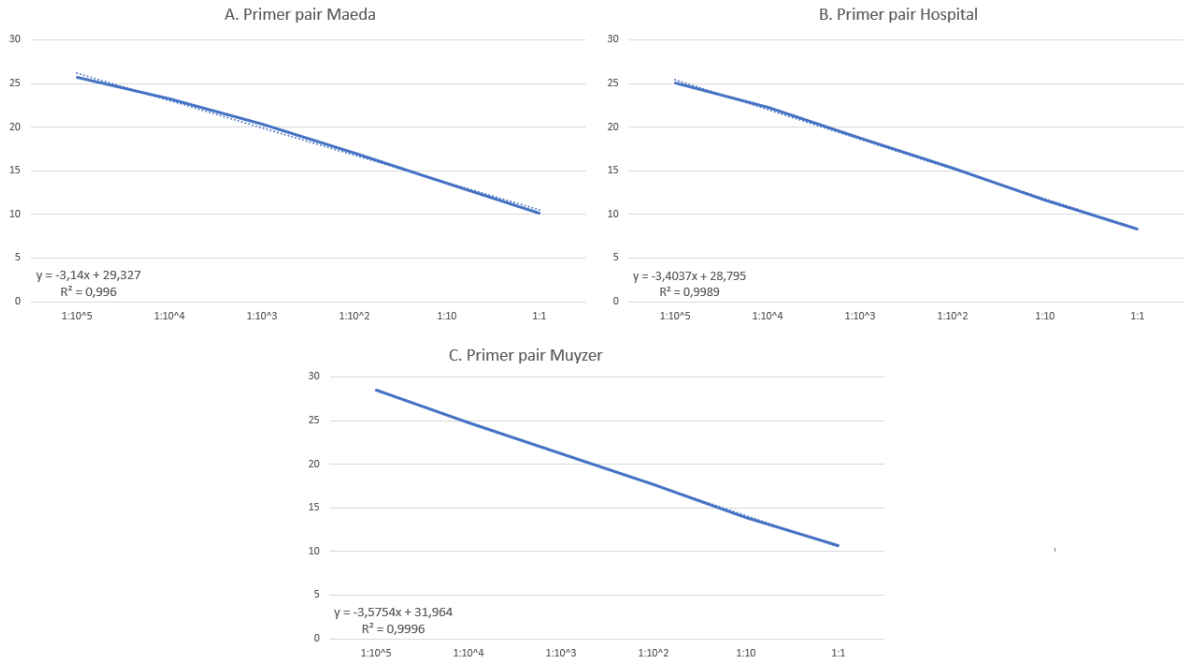

Figure 4.13 These are the regression lines for each primer pairs calculated based on Real-Time PCR amplification efficiency analysis run with undiluted DNA extracted by DNeasy. A: Primer pair Horz's regression line with a slope of -3.14. B: The regression line of primer pair Hospital with a slope of -3.4037. C: Primer pair Muyzer's regression line with a slope of -3.5754.

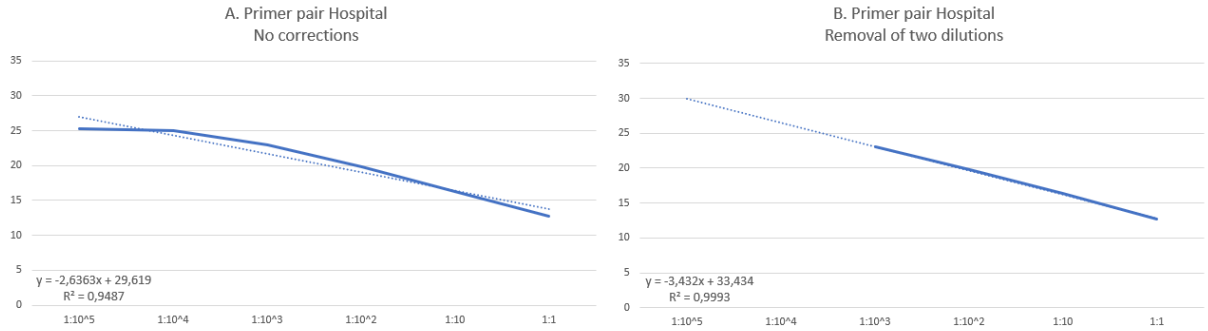

Figure 4.14 Two regression lines for primer pair Hospital from amplification efficiency analysis done after a boiling lysis protocol test using Protocol 1 (*3.4.2 Boiling lysis*). A. Without corrections the line curved at the top producing a slope of -2.6363. B. Removing the two most diluted samples produced a straighter regression line and the slope was -3.432.

4.4 Predicting S-I-R profiles based on growth curves and PCR

4.4.1 Time to differentiate, OD and Cq-values

Strains A-L were measured spectrophotometrically. Then the mean of the strains determined to be susceptible, and the mean of the strains determined to be resistant were calculated. The mean of resistant and the mean of susceptible strains were plotted in the same graph (*Figure 4.15*). At one hour the error bars of the two graphs were no longer overlapping, and some estimation of S-I-R profile was possible. Another graph was made by plotting all the growth curves into the same graph (*Figure 4.16*Figure 4.16). This second graph showed that 1 hour was not enough to positively predict all the S-I-R profiles. At 1.5 hours a prediction could be made as the resistant and susceptible strains had diverged significantly.

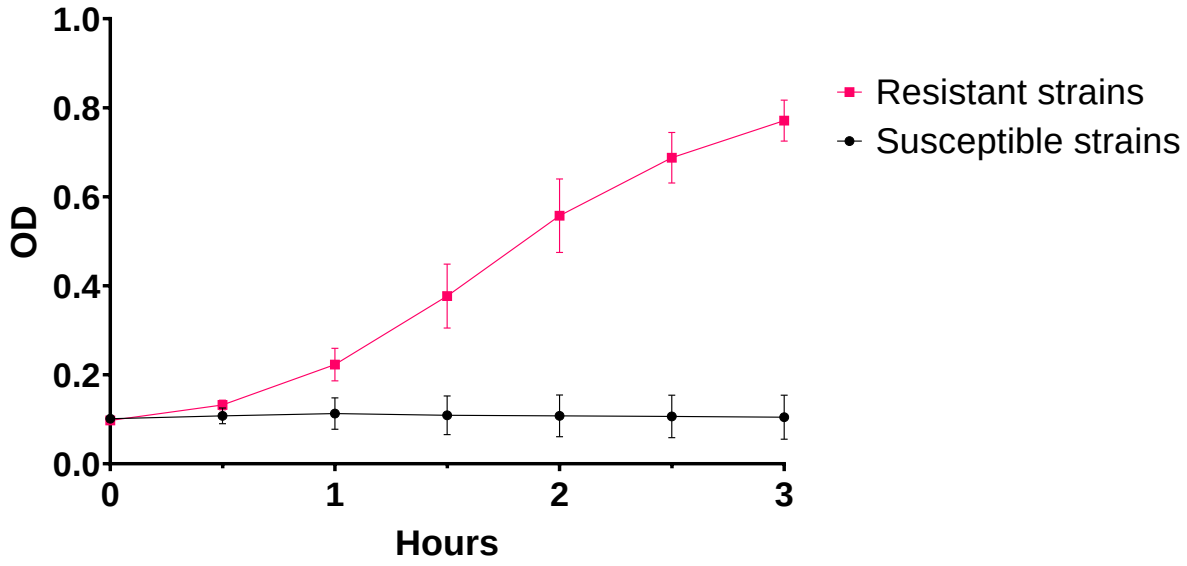

Figure 4.15 This shows a growth curve obtained by the mean of all strains determined to be susceptible (black) and the mean of all strains determined to be resistant (red) grown in gentamicin to determine the length of time needed to predict resistance in these *E. coli* strains. Error bars were calculated by 95% CI.

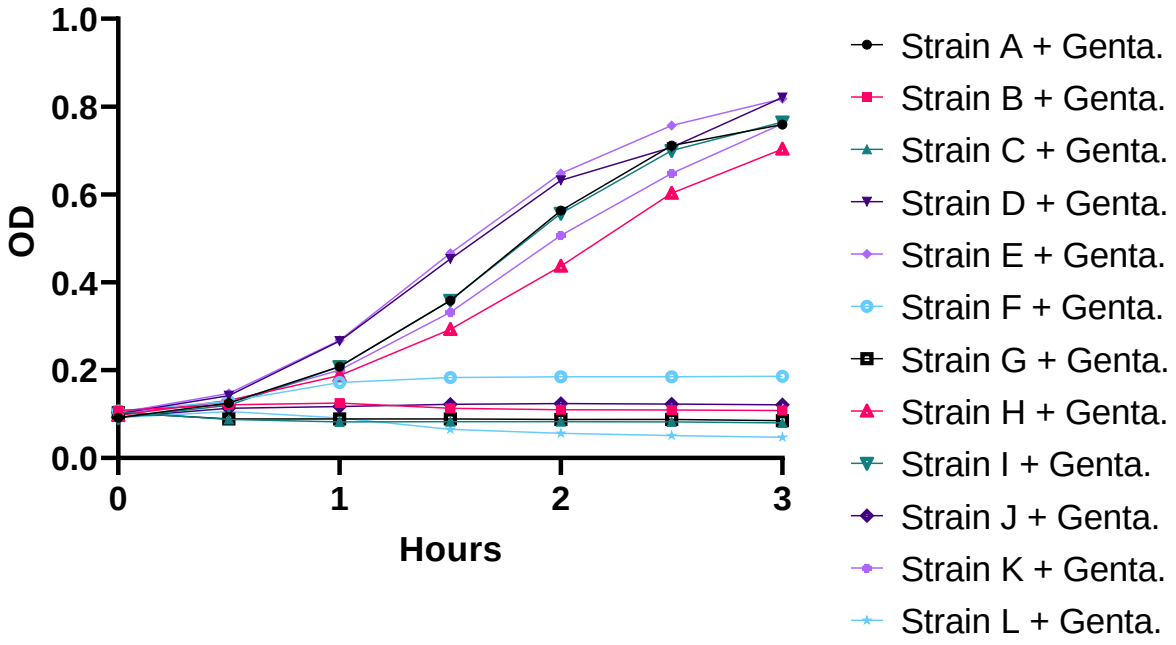

Figure 4.16 The graph shows every growth curve from strains A-L grown in gentamicin based on OD measurements.

When trying to determine the S-I-R profile from Cq-values it became harder than by OD600. The graphs didn't separate as early, and error bars were overlapping more often than with OD measurements. Growth curves of the Sus strain grown with or without gentamicin is included below as an example. The graph shows one curve derived from Cq-values and one derived from OD values (*Figure 4.17* and *Figure 4.18*). This was one of the better runs for the Real-Time PCR in regards to the stability of the growth curve based on Cq-values. It was not possible to estimate a S-I-R profile based on Cq-values before the 4 hour mark (*Figure 4.17*). The error bars are quite large and overlap at 0, 0.5, 1 and 3 hours. It did look promising from 1.5 hours, but at 3 hours the Sus strain grown without gentamicin suddenly rose in Cq value and the error bars overlapped again. Using OD measurements from the same experiment a prediction could be made already after 0.5-1 hour (*Figure 4.18*).

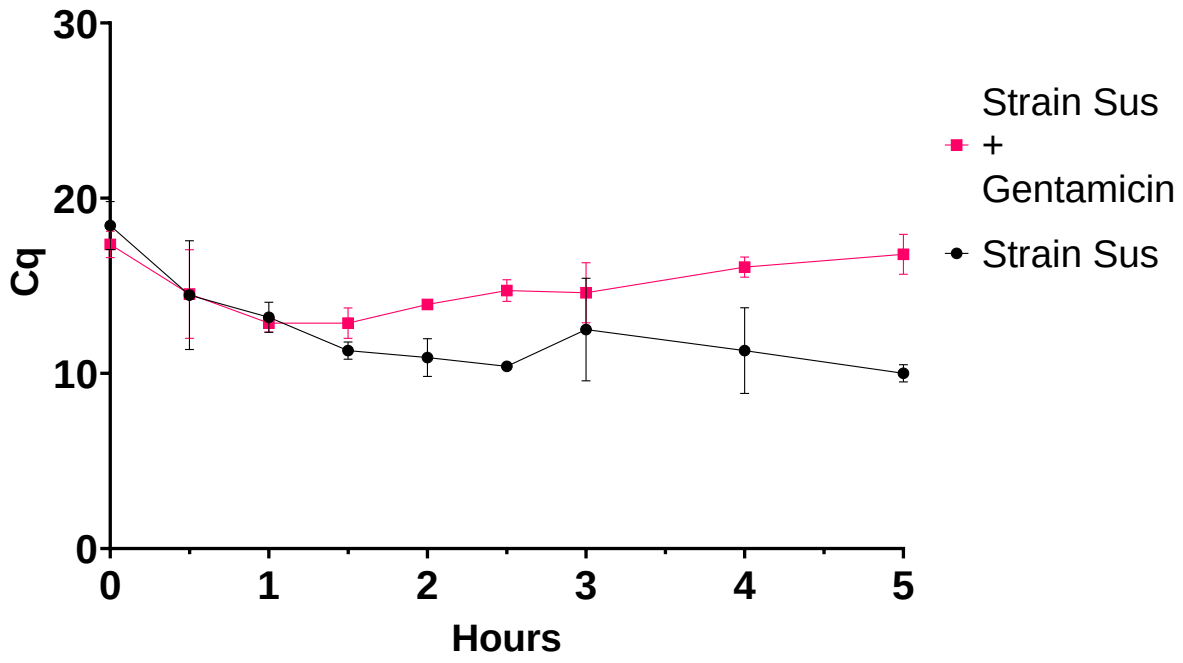

Figure 4.17 This graph shows a growth curve based on Cq-values from strain Sus grown with (red) and without (black) gentamicin. The samples were run in parallel in the Real-Time PCR. The error bars were calculated using 95% CI.

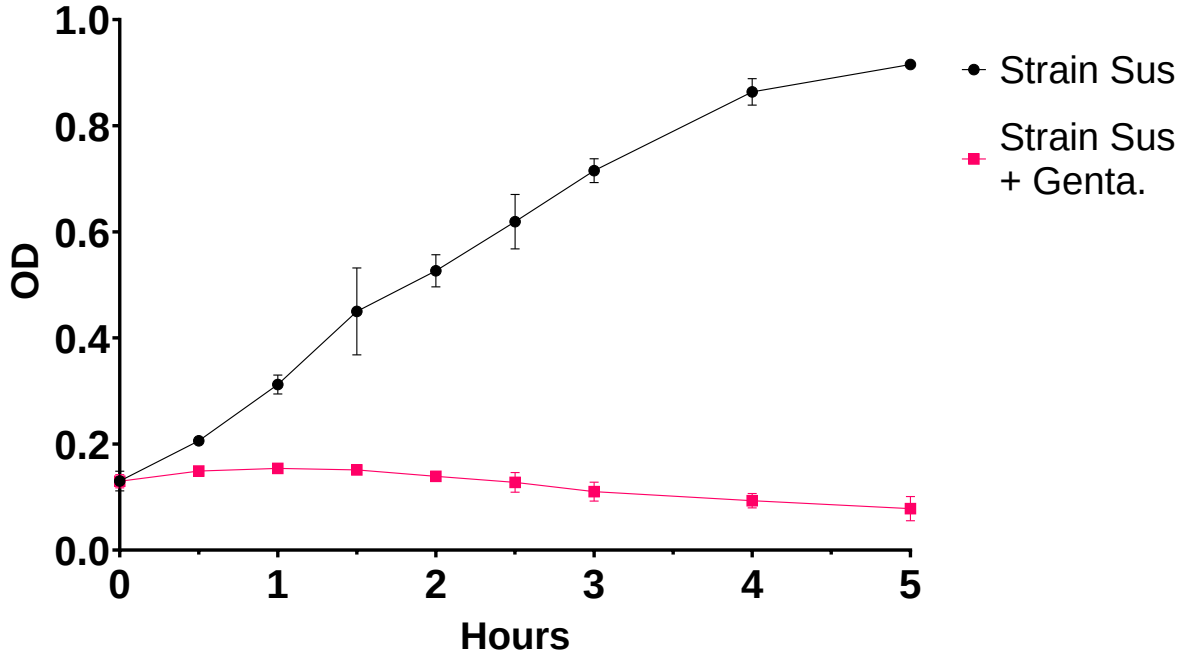

Figure 4.18 This is a growth curve based on OD measurements from strain Sus grown with (red) and without (black) gentamicin. Samples were run in parallel in the Real-Time PCR. Error bars were calculated using 95% CI.

4.4.2 Predicted S-I-R profiles of *E. coli* strains

Every strain's original S-I-R profile was confirmed by measuring their MIC-values using gentamicin MIC-test strips (*3.2.1 Minimum inhibitory concentration testing*). Results show all strains had the same profile as reported when acquired (*Table 4.11*). Seven strains were susceptible, and seven were resistant.

Growth curves based on OD measurements were used to determine if one could predict S-I-R profiles. The predictions of the S-I-R profile of strains A-L was blind, but not for the control strains Sus and Res. Predictions were correct for every strain (*Table 4.11*).

Table 4.11 Every E. coli strain used in this study, their S-I-R profile, MIC values (mg/L) and their predicted S-I-R profile based on the growth experiments.

***E. coli* strain**	**S-I-R profile**	**MIC (mg/L)**	**Prediction of S-I-R based on growth experiment**
Sus (CCUG 17620)	S	0.25	S
Res	R	16	R
A	R	16	R
B	S	0.25	S
C	S	0.50	S
D	R	32	R
E	R	64	R
F	S	0.25	S
G	S	0.50	S
H	R	16	R
I	R	16	R
J	S	0.25	S
K	R	16	R
L	S	0.50	S

5 Discussion

This discussion will deliberate on the four-part results presented above: growth curves with their lag and log phases, comparison of the two bacterial DNA preparations, boiling lysis and EZ1, Real-Time PCR struggles and predictions of S-I-R profiles. It also includes sections on limitations and further research.

5.1 Growth experiments

To accurately perform growth experiments it's essential for there to be growth, and in this case, optimal growth was the goal. After a failed attempt at making an ON culture, optimisation of growth vessels was done. This could have been avoided with better literature searches into bacterial culturing. In later stages of research some information about vessel size and broth volume were found, but by then the optimisation had already taken place. Even if this information had been available at the start, some optimisation would probably have been necessary to test the variables in our lab.

Having to remeasure the cuvette volume was a surprise. After struggling to obtain consistent OD measurements with the stated cuvette volume of 70 µl, several test volumes were run to optimise the cuvette volume. By almost tripling the volume to 200 µl the problem was solved. It seemed the problem was getting the meniscus in the same place each time. This could have been due to manual pipetting not getting the fluid in the same position each time. This could be improved by automated pipetting, or a more seasoned hand. Drops from the pipette would also cling to the side of the cuvette, maybe due to the static electricity of the plastic. It would appear the increased volume raised the meniscus out of the way of the light path so it would not interfere with the measurement. There were no further problems after changing the volume and OD measurements were consistent.

It was decided to follow the EUCAST recommendations for AST as closely as possible. This was done to the best of our ability, but some things were out of our hands, like the changing of broth supplier. As the Department of medical microbiology was our supplier, we were forced to change broth when they did. This was not optimal as it states in "*How to accelerate antimicrobial susceptibility testing*" by E.A. Idelevich and K. Becker [99] that the variables should not change throughout an experiment. If parameter were changed a new validation of the method should be performed [99]. There were no tests done to compare the new and old broth suppliers, but no changes in the experiment results were observed. An assumption was made that the two suppliers would be similar enough as they are both well known, large global manufacturers of laboratory supplies.

This project used plain MH broth, not cation-adjusted as suggested by the EUCAST methodology [49], a mistake by the researcher. By the time this was uncovered the experiments were already started and it was decided not to change yet another broth parameter as the supplier already had been changed.

Both OD600 and McFarland was measured for most growth experiments. McFarland was chosen for its semi-direct relationship to CFU/ml [65, 66]. OD was chosen because of its

ease of use, availability and it being commonly used for bacterial density determination. OD does have a connection to CFU/ml, but it should be confirmed through a standard curve using known suspensions of bacteria. This was not done, only a preliminary search online was done to look up approximate OD values and CFU/ml. It was probably redundant to have two measurements for turbidity. It would have been advantageous to just chose one from the beginning to save work in the process. In the end only OD measurements were used, and it is debatable if OD or McFarland would have been the best choice. OD was chosen because of the lower volume it needed for measurements, 200 µl instead of 2 ml. McFarland could have been a good choice too, especially regarding calculation of CFU/ml.

It was not important in this project to know the absolute number of bacteria present in a sample. What was of interest was the relative change to the first sample taken in a growth curve. Even though, a plate count could have been done to back up the OD and McFarland results. As it stands now, the number of bacteria in all samples is conjecture. This was not done due to time restraints.

The amount of bacterial cells from a positive BC would be diluted when put into broth. The cell numbers operated with here are therefore too high as they correspond to the amount of cells in a positive undiluted BC. It should have been diluted to replicate the starting concentration of a BC sample diluted in broth.

5.1.1 Lag phase and exponential phase

These phases were easily uncovered by looking at the growth curves. All the strains' lag phases were consistently between 1 and 1.5 hours. The exponential phases for all strains were 0.5 hour, except for one experiment were the Sus strain's exponential phase was 1 hour. This could be due to these measurements being done by eye, or other parameters in the growth experiment.

As measurements of lag and exponential phases were done by eye it might lead to some inconsistencies, even if it was done as accurately as possible. It might have been advantageous to have used a computer program or other calculating methods to get a more accurate result, avoiding human error. This was not done, due to lack of familiarity with such programs or any other alternative to observations by eye.

It was not optimal in terms of speed, that the lag phase lasted 1-1.5 hours. Some optimisation of the broth temperature had been done. Heating for 30 min was done but no temperature measurements were taken of the broth to confirm adequate heating. Another parameter to change to shorten the lag phase would be altering the medium. However, this would be in direct opposition of the EUCAST guidelines as it is not a standard broth and affects the reproducibility of the experiment [99].

5.2 Preparation of bacterial DNA

Boiling lysis and EZ1 were both seen as possible options for preparation of bacterial DNA. Here they are explored and compared to determine which would be the best choice for this Real-Time PCR method.

5.2.1 Boiling lysis

This boiling lysis method was developed to be a fast and easy to use as possible, with moderate success in both parameters. In terms of speed, it was not as fast as one could have hoped. It took up to 25 min to run nine samples. It was very hands-on work, meaning one person had to be present and could not really engage in other tasks before the lysis was finished. There were no advanced skills needed to perform this method as the hands-on work was basic pipetting and moving of tubes. The issue was the frequency of when the hands-on work was needed; every 5 min. A positive side was that there was no need to purchase kits or special equipment. All instruments used were readily available in the lab, and the materials used are relatively inexpensive.

The first thing done was to test and compare two boiling lysis protocols (*3.4.2 Boiling lysis*). Looking at the results in *Table 4.3*, one can see protocol 2 having the overall lowest Cq-values. Protocol 2 had an extra boiling step at the end, this would potentially make this method more cumbersome, but the potential gain might be worth it. Protocol 1 was chosen as the starting point for further testing as it used less time and was simpler. The results in *Table 4.3* make the choice of protocol 1 questionable.

Two temperatures were compared, 95 °C and 99 °C, with the final choice for the protocol being 95 °C. In both tables presented above, *Table 4.4* and *Table 4.5,* 99 °C had consistently lower Cq-values than 95 °C. The results were in their raw state in an excel file, and not ordered in tables, when the final boiling temperature was chosen. Had the temperatures been ordered, a different boiling temperature would have been chosen.

The 5 min boiling time seems to have been the correct choice based on the results. *Table 4.6* above, shows the lowest consistent Cq-values were found at 5 min boiling. Boiling for 10 minutes might not have worked due to the prolonged heat. This could have denatured the DNA, similar to the action in a PCR denaturing step at 95 °C.

Centrifugation time did not seem to affect the Cq-values greatly. As shown in *Table 4.6* there was only a 0.8 difference in Cq between 1 and 10 min centrifugation. Centrifugation did affect the formation of a visible pellet. Both 3 and 5 minutes were used, but 5 minutes seemed to work better at lower concentrations of bacterial cells.

Centrifugation speed was never optimised. The max speed of the centrifuge used was chosen. Centrifugation was originally only done after the boiling lysis step, and therefore there was no need to be gentle. The bacterial cells were already lysed, and the assumption was that a faster speed would sediment the cell debris faster than a slower speed. As a centrifugation step was included to remove the MH broth, it was decided to make it identical to the other centrifugation step for convenience. Now the potential down-side of such a harsh centrifugation speed could be problematic. There was a possibility of the cells breaking in centrifugation and releasing their DNA into the broth that would be removed. This did not seem to be a problem but should have been evaluated when establishing the second centrifugation step.

The shaking of the tubes when boiled was not a parameter tested on its own. As protocol 1 included shaking, no thought was put to it not being included. Theoretically one could argue that shaking was better as it moved the sample, allowing more of it to be in close contact with the heating element. Thus, this movement heated the sample more efficiently. Since this was not tested, it is only conjecture.

The method developed here has room for improvement. It has only been tested on one gram-negative bacterium, *E. coli*. Initial results were not properly organised, due to a

desire to get as much work done as quickly as possible. Decisions regarding protocol choice and boiling temperature were not made with a proper overview of the results. When compiling the results, organising, and making graphs and tables it became clear that some decision made were not reflecting the actual results. However, this was only a preliminary proof of concept. If any further research is to be done, this would be a good starting point to work from.

5.2.2 Automated DNA extraction, EZ1

This method was very fast, with little hands-on time. Running 14 samples took 17 minutes, excluding setup time. Hand-on time was limited to the beginning, meaning one could to other tasks in the 17 min the instrument ran. The setup was not complicated and there was no need for any special skills. One down-side was the cap of 14 samples each batch. There was no option to add addition samples when the run had started. This might not be a problem as the runtime is so fast.

With EZ1, Cq-values could indicate the Sus strain to be susceptible after 1.5 hours. When the Sus strain was grown with gentamicin there was a slight unexpected decrease. The drop is not large, so one could argue that it's not consequential. The culture could of course have continued to grow slightly in gentamicin, but there are no signs of this in either OD600 or McFarland measurements. In the Sus strain grown without gentamicin there was a clear sign of growth by the declining Cq-values. There was a surprising small increase in Cq-values up until 1 hour. This would indicate no growth, but again, nothing in the OD600 or McFarland measurements would indicate this. One could imagine the three first samples, 0, 0.5 and 1 hours, had been mixed up. It would explain why the three first samples grown with and without gentamicin did the exact opposite of what was expected. However, such a mix up is unlikely.

At the time of this project there were some problems acquiring kits for the EZ1 instrument. Using equipment where kits are an essential part of the instrument might lead to problems in times of shortage. In addition, the kits used for EZ1 are a lot more expensive than the ingredients needed for boiling lysis. This was one of the reasons why there were only one run performed in this project.

5.2.3 Boiling lysis and EZ1 compared

Several aspects were considered before a lysis method was chosen: cost, time and PCR results. Cost-wise, boiling lysis has the clear advantage. The method needs only a few basic components that are commonly found in any microbiological laboratory. EZ1 is clearly the more expensive option, but it can be seen as an investment. There is a large upfront expense in purchasing the instrument, and a running cost in maintenance and kit purchases. As experienced in this project, one can run into problems acquiring kits when relying on another party.

When it comes to time spent on each method, EZ1 is the best option. Running 14 samples only took 17 minutes (not including set up), and in those 17 minutes the operator was free to do other things. For boiling lysis, nine samples took 25 minutes, with the operator not being able to do other things due to the large amount of hands-on work. That was not optimal for a method that aspired to be fast and simple.

Comparing Cq-values from the Real-Time PCR, EZ1 achieved lower and more consistent Cq-values compared to boiling lysis (*Table 4.9*). The consistency of EZ1 compared to boiling lysis is seen especially well in *Figure 4.4*. The Cq-values from boiling lysis oscillate

from each time point to the next, not forming a line but a wave. The EZ1 Cq-values form a slightly downwards line, with only a small increase at 1.5 hours.

It would have been advantageous to test EZ1 more times to confirm the results in this project. Also, to test different parameters like other species. Based on these findings EZ1 would be a good option for an institution with the funds to back up the cost.

5.3 Primers and Real-Time PCR

When searching for primers for this Real-Time PCR, an important factor was the product length. It was decided to look for primers with a product length between 150-200 bp, because this method should be as fast as possible and it is a Real-Time PCR protocol. The reason why was that a short amplification product would be copied faster than a long one. There is also no need for a long product as the amplification product won't be used for anything, and would therefore only cost time and resources. This line of thinking ended up being irrelevant. The Hospital primer pair was included as a control for a good clinical primer, meaning the PCR program should amplify its 587 bp amplification product. This meant the Real-Time PCR protocol wasn't optimised for the shorter amplification lengths, but for a long one. Not optimising the PCR protocol for shorter lengths might contribute to the Hospital primer being chosen as the main primer for this project. One could however argue that the longer amplification products Real-Time PCR protocol had little effects on the amplification of the shorter lengths. When the shorter amplification length had been copied, nothing else would have happened to them.

There were no efforts optimise the reverse and forward primers separately. It's not always true that the best results are found with an identical concentration for both forward and reverse. In this project the choice was made to keep the two concentrations identical due to ease of use. It should still have been looked into, to confirm or deny if it would work better with dissimilar concentrations. As all concentrations tested in this project performed equally well it was decided to go with the lowest concentration. This was primarily to minimise cost, and as none were better than the other it was as good a concentration as any.

One might argue there should have been made new primers for this project. This was briefly considered, but ultimately decided against, because of time restraints and the author's inexperience. Instead, already published primer pairs and the primer pair used by the Hospital was chosen.

5.3.1 Real-Time PCR

The Real-Time PCR protocol corresponded to the Hospital primer pair. Since it was established from St. Olavs hospital no further testing was performed, trusting their protocol.

5.3.2 Identification of non-specific amplification products

The nun negative controls were a problem from the first run. They always seemed positive, with a C-value of approx. 30. The negative controls of the Hospital primer pair were also above the threshold, but they were always above Cq 30. This resulted in changing negative controls to be run with PerfeCta instead of water, as mentioned in the protocol for 16S Real-Time PCR by St. Olavs Hospital. Minimising the water content helped elevate the Cq-values. This might indicate some minor contamination in the water

or other reagent used. As 16S rRNA is found in every bacterium and the primers used here were binding to conserved regions, unselective amplification of any present bacterial DNA is likely. It is a known problem that reagents might contain small amount of bacterial DNA. One can treat reagents with UV-light or filter them, but there was no effort in this project to reduce the amount of bacterial DNA present.

5.3.2.1 Melt peak analysis

A melt curve analysis was included in the protocol to detect any nonspecific amplification products. The negative controls that were partially positive showed different mounting temperatures in the melt curve analysis than that of the samples. This means they were not amplifying the same product as the samples. The inclusion of melt curve analysis is a good option for quality control.

The challenge with 16S rRNA gene is its universal application. The amount of contamination present can be low, but if the 16S rRNA gene is there it will be amplificated. Therefore contamination minimalization is extremely important when using 16S rRNA PCR protocols.

5.3.2.2 Gel electrophoresis

The age of the gels affected some of the results on the first run. This was avoided in the following runs by not using the edge wells. After using all the old gels, new gels were bought. No edge wells were used on the new gels either, for consistency. They probably could have been used, but extreme caution was being used on the account of the gel electrophoresis equipment not being the newest on the market.

There were some inconsistencies with the product lengths concerning primer Horz. On the gels it appeared to be above 200 bp, but it should have been around 150 bp (*Figure 4.12*). This was not consistent in every run though. The reason could be the readers fault for not measuring correctly. The challenge using gel electrophoresis is comparing sample lines with the ladder.

5.3.3 Temperature gradient

A temperature gradient was done to find the optimal annealing temperature for the primers. As there were minimal changes seen in the gradient, all primers ended up having roughly the same annealing temperature, 58 °C.

There were no results for 65 °C for any of the negative controls, and no results for 63.3 °C for the negative control of the Hospital primer pair. Because of the lack of Cq-values at these temperatures they couldn't be plotted into the graph. The reason they were missing could be that no annealing took place as the amount of DNA template was too low resulting in the primers not binding.

The annealing temperature for the Hospital primer pair was set to 58 °C following the protocol form St. Olavs hospital. According to the temperature gradient it could have been set to 63.3-65 °C. These temperatures yielded low Cq-values for the samples, and no amplification for the negative controls. 58 °C was not tested in this gradient, but 57 °C and 59 °C were. In the samples there were no differences between the two temperatures. But in the negative controls there was an increase in Cq from 57 °C to 59 °C. By using the temperature in St. Olavs protocol some work of evaluating a potential annealing temperature change was avoided.

5.3.4 Amplification efficiency analysis

The best annealing temperature should yield a low Cq-value for specific amplification, without nonspecific amplification. Amplification efficiency turned out to be the deciding factor choosing a primer. The Hospital primer pair was the closest to 100 % efficiency most of the time, but only if the calculations were corrected. The last two dilutions were removed from the regression line because of their close values. This is especially apparent in *Table 4.3* were primer pairs Muyzer and Hospital are used to compare two boiling lysis protocols. Primer pair Muyzer has almost the same difference in Cq-value for all the dilutions. Primer pair Hospital had the same difference in Cq-value except the two lowest concentrations, $1{:}10^4$ and in $1{:}10^5$. The expected 3.32 rise in Cq was instead 0.47 and 1.03. This might point to primer pair Hospital having problems amplifying lower concentration of template. Alternatively, and contractionary to the first point, the primer might be good at amplifying template at low concentrations due to the decrease of PCR inhibitors.

The three primers were never run all in one Real-Time PCR. It is therefore a little difficult to compare as all were never in the same assay at the same time.

5.4 Predicting S-I-R profiles based on growth curves and PCR results

5.4.1 Time to differentiate, Cq-values and OD

When estimating S-I-R profiles, Cq-values were more difficult to interpret than OD values. Resistant and susceptible strains were similar to each other, and often there wasn't a clear difference in Cq until a few hours had passed. Looking at *Figure 4.17* and *4.18*, one can clearly see the problem. *Figure 4.18* with OD measurements show a difference as early as 30 min, and it only increases with time. After one hour one can determine this strain to be susceptible to gentamicin. The Cq-values from the same growth experiment (*Figure 4.17*), do not yield as clear results as the OD measurements do. The two parallels are almost indistinguishable at 30 minutes. Not until hour two do they start to part enough to make an estimation. But even then, the parallels are close at three hours, not presenting a stable graph.

The reason Real-Time PCR might fail to detect changes in CFU/ml early on is that the Cq-values are not directly correlated to this increase. A 10-fold increase in DNA template leads to a 3.32 decrease in Cq when the amplification efficiency of the PCR is 100 %. To reach a 10-fold increase in the 16S rRNA gene a 10-fold increase in cell density is needed, and this doesn't happen until 3 hours for most growth curves (*Figure 4.16*). As the generation time for E. coli is 20 min in optimal conditions, a 10-fold increase in cell density would take 1 hour and 5 minutes, but that's in perfect conditions. As seen in the OD measurements from *Figure 4.18*, in one hour the cell density has only doubled, not even near a 10-fold increase. This means Real-Time PCR might be unsuited to detect such minuscule changes under 10-fold increases happening in growth curves.

The difficulty of interpreting Cq-values might also be influenced by the DNA preparation method. In most cases boiling lysis was utilised for DNA prep., but as discussed in *5.3 Preparation of bacterial DNA*, changing the DNA prep. method might improve the outcome of Real-time PCR.

The OD measurements were used to estimate S-I-R profiles. McFarland values could probably have been used for this as well, but they were not available for the last growth experiment. When comparing resistant strains and susceptible strains it was quite easy to see what strains were resistant and what strains were susceptible. Susceptible strains would have little to no growth, and some even declined after a time. Some resistant strains did have a period where they seemed to be affected by gentamicin, but after adapting to the environment all resistant strains grew.

5.4.2 Predicted S-I-R profiles of *E. coli* strains

No particular hardships were discovered in predicting the S-I-R profile of the strains from the OD results. The predictions made were 100 % in line with the results from the MIC test. A factor that plays into this is that there were no intermediate strains chosen, as they do not exist by definition. Using NordicAST Breakpoint tables for interpretation of MICs and zone diameters there is no intermediate category as above 2 is resistant and below or equal to 2 is susceptible [98]. Expectations were then to find either resistant or susceptible strains, which made it easier to determine.

5.4.2.1 Bacterial strains and MIC-values

In this project *E. coli* was chosen as the model bacterium to test and optimise both Real-Time PCR and boiling lysis. The reason for this was the availability of the bacteria, combined with the immense amount of research material available. And most importantly it is relevant to the problem at hand, BSI. *E. coli* is the number one gram-negative cause of BSI. Of course, one would have liked to test more types of bacteria, like gram positive, but it is sufficient for a starting point.

The MIC results were very important to the project because they would be compared to the other results as a control template. That meant these results should be as close to clinical results as possible. To achieve this, a staff member at the Department of medical microbiology St. Olavs hospital was engaged to perform the test. He had training, and was doing such tests regularly, meaning the results would be as uniform and standardised as possible.

5.5 Limitations of the study

Many decisions were made before compiling the proper results, weakening those decisions. However, none of these decisions were directly detrimental to the project but does show a potential for personal improvement.

The diversity of the project was low. Only one bacterial species was tested against one antibiotic. This means its clinical significance is quite low, as there exists a large number of both bacteria and antibiotics in the clinical setting. A larger number of bacterial species would be preferable, as would a larger selection of antibiotics. However the project is a good starting point for others, as they can build upon this research with further optimisation and testing.

The goal was for this method to be able to test directly from BC samples, though the method was never tested with the inclusion of blood in any step. Blood's complex and diverse components may have an impact on many different aspects of this project, especially the Real-Time PCR. To be able to use the methods in this project on BC samples, there is a large amount of testing and optimising that needs to be done.

5.6 Further research

Discovering that Real-Time PCR might not be the perfect method of detection for bacterial growth detection was valuable knowledge. The solution of using OD or McFarland measurements instead makes the method more similar to broth micro dilutions. The difference is the total time of the assay. An interesting thought to explore would be the possibility of broth micro dilutions directly from BC samples with a shortened incubation time. As previously stated, the shorter the time of AST the faster one can start the patient on an appropriate antibiotic, and the probability of a positive outcome increases.

The boiling lysis protocol could be optimised again. Many of the first decisions were made on the wrong basis, so having a second look at the method might yield a completely different outcome. It was also only tested on *E. coli*. It would have been interesting to see how a Gram-positive bacteria would react, and what changes would be made to accommodate other types of bacteria.

A broader selection of antimicrobials would be beneficial to test. Gentamicin inhibits translation, whilst other antimicrobials can stop cell wall synthesis, inhibit cell membrane functions, nucleic acid synthesis and other metabolic processes. It would be interesting to see if other antimicrobials need longer to stop the bacterial growth, and if that would influence the time it takes to differentiate resistant and susceptible bacteria [99]. Testing a large selection of bacteria would also be beneficial. Not all species will react the same to every antimicrobial, so to test a large array of both could uncover more about the time it will take to differentiate susceptible and resistant isolates.

One last point to further explore would be the possibility to test directly from BC. Inoculum concentrations would have to be optimised and standardised [99], and any adverse effect of blood uncovered.

6 Conclusion

The boiling lysis method parameters were not chosen on the basis of properly displayed results. This lead to a method not supported by the results. The boiling lysis results were inconsistent. It is not recommended to use this boiling lysis method without further optimalisation. EZ1 is a good alternative. It was more consistent and had a faster run time than this boiling lysis method.

The use of Real-Time PCR to detect bacterial growth seems to be flawed. The relationship between growth and Cq-values is not direct, leading to too many uncertainties when measuring small changes in growth between strains. It took up to 4 hours to differentiate resistant and susceptible strains with Cq-values, making it too slow for this project.

OD measurements or McFarland might seem to be a better option for detecting bacterial growth. There is a semi-direct correlation between increased cell density and increased measurements. It would be more favourable to use OD or McFarland measurements to create growth curves for this project than Real-Time PCR Cq-values.

7 Sources

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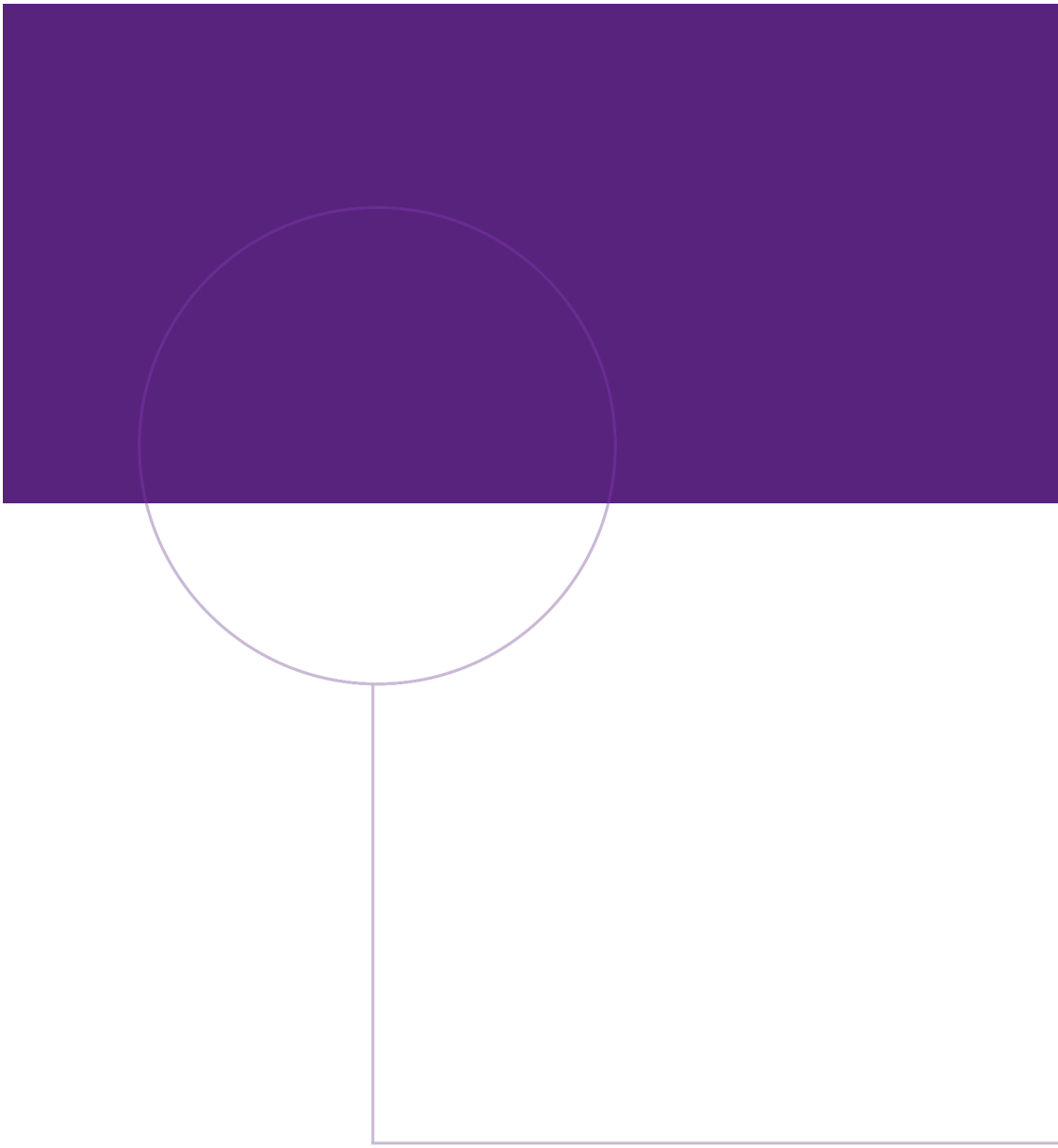

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