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High throughput pipeline for rapid antibiotic susceptibility testing and ID of bacteria from blood cultures

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Abstract

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Rapid and accurate species identification and antibiotic susceptibility testing are of great importance for patients with sepsis and to stop over- and misuse of antibiotics contributing to antibiotic resistance. QuickMIC™ is a rapid antibiotic susceptibility testig system based on a microfluidic technology solution developed by Gradientech that measure MICs on bacteria from positive blood culture bottles. By combining QuickMIC™ with a rapid system for detection and identification, the time to detection, identification and antibiotic susceptibiolity testing could be shortened with days compared to pipelines used today which could mean the difference of life and death for patients. The T2Bacteria® panel and T2Dx® instrument developed by T2 biosystems is an FDA-cleared test for rapid detection and identification of bacteria from whole blood based on magnetic molecular resonance technology. The time to result of the T2Dx® instrument is 3-4 hours and the time to result for QuickMIC™ is 2-4 hours. In this project, the possibilities and benefits of such a pipeline have been studied by comparison to a pipeline typically used today. Time, accuracy and practical aspects have been investigated during the project and the results are promising for future further studies.

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Populärvetenskaplig sammanfattning

Idag dör cirka 6 miljoner människor varje år av sepsis (WHO 2019). En patient som fått blodförgiftning kan lätt utveckla septisk chock och hypotoni som följd vilket är ett livshotande tillstånd. Efter att patienten fått hypotoni minskar överlevnadschanserna drastiskt för varje timme som går utan effektiv antimikrobiell behandling (Kumar *et al.* 2006). Idag breder även antibiotikaresistensen ut sig över världen vilket betyder att den antibiotika som normalt ges till patienter inte alltid kommer vara effektiv, vilket leder till ökad dödlighet.

Ökande behov för snabbtest på antibiotikaresistens är därför ett nödvändigt faktum. Gradientech AB utvecklar QuickMIC^{TM}, ett test för att på endast 2-4 timmar kunna mäta MIC, känslighet mot antibiotika, på bakterier som orsakat sepsis hos patienter. Hög känslighet mot antibiotika innebär att den kommer att ha en effekt mot de bakterier som orsakar blodförgiftning i patienten medan låg känslighet eller resistens innebär att antibiotikan inte kommer ha någon verkan alls. För att veta vilka patientprov som innehåller bakterier och därmed på vilka MIC-bestämning bör utföras på behövs ett identifikationssteg före QuickMIC^{TM}. I detta examensprojekt har en sådan pipeline undersökts.

Bakterietillväxt detekteras och identifieras i blodflaskor från patienter och de som visar sig vara positiva MIC-bestäms med QuickMIC^{TM}. Dessa resultat skulle sedan kunna influera den behandling en patient får genom att effektiv antibiotika skulle kunna ges och därmed öka chansen för överlevnad. Snabb resistensbestämning betyder även att överanvändning och felaktig användning av antibiotika minskar vilket leder till att de antibiotika som vi har idag kommer ha verkan längre och hastigheten i vilken antibiotikaresistensen utbreder sig skulle minska.

Table of contents

Abbrevations

AMK	Amikacin
AST	Antibiotic Susceptibility testing
CAZ	Ceftazidime
CIP	Ciprofloxacin
COC	Cyclic Olefin Copolymer TOPAS® 5013S-04
CST	Colistin
CTX	Cefotaxime
DMSO	Dimethylsulfoxide
ESKAPE	*E. faecium*, *S. aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa* and *E. coli*
EUCAST	The European Committee on Antimicrobial Susceptibility Testing
GEN	Gentamicin
HCCA	alpha-Cyano-4-hydroxycinnamic acid
HPS1R	Lexan healthcare resin HPS1R
IPM	Imipenem
K2EDTA	K2 ethylenediaminetetraacetic acid
MALDI-TOF MS	Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry
MEM	Meropenem
MIC	Minimum Inhibitory Concentration
MN211	Eastar™ Copolyester MN211 Natural
NAS30	Styrene Methyl Methacrylate NAS 30
PIP	Piperacillin
SAN31	Lustran Styrene Acrylonitrile 31
Styrolux	Styrene Butadiene Copolymer styrolux 684D
SXT	Trimethoprim/sulfamethoxazole
TAZ	Tazobactam
TGC	Tigecycline
TOB	Tobramycin

1 Introduction

This document is the master thesis *High throughput pipeline for rapid antibiotic susceptibility testing and bacterial ID of bacteria from blood cultures*, which is done as a final exam project at the master programme in molecular biotechnology engineering at Uppsala university, 30 hp. The master thesis is proposed by Gradientech AB.

Sepsis affects more than 30 million people worldwide and leads to approximately 6 million deaths every year (WHO 2019). For patients to receive appropriate treatment, antibiotic susceptibility testing (AST) is of great importance. For patients that develop septic chock from sepsis, delayed treatment with effective antimicrobial therapy increases mortality drastically (Kumar *et al.* 2006). Septic shock associated hypotension is a critical point of survival for the patient. Within the first hour of hypotension, the survival rate of the patient is 79.9% if effective antimicrobial treatment is initiated. The survival rate would decrease to 70.5 % if effective antimicrobial drugs are initiated in the time span of 1-2 hours after onset of hypotension. The survival rate then continues to drop every hour without effective antimicrobial treatment. Between 9-12 hours after onset of hypotension the survival rate had dropped to 25.4% (Kumar *et al.* 2006) when the correct antibiotic treatment is administered. This demonstrates the crucial importance of fast and accurate therapy of patients with septic shock.

Rapid AST is also of great importance in the fight against antimicrobial resistance (AMR) that is one of the greatest health threats to mankind today and in the coming decade. The mis- and overuse of antibiotics accelerate the spread of AMR and by decreasing the inappropriate use of antibiotics with rapid AST, the drugs we have today will last longer (O'Neill 2016) Already today at least 700 000 people die every year from resistant strains of common bacterial infections, HIV, tuberculosis and malaria, where nearly 200 000 of these people die from multiresistant and extremely resistant tuberculosis. These numbers increase rapidly with the rise of AMR and it is estimated that by 2050, 10 million people will die from AMR related diseases (O'Neill 2014). By developing rapid AST systems, the rate of AMR mortality can be slowed down.

Gradientech AB develops an ultra-rapid antibiotic susceptibility testing system called QuickMIC^{TM} to generate phenotypic AST results in only 2-4 hours (QuickMIC 2019) which is significantly shorter time than the culture based AST methods used today. Before culture based AST is conducted the positive blood samples are detected. The concentration of bacteria in blood is usually very low (less than 100 CFU/ml). Before culture-based AST is performed, the blood is incubated in a blood culture bottle to allow the bacteria to grow and increase in numbers. Detection of bacterial growth, that is a positive blood culture bottle, does not occur until several hours of incubation. To shorten the time of result to detection a new detection and identification method with higher sensitivity from T2 biosystems is used prior to the QuickMIC^{TM} assay. The T2Dx instrument and T2Bacteria® Panel by T2 Biosystems identify bacteria related to sepsis from whole blood without the need for blood culture enrichment (T2 Biosystems 2019). The idea is to combine the T2Bacteria® Panel with the QuickMIC^{TM} assay into a pipeline that yield rapid bacterial ID and AST results within hours.

The aim of this master thesis is thus to find the lowest detection limit of QuickMIC^{TM} while maintaining specificity, to examine the specificity and sensitivity of the T2Bacteria® Panel run with blood from blood culture bottles, and to combine the QuickMIC^{TM} assay with the T2Bacteria® Panel to establish a pipeline for rapid bacterial ID and AST results, leading to faster response time and higher survival rates of patients suffering from sepsis.

2 Background

2.1 QuickMIC™

QuickMIC™ is being developed by Gradientech and is an ultra-rapid antibiotic susceptibility testing system to generate phenotypic AST results in only 2-4 hours which is 20 hours faster than traditional culture based AST.

QuickMIC™ is based on a microfluidic technology solution to create stable gradients of antibiotics to generate minimum inhibitory concentration (MIC) values (QuickMIC 2019). The microfluidic cassette that is loaded into the instrument has 13 chambers. In 12 of them stable gradients of antibiotics and growth medium are formed (figure 1). One chamber is a control chamber without flow of neither antibiotics or growth medium.

Figure 1: QuickMIC™ cassette.

Bacteria is loaded together with agarose in all 13 chambers through an inlet before loaded into the instrument. Every ten minutes each chamber is photographed using a built-in microscope and light source. When the bacteria grow in the gel, the MIC can be interpreted depending on where in the antibiotic gradient they stop growing. A chamber in QuickMIC™ is illustrated in figure 2 at time point zero (cycle 0) and in the end of a run in figure 3 (cycle 23). The light intensity increases in the picture where bacteria grow in the chambers which can be converted to MIC values using scripts developed by Gradientech.

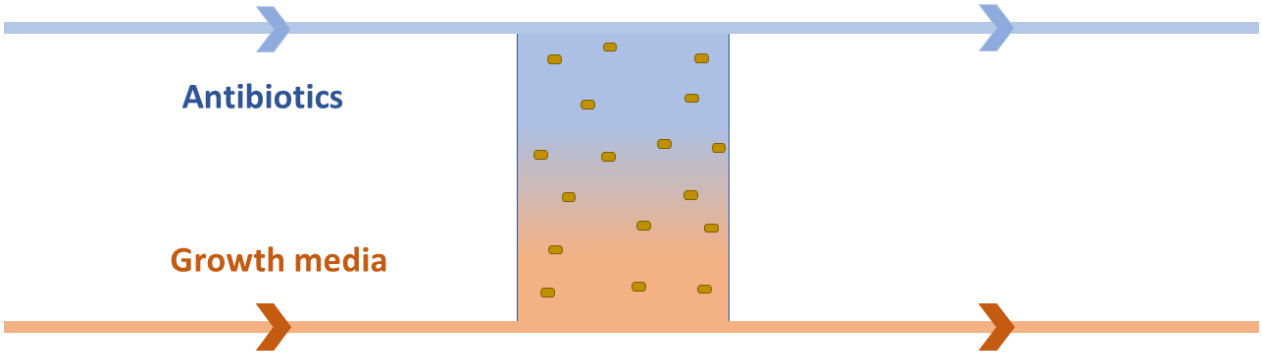

Figure 2: Illustration of a chamber at cycle 0.

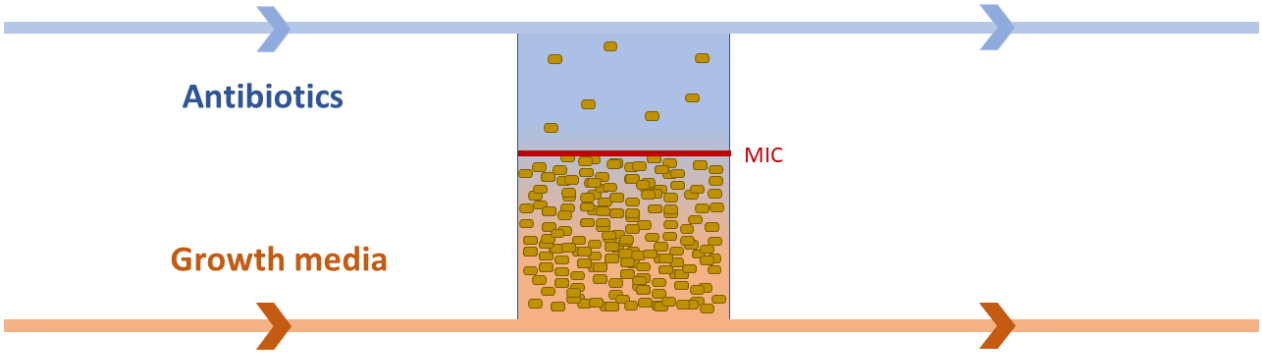

Figure 3: Illustration of a chamber at cycle 23.

2.2 The T2Dx® instrument and T2Bacteria® Panel

The T2Dx® instrument (figure 4) with the T2Bacteria® Panel is an FDA-cleared test developed by T2 Biosystems that identify bacteria related to sepsis from whole blood in K2 ethylenediaminetetraacetic acid (K2EDTA) tubes without blood culture enrichment (T2 Biosystems 2019). K2EDTA tubes are blood tubes loaded with 4 ml blood by negative pressure and contain the anticoagulant (K2EDTA). The panel is loaded into the T2Dx® Instrument that uses a technology based on magnetic molecular resonance for detection and can detect bacteria down to 1 CFU/ml or 1 bacteria/ml. The pathogens that can be detected with the T2Bacteria® Panel are common blood pathogens often associated with antbiotic resistance; *Enterobacter faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Escherichia coli* (ESKAPE). Detection and identification is achieved whitin 3-5 hours which is faster than culture based detection and identification methods that take from 6-24 hours using the Bact/Alert® Virtuo® system by bioMérieux combined with the matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS).

Figure 4: T2Dx® Instrument.

2.3 Traditional detection and identification of bacteria

The Bact/Alert Virtuo microbial detection system and matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) are common methods for detection and identification of bacteria from blood cultures.

2.3.1 Bact/Alert® Virtuo® microbial detection system

Blood culture is considered the gold standard of diagnosing blood stream infections (Lamy *et al.* 2016). Bact/Alert® Virtuo® microbial detection system is an automated blood culture system developed by bioMérieux. Whole blood is loaded into Bact/Alert® blood culture bottles and thereafter loaded into the Bact/Alert® Virtuo® microbial detection system for detection of blood infections (BioMérieux 2019a).

2.3.2 MALDI-TOF MS

Fast and accurate identification of bacteria is generally conducted using MALDI-TOF MS. The Microflex™ system from Bruker was used in this project and is a high performance bench-top system for basic applications. Samples in this project run in the Microflex™ system was prepared either with the MALDI sepsityper® kit from Bruker directly from positive blood cultures or by direct identification on subcultures from agar plates (Bruker 2019).

2.4 Traditional culture based AST

Culture based AST have long been the standard of AST but are time consuming methods that do not generate AST results fast enough to influence the antimicrobial treatment of patients suffering from sepsis, leading to many cases of improper use of antibiotics. The time consumption of traditional culture based AST is partly due to the requirement of subcultures, the transfer of a previous culture to fresh growth medium, that are incubated overnight and then resuspended accordingly to be used for AST and partly due to the incubation in the AST methods themselves.

2.4.1 Broth Micro Dilution

Broth micro dilution (BMD) is considered the reference method of antimicrobial susceptibility testing by the European committee of antimicrobial susceptibility testing (EUCAST). In BMD growth of bacteria are

visually investigated in serially diluted antibiotics that are incubated 16-20 hours. The standard inoculum of bacteria is 10^5 CFU/ml. The MIC is interpreted as the lowest concentration of antibiotic that completely inhibit growth. The breakpoints of susceptible, intermediate and resistant (S,I,R) are based on BMD (EUCAST 2019a).

2.4.2 Disk diffusion

Disk diffusion is one of the oldest but still a widely used method for AST. In this AST method zone diameters of cell growth around antibiotic diffusion disks are used to interpret susceptibility. SIR establishment by zone diameter are defined by EUCAST (EUCAST 2019b).

2.4.3 Etest®

The Etest® is produced by bioMérieux and a well-established method for AST. The Etest® is a plastic strip that has a predefined concentration gradient of antibiotics and is placed on an agar plate. The MIC is read by interpretation of the growth around the strip (BioMérieux 2019b).

2.5 Novel rapid AST methods

The rising demand of rapid AST methods in the world today have led to the development of many new inventive methods. Below are a few of the most promising methods today.

2.5.1 Accelerate Pheno™ system

The accelerate Pheno™ system provides both bacterial ID in approximately 90 minutes and rapid AST results in approximately 7 hours on both gram positive and gram negative bacteria. The bacterial ID is determined using automated fluorescence *in situ* hybridization technology and AST results are provided with morphokinetic cellular analysis. The disadvantage of the system is that the system requires ID of the bacteria before AST which is a time consuming step. Another disadvantage is that that the cassettes are both overly large in size and expensive (Charnot-Katsikas *et al.* 2018).

2.5.2 Single-cell morphological analysis (SCMA)

Single cell morphological analysis (SCMA) provides rapid AST results in less than 4 hours for all species of bacteria using an agarose chip with immobilized bacteria. Imaging of single cells are performed to interpret MIC values by studying the division of cells. An advantage of the system is that filamentary formation and swelling of the cells also are recognized as susceptible (Choi *et al.* 2014).

2.5.3 FASTinov® kit

The FASTinov® kit by FASTinov provides rapid AST results on gram negative bacilli in 2 hours. The method is based on flow cytometry where fluorochromes are used to examine lesions induced by antibiotics (Costa-De-Oliviera *et al.* 2016).

2.5.4 Microplate-based surface area assay

The microplate-based surface area assay yields AST results whitin 5 hours by binding of a universal small-molecule amplifier to measure the concentration of bacteria in different concentrations for different antibiotics. By binding of the small-molecule amplifier morphological changes of the bacteria can be examined (Flentie *et al.* 2019).

2.6 ESKAPE pathogens

ESKAPE is an acronym for the pathogens *Enterobacter faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterococcus* spp. These are the most common pathogens responsible for life threatening infections and the name also indicates that they are

characterized by escaping bactericidal effects of antibiotics. Throughout the exam project *Enterococcus* species are exchanged with *E. coli* since the T2Bacteria® Panel identifies *Escherichia coli* but not *Enterococcus* spp. (Santajit & Indrawattana 2016).

3 Project goal

The project goal is to find the lowest bacterial concentration that can be detected in QuickMIC™ without reducing the specificity of the assay, to examine the specificity and sensitivity of the T2Bacteria® Panel run with blood from blood culture bottles and to establish a pipeline with the QuickMIC™ assay and the T2Bacteria® Panel for rapid bacterial ID and AST results on the six most common blood pathogens (*E. coli*, *K. pneumoniae*, *P. aeruginosa*, *S. aureus*, *A. baumannii* and *E. faecium*). The MIC value and time until detection will be compared with traditional E-test or microdilution on pure cultures.

3.1 Effect goal

The effect sought by the project is to decrease response time to patients suffering from septic shock and thereby decrease septic shock mortality. A further effect is to limit the spread of AMR by reducing inappropriate use of antibiotics.

4 Material and method

4.1 Species verification with MALDI-TOF MS

The Microflex™ MALDI-TOF MS system from Bruker was used to verify the species of the chosen strains of ESKAPE bacteria. A colony was taken from each of the plates with ESKAPE strains and applied to a position on a microflex test plate. Each position was covered in 1 μl alpha-Cyano-4-hydroxycinnamic acid (HCCA) matrix within 10 minutes. The test plate was left to dry for approximately 5 minutes and was then analyzed on the microflex MALDI-TOF MS system.

4.2 Growth evaluation in the Bact/Alert® Virtuo® detection system

To evaluate time to detection and the concentration of the blood culture after alarm in Bact/Alert® Virtuo® system, spiked Bact/Alert® blood culture bottles were inserted in the Bact/Alert® Virtuo® microbial detection system. Citrated horse blood was collected from the fridge and heated in a water bath to 37°. Meanwhile, each of the ESKAPE strains were resuspended in physiological NaCl to 0.5 McFarland and diluted 10^{-2} times in phosphate buffered saline (PBS). 100 μl of each dilution was transferred to an eppendorf tube. The citrated horse blood was taken out of the water bath and approximately 10 ml was transferred to aerobic blood culture bottles with a syringe. With the same syringe, all of the solution from the eppendorf tubes with diluted ESKAPE strains were transferred to each blood culture bottle to a final concentration of approximately 10^3 CFU/ml. The blood culture bottles were mixed gently. Approximately 100 μl of solution from each blood culture bottle was diluted and plated on MH-II plates for inoculum control. The plates were incubated overnight. The blood culture bottles were then loaded into the Bact/Alert® Virtuo® microbial detection system. At the time of alarm of the Bact/Alert® Virtuo® microbial detection system, the blood culture bottles were unloaded and the time noted. A sample of 100 μl was diluted, plated on agar plates and incubated at 37°C overnight to determine the bacterial concentration in a positive blood culture bottle at the time of alarm.

A further test to investigate if the time to alarm was influenced by transferring blood from the blood culture bottle before alarm was conducted to investigate if one or two blood culture bottles had to be started in the whole pipeline test later in the project. Two bottles spiked with the gram negative *E. coli* and two bottles spiked with the gram positive *S. aureus* were prepared and loaded into the Bact/Alert® Virtuo® microbial detection system. The strains were taken from plates and suspended to 0.5 McFarland. They were thereafter diluted to 10^{-3} in PBS and 100 μl of each strain were transferred to an eppendorf

tube. 10 ml citrated horse blood was heated in a water bath to 37 °C for each bottle to be loaded and transferred into blood culture bottles using a 10 ml syringe. With the same syringe, the diluted strains were transferred into the bottle. Samples of 100 μl from each bottle were plated on agar plates and incubated at 37 °C overnight. All blood culture bottles were loaded into Bact/Alert® Virtuo® microbial detection system and the time was noted. After 4 h and 45 min - 5 h one bottle of each strain was taken out and plated on agar plates, that were incubated at 37 °C overnight, and then loaded again after approximately 10 min in the Bact/Alert® Virtuo® microbial detection system until alarm. The time at alarm was noted.

4.3 Runs with blood from blood cultures in the T2Dx instrument

Evaluation of runs with blood from aerobic blood culture bottles in the T2Dx instrument with the T2Bacteria® Panel were conducted using spiked citrated horse blood with *K. pneumoniae*. Three runs were conducted in this test. First, citrated horse blood from a blood culture bottle with *K. pneumoniae* was tested, secondly, whole blood from human in a blood culture bottle with *K. pneumoniae* was tested and thirdly, citrated horse blood in a K2EDTA tube with *K.pneumoniae* was tested. The preparation of the spiked blood culture bottles was conducted as described in section 4.2 and the K2EDTA tube was prepared with 4 ml citrated horse blood and the same inoculate as the blood culture bottles in previous tests. 1 ml of blood from the blood culture bottles were loaded into the loading unit of the T2Dx instrument by puncturing the aluminum foil and filling the chambers. The K2EDTA tube was loaded according to the original instrucions from T2 biosystems. The cassettes were then assembled and loaded according to instructions from T2 biosystems.

4.4 Broth microdilution

Broth micro dilution was performed on the ESKAPE strains to obtain their MIC to be able to compare it to the MICs from QuickMIC™. The comparison will determine the lowest detection limit in QuickMIC™ later in the project. The antibiotics amikacin, ceftazidime and meropenem was used for the gram negative strains and ciprofloxacin, tetracycline and gentamicin was used for the gram positive strains. The dilution series of the antibiotic for each strain was chosen around their epidemiological cut-off values (ECOFF) from (EUCAST 2013) or earlier broth micro dilution results from a particular strain if available. Ceftazidime was dissolved in 0.1 M HCl and ciprofloxacine was dissolved in dimethyl sulfoxide (DMSO). All other antibiotics were dissolved in deionized, sterile water. The dissolved antibiotics were then serially diluted in MH-II. 50 μl of each dilution was transferred to corresponding well in U-shaped microtiter plates. 50 μl MH-II was pipetted to the positive control wells and 100 μl MH-II was used as negative control. Each strain was then adjusted to 0.5 McFarland standard and diluted 100x. 50 μl of the diluted stains was transferred to associated wells and the positive control wells. 10 μl of each positive control was diluted in 10 ml NaCl for inoculum control. 100 μl of the diluted positive control was plated using glass beads and the results were analyzed using a mirror. Colony counting was executed on the inoculum controls that contained 50-100 colonies to be approved. The microtiter plates and inoculum controls were incubated overnight in 37 °C.

4.5 QuickMIC™ evaluation and verification

During the project some general evaluation and verification were conducted. New slider materials were tested to better adhere to the agarose gels which would yield more accurate results in the analysis step.

4.5.1 Optical and bactericidal properties of new slider materials

The gel loaded in the QuickMIC™ system moved during runs which potentially could lead to inaccurate results due to falsely detected regions in the chambers. If a region in one twentieth of the chamber is detected and the gel then move, it could be detected as a new region in another twentieth part of the chamber. Other slider materials than Lexan healthcare resin HPS1R (HPS1R), used today, were tested to improve the adhesion of the chamber walls to the gel. Optics and bactericidal effects of the materials were to be tested before replacing HPS1R. The materials to be evaluated were Eastar™ Copolyester MN211 Natural (MN211), Lustran Styrene Acrylonitrile 31 (SAN31), styrene Methyl Methacrylate NAS 30 (NAS30), Styrene Butadiene Copolymer styrolux 684D (styrolux), Cyclic Olefin Copolymer TOPAS®

5013S-04 (COC). Background noise of each slider material was measured by capturing 3 pictures of one of the chambers with different resolutions along the z-axis. Chamber 5 was used in all sliders with the z-coordinates 1600 μm, 3100 μm and -400 μm. The exposure time used was 6000μs, which is the exposure time used in all earlier runs with QuickMIC^{TM}. The pictures were then analyzed using imageJ and excel to conduct a pixel analysis. To briefly examine bactericidal effects, both gram negative and gram positive strains were run in the instrument with the sliders that showed the best optical properties. The species used were gram negative *E. coli* and gram positive *S. aureus*. Growth in the control chamber was interpreted as non-bactericidal.

4.5.2 Lowest detection limit

The lowest detection limit of QuickMIC^{TM} was evaluated to shorten the overall time of the pipeline to be able to get the earliest possible AST results. The species *P. aeruginosa* was chosen for the experiment because it is previously known to be difficult to analyze in QuickMIC^{TM} and high bacterial concentrations were needed to obtain a reliable result. *E. coli* was chosen because a lower bacterial concentration is known to be needed to reach reliable results and is therefore one of the easier species to analyze in QuickMIC^{TM}. The inoculates that were tested were 10^5 and 10^3 CFU/ml of *E. coli* and 10^6, 10^5 and 10^4 CFU/ml of *P. aeruginosa*. The species were chosen to get an overview of the detection limit to yield reliable results from the lowest to the highest limit of all ESKAPE strains. Amikacin, ceftazidime and meropenem were used in the runs as in the BMD in section 5.4. Twice the MIC from earlier BMD tests were used as the concentration of the antibiotic for each strain to ideally see growth in half the chamber. Four technical replicates were made for each inoculate concentration and antibiotic since there are 12 wells in one cassette and 3 antibiotics were used.

The detection limit was determined based on the number of detected regions in the inoculum controls and comparison of MIC values from earlier BMD (section 5.4). Earlier rough guidelines have indicated that at least 10 detected regions are needed in every twentieth part of the control chamber to yield reliable results.

4.6 Pipeline evaluation

The T2-QuickMIC^{TM} pipeline was evaluated against a current typical pipeline in the final step as described in figure 5 below. Three pipelines were run, two with the gram negative *E. coli* and one with gram positive *S. aureus*. The time consumption of each step was noted and inoculum controls were conducted at different stages. All were run with the same antibiotic panel. The panel is designed for gram negative bacteria but since both BMD and QuickMIC^{TM} runs were done, the MICs were still comparable.

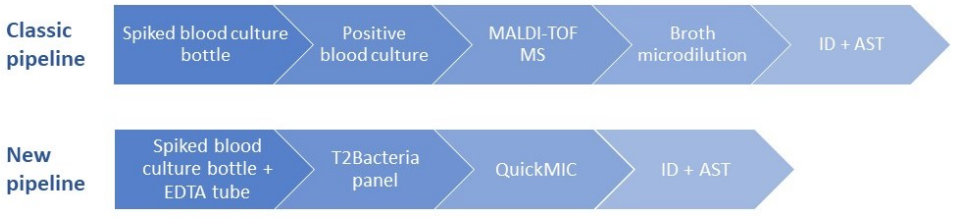

Figure 5: Flowchart describing the work flow of the pipeline evaluation.

4.6.1 Antibiotic panel preparation

An antibiotic panel designed for gram negative bacteria was prepared to be used in both QuickMIC™ and BMD. The concentrations of antibiotics were prepared 20 times their target concentration in QuickMIC™ which was 2.5 times the current resistance MIC breakpoints from EUCAST (table 1). The reason the target concentration is 2.5 times the breakpoint in QuickMIC™ is that 2 times the breakpoint ideally yield the breakpoint in the middle of the chamber. Broth microdilution that however is the reference method for AST have an accepted variation of 0.5-2 times the MIC, leading to that the concentration in QuickMIC™ was prepared over 2 times the breakpoint to cover variation both below and above the MIC. In total 5 BMD dilution steps are covered in the chamber.

Table 1: Resistance breakpoints for the antibiotic panel.

Antibiotic	Breakpoint (mg/l)
Amikacin	16.0
Cefotaxime	2.0
Ceftazidime	8.0
Ciprofloxacin	1.0
Colistin	2.0
Gentamicin	4.0
Imipenem	4.0
Meropenem	8.0
Piperacillin/Tazobactam	16.0
Tigecycline	0.5
Tobramycin	4.0
Trimethoprim/sulfamethoxazole	4.0

Twelve different antibiotics were prepared by first suspending them in appropriate suspension agent and then diluting them to 20x, 2.5x their breakpoint according to table 2. 100 μl was aliquoted to eppendorf tubes and freezed at -70 °C until use.

Table 2: Antibiotic panel preparation.

Antibiotic	Abbr.	Suspension agent	Suspension conc. (mg/ml)	Dilution agent	Target conc. (mg/l)
Amikacin	AMK	PBS	10	Water	40.0
Cefotaxime	CTX	PBS	10	Water	5.0
Ceftazidime	CAZ	PBS	5	Water	20.0
Ciprofloxacin	CIP	DMSO	10	Water	2.5
Colistin	CST	PBS	10	Water	5.0
Gentamicin	GEN	PBS	10	Water	10.0
Imipenem	IPM	PBS	2	PBS	10.0
Meropenem	MEM	PBS	10	PBS	20.0
Piperacilin/ tazobactam	PIP/TAZ	DMSO/PBS	10/10	PBS	40.0/4.0
Tazobactam	TAZ	PBS	10	PBS	4.0
Tigecycline	TGC	PBS	10	Water	1.25
Tobramycin	TOB	PBS	10	Water	10.0
Trimethoprim/ Sulfamethoxazole	SXT	DMSO/DMSO	10/10	PBS	10.0/190.0

4.6.2 Sample preparation

Two blood culture bottles and one K2EDTA tube were prepared for each pipeline run. The day before, each strain to be used was streaked onto a MH-II plate and left for incubation in 37°C overnight. The next day 10 ml citrated horse blood for each blood culture bottle to be prepared and 5 ml citrated horse blood for each K2EDTA tube to be prepared was warmed to 37°C in a water bath. The plates were taken out from incubation and resuspended to 0.5 McFarland. The suspension was diluted further to 10^{-5} and 100 μl of the dilution was mixed with 10 ml blood to each blood culture bottle and 50 μl was mixed with 5 ml blood to the K2EDTA tubes. The blood culture bottles were immediately loaded into the Bact/Alert® Virtuo® detection system. The time of loading was noted.

4.6.3 Current pipeline

Positive blood culture bottle
The blood culture bottle loaded into the Bact/Alert® Virtuo® detection system that was not used for QuickMIC™ was incubated until alarm and then taken out directly. With a monovette syringe, 4.5 ml blood was withdrawn from the bottle. 100 μl was diluted and plated on agar plates with glass beads and incubated overnight at 37°C to have a subculture for BMD and to be able to approximate the bacterial concentration.

MALDI-TOF MS
The MALDI sepsityper® kit from Bruker was used for identification of the pathogen when the blood culture bottle alarmed. In cases where the sepsityper kit did not work, a direct method was used instead by analyzing the subculture from the positive blood culture bottle. A monovette syringe was used to take 4.5 ml blood from the blood culture bottle directly after alarm. An eppendorf tube was filled with 1 ml blood from the monovette and 200 μl lysis buffer was added. The eppendorf tube was vortexed for 10 seconds and centrifuged for 2 minutes at 13300 rpm. The supernatant was removed with a pipette. 1 ml washing buffer was added and the pellet resuspended with a pipette. The tube was then centrifuged again for 1 minute at 13300 rpm and the supernatant was discarded. The pellet was then resuspended in 300 μl sterile water by pipetting up and down. Then, 900 μl 99.5% ethanol was added and the suspension was mixed. The tube was centrifuged for 2 minutes at 13300 rpm and the supernatant was discarded. The tube was then again centrifuged for 2 minutes at 13300 rpm and residual ethanol was removed by pipetting. The tube was left open to dry for approximately 5 minutes at room temperature. When the pellet had dried, the pellet was resuspended in 40 μl 70% formic acid. 40 μl acetonitrile was then added and the suspension was mixed with a pipette two to three times. The tube was centrifuged for 2 minutes at 13300 rpm and 1 μl of the supernatant was added to a MALDI target plate position and was allowed to dry for approximately 1 minute. Immediately after the sample on the MALDI target plate had dried, 1 μl HCCA matrix was added to the sample. The MALDI target plate was then added into the microflex MALDI-TOF MS system.

Broth microdilution
Broth Microdilution was conducted on the subcultures. The antibiotic panel was taken out of the fridge and thawed for approximately 20 minutes. All antibiotics were then diluted to 4x resistance breakpoint in MH-II in the first row of a U-shaped 96-well plate to the total volume of 100 μl in the same order as in table 2. In all other wells except the last row and column 9, 50 μl MH-II was added. In columnn 9, 50 μl diluted tazobactam (TAZ) to 4x the resistance breakpoint was added. Column wise, serial dilution was conducted with a multichannel pipette down to row G by transferring 50 μl between each row and discarding the final 50 μl from row G. In row H, positive and negative controls were added. In row H:1-6, 50 μl MH-II was added for positive growth control and in H:7-12, 100 μl MH-II was added for negative control. The subcultures of each strain were resuspended to 0.5 McFarland and then diluted to 10^{-2} in PBS. 50 μl was added to all wells except the wells for negative control. The plates were covered with sealing tape and incubated at 37°C for 16-18 hours.

4.6.4 T2-QuickMIC™ pipeline

Loading of the T2Dx instrument

The T2-QuickMIC™ pipeline was started with identification of the strain in the T2Dx® instrument with the T2Bacteria panel. The earlier prepared EDTA-tube containing blood was loaded by using standard protocol for loading of the instrument provided by T2 biosystems®. The time at start and alarm was noted.

Loading of QuickMIC™
When the T2Dx® instrument detected and identified the microbe, one of the blood bottles with each strain was unloaded from the Bact/Alert® Virtuo® system. Two QuickMIC™ runs were conducted for *E. coli* and *S. aureus* since the first run had too low bacterial concentration. The first run was instead used for growth rate evaluation to be able to approximate the time of sufficient growth for the second run. First, one eppendorf of each antibiotic was taken out of the -70 °C freezer and thawed for approximately 20 minutes. Each antibiotic was then diluted 20 times in MH-II media and 500 μl was loaded in the antibiotics reservoirs of the cassette according to table 2. In the other side of the cassette, 500 μl MH-II media was loaded in all media reservoiars except in the reservoir for chamber 9 with antibiotics piperacillin/tazobactam (PIP/TAZ) where MH-II containing TAZ was loaded. This created an even concentration of TAZ throughout the whole chamber. When all reservoirs were loaded, the cassette was primed by withdrawal of 2 ml air, 30 ml/min twice from the fluidic inlets. This filled all fluidic channels in the cassette and pushed out air that would affect the run. After priming, 4.5 ml of blood was collected from the blood bottles using a monovette syringe. From the monovette, 2 ml of blood was transferred to a falcon tube while 100 μl was saved for plating to determine the bacterial concentration. The falcon tube containing 2 ml blood was centrifuged at 150 rcf for 5 minutes to separate bacteria and blood components. As control, 100 μl supernatant was saved and 500 μl supernatant was transferred to a tube and mixed with 500 μl MH-II. Equal parts of supernatant dilution and 1% agarose was mixed gently without introducing micro bubbles and loaded into the cassette's sample inlet to all chambers. The cassette was loaded into the instrument and a run was started. The time of the start and end of the run was noted. The saved samples before and after centrifugation were plated using glass beads, incubated at 37°C overnight and counted the next day.

5 Results

5.1 MALDI-TOF MS

MALDI-TOF MS was conducted on all ESKAPE species with two biological replicates to verify their identity.

Table 3: The first MALDI-TOF MS identification verification on ESKAPE pathogens.

Strain	**Organism (best match)**	**Score**	**Organism (second best match)**	**Score**
E. faecium	*Enterococcus faecium*	2.43	*Enterococcus faecium*	2.42
S. aureus	*Staphylococcus aureus*	2.36	*Staphylococcus aureus*	2.33
K. pneumoniae	*Klebsiella pneumoniae*	2.35	*Klebsiella pneumoniae*	2.33
A. baumannii	*Acinetobacter baumannii*	2.43	*Acinetobacter baumannii*	2.26
P. aeruginosa	*Pseudomonas aeruginosa*	2.36	*Pseudomonas aeruginosa*	2.30
E. coli	*Escherichia coli*	2.34	*Shigella dysenteriae*	2.22

Table 4: The second MALDI-TOF MS identification verification on ESKAPE pathogens.

Strain	**Organism (best match)**	**Score**	**Organism (second best match)**	**Score**
E. faecium	*Enterococcus faecium*	2.44	*Enterococcus faecium*	2.42
S. aureus	*Staphylococcus aureus*	2.39	*Staphylococcus aureus*	2.37
K. pneumoniae	*Klebsiella pneumoniae*	2.40	*Klebsiella pneumoniae*	2.39
A. baumannii	*Acinetobacter baumannii*	2.38	*Acinetobacter baumannii*	2.37
P. aeruginosa	*Pseudomonas aeruginosa*	2.31	*Pseudomonas aeruginosa*	2.30
E. coli	*Shigella dysenteriae*	2.43	*Escherichia coli*	2.36

The results in table 3 and 4 show that all species were identified as expected except for *E. coli* which was identified as *Shigella dysenteriae* once. They do however have similar score values in both verifications and by consultation with staff at Uppsala antibiotic research center this happens often, which indicate a false negative and the strain was used in further experiments.

5.2 Bact/Alert® Virtuo® time to detection

Blood culture bottles with the ESKAPE pathogens were incubated in the Bact/Alert® Virtuo® detection system until it was detected positive. Colony counting to approximate concentration before and after incubation was carried out and the time to detection and concentration are summarized in the dot plot (figure 6 below. Since the concentration of bacteria after alarm is approximately between 10^6 - 10^{10} CFU/ml and QuickMIC™, it would be possible to run QuickMIC™ before alarm.

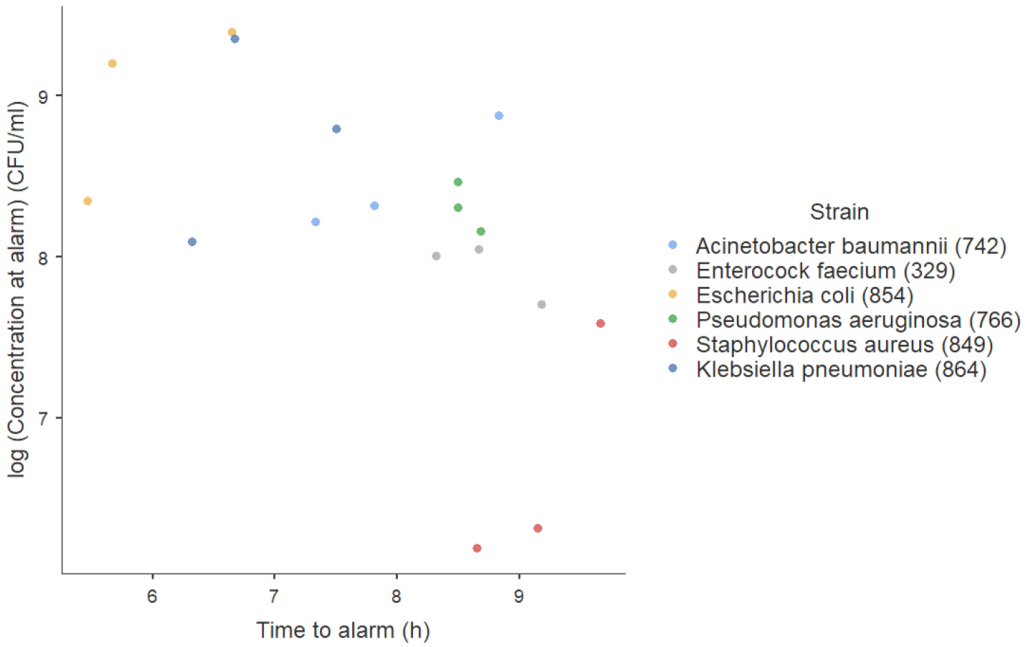

Figure 6: Time and concentration at alarm in Bact/Alert® Virtuo®

The time of alarm in the Bact/Alert® Virtuo® detection system was investigated for two strains, *E. coli* and *S. aureus*. Two bottles were started each time but only one of them was sampled after 5 hours to test if the time of alarm was affected by withdrawal of blood from the blood culture bottle.

Table 5: Time to detection in Bact/Alert® Virtuo® system depending on sampling after 5 hours.

Strain	Start inoculum (CFU/ml)	Sampling after 5 h (ml)	Time of alarm (h)
E. coli	130	4.5	7.30
E. coli	230	0	7.45
S. aureus	340	4.5	11.27
S. aureus	340	0	10.27

The results in table 5 indicate no significant difference in time to detection for *E. coli* but 1 h difference for *S. aureus*. The conclusion from this experiment was that two blood culture bottles were to be started for all strains in the whole pipeline runs to eliminate potential error sources in detection time because of sampling of blood from the blood culture bottle.

5.3 TDx® instrument with blood from bloodculture bottles

Three runs were conducted to evaluate if the T2Dx® instrument could be run with blood from blood culture bottles. The results are presented in table 6.

Table 6: Results from the T2Dx® instrument with the T2Bacteria panel.

Run	Species	Blood	Container	T2 result
1	*K. pneumoniae*	Citrated horse blood	Blood culture bottle	Invalid run
2	*K. pneumoniae*	Human whole blood	Blood culture bottle	Invalid run
3	*K. pneumoniae*	Citrated horse blood	K2EDTA tube	Positive

Both runs with blood from blood culture bottles ended up with invalid results. The reason was clogged pipettes in the system and was probably due to silicon beads in the blood culture bottles needed to absorb antibiotic residues from patient blood. The last run was positive, indicating that the citrate in the horse blood did not affect the detection and it was concluded that citrated horse blood in K2EDTA tubes was to be used in further tests on the whole pipeline.

5.4 Broth microdilution

Broth microdilution was executed to find the MIC of the ESKAPE strains to be used in further experiments. The MICs of the strains were interpreted according to EUCAST directions (EUCAST 2019b) (table 7 - 10) and could later on be compared to the MICs from QuickMIC™ to determine the lowest detection limit in QuickMIC™. *E. faecium* did not grow in the first experiment (table 9) which resulted in that no MICs could be interpreted.

Table 7: Broth microdilution test 1 for gram negative strains.

Strain	Amikacin	Ceftazidime	Meropenem
K. pneumoniae	16 mg/l	128 mg/l	16 mg/l
A. baumannii	>64 mg/l	>4 mg/l	32 mg/l
P. aeruginosa	2 mg/l	4 mg/l	0.25 mg/l
E. coli	8 mg/l	>128 mg/l	0.06 mg/l

Table 8: Broth microdilution test 2 for gram negative strains.

Strain	Amikacin	Ceftazidime	Meropenem
K. pneumoniae	16 mg/l	>128 mg/l	16 mg/l
A. baumannii	>64 mg/l	>4 mg/l	64 mg/l
P. aeruginosa	2 mg/l	4 mg/l	<0.125 mg/l
E. coli	4 mg/l	>128 mg/l	0.12 mg/l

Table 9: Broth microdilution test 1 for gram positive strains.

Strain	Ciprofloxacin	Tetracycline	Gentamicin
S. aureus	>8 mg/l	0.125 mg/l	0.25 mg/l
E. faecium	-	-	-

Table 10: Broth microdilution test 2 for gram positive strains.

Strain	Ciprofloxacin	Tetracycline	Gentamicin
S. aureus	>8 mg/l	0.125 mg/l	0.5 mg/l
E. faecium	<0.25 mg/l	4 mg/l	16 mg/l

5.5 Slider material evaluation

A pixel analysis was conducted to evaluate the optical properties of each slider material as an additional experiment to the project. Disturbing reflective lights from the material is considered noise and dark backgrounds yield better results. A lower pixel value indicates a darker pixel which means that HPS1R, which is the original material used, in QuickMIC^{TM} has the best optical properties of the materials tested as seen in figure 7. The material NAS30 had the second best optical properties, followed by SAN31 and MN211. The sliders used were not optimal since they had surface scratches on the optical surface from the production. To avoid misleading results from these marks, an area from each slider was chosen with as even background as possible. The chosen areas are shown in appendix 8.1.

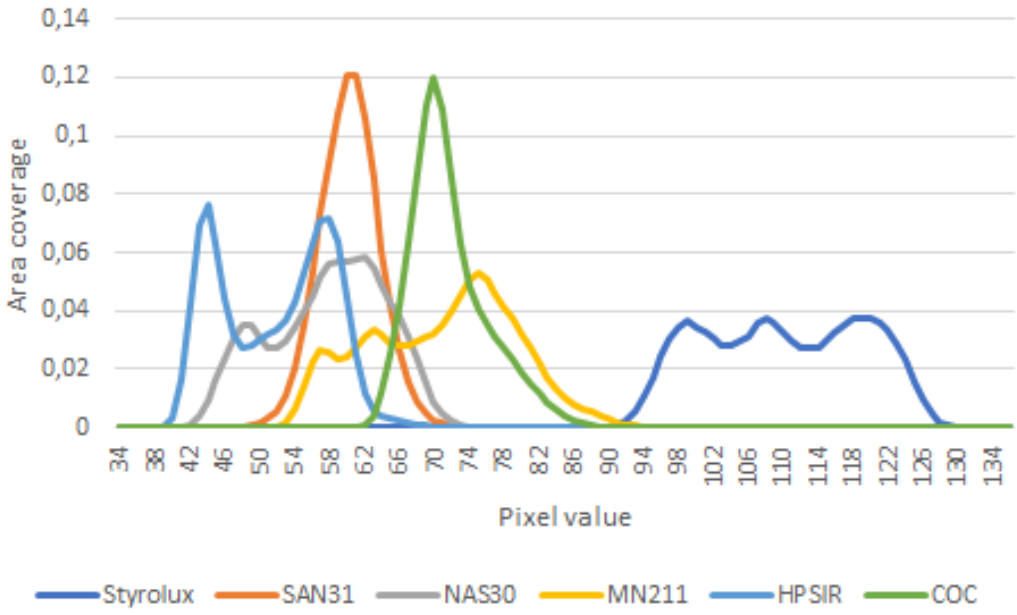

Figure 7: Pixel analysis of the slider materials.

NAS30 and MN211 were briefly evaluated further for bactericidal effects with gram negative and gram positive bacteria. *E. coli* was used for evaluation of gram negative bacteria and *S. aureus* was used for evaluation of gram positive bacteria. Growth was observed in both sliders with both gram negative and gram positive bacteria. Overexposure was a problem in the run with MN211 and *S.aureus* which made it uninterpretable. Pictures were instead taken manually with a modified exposure time several hours after the run ended. Growth was observed in these pictures but could not be compared with a chamber at time point zero as in the other runs. Pictures of the chambers at time point 0 and 18 are shown in appendix 8.2 Growth control in new slider materials. Significant bubble formation was also shown in the chambers, especially in chamber 6 of MN211 with *S. aureus*.

NAS30 showed the best adhesive and optical properties compared to HPS1R and was further investigated beside this project.

5.6 Lowest detection limit in QuickMIC^{TM}

To evaluate if QuickMIC^{TM} can be run directly after the T2Dx instrument identified a pathogen, the lowest possible bacterial concentration to run in QuickMIC^{TM} was determined for *E. coli* and *P. aeruginosa*. Three runs with *P. aeruginosa* were conducted at the inoculum concentrations 10^4, 10^5 and 10^6 CFU/ml and two runs with *E. coli* were run with the inoculum concetrations 10^5 and 10^3 CFU/ml. The control chamber (chamber 6) photographed at the last cycle in each run was used to count the number of detected regions. The number of the detected regions in each twentieth part of the chamber at the lowest detection

limit, determined by the number of correctly determined MICs, was used as threshold for later whole pipeline runs. In figure 8 - 12, the control chambers are divided into twenty parts and the number of detected regions are indicated in each part. The results showed that 10^5 CFU/ml was a sufficient bacterial concentration for *E. coli* but a higher than 10^6 CFU/ml was needed to yield accurate results for *P. aeruginosa*. The conclusion is that 5-10 detected regions in every twentieth part of the picture are sufficient for reliable results and are used as lower detection limit in the whole pipeline runs described in section 5.7. Problems with the antibiotic amikacin are observed during the experiment for *E. coli*, where the MICs are 0, which was unexpected and might be due to an error source such as a higher prepared antibiotic than planned.

5.6.1 *E. coli* with inoculum 10^3 CFU/ml

The run with approximately 10^3 CFU/ml ($4.1 * 10^3$ CFU/ml) is shown in figure 8 and 0-17 regions are detected in the twentieth parts of the picture outside the bubble.

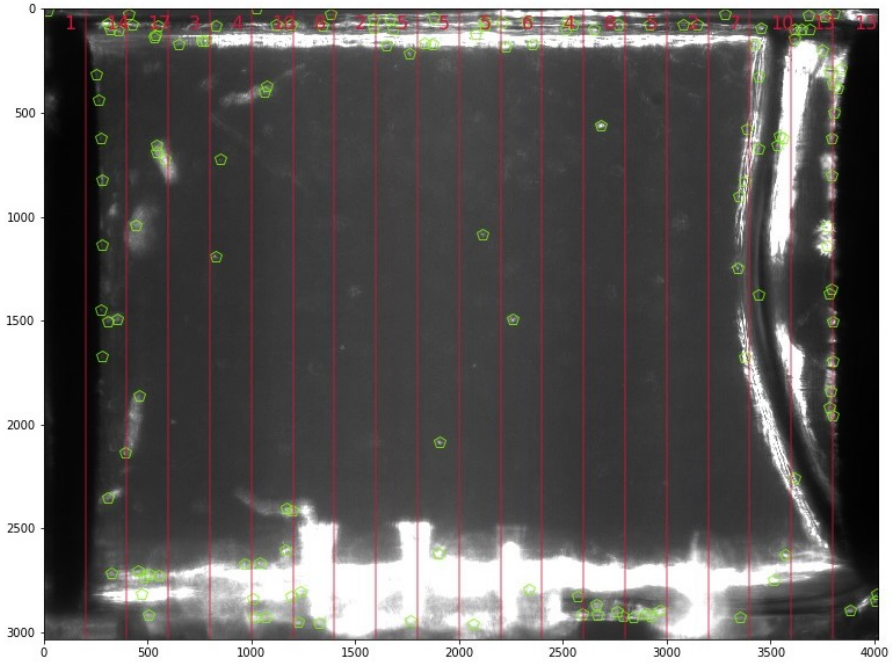

Figure 8: Detected regions in QuickMIC^{TM} chamber 6 (control chamber) and no antibiotics with *E. coli* with bacterial concentration 10^3 CFU/ml. The values on the x-axis and y-axis indicate the number of pixels.

The MICs were calculated in table 11 and was expected to be 2-16 mg/l with amikacin, higher than 128 mg/l with ceftazidime and between 0.03-0.24 mg/l with meropenem. Only two of the MICs with meropenem met the MICs from the BMD and the bacterial concentration was concluded to be too low to yield accurate QuickMIC^{TM} results.

Table 11: QuickMIC^{TM} run with *E. coli*, inoculum 10^3 CFU/l, and amikacin 16 mg/l, ceftazidime 512 mg/l and meropenem 0.24 mg/l.

Chamber	Growth to pixel	Growth to pixel/4000	MIC (mg/l)	Antibiotic
6	2100	0.525	-	None
0	0	0	0	Amikacin
3	0	0	0	Amikacin
7	0	0	0	Amikacin
10	0	0	0	Amikacin
1	0	0	0	Ceftazidime
4	0	0	0	Ceftazidime
8	0	0	0	Ceftazidime
11	0	0	0	Ceftazidime
2	0	0	0	Meropenem
5	0	0	0	Meropenem
9	700	0.175	0.042	Meropenem
12	500	0.125	0.03	Meropenem

5.6.2 *E. coli* with inoculum 10^5 CFU/ml

In the whole twentieth parts of the chamber, with the parts of the bubble not included, 4-12 detected regions are found with the inoculum concentration approximately 10^5 CFU/ml ($5.7 * 10^5$ CFU/ml) as seen in figure 9.

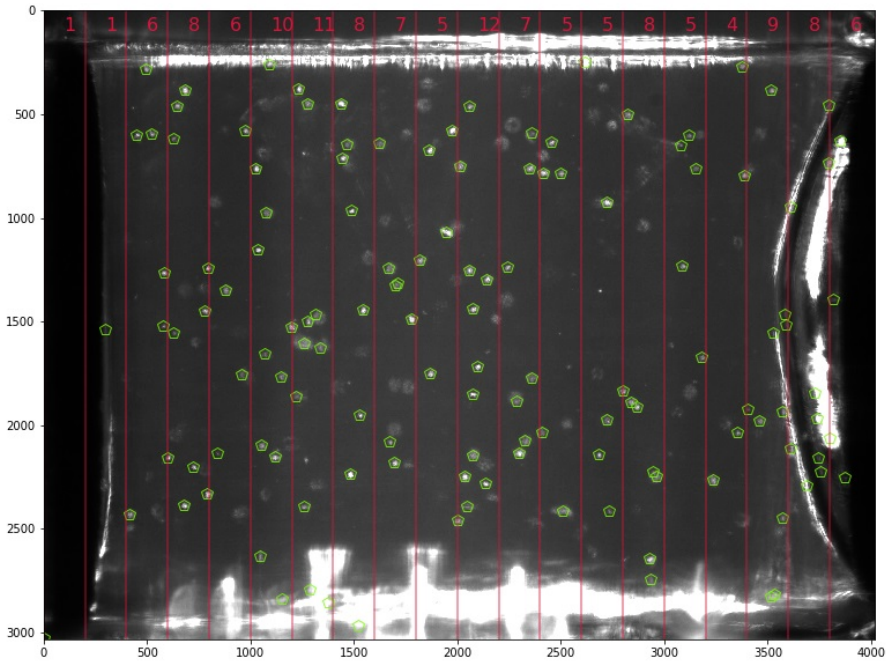

Figure 9: Detected regions in QuickMIC^{TM} chamber 6 (control chamber) and no antibiotics with *E. coli* with bacterial concentration 10^5 CFU/ml. The values on the x-axis and y-axis indicate the number of pixels.

The interpreted MICs from the run are summarized in table 12 and were expected to be 2-16 mg/l with amikacin, higher than 128 mg/l with ceftazidime and between 0.03-0.24 mg/l with meropenem based on earlier BMD. All MICs with ceftazidime and meropenem agreed with the MICs from BMD. All MICs with amikacin were 0 which was unexpected and might depend on improper preparation of the antibiotic.

Table 12: QuickMIC^{TM} run with *E. coli*, inoculum 10^5 CFU/l, and amikacin 16 mg/l, ceftazidime 512 mg/l and meropenem 0.24 mg/l.

Chamber	Growth to pixel	Growth to pixel/4000	MIC (mg/l)	Antibiotic
6	3700	0.925	-	None
0	0	0	0	Amikacin
3	0	0	0	Amikacin
7	0	0	0	Amikacin
10	0	0	0	Amikacin
1	2900	0.725	371.2	Ceftazidime
4	3100	0.775	396.8	Ceftazidime
8	3300	0.825	422.4	Ceftazidime
11	1900	0.475	243.2	Ceftazidime
2	1300	0.325	0.078	Meropenem
5	900	0.225	0.054	Meropenem
9	1700	0.425	0.102	Meropenem
12	500	0.125	0.03	Meropenem

5.6.3 *P. aeruginosa* with inoculum 10^4 CFU/ml

Very few regions were detected with bacterial concentration approximately 10^4 CFU/ml ($1.16 * 10^4$ CFU/ml) for *P. aeruginosa*, only 0-4 detected regions in every twentieth part of the picture without the bubble (figure 10).

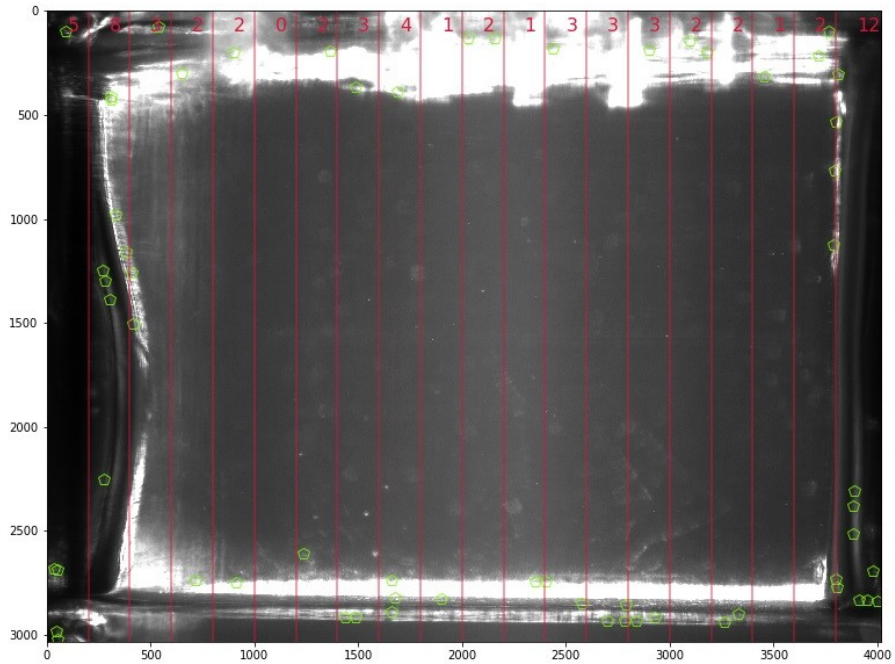

Figure 10: Detected regions in QuickMIC^{TM} chamber 6 (control chamber) and no antibiotics with *P. aeruginosa* with bacterial concentration 10^4 CFU/ml. The values on the x-axis and y-axis indicate the number of pixels.

The MICs are calculated in table 13 and are expected to be 1-4 mg/l with amikacin, 2-8 mg/l with ceftazidime and <0.125-0.5 mg/l with meropenem. Only one MIC with meropenem and one with ceftazidime were accurate and the bacterial concentration was concluded to be too low.

Table 13: QuickMIC^{TM} run with *P. aeruginosa*, inoculum 10^4 CFU/ml, and amikacin 4 mg/l, ceftazidime 4 mg/l and meropenem 0.5 mg/l.

Chamber	Growth to pixel	Growth to pixel/4000	MIC (mg/l)	Antibiotic
6	0	0	-	None
0	0	0	0	Amikacin
3	0	0	0	Amikacin
7	0	0	0	Amikacin
10	0	0	0	Amikacin
1	900	0.225	0.9	Ceftazidime
4	700	0.175	0.7	Ceftazidime
8	2900	0.725	2.9	Ceftazidime
11	0	0	0	Ceftazidime
2	0	0	0	Meropenem
5	0	0	0	Meropenem
9	0	0	0	Meropenem
12	500	0.125	0.0625	Meropenem

5.6.4 *P. aeruginosa* with inoculum 10^5 CFU/ml

In figure 11 with approximately 10^5 CFU/ml ($1.16 * 10^5$ CFU/ml) there are 0-2 detected regions which is a low number of detected regions.

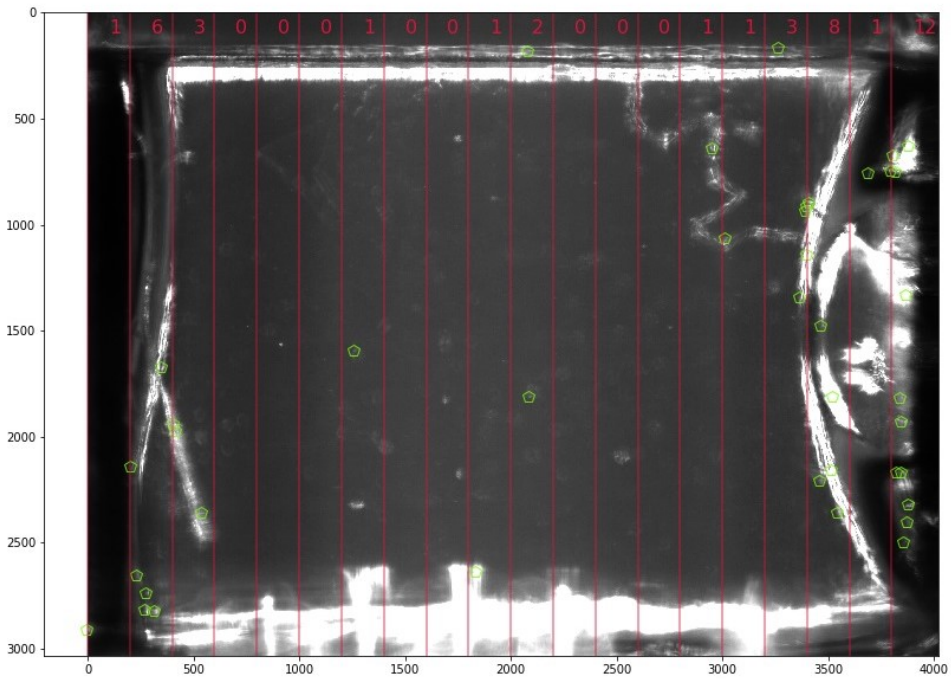

Figure 11: Detected regions in QuickMIC^{TM} chamber 6 (control chamber) and no antibiotics with *P. aeruginosa* with bacterial concentration 10^5 CFU/ml. The values on the x-axis and y-axis indicate the number of pixels.

The MICs were calculated and were expected to be 1-4 mg/l with amikacin, 2-8 mg/l with ceftazidime and <0.125-0.5 mg/l with meropenem in table 14. One MIC with amikacin and one MIC with meropenem were accurate which and the inoculum was concluded to be too low.

Table 14: QuickMIC^{TM} run with *P. aeruginosa*, inoculum 10^5 CFU/ml, and amikacin 4 mg/l, ceftazidime 4 mg/l and meropenem 0.5 mg/l.

Chamber	Growth to pixel	Growth to pixel/4000	MIC (mg/l)	Antibiotic
6	0	0	-	None
0	0	0	0	Amikacin
3	1100	0.275	1.1	Amikacin
7	0	0	0	Amikacin
10	700	0.175	0.7	Amikacin
1	0	0	0	Ceftazidime
4	0	0	0	Ceftazidime
8	0	0	0	Ceftazidime
11	0	0	0	Ceftazidime
2	0	0	0	Meropenem
5	0	0	0	Meropenem
9	0	0	0	Meropenem
12	700	0.175	0.0875	Meropenem

5.6.5 *P. aeruginosa* with inoculum 10^6 CFU/ml

The detected regions in the control chamber with bacterial concentration approximately 10^6 CFU/ml ($1.32 * 10^6$ CFU/ml) were 0-2 regions outside the bubble in figure 12.

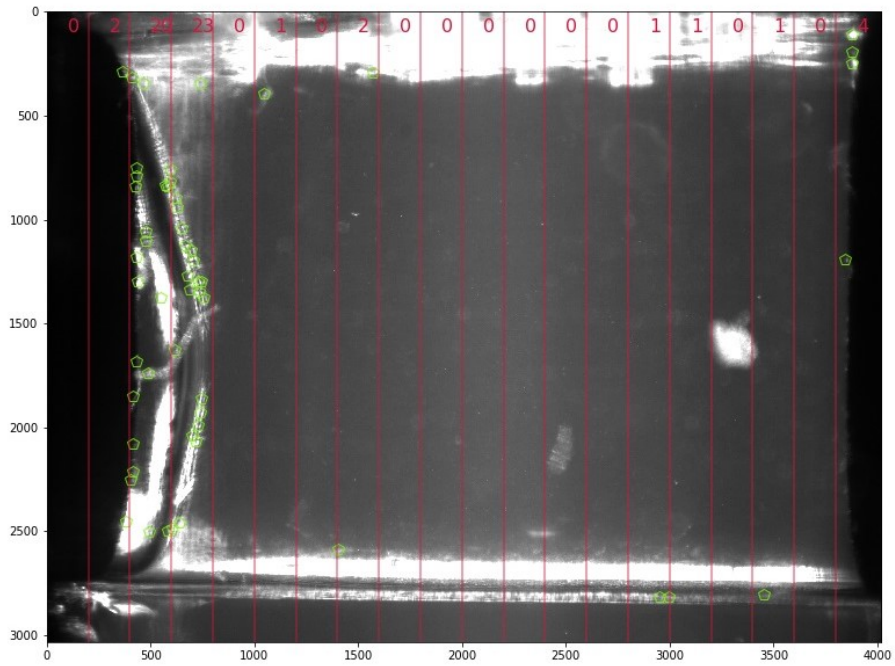

Figure 12: Detected regions in QuickMIC^{TM} chamber 6 (control chamber) and no antibiotics with *P. aeruginosa* with bacterial concentration 10^6 CFU/ml. The values on the x-axis and y-axis indicate the number of pixels.

In table 15, are the MICs from the run calculated and were expected to be 1-4 mg/l with amikacin, 2-8 mg/l with ceftazidime and <0.125-0.5 mg/l with ceftazidime. Only two of the MICs with meropenem agreed with previous results from BMD and 10^6 CFU/ml was concluded to be too low for accurate results.

Table 15: QuickMIC^{TM} run with *P. aeruginosa*, inoculum 10^6 CFU/ml, and amikacin 4 mg/l, ceftazidime 4 mg/l and meropenem 0.5 mg/l.

Chamber	Growth to pixel	Growth to pixel/4000	MIC (mg/l)	Antibiotic
6	0	0	-	None
0	0	0	0	Amikacin
3	0	0	0	Amikacin
7	0	0	0	Amikacin
10	0	0	0	Amikacin
1	700	0.175	0.7	Ceftazidime
4	0	0	0	Ceftazidime
8	0	0	0	Ceftazidime
11	0	0	0	Ceftazidime
2	700	0.175	0.0875	Meropenem
5	0	0	0	Meropenem
9	0	0	0	Meropenem
12	700	0.175	0.0875	Meropenem

5.7 Pipeline runs

The whole pipeline was tested with both *E. coli* and *S. aureus* and the goal was to compare the classical pipeline with the T2-QuickMIC^{TM} pipeline in the aspect of time and accuracy. In total three pipelines were tested with two species, *E. coli* an *S. aureus* as in table 16.

Table 16: Pipeline runs.

Pipeline	Species
1	*E. coli*
2	*E. coli*
3	*S. aureus*

5.7.1 Current pipeline

Time
The total time of the current pipeline was calculated in table 17 and compared to the time calculated for the T2-QuickMIC^{TM} pipeline in table 21. The bacterial concentrations at alarm for a positive blood culture bottle are also indicated in table 21.

Table 17: time for the current pipeline and bacterial concentration at positive blood culture. Pipeline 1 and 2 are run with *E. coli* and pipeline 3 with *S. aureus*.

Pipeline	Pos. blood culture (h)	CFU/ml	MALDI-TOF MS (h)	BMD (h)	Total time (h)
Pipeline 1	8.58	$2.90 * 10^8$	0.17 (direct)	17.00	25.75
Pipeline 2	8.08	$5.60 * 10^7$	0.50 (sepsityper kit)	17.83	26.41
Pipeline 3	23.53	$2.15 * 10^8$	0.50 (sepsityper kit)	17.83	41.86

MALDI-TOF MS identification
The MALDI-TOF MS runs on the strains were firstly done with the sepsityper kit from Bruker directly from positive blood culture bottles. If the analysis with the sepsityper kit failed, direct identification from subcultures was done instead the next day. The type of identification method and the results are presented in table 18.

Table 18: MALDI-TOF MS identification.

Pipeline	Type	Best match	Score	Second best match	Score
Pipeline 1	Sepsityper kit	No ID possible	1.39	No ID possible	1.31
Pipeline 1	Direct	*Escherichia coli*	2.37	*Escherichia coli*	2.22
Pipeline 2	Sepsityper kit	*Escherichia coli*	2.19	*Shigella dysenteriae*	2.18
Pipeline 3	Sepsityper kit	*Staphylococcus aureus*	2.32	*Staphylococcus aureus*	2.06

Broth microdilution on subcultures

BMD is the standard method for antibiotic susceptibility testing and was conducted on early subcultures from the blood culture bottles (figure 13 - 15) and analyzed according to EUCAST instructions (EUCAST 2019b). The antibiotic panel earlier prepared was used for both *E. coli* and *S. aureus*. Table 19 indicate in which columns in the microtiter plate the antibiotics were loaded and their concentration range indicated in number of times the brekpoint. The concentration of the antibiotics are reduced by a factor of 2 for each row in the plate. The breakpoints of each antibiotic can be seen in table 1.

Table 19: Antibiotic panel used for broth microdilution.

Column	Antibiotic	Concentration range
1	Amikacin	4xR - 0.0625xR
2	Cefotaxime	4xR - 0.0625xR
3	Ceftazidime	4xR - 0.0625xR
4	Ciprofloxacine	4xR - 0.0625xR
5	Colistin	4xR - 0.0625xR
6	Gentamicin	4xR - 0.0625xR
7	Imipenem	4xR - 0.0625xR
8	Meropenem	4xR - 0.0625xR
9	Piperacillin/tazobactam	4xR - 0.0625xR
10	Tigecycline	4xR - 0.0625xR
11	Tobramycin	4xR - 0.0625xR
12	Trimethoprim/sulfamethoxazole	4xR - 0.0625xR

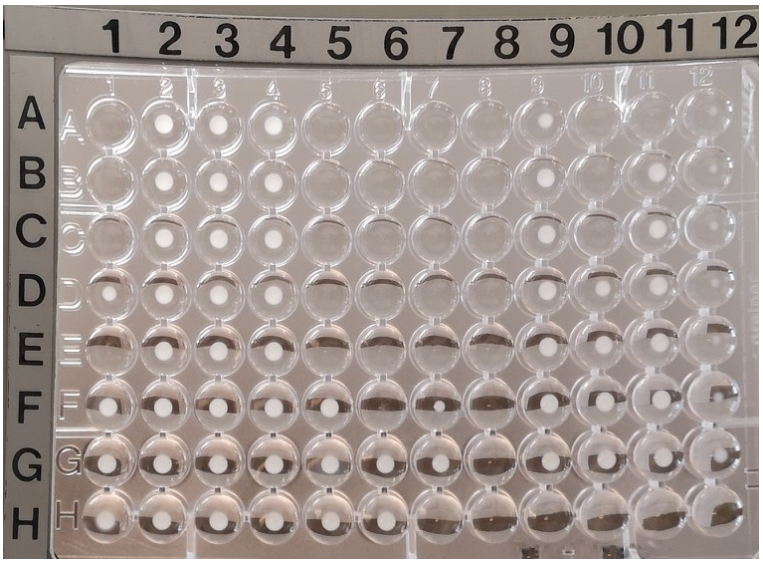

Figure 13: Broth microdilution with *E. coli*, pipeline 1, and the gram negative antibiotic panel. The gram negative antibiotic panel is loaded column-wise and the concentration is reduced by a factor of 2 each row.

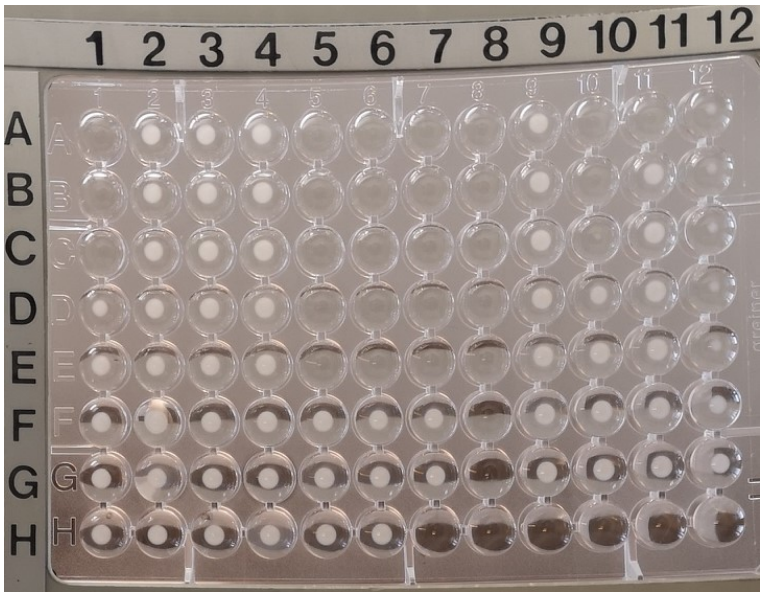

Figure 14: Broth microdilution with *E. coli*, pipeline 2, and the gram negative antibiotic panel. The gram negative antibiotic panel is loaded column-wise and the concentration is reduced by a factor of 2 each row.

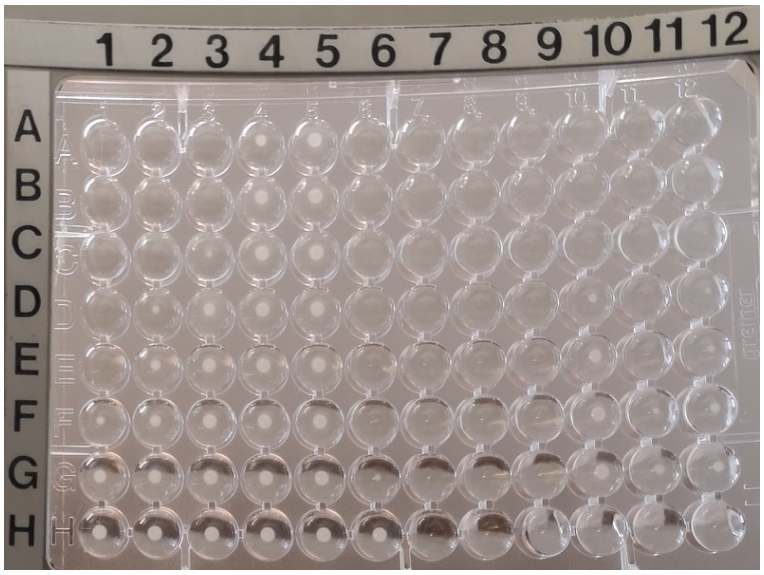

Figure 15: Broth microdilution with *S. aureus* and the gram negative antibiotic panel. The gram negative antibiotic panel is loaded column-wise and the concentration is reduced by a factor of 2 each row.

The MICs from BMD are summed in table 20 below. To get an overview, the MICs are shown in number of times the breakpoint (table 1). The first and second pipeline with the same strain of *E. coli* have similar MICs, validating the results. Only gentamicin and trimethoprim/sulfamethoxazole show different MICs, but since the variance in BMD was allowed to be 0.5-2 times the MIC, the broth microdilutions were interpreted as valid. All positive and negative controls were approved.

Table 20: Summary of the MICs from BMD in the whole pipelines. The MICs are indicated in number of times the breakpoint.

Nr	AMK	CTX	CAZ	CIP	CST	GEN	IPM	MEM	PIP/TAZ	TGC	TOB	SXT
1	0.5	> 2	> 2	> 2	0.125	0.063	0.125	< 0.031	> 2	0.5	2	> 2
2	0.5	> 2	> 2	> 2	0.125	0.125	0.125	< 0.031	> 2	0.5	2	2
3	0.125	0.5	1	> 2	> 2	0.063	< 0.031	< 0.031	< 0.031	0.5	0.125	0.25

5.7.2 T2-QuickMIC™ pipeline

Time
Records of the time during the T2-QuickMIC™ pipeline are presented in table 21 below. QuickMIC™ was at first conducted directly after alarm from T2, which would be the ideal case, but the bacterial concentration was way too low to yield results. Instead the growth rate was calculated and two new QuickMIC™ runs were started after longer incubation time as in table 22, based on the growth rate calculated.

Table 21: Time for the T2-QuickMIC™ pipeline.

Strain	T2 (h)	Incubation	QuickMIC™ (h)	Total time (h)
E. coli run 1	3.17	0	3.82	6.99
E. coli run 2	3.67	0	3.82	7.49
S. aureus	3.58	0	3.82	7.40

The time consumption for the second QuickMIC™ tests are displayed in table 22 below. The blood culture bottles were left in the Bact/Alert® Virtuo® detection system for a longer time than in the first run to obtain a higher bacterial concentration.

Table 22: Second QuickMIC™ run with longer incubation times.

Strain	Incubation (h)	QuickMIC™ (h)	Total time (h)
E. coli	5.1	3.82	8.92
S. aureus	13.6	3.82	17.42

T2Dx results
Many runs in the T2Dx® instrument with the T2Bacteria panel ended in "target not detected" as seen in table 23 even though the inoculum exceeded 1 CFU/ml which was the lowest detection limit according to T2 Biosystems. The runs with *S. aureus* resulted in "Target not detected".

Table 23: T2 run results and approximate inoculum in the loaded K2EDTA tubes.

pipeline	T2 run nr.	Result	Inoculum (CFU/ml)
1	1	Target not detected	7
1	2	*E. coli*	9
2	1	*E. coli*	8
3	1	Target not detected	14
3	2	Target not detected	11

QuickMIC™ results
In the first QuickMIC™ runs, no incubation between the T2 results and the start of QuickMIC™ was conducted which led to too few detected regions in the control chamber as seen for pipeline 1 in figure 16, pipeline 2 in figure 17 and pipeline 3 in figure 18. Only the detected regions outside of the bubble were used.

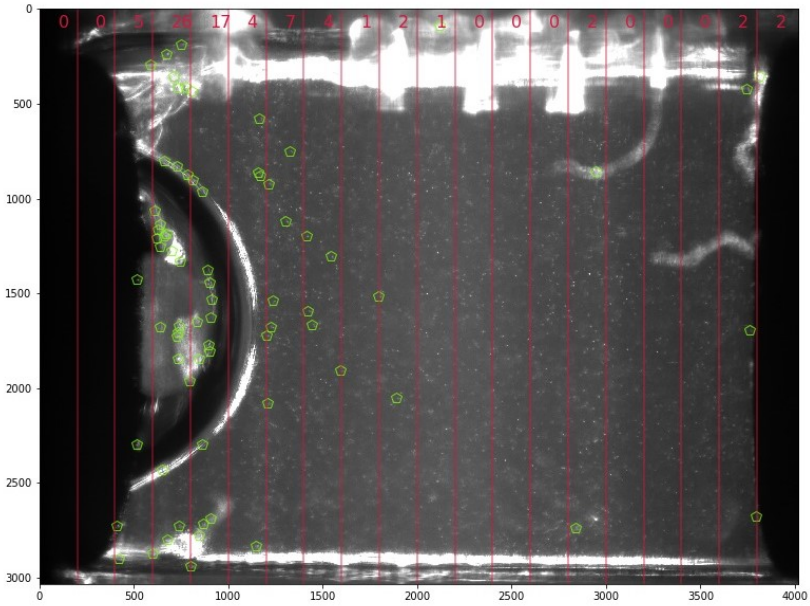

Figure 16: Detected regions in the control chamber in pipeline 1 with *E. coli*.

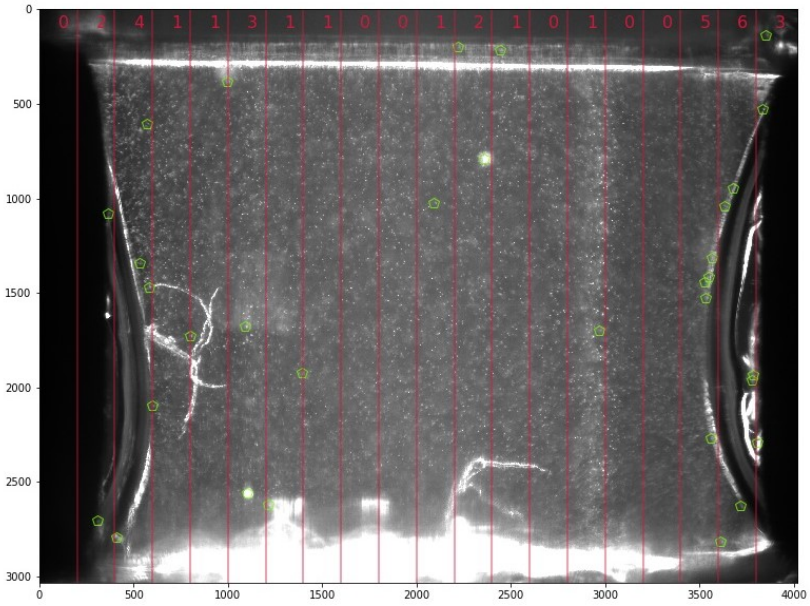

Figure 17: Detected regions in the control chamber in pipeline 2 with *E. coli*.

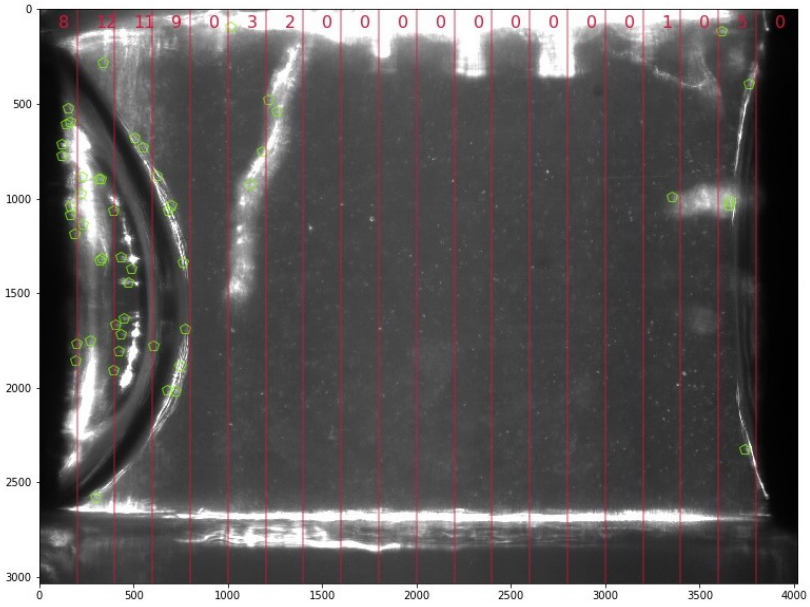

Figure 18: Detected regions in the control chamber in pipeline 3 with *S. aureus*.

The bacterial concentrations were calculated before and after centrifugation in the sample preparation of QuickMIC^{TM}. The results are shown in table 24 below. After centrifugation of the sample from the blood culture bottles, a lot of the bacteria was lost. No bacteria could be seen on the plates in pipeline 3 with *S. aureus* indicating that the bacterial concentration was below 10 CFU/ml and thereby way below the needed concentration to run QuickMIC^{TM} which previously at least for *E. coli* was shown to be 10^5 CFU/ml.

Table 24: Bacterial concentration at inoculum and before and after centrifugation in the sample preparation of QuickMIC^{TM}.

pipeline	Inoculum (CFU/ml)	Before centrifugation (CFU/ml)	After centrifugation (CFU/ml)	Loss (%)
1	2	$4.6 * 10^3$	$3.3 * 10^2$	92.8
2	2	$1.9 * 10^3$	$5.9 * 10^2$	68.9
3	4	<10	<10	-

Growth curves were calculated from previous results to evaluate the time the blood culture bottles were to be incubated in the Bact/Alert® Virtuo® detection system to reach the minimum bacterial concentration that was needed to run QuickMIC^{TM}. The growth rate was calculated using

$$y(t) = y_0 * e^{k*t}$$

Where $y(t)$ is the final bacterial concentration, y_0 is the start bacterial concentration, t is the time and k is the growth rate constant that indicate the number of generations per time unit. The bacterial concentration at alarm in Bact/Alert® Virtuo® detection system from table 17 was used when the growth rate for both *E. coli* and *S. aureus* was calculated. The growth rate for *E. coli* was calculated as

$$y(t) = y_0 * e^{2,1902*t}$$

and plotted in figure 19.

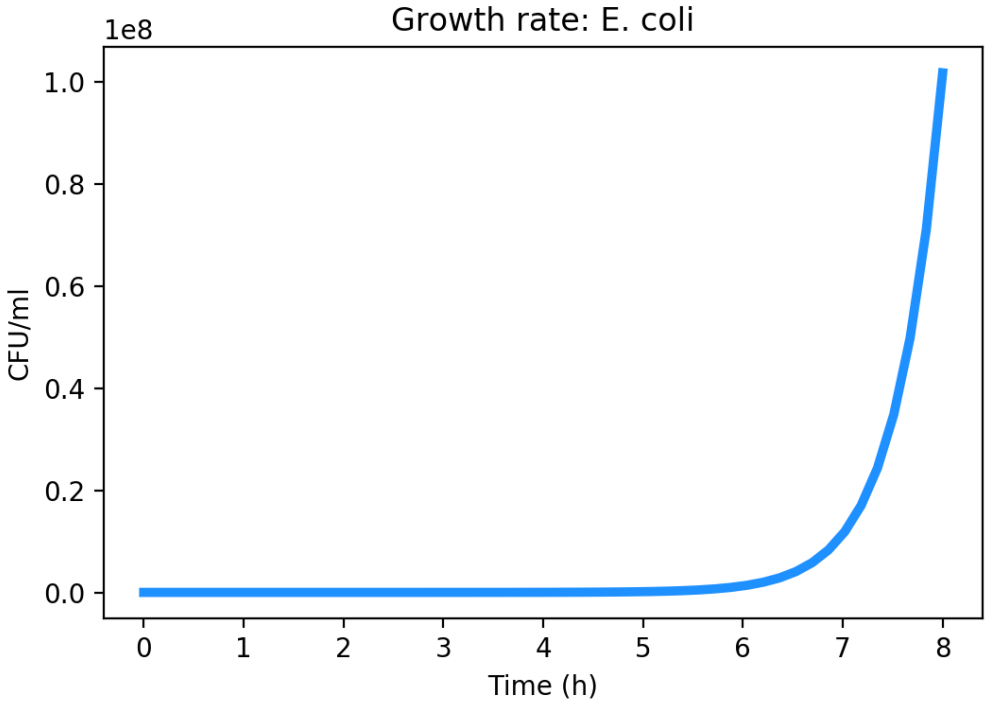

Figure 19: Growth rate of *E. coli* calculated from the bacterial concentration of the positive blood culture bottle in the current pipeline.

The growth rate for *S. aureus* was calculated as

$$y(t) = y_0 * e^{0,7565*t}$$

and plotted in figure 20.

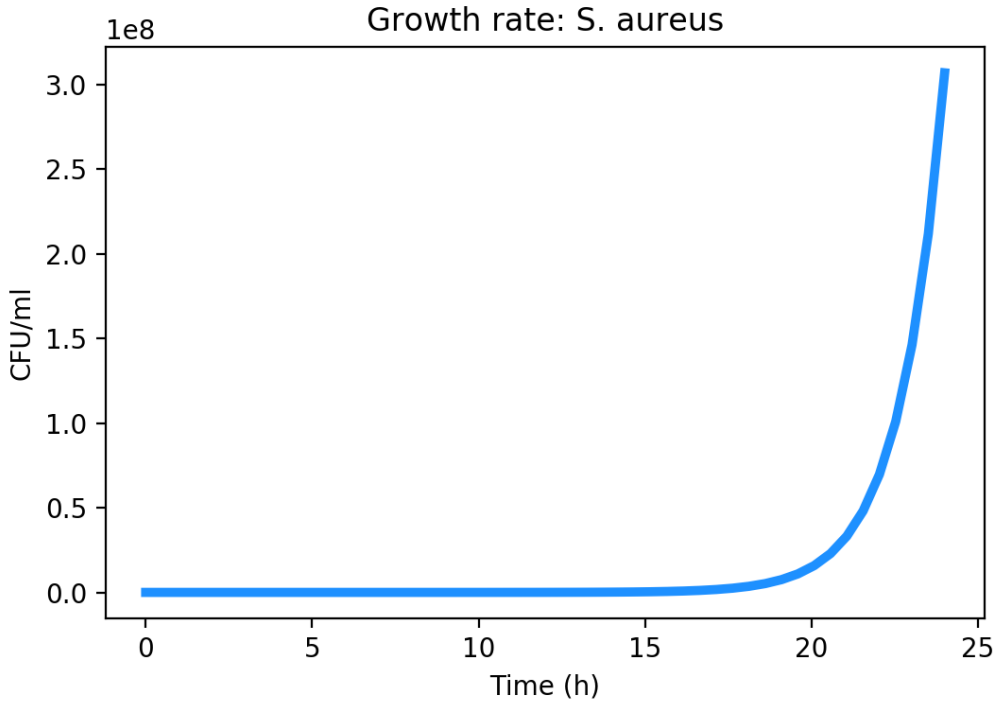

Figure 20: Growth rate of *S. aureus* calculated from the bacterial concentration of the positive blood culture bottle in the current pipeline.

From the calculated growth rates, the incubation time to reach 10^5 CFU/ml was calculated. With $y(t)$ as 10^5 CFU/ml and y_0 as 2.5 CFU/ml the incubation time for *E. coli* was calculated to 4,84 hours and for *S. aureus*, 14 hours. The time that the blood culture bottles were incubated at is summarized in table 22. In table 25 are the bacterial concentrations summarized for the second run of QuickMIC^{TM} with longer incubation times.

Table 25: Bacterial concentration at inoculum and before and after centrifugation in the sample preparation of the second QuickMIC^{TM} run.

Strain	Inoculum (CFU/ml)	Before centrifugation (CFU/ml)	After centrifugation (CFU/ml)	Loss (%)
E. coli	3	$1.3 * 10^4$	$7 * 10^2$	94.6
S. aureus	4	$8.8 * 10^5$	$2.9 * 10^4$	96.7

Table 25 indicates that the concentration before centrifugation was too low for *E. coli* and could have been sufficient for *S. aureus*. The loss of bacterial mass after centrifugation was the reason no results could be interpreted. The detected regions in QuickMIC^{TM} in the control chamber in the second run in figure 21 and figure 22 were still too few and the MICs could not be interpreted.

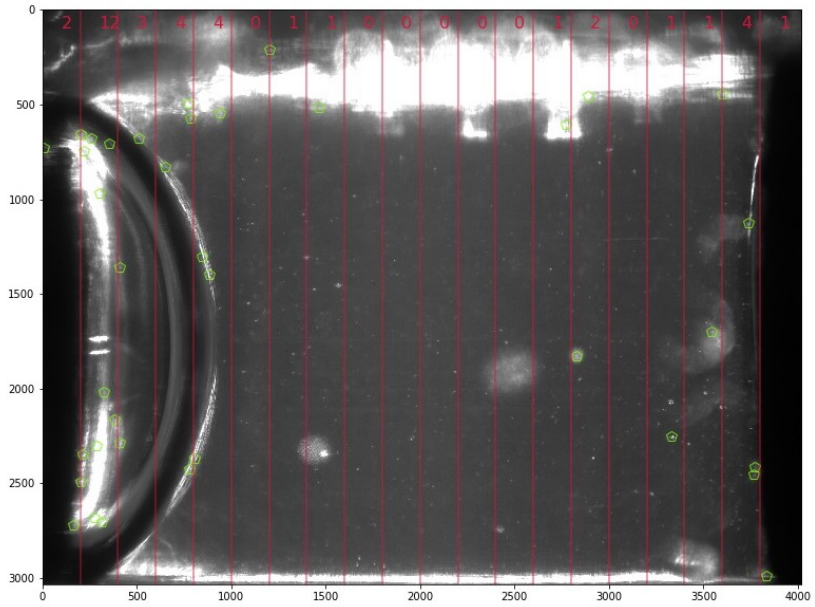

Figure 21: Detected regions in the control chamber in the second QuickMIC^{TM} run with *E. coli*

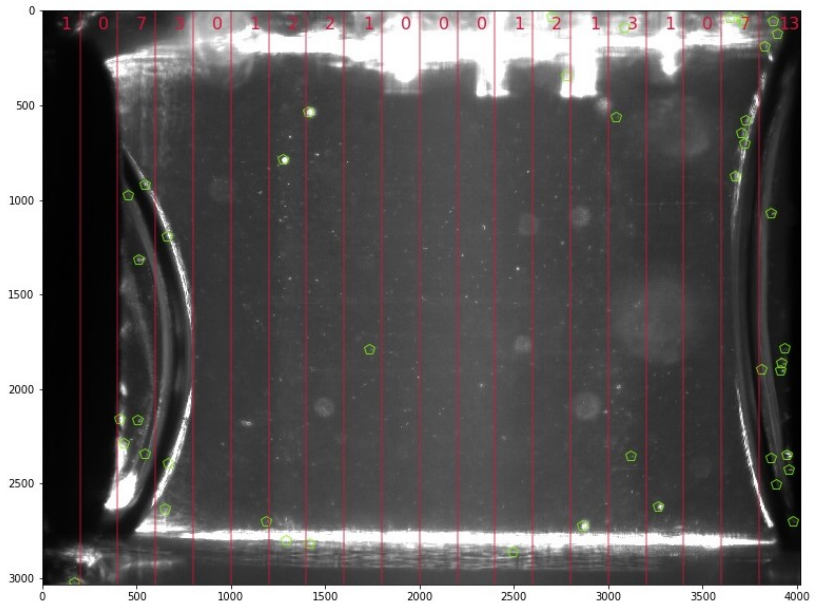

Figure 22: Detected regions in the control chamber in the second QuickMIC^{TM} run with *S. aureus*.

6 Discussion

The aim of this project was to investigate a new pipeline with future possibilities to be used in hospitals and clinics from patient sample to detection, ID and AST results. The easiest way to incorporate this pipeline would be to use Bact/Alert blood culture bottles that are used in most hospitals today. There were several experiments that needed to be conducted before the whole pipeline could be tested. These included to investigate if the T2Dx instrument could analyze blood from blood culture bottles, if the blood culture bottles could be analyzed in QuickMIC™ before alarm in the Bact/Alert® Virtuo® system, to investigate the lowest detection limit in QuickMIC™ and finally to test the whole pipeline at once.

6.1 T2Dx instrument run with blood from blood cultures

To know which blood culture bottles contained bacteria, the original plan was to use the T2Dx instrument but the T2Dx instrument was unable to analyze samples from blood culture bottles, since the silicon beads in the blood culture bottles clogged the pipettes. Therefore, K2EDTA tubes were used instead in order to detect and identify bacteria in the first step of the T2-QuickMIC™ pipeline. This was not optimal since it would be better to know in exactly which blood culture bottles the bacteria were growing since the bacterial concentration in blood is low and there is a risk that not all blood culture bottles from a patient contain bacteria.

A further experiment to test would be to filter the blood from blood culture bottles to eliminate the silicon beads from the sample and avoid clogging of the pipettes. Such an experiment would provide useful information about possible disturbances of the other additives in the blood culture bottle, such as the anticoagulant and growth medium.

It is also important to note that the T2Bacteria panel only detect the ESKAPE pathogens which means that blood culture bottles in the future at hospitals will have to be loaded in the Bact/Alert® Virtuo® detection system until alarm as well to be able to detect all species of bacteria.

6.2 Lowest detection limit in QuickMIC™

The experiments conducted in order to find the lowest possible bacterial concentration that could be run in QuickMIC™ indicated that a minimum of 5-10 detected regions in all twentieth parts of the chamber were necessary to yield a trustworthy result. The only run with an accurate result was *E. coli* at inoculum 10^5 CFU/ml. No run with *P. aeruginosa* yielded an interpretable result. These results led to the conclusion that the bacterial concentration to be used in further experiments on the whole pipeline was 10^5 CFU/ml or higher. It should be noted that the analysis only was conducted in one slice/resolution of the chamber in Z-axis, meaning that if the analysis was conducted in more than one slice, more regions could be detected which would increase the sensitivity and samples with lower bacterial concentration could yield interpretable results which is to be implemented in the future.

6.3 Bacterial concentration at alarm in the Bact/Alert® Virtuo® detection system

Investigating at which bacterial concentration the Bact/Alert® Virtuo® detection system detected pathogens was done to draw a conclusion if QuickMIC™ could be run before detection of a positive blood culture bottle. The results showed that all spiked blood culture bottles were detected at a bacterial concentration between $10^6 - 10^{10}$ CFU/ml. *E. coli* was detected first and *S. aureus* last. From these results the conclusion was that QuickMIC™ could be run before detection of a positive blood bottle in the Bact/Alert® Virtuo® detection system and that *E. coli* and *S. aureus* were to be used in experiments on the whole pipeline to obtain a time span between the fastest and slowest growing species. An experiment

was also conducted in order to investigate if the time to alarm in the Bact/Alert® Virtuo® detection system was influenced by sampling of 4.5 ml blood before alarm. This experiment was conducted to conclude if one or two blood culture bottles could be started in the beginning of a whole pipeline run. The results showed that the time to alarm with *E. coli* practically was uninfluenced by the number of bottles used but influenced with *S. aureus*, which led to the conclusion that two blood culture bottles were to be used in the whole pipeline runs.

6.4 Whole pipeline runs

The comparative whole pipeline study between the currently used pipeline and the T2-QuickMIC™ pipeline suggested that the T2-QuickMIC™ pipline has a great potential to be a faster option to detection, ID and AST than culture based pipelines used today. No significant results regarding the time of the T2-QuickMIC™ pipline could be obtained because of failed QuickMIC™ runs and a time contraint. The bacterial concentration after incubation reached a level that was barely high enough to yield interpretable results but the loss of bacteria in the centrifugation step led to that the run could not be analyzed. In further studies, the loss of bacteria after centrifugation in the sample preparation have to be considered when calculating the incubation time to reach sufficient microbial growth. The loss of bacteria in the centrifugation step has to be considered in future studies in order to fully compare the time consumption of the T2-QuickMIC™ pipline and current pipelines for bacterial detection, ID and AST.

6.5 Setbacks

There have been setbacks during the project, for example problem with leakage in the fluidics of the instruments. It was an error source in many tests which led to the results not being able to be used. The cause of the leakage was found in both the cassettes and in the instruments themselves. To seal the leakage in the cassettes cyanoacrylate glue was used. Cyanoacrylate is a bactericidal property (Romero *et al.* 2009), but since the leakage was found in the waste tanks of the cassettes, a conclusion was made that it would not affect the bacteria in the chambers. To seal the leakage in the instruments, new tubes were installed and all screws were tightened which solved the problem. Another setback during the project was micro bubbles forming in the agarose gels that expanded when the fluidics was started. Sometimes the expansion caused the agarose gel in the chambers to rip and move. The scripts for analyzing the chambers are dependent on that the gel stick properly in the chambers and also that no bubbles form and move since that is recognized as bacterial growth when analyzed. It was in sample preparation the samples were vortexed which introduced micro bubbles into the solution when in later steps it was mixed with agarose. By mixing with a pipette instead of vortexing, bubble formation was avoided.

6.6 Future work and prospects

This project have shown the potential of using the T2-QuickMIC™ pipeline for rapid detection, ID and AST that in the future have the possibility to save lives of people suffering from sepsis by fast and accurate influence of the patient treatment. More research is however needed before it can be used in hospitals and clinical settings. A solution to determine the incubation time to sufficient bacterial growth in the blood culture bottles have to be solved and the false negatives on the T2Dx instrument have to be investigated further. The study will continue in the future and a total of 77 strains will be examined and compared in the piplines. I am grateful to be able to continue to be a part of this project and convinced that it will make a difference for patients with sepsis in the future.

7 Acknowledgements

I would like to give a special thank you to my supervisor Christer Malmberg for his support in discussions throughout the project and for helping me plan this project and my experiments. A special thank you also to Pikkei Wistrand-Yuen who has been a great support in all laboratory work and in analyzing my results. I would also like to thank Po-Cheng Tang for helping me with the laboratory work to get my last results in time and Linus Sandegren who has given many helpful tips regarding the writing of this report.

Additionally, I would like to thank Sara Thorslund, together with everyone else at Gradientech and the unit of antibiotic research for making me feel welcome and for making the time during my exam project fun, educational and inspiring.

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8 Appendices

8.1 Slider material pixel analysis area

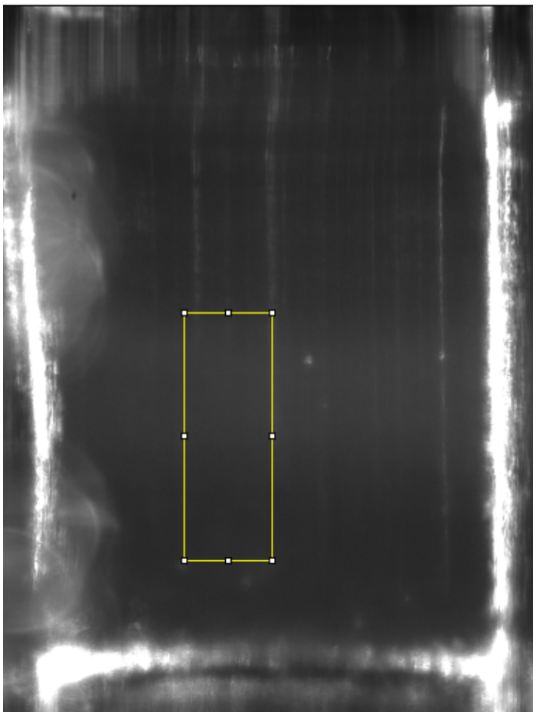

Figure 23: Analysis area of HPS1R for pixel analysis of slider materials.

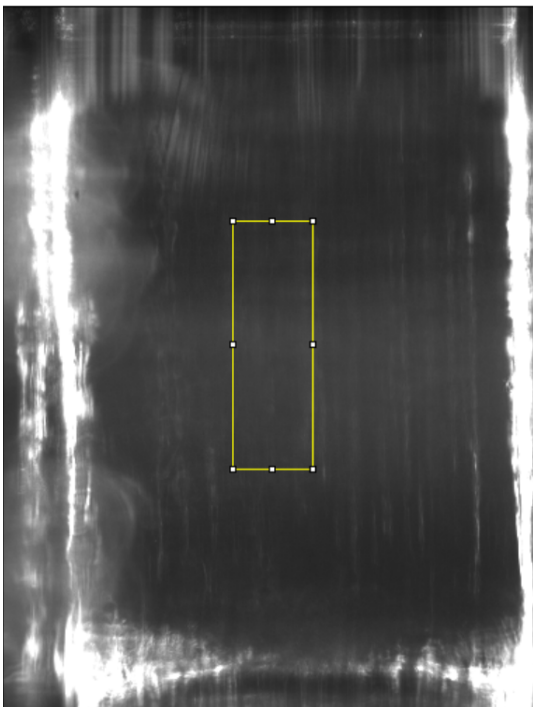

Figure 24: Analysis area of NAS30 for pixel analysis of slider materials.

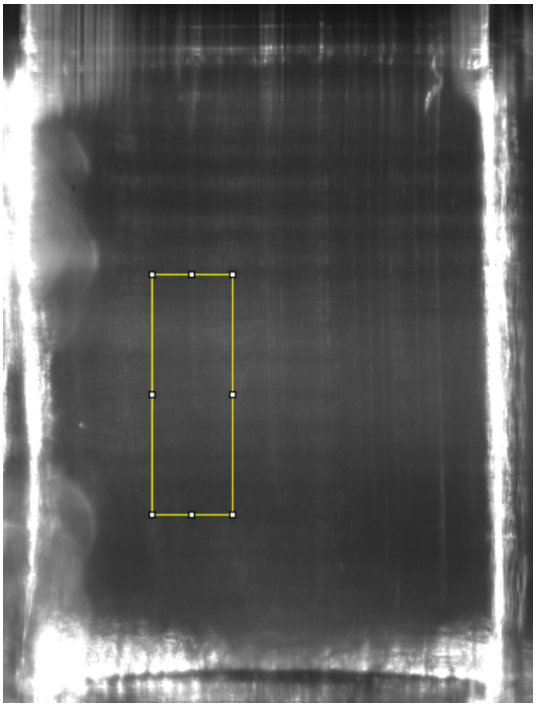

Figure 25: Analysis area of MN211 for pixel analysis of slider materials.

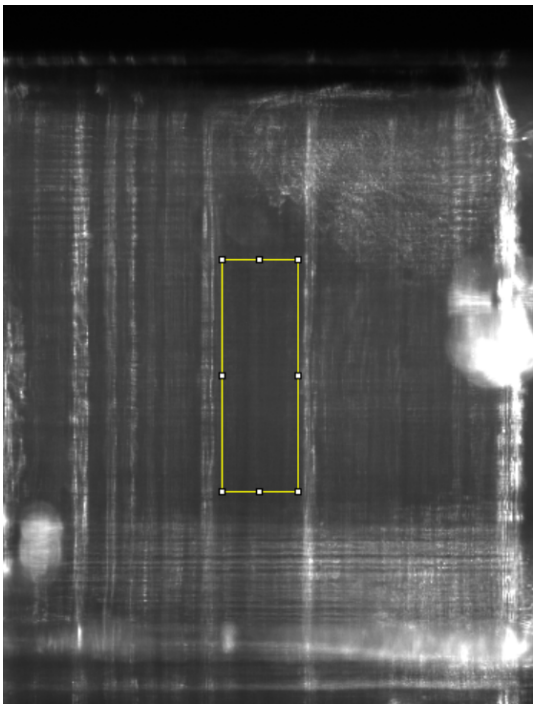

Figure 26: Analysis area of SAN31 for pixel analysis of slider materials.

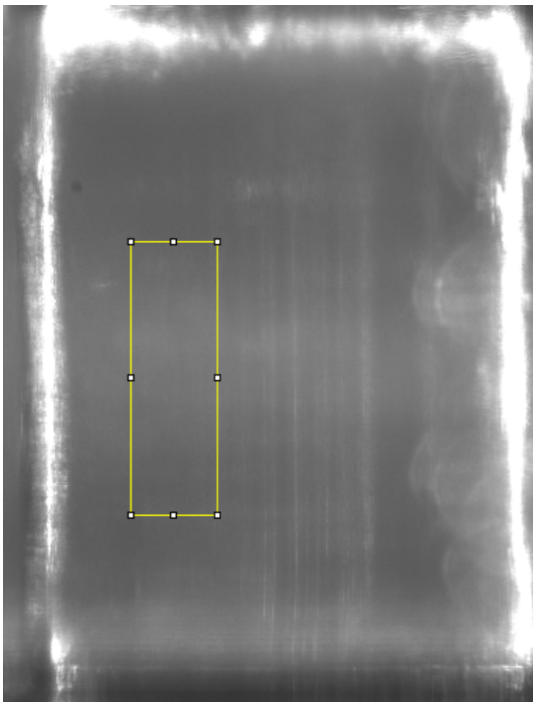

Figure 27: Analysis area of styrolux for pixel analysis of slider materials.

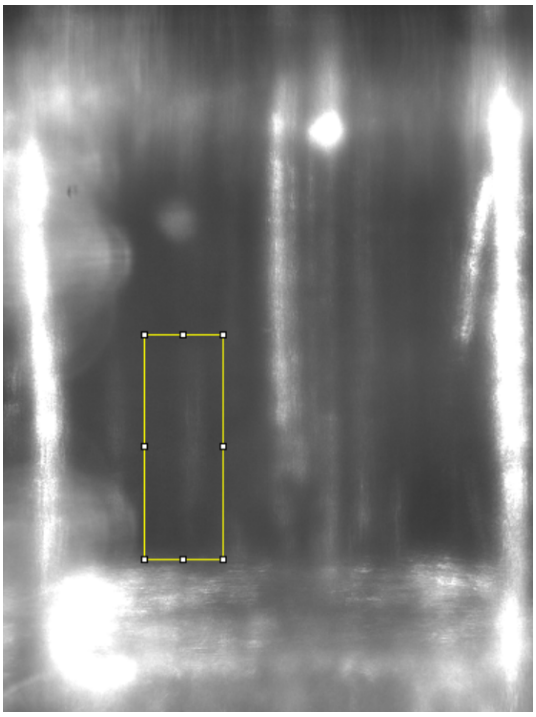

Figure 28: Analysis area of COC for pixel analysis of slider materials.

8.2 Growth control in new slider materials

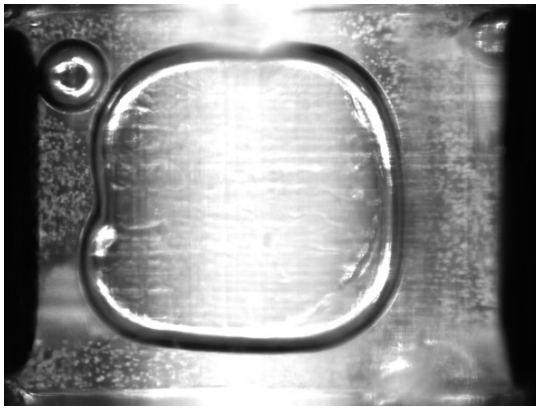

Figure 29: Growth control in chamber 6 of *S. aureus* in MN211 slider material.

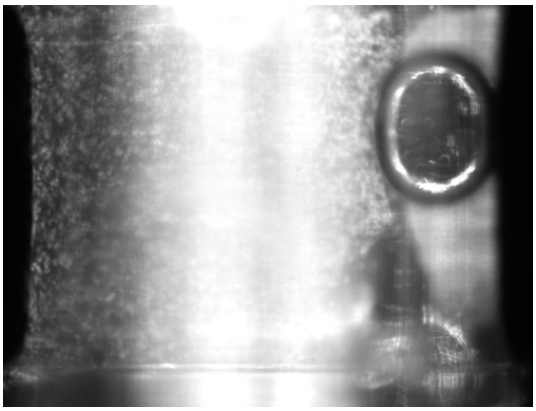

Figure 30: Growth control in chamber 8 of *S. aureus* in MN211 slider material.

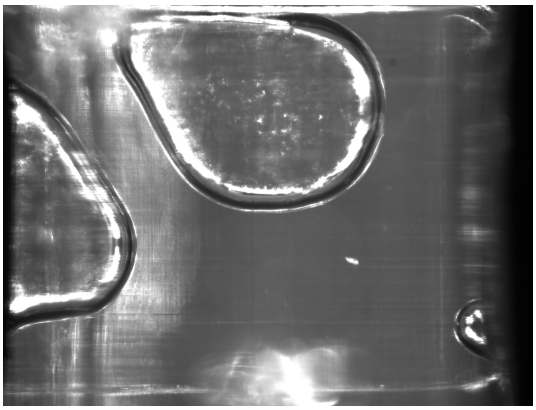

Figure 31: Growth control in chamber 6 of *E. coli* in MN211 slider material, cycle 0.

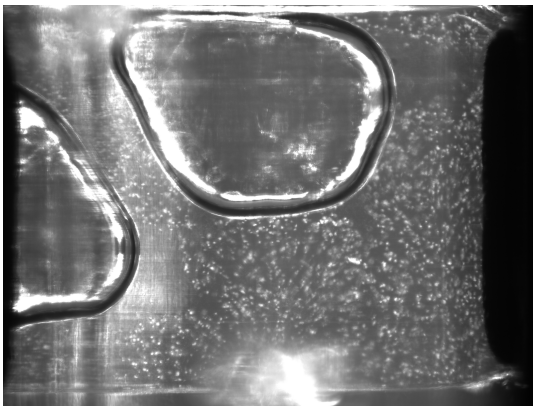

Figure 32: Growth control in chamber 6 of *E. coli* in MN211 slider material, cycle 23.

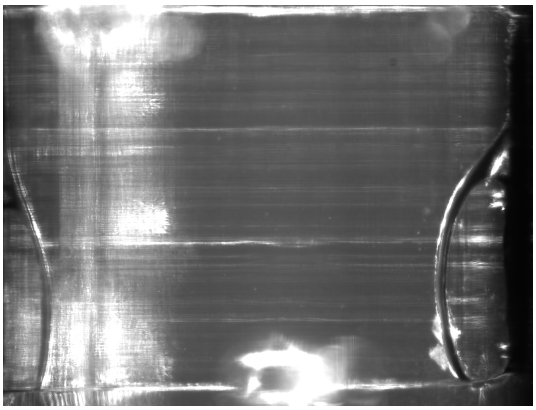

Figure 33: Growth control in chamber 6 of *E. coli* in NAS30 slider material, cycle 0.

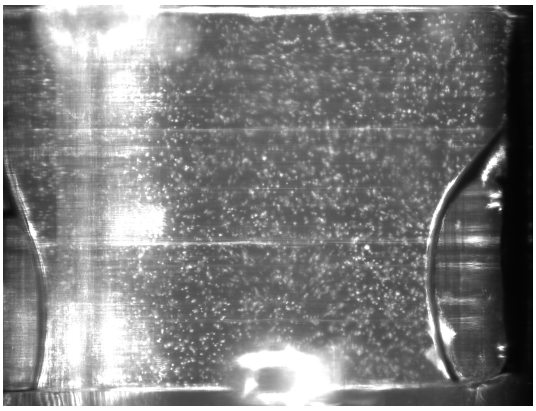

Figure 34: Growth control in chamber 6 of *E. coli* in NAS30 slider material, cycle 23.

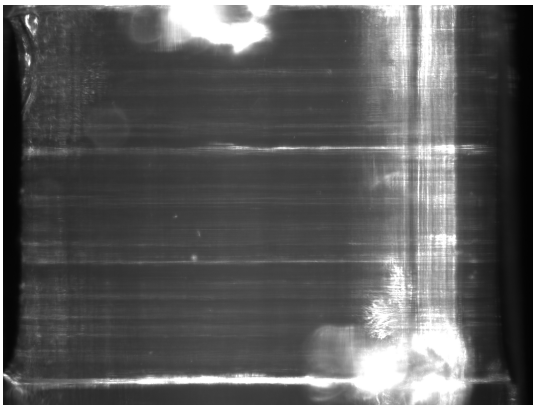

Figure 35: Growth control in chamber 6 of *S.aureus* in NAS30 slider material, cycle 0.

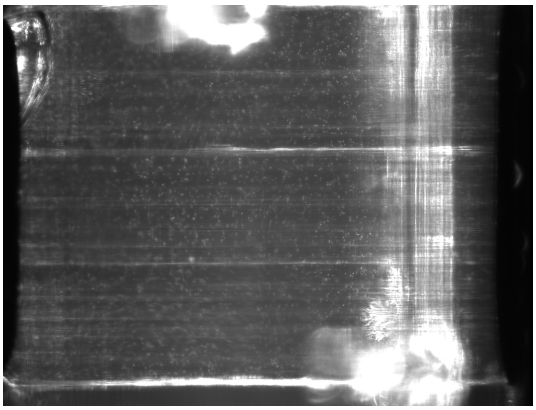

Figure 36: Growth control in chamber 6 of *S. aureus* in NAS30 slider material, cycle 23.