Tackling antimicrobial resistance in dairy production: using microfluidics and smartphone technology to monitor resistance through functional antibiotic sensitivity testing

Degree of Doctor of Philosophy

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AUTHOR'S DECLARATION

I confirm that this is my work and the use of all material from other sources has been properly and fully acknowledged.

MATTHEW MICHAEL LONG

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Contents

Abstract

Antimicrobial resistance (AMR) is a current and increasing threat to both animal and human health alike, and particularly common where farming is concerned. Of importance is the role that antibiotic treatment of mastitis in cattle plays in this health crisis. Improved diagnostics for bacterial detection and antibiotic resistance detection is one area that can be targeted to improve antibiotic prescribing and treatment regimes, optimising the use of antimicrobials. However, rapid, on-farm tools are needed to effectively make antibiotic-prescribing decisions. On-farm diagnostic tests are already in use, such as the California Mastitis Test (CMT), somatic cell counting (SCC) and infrared thermography. However, these generally rely on host biomarkers for mastitis rather than bacterial culture. This is due to the complex nature of the milk microbiome and slow culture-based methods increasing the time to treatment. New technology offers the promise of rapid and more complex diagnostics for on-farm diagnosis of mastitis and the detection of AMR. Here we aim to review the state-of-the-art and emerging approaches to detecting bovine mastitis, with a particular focus on novel on-farm bacterial and antibiotic resistance detection.

The detection and treatment of this disease can prove problematic and often time-consuming, leading to the inappropriate use and overuse of antibiotics in farming. Common diagnostic and AMR detection techniques, although standardised and highly sensitive, are often too laborious and require laboratory time and equipment. Meanwhile, the literature suggests that novel AMR detection systems need to be rapid, accessible, affordable and more information to be useful in aiding antibiotic stewardship. The overarching aim of this project is to address this problem by building on microfluidic technologies, combining traditional laboratory techniques with the use of microcapillary film (MCF) to carry out high-throughput investigations and perform testing that would usually be confined to a laboratory setting, on-farm. Of importance is the ability to carry out direct testing in milk from mastitis-infected cattle. The main objective was to produce a microfluidic test that can be used on-farm to detect bacteria growth and help indicate antibiotic resistance and therefore inform on a course of treatment. We describe a system by which we can detect the growth of bacteria within milk samples using resazurin dye in an assay imaging system.

The development of the PiRamid imaging system takes advantage of open-source computing allowing for automated imaging of a range of microbiological experiments including novel MCF and bacteria motility assays and conventional culture, as well as proving useful in chemistry analysis through the imaging of crystal formation. The effects of spoiled milk on a miniaturised

AST system were identified by the comparison of fresh, pasteurised milk and yoghurt as a simulated spoiled milk sample. It was also determined the effect on time to results by a milk sample that may contain both a pathogen of interest and commensal bacteria, indicating the potential for a period of false susceptibility in the presence of a high commensal bacterial load. It was also demonstrated that the resazurin-based assay can be adapted to accommodate the potential antimicrobial properties of resazurin dye, evaluating the use of lower concentrations of resazurin with reduced antimicrobial effect. The fluorescence intensities analysed allow for the detection of bacteria growth at lower concentrations of resazurin dye, with the time to detection of growth for all species more rapid than the standard laboratory culture method. These developments begin to pave the way for high-throughput, on-farm diagnosis of mastitis in cattle, reducing treatment time and eventually aiding in the choice of treatment antibiotics.

It is important to understand the challenges relating to the matrix effects of milk as a sample. Milk can become spoiled during storage, therefore a comparison of fresh, pasteurised milk and yoghurt as a simulated spoiled milk sample was made in a miniaturised AST system. Moreover, not only does a milk sample collected from a dairy farm potentially contain a pathogen of interest related to a mastitis infection, but it can also harbour commensal bacteria from the surrounding environment and the cow itself. It was hypothesised the potential for false susceptibility in the presence of a high commensal bacterial load. Investigation was required to evaluation time to results and the performance of this miniaturised AST system containing a simulated milk sample that may contain both a pathogen of interest and commensal bacteria, indicating. Sample dilution reduces the effects of milk on bacteria growth and observed MIC and reduces the interference of commensal bacteria on observed MIC and time to result in this system. It is possible to monitor Gram-negative bacterial growth colourimetrically even in the presence of milk and yoghurt (used to simulate spoiled milk samples), as long as this sample matrix was diluted 1:5 or more in growth medium. Growth detection kinetics using resazurin was not changed by milk at final concentrations of 20% or lower, but a significant delay was seen with yoghurt above 10%. The minimum inhibitory concentration (MIC) for ciprofloxacin and gentamicin was increased in the presence of higher concentrations of milk and yoghurt. When diluted to 1% all observed MIC were within range, indicating dilution may be sufficient to avoid milk matrix interfering with microfluidic AST. Finally, overlap is seen between mastitis-causing bacteria with commensal organisms proving difficulty to avoid false positive culture from the growth of commensals found in healthy milk. It is determined that a susceptible commensal would only be likely to mask the resistance of a pathogen in samples where the commensal cell density significantly exceeds the pathogen cell density.

Where previously the growth and AST of gram-negative bacteria has been validated in microcapillary film (MCF), it was important to understand if it can be used as a portable, microenvironment enabling the growth of *Streptococci*. Experimentally, 78% of *Streptococcus* strains were found to be resistant to resazurin dye at a concentration of ≥ 10µg/mL, where previously a concentration of 60µg/mL was shown to provide fluorescent intensity suitable for analysis of bacterial growth kinetics. Where the reduction in resazurin dye concentration may be required to allow Streptococcal growth, the difference in start and endpoint is also reduced, therefore making colourimetric detection unviable for Streptococcal growth. Compared to traditional microtitre plates (MTP) used for BMD, MCF has a decreased path length, decreasing the colour intensity of resazurin dye and decreasing the range between start and endpoint absorbance. Resazurin dye has been shown to be useful colorimetric indicator of the growth of bacteria in MCF. It was determined that haemolysis alone cannot indicate the growth of *Streptococcus spp*. in MCF where the difference between the absorbance calculated at 0h does not vary enough from the absorbance calculated at 20 h, where growth is present. Fluorometric detection with resazurin dye at 10µg/mL and 5µg/mL saw the difference between the highest fluorescent intensity and the background intensity as acceptable to detecting growth. In this system, it was determined that the maximum time to detection for *Streptococci* in this system was 9 h at a bacterial concentration of $2.5x10^4$ CFU/mL. All species had detectable growth between 5.5-9 h, considerably faster than the method deployed for traditional AST (> 24 hrs). In AST validation experiments, essential agreement for ampicillin minimum inhibitory concentration (MIC) across all the strains was 100% with all MCF MICs within ±1 doubling dilution of antibiotic. For benzylpenicillin, essential agreement was 80% within ±1 doubling dilution of antibiotic. Future resazurin-based microfluidic assays for the detection of bovine mastitis organisms require lower concentrations of resazurin dye and should potentially utilise fastidious Mueller Hinton broth (MHB-F) to incorporate the detection of *Streptococcus spp*. growth and AST detection.

Rapid AST in mastitis-infected milk still requires optimisation and careful consideration of the problems faced by such a complex media. With an extensive microbial community, we still see effects of false susceptibility, microbial depletion and pH changes that must be considered. Reducing time to testing and therefore time of storage will have a positive effect on the microbial community being tested and will provide the most accurate simulation of the pathogenic bacteria for AST. We have demonstrated reduced time to results sufficiently below common culture methods for AST of mastitis bacteria. Time to results < 10 h have been demonstrated, significantly faster than traditional culture methods of 24 – 48 h. There are still problems with

understanding the bacterial load of a mastitis infection in milk and currently insufficient consistent data on the bacteria inoculum found in milk of a mastitis infection to conclude a threshold for infection. Although the limit of the detection for a novel MCF assay is as low as 10^3 CFU/mL, complications surrounding the detection of false susceptibility in the presence of high loads of commensal bacteria may still arise. This device can provide a comprehensive screening for AMR in mastitis-infected milk. Moreover, if coupled with a rapid method of microbial identification, will prove a powerful tool for detecting AMR and informing effective antibiotic treatment of mastitis.

Thesis Flowchart

Chapter 1

- Determine the problems surrounding AMR in dairy cows
- Research the current advances in microfluidic microbiology and open-source hardware for use in antibiotic susceptibility testing (AST)

Chapter 2

- Determine how mastitis in cows is currently diagnosed and treated
- Evaluation of the current detection techniques and determination of what is required for on farm testing

Chapter 3

- Develop an open-source tool for automated imaging of microfluidic assays for mastitis detection and AST
- Validation of the device as a tool for mastitis detection for use in further experimental design

Chapter 4

- Determine the challenges of developing a rapid microfluidic AST for mastitis directly in cows' milk
- Conclude and validate optimal conditions for detection of mastitis bacteria in milk and determine AMR

Chapter 5

- Adapt the current method for microfluidic AST for detection and testing of fastidious organisms common to mastitis infections
- Determination of optimal conditions for the detection and AST of *Streptococcus spp.*

Chapter 6

- Evaluation of the challenges of developing an on-farm AMR detection tool for mastitis
- Overall conclusions of device development and the direction of future works/future work started

Supporting publications

Chapter 3 - The Development of a Tool to Automate Time-Lapse Imaging of Microfluidic Assays for Field Use

Long, M.M.; Diep, T.T.; Needs, S.H.; Ross, M.J.; Edwards, A.D. PiRamid: A compact Raspberry Pi imaging box to automate small-scale time-lapse digital analysis, suitable for laboratory and field use. *HardwareX* **2022**, *12*, e00377.

The extent of my contribution to this work:

- Conceptualisation
- Methodology
- Formal Analysis
- Investigation
- Writing – original draft and review and editing

Chapter 4 - Challenges in developing a rapid antibiotic susceptibility test for bovine mastitis directly from infected cow's milk

Long, M.M.; Needs, S.H.; Edwards, A.D. Dilution Reduces Sample Matrix Effects for Rapid, Direct, and Miniaturised Phenotypic Antibiotic Susceptibility Tests for Bovine Mastitis. *Antibiotics* **2023**, *12*, 1363. https://doi.org/10.3390/antibiotics12091363

The extent of my contribution to this work:

- Conceptualisation
- Methodology
- Formal Analysis
- Investigation
- Writing – original draft and review and editing

List of abbreviations

AMR	Antimicrobial resistance
AMU	Antimicrobial usage
AST	Antibiotic susceptibility testing
BMD	Broth microdilution
CFU	Colony forming unit
CLSI	Clinical and Laboratory Standards Institute
CMT	California milk test
DNA	Deoxyribonucleic acid
IRT	Infrared thermography
MCF	Micro capillary film
MDR	Multidrug-resistant
MHB	Mueller Hinton broth
MHB-F	Fastidious Mueller Hinton broth
MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistance *Staphylococcus aureus*
MTP	Microtitre plate
NSAID	Non-steroidal anti-inflammatory drugs
OIE	World Organisation for Animal Health
PCR	Polymerase chain reaction
PLA	Polylactic acid

POC	Point of care
PVOH	Polyvinyl alcohol
SCC	Somatic cell counting
UPS	Uninterruptible power supply
USST	Udder skin surface temperature
XDR	Extensively drug-resistant

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Chapter 1 – The issue of antimicrobial resistance in dairy cows and the potential for novel microfluidics and open-source hardware to be used for the detection of mastitis and antibiotic susceptibility testing (AST)

1.1 Antimicrobial resistance in cows

Antimicrobial resistance (AMR) is a major global public health risk with the widespread usage of antimicrobials considered the greatest enhancement of AMR (Zhang et al., 2019). Of particular importance is the growing effect of AMR on the dairy industry, and in turn, on human health. A dairy animal may pose an alarming risk of transmitting resistant bacterial strains to humans and the environment around them (Sharma et al., 2017), making it clear that industrial antibiotic use is a driver for a rise in 'superbugs' spreading across the world, including in humans. As well as becoming a serious threat to human health and public safety, treatment of our dairy herds becomes ever harder as antibiotics become less effective, with the overapplication of antibiotics playing a major role in the rise of resistant strains (Sharma et al., 2017; Woolhouse et al., 2015). AMR is common in mastitis pathogens making it difficult to treat and less susceptible to the more common antimicrobials used. Research has evidenced the great threat that AMR plays in the dairy industry. A bacterial infection of the udder that can infect around 40% of cattle in the UK (Donadeu et al., 2020), mastitis is an inflammatory reaction of the udder tissue. Historically it has been documented as a major economic burden on the dairy industry, affecting milk production and milk quality (Cheng et al., 2019; Hogeveen & Van Der Voort, 2017). Leukocytes are released into the mammary tissue in response to the bacteria. Some common mastitis-causing bacteria include *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella spp*., *Pseudomonas aeruginosa*, and *Streptococcus agalactiae*. When looking at the treatment of these pathogens, of highest importance is to consider some of the antibiotic classes that have been labelled as the most critically important antibiotics in dairy cattle include aminoglycosides, fluoroquinolones, 3rd, and 4th generation cephalosporins and tetracyclines, as identified by World Organisation for Animal Health (OIE), something to be considered when carrying antibiotic sensitivity tests on clinical mastitis samples and bacterial isolates.

1.2 The One Health problem and antimicrobial usage on dairy farms

The problem we are facing is the insurgence of AMR and the transfer of resistant bacterial strains from animals to humans, via direct contact with bacteria on infected sites or consumption of

infected dairy products. A major route of AMR from farm to human is through food production, in the case of unpasteurised dairy products, through milk and cheese products. Although not a major problem in the UK, globally, dairy products can often be left untreated, particularly where raw milk is purchased and consumed directly from farms. This is more often seen in less economically developed countries where producers and consumers of dairy products do not have access to appropriate methods of pasteurisation. Moreover, there is potential for AMR pathogens and commensal organisms to disseminate to humans via direct contact with animals, introducing public health problems (Call et al., 2008). When considering the different ecologies that act towards this link between human and animal biota, we see not only the movement of bacteria between species but also the mobile genetic elements and often the active ingredient of the drugs themselves (Woolhouse et al., 2015). With this in mind, further research is needed to find safer and more cost-effective alternative treatments, alongside discovering new antibiotics. It is well documented the effects that antimicrobial usage has on the accumulation of AMR-resistant genes in bacteria (Kawai et al., 2017). The complexities of AMR epidemiology are often exacerbated by the challenge of collecting comprehensive antimicrobial usage data in the UK (Graham et al., 2019). Research has shown the relationship between the usage of certain antimicrobial classes and the harbouring of resistant genes to those classes, with AMU-associated AMR a major public concern (Mesa Varona et al., 2020), with the most critical classes identified as fluoroquinolones and 3rd generation cephalosporins. Farms are complex systems; animals can become exposed to large quantities of antibiotics and act as a reservoir for resistance genes (Silbergeld & Dailey, 2017; Silbergeld et al., 2008; Xiong et al., 2018) Although responsibly restricted, concerns remain that antibiotic use in food animals increases the risk of selected resistance and the emergence of AMR bacteria. Indeed, although efforts to improve this are being made across the farming sector, better antimicrobial stewardship has proven difficult to achieve on farms (Argudin et al., 2017; Hoelzer et al., 2017), given the extensive role that antibiotics play in agriculture and the lack of acceptable alternatives. According the VARSS report 2023, since 2014, the total amount of highest priority critically important antibiotics (HP-CIAs) sold for use in food-producing animals in the UK has reduced by 84%, with a particular reduction seen in 3rd and 4th generation cephalosporins (94%). The Veterinary Medicines Directorate (VMD) is working to improve sector-led data collection to monitor antimicrobial usage (Veterinary Medicines Directorate, 2023). According to VARSS ruminant (including cattle) usage data is generally low and is not representative of the national sectors, inclusive of the dairy sector. Give that usage data is voluntary; it can often be difficult to determine the true amounts of antimicrobials used.

Resistance genes within bacteria can be introduced to the environment through the spread of waste and effluent on livestock and arable land (Graham et al., 2019), proving difficult to control and record in terms of the ecological spread of resistance. Moreover, the understanding of the epidemiology of AMR is often skewed by the lack of comprehensive antimicrobial usage (Kawai et al., 2017) data we collect in the UK, with AMU-associated AMR being a major public concern (Mesa Varona et al., 2020). AMR can be driven human behaviour within industrial agriculture through the worldwide negligence of recorded antibiotic usage to the extensive prescription of antibiotics for disease control and prevention (Bennani et al., 2020). Although there is headway being made in the UK to record this kind of data, antimicrobial sales often do not directly correlate to the volume of antimicrobials being used. Therefore, the surveillance of AMU is one of the suggested strategies for a better understanding of this consumption of antibiotics (Sharma et al., 2017).

Antimicrobial treatment is indispensable to balancing animal welfare and economic aspects, however, AMU in livestock production is an increasingly talked about subject. Reviews have discussed the urgent need for the reduction of AMU within the dairy industry and the implementation of evidence-based mastitis treatment concepts for the most effective method of decreasing AMU (Kromker & Leimbach, 2017). However, conventional bacteriological culture and PCR methods, although the most common and reliable tools used, do not allow for timely treatment decisions (Duarte et al., 2015; Kromker & Leimbach, 2017) and therefore less well informed treatment with antibiotics is still made.

1.3 Testing for mastitis and current antibiotic susceptibility testing (AST)

With the idea of safer and more cost-effective alternative treatments to tackling mastitis and antimicrobial resistance (AMR), we require a focus on how we test for mastitis on farms and how we inform decision-making on antibiotic therapy. When it comes to the diagnoses of mastitis, often tests are deployed that measure the level of somatic cells produced in response to a bacterial infection, rather than detecting an actual pathogen. An example of this is the California Milk Test, where milk from individual quarters can be tested to indicated somatic cell counts (SCC) above an expected value. Veterinary surgeons will implement the treatment of the disease with antibiotics based on high somatic cell counts, or clinical signs and symptoms either diagnosed in person or by information from the farmer. Often anti-inflammatory medication is carried out, particularly where clinical symptoms are displayed. For the reduction of antimicrobial usage, it is important to understand the microbial load of an infection and the types of bacteria you are dealing with to effectively inform treatment. This would indicate the type of

antimicrobial required, potentially the dosage, and reduce the use of ineffective antimicrobials where mastitis-causing bacteria may be resistant. It is also important to understand the safety of raw milk. Currently, the European Union legislation (Commission, 2021) stipulates that only milk from healthy animals can be used for human consumption with a specific limit of <500 CFU/mL^{-1} for *S. aureus* and <100 CFU/mL^{-1} for *E. coli* (coliforms) in raw milk for drinking (Council Directive 92/46/EEC) (Asif et al., 2020). At current, this is usually achieved by cell-culture-based methods and growth inhibition assays which are cumbersome, expensive and time-consuming (Ashraf et al., 2017; Jorgensen & Ferraro, 2009). Culture-based testing all have similar time to results, of 24-48 h, which although generally this has been accepted as fast enough to inform decision making, farmers would prefer to for less delay with a time to treatment from one milking to the next (Malcata et al., 2020), therefore within 12 h. This is why there has been greater interest in rapid on-farm testing. Alternatively, PCR (Ashraf et al., 2017) and ELISA techniques have been used for the detection of these pathogens in milk, but they require the availability of a dedicated laboratory facility, highly trained personnel, stable reagents, and multistep sample handling (Asif et al., 2020). Therefore, the introduction of on-farm, novel, phenotypic assays, capable of microbial detection, have begun to highlight the usefulness of using AST as a tool cow side to diagnosing mastitis, reducing the future over-usage of antimicrobials and in turn, aiding the reduction of antimicrobial resistance. Particularly, microfluidic-based phenotypic detection methods highly promising for the development of cheap, convenient, and efficient diagnostic tools that can be used to determine antibiotic susceptibility. Moreover, indicating the importance of reducing the time to treatment and making AST more accessible and cost-effective to farmers. Although most current point-of-care (POC) testing is often culture-based also, relying on modification of agar plate, petrifilm or tube-based culture systems, such as MastDecide (MastDecide, Quidee GmbH, Homberg, Germany), they provide positive results for mastitis in >14 h, compared to the conventional 24 – 48 h lab turn around. However, some POC AST mastitis detection and AST devices, such as Point-of-Cow and Mastatest, still require expensive and sophisticated lab consumables and equipment to improve and ease use, which will often not be accessible on farm and in low-budget areas.

1.4 Microfluidic devices for microbiology assays

Microfluidic devices are becoming more common in the detection of pathogenic species of bacteria, particularly within the development of novel assays. They often allow for faster detection of disease and require fewer and cheaper lab consumables compared to traditional methods. The idea of a lab-on-a-stick process has been noted as ideally suited to performing

assays on prokaryotic cells such as bacteria, for example, the phenotypical identification of bacteria and the quantitative measurement of antibiotic susceptibility (Reis et al., 2016). This may be extrapolated to the identification of mastitis-causing bacteria from infected milk. Moreover, it will aid in the prescription of the correct antibiotic for treatment by rapidly indicating antibiotic susceptibility in specific strains of bacteria, improving the quality of on-farm treatment. Overall, this method allows for bacterial assays to be performed outside the lab and into the field for more rapid results. Microfluidic devices have previously been deployed for bacterial assays. MicroPADs provide a paper-based analytical device requiring little to no specialised lab equipment and therefore a point-of-care alternative (Chen et al., 2024), proving cost-effective, stable and portable methods of detection.

With this idea of on-farm treatment and diagnostics in the field, we consider the use of smartphones and portable high-quality cameras to read functional cellular assays for measuring bacterial growth or death when combined with antibiotics (Donmez et al., 2020). The combination of current research into the use of low-cost microcapillary devices and smartphone imaging provides a potential tool for portable AMR detection. Research has shown that with the design of certain accessory attachments on smartphones, we can carry out analytical biosensing (Huang et al., 2018) due to the ever-increasing capabilities and power of conventional smartphones. When phenotypically identifying bacterial species and quantifying antibiotic susceptibility, assays rely on the identification of a colour change for a positive result. Smartphones can be used as colourimetric biosensors, used to directly capture images with colourimetric changes (Huang et al., 2018), allowing the production of growth curves of bacteria in different antibiotic conditions and the identification of the correct class and concentration of antibiotics to be administered to those cases of mastitis.

In recent years, high-throughput devices have been developed to provide greater flexibility in analysing the problem of AMR (Long et al., 2023; Needs et al., 2019; Needs et al., 2022). Where an automated process for microbiological techniques can improve sample time, the use of microfluidic techniques to detect pathogens and measure AMR can aid such speed required for the development of a point-of-treatment technique. This is especially important where mastitis bacterial identification is often carried out in a laborious and time-consuming manner within laboratories. Our group developed a simple, low-cost example microfluidic device that can be used to detect bacterial growth and measure multiple antimicrobial resistance profiles of bacteria using the metabolic sensitive dye, resazurin able to detect bacterial growth by a colour change from blue to pink. We build on the development of a simple, low-cost microfluidic device

that is capable of detecting bacterial growth through the use of a metabolic-sensitive dye, resazurin. Recent studies have demonstrated this simple and high-throughput technique and used it to identify uropathogenic species of bacteria and show that we can accurately predict bacterial concentrations (CFU/mL) and identify resistance strains throughout MIC (Needs, Donmez, et al., 2021; Needs, Osborn, et al., 2021).

1.5 Customisable automated imaging and time-lapse

Digital photography can be useful to many laboratory methods and assays for capturing and quantifying results and digitising processes. However, without sophisticated imaging systems, this can be time-consuming and is often not automated. The most common examples of these systems are usually expensive, limited to laboratory use, and almost always highly specialised to a certain technique i.e. gel electrophoresis. This unfortunately means that often these expensive benchtop devices are unsuitable to be carried outside of the lab and used at point-of-care. This particularly affects smaller-scale labs or research areas that do not regularly use standardised methods or are developing novel devices (Morris et al., 2022; Walzik et al., 2015). However, smartphones and digital camera modules are now often used as digital sensors for colour-based bioassays, to directly capture images to digitise colourimetric and fluorescence changes (Huang et al., 2018; Rodoplu et al., 2021), offering the potential for portable field imaging, particularly for microbiological assays.

The limited availability of expensive instrumentation means that flexibility is key (Walzik et al., 2015). This is also the case when labs are developing an array of different methods and requiring analysis. Therefore, customisable systems that can be modified to suit a user's needs are advantageous. A particular example is in the development of assays used for antibiotic susceptibility testing of mastitis. This process requires a range of distinct microbiological assays potentially more so than in human clinical samples, due to the diverse range of bacteria present. However, at their core, these methods all rely on the quantitation of colour changes or fluorescence emittance for the indication of microbial growth, from assays in microtitre plates to chromogenic agar in Petri dishes. This makes customisable equipment so important for low-funded and smaller-scale laboratory methods whereby a single device can have many uses. It also highlights the requirement for simplicity in the way we image and quantify assays outside of extensively funded large-scale labs.

With the recent move towards open-source hardware, innovations are showing that traditional large and expensive devices can be replaced with smaller, open-source and cheaper devices,

inclusive of 3D printed labware (Diep et al., 2022). This coupled with the ever-improving market of cheap digital cameras, offers a simplified approach to producing scientific imaging devices that remain sensitive yet cost-effective and simple to operate. Although a lot of high-quality digital photography is limited to manual operation, and often built-in firmware for automation can prove tricky to operate and produce unreliable time-lapse outcomes. Therefore, automation of these processes is of high importance, with the ability to greatly increase the number of time points and reduce the time needed for manual operation. This can reduce time in the field, making these types of imaging and microbiological methods more appealing, and allow for the preservation of data integrity (Ouyang et al., 2022), reducing human error. Specifically in advancing the field of reducing AMR, where an automated process for microbiological techniques can improve sample time (Morgans, 2020), the use of microfluidic technology to detect pathogens and measure AMR may offer a point-of-treatment technique. This might avoid mastitis bacterial identification in a laborious and time-consuming manner within laboratories (Chakraborty et al., 2019). However, these devices require a vast amount of validation against more traditional and currently deployed lab-based techniques to be comparable and would require acceptance by the veterinary and farming industries, who will ultimately be the end user of any on-farm open source microbial detection techniques.

1.6 Research aims and objectives

It was hypothesised that bovine mastitis and antimicrobial resistance can be detected directly in milk using a novel, microfluidics-based assay. Through this research, the aim was to develop a portable on-farm microfluidic test for the detection of mastitis bacteria and antimicrobial resistance (AMR) directly in milk.

An initial objective was to evaluate the current situation regarding the testing of mastitis on farms through the detection of pathogenic bacteria, with particular interest in AST. The current commercial on-farm devices capable of mastitis detection were critically reviewed and researched the novel devices and assays being developed by researchers to understand the direction in which on-farm AST is heading for mastitis in dairy cows.

A further objective was to produce an imaging device capable of portable time-lapse imaging outside of the lab to determine the growth kinetics of bacteria under different environments and carry out time-lapse imaging to determine growth kinetics of bacteria in milk using colourimetric and fluorescent detection. The ‘PiRamid’ was developed, a device that was designed to take advantage of low-cost 3D printing and simple Python script programming to produce a compact,

low-cost, high-performance system for automated laboratory imaging. With this, it has provided the capacity to take digital imaging of assays outside of the lab, a step closer to performing microfluidic mastitis detection and AST on a farm, as well as providing portable and flexible imaging for several other microbiological methods, from growth assays to conventional culture and bacteria motility.

Moreover, it was an objective to successfully design a test for the detection of bacteria in milk using microcapillary film (MCF) and to detect antimicrobial resistance in bacteria found in a milk sample directly using MCF. Some of the challenges that must be overcome to develop rapid and miniaturised devices with on-farm capabilities for detecting mastitis-causing pathogens were explored alongside the ability to carry out minimum inhibitory concentrations assays for the identification of resistant bacteria directly in milk samples, showing that milk can be directly tested using a growth indicator dye—resazurin in low-cost microfluidic devices capable of detecting bacterial growth and determining antibiotic resistance. This, in turn, fulfilled the objective of being able to accurately determine the presence of mastitis-causing bacteria in a milk sample using MCF.

With this, milk sample matrix interference on growth detection and direct antibiotic resistance testing was investigated, looking at how commensal bacterial counts in healthy samples could be expected to interfere with pathogen detection and testing if false susceptibility could be observed in a sample containing a mixed population of bacteria. This aided in the determination of the abundance of bacteria present in an average mastitis milk sample and the determination of the presence and abundance of commensal bacteria in milk without mastitis infection to decide appropriate sample dilution.

Alongside this, an objective was to develop a resazurin-based microfluidic AST assay for fastidious organisms in MCF. The inclusion of methodology for the growth of fastidious *Streptococci* was explored, looking at the challenges regarding the addition of fastidious-specific growth media and the possibility of the bactericidal effects of resazurin dye, leading to modification of dye concentration for detection. This research serves as an important step to improving the detection and AST of mastitis on farms, reducing the need for laboratory culture and minimising costs, providing faster and more accessible methods for improving the treatment of mastitis and, ultimately, antibiotic stewardship on dairy farms.

Finally, some initial investigation was undertaken to designing a system where Gram-discrimination can prevent the growth of certain types of bacteria to help determine species and

resistance mechanisms. However, this objective requires further work and development to finding an appropriate systems to determining whether organisms are Gram-negative or -positive and helping to aid the AST and therefore more informed antibiotic treatment of bacterial infection.

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Chapter 2 – Bacterial detection of bovine mastitis and antimicrobial resistance (AMR): A review of emerging technologies and challenges of on-farm testing compared to conventional methods

Abstract

Mastitis, often caused by a bacterial infection of the mammary gland, is one of the leading causes of poor cattle health and decreased milk yield on dairy farms. Antimicrobial resistance (AMR) is where a pathogen becomes resistant to a form of antimicrobial and will no longer respond to treatment. Specifically, bacteria can become resistant to antibiotics. In pathogenic bacteria, AMR is a major global health challenge in which animal, environmental and human health are all linked, and a one-health approach is needed to tackle rising AMR levels (Velazquez-Meza et al., 2022). Improved diagnostics for bacterial detection and antibiotic resistance detection is one area that can be targeted to improve antibiotic prescribing and treatment regimes, optimising the use of antimicrobials. However, rapid, on-farm tools are needed to effectively make antibiotic-prescribing decisions.

On-farm diagnostic tests are already in use, such as the California Mastitis Test (CMT), somatic cell counting (SCC) and infrared thermography. However, these generally rely on host biomarkers for mastitis rather than bacterial culture. This is due to the complex nature of the milk microbiome and slow culture-based methods increasing the time to treatment. New technology offers the promise of rapid and more complex diagnostics for on-farm diagnosis of mastitis and the detection of AMR. Here we aim to review the state-of-the-art and emerging approaches to detecting bovine mastitis, with a particular focus on novel on-farm bacterial and antibiotic resistance detection.

2.1 Introduction

Antimicrobial resistance (AMR) is a growing challenge that requires a ‘One Health’ approach, which is multi-sectoral, aiming to reduce AMR across the environment and human and animal health. The improper dissemination and management of antimicrobials and the presence of antibiotic-resistant bacteria within livestock through overuse or unsuitable treatment are of significant concern and pose potential risks to human health (Pandey et al., 2024). This dissemination can be ascribed to the ultimate overspill of antibiotic residues into the

environment and food systems from livestock production and waste (Figure 2.1), and the lack of effective antibiotic stewardship globally (Manyi-Loh et al., 2018). AMR The 'One Health' approach advocates for the collaboration of human and animal health sectors in addressing issues such as AMR. The One Health initiative within dairy farming is aiming to mitigate the spread of AMR with improved monitoring and surveillance of AMR on farms, through early and proper diagnosis of bacterial species, as well as the development of alternative mastitis treatments not reliant on antibiotics (Panchal et al., 2024). With this, comes an emphasis on the development of novel diagnostics and treatments, such as rapid testing, biosensors and phage therapy.

Mastitis is an inflammation of udder tissue and can be caused by a bacterial, fungal or mycoplasma infection with an incidence rate of ~40% of dairy cattle in the UK (Donadeu et al., 2020). The most common source of bovine mastitis is considered to be bacterial intra-mammary infection (Gonzalo et al., 1993) which can occur from two major sources, from cow to cow and from the environment (Cheng & Han, 2020). Historically it has been documented as a major economic burden on the dairy industry, affecting milk production and milk quality, often causing a loss of 1000L of milk per cow per infection and costing the UK dairy more than £200m every year (Cheng et al., 2019; Hogeveen & Van Der Voort, 2017). The dairy industry worldwide has adopted standards to control milk quality, particularly involving the levels of somatic cells, and microbial contaminants (Murphy et al., 2016), with mastitis pathogens also a potential public health concern due to their potential dissemination into human food systems. Where antibiotic-resistant bacteria are present in milk due to the overexposure of these bacteria to antibiotics, those that consume cow's milk products are inadvertently exposed to AMR genes downstream due to improper pasteurisation techniques, consumption of raw milk, or the survival of bacterial plasmid mediated AMR genes through the pasteurisation process (Taher et al., 2020).

The treatment for mastitis accounts for around 60-70% of antimicrobials that are currently administered by dairy farmers (Tommasoni et al., 2023; Abdi et al., 2018), making it a potential target for improved antibiotic stewardship. AMR is common in mastitis pathogens making it difficult to treat and is less susceptible to the more common antimicrobials used. Some studies have shown that up to 45.5% of bacterial strains isolated from mastitis cases are resistant to at least one common antimicrobial used in the treatment of mastitis (Holko et al., 2019), with others reporting specific resistance to beta-lactams and penicillin to be over 50% of isolated bacterial strains in countries such as Finland, Italy and Germany (Naranjo-Lucena & Slowey, 2023). Moreover, common mastitis causing bacteria, such as *Staphylococcus aureus*, are capable of forming biofilms in the udder protecting the bacteria from antibiotic treatments.

Therefore, even where commonly effective antibiotics are administered, they may not effectively penetrate the biofilm, leading to chronic or recurrent infections (Pedersen et al., 2021). Biofilm-producing bacteria are harder to treat with standard antibiotics because they require higher doses or alternative therapies, which are not always available or feasible (Sharma et al., 2023). While farm-related factors (such as hygiene, nutrition, and milking practices) certainly play a crucial role in the incidence of mastitis cases, and create difficulties in treating cases, the ability of certain bacteria to form biofilms and develop resistance, have made the management of mastitis in cows increasingly challenging. Thus, the connection between resistance and treatment difficulties cannot be overlooked, even when farming practices are considered.

2.1.1 Diagnosis of mastitis and detection of antimicrobial resistance (AMR)

Mastitis can be broadly categorised into two types of cases: clinical and subclinical. Clinical mastitis can be characterised by signs such as changes in milk appearance and composition, with samples often being discoloured or of a thick and coagulated consistency, with higher levels of somatic cells. Clinical symptoms of the cow will often include red and inflamed udders, poor milk yield and lethargy (Cheng & Han, 2020). Subclinical mastitis is the most common form of the disease found across farms worldwide (Pascu et al., 2022) and often does not exhibit any of these changes in milk or the cow, other than potentially a reduction in milk yield, and slight milk quality deterioration that can go unnoticed. This is particularly a problem whereby subclinical cases can often be 15 to 40 times more prevalent in cow herds than a case of clinical. Subclinical cases are often difficult to quantify, but can often account for greater financial loss in a herd than clinical cases (Cheng & Han, 2020). Subclinical mastitis however usually exhibits an increased somatic cell count (SCC) (Cheng & Han, 2020).

Clinical cases are often the most severe, with mortality rates as high as 34.5% in severe cases (Krebs et al., 2023). In severe cases, *E. coli* isolates are most commonly present, followed by *Streptococcus spp*. and *Klebsiella spp*. One study suggests that over half (50.6%) of species isolated from cases considered as severe, are *E. coli,* with milder cases exhibiting a greater deal of variability in the pathogens present (38.4% mixed infections) (Krebs et al., 2023). It is important to understand the epidemiology of specific bacteria to better understand the types of pathogens closely associated with infection and better inform therapy.

Major indications of mastitis infections include host response factors, such as immune response in the form of somatic cell counting, combined with signs and symptoms displayed by the infected animal. In particular, these indicators do not indicate the pathogenic bacteria causing

the infection, alongside antimicrobial susceptibility testing (AST) to decide whether the bacteria may harbour any resistance to antimicrobial treatments. This results in both clinical and subclinical cases treated empirically with antibiotics, often without information on the type of bacteria present and their resistance profiles to the most common first-line antibiotics. This emphasises the more responsible use of antibiotics in dairy farming and the importance of a One Health initiative in developing new and more informative diagnosis techniques for the disease.

The complexities of AMR epidemiology are often exacerbated by the challenge of collecting comprehensive antimicrobial usage (Kawai et al., 2017) data in the UK (Graham et al., 2019). Research has shown there is a conclusive relationship between the usage of certain antimicrobial classes and the harbouring of resistant genes to those classes (Kawai et al., 2017). Antimicrobial usage-associated AMR is a major public concern (Mesa Varona et al., 2020), with the most critically important classes identified by the WHO as fluoroquinolones and 3rd and 4th generation cephalosporins. The World Organisation for Animal Health (OIE) goes further to detail Veterinary Critically Important Antimicrobial Agents (VCIA) which include aminoglycosides, macrolides, fluoroquinolones, 3rd, and 4th generation cephalosporins, sulphonamides and pencillins. The cross over of critically important antibiotics for both human and animal health emphasises the need for a One Health approach to reducing antimicrobial usage. Although responsibly restricted by veterinarians and government guidance, concerns remain that antibiotic use in food animals increases the risk of selected resistance and the emergence of AMR bacteria. Farms are complex environments; animals can become exposed to large quantities of antibiotics through activities to control and prevent disease (Bennani et al., 2020) and act as a reservoir for resistance genes (Xiong et al., 2018; Silbergeld & Dailey, 2017; Silbergeld et al., 2008). Efforts to improve antibiotic usage are being made across the farming sector. According the VARSS report 2023, since 2014, the total amount of highest priority critically important antibiotics (HP-CIAs) sold for use in food-producing animals in the UK has reduced by 84%, with a particular reduction seen in 3rd and 4th generation cephalosporins (94%). Although progress has been seen in the UK, better antimicrobial stewardship is difficult to achieve on farms (Argudin et al., 2017; Hoelzer et al., 2017), given the extensive role that antibiotics play in agriculture and the lack of acceptable antimicrobial alternatives. According to VARSS ruminant (including cattle) usage data is generally low and is not representative of the national sectors, inclusive of the dairy sector. Give that usage data is voluntary; it can often be difficult to determine the true amounts of antimicrobials used and the Veterinary Medicines Directorate (VMD) is working to improve sector-led data collection to monitor antimicrobial usage (Veterinary Medicines Directorate, 2023). Finally, The VARSS report also provides an idea

of the types of resistance we see in common mastitis causing bacteria such as *E.coli*. 24% of *E.coli* isolates collected from cattle samples were resistant to four or more individual antibiotics. Resistant genes within bacteria can be introduced to the environment through the spread of waste and effluent on arable land and land used to house livestock (Graham et al., 2019), making it difficult to control and record in terms of the ecological spread of resistance.

Moreover, the understanding of the epidemiology of AMR is often skewed by the lack of comprehensive antimicrobial usage (Kawai et al., 2017) data collected in the UK (Kawai et al., 2017), with AMU-associated AMR being a major public concern (Mesa Varona et al., 2020). Although the VARSS reports provides a comprehensive idea of the types and numbers of antibiotics sold in the UK, this doesn't necessarily correlate to the amount of antibiotics actually used (AMU). The surveillance of AMU is one of the suggested strategies for a better understanding of this consumption of antibiotics (Sharma et al., 2017).

Antimicrobial treatment is indispensable to balancing animal welfare and economic aspects, however, with AMR of particular interest to both human and animal welfare, AMU in livestock production is an increasingly talked about subject. Reviews have discussed the urgent need for the reduction of AMU within the dairy industry and the implementation of evidence-based mastitis treatment concepts for the most effective method of decreasing AMU (Kromker & Leimbach, 2017). However, conventional bacteriological culture and PCR methods, although the most common and reliable tools used to identify mastitis-causing pathogens, do not allow for timely treatment decisions (Kromker & Leimbach, 2017; Duarte et al., 2015). The detection of resistance bacterial strains in mastitis cases before treatment would allow for a more informed course of treatment with antimicrobials, understanding what kinds of antimicrobials are not going provide effective treatment. This not only improves treatment but may potentially reduce recovery periods and reduce the use of ineffective antimicrobials.

2.1.2 The epidemiology of mastitis bacteria and antimicrobial resistance in dairy cattle

E. coli is considered the most common Gram-negative bacteria that causes mastitis infection in cattle, due to the predominant isolation of this bacteria from mastitis infections (Ali et al., 2017; Kempf et al., 2016). It is the most common cause of toxic mastitis that results in high rates of culling (Timofte et al., 2014), leading to great economic losses to farmers. As a way of avoiding the severe economic losses globally in dairy cows as a result of culling, mastitis caused by this bacterial infection is often treated with potent antibiotics such as extended-spectrum cephalosporins, a trend that may be associated with the rise of bacterial resistance to these

antibiotic classes, particularly in species such as *E. coli* (Ali et al., 2017; Ajiboye et al., 2009). Some studies have shown strains of *E. coli* identified in mastitis clinical samples to be most highly sensitive to azithromycin and chloramphenicol, whilst being most resistant to common farm antibiotics such as penicillins (52.17% of isolates), ampicillin (70.65% of isolates) and tetracyclines (44.2% of isolates), often carrying plasmid-mediated resistance genes such as *ampC,* with 92.85% prevalence and *tetA, tetC* (Rana et al., 2022; Cheng et al., 2019; Fazel et al., 2019; Karanvir et al., 2018).

Alongside *E. coli*, *Pseudomonas aeruginosa* is reported to be among the three most prevalent that is cultured from bovine milk samples (Bannerman et al., 2005). It can easily acquire resistance to various antimicrobials through genetic mutation leading to the hyperproduction of cephalosporinases, through the overproduction of β-lactamases caused by mutations in a β-lactamase inducible gene *ampC* (Pachori et al., 2019; Pang et al., 2019). Intrinsically multidrug-resistant (MDR), but clinically important in human health and transfer of resistance to humans (Kelly & Wilson, 2016; Ohnishi et al., 2011), *Pseudomonas spp*. can cause opportunistic infections and nosocomial outbreaks within human hospitals, providing a One Health concern through the potential transmission of resistance *Pseudomonas spp*. from mastitis infected cows, to humans. This is of concern in countries where humans will consume raw milk. *Pseudomonas spp*. being psychotropic, their growth isn't fully inhibited in refrigerator temperatures, with species having been isolated from refrigerated raw milk, yoghurt and cheese products (Atia et al., 2023). Where milk is pasteurised, the transmission of *Pseudomonas* from milk to humans is rare. However, antibiotic resistant *Pseudomonas* can be spread from infected udders to the environment through contaminated areas like soiled bedding, manure, water, and milking equipment, potentially infecting other animals, including companion animals where *P. aeruginosa* has been demonstrated to be etiological cause of infections such as otitis, dermatitis, and urinary tract infection (Schauer et al., 2021). In cattle*, Pseudomonas aeruginosa* strains that cause mastitis often possess type III secretion systems that increase the presence of somatic cells upon infection (Park et al., 2014), however, the resultant symptoms caused by *Pseudomonas spp*. infection are often relatively mild and subclinical and therefore difficult to detect early on (Ohnishi et al., 2011).

Of the causative agents of mastitis, *Streptococcus uberis (S. uberis)*, is often considered the most commonly isolated species of Gram-positive bacteria from milk samples (Timofte et al., 2014). This bacteria is shown to have good susceptibility to penicillin, often the first line of antibiotic defence for intramammary treatment (Kappeli et al., 2019). This is particularly

important when considering we see high levels of resistance to penicillin in *E. coli* isolates from mastitis infections (Rana et al., 2022), indicating the importance of identifying the infection-causing pathogen before effective treatment. However, with the continued extensive use of this class of antibiotic in both human and veterinary medicine, and a rise in the number of other resistant strains, worldwide surveillance programmes are employed to reduce the risk of these isolates entering the food chain, either through poor milking hygiene or through environmental factors. Although pasteurisation is deployed for the destruction of pathogenic microorganisms, pasteurisation often fails to decrease the prevalence of plasmid-mediated AMR genes (Taher et al., 2020).The use of this continued surveillance has shown increased resistance of these bacteria to beta-lactam antibiotics such as Penicillin G, a first-line treatment for bovine mastitis, through sequence-based prediction using penicillin-binding protein transpeptidase signatures (Haenni et al., 2018). Although most studies show isolates that are still considered susceptible to beta-lactam antibiotics, this slow-growing but clear shift towards decreased susceptibility poses a threat to future antibiotic treatment of mastitis caused by *Streptococcus spp*. infection (Kappeli et al., 2019; Haenni et al., 2018). *Staphylococcus aureus* is a frequent and contagious mastitis bacterial pathogen (Abdi et al., 2018; Sheet et al., 2016) and has the potential for transmission into human medicine with the detection of methicillin-resistant *Staphylococcus aureus* (MRSA) in raw milk and milk products (Titouche et al., 2022). This is due to its ability to gain MDR alongside resistance to last-resort antimicrobials such as methicillin and vancomycin, with the potential to become XDR (extensively drug-resistant) (Abdi et al., 2018). Studies in recent years have indicated the recommended priorities for research to be centred around improved diagnostic techniques, particularly early detection, and intervention (Rainard et al., 2018). Organisms most associated with bacterial mastitis are summarised in Table 2.1.

Table 2.1 Summary of potential mastitis-causing pathogenic bacteria.

Bacteria	Types of infections	Types of treatment	Microbiological testing
Escherichia coli	Toxic mastitis – high rates of culling (Timofte et al., 2014)	Non-steroidal anti-inflammatory drugs. Extended spectrum cephalosporins Azithromycin, chloramphenicol	Easily grown on most media. Identified through disease-specific Chromagar and MacConkey's Agar

		Great resistance to non-ESC β-lactams, tetracyclines and amoxicillin (Cheng et al., 2019; Karanvir et al., 2018)	
Pseudomonas aeruginosa	Often mild and subclinical (Ohnishi et al., 2011).	Intrinsically multidrug-resistant (MDR) – particularly when spread to humans (cystic fibrosis) (Kelly & Wilson, 2016; Ohnishi et al., 2011)	Easily grown on most media. Often difficult to isolate due to the swarming effect on semi-solid media and difficulty in inhibiting
Streptococcus uberis	Mild to severe clinical cases as well as some subclinical	Penicillin – has good susceptibility (Kappeli et al., 2019) however, some resistant strains becoming apparent. Growing susceptibility to beta-lactam- antibiotics (Kappeli et al., 2019; Haenni et al., 2018)	Fastidious organism – requires a specific environment for isolation and enumeration
Streptococcus agalactiae	Mild to severe clinical cases as well as some subclinical	Penicillin – has good susceptibility (Kappeli et al., 2019) however, some resistant strains becoming apparent. Growing susceptibility to beta-lactam- antibiotics (Kappeli et al., 2019; Haenni et al., 2018)	Fastidious organism – requires a specific environment for isolation and enumeration.
Staphylococcus aureus	Majorly contagious clinical cases (One Health problem) (Abdi et al., 2018; Sheet et al., 2016). Reduction in prevalence in the UK.	Resistance to beta-lactam-antibiotics. Intrinsically MDR and resistant to last-resort drugs such as methicillin and vancomycin (Abdi et al., 2018)	Easily grown on most media, Identified on Baird Parkers agar or disease-specific Chromagar.

2.2 Current mastitis detection techniques

Current techniques deployed to identify the presence of pathogenic bacteria are often expensive and time-consuming, ideally requiring specialist laboratory equipment and facilities for the aseptic processing of samples. When considering the detection of pathogens for the optimisation of treatment, time-to-detection becomes a key limitation (Ferone et al., 2020). Commercially available mastitis diagnostics are summarised in Table 2.2 and further discussed in Section 3.1

Table 2.2 Current mastitis detection techniques deployed within dairy farming

Type of testing	Commercial products	Time to mastitis diagnosis	Characteristics and methods
Somatic Cell Counting (SCC)	DeLaval™ cell counter Fossomatic™ PortaCheck™ - PortaSCC Somaticell® CSS	DeLaval™ cell counter: 45 sec Fossomatic™: up to 600 test results/h Portacheck - PortaSCC: 45min; quick test 5min Somaticell® CSS: < 3min	DeLaval™ cell counter: SCC measure optically by taking an image of cells stained with a DNA-specific fluorescent marker. Fossomatic™: Sensors detect fluorescence signals from individual somatic cells. High throughput and automated method testing 100-600 samples/h. This is the most commonly used somatic cell counting device commercially by milk processing companies for cows and ewes milk due to high throughput. PortaCheck™ PortaSCC: Test strips; manual, low throughput, add-on drop of milk and colour change indicate a range in SCC. Quick test comparison to colour chart; longer test semi-quantitative with a reader Somaticell® CSS: 2ml of milk added to a graduate vial with the same volume of reagent and inverted. Its maximum and minimum values are 1,970,000 cells/mL^{-1} and 69,000 cells/mL^{-1} respectively.

Infrared Technology (Thermal Imaging Cameras)	Commercially available thermal imaging cameras are used but usually utilised by veterinarians or research scientists with expert set-up and image analysis (Zhang et al., 2023)	Immediate diagnosis with veterinary expertise	Thermal imaging to look at the udder surface temperature. (Wang et al., 2022) Positive correlation between udder skin surface temperature (USST) and somatic cell count (SCC), with USST increasing with SCC (Machado et al., 2021)
California Milk Test	Commercially available and used regularly on farms. Used globally largely in the UK and Canada as a simple, cow-side indicator. Also widely deployed in less-economical developed countries.	Immediate indication of colour change	Sodium lauryl sulphate (SLS) ruptures leukocytes DNA is released from their nuclei which results in gel formation. The formation of a gel indicates a leucocyte count of between 200,000 and 5,000,000, meanwhile, the thickness of the gel indicates the severity of inflammation. This test provides a positive or negative outcome based on the expected range of leukocytes found in a positive mastitis case.
Conventional culture	Not commercially available; requires laboratory expertise	18-24 h	Culture of bacteria on suitable agar plating; consider Chromagar for identification of specific species of bacteria and Plate Count Agar for total enumeration of bacteria in the milk sample. Diluted milk sample inoculated onto an agar plate and incubated overnight at 37°C. Bacteria enumerated between 18 and 24 h.

2.2.1 Somatic Cell Detection

Somatic cell counts (SCC) are one of the main techniques deployed to diagnose mastitis. SCC results can vary depending on whether the mastitis is caused by bacterial infection, what type of bacteria are present, and the age and lactation stage of the cow. However, the presence and type

of bacteria are the principal factors of variation in the severity of the results (Sumon et al., 2020) Often, the distribution and concentration of bacteria in a milk sample can differ significantly from the bacterial population found upon culture, with low SCCs often correlating with a high presence of bacteria (Hisira et al., 2023). Leukocytes are released from the blood into the mammary tissue in response to the bacteria during infection and therefore SCC has been consistently used as a measure for the regulation of milk quality both domestically and internationally. According to UK legislation, the conditions for raw cow's milk in the UK are that SCC remains below 400,000 cells/mL (Hisira et al., 2023). This also falls in line with the current European Union Health Certification Program, which also specifies a SCC threshold of 400,000 cells/mL for companies exporting products into the EU. The legislation suggests that this correlates with an average bacterial plate count of >100,000 CFU/mL.

There are a range of commercially available SCC tests that are deployed across the dairy industry. Some of these tests require significant investment in equipment but are high throughput and highly automated, while others are single sample, manual tests that can be done by a single person on a farm with minimal cost and no investment in large-scale automation. Some examples include the DeLaval™ cell counter (DCC, DeLaval International AB, Tumba, Sweden) and the Fossomatic (Fossomatic TM FC, Denmark), which are cell counters that accurately measure the herd, cow, or cow quarter SCC. These instruments automatically count the somatic cells optically using fluorescent markers. The DeLaval cell counter is a portable optical device that can be filled with a milk sample and a reagent, propidium iodide. In contact with somatic cells, the reagent provides a fluorescent signal that is detected by the device. Cell density is determined by fluorescence intensity. Although rapid, the DeLaval cell counter is often costly and requires expertise to operate. Moreover, the device requires calibration, and without so may yield incorrect results. The Fossomatic determines SCC in a similar way, whereby the reagent, ethidium bromide, from a fluorescent complex with the nuclear DNA of each cell, emits a fluorescence intensity reading (Gonzalo et al., 1993). Some studies go as far as to use the Fossomatic as a 'gold standard' for the comparison of other SCC devices (Hisira et al., 2023). Similarly, although more accurate, the Fossomatic is often found in large scale milk production companies; it requires expensive equipment and expertise to use and therefore is not suitable for on-farm usage. Some on-farm SCC tests include Somaticell® CSS (Somaticell, IDEXX Laboratories Inc. Maine, USA), which is a counting device that has been deemed comparable to electronic counting performed in laboratories. Somaticell® CSS has 41 outcomes for milk SCC read from the presence of a coagulated milk sample left in the vial at the end of the testing process. The value directly correlates with a SCC (Langoni et al., 2012). The device is practical

and efficient giving you time to results within minutes and also has low running costs. However, the result can be easily influence by a number of factors including milk temperature, fat content and sample handling, and the semi-quantitative interpretable results introduces human error and bias. Finally, Portacheck – which can also detect subclinical ketosis as well as mastitis, and PortaSCC Quick Test for Cows (Porta SCC, PortaCheck, Inc., White City, USA), where the test involves a reaction with milk which causes the esterase-containing test strip to change based on the level of somatic cells found in the milk sample. This then relates to a colour chart with varying degrees of severity. The intensity of the resulting blue colour directly correlates to the SCC in the milk. Results are moderately rapid when compared to conventional culture, with results in uder 45 minutes and a low cost per test, however, the test has been seen to have reduced sensitivity with higher fat content milk and struggles to differentiate immune-activate white blood cells from other cells in the milk, lacking specificity.

The California milk test (CMT) is a common, low-cost, and rapid determination of mastitis and is common in diagnosing sub-clinical cases. Milk is collected usually in a four-sectioned paddle, usually representing the four quarters from which the milk has been drawn. Sodium lauryl sulphate (SLS) is added to the milk, rupturing somatic cells in the sample including leukocytes and thus deoxyribonucleic acid (Kupczynski et al., 2023) is released from their nuclei. This results in a gel formation indicating a leucocyte count of between 200,000 and 5,000,000. Meanwhile, the thickness of the gel indicates an increased number of cells in the sample and therefore the severity of inflammation. Studies have demonstrated a significant correlation between SCC and CMT, whereby an elevation in SCC corresponded with positive CMT results or higher scores. The use of the CMT to identify infected quarters has been extensively validated in cows that were not in early lactation. However, when considering the viability of CMT testing as a form of diagnosis, the CMT has been observed as less accurate (87.4–90.8%), less sensitive and specific than other tests like SCC which has shown a sensitivity of 94.9–99.5% and specificity of 48.1–87.1%. Although the CMT provides positive or negative results for mastitis infection, it fails to provide an indication of the type of infection at hand, the pathogen that may be causing the infection, and therefore a suggestion of the type of antimicrobial may be key in fighting the infection. It provides a suitable first line of diagnosis but is limited in its use for treatment. Moreover, the results is only semi quantitative and is subjective it it's interpretation.

SCC provides a key indication of the presence, and the severity of mastitis cases, and the presence of somatic cells is used worldwide to determine the quality of a milk sample and the presence of mastitis. SCC is also a substantially quicker diagnostic than cell culture, with testing

being carried out on farms with almost immediate results and therefore rapid decision making, in comparison to 24 h lead time for traditional culture. However, SCC lacks key information about the type of bacteria present. Therefore, for more informed antimicrobial treatment, culture methods and standard AST methods would need to be carried out alongside.

2.2.2 Infrared Technology

Infrared thermography (IRT) can be used for detecting inflammation, an indicator for mastitis (Machado et al., 2021), which may offer an alternative to the California mastitis test. This method may also offer additional benefits such as differentiating clinical mastitis from subclinical mastitis cases. IRT detection methods are based on infrared thermal images, particularly looking at udder skin surface temperature (USST). IRT is often used for the prediction of ovulation, assessment of general animal welfare, and inflammation from various infections and lameness. With mastitis being an inflammatory response to a bacterial infection, the release of prostaglandins can increase the udder surface temperature, therefore triggering and response in IRT results. These studies have shown a positive correlation between USST and SCC, with USST increasing with SCC, with little effect from external environmental factors (Wang et al., 2022). Overall, IRT can provide a useful diagnostic tool for mastitis. IRT kits can be a costly investment, however, devices are reusable and can be used to screen an entire herd regularly over many years. Time to results is instantaneous, meaning diagnosis is quick and therefore time to treatment can be significantly reduced. Moreover, advancing technology means that almost anybody can use and receive the results from IRT straight to a smartphone. IRT however, does not provide any microbiological information. What cannot be deduced is the type of infection present and therefore, cannot inform the course of treatment that should be selected. Potentially, this may harm antimicrobial stewardship on farms, leading to the treatment of other non-infections inflammatory udder conditions with antibiotics.

2.2.3 Conventional Microbiology: culture and AST

Conventional methods of culturing are still considered the gold standard, and any new methodological developments in the detection of bacteria are likely to require validation against these quality control methods. Generally, these methods are inexpensive and reliable at detecting pathogens and often genus-level identification (selective media, chromogenic media, appearance and morphology), however, in conjunction with biochemical testing, it becomes very time-consuming. Instead, laboratories are turning to more sophisticated techniques of identifying pathogen bacteria, such as Matrix-assisted laser desorption/ionization time-of-flight

mass spectrometry (MALDI-TOF MS), which proves to be more rapid and reliable with an ever-expanding database of common mastitis-causing organisms (Nonnemann et al., 2019). This can provide rapid identification of previously cultured bacteria from infected milk samples, however, has also been proven useful in identification from direct testing of infected milk (Barreiro et al., 2018). When considering these techniques as a point-of-treatment method of identifying disease-causing bacteria, particularly in a farming environment, they become less useful where rapid treatment is so important with accessibility on the farm key in delivering this. When identifying mastitis-causing bacteria, the majority of positive cases are detected by visually inspecting milk and identifying symptomatic cases, meanwhile, the cause of infection is confirmed by expensive laboratory testing by milk culture, often identified as adopting such approach for routine testing of cows (Godden et al., 2017). Conventional culture-based AST allows for the determination of the resistance profile of a given bacterium. AST is usually performed using phenotypic methods of broth microdilution or standardised disk diffusion with reference methods based on the Clinical and Laboratory Standards Institute (CLSI) guidelines. AST can be important in determining the appropriate cause of treatment for a pathogenic bacteria. Although these conventional AST methods are considered the gold standard for testing bacteria susceptibility, they require expertise, and laborious lab-based methodology, and can be time-consuming, requiring lengthy culture times of up to 24 h. The combination of conventional bacterial culture and AST is powerful at determining the pathogenic cause of an infection and informing antimicrobial treatment by determining the susceptibility status of a given antimicrobial. However, these methods are greatly restricted to a laboratory setting, require microbiology expertise to deliver, and will often not be carried out in time to inform the timely treatment of mastitis.

2.3 Emerging technologies for the on-farm detection of mastitis-causing bacteria

2.3.1 The potential of on-farm bacterial detection

It is well understood that an accurate diagnosis at the point-of-care (POC) or point-of-decision-making can inform more targeted use of antimicrobials and limit antimicrobial use where there is no demonstrable clinical benefit (Zadoks et al., 2023; Singh et al., 2022; van der Pol et al., 2022). With this, when considering the short-term financial burden of mastitis on dairy farmers, there is an increasing recognition that the antibacterial treatment of mastitis and the effects that that has on the ever-growing selection for AMR do not outweigh the risks of not using antibacterial treatment. Therefore, farmers and veterinarians can seek to treat cattle before formal cell culture

and AST, with clinical indications leading to treatment. The occurrence of AMR in this case is likely to be established in the target pathogen and spread to the surrounding environmental organisms (Zadoks et al., 2023). Indeed, the use of on-farm bacterial diagnostics and AST to determine the presence of bacteria and inform treatment decisions is feasible with the potential to reduce antimicrobial use without compromising animal health and welfare, milk quality or yield, and to be economically viable, depending on circumstances that include pathogen profiles.

2.3.2 Bacterial detection and mastitis

All previous on-farm diagnostic methods are not capable of determining bacterial infection and indicate host factors of inflammation. However, bacterial culture-based tests are becoming available to determine the class of bacteria causing the infection allowing better prescription decisions.

Phenotypic rapid testing kits, such as MastDecide® (MastDecide, Quidee GmbH, Homberg, Germany), rapidly detect mastitis-causing pathogens (Morgans, 2020). The test contains a direct sample tube test system providing positive results in 8-14 h (Leimbach & Kromker, 2018) in comparison to conventional agar growth of 24–48 h. Milk from a suspected infection is added directly to two test tubes per test. The test provides both diagnostic information and information to inform on a course of treatment. Upon both tubes turning a pink colour, there is no bacterial growth. One tube turning pink means that the bacteria present is Gram-negative. MastDecide® suggest that due to this, the course of treatment be non-steroidal anti-inflammatory drugs (NSAID) instead of antibiotics, therefore helping to inform treatment. If both tubes turn white, there is a Gram-positive infection and therefore they suggest treatment with both NSAID and a course of antibiotics. The idea is that potentially this is reducing the number of antibiotic-treated cases by informing whether antibiotics are appropriate for the type of pathogen present. MastDecide ® enables evidence-based treatment decisions, improving antimicrobial stewardship, whilst no requiring a lab and providing results on-farm. However, the test is not able to indicate specific pathogens, only providing a recommendation on pathogen class, meanwhile although cheaper than lab diagnostics, it may be cost prohibitive, and the per-test cost is much higher than more basic SCC tests or CMT.

BACT by Point of Cow (FluimediX APS), relies on a disposable plastic chip where a single drop of milk can be applied. The chip can indicate bacterial growth and differentiate between Gram-positive and Gram-negative therefore helping to indicate a course of antibiotic treatment. It is

noted for use as a routine decision support tool, with farmers' findings used to help the preparation of treatment plans by veterinarians. The chip relies on a microfluidic technology with integrated reagents for Gram- identification, with results within 14-16 h upon incubation at 37°C. The accuracy for detection of bacterial growth and Gram-positive growth was 91 and 89%, respectively. The BACT test could provide an accurate and relatively fast decision tool for farmers to aid in antimicrobial treatment decisions in cases of clinical mastitis. This test support antimicrobial stewardship by indicating which bacteria causes the infection and potentially removing the need for antibiotic treatment. However, it has limited pathogen specificity, can only provide results in a little as 14-16 h and has shown to incorrectly classify up to 7.8% of cases tested.

A further on-farm test that has been developed is known as Mastatest®. Their unique system can not only identify the species of bacteria present in a milk sample but also indicate which antibiotic the strain will be most sensitive to, helping to target antibiotic usage (Jones et al., 2019). A collected milk sample is poured into a disposable cartridge containing multiple wells containing different antibiotics. The cartridge is incubated, and a colour change is automatically detected identifying bacterial growth in the different conditions resulting in bacterial identification and antibiotic resistance. The system contains two types of 'cartridges', for both clinical and subclinical cases. The clinical 'cartridge' will indicate the presence of bacteria as well as species, categorised as *E. coli*, *Klebsiella*, *Streptococcus uberis*, *Streptococcus dysgalactiae*, *Streptococcus species*, *Staphylococcus aureus*, coagulase-negative Staphylococci (non-*aureus* Staphylococci) and other Gram-positive bacteria. The test can identify organism IDs and results are automatically emailed within 24 h. Studies have shown that the Mastatest® system is capable of reliably identifying the presence of *S. aureus* in quarter milk and pooled cow-level milk from cows with an SCC ≥150,000 cells/mL. It can also reliably detect the presence of other coagulase-negative Staphylococci in pooled milk samples and can positively identify cows with as low as 10 CFU/ 10µl of bacteria in their milk (1 x 10^3 CFU/mL). Compared to gold standard culture methods, the system is rapid with results being returned within 24 h of the 'cartridge' being filled (Saila et al., 2023). However, it can be noted that most other conventional plate-based on-farm bacterial diagnostic systems can also provide reliable and easily interpretable results within the same time frame. Moreover, although Mastatest® can accurately predict pathogen type and the potential use of some antimicrobials, it fails to recognise the importance of *in vivo* antimicrobial performance, therefore veterinarian advice would need to be considered in conjunction with the results. Finally the initial investment of the

'cartridge' reader can be a barrier for some, though potential savings in treatment cost may offset this over time.

There has been some move towards the use of microfluidics in the detection of somatic cells, with the production of spectroscopic microfluidic chips for mastitis detection in raw milk (Phiphattanaphiphop et al., 2023). These devices were designed to screen somatic cells and identify normal, sub-clinical, and clinical mastitis within seconds. A staining process is added to identify somatic cells using single-cell process analysis, with a portable spectrophotometer used to identify the infection status of the milk by fluorescence detection. Table 2.3 summarises the current novel on-farm bacteria detection and AST tests.

Table 2.3 Novel on-farm bacteria detection and AST tests. There are a number of new rapid phenotypic testing kits for use on farm.

Name of kit (and company)	Characteristics and methods	Time to mastitis diagnosis	Identification of pathogens	Detection of antibiotic resistance
MastDecide® - MastDecide, Quidee GmbH	Rapid, direct sample, tube test system where milk is added directly to two test tubes containing reagents. Test tubes to be incubated.	14 h	Identifies if pathogens are Gram-negative or Gram-positive	No
BACT Point-of-Cow® - FluimediX	A disposable plastic chip where a single drop of milk can be applied. The chip can differentiate between Gram-positive and Gram-negative. The chip relies on a microfluidic technology with integrated reagents for Gram- identification, with results within 14-16 h upon incubation at 37°C.	14-16 h	Identifies if pathogens are Gram-negative or Gram-positive	No

Mastatest® - Mastaplex	A collected milk sample is poured into a disposable cartridge containing multiple wells containing different antibiotics. The cartridge is incubated, and a colour change is automatically detected identifying bacterial growth in the different conditions resulting in bacterial identification and antibiotic resistance. The systems contain two types of 'cartridges', for both clinical and subclinical cases.	≤ 24 h	*E. coli*, *Klebsiella*, *Streptococcus uberis*, *Streptococcus dysgalactiae*, *Streptococcus species*, *Staphylococcus aureus*, coagulase-negative Staphylococci (non-*aureus* Staphylococci) and other Gram-positive bacteria	Yes. Phenotypic colour change. Each Mastatest® cartridge can provide MIC data for three antibiotics. Antibiotics are dried in wells of the cartridge.
VétoSlide – Vetoquinol	VétoSlide is a dual slide set within a test tube containing two types of culture media. The collected milk sample is streaked onto the different types of culture media on each slide. There are three different media, one specific to Gram-positive bacteria, one specific for Gram-negative bacteria and a total count media.	8-24 h	Two culture media identifying Gram-positive and Gram-negative pathogens. The test turns red to identify Gram-positive growth and green to identify Gram-negative growth. *E. coli* will produce reddish colonies whereas all other Gram-negative species will produce white colonies.	No

2.4 Challenges of on-farm detection of mastitis bacteria

As previously described, rapid culture-based tests often use direct samples in liquid samples, simplifying sample processing and time to results. This can be appropriate for determining if

bacteria are present in a sample, however, gold standard laboratory antibiotic susceptibility tests such as broth microdilution, require a pure culture of the microorganism and specific media composition to be accurate. This standardised method is made more challenging for veterinary samples due to unknown, missing or incomplete breakpoints for veterinary samples. Often there are missing species-specific breakpoints for many treatment combinations of bacteria and antibiotics, and therefore breakpoints used for veterinary samples are extrapolated from other similar antibiotics or closely related species of bacteria and are usually based on breakpoints from human-derived bacteria.

2.4.1 Sample composition

Raw cow's milk has been shown to contain a host of microorganisms contributing to a complex microbiota potentially affecting the efficacy of rapid testing for pathogen mastitis-causing bacteria. It was once widely considered that healthy cow mammary glands are sterile and that the potential for bacteria in milk was a result of improper milk sampling, advocating precaution in aseptic milk collection (Metzger et al., 2018; Rainard, 2017). The milk microbiome in bovine mammary quarters free from intramammary infection and inflammation, with a low milk SCC, is even more diverse than that seen in quarters with clinical mastitis presenting a problem for a phenotypic and indiscriminate on-farm test for the presence of mastitis infection. Many studies have investigated the complex milk microbiome, with the conventional aseptic collection of cow's milk still harbouring species of bacteria including *Pseudomonas spp., Streptococcus spp. Corynebacterium* and *Staphylococcus spp*. (Guo et al., 2024). Moreover, with the purpose of cow's milk to provide early gut development for calves, probiotic phyla are dominant, including Proteobacteria, Firmicutes, Bacteroidetes and Actinobacteria, with genus *Bifidobacterium* and *Lactobacillus* present (Guo et al., 2024; Hoque et al., 2019; Skeie et al., 2019). These can all provide potential problems when trying to identify the presence of a pathogenic bacteria by culture method alone or by phenotypic identification in a rapid test.

Most emerging mastitis bacteria detection tests are focused on the ability to test individual cows, requiring cow-level and even quarter-level milk collection, more often this is done manually. With this, comes the added pressure of environmental contamination. Often milk samples are collected and pooled before testing to confirm milk quality in bulk tank measurements. However, the dilution effect from pooling milk samples can dramatically reduce the limit of detection (Long et al., 2023). This has prompted researchers and udder health specialists to recommend the collection and testing of separate quarter-level samples. However, pooled samples are still often used due to affordability and time constraints. Moreover, to provide the most accurate and

actionable culture results, special care must be taken to ensure samples are collected aseptically, to avoid the risk of contamination. In practice, this is difficult to do given the many opportunities for contamination that occur within a commercial dairy parlour.

There are potential factors that have been discussed to minimise the contamination of milk samples when individually collected from the udder quarters. It is often recommended to collect a post-milking sample as opposed to a pre-milking sample. Results have shown that post-milking samples are less likely to be contaminated because teats and teat canals are washed during the milking process (Sears et al., 1991). However, to aid with the aseptic collection of quarter milk samples, appropriate training of sampling personnel and careful attention to the sampling procedure used are important (Zadoks et al., 2023), as some studies have found very few contaminated samples among either the pre-milking or the post-milking samples when strict sampling procedures are adhered to.

2.4.2 Equipment costs, resources and adoption drivers

Although we understand that on-farm bacteriological testing may be effective in reducing antimicrobial drug usage, it must be cost-effective as well as accurate to be adopted. We know that mastitis has a significant economic impact on farmers, therefore the cost of using a rapid on-farm detection system must outweigh the financial burden of failing to diagnose and treat a case and must be more cost-effective than simply treating suspected cases with a broad spectrum of antibiotics. Often antimicrobials are cheaper than diagnostic assays. A wider use of diagnostics alongside treatment will initially incur greater costs to farmers, and for bacteriological testing and AMR detection to be viable, the cost of introducing diagnostics needs to be outweighed by the potential savings made from not having to treat all cases with antibiotics (Zadoks et al., 2023).

A study has looked specifically at whether an on-farm culture detection for mastitis is cost-effective when compared to financial loss. They concluded that the proportion of Gram-positive mastitis bacteria and the difference in bacteriological cure risk between the on-farm culture approach and the more conventional SCC and clinical symptoms will have the most impact on whether an on-farm culture system will be cost-effective (Lago et al., 2011). This means, that at present, research suggests that on-farm culture is only suitable for farms that present mostly Gram-positive cases. Moreover, it emphasises that a reduction in bacteriological cure risk may be associated with on-farm culture unless the time to treatment can be reduced from the 24 h delay that is associated with bacterial culture (Down et al., 2017). This places a greater emphasis

on not just developing on-farm culture, but also making sure testing is rapid. However, it was noted, that milk price and therefore the reduction in yield presented by a case of mastitis, had very little effect on the cost-effectiveness of an on-farm culture technique (Down et al., 2013). It has been demonstrated previously that although the price of milk and milk yield will have an overall effect on the profitability of a farm, it plays a minor role in terms of the cost of a clinical mastitis case (Smith & Thanassoulis, 2015).

Finally, it has been shown that farmers are more likely to adopt a test if it were rapid and efficient, and fit into regular farming practices. Based on farmer interviews conducted in The Netherlands, the ideal POC test for mastitis would provide a result prior to the next milking, which would be 8–12 h later in herds milking 2 or 3 times daily (Griffioen et al., 2016). Farmer engagement and opinion should be at the forefront of developing an on-farm test of any kind, where the key stakeholder will be the vet or the farmers themselves. Emphasis should be made to communicate with farmers to understand the key drivers to adopting novel on-farms tests. Zadoks et al., (2023) claims that currently there is little to no social science research addressing drivers or barriers of uptake of specific on-farm tests for mastitis.

2.4.3 Expertise and Training

On-farm or POC tests must be developed with the intended use in mind, potentially by people who have limited or no prior knowledge of diagnostic testing. It will not always be possible to have trained veterinary staff to carry out on-farm cultures. A significant challenge to developing one of these tests is to make it useable with as little training and expertise as possible (Zadoks et al., 2023). An example of this would be the development of a simplified dipstick model, whereby a diagnostic media is dipped into a suspected milk sample, with overnight incubation to retrieve a positive result, potentially as simple as a growth or no growth result (Malcata et al., 2021). We are already seeing the emergence of this simplified diagnostic technique through devices such as VetoSlide, whereby the already prepared dip slides can be easily inoculated with a direct milk sample and simply incubated to provide results. Such rapid on-farm tests need to be simple enough that they will take little expertise in microbiological methods, and little time, where farmers and potentially veterinarians do not want to spend too much labour where microbiological testing can be outsourced to laboratories.

2.5 Conclusion and recommendations for future on-farm tests: what do we need?

Culture-based tests are all constrained by the time taken to detect bacterial growth in a sample and usually have an overnight incubation step resulting in a time to result of 24 h. While this is more rapid than sending samples for microbiological analysis by a laboratory, this time to result is still not optimal for informing decision-making and requires a new animal management process (Malcata et al., 2020; Griffioen et al., 2016), which may indicate the more popular on-farm mastitis tests detecting host factors which can be detected in minutes compared to hours and days. However, these tests do not provide information on antibiotic resistance. There is also a question which must be raised, if while antibiotics are broadly effective, are the cons of overprescribing antibiotics empirically more important to consider, than the cons of developing and implementing new on-farm methods of testing?

Developing a successful on-farm bacterial detection test for the diagnosis of mastitis can prove challenging. There are technical and market complications including sample composition and potential contamination, costs incurred from developing the test and deploying it. Several important factors should be considered when developing an on-farm bacterial and AMR detection device.

1. **Rapid**; certainly, this will need to be less than 24 h from collection/sampling to results, and below the standard 18 h minimum detection for the gold standard minimum inhibitory concentration of antibiotics in laboratory testing. Ideally, these would be same-day results, i.e. tests taken in the morning should see no more than 6-8 h to results to inform treatment the same day.

2. **Accessible** across all dairy farms; the testing and interpretation needs to be as uncomplicated as possible, such as a simple dip test. The results need to be easy to interpret, an example of this could be a simple colour change for a positive presence of a bacterial species to report to veterinary professionals.

3. **Affordable** and easy to disseminate; veterinary professionals and particularly farmers are not going to be invested in a new form of detection if the tests are more expensive than just the already simple test deployed and a course of antibiotics. We need a test that is going to be cheap to carry out and then ultimately reduce the number of antibiotics needed and thus: (Cost of test + cost of new antibiotics) < cost of the initial course of antibiotics. The costs

incurred by a farmer for testing, and potentially a better informed course of antibiotics needs to be less than an initial less well-informed course of antibiotics without testing.

4. **More informative** than other available on-farm tests; the test needs to provide more information on the presence of disease than can currently be provided and the ability to record data for evidence-based decision-making on a course of treatment. This will include the types of bacteria present (if applicable) and even potentially what antibiotics are going to be effective in treating the disease. There are already plenty of detection techniques that confirm a case, we need to know what is causing the case to make more informed treatment decisions.

Mastitis continues to be a major problem for dairy farmers and resistance in mastitis-causing pathogens is increasing (Morales-Ubaldo et al., 2023). Some headway is being made in the development of new bacterial detection techniques on farms for mastitis. However, there is still a lot of work to be done and challenges to overcome in producing a testing protocol that is going to suit the needs of farmers and veterinarians in making informed decisions for mastitis treatment with or without antibiotics and which antibiotics.

The vast majority of developed on-farm tests required rigorous laboratory testing before deployment and are often peer-reviewed based on laboratory success rather than on-farm. This pulls into question the actual success that some of these on-farm tests will have in diagnosing a mastitis case and providing useful information for a course of treatment when handled by a less experienced handler. This also harks back to the potential for sample contamination as well as the potential for misinterpretation of results.

Based on the conclusions from this literature review the PiRamid imaging device was designed as an accessible, more affordable and more informative way of allowing time-resolved imaging of microbiological testing. As this was designed to fit in smaller-scale incubators such as table-top incubators, it could be transported to on-farm settings alongside cheaper tabletop incubators avoiding the need for large scale walk in incubators and vast lab space. The device was designed to simplify sample imaging by reducing the need for moving parts and being able to make the device portable. Similarly, standard spectrophotometer plate readers can provide time-lapse data, however for much lower numbers of conditions and at much greater initial expense, with conventional plate readers costing upwards of 30 times more than the construction of a PiRamid, making the device more affordable to the user. Moreover, the device

was designed to allow time-resolved imaging, providing more data than a simple endpoint, allowing for automation, and enabling the detection faster time-to-result. Similarly, the use of MCF as a microfluidic assay can allow for simple dip-test style use making a device that is more accessible and easier to use, but also more informative, containing 10 capillaries to provide results for up to 10 different conditions. Therefore, this device has been carried forward in developing of an on-farm mastitis detection and AST.

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Chapter 3 – The development of a tool to automate time-lapse imaging of microfluidic assays for on-farm mastitis detection and antibiotic susceptibility testing (AST)

With an objective to rapidly image and analyse the growth kinetics of bacteria in a miniaturised AST (antibiotic susceptibility test) system using MCF, a solution was required to take this system outside the lab environment. Moreover, given that the assays we carry out utilise custom microfluidic chips in the form of MCF, we required a device that could be customised to suit the imaging of these chips. While many technologies provide an end-point result, such as lateral flow, or conventional microtitre plate readers, there was also a requirement for time-lapse imaging of these ASTs on a smaller scale. Time-lapse is important to understand the growth kinetics of the bacteria and the effect of antibiotics on growth to help reduce the time to result and therefore time to treatment. The basic requirements of an imaging system are to be incubated at 37°C, to be small and portable enough for field use, to be automated for long growth periods, and to provide time-lapse. More sophisticated imaging systems can switch emission filters to allow imaging of different wavelengths of light and, the use of dichroic mirrors for the splitting of different wavelengths or different coloured light illumination.

Before this, time-lapse imaging of AST experiments in MCF was carried out using the POLIR robot that was developed by Dr Sarah Needs (Needs et al., 2019). The POLIR is a robotic, automated imaging system with a moveable x-y gantry holding a camera that is controlled by a Raspberry Pi single-board computer. The camera gantry is moveable using an Arduino single-board microcontroller programmed to respond to GCODE, the same coding language used to control the movement of 3D printers. The device has a large imaging area allowing for adaptation for the imaging of up to six microtiter 96-well plates and up to 18 combs of MCF microfluidic chips totalling 216 chips. While this camera system was able to sit in a laboratory walk-in incubator, we wanted to develop this with a self-sufficient incubation system that would be suitable for transportation outside of a laboratory y environment and potentially on a farm. One of the main drawbacks of the POLIR was the size, and although the device proved successful for high throughput experimentation, a smaller device was realised to suit the potential lack of expansive incubator space on farms and in smaller-scale laboratories. The challenges included the construction and size of the device, which made the inclusion of an incubator that was efficient enough to heat the entire system difficult. Based on this we looked at developing a smaller-scale

device that would alleviate these problems. The PiRamid was designed to fit in smaller-scale incubators such as table-top incubators that, if needed, could be transported alongside the imaging device. This simplified sample imaging by reducing the need for moving parts and being able to make the device portable. Even though the sample throughput was much less, the cost of materials was comparable when considering the construction of multiple PiRamid devices to provide the same throughput of results, and cheaper when constructing an individual device.

The PiRamid imaging system takes advantage of open-source computing, in the form of a Raspberry Pi single-board computer, which allows for the inclusion of an automated imaging script using Python. This device was designed to be cheap to construct, using PLA 3D printed parts, modular, to incorporate a level of customisation dependent on the user's needs, and easy to use, with a standardised Python script for automated time-lapse imaging. The PiRamid was extensively tested using a range of microbiological experimentation including novel MCF and bacteria motility assays and conventional culture, as well as proving useful in chemistry analysis through the imaging of crystal formation.

The PiRamid is capable of a lower capacity of microfluidic chips. The means a requirement of multiple devices for a substantial number of conditions to be analysed. However, for smaller-scale experimentation, data collection is simpler as all data can be collated from a single image taken by a single device of all the required conditions. This makes image analysis easier, and less time-consuming and reduces RAM consumption for the Raspberry Pi computer. It has also meant that with table-top incubation, data collection can be carried out in the field rather than being limited to the lab. Moreover, the PiRamid has allowed it possible to reduce the time to results for AST by delivering time-lapse images and allowing for MIC determination faster and less time-consuming than reading endpoint at 18-24 h or manually recording results by eye at different time points. Similarly, standard spectrophotometer plate readers can provide time-lapse data, however for much lower numbers of conditions and at much greater initial expense, with conventional plate readers costing upwards of 30 times more than the construction of a PiRamid.

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Author contributions:

The CAD files were initially designed and developed by Dr Alexander Edwards in OpenSCAD with 3D printing and adjustments made by Matthew Long in OpenSCAD for the use of light panels and

the attachment of a Raspberry Pi computer and touch screen. The Python script to control the camera and lighting panels was written by Matthew Long. Raspberry Pi setup, wiring of light panels and camera and resolution correction were carried out by Matthew Long. Construction of the device was carried out by Matthew Long. The creation of the Zenodo repository was carried out by Matthew Long.

Initial validation experiments using microfluidic chips with backlit imaging were carried out by Matthew Long. 3D printed agar dip slides were designed by Dr Tai Diep with dip slide and Petri dish agar growth imaging experiments carried out by Matthew Long and Dr Tai Diep. Motility testing experiments were designed by Dr Tai Diep and carried out by Matthew Long and Dr Tai Diep. Experiments using crystals were performed by Matthew Long, with a method developed using the information provided by Dr Marta Ross. The inclusion of epi-illumination by top light was developed by Dr Sarah Needs who analysed growth kinetics in microfluidic chips and imaged lettuce leaves.

Initial manuscript draft and figures were produced by Matthew Long and continued writing and review was carried out by Matthew Long, Dr Alexander Edwards and Dr Sarah Needs. Dr Sarah Needs provided Figure 9 and details regarding the method of validation for top light inclusion in the methodology. Final review and reading of manuscript carried out by all authors listed. Project supervised by Dr Alexander Edwards.

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PiRamid: A compact Raspberry Pi imaging box to automate small-scale time-lapse digital analysis, suitable for laboratory and field use

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ABSTRACT

Digital imaging permits the quantitation of many experiments, such as microbiological growth assays, but laboratory digital imaging systems can be expensive and too specialised. The Raspberry Pi camera platform makes automated, controlled imaging affordable with accessible customisation. When combined with open source software and open-source 3D printed hardware, the control over image quality and capture of this platform permits the rapid development of novel instrumentation. Here we present "PiRamid", a compact, portable, and inexpensive enclosure for autonomous imaging both in the laboratory and in the field. The modular three-piece 3D printed design makes it easy to incorporate different camera systems or lighting configurations (e.g., single wavelength LED for fluorescence). The enclosed design allows complete control of illumination unlike a conventional digital camera or smartphone, on a tripod or handheld, under ambient lighting. The stackable design permits rapid sample addition or camera focus adjustment, with a corresponding change in magnification and resolution. The entire unit is small enough to fit within a microbiological incubator, and cheap enough (~£100) to scale out for larger parallel experiments. Simply, Python scripts fully automate illumination and image capture for small-scale experiments with an ~110×85 mm area at 70–90 μm resolution. We demonstrate the versatility of PiRamid by capturing time-resolved, quantitative image data for a wide range of assays. Bacterial growth kinetics was captured for conventional microbiology (agar Petri dishes), 3D printed custom microbiology labware and microfluidic microbiology. To illustrate application beyond microbiology, we demonstrate time-lapse imaging of crystal growth and degradation of salad leaves. Minor modifications permit epi-illumination by addition of a LED ring to the camera module. We conclude that PiRamid permits inexpensive digital capture and quantitation of a wide range of experiments by time-lapse imaging to simplify both laboratory and field imaging.

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Specifications table

Hardware name	PiRamid (Pyramid Imaging Rig)
Subject area	• Microbiology • Environmental science • Agriculture Science • Chemistry • Crystallography and crystal engineering
Hardware type	• Imaging tool • Quantitative imaging
Closest commercial alternative	Gel documentation and laboratory imaging boxes
Open Source license	CC-BY 4.0 Creative Commons Attribution-ShareAlike 4.0
Dimensions of hardware	190 mm (L) × 140 mm (W) × 140 mm (H) (incl. Raspberry Pi)
Cost of hardware	£107.98 (£158.97 incl. touchscreen and UPS)
Source file repository	Digital Object Identifier DOI https://doi.org/10.5281/zenodo.7090151URL : https://zenodo.org/record/7090151

Hardware in context

Many current standard laboratory methods and assays can benefit from digital photography to capture, quantify, and digitise results but this can be time-consuming and not automated without dedicated imaging systems. While some commercial systems exist for specific common applications, such as electrophoresis gel documentation, these can be expensive and are typically tailored to particular setups (e.g., UV or blue-fluorescent illumination). This limits digital imaging to particular assays, the cost can be a barrier to access, and large expensive benchtop instruments are unsuited to carry outside of the lab and into the field. The number of uses of digital imaging - often exploiting consumer products such as smartphone cameras is rapidly expanding within many biological and chemical sciences. For example, smartphone colourimetry [1], illustrates how analysis of spectral changes in reporter dyes used widely in biological and chemical assays, can be transported away from spectrometers, imaging processing software quantify colour from RGB digital images and convert them into absorbance values. Within microbiology, colourimetry and turbidity can be used to detect and measure bacterial growth. Likewise in chemical analysis [2], where once semi-qualitative approaches to comparing colour change by eye can be replaced with more sophisticated and quantitative image analysis using digital imaging. The development of Digital Image Correlation (DIC) techniques is becoming popular in fields including crystallography [3]. As digital cameras become cheaper, a move from laboratory instruments (e.g. CCD cameras) to consumer cameras offers a simplified approach to producing scientific imaging devices, remaining sensitive yet cost-effective in a resource-limited setting, from chemical sensors [4] to phenotypic identification of bacteria [5]. Often, however, data quality is restricted by manual camera operation to take images with a digital camera or smartphone camera. Although the use of a tripod or frame to support the camera can stabilise image capture, consumer cameras with proprietary control firmware can be tricky to control offering limited or unreliable time-lapse options. Automation of these processes can therefore greatly increase the number and quality of images recorded and reduce the hands-on time needed to take the images. There have already been several advances across different fields highlighting how automation reduces time in the field and allows for the preservation of data integrity [6,7]. Another trend is toward portability, to take lab measurements outside the lab and into the field [1]. Indeed, the increasing popularity of smartphone capture for many diverse assays [8,9] suggests that the rapid development of customisable, precise imaging devices will offer a portable format for experimental imaging in many experimental fields.

Screening and imaging are often found within clinical microbiology labs, but although automation has been extensively adopted in clinical diagnostic labs, it remains expensive and/or laborious in smaller labs or research areas that do not use the core standardised assays required in clinical testing [10,11]. Here we introduce why microbiology methods are important, and yet outside the best-funded clinical labs, limited instruments are available, and flexibility is key. Due to the specialism of many microbiological samples, and the range of different ways in which experiments are carried out, customisable imaging and analysis systems are vital. A range of different sample formats may be required for analysis, even when core methods remain the same. For example, testing mastitis milk samples in an agricultural setting will require a distinct set of analytical microbiology assays to identify and test antibiotic susceptibility of bacterial pathogens, to human clinical samples. Yet in both cases, core assay equipment, multi-well plates, and Petri dishes remain identical requiring quantitation of colour changes that indicate microbial identification, in a range of different devices and conditions. Recent innovations have shown that traditional large devices can be replaced by smaller devices including custom 3D printed labware [12] down to the smallest sample volumes being assessed within microfluidic devices [13,14] and droplet microfluidics [15,16]. Flexibility and customisation are therefore vital for a lab imaging platform to be useful for as many different applications as possible. One of the most important analytical microbiology methods is the identification of antimicrobial resistance (AMR) in a wide range of samples. Currently, these assays either require skilled laboratory technicians or expensive automated instrumenta-

tion, both of which are operated within an appropriate lab environment. This highlights that simplicity in the way we image and analyse assays is required outside of larger-scale labs where funds are extensive, and the sample throughput is high.

When addressing problems such as AMR on farms, treatment is almost always carried out before microbiological testing, days before AMR would be identified. This historically has contributed to a continual build-up of AMR on farms due to the overuse or inappropriate use of antibiotics [17,18]. A more flexible and rapid approach is required to tackle these problems. Flexible time-resolved automated imaging could therefore become important in agriculture, for example supporting the development of more rapid AMR tests ideally measuring bacterial resistance to antibiotics directly from the sample. For use in the dairy industry, it would be ideal to analyse milk samples from dairy cows with mastitis for antibiotic resistance. Potential use for this device would be to image antibiotic susceptibility assays in mastitis milk samples. Portable, timelapse imaging would therefore be able to determine AMR more rapidly on-farm, highlighting the usefulness of PiRamid outside of the lab. Moreover, the importance of kinetics in microbial growth analysis has been shown by the existence of much more costly and sophisticated in-house laboratory imaging systems, however, the simplicity and automation of PiRamid may prove useful for a much quicker and simpler way of analysing AMR on dairy farms in the future.

Microfluidic devices are now more widely used in the detection of pathogenic species of bacteria, with their use allowing for greater speed of detection and the development of point-of-care diagnostics, able to be used in the field. Whether this be within the detection of urinary tract infections (UTIs) [13] or for the portable detection of bacteriophage lysis [9]. Our group developed a simple, low-cost example microfluidic device that can be used to detect bacterial growth and measure multiple antimicrobial resistance profiles of bacteria using the metabolic sensitive dye, resazurin [14] able to detect bacterial growth by a colour change from blue to pink. The devices used are made from a melt-extruded highly transparent fluorinated ethylene propylene co-polymer (FEP-Teflon) microcapillary film (MCF) comprising 10 capillaries along its length. This method allows for the use of high-throughput microfluidic devices, termed 'lab-on-a-comb', compatible with existing laboratory equipment such as 96 well microtitre plates [9,14,19]**.** The use of microfluidic devices can provide detailed information on bacterial growth, morphology, and kinetic effects of substances on bacterial species, which can prove beneficial to phenotypic analysis for both research and clinical applications (e.g., identify pathogens and choosing antibiotic treatment for UTIs, and AMR surveillance). Previously the reliance on expensive plate readers to collect this data was time-consuming and labour-intensive, replacing plate-readers with time-lapse cameras could easily increase throughput, reduce labour time and increase flexibility since different formats can be tailored to specific needs.

To address this, we have seen the development of open-source imaging devices, exploiting the use of 3D printing and utilising robotics [6,19]. Devices such as the one described by Needs et al. [19] allow for fully customisable and high throughput imaging of both low-cost microfluidics and conventional MTP and Petri dishes. Others have developed open-source hardware to obtain the same outputs at lower cost, or when proprietary commercial instruments are not suited to novel configurations [20–22]. These could replicate or improve on established analytical microbiology systems based on reagent-loaded 96 wells plates, including those for kinetic analysis of single plates or up to 50 microtitre plates, which are monitored every 15 min [23]. The success of these commercial devices proves the value of kinetic microbial growth analysis, but the instrumentation is not widely available to most labs due to cost and specialism of experiment format, as only a few labs process enough plates to justify the capital investment in this dedicated instrument.

We propose "PiRamid" an imaging system that was designed to exploit low-cost desktop fused-filament deposition (FFD) 3D printing and simple python script programming to produce a compact, low-cost, high-performance system for automated laboratory imaging. The design centres around the simple to use and low-cost Raspberry Pi single-board computer system and associated camera. LED sample illumination is powered by GPIO pins. The system is controlled by basic Python scripts based on the widely documented PiCamera camera control library. The device is fully customisable, with the 3D printed case stacking for ease of opening, designed using OpenSCAD open-source CAD software. This design is compact and can be portable for use in the field, whilst maintaining the same automation as when used in the laboratory if powered using inexpensive consumer lithium battery packs sold typically as smartphone chargers. To make it compact, the device is scaled down to allow for easier transportation and storage; this does limit the imaging area and restricts the number of samples that can be simultaneously imaged. Importantly, the small size allows it to be used inside standard table-top microbiological incubators for controlled temperature, without requiring either a large incubator facility or built-in heading and temperature control. Although open-source incubator designs exist, and low-cost PID controlled incubators can maintain a suitable temperature for microbiological experiments [24] we avoided adding an incubator to the PiRamid to make it as simple to assemble and program as possible.

The simple system is capable of taking time-resolved images of samples of different microbiological based assays that can be performed in microfluidic devices, strip wells, and custom agar device designs. We provide validation data of its uses within different scientific fields and across different methods. With the use of microfluidic MCF, PiRamid can image up to 24 different bacterial isolates, with up to 240 different conditions (10 capillaries per isolate). Due to the ease of production and use, it would be more than appropriate to build and use multiple devices for larger experiments. This would still be cost-effective, more flexible in use and provide greater portability. Even with the average cost of an individual device ranging from ~£120 to ~£180, we can expect the production of four of these devices to cost ~£480 to ~£720, and capable of imaging up to 960 conditions. Conventional lab-based assay readers and spectrophotometers can cost far in excess of £3000 each, often analysing one individual 96-well plate at a time and incapable of capturing growth kinetics; plate readers with inbuilt incubation plus time-resolved scanning might even cost £10,000 or more, and most plate readers are too bulky to fit in microbiological incubators.

Hardware description

The PiRamid device is a simple, compact enclosure for a small CMOS camera (Figs. 1 and 2), designed for the Raspberry Pi Camera Module v2, an affordable yet high-quality CMOS image sensor with a fixed focus lens capable of producing 3280 × 2464 pixel static images. Whilst the lens has a fixed focus, this can be adjusted by screwing in or out of the casing, permitting it to be used for macro photography simply by unscrewing the lens to allow closer focus. Despite the low cost (£22 for a camera sensor with lens), this v2 camera takes excellent images and has been extensively adapted for digital microscopy [11,25,26]. Moreover, with use in incubators, producing humid environments, no issues of droplet formation within or upon the lens of the camera have been noted by users. The camera is mounted within the top section (cap) of the PiRamid and forms the lid of an interlocking 3-part pyramid of 3D printed parts. This enclosed design allows for total control of lighting for the images produced, by eliminating external ambient light. The device contains a 3D printed imaging stage that forms the bottom section of the PiRamid. The stage was designed to reduce the risk of contamination of biological materials into wiring and onto the light source and allows for easy addition and removal of samples, with the optional installation of a small knob to form a handle. All three stacking parts forming the enclosure are inexpensive and can be printed, without supports, within a standard bed of a low-cost consumer 3D printer (<200x200mm bed). The rectangular-base pyramid shape reflects the camera imaging space, and at the same time allows printing without supports since most desktop, FFD 3D printers can produce slopes of up to 45°. We printed using PLA and found no problems with mechanical or optical properties in our example applications, however, we expect the design could be printed with other filaments for example with higher melting temperatures. We and others have previously shown PLA can be sterilised using 70 % Ethanol, important for some life science applications [12,27]. We added above the imaging stage design support for a 100 × 95 mm sheet of light-scattering white translucent acrylic to make the backlight of the imaging stage more even in brightfield mode. The entire

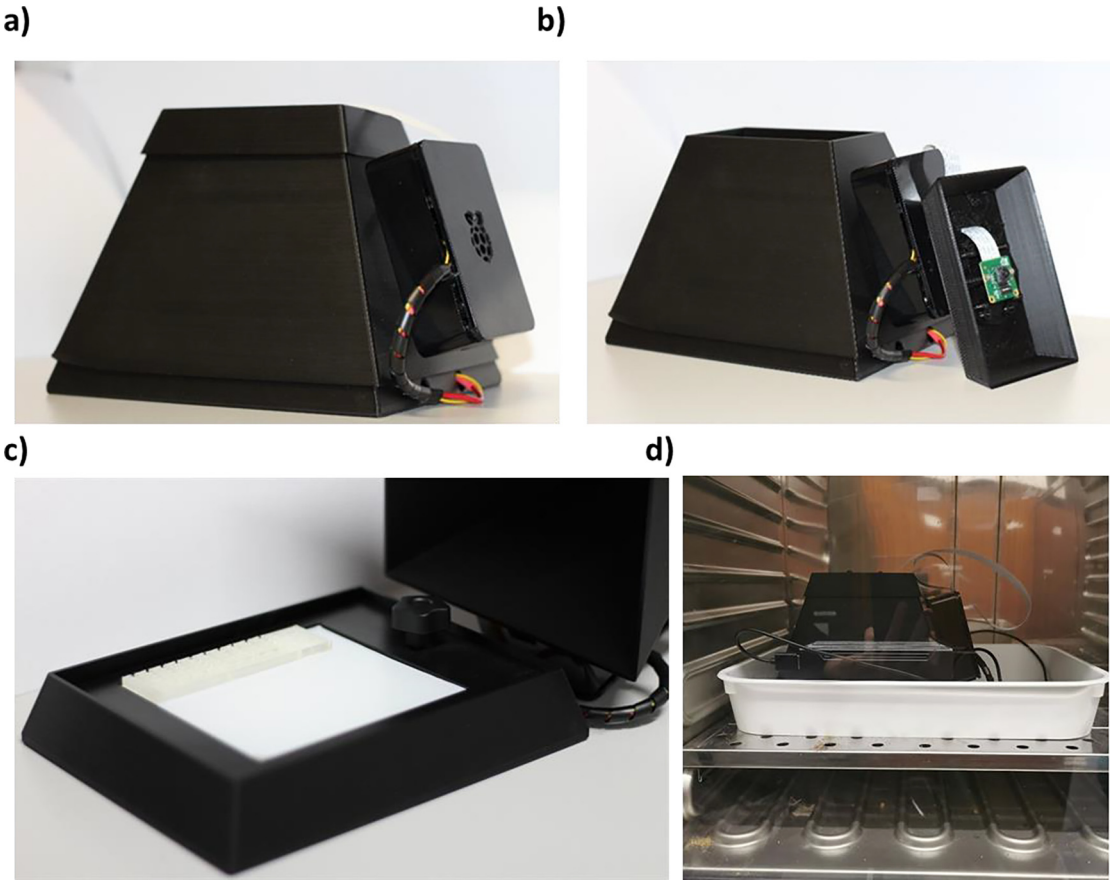

Fig. 1. Images illustrating PiRamid design, formed from three stacking 3D printed parts. (a) Compact unit in use. A Raspberry Pi computer is attached to the middle section of the box, with LED light strips wired and powered via the GPIO pins on the Raspberry Pi. (b) Raspberry Pi v2 camera is attached within the inside of the top cap section. (c) Imaging stage design. A 3D printed sample tray that slots within the bottom section of the PiRamid, preventing leakage of biological materials and for ease of samples addition. A white acrylic diffuser plate offers backlight for brightfield imaging. (d) Demonstration of compact size and portability allowing use within a tabletop incubator, powered here by an optional UPS battery unit visible in front of the PiRamid unit providing 5 V to the Raspberry Pi; external power can also be provided via USB with a power cable fed through incubator door seal.

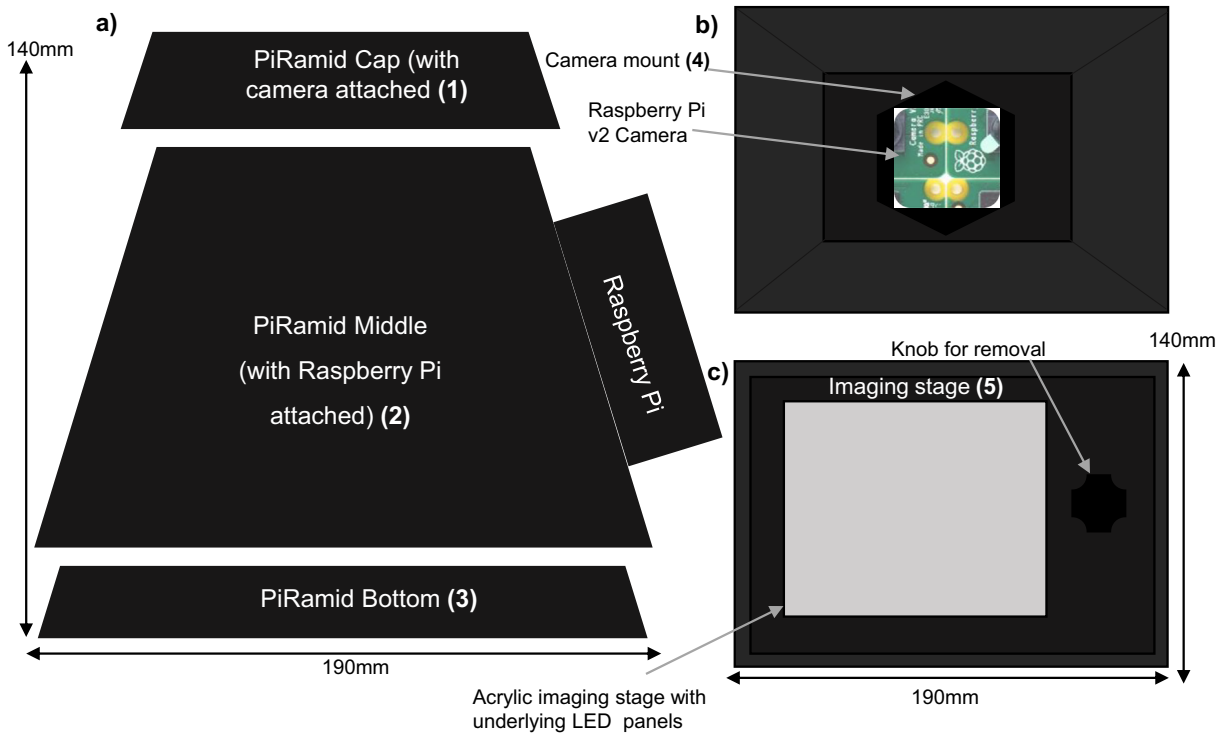

Fig. 2. Diagram of the PiRamid imaging system. (a) Side view indicates the three stacking parts with overlap between tapered section ensuring external light is blocked, with camera inside top, plus attachment of Raspberry Pi to the side with space to add a small touchscreen. (b) Inside view of the PiRamid cap demonstrating Raspberry Pi v2 camera attachment on a simple adjustable 3D printed camera mount. (c) Top view of PiRamid base tray section containing sample area and removable imaging stage with acrylic diffuser underneath which two LED light panels are placed with wiring underneath and knob for removal of sample tray.

system is controlled by a Raspberry Pi single-board computer. Two LED backlight panels are positioned in the bottom section of the PiRamid, at the base of the imaging stage, in a fixed position. These LED strips are wired to Raspberry Pi GPIO pins which are programmable to switch on and off using commands in Python script. The LED backlight units were wired with series 33-ohm resistors, drawing approximately 10 mA from the 3.3 V GPIO switched pins. The device can be operated via a remote desktop or SSH connection, which can be viewed or typed directly via a mobile phone or PC by connecting the Pi computer to WIFI or a network hotspot or using a LAN cable. PiRamid can be provided with an inbuilt screen and interface by the addition of an optional 3.5-inch touch screen which enables complete remote operation without a network connection for virtual desktop or SSH access. The device can be powered by external mains power (120–240 AC) using an inexpensive USB power supply (5 V > 2A); the addition of an uninterruptable power supply (UPS) offers greater portability (costing an additional ~£60). When required, external power (12 V adapter plugged into AC mains) can both recharge the internal UPS battery, and power the PiRamid. The USP allows the sample to be set up, Raspberry Pi started up and imaging scripts run on a desktop or near a PC, before transfer to an incubation location (e.g., inside an incubator) where it can be plugged in.

Being a fixed focus camera, the camera module lens was manually rotated to focus on the sample before use, taking care to focus on the experimental sample. A USAF 1951 high-resolution target (Edmund Optics #38-257, Edmund Optics, York UK) was used in the imaging stage when focussing to ensure the best resolution possible for the camera module. Images were taken at the centre of the imaging stage and in all four corner to compensate for distortions around the edges whilst using a stationary camera. The resolution target indicated that the resolution of the centre of the image was at or better than 7.13 lp/mm (with group 2 element 6 showing clearly separated lines), with approximately 70 μm lines spaced 70 μm apart being the smallest element visible (Fig. 3). When the resolution target was towards the corners of the image, we see reduced resolution to 5.66 lp/mm, with approximately 90 μm lines spaced 90 μm apart resolved. Even at the corners of the image, this is more than sufficient to image many targets such as microfluidic systems.

The PiRamid design mounts the camera 95 mm away from the sample, giving a field of view for samples of 116 mm × 86 mm, illuminated in a brightfield with a white backlight area of 100 × 850 mm. Note that the 3280 × 2464 pixels resolution and 116 × 86 mm corresponds to a theoretical maximum possible resolution of 35 μm per pixel, so the achieved resolution of 70–90 μm across the whole imaging area could potentially be improved somewhat for example by better focus, but not great. The achieved resolution across the whole imaging area was more than sufficient to resolve the individual microcapillaries within our microfluidic devices which are ~260 μm wide and separated by ~300-μm gaps.

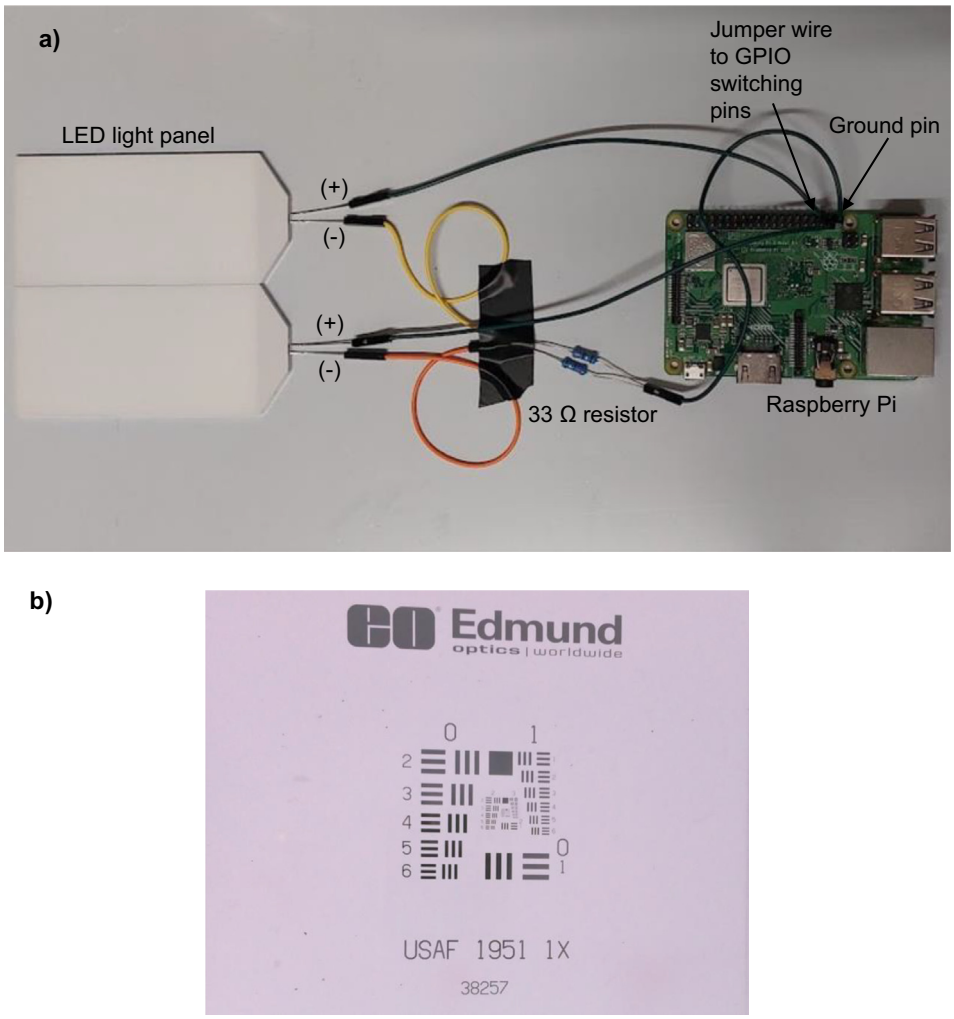

Fig. 3. (a) Wiring guide for LED light panels to Raspberry Pi (b) Demonstration of PiRamid resolution. A high resolution glass USAF 1951 resolution target was used on the imaging stage to ensure the best resolution possible for the PiCam. When using an image of the resolution target it was identified that the resolution of the image was around 70 μm with 7 lp/mm being the smallest line dimensions that could be resolved clearly (group 2 element 6 of the target).

In this current format, the PiRamid is capable of imaging one 96-well plate, one petri dish, four custom dip-slides [12], four 8-well ELISA strips, or three combs of 8 MCF strips for microfluidic testing corresponding to 240 individual 1 microliter assays (each strip contains 10 microcapillaries). The 3D printed open-source design allows for full customisation, with individual parts adaptable to varying experimental designs and required fields of view. For example, making a taller pyramid shape with a larger base section will move the camera higher, giving a larger field of view. There is a direct trade-off of resolution vs imaging area. Thus, for higher resolution images of smaller samples or fewer devices, a smaller PiRamid would work.

The system can, however, be adapted for use of Raspberry Pi's HQ camera module. This addition will allow for the capture of higher resolution images and where required, the adaptability to use with any standard C- or CS-mount lens. The HQ camera can be attached to the camera mount found in the design files. The length of the HQ camera however would require adjustment and careful lens selection would be needed as the HQ camera can be configured with different imaging angle and field of view, ranging from wide angle to telephoto; unlike the v2 camera with a single fixed lens. However, the size and shape of the PiRamids 3D structure can also be easily altered within the OpenSCAD design to accommodate a different camera distance and sample bed size, therefore making the addition of this higher budget and more sophisticated camera possible.

To take images a simple Python script was configured with the required picamera library settings (e.g. image exposure, resolution, time) and looped using Python if commands to take a specified number of images for a required length of time (the picamera library documentation is available at https://picamera.readthedocs.io/en/release-1.13/). The Raspberry Pi Foundation recently replaced the picamera library with a new camera control system following the release of "Bullseye" Raspbian operating system. Our scripts are compatible with the legacy camera control system included with the "Buster" Raspbian release. The legacy camera settings found in the previous "Buster" release can be enabled in the newer "Bullseye" release to allow for execution of our script (see Python script for further instructions). The length of time between image capture in each loop can be altered depending on the number of images required, the interval between images and the time frame of the experiment being analysed. For example, certain crystallography demonstrations require much shorter overall experiment times, with shorter imaging intervals. The images are then stored onboard the Raspberry Pi SD card and can be accessed remotely by file transfer protocol (FTP) or saved to a removable USB memory device. The Python script was also configured so that the LED strips switch on immediately before the images are taken and switch off immediately after (see design files for a text file with example script). This command is also looped alongside the imaging. Sufficient time for the camera to adjust exposure times to lighting is required after switching the LED on, alternatively, the camera exposure settings can be fixed in the script. The system was evaluated here using the Raspberry Pi's built-in VNC viewer via wireless networking to a laptop or by starting python scripts via the small touchscreen.

Main properties

1. The system is automated; the PiRamid device is capable of imaging with time intervals as short as seconds and experiment lengths up to several days.
2. The design centres around the use of simple and low-cost single-board Raspberry Pi computers, camera modules and LED backlight controlled by basic scripts using Python language; these are easy to control, and open access for customisation.
3. The device can be fully customisable, with an enclosed structure entirely consistent with low-cost desktop 3D printing, adaptable using open-source design software and enclosed to allow total control over lighting conditions.
4. This design is compact and can be considered portable for use in the field, with the addition of battery power from a UPS.
5. The device is small allowing use inside inexpensive table-top or portable incubators, for example, simple microbiology incubators, without the need for a large incubator facility or laboratory equipped with a walk-in incubator room, reducing costs, and avoiding the need for laboratory access.

Limitations

1. The device still requires the use of a tabletop or walk-in incubator for microbiological and temperature-sensitive experiments. This could be solved with a built-in incubation system to be fully compatible with field use; yet this would significantly increase.
2. The system is driven by a python script that requires some basic programming skill to modify. This could be solved with dedicated software providing a more intuitive user interface. However, most Raspberry Pi users have sufficient python familiarity to use and modify these scripts, and extensive documentation and training is available freely online, for example from the Raspberry Pi foundation.
3. As mentioned, although we can adapt the size and shape of the device, because it uses a fixed-position camera, we see a trade-off between resolution and imaging area. However, this could be addressed by customisation for the higher-cost, higher performance version HQ camera module. The device is low enough cost that multiple devices can be made to increase the area imaged or the number of samples tested per experiment.

Design files summary

The design entirely consists of customised 3D printed parts designed to stack together creating a completely closed and light-controlled environment, with the pyramidal form reflecting the minimal enclosed space for digital imaging, and the stack permitting very rapid and easy sample addition and/or camera adjustment. No glue or fasteners are required to close the device and the simple tapered overlaps between the three components eliminate external light without needing tight tolerances to ensure a close fit. We provide here the CAD design files (OpenSCAD and STL mesh) for 3D printing direct or for modification of the three main structural components to fit the user's differing needs e.g., different sample sizes, lighting configurations, or camera types. Also provided are the final STL mesh designs that can be used for 3D printing the same device configuration used in this article. This includes a camera mount design for the simple and adjustable attachment of the Raspberry Pi v2 camera module. The following design information includes all required for procuring and/or making the parts for the PiRamid and its assembly for use.

Design part name (see Fig. 2. for part positioning)	File type	File name and Location Repository location: https://zenodo.org/record/7090151Repository permanent location (Digital Object Identifier) : https://doi.org/10.5281/zenodo.7090151
PiRamid Cap **(1)**	STL	PiRamidCap.stl https://zenodo.org/record/7090151/files/PiRamidCap.stl
PiRamid Middle (with Pi attachment) **(2)**	STL	PiRamidMiddle.stl https://zenodo.org/record/7090151/files/PiRamidMiddle.stl
PiRamid Bottom **(3)**	STL	PiRamidBottom.stl https://zenodo.org/record/7090151/files/PiRamidBottom.stl
PiRamid three-part imaging device	OpenSCAD	PiRamid3Part.scad https://zenodo.org/record/7090151/files/PiRamid3Part.scadNote : this design file contains all three components in the stacked pyramid; each component needs to be rendered to view or edit and should be rendered individually to output single pieces for 3D printing.
Camera module mount **(4)**	STL	CameraModuleMount.stl https://zenodo.org/record/7090151/files/CameraModuleMount.stl
PiRamid imaging stage **(5)**	STL	PyramidImagingStage.stl https://zenodo.org/record/7090151/files/PyramidImagingStage.stl
PiRamid imaging stage **(5)**	OpenSCAD	PyramidImagingStage.scad https://zenodo.org/record/7090151/files/PyramidImagingStage.scad
PiRamid Python Script	Text	PiRamidPythonScript.txt https://zenodo.org/record/7090151/files/PiRamidPythonScript.txt
White semi-transparent plastic sheet to diffuse light, such as White Opal 3 mm Acrylic	No design file- a simple rectangle	100 × 95 mm rectangle – can be laser cut, cut by hand or machined depending on local supply and machining options.

Design files are also available within a public GitLab repository: https://gitlab.com/mattlong29/PiRamid.

These components of the device were all designed in the open-source OpenSCAD computer-aided design package (https://openscad.org/), and the STL files exported from OpenSCAD were then sliced using PrusaSlicer 2.3.0 software and transferred via SD card to a 3D printer, to print the components using default printer parameters. We used the following printer and configuration options:

- 3D printer: Prusa I3 MK3
- Black PLA (Polylactic Acid): 1.75 mm
- Printing conditions 0.2 mm layer height, infill 20 %, and other conditions as recommended by the slicer software.
- No support
- Brim may be required to improve bed adhesion

We have successfully printed PiRamid parts on a Creality Ender 3 with a variety of PLA colours from different suppliers. Most coloured filament did affect colour of light within the box and we therefore advise using black (or white) PLA.

Bill of materials

Component	Qty/ unit	Cost per unit (GBP; price in November 2021)	Source of material
Raspberry Pi 3B+ (Note: other models of Raspberry Pi can be used, depending on the application)	1	28.25	RS Components; https://uk.rs-online.com/web/p/raspberry-pi/1373331
Raspberry Pi, v2 Camera Module, CSI-2 with 3280 × 2464 pixels resolution	1	20.25	RS Components; https://uk.rs-online.com/web/p/raspberry-pi-cameras/9132664/
Raspberry Pi Power Supply, Micro USB Type B with UK Plug Type 1.5 m	1	6.45	RS Components; https://uk.rs-online.com/web/p/raspberry-pi-power-supplies/1770225/
SanDisk Micro SD Card 32 GB	1	6.08	RS Components; https://uk.rs-online.com/web/p/micro-sd-cards/1231040/
DesignSpark ABS Case for use with Raspberry Pi 3B	1	4.62	RS Components; https://uk.rs-online.com/web/p/raspberry-pi-cases/1677047/
Raspberry Pi Camera Ribbon Cable – 300 mm - CTLCAMCABLEASSY-300 mm	1	1.19	CPC; https://cpc.farnell.com/pro-signal/ctlcamcableassy-300mm/cable-for-pi-camera-300mm-formed/dp/SC13282?st=raspberry%20pi%20ribbon%20cables
LED backlight panel 86 × 45 mm (manufacturer part number KWB-R8445W/1W)	2	2.98	Cool Components; https://coolcomponents.co.uk/products/white-led-backlight-module-large-45mm-x-86mm
6 in Breadboard Jumper Wires female-to-female (pack of 5)	1 pack	3.92	RS Components; https://uk.rs-online.com/web/p/breadboard-jumper-wires/1947654
RS PRO Single Core Control Cable, Black, 1.5 mm2 CSAm 1000 V, 30/0.25 mm Core Strands, 25 m (Optional if LED modules are wired permanently by soldering)	1	11.67	RS Components; https://uk.rs-online.com/web/p/hook-up-wire/8114429
RS PRO 1.75 mm PLA 3D Printer Filament, 250 g, Black (Black PLA excludes external light most effectively, but we found different brands or colours of PLA printed successfully.)	1	9.99	RS Components; https://uk.rs-online.com/web/p/3d-printing-materials/8320406
Diffuser sheet of opal white semi-transparent acrylic. Product name "3mm Heavy Opaque Acrylic" option "Opal" 100 mm × 95 mm rectangle	1	1.18	Can be cut to size or ordered e.g. from "Cut my plastic"; https://www.cutmyplastic.co.uk/acrylic-sheet/heavy-opaque/3mm/non-recycled/L100-W95/
Optional components			
3.5″ IPS Touch Screen for Raspberry Pi (GPIO/SPI)	1	24.00	The Pi Hut (Waveshare); https://thepihut.com/products/spi-3–5-320x480-ips-touch-screen-gpio
Universal Power Supply or USB power bank with pass-through function GM322 Mini UPS 7800MAH 12 V 2A – KTC5336FBA, (15.4 × 13.6 × 4.6 cm; 340 Grams) Any suitable power bank or battery could be used, but it must output 5 V at 2A via USB to power Raspberry Pi, and must be possible to plug into the mains charger without interrupting the output	1	26.99	Amazon https://www.amazon.co.uk/Docooler-Protection-Charger-Portable-Applications-White/dp/B07BF4SR6S
Elesa 69,811 Black Multiple Lobes Clamping Knob	1	1.56	RS Components; https://uk.rs-online.com/web/c/engineering-materials-industrial-hardware/knobs-levers-handles/clamping-knobs/

(*continued on next page*)

(*continued*)

Component	Qty/ unit	Cost per unit (GBP; price in November 2021)	Source of material
Epi-illumination Version			
LISIPAROI White LED Camera Light for Raspberry Pi	1	13.84	Farnell; https://uk.farnell.com/cyntech/lisiparoiwht-01/lisiparoi-white-led-camera-light/dp/2840710

The total cost for this PiRamid, including the 3D printer material PLA is ∼£120, without the 3.5-inch touchscreen or UPS. The performance of this product will not differ with the addition of the touchscreen, but it improves usability by allowing experiments to be started and progress checked without a remote desktop via a network connection. Network connections can be more difficult to access in a laboratory environment, for example, Wi-Fi radio signals only poorly penetrate metal incubators. The device can also be powered with a UPS or USB power bank for increased portability with an additional cost of £26.99. The PiRamid with a touchscreen can have a cost of ∼£145 with the additional inclusion of a UPS rising to ∼£180.

Build Instructions

The following steps provide a step-by-step guide for building and assembling the PiRamid. Please see Fig. 2. for a diagram of the assembly.

3D printed parts assembly and Pi attachment:

1. Slice the STL files downloaded from the repository using the preferred slicer programme for your 3D printer. Transfer files using an SD card to the 3D printer and allow to print using chosen colour PLA (black PLA is suggested to allow for better light control).
2. Install the later version of Raspberry Pi software Raspbian onto a formatted SD card for use on your Raspberry Pi (see Raspberry Pi website for instructions). With this, you will be required to understand some basic Python coding skills. If you wish to use the picamera Python library ensure the operating system selected supports this, either by installing Buster or an earlier release or by enabling legacy camera control as advised by the Raspberry Pi Foundation. The basic Python script for using the PiRamid is available within the file repository and can be edited as required.
3. Attach the 3D printed camera mount to the inside of the cap part of the PiRamid (as shown in Fig. 1 and Fig. 2.) using four M1 screws and nuts. Attach the camera module to the camera mount using two M1 screws. At this point, the camera module can also be attached to the mount and the Raspberry Pi using either the stock ribbon cable or the ribbon cable purchased in the bill of materials (different lengths for personal preference). There is a slit in the cap of the PiRamid to thread the ribbon cable. Please follow the manufacturer's instructions (Raspberry Pi instructions) for camera attachment and enabling the hardware in operating system options.
4. Attach the bottom half of the Raspberry Pi case using screws to the two screw holes on the side of the middle section of the PiRamid. Alternative cases can be used according to preference, or a case can be 3D printed using various open-source designs; we recommend the use of a case to enclose the Raspberry Pi to reduce the risk of short-circuiting electronics or exposing it to laboratory spillages.
5. The imaging stage requires a 100 mm × 95 mm rectangular piece of white diffuser plastic sheet, such as 3 mm opal acrylic. This should be purchased or cut using a laser cutter and placed into the rectangular space in the centre of the imaging stage piece.
6. Optionally, using a drill, make a 5 mm diameter hole in the top of the imaging stage for attachment of the lobe clamping knob. This will act as a handle to make it easier to remove the imaging stage when required for example to change samples (see Fig. 2. for placement).
7. Slot the imaging stage over the top of the light panel housing and stack the 3 interlocking PiRamid sections together.
8. Optionally, the UPS can be attached to the Raspberry Pi via a suitable USB cable, or other Raspberry Pi power supply systems are available.
9. Optionally, for the epi-illumination version, follow instructions for wiring the LISIPAROI White LED camera light for Raspberry Pi. The PiRamid Bottom (3) can be printed and used as a base. For this option, we recommend printing in white PLA to have a white background.

Electrical wiring

1. Slot the LED light panels into the housing in the bottom section of the PiRamid. Make sure the connection wires are sticking out of the two holes printed at one end of the housing. Take care with the LED wires as they can break if bent repeatedly.
2. Using jumper wires, wire the positive (long wire) of each LED panel to a 33-ohm resistor. Users can attach these using jumper wires for speed and convenience, or permanent use by soldering using the appropriate wire. A 33-ohm resistor was found to deliver suitable brightness for the LED backlight panels used here, however, different resistors could be chosen depending on the voltage drop and target current if different LED modules are used. The switched GPIO pins deliver 3.3v and care should be taken to ensure the LEDs and series resistors do not draw more than the specified 16 mA maximum current from these pins.
3. Using jumper wires again, attach the ends of each resistor to one of the GPIO switching pins on the Raspberry Pi board. Make sure to note the number of the switching pin as this will be reflected later in the Python script. The numbers of the pins used in the current example Python script are GPIO 5 and 17. If a touchscreen is to be attached using a GPIO connection, make sure different pins are used than those used to power and communicate with the touchscreen. This can be edited on the Python script depending on which pins you use.
4. Using similar jumper wires, you can attach the two negative wires of the LED panels to the Ground pin on the Raspberry Pi computer GPIO.
5. When switching LED on or off using the Python script, you independently switch the two LED panels by controlling the different pins you decide to wire these to. Although both LED panels could be powered by a single pin, care should be taken to avoid drawing more than the maximum 16 mA specified for each pin; an advantage of using two independent pins for the two LEDs is that each one can draw more current to be brighter.
6. Optionally, the 3.5-inch touchscreen can be attached directly to the Raspberry Pi. To do so, the positive wires for the LED panels must be positioned within the top six GPIO pins. This is because the screen will slot directly onto the remaining GPIO pins.

Operation Instructions

This PiRamid imaging device can be used to image a range of life science laboratory experiments from monitoring crystal growth, food and nutrition research and analytical microbiology. The device can be allowed to run for seconds up to days with image intervals as low as fractions of seconds for time-lapse imaging. We have found it possible to record high-resolution images up to around 4 per second, even at the maximum image resolution; faster frame rates are possible with lower image resolution. User instruction is as follows:

1. Carry the device to the desired location for imaging, be it a walk-in incubator, smaller incubator, or benchtop.
2. Plug the Raspberry Pi controlling the device either into the mains power with the appropriate power supply or into the UPS and allow Raspberry Pi to boot.
3. The device will run with a fully charged UPS, however, can be powered by the mains through the UPS and moved/-transferred by removing from mains power and running solely on the UPS.
4. Load either the Raspberry Pi command line or Python integrated development software (IDE) such as Thonny. Here, load the desired Python script that will run your imaging. The basic Python script for running the device can be found in the repository (labelled as PiRamidPythonScript.txt). Instructions on how to edit the Python script to take the desired number of images with intervals can be found embedded within the Python script itself. The commands are described as image_count and wait_time.
5. Edit the Python script to the desired length of time between images, and the required number of images to set for a whole experiment. For example, this can be an image taken every 15 mins for 24 h, or simply an image every 30 s for 10 mins.
6. Remove the imaging stage from inside the device to add samples, making sure any samples or areas of the sample to be imaged remain within the diffuser light window on the imaging stage to illuminate.
7. Return the imaging stage to the base unit of the PiRamid taking care to not spill any biological materials or liquid from your samples.
8. On the first operation, it is important to carefully focus the v2 camera lens, this benefits from the remote desktop operation of the Raspberry Pi or connecting a large HDMI display, to see images in full definition to ensure sharp images. To focus the v2 camera module, a small plastic ring device (typically supplied with the camera module) is used to rotate the lens; unscrewing the lens is required to focus closer than when supplied, where these modules are typically supplied focussed near infinity to image objects at a significant distance. Depending on the height of the holder in the bottom tray, and the thickness of the sample, small focus adjustments might be needed for different tests.
9. The device can either be left on a shelf in a walk-in incubator (either plugged into mains or via UPS) or can be placed in a bench-top incubator when used for temperature-controlled experiments. Similarly, the device can be placed in any temperature-controlled room.

10. Make sure the cap containing the camera module is placed carefully on top of the device and that the device is running the Python script. When the experiment has started, check that images are being taken of the correct area and with suitable image quality by looking in the folder destination set within the Python script. Images are saved as soon as they are taken, allowing remote desktop or FTP to check the initial images before leaving the experiment to run e.g., overnight.
11. All 3D printed parts can be completely cleaned after use with detergent or antimicrobial such as 70 % alcohol to ensure sterility before and after use. Damaged or contaminated parts can be reprinted and replaced easily.

Validation and characterisation

To demonstrate the application of this device we compared a wide range of different experimental methods that might benefit from controlled, programmable time-resolved imaging. We explored different microbiology applications including bacterial growth assays in microfluidic devices and conventional agar colony culture using dip slides and Petri dishes, soft agar bacterial motility testing. Finally, we imaged crystal growth in a petri dish and also degradation of salad leaves, illustrating more diverse applications of time-lapse laboratory imaging. In these demonstrations we operated the PiRamid from battery or mains powered, and with minimal modification we achieved epi-illumination simply by adding an off-the-shelf LED ring for the Raspberry Pi camera, although most applications were brightfield illuminated with a white LED backlight.

We explored whether colourimetric analysis could be recorded successfully in microfluidic assays of resazurin growth indicators to produce growth curves, using a quality control reference strain of *E. coli* (ATCC 25922). To do this, MCF was coated internally with a hydrophilic layer of 5 mg/mL polyvinyl alcohol (PVOH) solution in water and incubated at room temperature for >2 h [28]. The coated MCF was then washed with 0.5 % Tween solution to remove any residual PVOH and left to dry on a vacuum manifold for 20 min. Individual MCF strips measured 17 mm in length with an internal capillary volume of 1 μL. In this case, six strips were clipped into 3D printed reusable 'combs', allowing the strips to be handled and dipped in a row of a 96-well plate and draw up the samples within the wells [14]. 3D printed end covers were filled with Dow Corning vacuum grease to prevent sample evaporation and slid over the ends of the MCF strips.

Reference strain *E. coli* 25922 was cultured on LB agar overnight at 37 °C. Colonies were scraped from the plate and suspended in Mueller-Hinton (MH) broth. Bacteria were grown for several hours until turbid and the final bacteria inoculum was diluted according to a 0.5 McFarland standard. MH broth was spiked with a bacterial suspension grown overnight at 37 °C in a 96-well plate with resazurin by 5-fold serial dilution using a micropipette. The bacterial concentrations ranged from 10^6 > to 10^3. The dipping of test strips into each well allowed for the sample to be taken up by capillary action. Samples were incubated overnight at 37 °C. Growth kinetics were able to be recorded using timelapse imaging from the PiRamid; resazurin conversion to the resorufin (dark blue to pink colour changes) was recorded every 15 min over a 20 hr incubation period. Images were analysed using MATLAB scripts to provide absorbance values which can be plotted against time. With resazurin dye reduction colour change from dark blue to pink, we see a change in absorbance values, allowing the plotting of a growth curve (Fig. 4.). Generation time can be calculated and used to estimate starting cell density in a sample (CFU/mL).

Alongside microfluidic bacterial growth assays, we show that we can image bacterial growth on agar and can identify individual colonies on 3D printed customised multi-sample dip-slides [12] and 50 mm diameter Petri dishes. For the custom dip slides, agar at 0.8 % in LB broth was autoclaved and, once cooled to 50℃, supplemented with a final concentration of

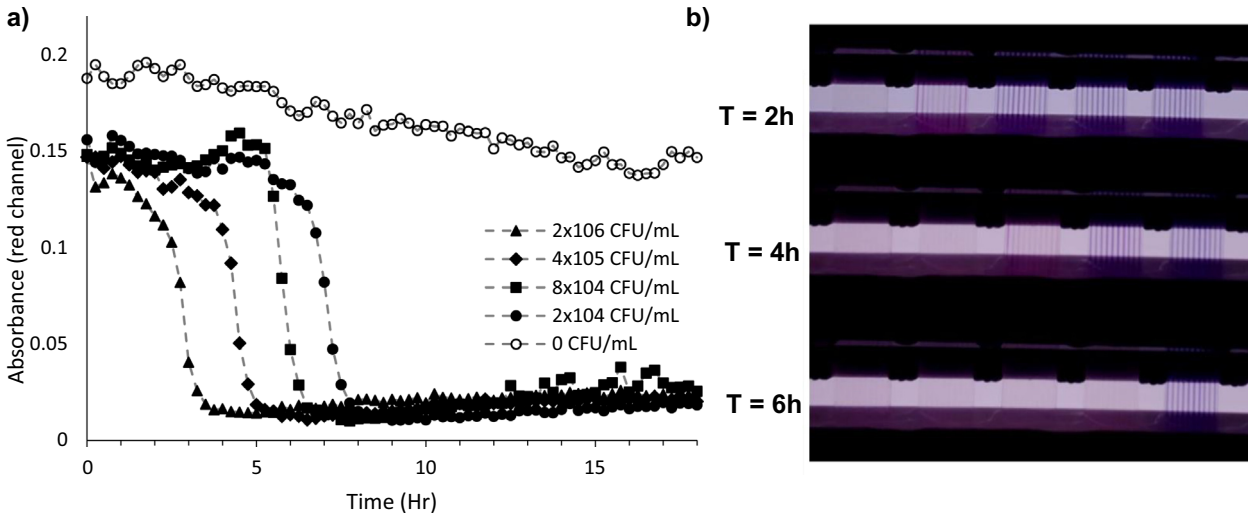

Fig. 4. Microfluidic colourimetric growth kinetics measurement of *E. coli* using resazurin dye, from time-lapse images taken in PiRamid (a) Growth curves for *E. coli ATCC 25922* in Mueller-Hinton broth were plotted for the indicated starting bacteria concentration (CFU/mL) in mirocapillary test strips. (b) PiRamid mages of MCF strips held in a 3D printed comb and containing 5-fold serial dilutions of E.coli ATCC 25922 grown in MH broth and resazurin dye. Four serial 5-fold dilutions of cell densities ranging from $2x10^6$ – $4x10^3$ CFU/mL from left to right; the final strip contains no bacteria and no growth was detected.

0.1 mg/mL Triphenyl tetrazolium chloride (TTC, Sigma Aldrich) which produces a dark stain following microbial growth, making colonies strongly coloured for brightfield imaging. Agar was added by micropipette into the wells of dip slides. Bacteria were grown overnight in MH broth and normalised to 0.5 McFarland standard and diluted to 1 in 10,000. Each dip slide was dipped into a 50 ml falcon tube containing diluted bacteria inoculum and then placed inside the PiRamid which was incubated overnight at 37C with images collected at regular intervals. Custom transparent covers for dip slides were used to prevent evaporation. The images were taken every 15 min over 20 h using the PiRamid allowing for the creation of a time-lapse video of bacterial growth with colonies stained by the TTC dye, used for the enumeration of bacterial colonies on solid culture media (Fig. 5). Time-lapse imaging allows clear visualisation of organism growth kinetics, with the potential to compare and quantify the growth of different species and in different growing conditions.

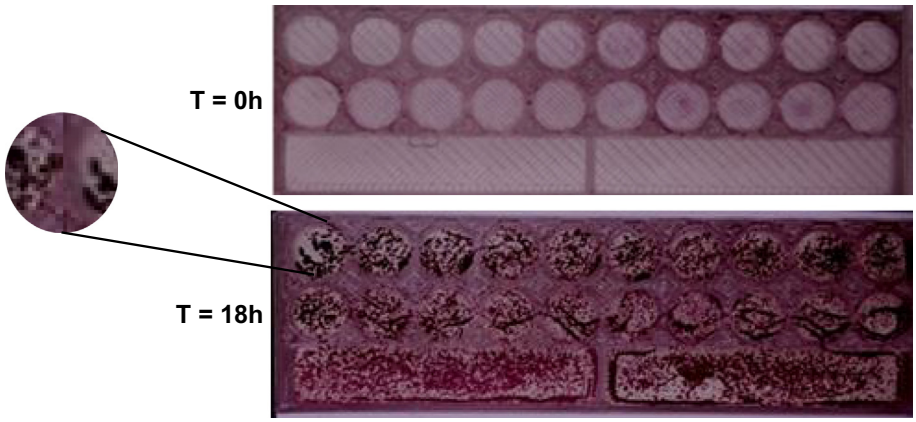

Fig. 5. Time lapse imaging of custom 3D printed agar dip slides (T. T. Diep et al., 2022). These 3D printed dip-slide devices have the potential for bacterial enumeration and identification for rapid testing in the field. Imaging using the PiRamid permits time-resolved imaging of microbial growth.

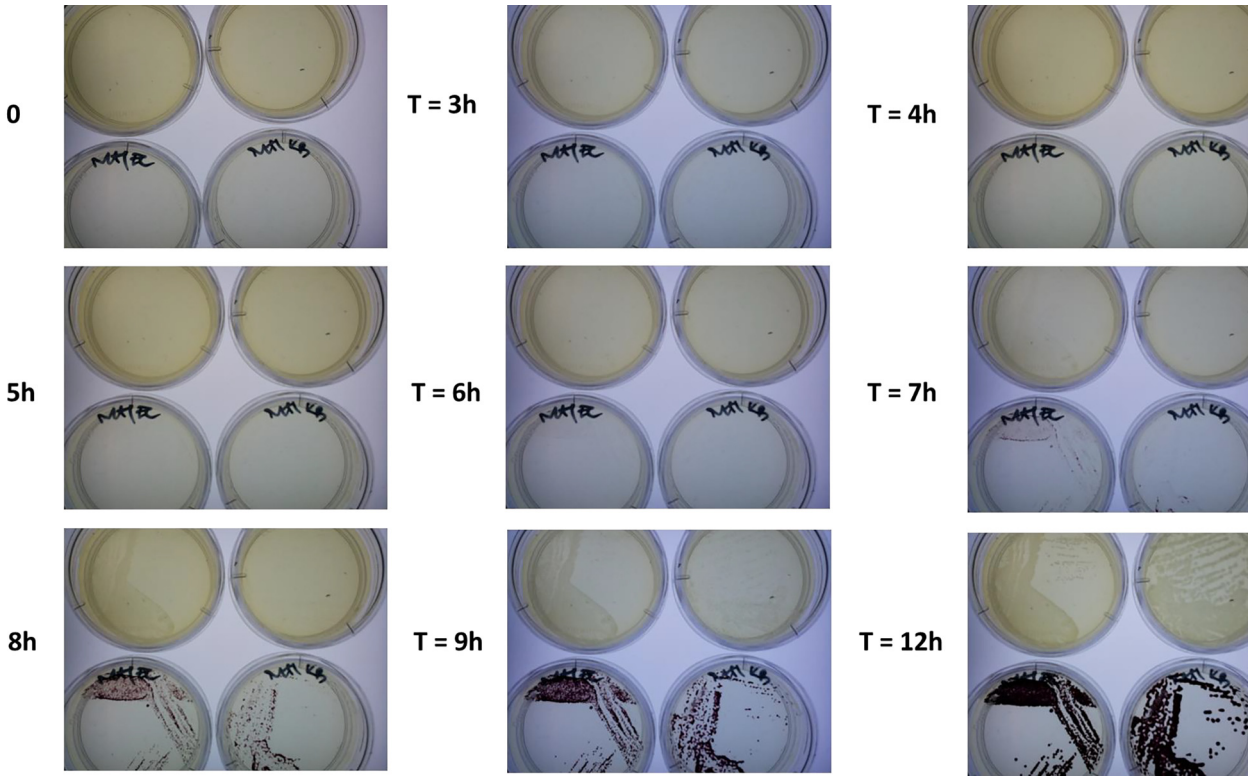

Fig. 6. Time lapse imaging of bacteria growth in petri dish. Images of *E.coli* (left) and *Klebsiella* (right) streaked on agar (top plates) and onto MH agar containing 0.5 mg/mL TTC dye to provide dark staining of microbial growth.

To observe traditional solid media culture, *E.coli* and *Klebsiella* colonies suspended in MH broth (normalised to 0.5 McFarland standard) were streaked onto 50 mm diameter Petri dishes. Images were taken every 10 min over 20 h using the PiRamid delivering a time-lapse video of bacterial growth either with unstained colonies, or the farm more clearly visible colonies when stained by TTC dye, often added for the enumeration of bacterial colonies on solid culture media (Fig. 6).

The final microbiological application we describe is motility testing. While conventional motility assays are interpreted at an endpoint, kinetic analysis might provide more detailed analysis of bacterial motility. For example, the effect of stimuli or inhibitors on growth and motility, such as inhibition, delays, or increased movement. Bacterial suspensions were stabbed into LB agar (0.4 %) containing TTC dye at a final concentration of 0.5 mg/ml in sterile polystyrene strip wells. TTC dye

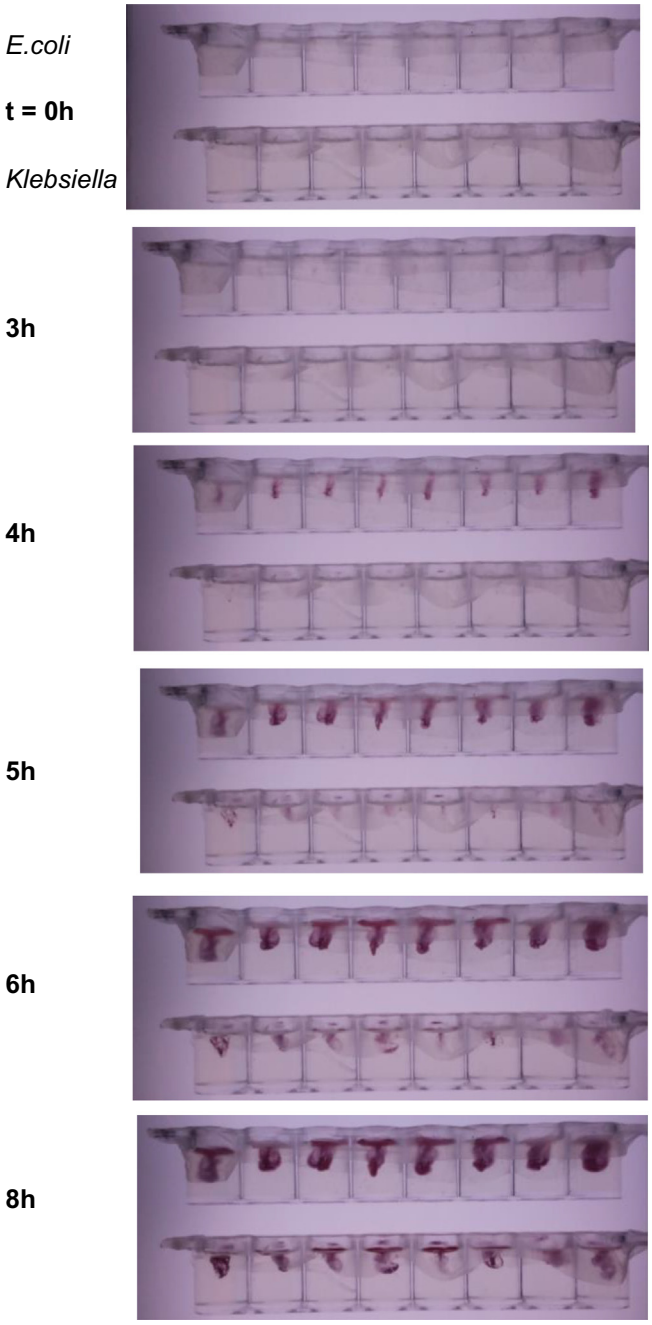

Fig. 7. Time lapse imaging of bacteria growth motility assay. Two species of bacteria (*E. coli* and *Klebsiella*) were stabbed into soft LB agar containing TTC dye at a final concentration of 0.5 mg/ml, which produces vivid dark staining where bacteria are present. This distinguishes motile non-motile bacteria enabling phenotypic identification. Here, *Klebsiella* is noted to be non-motile as only the immediate area inoculated with bacteria become stained; in contrast, a cloud of motile bacteria emerge from the stab site for *E. coli*.

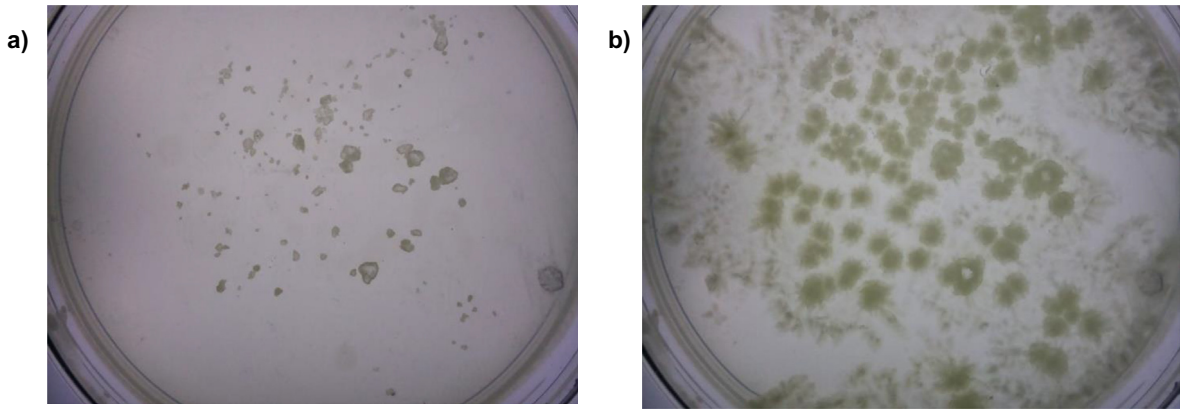

Fig. 8. Start and finish images of methyl anthranilate crystal formation Image of methyl anthranilate in water at start (a) with crystal growth appearing after 5 mins incubation at room temperature (b). Details of crystal growth can be seen with associated time lapse video available in the project repository https://zenodo.org/record/7090151.

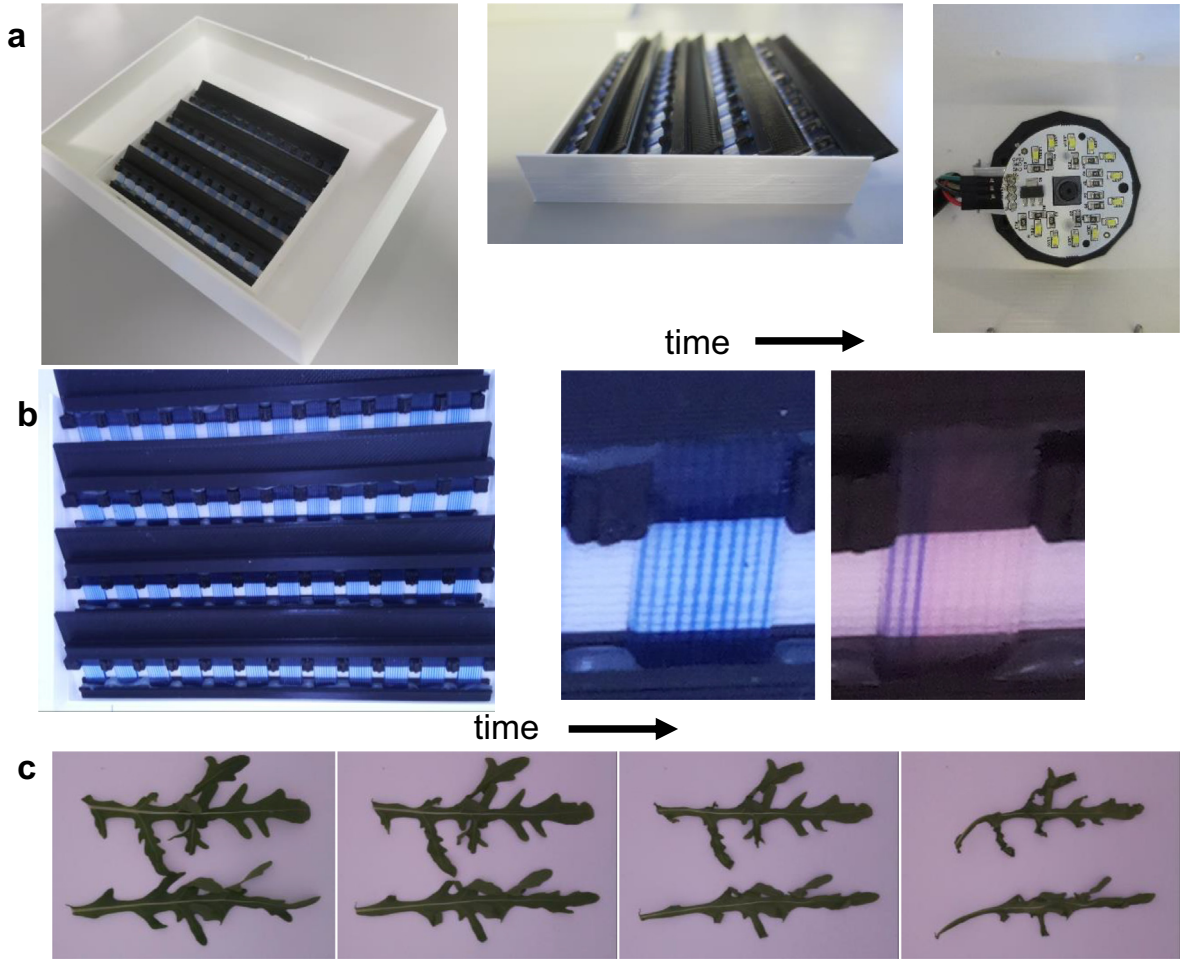

Fig. 9. Simple rapid customisation illustrated by adaptation for epi-illumination using LED light ring added to v2 camera module. a) Images of simple 3D printed base tray for 48 microfluidic test strips and LED ring light attachment inside top of PiRamid. b) Example image of 48x 10-capillary microfluidic bacterial growth strips (left), each containing a panel of antibiotics. Colorimetric growth by change over time from blue resazurin to pink-white can clearly be detected as well with top-light on white PLA 3D printed base tray (right) as with brightfield using LED panels under diffuser (Fig. 4). c) Toplight illumination clearly records deterioration of salad leaves over time, illustrating a broader range of samples can be imaged using distinct lighting configuration.

was added to stain bacteria improving image contrast. The images obtained clearly demonstrate the different behaviour of motile vs non-motile bacteria (Fig. 7). As expected, *Klebsiella* is non-motile with staining limited to the stab site; in contrast a cloud of *E. coli* moves away from the stab, indicating the ability to swim through the soft agar.

To explore alternative applications, we asked if the system could record dynamics of crystal growth, using methyl anthranilate. This is an ester of anthranilic acid and is widely used as a flavouring and scenting agent in food and drink products. Methyl anthranilate was chosen as a simple organic compound (melting point 24 ℃) that has been found to grow crystals readily (<5 mins). Other crystal systems take significantly longer to form crystals, which would benefit from automated viewing. Solid methyl anthranilate was heated at 37 ℃ in an incubator in a laboratory petri dish of water, followed by cooling to 4 ℃. When placed in the PiRamid at room temperature, a small amount of solid methyl anthranilate was added to seed crystallisation. The resulting crystal growth was imaged every-five seconds for up to five minutes in total and a time-lapse video was produced from the resulting images, with crystal growth clearly visible by comparing endpoint with start image (Fig. 8). This setup could be combined with real-time image analysis software to notify scientists when crystals have grown large enough for analysis. This could be a useful automation tool for panels of crystallisation samples without in-person supervision.

Finally, to demonstrate the flexibility of the system, we added an off-the-shelf LED ring module to the v2 camera, and replaced the base tray with a deeper tray, printed in white PLA to provide epi-illumination (Fig. 9). We tested this top-light configuration with a panel of 48 microfluidic microbiology test strips (480 × 1 µL samples) and with two salad leaves. Time-resolved imaging of the microcapillaries allowed bacterial growth kinetics to be detected, with similar results to the brightfield setup with backlight. Similarly, degradation of the salad leaves was clearly visible over time, with great potential to quantify shelf-life properties of food products. The rocket leaves were imaged every hour for 22 h at room temperature and the wilting of the leaves can be clearly observed.

In conclusion, PiRamid is useful as a compact, small experiment imaging device, and is limited by its size and therefore the number of samples capable of being imaged. However, for larger studies needing more samples its affordability and easy construction would allow the user to purchase the materials needed and construct with ease multiple devices that could be used in parallel. For example, for bacteriology and microfluidics, you could run experiments with greater numbers of isolates by using a cluster of multiple PiRamids.

For all above experiments, time-lapse videos can be located in the Zenodo repository, as indicated in Table 1.

Table 1

Figure	File name within published repository: https://zenodo.org/record/7090151 https://doi.org/10.5281/zenodo.7090151
Figure 5. Time lapse imaging of custom 3D printed agar dip slides.	**E.coliGrowthDipSlide**
Figure 6. Time lapse imaging of bacteria growth in petri dish.	**E.coli + KlebPetriDish**
Figure 7. Time lapse imaging of bacteria growth motility assay.	**E.coli + KlebMotility**
Figure 8. Time lapse imaging of methyl anthranilate crystal formation.	**MethylAnthranaliteCrystalFormation**

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Alexander D. Edwards With a background in fundamental immunology combined with expertise in biochemical engineering, I am an interdisciplinary researcher focussed on solving current and future healthcare challenges using an engineering science approach that combines a range of fields from biology, biochemistry, chemistry and physics. I work at the interface between academic technology discovery and industrial development and have experience of both fundamental research and the commercialisation of new technology. A major current focus is portable, affordable, microfluidic microbiology tests.

Chapter 4 – Challenges in developing a rapid antibiotic susceptibility test for bovine mastitis direct from infected cow's milk

Antibiotic susceptibility testing (AST) is a useful tool to help determine the resistance status of a potential mastitis-causing pathogen and inform the best course of treatment. Time to result is a key factor in aiding quicker and more effective treatment of mastitis and reducing the use of ineffective antibiotic treatment before bacterial detection. Therefore, a novel AST system must be rapid. A potential direction for this is to test directly from an infected sample, such as milk, to avoid laborious culture and bacterial isolation methods used in standard broth microdilution and disk diffusion AST. This is something that has been widely investigated in human medicine with direct AST being tested in human urine and blood culture, greatly reducing time to results and eliminating steps of culture and isolation (Needs et al., 2023; Jacobs et al., 2022). Therefore, this is a necessary step towards developing a rapid AST system for the treatment of bovine mastitis.

To begin to develop a rapid and miniaturised phenotypic AST directly from an infected milk sample, it was important to understand the challenges relating to the matrix effects of milk as a sample. Milk is often collected and stored in varying conditions and therefore can become spoiled, therefore this was also taken into consideration by the comparison of fresh, pasteurised milk and yoghurt as a simulated spoiled milk sample in a miniaturised AST system. It was decided that a spoiled milk sample be considered to simulate poor storage conditions, as some milk that may be tested will not be refrigerated or may be stored for longer periods. Spoiled milk may exhibit a similar drop in pH conditions to yoghurt, which in turn effects antibiotic activity. Yoghurt, however, does contain high levels of lactobacilli, which has the potential to interfere with resazurin reduction, but it generally susceptible to most antibiotics. Although it has its limitations, the use of yoghurt provides an interesting look into what can happen to AST when carried out directly in milk samples that are not fresh from a cow or refrigerated to reduce spoilage and pH changes. Moreover, not only does a milk sample collected from a dairy farm potentially contain a pathogen of interest related to a mastitis infection, but it can also harbour commensal bacteria from the surrounding environment and the cow itself (Oikonomou et al., 2014; Kuehn et al., 2013). Finally, it was decided to investigate the effect of result time on the performance of this miniaturised AST system on a simulated milk sample that may contain both a pathogen of interest and commensal bacteria, indicating the potential for false susceptibility in the presence of a high commensal bacterial load.

It was an objective to successfully design a test for the detection of bacteria in milk using microcapillary film (MCF) and to detect antimicrobial resistance in bacteria found in a milk sample directly using MCF. Some of the challenges that must be overcome to develop rapid and miniaturised devices with on-farm capabilities for detecting mastitis-causing pathogens were explored alongside in this chapter, including the effect of milk on the resazurin dye-based microfluidic assay, the presence of commensal organisms and their effect on AST in this device. Alongside, the aim was to validate the devices ability to carry out minimum inhibitory concentrations assays for the identification of resistant bacteria directly in milk samples, showing that milk can be directly tested using a growth indicator dye—resazurin in low-cost microfluidic devices capable of detecting bacterial growth and determining antibiotic resistance.

It was determined that sample dilution reduces the effects of milk on bacteria growth and observed MIC and reduces the interference of commensal bacteria on observed MIC and time to result in this system. It is possible to monitor Gram-negative bacterial growth colourimetrically even in the presence of milk and yoghurt (used to simulate spoiled milk samples), as long as this sample matrix was diluted 1:5 or more in growth medium. Growth detection kinetics using resazurin was not changed by milk at final concentrations of 20% or lower, but a significant delay was seen with yoghurt above 10%. The minimum inhibitory concentration (MIC) for ciprofloxacin and gentamicin was increased in the presence of higher concentrations of milk and yoghurt. When diluted to 1% all observed MIC were within range, indicating dilution may be sufficient to avoid milk matrix interfering with microfluidic AST. Finally, overlap is seen between mastitis-causing bacteria with commensal organisms proving difficulty to avoid false positive culture from the growth of commensals found in healthy milk. It is determined that a susceptible commensal would only be likely to mask the resistance of a pathogen in samples where the commensal cell density significantly exceeds the pathogen cell density. It was determined that a trade-off may be needed between the endpoint time chosen, and the accuracy of results obtained using direct milk testing, with earlier AST results reading possibly presenting more risk of false susceptibility. Longer time to results may be required. False susceptibility may lead to treatment with an inappropriate course of antimicrobial, therefore not treating the condition. Although time to treatment may be faster, it is essential that the information provided by the point-of-case AST is successful in informing treatment. This could potentially delay treatment with the correct antimicrobials , therefore further optimisation is needed to mitigate the effects of commensal organisms on this method of AST.

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Dilution Reduces Sample Matrix Effects for Rapid, Direct, and Miniaturised Phenotypic Antibiotic Susceptibility Tests for Bovine Mastitis

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Abstract: The time-consuming nature of current methods for detecting antimicrobial resistance (AMR) to guide mastitis treatment and for surveillance, drives innovation towards faster, easier, and more portable technology. Rapid on-farm testing could guide antibiotic selection, reducing misuse that contributes to resistance. We identify challenges that arise when developing miniaturized antibiotic susceptibility tests (AST) for rapid on-farm use directly in milk. We experimentally studied three factors: sample matrix (specifically milk or spoiled milk); the commensal bacteria found in fresh bovine milk; and result time on the performance of miniaturised AST. Microfluidic "dip-and-test" devices made from microcapillary film (MCF) were able to monitor Gram-negative bacterial growth colourimetrically even in the presence of milk and yoghurt (used to simulate spoiled milk samples), as long as this sample matrix was diluted 1:5 or more in growth medium. Growth detection kinetics using resazurin was not changed by milk at final concentrations of 20% or lower, but a significant delay was seen with yoghurt above 10%. The minimum inhibitory concentration (MIC) for ciprofloxacin and gentamicin was increased in the presence of higher concentrations of milk and yoghurt. When diluted to 1% all observed MIC were within range, indicating dilution may be sufficient to avoid milk matrix interfering with microfluidic AST. We found a median commensal cell count of 6×10^5 CFU/mL across 40 healthy milk samples and tested if these bacteria could alter microfluidic AST. We found that false susceptibility may be observed at early endpoint times if testing some pathogen and commensal mixtures. However, such errors are only expected to occur when a susceptible commensal organism is present at higher cell density relative to the resistant pathogen, and this can be avoided by reading at later endpoints, leading to a trade-off between accuracy and time-to-result. We conclude that with further optimisation, and additional studies of Gram-positive organisms, it should be possible to obtain rapid results for microfluidic AST, but a trade-off is needed between time-to-result, sample dilution, and accuracy.

Keywords: mastitis; milk; microfluidics; bacteria; AMR; resazurin

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1. Introduction

Increasing evidence shows that transmission of resistant bacterial strains from dairy farms to humans and the environment is possible [1], confirming that industrial and agricultural antibiotic use is one major driver for the endless rise in antimicrobial resistance (AMR) worldwide. As well as the serious threat to human health and public safety, treatment of our dairy herds becomes ever harder as antibiotics become less effective, with overuse and misuse of antibiotics recognised as areas for improvement [1,2]. Routes of AMR from farm to humans include food production—in the case of dairy, through milk and cheese products—and via direct contact with animals [3]. Safer and more cost-effective alternative treatments, combined with innovation in diagnostics and discovering new antibiotics, have been prioritised to tackle AMR. Of highest importance is to consider some

of the antibiotic classes that have been labelled as the most critically important antibiotics in dairy cattle including aminoglycosides, fluoroquinolones, third-, and fourth-generation cephalosporins and tetracyclines, as identified by the World Organisation of Animal Health (OIE) [4,5]. Equally important is maintaining appropriate testing and surveillance, so antibiotic susceptibility tests on clinical mastitis samples and bacterial isolates must be kept up-to-date to match current treatment practices and resistance profiles found in herds. Many emerging technologies are being developed that offer new ways to measure antibiotic susceptibility, which are needed to increase the frequency of testing, and ideally offer faster time-to-result.

Raw cow's milk has been shown to contain a host of microorganisms contributing to a complex microbiota potentially affecting the efficacy of rapid testing for pathogenic mastitis-causing bacteria. It was once considered that healthy cow mammary glands are sterile and that bacteria found in milk resulted from non-sterile milk sampling, advocating precaution in aseptic milk collection [6,7]. However, studies in the microbiota of milk and the application of more advanced detection methods have overturned this assumption, identifying the frequent presence of bacterial species in a healthy milk sample, and differentiating distinct groups of normal milk samples from healthy cows by the distribution of common species present [8]. High-throughput sequencing techniques and metagenomics permit the investigation of microbial communities—termed microbiomes—of which bovine milk has become of particular interest [9,10]. The status of these microbiomes can affect outcomes after mastitis treatment [11], and conversely, antibiotic treatment can alter the microbiome [12]. With this microbial complexity in mind, some of the most common pathogen genera isolated in mastitis milk samples are *staphylococci*, *enterobacteria* and *streptococci*, together causing the majority of clinical intramammary mastitis infections [13] with mastitis-infected quarters often demonstrating a much higher bacterial load than healthy quarters [9]. However, the milk microbiome in bovine mammary quarters free from intramammary infection and inflammation, with a low milk somatic cell count, has been found to be even more diverse than that seen in quarters with clinical mastitis [14,15] presenting a problem for rapid phenotypic and direct on-farm test for the presence of mastitis infection.

With a move towards on-farm diagnostics and the need for more convenient and portable diagnostic tools, innovations are emerging such as infrared thermography (IRT) for detecting inflammation, an indicator for mastitis [16], which may offer an alternative to the California mastitis test and may also offer additional benefits such as differentiating clinical mastitis from subclinical mastitis cases. IRT detection methods are based on infrared thermal images, particularly looking at udder skin surface temperature (USST). These studies have shown a positive correlation between USST and somatic cell count (SCC), with USST increasing with SCC, with little effect of external environmental factors [17]. New phenotypic rapid testing kits, such as MastDecide (MastDecide, Quidee GmbH, Homberg, Germany), are aiming to reduce the future over-usage of antimicrobials in dairy cows with the greater aim to decrease antimicrobial resistance. Rapid detection of mastitis-causing pathogens is necessary for early detection and treatment [18], with rapid direct sample tube test systems such as MastDecide providing positive results in >14 h [19] in comparison to conventional agar growth of 24–48 h or longer to result. Current point-of-care (POC) testing is often culture-based and includes some form of a modified agar plate, petrifilm or tube-based system, such as MastDecide. However, other more recent POC devices, such as Point-of-Cow and Mastatest [20], utilise consumables and equipment that are more sophisticated and more expensive to further improve ease of use. Culture-based tests all have similar time to results of 24–48 h. Although this is fast enough to inform decision making, farmers would prefer less delay with turn-around from one milking to the next [21,22], leading to greater interest in developing rapid on-farm tests.

Our group developed a "lab-on-a-stick" concept for performing functional assays on cells including bacterial growth assays, allowing both phenotypical identification and the quantitative measurement of antibiotic susceptibility [23]. This may be extrapolated to the identification of mastitis-causing bacteria from infected milk. If adapted for rapid

susceptibility testing, this technology could aid in the prescription of the correct antibiotic for treatment by rapidly indicating antibiotic resistance or susceptibility in specific strains of bacteria, improving the quality of on-farm treatment. The targeting of antibiotic treatment based on an antibiotic susceptibility test is helpful in avoiding antibiotic change due to inefficacy. However, it is important to note that any use of antibiotics in mastitis treatment will promote AMR. This technology has the potential to transport bacterial assays outside the lab and into the field for more rapid results. With this idea of on-farm treatment and diagnostics in the field in mind, we considered the use of smartphones and portable high-quality cameras to read functional cellular assays for measuring bacterial growth or death when combined with antibiotics [24,25]. The combination of low-cost microcapillary devices with smartphone imaging offers the potential for portable and field AMR detection. When phenotypically identifying bacterial species and quantifying antibiotic susceptibility, many microbiological assays rely on the identification of a colour or fluorescence change. Smartphones and digital camera modules can be used as digital sensors for colour-based bioassays, to directly capture images to digitise colourimetric and fluorescence changes [24,26]. When automated with time-lapse imaging, monitoring the growth of bacteria in different antibiotic conditions allows the identification of the correct class and concentration of antibiotics that would need to be administered to each case of mastitis. High-throughput laboratory devices have likewise been developed to provide greater flexibility in analysing the problem of AMR [27,28]. Where an automated process for microbiological techniques can improve sample time [18], the use of microfluidic technology to detect pathogens and measure AMR may offer a point-of-treatment technique. This might avoid mastitis bacteria identification in a laborious and time-consuming manner within laboratories [29]. Miniaturized devices analyse small sample volumes reducing reagent consumption and therefore potentially lowering cost [30]. Reducing space requirements compared with large agar Petri dishes may also simplify high-throughput screening even in smaller laboratories or on-farm. Simple and high-throughput microcapillary techniques can be used to test uropathogenic species of bacteria, quantify bacterial concentrations (CFU/mL) and detect resistance through minimum inhibitory concentration MIC AST [31,32], raising the possibility that point-of-care testing for mastitis infections may be possible using test volumes as small as 1 microlitre.

Here, we explore some of the challenges that must be overcome to develop miniaturized and/or rapid devices with on-farm capabilities for detecting mastitis-causing pathogens and to carry out minimum inhibitory concentrations assays for the identification of resistant bacteria directly in milk samples. We show that milk can be directly tested using a metabolic-sensitive growth indicator dye—resazurin—in low-cost microfluidic devices capable of detecting bacterial growth and determining antibiotic resistance. We explore the milk sample matrix interference on growth detection and direct antibiotic resistance testing. As fresh milk even from healthy cows contains an abundance of commensal bacteria that can interfere with the detection of mastitis pathogens, we explore if the commensal bacterial counts in healthy samples could be expected to interfere with pathogen detection. Finally, we test if false susceptibility could be observed in a sample containing a mixed population of bacteria, specifically testing simulated samples containing a susceptible commensal organism alongside a resistant pathogen.

2. Results

2.1. Outlining Challenges with Direct Rapid Field Antibiotic Susceptibility Testing

We examined the current diagnostic pathway to identify major challenges that need to be overcome to achieve rapid direct antibiotic susceptibility testing (AST) in milk from cows suffering from mastitis. We then experimentally tested if these challenges arise using a low-cost, scalable microfluidic platform. Currently, diagnosis relies on clinical presentation combined with tests such as somatic cell count or the California milk test (Figure 1a). Milk samples from cows with diagnosed mastitis are typically subsequently tested in laboratories using standard culture methods (Figure 1b). To be effective, direct AST methods need

to avoid the milk sample matrix affecting results, avoid incorrect susceptibility scoring through interference by commensal organisms present in milk, and maintain accurate results at earlier time points and/or with smaller test volumes (Figure 1c). We explored the impact of these parameters using a low-cost and scalable microfluidic method that uses microcapillary film (MCF) test strips (Figure 1d) to kinetically monitor microbial growth using resazurin colourimetric conversion.

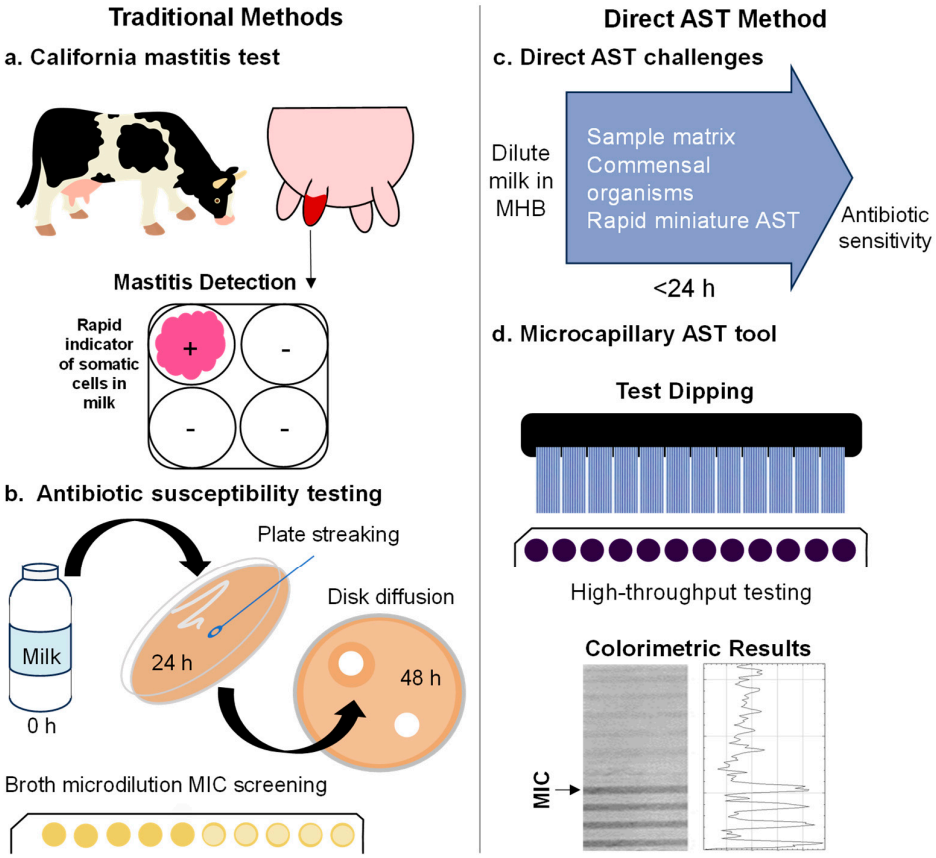

Figure 1. Direct microfluidic antibiotic susceptibility testing and viable cell quantitation using resazurin in cow's milk compared to traditional methods of mastitis diagnosis and antibiotic susceptibility testing. (**a**) Traditional methods of diagnosing mastitis in cattle include the California milk test, indicating the presence of a high number of somatic cells within an infected milk sample. (**b**) Confirmation of infection and antibiotic susceptibility is carried out in lab using traditional plate count methods for enumeration of cells and disk diffusion or full MIC in well plates for antibiotic susceptibility. (**c**) Microfluidic methods described can be used to directly test milk samples, with simple dilution of milk and addition of resazurin dye. Antibiotic–coated MCF strips can then be dipped into individual milk samples. (**d**) Results allow for the enumeration of bacterial content and antibiotic susceptibility by MIC determination. Colour change can be detected by eye and confirmed by absorbance values from image analysis (ImageJ, Version 1.53t).

2.2. Colourimetric Microfluidic Bacterial Growth Detection in the Presence of Milk

Firstly, milk is strongly light scattering and opaque, so we tested milk matrix interference on colourimetric detection, to determine whether bacterial growth can be monitored by time-lapse imaging directly in milk. Pasteurised, homogenized supermarket milk—chosen for its low bacterial load and to improve experimental repeatability, significantly altered the visual appearance of microcapillary devices and digital images compared to Mueller–Hinton (MH) broth alone. We presumed this is due to strong light-scattering properties. Some differences in calculated absorbances within individual capillaries were detected (Figure 2a,b). Surprisingly, although visually different, with the transillumination imaging

setup used here [33], clear changes in red channel absorbance still indicated that metabolic conversion of resazurin is easy to quantify colourimetrically in the presence of milk. Only the highest milk concentrations (50% final concentration) gave a large enough increase in baseline absorbance to reduce the growth detection measurement range. Equivalent concentrations of natural yoghurt were used to simulate spoiled milk and explore potential problems if poorly stored samples affected growth detection. We used yoghurt as a consistent representation of aged milk samples where microbial growth may affect composition for example by decreasing pH. Yoghurt affected colourimetric growth detection more strongly than milk, with altered absorbance values between time 0 h and 16 h in yoghurt not only at 50% but also at 20%. This suggests that at least 1:10-fold dilution may be needed, to reduce the risk of milk matrix interference, if there is any risk that samples may have spoiled prior to testing.

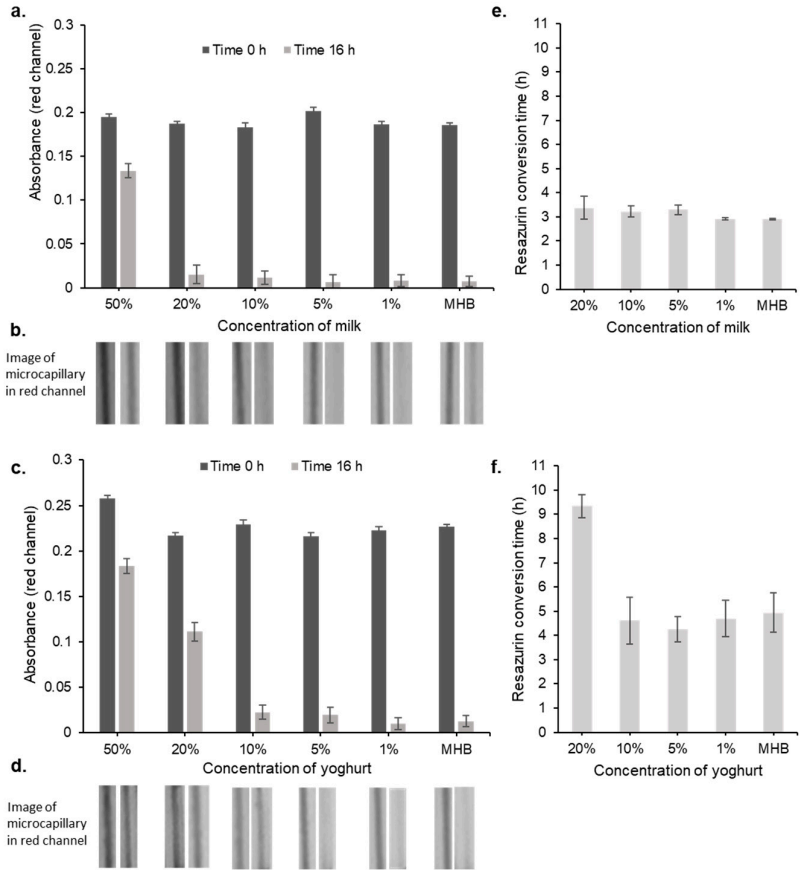

Figure 2. Milk sample matrix effect on absorbance of resazurin dye. Mean absorbance values and representative capillary images of resazurin dye before and after overnight incubation with *E. coli* 25,922 in the presence of milk (**a**,**b**) or (**c**,**d**) yoghurt. Bacterial growth results in resazurin conversion and decrease in absorbance. Time to resazurin conversion in the presence of milk (**e**) or yoghurt (**f**) diluted in Mueller–Hinton (MH) broth was recorded when spiked with 5×10^5 CFU/mL *E.coli* 25,922. Error bars indicate ± standard error of the mean (n = 3).

Time-lapse images permit growth kinetics to be estimated [34]. When growth kinetic in 20% or lower milk concentrations were compared, no delay in resazurin conversion was detected. The growth rate of *E. coli* 25299 and time to resazurin conversion was independent of the concentration of milk for 20% milk and lower, indicating that 1:5 sample dilution may be enough to avoid milk matrix interference in growth detection even at earlier endpoints for faster time-to-result (Figure 2e). However, a greater effect on time to resazurin conversion was found with yoghurt, with 20% yoghurt taking >9 h for growth to be detected (Figure 2f). With 1:10 dilution or lower, there was no delay.

2.3. Impact of Milk Sample Matrix on Antibiotic Minimum Inhibitory Concentration Measurement

Having established that growth can be detected colourimetrically in the presence of supermarket milk, we examined if milk affects the observed MIC of gentamicin and ciprofloxacin on *E. coli* 25922. At 1:5 or 1:10 dilution, milk increased the MIC of gentamicin above the expected range, but no effect was seen of 20% final concentration of milk on ciprofloxacin, with MIC values in the target range between 0.016 and 0.004 mg/L (Figure 3). Yoghurt had a greater impact on the observed MIC of *E. coli* 25922 for both ciprofloxacin and gentamicin. All concentrations of milk did not affect the MIC falling in the expected range for ciprofloxacin. This indicates that although milk may not affect some antibiotics, it may affect others, and it would be important to dilute direct milk samples to carry out MIC determination. At 1% milk the MIC for gentamicin is within the expected range (1–0.25 mg/L) suggesting it may be necessary to use dilutions of 1:100 to reduce the risk that milk affects MIC determination. Yoghurt showed a much greater effect on AST for both antibiotics.

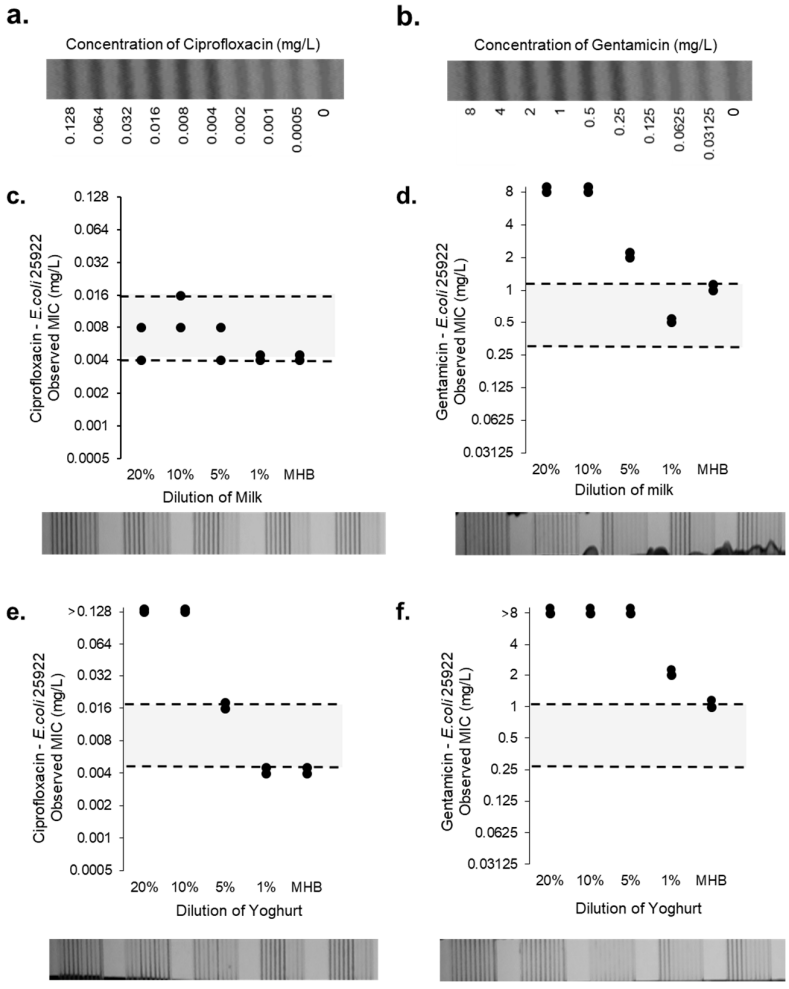

Figure 3. MIC determination for ciprofloxacin and gentamicin for *E. coli* 25922 QC strain in differing dilutions of milk and yoghurt in MCF (n = 2). MIC test strips concentrations for ciprofloxacin (**a**) and gentamicin (**b**) performed by spiking *E.coli* 25922 (5×10^5 CFU/mL) in differing dilutions of pasteurised sterile (**c**,**d**) milk and (**e**,**f**) yoghurt diluted in MHB. Grey area indicates the acceptable MIC range for *E. coli* 25922. Bacterial growth results in resazurin conversion and decrease in absorbance. Images of milk and yoghurt MIC experiments are shown below. Images indicate the microcapillary test strips after overnight incubation.

2.4. Exploring Whether Commensal Organisms in Milk Might Affect Direct Microfluidic AST

Fresh milk samples even from healthy cows have a significant bacterial load, and we first measured this to help understand how the presence of these organisms might affect rapid direct antibiotic susceptibility testing. Commensal organisms were counted and isolated from 40 normal farm milk samples from a dairy farm (CEDAR). Plate counts were as high as 10^7 CFU/mL but with a median bacterial count of 6×10^5 CFU/mL (Figure 4).

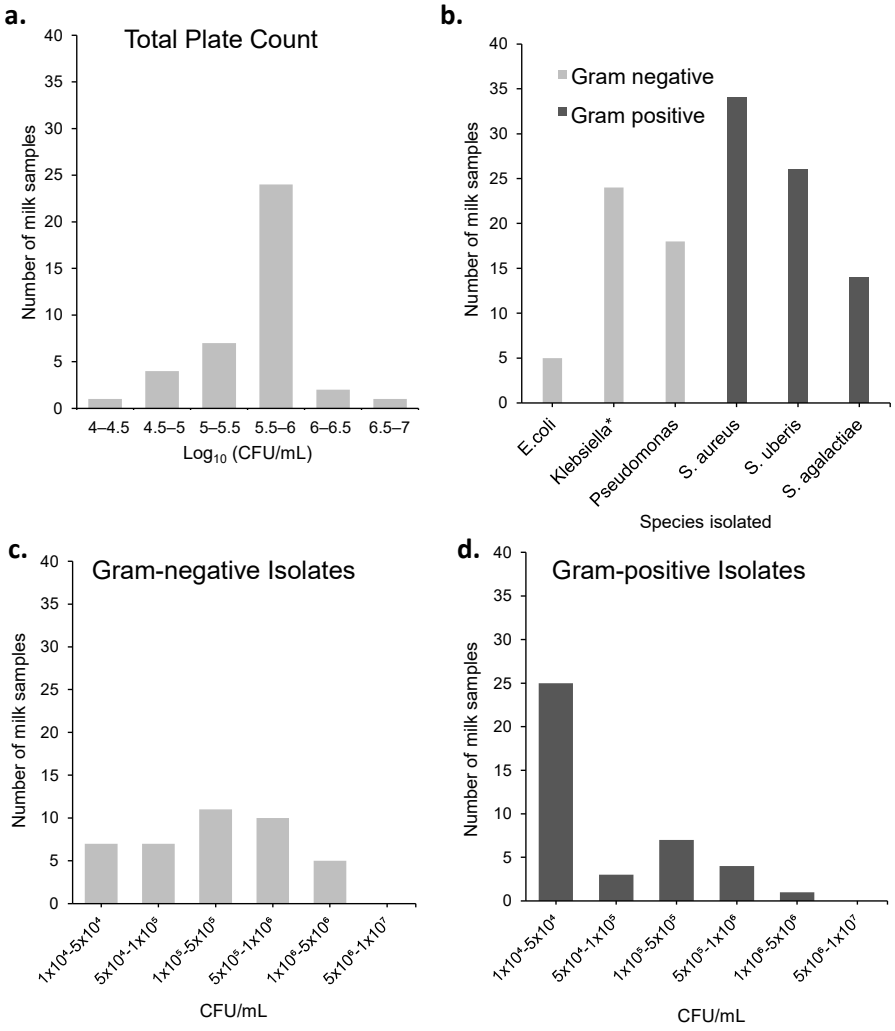

Figure 4. Counts in CFU/mL of bacteria isolated from 40 individual milk samples on plate count agar. (**a**) A bar chart of total plate counts from 40 individual milk samples from dairy cows. (**b**) Species of organisms isolated. * Indicates coliform species identified using CHROMagar Mastitis GN (Paris, France), presumed *Klebsiella* spp. (**c**) CFU/mL of Gram-negative organisms isolated. (**d**) CFU/mL of Gram-positive organisms isolated.

Many different species were identified indicating the broad range of organisms present, most of which can be detected with resazurin. The majority of organisms were identified as Gram-positive, with the most frequent Gram-positive being *Staphylococcus aureus*. The most abundant Gram-negative commensals were *Klebsiella* spp. and other coliforms. The least common organism across all milk samples was *E.coli*. Although Gram-positive bacteria were the most abundant across all samples, they typically had lower cell densities with the majority of samples containing 1–5 $\times$ 10^4 Gram-positive CFU/mL. On the other hand, Gram-negative bacterial cell densities were typically between 1×10^5 and 1×10^6 CFU/mL.

The limit of growth detection for microcapillaries is 1 CFU [34] with a test volume of 1 microlitre; thus, if tested without dilution, all of these milk samples would eventually

show detectable bacterial growth through resazurin conversion. However, the median cell densities are—as expected—somewhat lower than levels expected with clinical mastitis samples, where around 10^6 CFU/mL appear to be typical [35]. Given that dilution is needed to avoid the milk matrix interfering with AST, it may be possible to optimize the dilution to differentiate between these different ranges of bacterial cell densities between pathogen and commensal. But as there is limited correlation between bacterial cell loads and infection, diagnosis of mastitis should not rely on bacteriological culture methods, instead clinical signs, and diagnostic tests such as the California mastitis test are more appropriate. Instead, the focus of rapid tests should be on the antibiotic susceptibility of the organisms present.

As well as considering if growth might be detected through the presence of commensal organisms found in healthy milk samples, it is critical to understand if the presence of commensals alongside pathogenic bacteria could lead to incorrect antibiotic susceptibility scores. Of particular concern is false susceptibility—where an ineffective antibiotic might be selected based on test results, leading to treatment failure and antibiotic misuse. If an AST indicates false susceptibility compared to reference standard methods, this is classified as a very major error. We predicted growth detection curves for mixed bacteria samples to determine if false susceptibility results could theoretically occur, then used simple experiments to test if this can occur in practice.

We considered theoretically what would happen in a direct test where the sample contained a fast-growing susceptible commensal organism in higher abundance than a resistant pathogen. Simulated growth curves were produced to give a visual representation of expected resazurin conversion (Figure 5). For a window of time, there could be an absence of growth detection in the presence of antibiotics during which time the test may indicate a susceptible organism; however, at later timepoints when the less abundant pathogen growth is detected, the resistance will become clear. This problem could be particularly challenging in samples where the commensal organism(s) are faster growing than the pathogen. Although it is also possible to observe false resistance with a susceptible pathogen mixed with resistant commensal, false resistance is less risky in practice, as this result would only result in avoiding the use of a potentially effective antibiotic, not lead to misuse of an ineffective treatment.

To determine experimentally if this predicted false susceptibility can occur, we selected one resistant pathogen and one susceptible commensal isolate from farm milk samples, and mixed them at different ratios, keeping the overall cell density constant. When these simulated samples were tested using direct microcapillary AST, we plotted growth detection kinetics in the presence or absence of the minimum inhibitory concentration of antibiotic. As predicted (Figure 5) we found that if a susceptible commensal isolate is included at far high concentrations than a resistant pathogen, for a window of time false susceptibility is apparent (Figure 6). Note the organisms selected for this test have different growth rates, with a fast-growing *Klebsiella* isolate and a slower-growing *Pseudomonas* isolate. Growth rates within microcapillaries were calculated by comparing resazurin conversion times for 10-fold dilutions of each organism as described previously [34], with observed doubling times of 20.5 min (*Klebsiella*) and 34.9 min (*Pseudomonas*), which is why growth was detected later for the latter (6–8 h) than the former (2–4 h) at the same starting density (Figure 6a,b). When the two organisms are combined 1:1 (Figure 6b) there is as expected a narrow time period when false susceptibility is observed, before growth of the resistant *Klebsiella* can be detected (between 2 and 4 h, Figure 6c). The colour intensity recorded with this imaging system can drift during an experiment, as seen with resistant *Klebsiella* in the presence of antibiotics (Figure 6b) but never falls below 0.6 of starting absorbance without bacterial growth. The longest period of false susceptibility was, as predicted, seen with a far lower inoculum of resistant *Pseudomonas* than susceptible *Klebsiella* where growth was only detected at 12–14 h (Figure 6d).

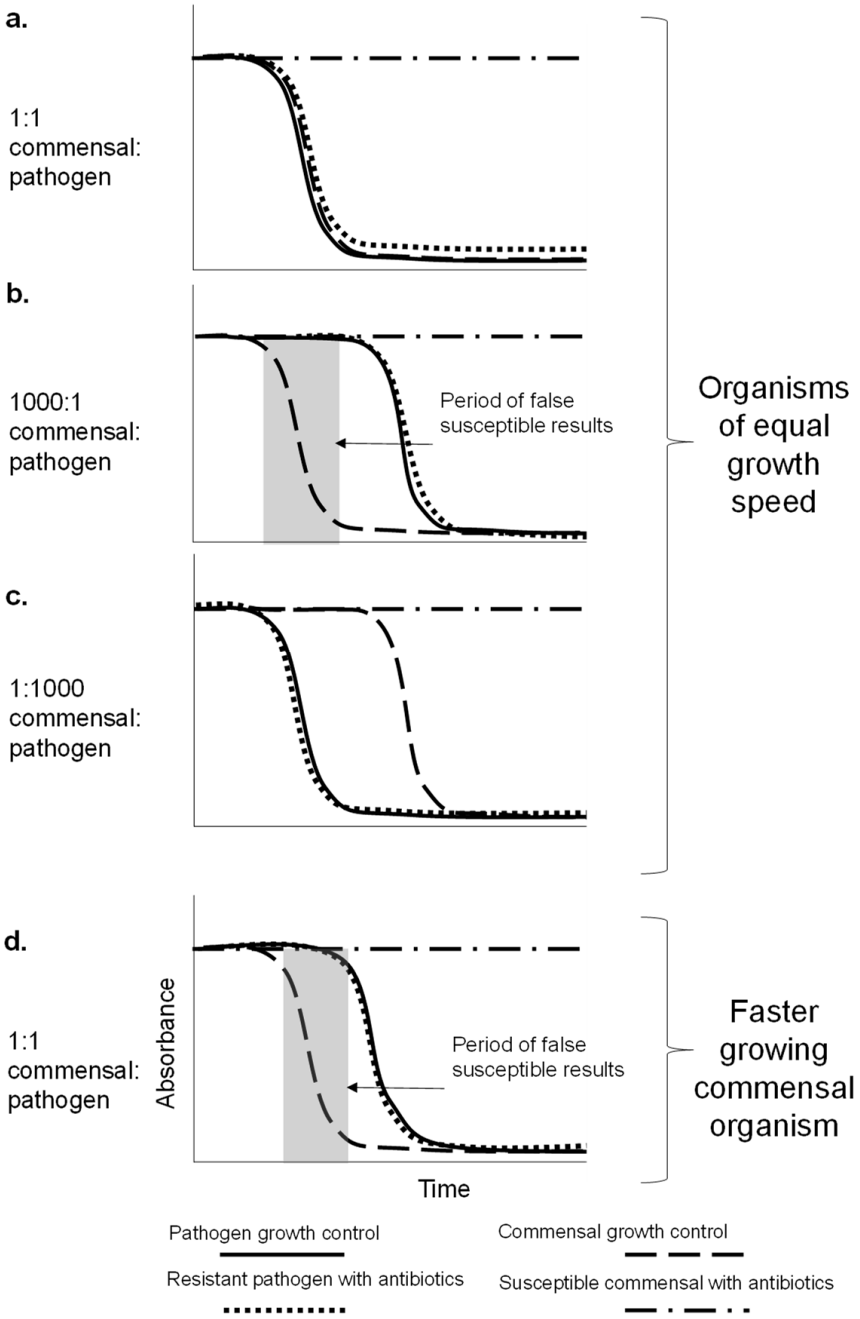

Figure 5. The potential problem of false susceptibility for direct antibiotic susceptibility testing of a milk sample containing a pathogen and commensal bacterial organisms. (**a**) No false susceptibility would be observed when the resistant pathogen and susceptible commensal are at the same concentration (1:1) and grow at equal speeds. (**b**) False susceptibility is observed where the susceptible commensal has a higher concentration than the resistant pathogen as no growth will be seen at early time points (1000:1 pathogen to commensal) and grow at equal speeds. (**c**) No false susceptibility resistant pathogen has a higher concentration than the susceptible commensal (1000:1 pathogen to commensal) and grow at equal speeds. (**d**) False susceptibility is observed when the resistant pathogen and susceptible commensal are at the same concentration (1:1) and the commensal is a faster-growing organism. Bacterial growth results in resazurin conversion and decrease in absorbance.

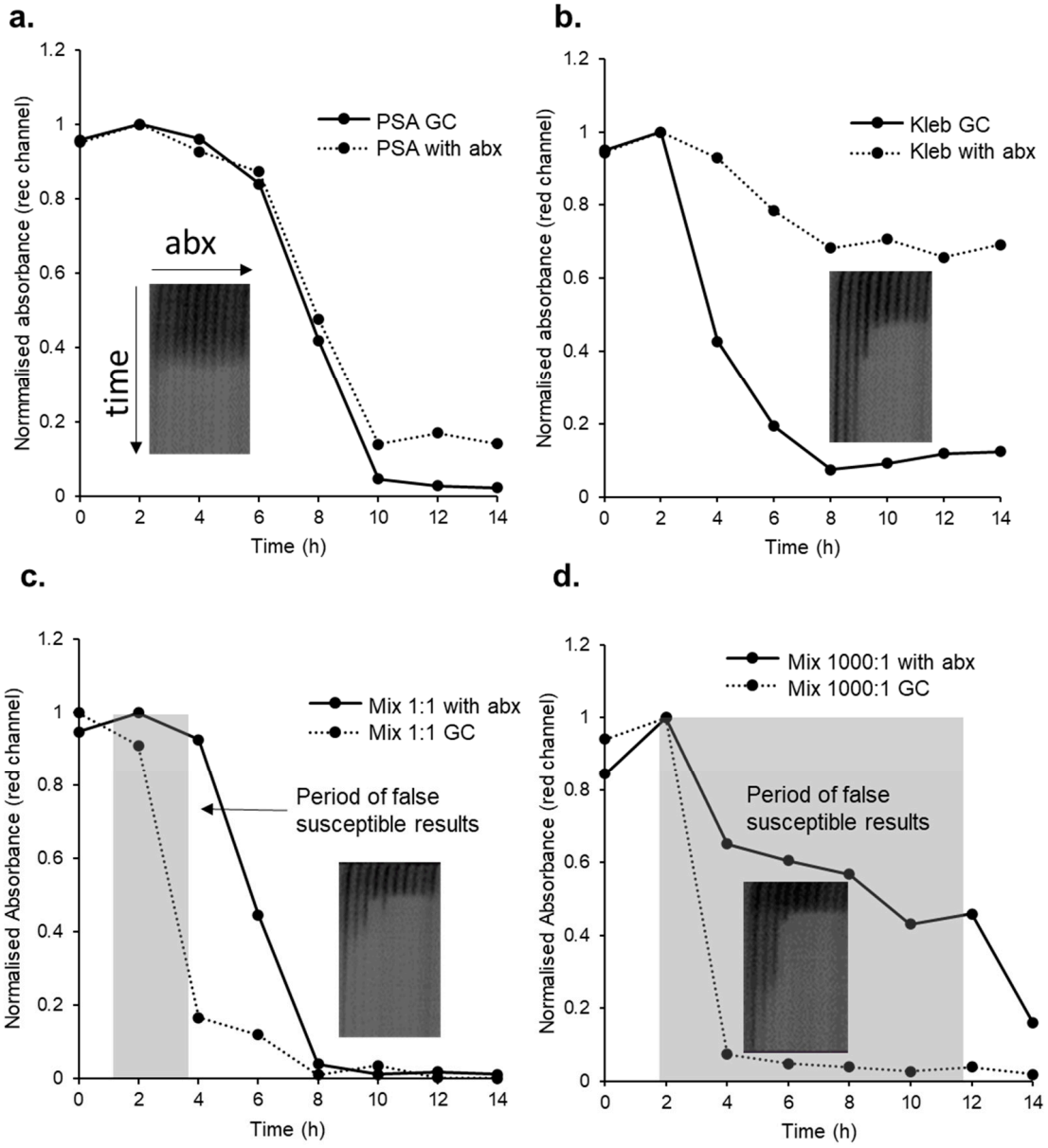

Figure 6. False susceptibility when a susceptible coliform (Kleb; *Klebsiella* spp.) commensal organism in is higher abundance than a resistant mastitis-causing pathogen (PSA; *Pseudomonas* spp.) in the presence of ciprofloxacin (n = 3). Inset images show the full MIC test strip with 9 antibiotic concentrations, capillary 1 indicates 0.5 mg/L ciprofloxacin with decreasing in $\log_2$ dilutions from left to right plus no antibiotic—termed growth control (GC) at the far right. Time course images indicate 18 h incubation time. Bacterial growth results in resazurin conversion and decrease in absorbance. (**a**) Growth kinetics of the resistant pathogen alone with and without ciprofloxacin at the MIC for the commensal (0.125 mg/L, capillary 3); GC indicates growth control without the presence of antibiotics. (**b**) Growth kinetics of commensal with and without ciprofloxacin at the MIC for the commensal (0.125 mg/L). (**c**) Pathogen and commensal at equal inoculum densities with and without ciprofloxacin at the MIC of the commensal (0.125 mg/L). Period of false susceptibility present. Total plate count is 8×10^4 CFU/mL (**d**) Growth kinetics of the resistant pathogen spiked at 10^4 CFU/mL and susceptible commensal at 10^7 CFU/mL, total plate count is 3×10^6 CFU/mL, with and without ciprofloxacin at the MIC of the commensal (0.125 mg/L). Period of false susceptibility present. Colour change caused by growth of the resistant pathogen in the presence of antibiotics not evident until after 10 h. abx = antibiotics.

3. Discussion

Despite the vital drive towards improved antibiotic stewardship, it has proved hard to deliver cost-effective and successful rapid AST that can inform treatment decisions by providing results fast enough to avoid days of treatment delay waiting for conventional laboratory culture methods. This is, in part, because the standard method has been highly optimized, and so changes to these methods, which might include mixed populations and/or interfering sample matrix components, can make the results more difficult to interpret. Here, we used an inexpensive microfluidic AST to examine these challenges in detail.

Direct from sample AST can potentially offer significant time saving by removing at least one overnight incubation step. Depending on the testing protocol it will also simplify sample preparation and cut handling time, offering the additional benefit of allowing tests to be performed outside of a lab, for example facilitating on-farm testing and avoiding sample transportation. We suggest that one of the simplest sample preparation options would be dilution into Mueller–Hinton broth medium. In this workflow, the dilution factor must balance reducing sample matrix interference with the uncertainty of an unknown inoculum population and density. We observed that with lower dilution factors (1:2 and 1:5) of milk or yoghurt (simulating poorly stored or spoiled sample) the colourimetric readout of resazurin was affected and growth delayed, thus further dilution may be essential to avoid sample matrix interference. It is also likely that for higher bacterial density samples, higher dilutions would be needed to avoid inoculum effects that we previously found can interfere with microfluidic AST [36]. We must also consider the potential bacteriostatic effects of lactic acid-producing *Lactobacilli* that will be particularly present in spoiled samples as in yoghurt, which by increasing membrane permeability will inhibit the growth of some Gram-negative species [37].

When considering the average bacterial cell count of a mastitis milk sample, it is difficult to find a definitive number and sample-to-sample variation is high. However, for commensal bacteria, we see legislation within the EU suggesting that 'healthy' raw milk can have up to 10^5 CFU/mL before processing [38,39]. We would expect milk from an infected sample to have much higher numbers of bacteria compared to normal. However, in the case of subclinical mastitis, lower bacterial loads may remain undetected and fall within the normal bacteria microbiome found in a healthy milk sample. The standard methods for AST use an inoculum range of 2–8 $\times$ 10^5 CFU/mL, which suggests that a mastitis sample would need to be diluted by at least 1:10 or more, reducing any risk of the milk sample matrix interfering with bacterial growth detection or susceptibility scores, given we found no effect on MIC values of milk diluted this much (Figures 2 and 3).

The commensal bacteria loads that we measured in the fresh milk samples were representative of fresh uninfected milk samples. This was carried out to provide information on the commensal populations present in fresh milk. Almost all were below these limits, suggesting the abundance of these organisms is as expected. Although careful sampling of milk is a prerequisite and will help to reduce the bacterial load of commensals, differing farming practices may make sample collection for an on-site rapid test difficult to control universally. The distribution of organisms that were found in a normal healthy milk sample is quite typical with the occurrence of commensal *Streptococcus* species particularly apparent in cow's milk [40]. Although some species detected are associated with infection, such as *E.coli*, *Staphylococcus aureus*, and *Klebsiella*, it is not uncommon for milk to become contaminated with these bacteria through contact of the udder with other areas of the dairy farm environment [41,42], manure, bedding, and other farm equipment [43]. *Klebsiella* spp. is also fast becoming one of the most common contaminants of milk samples, coming second in prevalence only to *E.coli* of Gram-negative bacteria in cow udders [43]. *Pseudomonas aeruginosa* is one of the pathogenic bacteria responsible for bovine mastitis, often with all isolates studied in one study having a genotype associated with increased SCC [44]. Although it is opportunistic and infections are infrequent, clinical mastitis caused by *Pseudomonas* species is often sporadic and severe with high rates of mortality [45,46],

indicating a potential clinical significance as a pathogenic organism. Further work would benefit from the study of milk samples with both clinical and subclinical mastitis alongside healthy samples, to better understand the distribution of organisms during an infection, requiring access to a suitable number of samples. Consideration of the "resistome" of the milk microbiome during clinical mastitis is also critical [47] and it is possible that direct measurement of mixtures containing commensals plus pathogens would indicate the response to antibiotic treatment better. However, for the purpose of developing a direct test, at this early stage using a mastitis sample with a varied bacterial environment would provide conditions that are sufficiently complex that it may be too difficult to control enough to deliver robust results.

Standard AST methods require specific media, MH broth, selected to minimise interference with antibiotic activity. It is clear that changing this medium can affect observed MIC results, including pH [36,48]. Our findings indicate that dilution of milk 1:5 or 1:10 may be sufficient to reduce any impact of the milk sample matrix on growth detection and changes in MIC.

Given there is an overlap between bacterial cell densities found in mastitis samples with commensal organisms it may prove harder to avoid false positive culture from the growth of commensals found in healthy milk. If the only purpose of testing is to measure susceptibility, however, and other tests are used to diagnose infection, rapid testing should not be used to detect microorganism growth. Instead, there remains a risk of additional organisms other than the pathogen (including commensal microbiota and any contaminants during sampling) affecting the susceptibility test. We determined that a susceptible commensal would only be likely to mask the resistance of a pathogen in samples where the commensal cell density significantly exceeds the pathogen cell density.

Finally, a trade-off may be required between the endpoint time chosen, and the accuracy of results obtained using direct milk testing, with earlier readouts possibly presenting more risk of false susceptibility. Further extensive analysis of milk samples from mastitis cases is now planned with this technology, to determine how frequently such very major errors in susceptibility might occur. Moreover, further optimization of the methodology is ongoing to better understand the behaviour of Gram-positive organisms. This will be essential to ensure that Gram-positive bacteria can also be measured in milk, which is vital for application to mastitis sample testing given how common Gram-positive infections are.

4. Materials and Methods

4.1. Bacterial Isolates and Reagents

Escherichia coli 25922 was used as our quality control strain. *Pseudomonas aeruginosa* ATCC 12903 was used as our mock pathogen and a *Klebsiella* spp. was isolated from fresh farm milk. Ciprofloxacin and gentamicin were purchased from Sigma Aldrich (Gillingham, UK). Pasteurised supermarket milk from The Cooperative Food Company (Manchester, UK) was chosen to standardise experiments, and because it has a low bacterial load following processing. Yeo Valley natural yoghurt was purchased from The Cooperative Food Company. Individual milk samples were collected from healthy cows from the Centre for Dairy Research (CEDAR) (Reading, UK) farm (n = 40). Milk samples were tapped off of the automated milking parlour system into sterile collecting pots and labelled according to cow number. Milk samples were immediately transported back to the microbiology lab (~15 mins transportation time).

4.2. Preparation of Antibiotic Microcapillary Dip-Stick Test Strips

Microcapillary antibiotic test strips were prepared as previously described [31]. Briefly, MCF of 1 m lengths were coated internally with a hydrophilic layer of 5 mg mL^{-1} solution of polyvinyl alcohol (PVOH, MW 146,000–186,000, >99% hydrolysed, Sigma-Aldrich) in water and incubated at room temperature for 2 h. Coated strips were washed with 0.5% Tween 20 in water (Sigma-Aldrich, UK) to remove residual PVOH, and dried on a vacuum manifold for 20 min. Ciprofloxacin and gentamicin antibiotic stock solutions were prepared.

Final concentrations of antibiotics indicated in the text, diluted in sterile Mili-Q water, were injected into individual capillaries using a sterile 30 G needle. The MCF was cut into 17 mm individual test strips and frozen overnight at −80 °C. Test strips were freeze-dried for >4 h on an Edwards Modulyo freeze drier. Test strips were placed into custom 3D-printed holders and were vacuum packed and stored at −20 °C until use.

4.3. Microcapillary Antibiotic Susceptibility Test

Bacterial strains were routinely grown from glycerol stocks overnight on LB agar. Approximately 4–5 colonies were grown in Mueller–Hinton (MH) broth for several hours until turbid and adjusted to 0.5 McFarland standard, according to CLSI guidelines. Bacterial suspensions were diluted a further 1:150 in MH broth and 100 µL of bacterial suspension was added to 100 µL 0.5 mg/mL resazurin in MH broth to give a final concentration of 0.25 mg/mL resazurin and 5×10^5 CFU/mL bacteria in a 96 well plate. Microcapillary test strips were dipped into the wells and the strips sealed with silicone grease to avoid evaporation. Samples were incubated at 37 °C overnight, colour change was time-lapse imaged using Raspberry Pi-operated automated imaging devices PiRamid [33]. The MIC was determined based on the lowest concentration that inhibited the reduction in resazurin from blue to pink after overnight incubation.

The effect of different milk samples on assay results was also studied. The antibiotic susceptibility test was performed as before with the addition of pasteurized milk or natural yoghurt, to simulate changes in spoiled milk. The concentrations of milk/yoghurt were prepared: 100%, 40%, 20%, 10%, and 2%. Then, 100 µL of bacteria solution and 100 µL of each milk dilution were pipetted into rows of a 96-well plate, with a final resazurin concentration of 0.25 mg/mL. The final estimated cell density of *E. coli* was CFU/mL of 5×10^5 and dilutions of milk were 50%, 20%, 10%, 5%, and 1%, with a MH broth control.

MIC measurements were taken in duplicate with two antibiotic-coated strips per dilution of milk or yoghurt. The MIC was recorded as the lowest concentration of antibiotic that did not show resazurin conversion in the capillaries. For tests that varied in duplicate measurements, the highest MIC was recorded. A growth control (no antibiotic) capillary was included for all test strips.

4.4. Total Plate Count and Bacterial Identification of Fresh Milk Samples from Healthy Cows

Milk samples from healthy cows collected from CEDARs were diluted 1:100 in 9% saline solution and 10 µL was pipetted and spread onto two individual sterile plate count agar plates for cell enumeration (Sigma-Aldrich, UK). For presumptive identification, 10 µL of each milk sample was pipetted and spread onto a single CHROMagar Mastitis GN and GP agar plate for identification of species of bacteria (CHROMagar™ Mastitis). CFU/mL was calculated for each sample after overnight incubation on plate count agar. CHROMagar plates were examined, and species present were confirmed by colour scoring of visible colonies, according to CHROMagar (CHROMagar™ Mastitis).

4.5. MIC Determination in Simulated Mastitis Samples with Mixed Bacterial Populations

Antibiotic strips containing ciprofloxacin were prepared as above with 2-fold dilutions from a maximum concentration of 0.5 mg/mL. MIC for bacterial species *Pseudomonas aeruginosa* ATCC 12903 and a *Klebsiella* spp. were performed with a total bacterial density of 5×10^5 CFU/mL and varying mixtures of the two were determined as indicated in the text. These represented a potential mastitis sample that contained both pathogen plus non-pathogenic commensal (or contaminant). Bacterial density was adjusted based on turbidity, with 0.5 McFarland estimated at 10^8 CFU/mL and confirmed by overnight plate counts. Bacteria were diluted in MH broth and resazurin at a final concentration of 0.13 mg/mL. Ciprofloxacin-loaded MCF strips were dipped into each of the wells and end covers filled with silicone grease were placed on each end to stop sample evaporation. Samples were incubated overnight (20 ± 4 h) at 37 °C and colour change was time-lapse imaged using

PiRamid. Time-lapse data were retrieved to evidence the difference in growth time between species and inoculum densities and demonstrate occurrences of false susceptibility.

4.6. Imaging and Image Analysis

All time-lapse imaging was carried out on a low-cost open-source imaging system designed for time-lapse imaging of colourimetric assays [33]. Image J was used to analyse the time-lapse image series of bacterial growth in the microcapillary test strips. Colour images were split into blue, blue and green (RGB) channels and the red channel was chosen for intensity. A line across the centre of each test strip was highlighted and re-sliced across an image stack of all timepoints to produce a composite image with the x-axis representing position across the strip of 10 capillaries, and a vertical axis representing incubation time. Absorbance (A) values at chosen timepoints were calculated with the sample (I) and background (I_o) intensity values using:

$$A = log_{10}\ (I_o / I)$$

The time for resazurin conversion was calculated based on the image timepoint when resazurin absorbance reached a threshold conversion: (mean signal for no bacteria absorbance) + 3 × (standard deviation of control absorbance).

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Conflicts of Interest: A.D.E. is one of the inventors of patent protecting aspects of the novel microfluidic devices tested in this study and is a director and shareholder in Capillary Film Technology Ltd., a company holding a commercial license to this patent: WO2016012778 "Capillary assay device with internal hydrophilic coating" inventors AD Edwards, NM Reis.

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Chapter 5 – Evaluating the adaptation of a rapid microfluidic mastitis and AMR diagnostic tool for fastidious *Streptococcus spp.* using fluorescence detection.

Abstract

Streptococci bacteria are one of the major pathogen groups inducing bovine mastitis. With this, S*treptococcus uberis* can be one of the most prevalent causes of clinical mastitis cases in the UK. The use of microcapillary film (MCF) can provide a portable, microenvironment enabling the growth of *Streptococci* using similar methods to CLSI guidelines broth microdilution and therefore providing an optimal condition for antimicrobial susceptibility testing (AST) with metabolic activity of growing bacteria reducing resazurin from a blue colour to pink. Previously, growth of gram-negative organisms has be demonstrated using colourimetric and fluorometric detection with resazurin dye, with fluorometric detection requiring considerably lower concentrations of resazurin dye. Experimentally, 78% of *Streptococcus* strains were found to be resistant to resazurin dye at a concentration of ≥ 10µg/mL, where previously a concentration of 60µg/mL was shown to provide fluorescent intensity suitable for analysis of bacterial growth kinetics. Where the reduction in resazurin dye concentration may be required to allow Streptococcal growth, the difference in start and endpoint is also reduced, therefore making colourimetric detection unviable for Streptococcal growth. Compared to traditional microtitre plates (MTP) used for BMD, MCF has a decreased path length, decreasing the colour intensity of resazurin dye and decreasing the range between start and endpoint absorbance. Resazurin dye has been shown to be useful colorimetric indicator of the growth of bacteria in MCF. It was determined that haemolysis alone cannot indicate the growth of *Streptococcus spp*. in MCF where the difference between the absorbance calculated at 0h does not vary enough from the absorbance calculated at 20 h, where growth is present. Fluorometric detection with resazurin dye at 10µg/mL and 5µg/mL saw the difference between the highest fluorescent intensity and the background intensity as acceptable to detecting growth. In this system, it was determined that the maximum time to detection for *Streptococci* in this system was 9 h at a bacterial concentration of $2.5x10^4$ CFU/mL. All species had detectable growth between 5.5-9 h, considerably faster than the method deployed for traditional AST (> 24 hrs). In AST validation experiments, essential agreement for ampicillin minimum inhibitory concentration (MIC) across all the strains was 100% with all MCF MICs within ±1 doubling dilution of antibiotic. The bias saw.

For benzylpenicillin, essential agreement was 80% within ±1 doubling dilution of antibiotic. . Future resazurin-based microfluidic assays for the detection of bovine mastitis organisms require lower concentrations of resazurin dye and should potentially utilise fastidious Mueller Hinton broth (MHB-F) to incorporate the detection of *Streptococcus spp*. growth and AST detection.

5.1 Introduction

5.1.1 The role of *Streptococci* in dairy cow mastitis infections

Mastitis is one of the most common and costly diseases in the dairy industry, negatively impacting farmer profit due to milk loss and antimicrobial therapy costs. This being said *Streptococci* bacteria are one of the major pathogen groups inducing bovine mastitis. S*treptococcus uberis* can be one of the most prevalent causes, with 23.5% of clinical mastitis cases attributed to *S. uberis* in the UK (Sherwin et al., 2021). Across Europe, the proportion of mastitis induced by *S. uberis* achieved more than one-third on a global average, with around 38% of *Streptococci*-induced mastitis infections being positively diagnosed as *S. uberis*. Although usually primarily of environmental source *S. uberis*, is the most commonly isolated species of Gram-positive bacteria from milk samples (Timofte et al., 2014). The species is commonly detected in bedding material, in contaminated straw at a higher magnitude than sand or sawdust, usually found in conjunction with exposure to faecal matter (Sherwin et al., 2021). This brings to light the importance of animal management in reducing the prevalence of infection from *Streptococci* organisms. Lying in infected bedding areas or infected pastures are confirmed entry points for the bacteria into the udder and are typically characterised by a high bacterial load (Kupczynski et al., 2023). Other more relevant *Streptococcus spp*. bacteria inducing bovine mastitis include *S. agalactiae* and *S. dysgalactiae*. *S. agalactiae* is another contagious pathogen known to cause bovine mastitis but is also commonly found in the gastrointestinal tract of a cow. In a cow's mammary gland, *S. agalactiae* is known to form biofilms enabling survival and is often heavily associated with subclinical cases of mastitis (Rosini & Margarit, 2015). The bacterium is generally considered beta-haemolytic, but some non-haemolytic strains have been observed. *S. agalactiae* is primarily found in the udder of the cow, with recent investigation suggesting that transmission to the udder may occur infrequently through contact with bedding or the environment and even potentially in contaminated food and water sources, with numbers of the bacteria detectable in the gastrointestinal tract (Cheng & Han, 2020).

Cow age, lactation stage and types of milking practices can influence how susceptible a cow is to a *Streptococci* infection (Kabelitz et al., 2021). *Streptococci* infection is 24% more common in cows >10 years of age compared to cows <6 years of age. Meanwhile, cows in the earlier stages of lactation (first 3 months) have a higher risk of developing Streptococcal mastitis than those that have been milked longer. This is expected due to immunosuppression due to increased oxidative stress early on in the lactation process, as well as a lack of teat flushing through milking (Kabelitz et al., 2021; Bradley, 2002). Meanwhile, the same cows appear to have a 5-fold increase in Streptococcal mastitis when going into the drying-off period. Interestingly, shorter teat length and slower closure time post milking have been shown to positively influence the incidence of Streptococcal mastitis. Nearly 70% of all cow teats test positive for *Streptococcus spp.* before milking (Kabelitz et al., 2021), therefore leaving the teat to become a major entry point for pathogens, particularly those individuals with shorter teat length and slower teat closure time, where effective pre- and post-milking teat disinfection isn't carried out (Gleeson et al., 2018).

The resistance profiles of *Streptococci* are difficult to determine and can differ greatly depending on species and source (Kromker & Leimbach, 2017). Although general resistance has been maintained over the last 10-15 years, there have been gradual increases in resistance towards tetracycline across all species, and alarmingly, an increase to 15% multidrug resistance (MDR) in *S. uberis* (Boireau et al., 2018). This bacterium has previously been shown to have good susceptibility to penicillin, often the first line of antibiotic defence for intramammary treatment (Kappeli et al., 2019). However, the use of continued surveillance has shown increased resistance of these bacteria to beta-lactam antibiotics such as Penicillin G, a first-line treatment for bovine mastitis, through sequence-based prediction using penicillin-binding protein transpeptidase signatures (Haenni et al., 2018). Although most studies show isolates are still considered susceptible to beta-lactam antibiotics, this slow-growing but clear shift towards decreased susceptibility poses a threat to future antibiotic treatment of mastitis caused by *Streptococcus spp.* infection (Kappeli et al., 2019; Haenni et al., 2018). When diagnosing and treating a *Streptococci* mastitis case, it is important to detect the pathogen quickly and be able to accurately inform a treatment path. Often intramammary antibiotic therapy can help treat a cow with an environmental *Streptococci* infection, however, this is not recommended for cows with *E. coli*-associated mastitis due to the ability of *E. coli* to form biofilms in intramammary tissue making it inherently resistant to these forms of therapy (Goulart & Mellata, 2022; Pedersen et al., 2021).

5.1.2 Traditional *Streptococci* culturing conditions

When considering the antibiotic susceptibility testing (AST) of fastidious organisms, and particularly when developing a novel method of AST some challenges must be addressed. Fastidious organisms such as *Streptococcus spp*. require Muller Hinton medium supplemented with additional nutrients (5% sheep blood, β-NAD) as a minimum requirement to grow in a gold standard broth microdilution method according to CLSI guidelines (Feßler et al., 2023). With this, traditional broth microdilution (BMD) results for *Streptococci* often require the detection of haemolysis to determine growth. The organism is mostly alpha-haemolytic, carrying out partial haemolysis during growth, however, often no haemolysis occurs (Kabelitz et al., 2021). Moreover, to culture most *Streptococcus spp*. on agar requires the use of blood agar and a simulated anaerobic environment. With the detection of haemolysis as an indicator of bacteria growth, it is possible to measure a change in absorbance. Haemoglobin absorbs light strongly at 415 nm. Haemoglobin is released from blood cells during haemolysis leading to a potential increase in light absorption in this wavelength range. Before introducing the need for another indicator, such as resazurin, for growth detection of *Streptococci*, it may be possible to measure a change in absorbance through haemolysis by analysing images of positive growth MCF capillaries using a shorter wavelength (415 nm).

5.1.3 Challenges in adapting a resazurin-based microfluidic assay for *Streptococci*

Microcapillary film (MCF) can provide a portable, microenvironment enabling the growth of *Streptococci* using similar methods to CLSI guidelines for BMD, therefore providing an optimal condition for growth and determination of MIC, but removing the need for an anaerobic environment, seen with gold standard agar plate disk diffusion. Previously, colourimetric detection of resazurin colour change in MCF has been used for the detection of bacteria growth, with the change in absorbance of light between the blue and pink colours providing growth kinetics quantitatively. Resazurin being a cell-permeable redox indicator, viable bacterial cells can reduce resazurin into resorufin, providing a colour change from blue to pink or from non-fluorescent to fluorescence. Bacteria reduce resazurin to resorufin as part of their metabolic process and only occurs intracellularly of metabolising cells. The process involves dehydrogenase enzymes that transfer electrons from NADH or NADPH to resazurin (Figure 5.1).

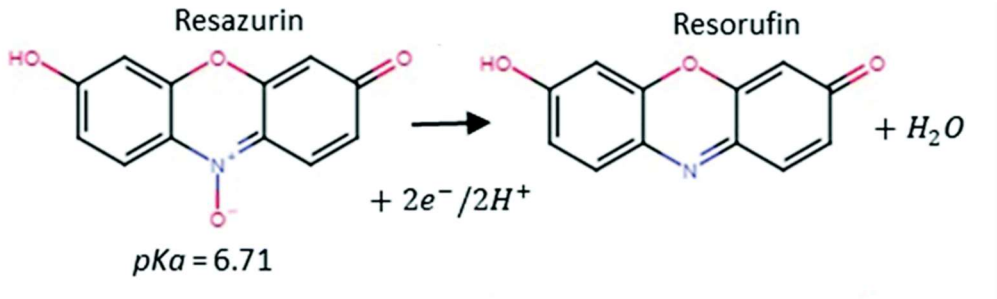

Figure 5.1 Reduction of resazurin dye to resorufin by metabolising bacterial cells. Dehydrogenase enzymes from metabolising bacterial cells transfer electrons from NADH or NADPH to resazurin reducing it to resorufin and exhibiting a colour change from blue to pink (Crane et al., 2021).

However, resazurin has also been shown to be cytotoxic to some bacteria (Schmitt et al., 2016; Schmitt et al., 2013). There is evidence to suggest that both resazurin and resorufin can suppress the growth of bacteria, and exposure to resazurin can change the morphology of cells and interfere with normal cell function depleting reduced forms of nucleotides and causing cell death (Riss et al., 2013). Compared to traditional microtitre plates (MTP) used for BMD, MCF has a decreased path length, decreasing the colour intensity of resazurin dye and decreasing the range between start and endpoint absorbance. Where the reduction in resazurin dye concentration may be required to allow Streptococcal growth, the difference in start and endpoint is also reduced, therefore making colourimetric detection unviable for Streptococcal growth. When developing a resazurin-based assay, it is therefore important to consider how the concentration of resazurin can affect the growth rates of different species, and how best to control these conditions to reduce the effect. This study aims to evaluate the use of a resazurin-based microfluidic assay by determining the effects of resazurin dye on the growth of fastidious organisms, evaluating the incorporation of broth supplementation for fastidious growth on the assay and indicating the viability of the device for the detection and antibiotic susceptibility testing of mastitis fastidious *Streptococci* organisms.

5.2 Methods

5.2.1 Bacterial isolates

S. pneumoniae NCTC 12973 was used as a quality control strain. *Streptococcus spp.* were isolated from milk samples collected from cows with confirmed cases of mastitis from the Centre for Dairy Research (CEDAR, University of Reading). Milk samples were refrigerated for no longer than 2 hours before plating and bacterial isolation. Upon collection from the farm, milk

samples were streaked on Mastitis Chromagar®, and species of bacteria were determined by colony colour, following the manufacturer's instructions, allowing presumptive identification. 9 Suspected *Streptococcus spp*. were then plated on blood agar and grown in anaerobic conditions. These *Streptococcus spp*. were only identified as *Streptococcus*, this could be either *S. uberis* or *S. agalactiae,* using Mastitis Chromagar®. Both species were considered applicable to mastitis infections, therefore no further identification techniques were deployed to differentiate the species. Therefore, they are all referred to as *Streptococcus spp*. All species were grown and then resuspended in Mueller Hinton broth with 5% lysed horse blood and incubated until turbid. The turbid bacterial suspension was then diluted 1:1 with 50% glycerol in water and frozen at -80°C to create stored glycerol stocks. Upon use, *Streptococcus spp*. were replated from glycerol stocks onto blood agar plates and grown in anaerobic conditions. A total of eight *Streptococcus spp*. isolated were used in this investigation.

5.2.2 Microfluidic strip production

The fluorinated ethylene propylene microcapillary film (MCF) was manufactured by melt-extrusion by Lamina Dielectrics Ltd (Billingshurst, West Sussex, UK) from a highly transparent fluorinated ethylene propylene co-polymer (FEP-Teflon®) and comprises a ribbon containing an array of 10 capillaries along its length with an average diameter of 270 µm. For each batch, 1–5 m MCF lengths were internally coated by incubation with a 5 mg mL^{-1} solution of PVOH in water, >99% hydrolysed (Sigma-Aldrich, UK) at room temperature for a minimum of 2 h. Coated strips were washed with 5 ml of PBS with 0.5% Tween 20 (Sigma-Aldrich, UK) to remove residual PVOH, and dried by attached one end of the film to a vacuum manifold and air dried for 20 minutes.
For antibiotic-loaded strips, individual microcapillaries of lengths of MCF up to 5 m long were filled using a 30G needle with freshly prepared antibiotic solutions of ampicillin and penicillin G. Individual test strips were cut to 17 mm lengths, frozen for at least 1 h at −80°C and freeze-dried using a Labconco FreeZone® freeze dry system. Test strips were vacuum sealed and stored at −20°C before use.

5.2.3 Agar Dilution MIC determination with resazurin

Concentrations of resazurin ranging from 10µg/mL to 160µg/mL were added to autoclaved nutrient agar containing 5% lysed horse blood. A blood agar plate without resazurin was used as a control. Each concentration was poured into sterile square petri dishes. 9 *Streptococcus spp*. isolates and *S. pneumoniae* 12973 were grown overnight on blood agar in anaerobic conditions from glycerol stocks at -80°C. Individual colonies were resuspended in MHB and were diluted to

0.5 McFarland corresponding to approximately 1 × 10^8 CFU mL^{-1}. Bacteria were further diluted 1:5 and 10µL of each bacterium was spotted onto each resazurin plate, with each spot corresponding to 2 × 10^5 CFU mL^{-1}/spot, per CLSI guidelines for inoculation of agar dilution plates for anaerobes. Spots were plated in triplicate. Bacteria were again grown overnight at 37°C in anaerobic conditions. Growth of each bacteria was noted at each concentration of resazurin to allow for the determination of resazurin MIC for each isolate. Results determined the maximum concentration that could be used for the detection of all *Streptococcus spp.* in the presence of resazurin.

5.2.4 Determination of fastidious organism growth in MCF

Streptococcus spp. were grown overnight on blood agar in anaerobic conditions from glycerol stocks at -80°C. Individual colonies were resuspended in MHB and were diluted to 0.5 McFarland corresponding to approximately 1 × 10^8 CFU mL^{-1}. Four different conditions were prepared for growth in MCF. Bacteria were simultaneously diluted 1:200 in MHB alone, MHB with 5% lysed horse blood and 20mg/L β-NAD (Mueller-Hinton fastidious broth (MH-F)), MHB with 10µg/mL resazurin dye and MHB with 5% lysed horse blood and 20mg/L β-NAD with 10µg/mL resazurin dye. Samples were also tested with varying concentrations of resazurin as indicated in the text. Samples were tested by dipping test strips into each condition for a few seconds until all capillaries had filled and the samples had reached the top of the device. After sealing the end by adding an end cap and vacuum grease, those strips containing resazurin were placed inside a PiRamid imaging device designed to image changes in fluorescence and those strips without resazurin were placed inside a PiRamid imaging device designed to image colourimetric changes. Test strips were placed in an incubator at 37°C overnight. Under CLSI guidelines for broth microdilution methods, test strips were not required to be incubated in anaerobic conditions.

5.2.5 Antibiotic MIC determination

Streptococcus spp. were grown at 37°C overnight (18 h) on blood agar in anaerobic conditions from glycerol stocks at -80°C. Individual colonies were resuspended in MHB and were diluted to 0.5 McFarland corresponding to approximately 1 × 10^8 CFU mL^{-1}. MIC determination was simultaneously carried out in a microtitre 96-well plate following the CLSI guidelines for broth microdilution of fastidious organisms. Stock solutions of ampicillin and penicillin G were prepared and 10 serial dilutions of each antibiotic were prepared in a microtitre plate. Each well

contained 50µL of antibiotic diluted in MHB, twice as concentrated as required. The addition of bacteria suspension would produce the correct antibiotic dilution.

For microtitre broth microdilution, bacteria were diluted 1:100 in MHB with 10% lysed horse blood and 40mg/L β-NAD. Upon addition to microtitre plate wells, 50 µL of bacterial suspension is added to 50 µL of each antibiotic leading to a 1:2 dilution of the bacteria suspension. For microcapillary broth microdilution bacteria were diluted 1:200 in MHB with 5% lysed horse blood and 20mg/L β-NAD, with 5µg/mL resazurin dye. Samples were sealed and imaged as before. Samples were incubated at 37°C overnight.

For broth microdilution of fastidious organisms in MH-F broth, haemolysis of blood is visible where bacteria growth is present. MIC was noted at the antibiotic concentration where no haemolysis was visible. For MCF strips, growth was indicated by the visible fluorescence upon image analysis from the PiRamid. MIC was noted at the antibiotic concentration where no fluorescence signal was visible.

5.2.6 Data analysis

Images were analysed in Image J. Determination of the growth of bacteria in blood broth alone was first attempted visually and then analysis was attempted in Image J. For colourimetric analysis or haemolysis, images are imported into ImageJ in a stack and resliced, providing an individual for each image in the time-lapse. The resliced image is then split into the different colour channels (RGB). Images without resazurin that were imaged colourimetrically were experimentally analysed in different channels to determine whether haemolysis of blood and therefore growth of bacteria was visible in MCF. It was determined that the green channel was used for analysis. The absorption of green light by blood is about 20 times greater than for red light, therefore any change in the colour of the blood (haemolysis) will be more easily detectable when analysing images from the green channel. The intensity value for capillaries was recorded and absorbance for each capillary was calculated.

The growth of bacteria in resazurin was determined by the visibility of a fluorescence signal emitted from the MCF. Test strips that contained resazurin were imaged in a PiRamid device designed to image fluorescent excitation of converted resazurin (resorufin) by green light at 530 nm. The adapted PiRamid design contained strips of green LED lights. A 590nm longpass glass filter is positioned over the camera lens, selectively allowing light with longer wavelength to pass through to the camera. Images again were analysed in ImageJ. Images were split into RGB, and

fluorescent intensity values were recorded for each capillary. When calculating the peak intensity at which bacteria growth reaches the end of the log phase, the intensity was normalised to a reference blank test strip. For all capillaries, time to resazurin conversion was calculated when fluorescence intensity rose above half the maximum intensity.

5.3 Results

5.3.1 MIC determination of resazurin for *Streptococcus spp.* using agar dilution

Although resazurin is commonly used to measure bacterial cell growth through reduction to fluorescent resorufin, resazurin can also have antimicrobial properties for certain types of bacteria. Previously work has suggested that this be the case, particularly for Gram-positive organisms, with *Streptococcus spp*. being affected by this. Therefore, to continue developing a resazurin-based microfluidic assay, it was important to understand how these antimicrobial properties affect the growth of the reference organism *S. pneumoniae* 12973 alongside some mastitis-derived *Streptococci*. MIC for resazurin concentration was determined for all *Streptococcus spp*. Isolated from mastitis-infected milk including the reference organism *S. pneumoniae* 12973. 2 strains of *Streptococcus spp*. did not grow in the lowest agar dilution of resazurin dye (10µg/mL) (Figure 5.1). The 7 remaining strains, including *S. pneumoniae* 12973 had MICs >10µg/mL. The number of strains that were resistant to resazurin decreased with increasing concentration. An MIC of >160µg/mL was recorded for one of the strains. *S. pneumoniae* 12973 had an MIC of 40µg/mL. MIC determination allowed for the use of resazurin as a reagent for the determination of growth of bacteria in microcapillary film (MCF) for *Streptococcus spp*. 78% of the strains used in this test were resistant to resazurin at a concentration of 10µg/mL. Previously, within a microfluidic system utilising fluorescent detection of growth, a concentration of 60µg/mL was shown to provide fluorescent intensity suitable for analysis of growth kinetics of *E. coli* isolates (Needs et al., 2021).

Table 5.1 Resazurin MIC for *S. pneumoniae* 12973 and *Streptococcus spp*. isolated from mastitis milk samples.

MIC was determined using agar dilution of resazurin dye in blood agar ranging from 10-160µg/mL. Resazurin MIC measure from <10->160 µg/mL. MIC of *S. pneumoniae* 12973 QC strain noted. In total, nine strains of *Streptococci* were used. 5 x 10^7 CFU/mL inoculum plated in 10µL spots onto agar resulting in 5 x 10^5 CFU/mL spots (n=3). MIC was determined as the first dilution of resazurin at which each bacteria growth was inhibited.

Number of *Streptococcus spp*.	Minimum inhibitory concentration (MIC) of resazurin dye (µg/mL)
1	>160
1	160
1*	80
2	40
2	20
2	<=10

** S. pneumoniae 12973*

5.3.2 Determination of *Streptococci* growth through haemolysis in MCF

Growth of *S. pneumoniae* in MCF with 5% lysed horse blood and 20mg/L β-NAD (Mueller-Hinton fastidious broth (MH-F)) was analysed colourimetrically to identify whether growth of bacteria could be determined by haemolysis of blood in MCF to eliminate the need for resazurin dye as an indicate for fastidious organisms. It was determined that the difference between the absorbance calculated at 0h did not vary enough from the absorbance calculated at 20 h, where growth was present (Figure 5.2). Haemolysis alone cannot indicate the growth of *Streptococcus spp*. in MCF.

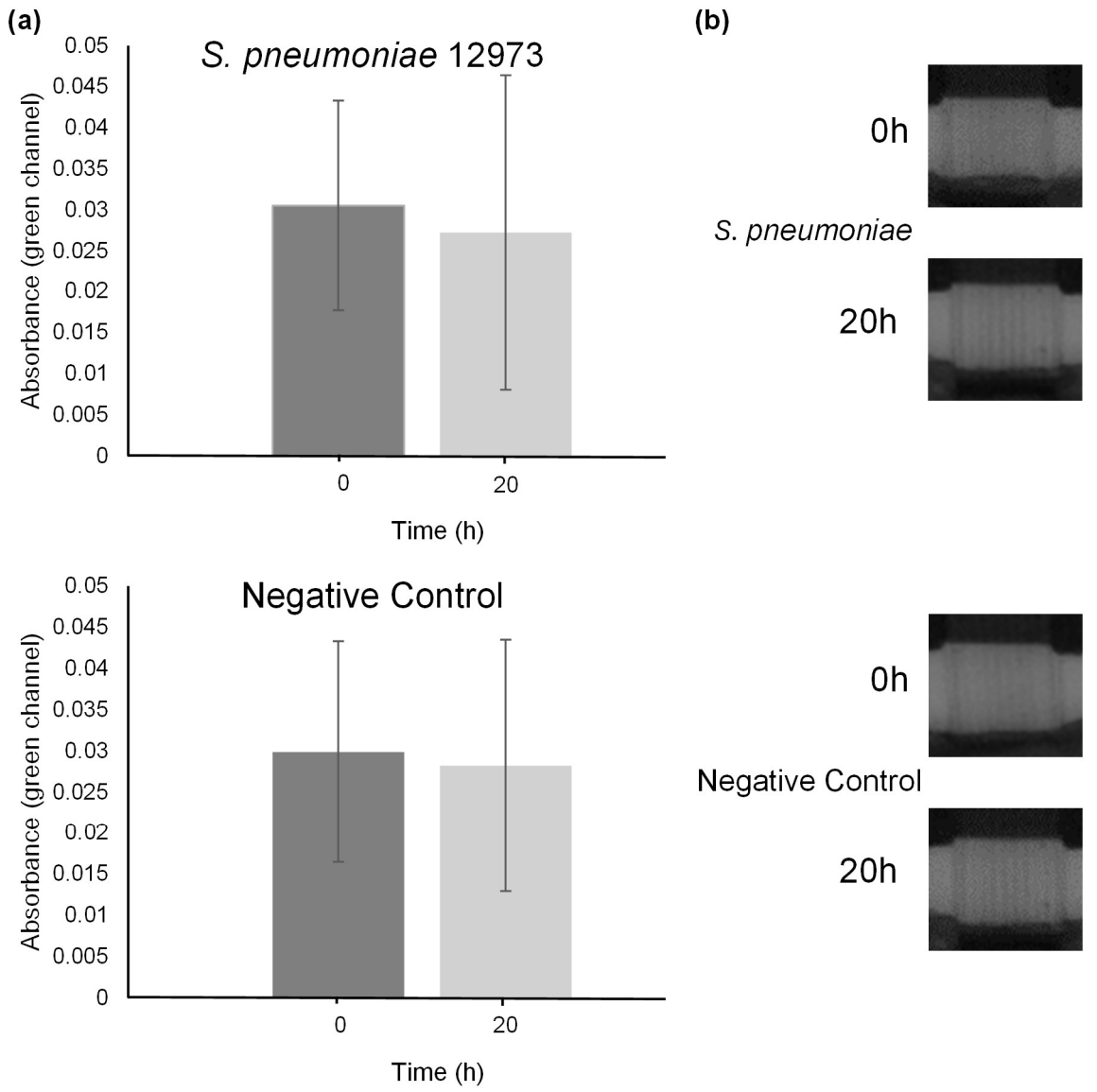

Figure 5.2 Haemolysis of blood in the presence of *Streptococci* growth is not visible in MCF. **(a)** Average absorbance values were recorded for MCF containing MHF broth with 5% lysed horse blood. Absorbance was noted for blood broth with and without *S. pneumoniae* 12973 QC strain at an inoculum density of 5 x 10^5 CFU/mL. Absorbance was recorded at time start and endpoint of 20 h. **(b)** Images of MCF containing MHF broth and either *S. pneumoniae* 12973 or negative control (no bacteria). Images show that there is no difference between MCF with bacterial growth and the negative control when using haemolysis for detections. Images are of the green channel.

5.3.3 Optimisation of resazurin-based MCF growth assay for *Streptococcus spp.*

Where MIC for resazurin in some *Streptococcus spp*. was <10µg/mL, it was important to determine the limit of detection for resazurin concentration in MCF and whether detection of bacteria growth was possible with resazurin concentration below this. When developing a

microfluidic assay, specifically with MCF, identification of individual capillaries is key. Previous research has determined that if the resolution of an image is sufficient to identify the individual capillaries, most imaging devices and cameras can quantify a range of concentrations of fluorometric dyes (Jegouic et al., 2021). The difference between the highest fluorescence intensity value detected and the background intensity value was determined for five *Streptococcus* isolates, including *S. pneumoniae* 12973. When the difference between the peak fluorescent intensity and the background intensity gets lower, it not only becomes visibly difficult to detect growth, but also the images become harder to analyse to produce growth kinetics, determine resazurin conversion time and growth kinetic data can become noisy. *S. pneumoniae* 12973 growth was detected as low as 1.25µg/mL resazurin concentration with the difference in fluorescent intensity compared to the background being 21.8. The standard error for the difference in fluorescence did not overlap the difference in intensity with no resazurin. However, this was not the case for isolate FL2819, where the difference in intensity at 1. 25µg/mL was not different to the difference in intensity with no resazurin present, where no growth would be detected. For all other strains, the difference in fluorescent intensity was below 20 at 1.25µg/mL resazurin concentration, with the standard errors overlapping that of isolate FL2819 at 0µg/mL. This would make it difficult to distinguish between actual bacteria growth and fluorescence emitted by resazurin conversion in the capillary and any background fluorescence. At 10µg/mL and 5µg/mL, the difference between the highest fluorescent intensity and the background intensity was acceptable, whereby the difference in fluorescent intensity compared to the background intensity was significantly higher than the standard error determined for 0µg/mL resazurin for all strains.

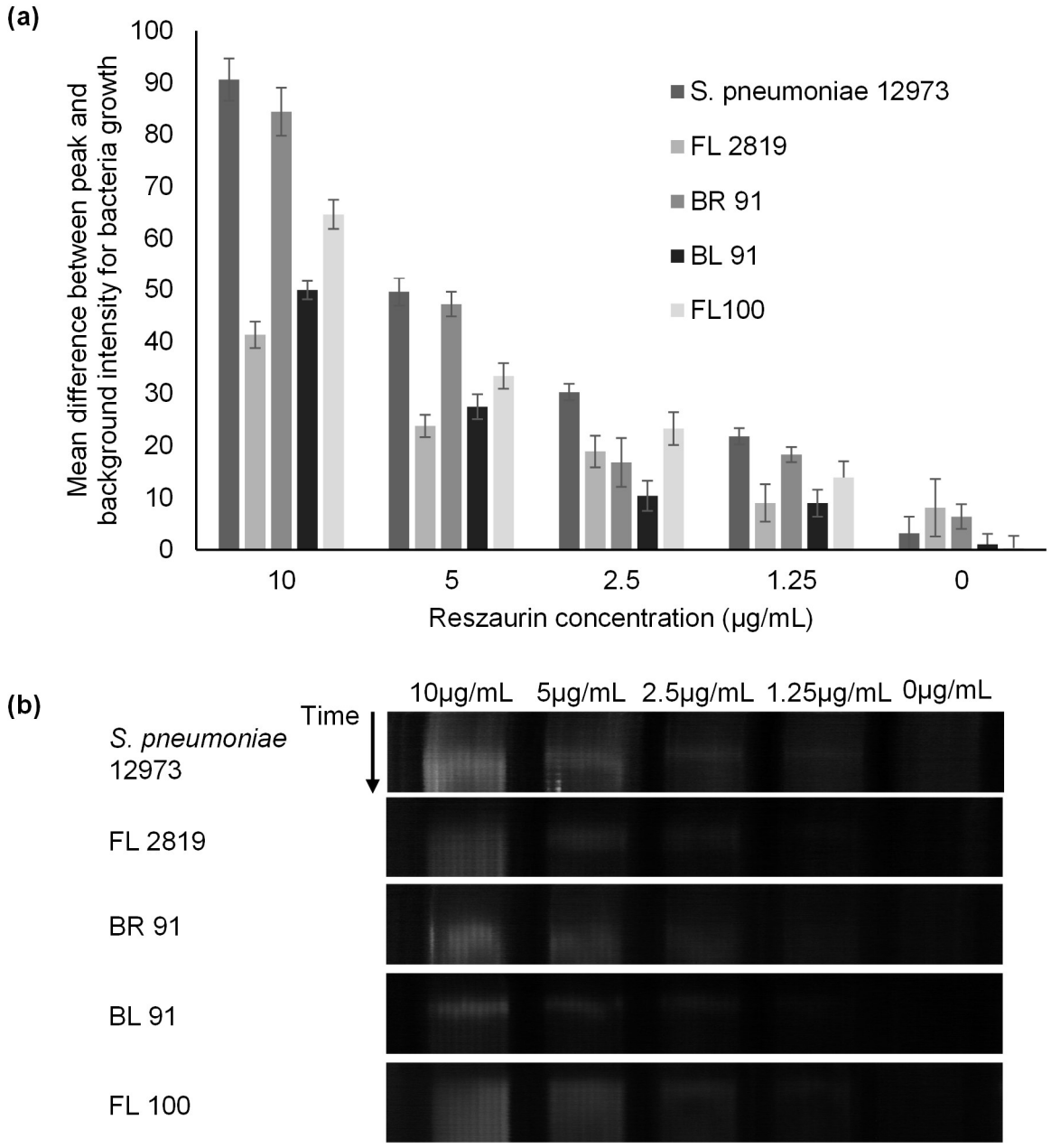

Figure 5.3 Mean difference between peak and background intensity values of fluorescence for resazurin conversion by *S. pneumoniae* 12973 and *Streptococcus spp*. isolated from mastitis milk samples.

(a) Peak intensity values determined using a dilution of resazurin dye in MHF broth ranging from 1.25-10µg/mL. 5 x 10^{5} CFU/mL inoculum used. The mean difference represents the difference between the mean peak conversion of resazurin seen by each of the test bacteria and the background intensity. Errors bars represent standard error (n=10). **(b)** Resliced mages of MCF strips with different concentrations of resazurin dye in MHF broth ranging from 1.25-10µg/mL. 5 x 10^{5} CFU/mL inoculum was used for each of the test bacteria. Images show the red channel.

Each pixel of the reslices represents a 5-minute time point. The time of the image increases vertically down the image for each MCF strip.

We need to know the time taken for bacteria at a known inoculum to grow and convert resazurin to a detectable fluorescence intensity, therefore being able to set the earliest endpoint for measurability. For this, we explored whether at different inoculum densities, we see a difference in growth kinetics and therefore a difference in time taken to convert resazurin. Growth curves were recorded by time-lapse imaging of resazurin conversion over time for each of the *Streptococcus spp.* over a range of starting cell densities using serially diluted inoculums from a starting solution of 5 x 10^5 CFU/mL in MCF (Figure 5.4). These growth curves highlight the direct relationship between inoculation density and the time taken for the conversion of resazurin to resorufin. Where inoculation density decreases, the time taken to fully convert resazurin increases. Images of the MCF strips were taken every 5 minutes for 20 h incubation at 37°C using the PiRamid imaging device (Long et al., 2022). The time taken to reach a threshold of resazurin conversion was determined by analysing growth curves and plotting against CFU/mL determined from colony counts on MH-F agar. Time to resazurin conversion was determined when fluorescent intensity first rose above half the peak intensity value. The time to resazurin conversion for 2-fold dilutions was compared to calculate generation times for each isolate. The generation time for *S. pneumoniae* 12973 was 53 minutes, 60 minutes for FL2819, 80 minutes for BL91 and 63 minutes for FL100.

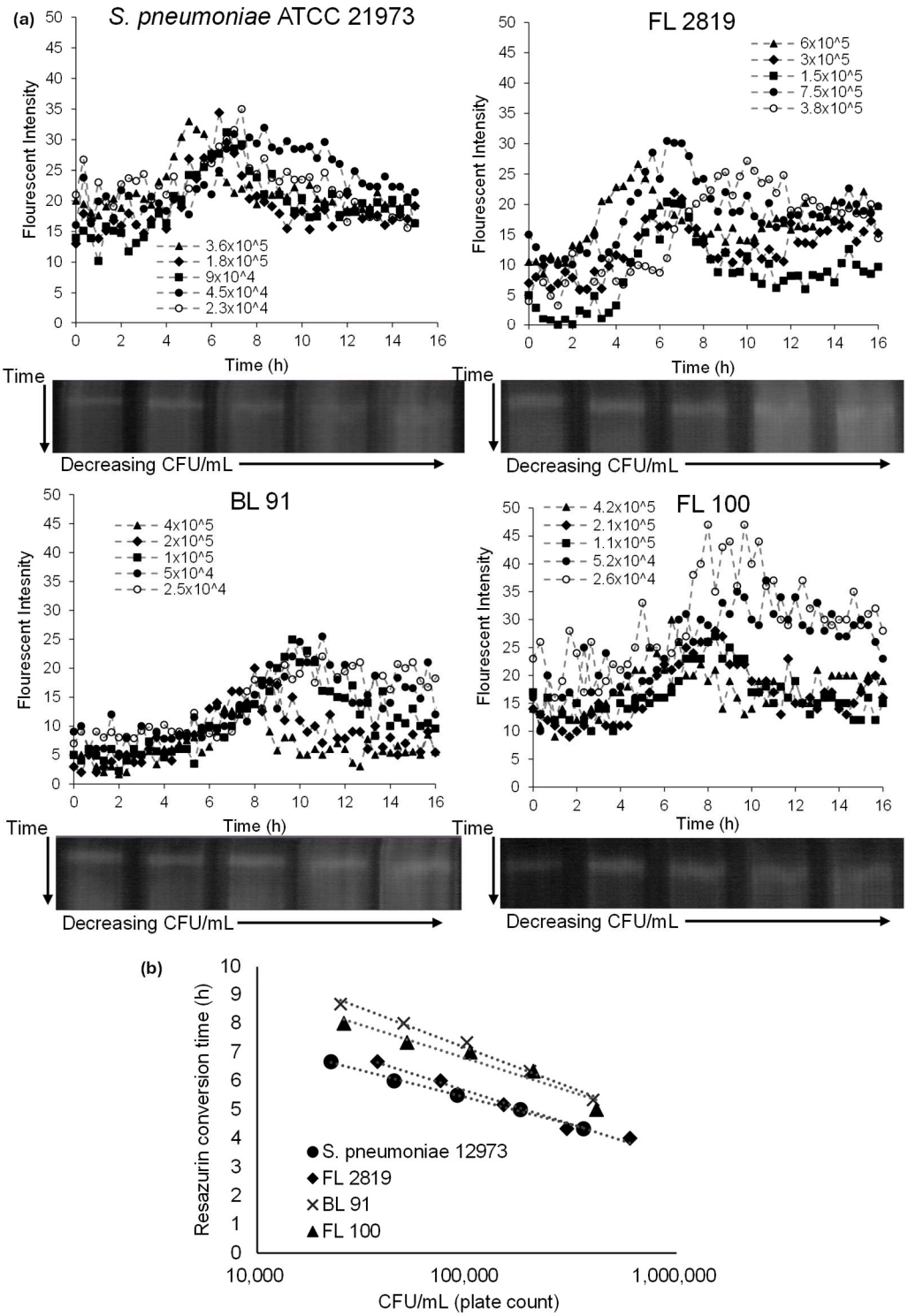

Figure 5.4 Resazurin conversion kinetics is dependent on bacterial concentration.

(a) Growth kinetics for *S. pneumoniae* 12973 and *Streptococcus spp.* FL 2819, BL 91 and FL 100 at five different inoculum densities. Bacteria were grown in MHF broth with 5µg/mL resazurin. A predicted starting concentration of bacteria of 5 x 10^5 CFU/mL was used. MCF was dipped into wells at 1:2 serial dilutions. The starting dilution of each of the test bacteria was diluted at 1:100 and 10 µL/mL was spread on blood agar. 5 µL spots of each serial dilution were plated on blood

agar. Image of MCF containing 1:2 serial dilutions with a starting concentration of bacteria of 5 x 10^5 CFU/mL for *S. pneumoniae* 12973. **(b)** Analysis of resazurin conversion kinetics by *S. pneumoniae* 12973 (R^2 = 0.9844) and *Streptococcus spp.* FL 2819 (R^2 = 0.9966), BL 91 (R^2 = 0.9889) and FL 100 (R^2 = 0.9432) were isolated from bovine mastitis milk samples. Bacteria CFU/mL log transformed. Time to resazurin conversion was set at a threshold fluorescence intensity of 50% that of the maximum intensity indicative of complete organism growth.

5.3.4 MIC determination for fastidious *Streptococcus spp.* in MCF

To validate this method for AST of fastidious organisms, a proof-of-concept investigation was carried out whereby MIC was determined for four mastitis *Streptococcus spp.* alongside the *S. pneumoniae* QC strain. Broth microdilution in microtitre plates was used as a reference test to determine MIC. Two antibiotics for the treatment of mastitis were used with a full screen of nine doubling dilutions of each antibiotic-loaded into the capillaries of MCF. MIC was determined where the bacteria was susceptible and where the resazurin was not converted to resorufin and therefore no fluorescence was detected upon image analysis. As previously, growth curves were recorded by time-lapse imaging and plotted to determine the difference in fluorescence intensity for capillaries where bacteria grew and the intensity of the first capillary where no growth was detected (Feßler et al., 2023) (Figure 5.4). The essential agreement was determined as MIC ±1 dilution and distribution of dilution differences between test and reference MIC results according to ISO 20776-2:202 (Table 1.).

Table 5.2 Essential agreement and distribution of dilution differences between MCF fastidious organism test method and reference BMD MIC determination for *Streptococcus spp.*

	N	Essential Agreement (%)	≤-2	-1	0	+1	>+2
Ampicillin	5	100	0%	0%	60%	40%	0%
Benzyl Penicillin	5	80	0%	0%	40%	40%	20%

Essential agreement for ampicillin MIC across all the strains was 100% with all MCF MICs within ±1 doubling dilution of antibiotic. The bias saw 40% of organisms with an MIC of +1 dilution. For benzylpenicillin, essential agreement was 80%, with a bias of 40% of organisms with an MIC of +1 dilution and 20% ≥2 doubling dilutions above the reference MIC (Tale 5.1).

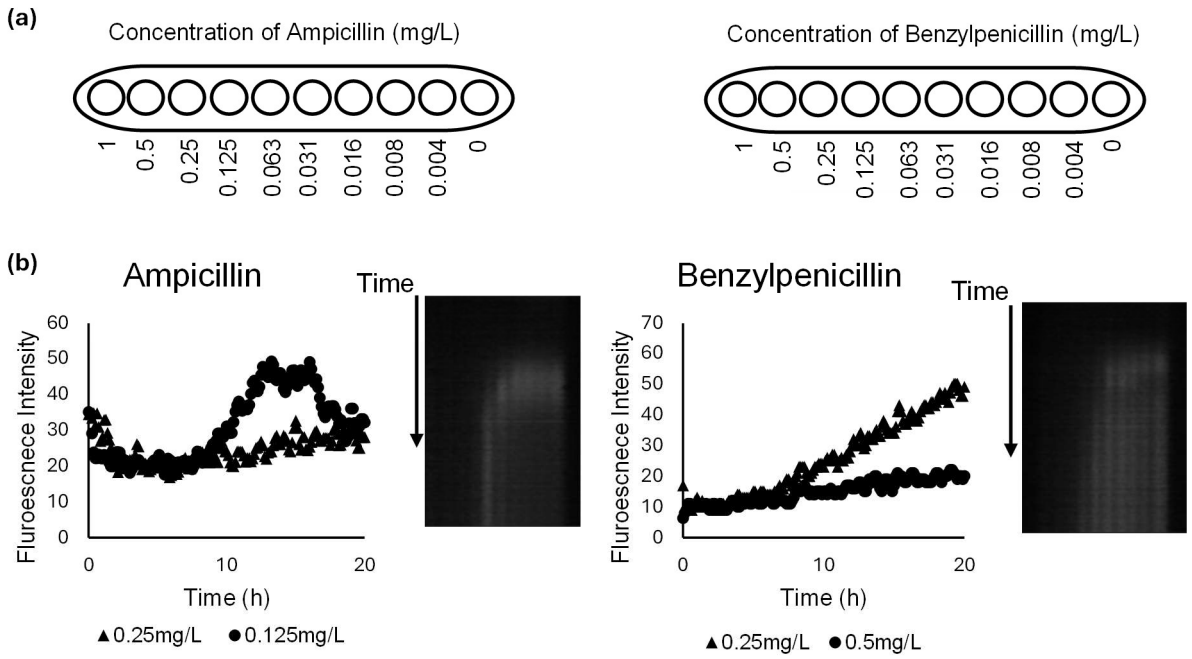

Figure 5.5 Example growth curves for *S. pneumoniae* 12973 allowing for MIC determination. (a) Diagrams depicting the serial dilutions of each antibiotic with different concentrations per capillary. **(b)** MIC was determined for ampicillin and benzylpenicillin. Bacteria were grown in MHF broth with 5µg/mL resazurin. A predicted starting concentration of bacteria of 5 x 10^5 CFU/mL was used. Antibiotic-loaded MCF strips containing nine serial doubling dilutions of antibiotic with the highest concentration of antibiotic in the furthest left capillary and a no antibiotic growth control capillary furthest right. Images were analysed in ImageJ and presented images represent the red channel (RGB).

5.4 Discussion

The development of a successful microfluidic antibiotic susceptibility test (AST) to inform treatment decisions for bovine mastitis has proven difficult already, with challenges including time to result, samples containing multiple organisms or other interfering factors such as sample matrix. In this study, we aimed to identify if a potential microfluidic system could be used for one of the more difficult to culture mastitis pathogens. We have identified that accurate AST results can be achieved for Streptococcal bacteria isolated from mastitis milk samples in our microfluidic AST system. With small adjustments to media additives and reducing resazurin concentration, we can detect Streptococcal growth and inhibition by antibiotics. This provides proof of concept that by the addition of supplemented MH-F broth to the microfluidic assay, we can achieve MIC determination for fastidious organisms that may otherwise remain undetected in MHB alone. Moreover, with growth detection between 5.5-9 h, this more rapid MIC

determination will provide a more proactive inference of treatment. A key objective of a rapid AST test is to allow for faster time-to-results to inform treatment and reduced the lag introduced by requiring lab culture techniques. Indeed, this seems comparative with the current novel bacterial detection devices. It was noted that phenotypic rapid testing kit MastDecide® (MastDecide, Quidee GmbH, Homberg, Germany), provides positive results in 8-14, whilst BACT by Point of Cow (FluimediX APS), wields results within 14-16 h upon incubation at 37°C. Meanwhile, Mastatest® system reports to provide results within 24 h of the 'cartridge' being filled. This suggests that the MCF resazurin-based assay can provide similar, if not better time-to-results as the current bacterial detection tests being deployed. With *Streptococci* bacteria causative of up to 38% of cases in Europe, the rapid detection and treatment of *Streptococci* mastitis will aid in more effective treatment, and potentially a reduction in inappropriate antibiotic use.

5.4.1 Fastidious organism-specific media in a novel bacteria detection system

With a disease as complex as bovine mastitis, populations of commensal bacteria and the broad spectrum of mastitis-causing pathogens reduce the success of a novel on-farm AST devoid of traditional laboratory culture methods. The development of a rapid, on-farm AST device that is not only successful in detecting a mastitis case but also providing susceptibility information for varying species of bacteria to the most commonly used antibiotics, has proven difficult. This is particularly difficult whereby fastidious organisms, particularly *Streptococci spp*. require special conditions to successfully grow. AST for fastidious organisms is one of the few times that the gold standard method of broth microdilution (BMD) allows for supplementation (Humphries et al., 2023). The use of defibrinated lysed horse blood at a concentration of 3-5% v/v is accepted and commonly used for the growth of fastidious organisms in broth. This provides a complication whereby during the development of a novel AST device, to detect fastidious organisms a device would either need a separate test or provide a test that always has the presence of defibrinated lysed horse blood when testing a range of organisms. The presence of a test with lysed horse blood would require refrigeration and would limit shelf life with recommended storage for up to 56 days. Moreover, the presence of lysed horse blood for the testing of organisms other than *Streptococci* deviates from the gold standard methods set by CLSI and therefore would require more testing to validate whether appropriate to determine MIC. Often, MIC for fastidious organisms is read by the detection of haemolysis rather than turbidity, usually by eye. The antibiotic concentration at which no haemolysis is noted is the MIC. Although the presence of lysed blood in an MCF microfluidic test strip is visible by the eye and by colourimetric image

analysis, there is no change in absorbance when analysing images in all channels (RGB), and therefore it is impossible to detect growth with this method (Figure 5.2).

5.4.2 Requirements for a resazurin-based assay for the detection and AST of *Streptococcus spp.*

For the techniques we have developed for detecting bacteria growth, using the conversion of resazurin dye to fluorescent resorufin, the type of bacteria is key to determining the optimal amount of dye to use. We suggest the use of a lower concentration of resazurin dye than has been previously used for cell viability assays. For example, CellTiter-Blue® cell viability assay requires an end concentration of resazurin of 50µg/mL (Riss et al., 2013), which is 10-fold higher than the concentration used in this study and would potentially be cytotoxic to Streptococcal cells. Whereby the path length in MCF is a lot shorter than traditional MTP, the changes in colourimetric changes between resazurin and resorufin are less easily detectable, and the method of detection has to be changed from colourimetric to fluorescence. The complications surrounding the growth of Gram-positive bacteria, and more importantly *Streptococcus spp*., in the presence of resazurin dye, mean that the methodology of using resazurin dye concentration higher than 10µg/mL, could lead to up to 78% of *Streptococci* related infections remaining undetected by rapid MCF detection using resazurin dye (Figure 5.1). These antimicrobial effects of resazurin dye require a lower concentration of resazurin dye and therefore, potentially jeopardising the fluorescent detection of growth through resazurin conversion to resorufin. When considering fluorescence intensity detected by imaging MCF, it was hypothesised that the lower the concentration of resazurin dye, the closer the detected fluorescence intensity for positive growth would be to the fluorescence emitted from the surrounding plastic strip. It was, therefore, important to determine at what resazurin concentration we stop seeing a difference in fluorescence intensity to the surroundings when bacteria has grown. We expect most *Streptococcus spp*. to grow in resazurin concentration below 10µg/mL, with complete growth of all species with a minimum inhibitory concentration (Feßler et al., 2023) no lower than 5µg/mL. At 5µg/mL resazurin concentration, fluorescence intensity for bacteria growth is at an acceptable level to be distinguished from any background intensity. Given there is an overlap in error between the intensity values detected from the MCF containing no resazurin and those containing 2.5µg/mL, the current methodology would not allow for the detection of *Streptococci* growth at this concentration of resazurin.

5.4.3 Optimising bacterial detection assays for the detection of *Streptococcus spp.*

For the viability of an AST, bacteria must be able to grow in optimal conditions and match growth kinetics and doubling times found in standard methods. *Streptococcus spp*. appear to have wildly different generation times depending on the type of media and species. For example, generation time for Group B *Streptococcus* (GBS) can be ~35min in Todd-Hewitt broth with supplemented yeast, and for Group A *Streptococcus* (Morris et al., 2022) 40min – 1h in rich media and grown anaerobically (Joyce et al., 2021; Sitkiewicz et al., 2009). This is from limited reported data on generation times for *Streptococci*. All *Streptococcus spp*. grown in Mueller-Hinton Fastidious broth (MH-F) with 5µg/mL resazurin dye in MCF had a generation time of between 53min and 1.2 h. All species are suspected GBS species, therefore the generation times observed are much greater than the expected generation time in the optimal media. When looking at the relationship between the conversion of resazurin and the bacterial concentration in MCF for individual isolates, we saw that the time to resazurin conversion was dependent on the number of bacteria present. However, this did not correlate between isolates and therefore, we would not be able to predict bacterial concentration for *Streptococcus spp*. from time to resazurin conversion. We also note that the maximum time to detection for *Streptococci* in this system was 9 h at a bacterial concentration of $2.5x10^4$ CFU/mL. Within the bacterial concentration range required to carry out a BMD, all species had detectable growth between 5.5-9 h, considerably faster than the method deployed for traditional AST.

5.5 Conclusions

This study has provided an evaluation of a microfluidic rapid AST device for the detection and susceptibility testing of fastidious *Streptococcus spp*. commonly found to be causative of bovine mastitis. We show that the resazurin-based assay can be adapted to accommodate the potential antimicrobial properties of resazurin dye by determining the growth rates of different species of bacteria in varying concentrations of resazurin and evaluating the use of lower concentrations of resazurin with reduced antimicrobial effect. A move from colourimetric to fluorescence detection is required to best exploit the lower concentrations of resazurin used. We showed that time-to-results is heavily dependent on the cell density of inoculated bacteria, however, the rate of resazurin conversion is positively correlated to the rate of bacteria growth. The fluorescence intensities analysed allow for the detection of bacteria growth with as low as 5µg/mL resazurin dye, with the time to detection of growth for all species tested within 5.5-9 h, much faster than the standard laboratory culture method. Determination of MIC within this system will allow for the rapid detection of antimicrobial resistance providing an accurate AST profile for fastidious

Streptococci spp. comparable to reference methods. This, in turn, will allow for more accurate and rapid informing of treatment for Streptococcal mastitis infections on farms. However, these results suggest that future resazurin-based microfluidic assays for the detection of bovine mastitis organisms should use lower concentrations of resazurin dye and should potentially utilise fastidious Mueller Hinton broth (MHB-F) to incorporate the detection of *Streptococcus spp*. growth and AST detection.

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Chapter 6 – Challenges in developing an on-farm AMR detection tool for mastitis and future work

6.1 Research in context and conclusions

Key to being able to determine AST on a farm is having suitable equipment that is both cost-effective and practical for use. The World Health Organisation (WHO) have previously identified that novel diagnostic techniques for on farm used be evaluated based on accuracy, sensitivity, specificity, user-friendliness, being rapid or robust, equipment-free and being deliverable (ASSURED) (Zadoks et al., 2023), where farms are considered as low-resource settings for their capacity to undergo microbiological testing. The most common on-farm methods of determining bacterial mastitis do not identify bacteria, but identify the presence of somatic cells, therefore providing no information on the type of infection or how best to treat it. Culture-based tests are all constrained by the time taken to detect bacterial growth in a sample and usually have an overnight incubation step resulting in a time to result of 24 h. While this is more rapid than sending samples for microbiological analysis by a laboratory, this time to result is still not optimal for informing decision-making and requires a new animal management process (Malcata et al., 2020; Griffioen et al., 2016), which may indicate the more popular on-farm mastitis tests detecting host factors which can be detected in minutes compared to hours and days. Even where bacterial detection is being developed for on-farm testing, generally this provides a positive result for the presence of bacteria, or at most whether the pathogen is Gram-positive or Gram-negative, without further information on susceptibility. Mastatest® however, does begin to provide information on MIC for a limited number of antibiotics, but these tests are not known to be rapid (Chapter 2, Table 3). Going forward, phenotypic assays are at the forefront of novel technologies being developed for the detection of AMR. However, these tests do not provide information on antibiotic resistance. There is also a question which must be raised, if while antibiotics are broadly effective, are the cons of overprescribing antibiotics empirically more important to consider, than the cons of developing and implementing new on-farm methods of testing?

Developing a successful on-farm bacterial detection test for the diagnosis of mastitis can prove challenging. There are technical and market complications including sample composition and potential contamination, costs incurred from developing the test and deploying it. Several important factors should be considered when developing an on-farm bacterial and AMR detection device.

Often these novel tests are developed based on the gold standard disk diffusion method or broth microdilution (BMD) for MIC detection, which is widely accepted for predicting resistance patterns (Yee et al., 2021). These kinds of detection models can also be more widely used in lower-income and more rural settings where access to complicated genetic sequencing materials is not viable.

6.1.1 Time-resolved automated imaging

As part of optimising any method for microbial detection and AMR measurements, it is important to understand the growth kinetics of bacteria, meaning that flexible time-resolved automated imaging is useful. Often automated digital imaging comes with a cost barrier, with large and expensive benchtop instruments impractical for use in the field. Cheap electronics and open-source hardware, along with advancements in digital imaging, allow for the quantification of experiments, particularly microbiological growth assays. This sort of instrumentation can be developed in less well-funded areas and can cover screening and imaging of a range of specialist microbiological methods. The development of the PiRamid was derived from the idea of wanting to take this ability of time-resolved imaging outside of the lab, and create a device that is cheap, easy to assemble and use, and easy to store, transport and fit inside most commercial tabletop incubators for optimal bacteria growth (Chapter 3) (Long et al., 2022). The PiRamid, in its varying formats, can be modified for backlit imaging, demonstrated by a time-lapse image of cell culture on agar plates and bacterial motility in agar. It was designed to colourimetrically image the conversion of resazurin to resorufin in the presence of bacteria growth. With the modifications of adding green LED strip lights and emission filters to the camera, it has been made capable of detecting fluorescence quenching of the same assay in lower concentrations of resazurin. The ability to modify this device has proven important to the continued development of an on-farm assay for mastitis detection. Although colourimetric imaging of resazurin conversion is possible with certain types of bacteria, for example, *E. coli* that can withstand the antimicrobial effects of resazurin, to some Gram-positive organisms resazurin is cytotoxic therefore it is important to use a lower concentration of resazurin (Schmitt et al., 2016; Schmitt et al., 2013), yielding a lower difference in absorbance between start and end point. The adaptation to fluorescent detection allowed for a much lower concentration of resazurin to be used and therefore allowed the growth of affected organisms such as *Streptococcus spp.* and some *Staphylococcus spp*. Indeed, the portability of the PiRamid and the ability to use it in tabletop incubators allows for use on farms and in clinical settings where maybe usual lab facilities such as walk-in incubators aren't

appropriate or available. It has long been discussed the use of on-farm diagnostics needed to inform treatment decisions for mastitis, but also the requirement for these diagnostics to be economically viable (Zadoks et al., 2023). The PiRamid can be a tool for bacterial detection, but also in the form of growth kinetics and aid in the detection of AMR through time-lapse imaging of AST assays, making it important to getting rapid microfluidic assays into the field and achieving more timely and cost-effective disease and AMR detection.

6.1.2 Antibiotic susceptibility in a mixed bacterial sample and detection of different species

Some studies have suggested that to reliably perform antibiotic susceptibility testing (AST) of a pathogenic disease, we must look carefully at the complex bacterial community from which the pathogenic bacteria comes. However, many of these phenotypic detection techniques deployed, rely on the isolation of a single organism. With different species interactions being unclear and unknown, are there effects on the resistance of the pathogen (Kandavalli et al., 2022)? It was important within this study to determine the effects of a mixed community of bacteria on phenotypic assays and how this could produce results of false susceptibility and potentially harm the treatment outcome of a disease.

For the development of an assay capable of AST, one of the biggest struggles is the ability to carry forward the test on-site for point-of-care (POC) (Gajic et al., 2022; St John & Price, 2014). Particularly where the treatment of disease in animals is concerned, farms can be difficult places to perform complicated microbiological methods with expertise and sterility (Saila et al., 2023). Moreover, the complex bacterial community on farms and in animal samples can contaminate samples and greatly interfere with results informing treatment, with a high potential for contamination of samples when inadequate collection and handling is involved (Saila et al., 2023). It was important for us to understand the effects that these sorts of problems would have on the results from a phenotypic microfluidic assay, whereby often you would be focusing on the detection and resistance profiling of a single disease-causing pathogenic bacteria, amongst a community of different environmental bacteria. Indeed, investigations into what a ‘normal’ uninfected milk sample provided an idea of the abundance of bacteria found even when there is not a mastitis infection present. Although not meant to representative across of all dairy cattle populations, across 40 fresh and health milk samples from the same farm, the different bacterial species and counts were vast (Chapter 4; Figure 4). It is likely that this will vary more greatly across farms, where different farming environments will harbour different species of bacteria. This will also be affected by husbandry practices. Particularly within mastitis, pathogen bacteria

are expressed in the cow's milk often shed from the inside lining of a cow's teat. Cow's milk is an extremely complex environment, not only containing a potential pathogen, but also an abundance of commensal organisms, skin flora, probiotic bacteria, and often high levels of environmental organisms depending on animal husbandry (Quigley et al., 2013). Using milk as a sample for the detection of mastitis is a common practice for such methods as somatic cell counting (SCC), however, the detection of bacteria and AST directly from these types of samples without bacterial culture and steps to isolate individual species, are uncommon. For example, standard AST methods require specific media, MH broth with no added supplementation, selected to minimise interference with antibiotic activity. It was clear in our investigations that changing this medium can affect observed MIC results. This included changing the pH, which was observed using yoghurt as a simulated direct sample versus normal milk. When looking at milk specifically for direct testing, our findings indicated that dilution of milk 1:5 or 1:10 may be sufficient to reduce any impact of the milk sample matrix on growth detection and changes in MIC. pH changes are particularly important to consider when looking into matrix effects on an AST. Studies into the effects of pH on the AST of uropathogenic *E. coli* have shown that important antibiotics such as erythromycin, ciprofloxacin and gentamicin are more effective against *E. coli* in a more alkaline environment, whereas ampicillin demonstrated a more potent effect on *E. coli* in acidic conditions (Kincses et al., 2021). Moreover, there is evidence to suggest that beta-lactam activity is increased in acidic conditions (Thomas et al., 2012). This can be related to the activity of the antibiotic itself, or such virulence factors as quorum sensing and the formation of biofilms in bacterial strains. With sample matrix having a particular effect on upregulating antibiotic activity and artificially increasing antibiotic susceptibility, the resulting MIC could introduce false susceptibility and misinform treatment. This is supported by the MIC determination for both ciprofloxacin and gentamicin in a simulated spoilt milk sample (yoghurt) whereby the pH would be more acidic, and the antibiotic efficacy is therefore reduced, resulting in MIC values much higher than the expected range (Chapter 4; Figure 3).

Many novel phenotypic bacterial detection and AST devices will face a similar problem of indiscriminate detection of bacteria and our MCF microfluidic assay is not dissimilar. The limit of growth detection for capillaries in MCF is 1 CFU with a test volume of 1 microlitre. If an uninfected fresh cow's milk sample is tested via this method without dilution, all milk samples will eventually show detectable bacterial growth through resazurin conversion. Dilution was therefore needed to reduce the noise created by commensal organisms (Figure 4.2), with a focus on optimising the dilution factor to bring the level of commensal bacteria down below the limit of bacteria growth detection for the device but still allow for the detection and AST of a pathogenic

mastitis bacteria (Long et al., 2023). Theoretically, we determined that if left undiluted, the testing of a direct milk sample may have periods of false susceptibility, whereby the greater presence of a susceptible commensal organism may overshadow the presence of a slower-growing pathogen with lower cell densities. This would therefore increase the time to results and introduce a period where results suggest the susceptibility of a sample. This can lead to occurrences of misinformed treatment. Even in the instance that a susceptible commensal bacterium and the resistant pathogen have similar cell densities, if the commensal bacteria have a lower generation, there may still be a short period of false susceptibility where resistance is recorded later after slower growth of the pathogen. This was demonstrated experimentally, whereby AST to ciprofloxacin was carried out for mixed samples containing a resistant bacterium and a susceptible bacterium at different inoculum densities. As predicted, where the resistance organism was at a much lower density, resistance was detected much later in the presence of ciprofloxacin. Resistance wasn't detected until 10 h in the case of a much lower cell density of the resistant organism. This stresses the importance of understanding the bacterial environment of the sample used for direct testing. In this case, milk is very complex but desirable as media for direct AST for mastitis due to its harbouring of mastitis pathogens. Indeed, we determined a trade-off between time to results and the accuracy of the susceptibility readout, with earlier readouts having the potential to indicate false susceptibility.

With *Streptococci* bacteria causative of up to 38% of cases in Europe, it is important that we develop more rapid detection and treatment of *Streptococci* mastitis. Moreover, the majority of novel on-farm microbial detection tests are capable of identifying such Gram-positive bacteria as *Streptococcus,* whether this be by some form of reagent, in the case of MastDecide and Mastatest, or by Gram-specific media, in the case of VétoSlide. Previously the growth and AST of gram-negative bacteria has been validated in microcapillary film (MCF), it was important to understand if it can be used as a portable, microenvironment enabling the growth of *Streptococci*. The stablished method of bacterial detection in MCF however, required resazurin as an indicator. However, resazurin has also been shown to be cytotoxic to some bacteria (Schmitt et al., 2016; Schmitt et al., 2013). There is evidence to suggest that both resazurin and resorufin can suppress the growth of bacteria, and exposure to resazurin can change the morphology of cells and interfere with normal cell function depleting reduced forms of nucleotides and causing cell death (Riss et al., 2013). To carry this method forward, therefore, it was important to understand how much resazurin could be deployed as an indicator, without having cytotoxic effects on the mastitis sample.

Resazurin has posed a problem with only 78% of *Streptococcus* strains were found to be resistant to resazurin dye at a concentration of ≥ 10µg/mL, where previously a concentration of 60µg/mL was shown to provide fluorescent intensity suitable for analysis of bacterial growth kinetics. This means that at with a resazurin concentration of ≥ 10µg/mL, up to 22% of *Streptococcus* strains may be killed. Where the reduction in resazurin dye concentration may be required to allow Streptococcal growth, the difference in start and endpoint is also reduced, therefore making colourimetric detection unviable for Streptococcal growth. With the inability to detect growth colourimetrically, the use of fluorescence detection limits capturing results to an imaging device over reading by eye. The adaptation of the PiRamid to enable fluorescence provided a low-cost and flexible solution to this, that still allows automation and deployment on farms, given the PiRamids portability. In order to reduce the impact of resazurin it was hypothesised that positive growth may be detected by haemolysis. AST for fastidious organisms is one of the few times that the gold standard method of broth microdilution (BMD) allows for supplementation (Humphries et al., 2023). The use of defibrinated lysed horse blood at a concentration of 3-5% v/v is accepted and commonly used for the growth of fastidious organisms in broth. Often, MIC for fastidious organisms is read by the detection of haemolysis rather than turbidity, usually by eye. The antibiotic concentration at which no haemolysis is noted is the MIC. Interestingly, it was determined that haemolysis alone cannot indicate the growth of *Streptococcus spp*. in MCF where the difference between the absorbance calculated at 0h does not vary enough from the absorbance calculated at 20 h, where growth is present. Although the presence of lysed blood in an MCF microfluidic test strip is visible by the eye, presence of haemolysis is not detectable. This still demonstrates for the requirement of an indicator for growth.

Positively, all species had detectable growth between 5.5-9 h, considerably faster than the method deployed for traditional AST (> 24 hrs). A key objective of a rapid AST test is to allow for faster time-to-results to inform treatment and reduced the lag introduced by requiring lab culture techniques. Indeed, this seems comparative with the current novel bacterial detection devices. It was noted that phenotypic rapid testing kit MastDecide® (MastDecide, Quidee GmbH, Homberg, Germany), provides positive results in 8-14, whilst BACT by Point of Cow (FluimediX APS), wields results within 14-16 h upon incubation at 37°C. Meanwhile, Mastatest® system reports to provide results within 24 h of the ‘cartridge’ being filled. This suggests that the MCF resazurin-based assay can provide similar, if not better time-to-results as the current bacterial detection tests being deployed.

6.2 Continued work and future directions

6.2.1 Validation of a novel microfluidic AST device on farm and stakeholder consultation

To move the use of a novel microfluidic AST device it is important that validation be carried out on farms and with a greater sample of mastitis cases and infected milk. The device would first need to be validated using more mastitis cases. We recommend that these detection techniques be carried out cow side and immediately after milk collection to reduce the effects of storage. Moreover, it would be important to consider inter- and intra-operator variability for all steps involved in the device development, including antibiotic MCF strip production, steps for appropriately diluting milk samples and carrying out the microdilution methods for AST. Secondly, these methods must be carried out in a farm setting, from milking to results, within an appropriate time frame to ensure successful detection of any resistance and then inform effective treatment. This would require taking the device out of the lab, collecting fresh and infected milk samples directly from the cow or milking system, carrying out the assay in a farm setting, and collecting results to inform treatment. At first, this would be carried out by personnel with experience in carrying out microbiological assays. It would then be important for both farmers and veterinarians to be able to understand the methodology of the device and get hands-on with using the device to ensure limited inter-operator variability between different types of users. Finally, we would want to ensure that results inform effective treatment. This may be considered where AST results inform the type of antibiotic therapy deployed, leading to successful treatment of mastitis in a cow. This would require extensive experimentation on farms, working with farmers and veterinarians to determine whether veterinarians consider the device suitable for informing treatment and following up on the informed treatment to determine its success *in vivo*.

A key limitation to this project was the lack of farmer and veterinary involvement in the development of the methodology. It is important to understand who it is that will used such rapid mastitis detection and AST tests and what exactly it is that the need to know. Farmers weigh up decisions about on-farm mastitis diagnostics as part of mastitis management practices. From a farmer's perspective this may be that they want a test that is affordable, less time consuming and provides them with information that inform treatment. For example, a study interviewing farmers in the Netherlands determined that ideal rapid test for mastitis would provide a result prior to the next milking, which would be 8–12 h later in herds milking 2 or 3 times daily (Griffioen et al., 2016). Zadoks et al. (2023) highlights the importance of directing attention to farmer's perspectives on the role of mastitis diagnostics, and whether they believe development of novel

rapid tests will benefit their farming practices. It would also have been useful to know what kinds of infections and bacteria farmers and vets are concerned about the most. Do farmers see more clinical cases that sub-clinical? Is there a higher prevalence of a particular type of pathogen? Where there are more cases of Gram-positive, *Streptococcus* mastitis, there should be a focus on optimising a test that specialises in differentiating Gram-postive bacterial species and determining antibiotic susceptibility. Therefore, validation of the test on-farm will need to heavily involve the stakeholders, farmers and vets, as to what they would deem necessary and effective as a rapid on-farm microbial detection and AST.

6.2.2 Stability of farm milk and the effects of storage

To study more carefully the change in stability of raw milk samples ensure suitable milk storage, it is key to understand the concentration of the bacterial community in both uninfected and infected raw milk samples. Initial steps were taken to understanding this, however, further work is needed to provide a more comprehensive idea of what sort of bacteria milk samples contain and how best to store these milk samples to provide the most accurate representation of an infection at the time of treatment.

Importantly, it has been determined that the median cell densities of fresh uninfected milk samples, unsurprisingly fall below the cell densities expected for a mastitis infection, with the majority of cell counts falling below 1×10^6 CFU/mL. When considering a positive mastitis case, it is common for cell densities to be $\geq 10^6$ CFU/mL. Retrospective to the investigation of milk matrix effects on our microfluidic assay, 7 milk samples positive for mastitis infection were acquired, and plate counts to determine the CFU/mL for each sample were carried out. Cell densities differed greatly between samples with a range from $2 \times 10^8 - 2 \times 10^4$ CFU/mL. This indicates the potential for great overlap in the bacterial cell densities of pathogens found in mastitis samples with commensal bacteria and consolidates the difficulty in avoiding false growth of commensals from healthy milk. In this case, if the use of the device is simply for measuring susceptibility, other tests should be considered for the detection of mastitis that does not exploit microorganism growth.

Not only are there complications with the initial bacterial concentration and the types of bacteria present, but also potential for a decrease in the number of viable bacteria for AST in a milk sample. As soon as a milk sample is taken for testing, the composition is changing. Commensals and pathogenic bacteria will continue to multiply, nutrients will be used up and eventually, some bacteria will die if stored for too long. Commonly, milk is stored in fridges and freezers before

testing (O'Connell et al., 2016). Even at a refrigerator temperature of 4°C, bacterial richness is higher in raw milk on day 0, compared to microbiota analysis after 7 days of storage, showing a clear depletion in the number of organisms viable for testing (Ban et al., 2023). Meanwhile, other studies have shown that at temperatures of 6°C, total bulk milk bacteria are shown to increase, particularly psychotropic bacteria including coliforms and *Streptococci* (O'Connell et al., 2016), indicating how variable and unpredictable changing bacterial communities can be in milk, especially where storage is inappropriate. Where an on-farm test is not available, and laboratory investigations are required to determine the cause and susceptibility status of a mastitis case, milk will undergo different types of storage and transportation. Alongside on-farm refrigeration of samples, increased length of storage during transportation and decreasing temperatures through freezing have been known to decrease the numbers of coliform bacteria, such as *E. coli*, and sometimes increase the number of samples known to contain *Staphylococcus spp.* (Schukken et al., 1989). The stability of milk samples that had been stored for known periods in -20°C conditions were studied. With eventual access to positive mastitis samples, a short investigation was carried out to determine the effects of freezing milk for longer periods, over 2 weeks. Initial plate counts were recorded upon sample collection and then subsequently after 1 week and then 2 weeks of freezing at -20°C. A substantial drop in bacteria was noted, with some samples seeing total plate count dropping by 100-fold over the periods of freezing (Annex 1, Figure 6.1).

This indicates the importance of time to testing and sample storage when carrying out a direct in-milk AST test. This also provides a problem when validating a new direct in-milk AST where gaining access to fresh mastitis samples that haven't been stored in some way can be difficult. Where stability is difficult to determine, without validating cow side, mastitis milk samples will almost always see a change in the bacterial community before being tested. With rapid testing, it would be important to reduce laborious and time-consuming tests such as traditional culture, and with freezing having unpredictable effects on sample cell density, it would be important to remove any steps of storage or freezing of milk samples before carrying out rapid AST to avoid a change in bacterial concentration. Testing a milk sample immediately after milking will yield the most accurate results in terms of bacteria species, and concentration and therefore identifying potential patterns of resistance within mastitis bacteria. With milk storage before AST, we risk missing potentially pathogenic bacteria, reducing bacteria below limits of detection and making samples unviable for AST or at risk of reporting false susceptibility.

6.2.3 Bacterial identification

Going forward, the idea of developing a microfluidic tool for the identification of bacterial species alongside AST is considered. The treatment of mastitis with antibiotic therapy is reliant on the determination of the pathogen causing the infection. Various antibiotics are used empirically and are known to be successful at treating the most common mastitis-causing bacteria. However, with increasing levels of resistance to the most used antibiotics, it is becoming more appropriate to determine the cause of infection and therefore aid a more informed course of treatment.

Whilst direct genotypic detection has become the most practised method within labs, including the use of 16S metagenomics and MALDI-TOF molecular testing, these methods often rely on costly equipment, (Franco-Duarte et al., 2019) requiring access to lab services and not designed to be used at point-of-care. Meanwhile, the most common method of indirect identification through cell culture is chromogenic media, for example, Chromagar™ Mastitis is a media of two parts specifical for Gram-positive and Gram-negative bacteria, with each media containing chromogens that often consist of a colourless substrate and a coloured chromophore. The growth of a particular bacteria with specific enzyme activity causes the cleaving of the coloured chromogen molecule, allowing the colour to become visible. This is generally very useful media and is frequently used in the isolation of bacterial genera. However, this method is time-consuming and requires laborious culturing techniques. The requirements of a rapid test for mastitis are usually faster than the time frame for a traditional 18-24 h culture, for example, the Netherlands require a POC test for mastitis to be within 8-12 h, within the time between milking (Griffioen et al., 2016).

Performing identification at a species level often then requires biochemical tests, before the overnight culture of bacterial colonies. The more commonly used tests are a phenotypic colour change exploiting the differences in protein expression, containing a host of reagents and providing a protein expression fingerprint (e.g. Api, bioMérieux). There are also automated tests, such as the OmniLog ID system from Biolog, which identifies bacteria based on their ability to utilise up to 94 different carbon-based compounds as a substrate, then oxidising tetrazolium-redox dye for identification. To reduce this time to identification, we hypothesised that in the same way we load antibiotics into microcapillary film (MCF), there is the potential to also load reagents carefully selected to inhibit the growth of certain organisms. A combination of these reagents could provide a fingerprint allowing for the identification of an organism in the MCF. An initial search for reagents was carried out that affected some major organisms found to cause mastitis cases, *E. coli, S. aureus, Streptococcus spp.* and *Pseudomonas spp.*, including a urinary

pathogenic *E. coli* (UPEC). A broth microdilution method was carried out to determine the MIC for each reagent using the quality control strains of the organism and four two-fold dilutions of the reagents; sodium chloride, deoxycholic acid, bile salts, potassium tellurite, nalidixic acid, urea, sodium azide and phenyl alcohol. The resulting MICs provided an initial idea of the reagent concentrations that could be used as a fingerprint to determine the species of bacteria present in a direct sample (Annex 1, Table 6.1). The MIC for all organisms tested in the presence of deoxycholic acid was above the highest concentration used and was determined as resistant, therefore this reagent could not be used as part of the fingerprinting system.

This method provides a very basic method of genus identification and is a starting point for introducing bacterial identification into a microfluidic assay device. The more common Api strips and automated systems provide a more comprehensive fingerprint down to species level, however, genus level identification, and even just identification of Gram-positive or -negative would allow for more informed AST of appropriate antibiotics for treatment of those organisms and would allow for more appropriate methods of bacteria growth, such as the introduction of fastidious media for identification of *Streptococcus spp*. More investigative work is required to ascertain whether this method is useful in identifying species in a mixed bacteria environment. This would include testing more bacterial isolates gathered from mastitis infections and screening against QC strains. Only one isolate was tested outside of the QC strains (*E. coli* UPEC 328.1), of which the fingerprint did not have 100% agreement with its reference QC strain (*E. coli* 29522). Further literature searching could be done to identify more reagents that will provide a more substantial fingerprint for each bacterium and still be viable within a resazurin-based microfluidic assay. Moreover, procedures for loading MCF with the required reagents would need to be developed, potentially similar to the freeze-drying process used to load antibiotic solutions. This would have to ensure a limited effect on the hydrophilic properties of the MCF and limited interaction with antibiotics if used in conjunction or with the same capillaries.

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Appendix

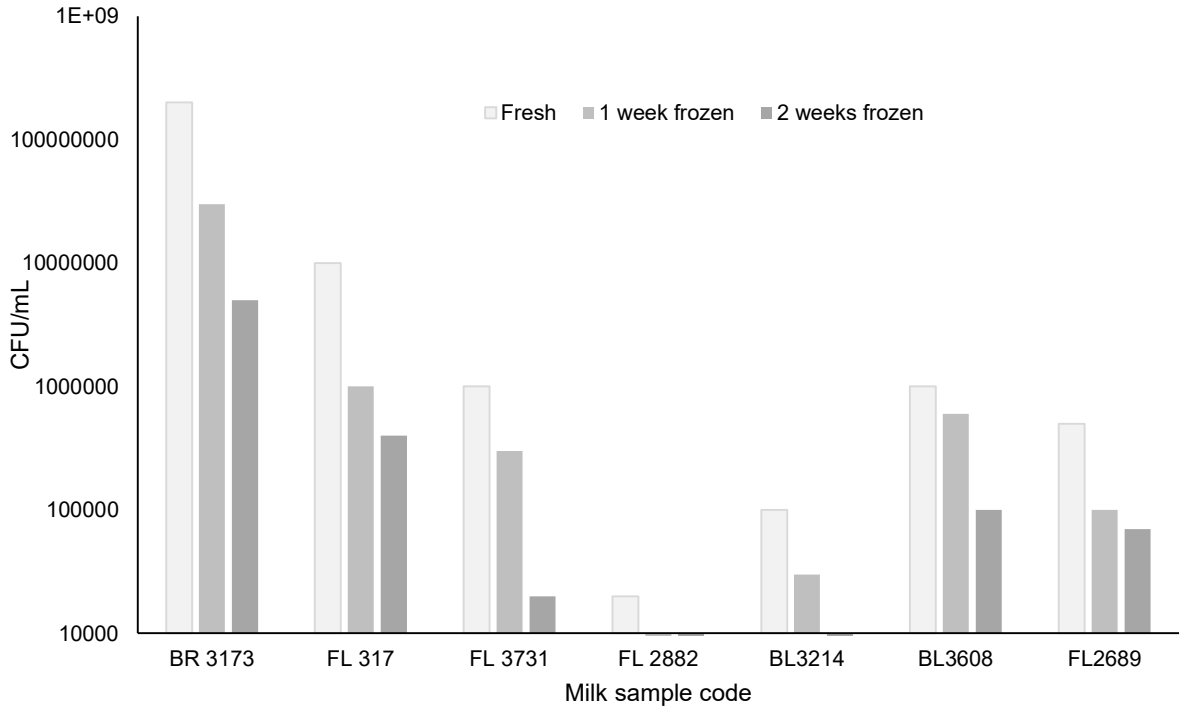

Figure A.1 Enumeration of bacteria present in confirmed mastitis milk samples before and after freezing.

Samples were diluted and streaked on Plate Count Agar the same day of sample was collected from a cow. At the point of first streaking, samples have been refrigerated but not frozen. Sample aliquots were then frozen at -20°C for 1 week and 2 weeks after collection and streaked on Plate Count Agar. Calculation of CFU/mL was made by counting colonies and multiplying by the dilution factor at the time of streaking. CFU/mL > 1 x 10^4 is below the limit of detection for this experiment**.** Sample FL 2882 fell below this limit of detection during the investigation.

Table A.1 The use of different reagents can produce a fingerprint to identify the species of bacteria present in a sample.

Four two-fold dilutions of each reagent were prepared in a 96-well plate. Wells were inoculated with a starting concentration for each species of bacteria of 5 x 10^5 CFU/mL. The table indicates the average MIC for each species under each reagent condition (n=3). A concentration of MIC is given for each reagent. MICs shaded grey were above the highest concentration for these reagents and were determined resistance in this test. MIC90 values were identified. MIC values

for *S. pneumoniae* were determined by haemolysis. MIC values for all other organisms were determined by spectrophotometer.

	NaCl	**Deoxycholic Acid**	**Bile Salts**	**Potassium Tellurite**	**Nalidixic Acid**	**Urea**	**Sodium Azide**	**Phenyl Alcohol**
***E. coli* (ATCC 29522)**	5%	>0.2mg/L	>0.2 mg/L	>0.005%	<155mg/L	>1.6M	0.025%	5mg/L
***S. aureus* (NCTC 12973)**	>10%	>0.2mg/L	>0.2 mg/L	>0.040%	625mg/L	>1.6M	0.1%	10mg/L
***S. pneumoniae* (NCTC 12977)**	>10%	>0.2mg/L	0.1mg/L	0.02%	1250mg/L	<0.2M	0.05%	<1.25mg/L
***P. aeruginosa* (NCTC 12903)**	>10%	>0.2mg/L	<0.025mg/L	>0.005%	<155mg/L	<0.2M	<0.025%	<1.25mg/L
***E. coli* (UPEC 328.1)**	10%	>0.2mg/L	>0.2 mg/L	>0.005%	155mg/L	1.6M	<0.025%	>10mg/L